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Pesticides in the Modern World

Effects of Pesticides Exposure

Edited by Margarita Stoytcheva

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Contributors

Jeff Bronstein, Arthur G Fitzmaurice, Bruno Sabino, Hannah Rozenbaum, Adriana Sousa, Khaled A. Osman, Nilesh Kumar Mitra, Yongning Wu, Hong Miao, Sai Fan, Kiran Dip Gill, Binukumar Bk, Kamil Musilek, Ondrej Holas, Anna Horova, Miroslav Pohanka, Jana Zdarova-Karasova, Daniel Jun, Kamil Kuca, Ozlem Ozmen, Anthon Alvarez Arredondo, Alba Delia Aby Campaña-Salcido, Zhijun Zhou, Lidia Caporossi, Bruno Papaleo, Ewa Rembiałkowska, Maciej Badowski, Narayan C. Rath, Komal Singh Rasaputra, Gerry Huff, W. E. Huff, Richard Wang, Maria Angela Montesano, Burkard Watermann, Katarina Gnass, Heike Kolodzey, Anja Thomsen, Benjamin Alewu, Nosiri Ijeoma Chidi, Maria del Pilar Diaz, Maria Josefina Lantieri, Mariana Butinof, Ricardo Fernández, Maria Ines Stímolo, Marcelo Blanco, Paloma Beamer, Wu-Chien Chien, Ching-Huang Lai, Lu Pai, Senyeong Kao, Jin-Ding Lin, Yu-Chen Hung

Notice

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Meet the editor

Professor Margarita Stoytcheva graduated from the University of Chemical Technology and Metallurgy of Sofia, Bulgaria, with titles of Chemical Engineer and Master of Electrochemical Technologies. She has a Ph.D. and DSc. degrees in chemistry and technical sciences. She has acted in research and teaching in several Universities in Bulgaria, Algeria and France. From 2006. to the present she has participated in activities of scientific research, technological development and teaching in Mexico at the University of Baja California, Institute of Engineering, Mexicali, as a full time researcher. Since 2008. she has been a member of the National System of Researchers of Mexico. Her interests and areas of research are analytical chemistry and biotechnology.

Contents

Preface

The introduction of the synthetic organochlorine, organophosphate, carbamate and pyrethroid pesticides by 1950's marked the beginning of the modern pesticides era and a new stage in the agriculture development. Evolved from the chemicals designed originally as warfare agents, the synthetic pesticides demonstrated a high effectiveness in preventing, destroying or controlling any pest. Therefore, their application in the agriculture practices made it possible enhancing crops and livestock's yields and obtaining higher-quality products, to satisfy the food demand of the continuously rising world's population. Nevertheless, the increase of the pesticide use estimated to 2.5 million tons annually worldwide since 1950., created a number of public and environment concerns.

This book, organized in two sections, comments on the major aspects of the pesticides risk, integrating pesticides exposure and pesticides health effects.

Chapter 1 covers the background information and the epidemiological evidence on the long-term pesticides exposure, commenting on the genetic susceptibility to pesticide toxicity, on the developmental toxicity and neurotoxicity, and on the neurobehavioral impairments provoked by pesticides exposure.

Chapter 2 explains how dermal exposure to sub-toxic amount of chlorpyrifos is connected to neurotoxicity. Most occupational exposures are dermal. The authors conclude that high dosage of chlorpyrifos can result in significant neurotoxicity, while low dosage produces a reduced level of neurotoxicity.

Chapter 3 inventories the typical sources and routes of occupational pesticides exposure, focusing on the ability of pesticides to interfere with the endocrine system and to cause adverse effects, and discussing the approaches to be applied for prevention and protection.

The effects of long-term low-level exposure to organophosphorus pesticides in both general and occupational population in China, exposure assessment, and studies on the mechanism of the organophosphorus pesticides action are considered in Chapter 4.

Chapter 5 reviews the exposure pathways and the neurodevelopment injuries in children of farm workers in U. S., associated with chronic pesticides exposure.

Effective interventions conducting to increasing of the farm workers self-protective behaviours and perception of control are discussed, too.

The alteration of the erythrocyte cholinesterase activity, and the affection of the process of hemostasis in migrants workers exposed to the action of organophosphorus pesticides in the Mexican state Sinaloa is the objective of the investigations, presented in Chapter 6.

Chapter 7 addresses the work practices of the pesticide applicators in Cordoba province, Argentina. Exposure indexes and scales proposed in this work are helpful tools for the assessment of occupational risks related to pesticide exposure.

In Chapter 8 are discussed the various methods of chiral separation of synthetic pyrethroids. It has been demonstrated that chiral isomers exhibit different biological activities and toxicities, and thereby the residues and metabolisms in the environment and biological organisms also vary.

Chapter 9 illustrates the importance of biological monitoring in the assessment of human occupational exposure to pesticides, facing in particular the exposure to ethylenebisdithiocarbamates. It comments on the main factors in biomonitoring such as sampling methods, analytical determination, and interpretation of the results.

Chapter 10 presents numerous evidences confirming that the presence of pesticide residues in organic food is lower than in conventional products. Thus, the risks associated to pesticides exposure are reduced.

The main topics discussed in Chapters 11 and 12 include hazard identification, exposure assessment, dose-response assessment and risk characterization associated with pesticides exposure and human health.

The forensic aspects of pesticides poisoning in Brazil, and the forensic analytical chemistry of pesticides are commented in Chapter 13.

Chapter 14 is intended to examine the characteristics and trends of unintentional pesticide poisoning mortality and hospitalization in Taiwan. Taking into consideration that currently no authority in Taiwan is in charge of pesticide poison surveillance, the present work is the most complete nationwide population-based study conducted to assess the risk of pesticide poisoning.

Chapters 15 and 16 provide pathological findings on endosulfan toxicity to human and animals, and epidemiologic evidences strengthening the hypothesis that exposure to pesticides could increase the risk of developing Parkinson's disease.

The objective of Chapter 17 is to review and to highlight some of the recent findings on the effects of dialkyl dithhiocarbamates and ethylene-bis-dithiocarbamates pointing out on studies of the avian system, which has not been a focus of earlier literature. It is

demonstrated that cells exposed to dithiocarbamates experience increased oxidative stress and metabolic disregulations leading to tissue damage and apoptosis.

Chapter 18 provides information on the potency of the commercially available acetylcholinesterase reactivators (pralidoxime, methoxime, trimedoxime, obidoxime, asoxime, etc.) developed against organophosphorus pesticides intoxication, and on the reactivation capability of some promising novel reactivators produced in the last decade, such as the mono-oximes from the K-compound series.

Chapter 19 reports investigations on the effects of several pesticides on the reproductive system of echinoderms, crustaceans, molluscs, fish, one amphibian and one mammalian species. The concentrations, selected in the range of the environmental concentrations, are discussed with respect to ecotoxicological impacts.

The book is a compilation of works, addressing the various aspects of the pesticides exposure and the related health effects. It offers a large amount of practical information to the professionals interested in pesticides issues. The commitment of each of the contributing authors with the present project is gratefully acknowledged.

Margarita Stoytcheva
Mexicali, Baja California
Mexico

Part 1

Pesticides Exposure

1

Chronic Exposure to Pesticides- Neurological, Neurobehavioral and Molecular Targets of Neurotoxicity

Binukumar B.K and Kiran Dip Gill
Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh, India

1. Introduction

There is an increasing concern regarding the widespread use of pesticides and their potential impacts on public health. Pesticides differ from other chemical substances because they are toxic chemicals deliberately spread into the environment with the aim of controlling undesired living species. Since their toxicity may not be completely specific for the target organisms, their use may pose a risk to human health. Pesticide poisoning remains a serious public health problem worldwide. More than 5 billion pounds of pesticides are used annually worldwide, with about 25% being used in the United States (US Environmental Protection Agency 2001, 2002). Pesticide exposure occurs during their application, via their drainage into water supplies, and through the consumption of food. According to the World Health Organization's estimate, 3 million cases of pesticide poisoning occur every year, resulting in more than 250,000 deaths. This number also accounts for a substantial fraction of the almost 900,000 people worldwide who die by suicide every year. Organo-phosphorus pesticides (OPs) are currently the most commonly utilized pesticides in the world, consisting of nearly 40 different chemical members registered by the US-EPA (www.epa.gov). About 73 million pounds of OP pesticides were used in the United States above in 2001 (70% of all insecticides; Kiely et al.,2004).

Pesticide poisonings are relatively common in countries such as Sri Lanka, Venezuela, Indonesia, South Africa, and Brazil. Among numerous pesticides that can result in death, organophosphate insecticides are the most common culprit agents because of their high toxicity. In developing countries, in which the use of OP compounds is particularly widespread because the hot climatic conditions, the number of deaths may be high. There is an increasing concern regarding the widespread use of pesticides and their potential impacts on public health. In the United States, a mixture of pesticide residues are detected in blood and/or urine of nearly all persons sampled (Barr et al., 2005). During the 1990s some 2.5–5.0 million agricultural workers were exposed to OPs, which are used as insecticides around the world (Abou-Donia, 2003; Das et al., 2001; Farahat et al., 2010; London et al., 1997). Although OPs are increasingly restricted for use in the US (EPA, 2002), many of the pesticides that are no longer available in the US and other developed countries are still being produced and used in agricultural or urban applications in developing countries.

The mechanism of action of pesticides frequently involves a neurotoxic effect: organophosphorous compounds act through the inhibition of central nervous system cholinesterase (Jeyaratnam and Maroni, 1994; Machemer and Pickel, 1994); pyrethroids affect the sodium channels of the nerve membrane, keeping them open for more than the few milliseconds needed for the generation of the action potential (He, 1994); organochlorinated compounds in general act as central nervous system stimulants, but the mechanism of action varies for the different active ingredients (Tordoir and Van Sittert, 1994); morpholine derivatives alter the balance between excitatory and inhibitory threshold in neurons, impairing the function of the nervous system (Barbieri and Ferioli, 1994), while formamidines have an agonistic action on the alpha-2 catecholamine receptor (Xue and Loosly,1994).

Organophosphate (OP) pesticides can produce several distinct neurotoxic effects depending on the dose, frequency of exposure, type of OP, and host of other factors that influence susceptibility and sensitivity. These effects include acute cholinergic toxicity, a delayed ataxia known as organophosphorus ester-induced delayed neurotoxicity (OPIDN), chronic neurotoxicity and developmental neurotoxicity. Acute cholinergic syndrome, due to the inhibition of ace-tylcholinesterase activity, which occurs within minutes or hours following exposure, usually subsides within days or weeks, and plasma or erythocytic acetylcholinesterase activity are used for monitoring acute exposure to OP (Lessenger and Reese, 1999); Acute OP pesticide exposure can involve wide range of both central and peripheral neurologic symptoms. Increased neurologic symptom prevalence may provide early evidence of neurologic dysfunctions, before clinically measurable signs are evident.

Rastogi et al (2010) analyzed the cross-sectional data on neurologic signs and symptoms from 225 rural children, both males (n = 132) and females (n = 93) who were occupationally and paraoccupationally exposed to methyl OPs (dichlorvos, fenthion, malathion, methyl parathion) and ethyl OPs (chlorpyrifos, diazinon, ethyl parathion) as they belonged to agricultural families handling, mixing, and spraying the OP pesticides. Among all the neurologic self-reported symptoms, headache, watering in eyes, and burning sensation in eye/face were the most important clinical manifestations attributed to OP pesticide exposure. These symptoms could probably be the consequence of chronic effects of most pesticides on the central nervous system. The high frequency of neurologic symptoms observed in the study may be due to parasympathetic hyperactivity due to the accumulated ACh resulting from AChE inhibition (Rastogi, 2010).

Intermediate syndrome, which usually starts 24 to 96 hours after the acute syndrome and is characterized by respiratory paresis, weakness, depressed tendon reflexes, and transient extrapyramidal symptoms, without response to treatment with the cholinergic receptor antagonist atropine (Bhatt et al., 1999; Mileson et al., 1998; Senanayake and Johnson, 1982; Shahar and Andraws, 2001); Organophosphate-induced delayed neuropathy, which is a symmetric distal neuropathy, usually occurring weeks following an acute exposure probably related to the inhibition of the enzyme neuropathy-target esterase present in the nervous system (Aiuto et al., 1993).

Long-term exposure to relatively low levels of OP agents occurs in a variety of environments. Pesticides are often applied in a combination with several classes of compounds featuring synergistic interactions. One of the neurological functions for which an adverse effect of neurotoxic pesticides has been repeatedly hypothesized is behaviour. Behaviour is the product of various sensory, motor and associated functions of the nervous system, and the hypothesis is that neurotoxic substances can adversely affect one or more of these functions, disrupt learning and memory processes, or cause detrimental behavioural

effects (IPCS/WHO, 2001). Since behaviour is a very complex system, made of several different functions and biochemical activities, it can be studied only based on a very complex approach, in which different tests are performed, addressed at a large spectrum of functions, in some cases with different approaches for different population subgroups (Anger et al., 2000; Cassitto et al., 1990; Fiedler et al., 1996; Krasnegor et al., 1995; Wetherell, 1996), and conclusion can be drawn only from an integrated evaluation of the available data. Because of this complexity, not surprisingly, different approaches have been chosen by different researchers, making comparisons between different studies very difficult. However, neurobehavioral toxicity is a very important issue for prevention, because some of the compounds thought to be involved are largely used in agriculture, and large sections of the human population are occupationally and/or environmentally exposed, including possible vulnerable subgroups such as children or pregnant women. (Colosio et al, 2009). An increasing number of papers have been and are being published on neurobehavioral effects of pesticides. However, besides what is well established (e.g. acute effects; OP induced delayed polyneuropathy; intermediate syndrome) (Jayawardane et al., 2009; Lotti, 2001; Lotti and Moretto, 2005), several uncertainties still remain on the real risks for workers and consumers of developing neurobehavioral changes after long-term exposures to low doses of neurotoxic pesticides (Colosio et al., 2003; Moser, 2007). Experimental data on neurotoxicological outcomes in animals are abundant, but relatively few are those studies dealing with long-term exposures (for a review see Moser, 2007). In fact, most reports in the literature deal with repeated exposures to pesticides, mainly OPs, as short as five days and rarely longer than three months. In addition, an even lower number of studies assessed neurobehavioral performance days or weeks after end of exposure.

In southern Brazil, agricultural workers involved in tobacco plantation use a combination of OP (chlorpyrifos and acephate), herbicides (glyphosate and clomazone), plant growth regulators (flumetralin), fungicides (iprodione), and insecticides (imidacloprid). Exposure to OP is known to induce clinical syndromes and biochemical alterations in humans. Besides acute cholinergic symptoms, which are related to the inhibition of acetylcholinesterase activity, acute or chronic OP exposure can also induce delayed toxic and behavioral effects not clearly related to the inhibition of esterases (Brown and Brix, 1998; Jamal, 1997; Mileson et al., 1998; Peter and Cherian, 2000; Sudakin et al., 2000). Most of the actions of OP on the nervous system seem to be related to organophosphorylation of protein targets, as acetylcholinesterase and neuropathy target esterase, or directly to binding of OP to nicotinic receptors (Mileson et al., 1998).

Chronic organophosphate-induced neuropsychiatric disorders (COPIND) are a less well-characterized syndrome in chronic OP poisoning. COPIND may be caused by chronic low-level exposure to OP, without cholinergic symptoms (Ray and Richards, 2001). The underlying mechanisms are not established, but are not dependent on inhibition of esterases (Levin et al., 1976) The most common clinical symptoms include impairment in memory, concentration, and learning; anxiety, depression, psychotic symptoms, chronic fatigue, peripheral neuropathy, autonomic dysfunction and extrapyramidal symptoms such as dystonia, resting tremor, bradikynesia, postural instability and rigidity of face muscles; and nonresponsiveness to levodopa treatment. Regarding psychiatric symptoms, neurobehavioral effects of low-level pesticide exposure have not been extensively studied with standardized, quantitative neuropsychologic batteries.

OPs do not accumulate in living organisms and the acute signs and symptoms disappear as the AChE activity returns to normal level. Therefore, they are regarded as relatively safe.

However, as some literature data suggest, after either acute or prolonged exposure to OPs subtle neurobehavioral impairments may persist long after normalization of AChE activity. The possibility that OPs exposure may induce such long-term effects is nowadays a problem of great concern for the regulatory agencies. Rodnitzky et al, (1975) and Durham et al, (1965) in their cross-sectional epidemiologic studies using neurobehavioral tests have suggested that subtle behavioural impairments among pest control workers, farmers, and manufacturing workers are related to low level pesticide exposure or are persistent effects of severe acute pesticide poisoning (Metcalf and Holmes, 1969; Burkhart et al, 1978; Korsak and Sato, 1977; Levin et al, 1976; Xintaras et al, 1978; Savage et al, 1983, reviewed by; Johnson and Anger, 1983). There are also numerous case reports and case registries indicating that 4-9% of individuals with acute organophosphate poisoning experience delayed or persistent neuropsychiatric effects, including depression, weakness, nervousness, irritability, fatigue, insomnia, forget fulness, confusion, and schizoid and depressive reactions (Gershon and Shaw, 1961). Behavioural impairments due to pesticide exposure have also been implicated in serious accidents among agricultural workers (Redhead, 1968; Wood et al, 1971; Smith et al, 1968).

Amr et al. (1997) found that, compared to controls, subjects heavily exposed to pesticides (40 h/week, 9 months/year) had a significant increase in the frequency of psychiatric disorders, especially depressive neurosis and dysthymic disorder (DSM-III-R). These results left unresolved issue of reversibility of psychiatric symptoms after a pesticide-free period and the occurrence of syndrome in subjects not so heavily exposed to OP compounds. Another confounding factor in these studies has been the exposures to several types of pesticides which has been shown to reproduce features of Parkinson's disease (Binukumar et al, 2010). Some of the factors which have been shown to influence the feasibility of an epidemiologic appraisal of CNS abnormalities among pesticide workers depend upon: 1) the extent to which exposure can be quantified; 2) the multiplicity of chemical exposures; 3) the sensitivity and specificity of the neurobehavioral test; and 4) the time taken to conduct the test. Stephens, et al (1995) studied the relationship between chronic (nonreversing) neuropsychological effects and acute exposure effects and investigated 77 organophosphate-exposed male sheep-dippers. Acute exposure effects were assessed prospectively using a purpose-constructed symptoms questionnaire administered pre-, and 24 h post exposure. Urine was analysed for dialkylphosphate levels to confirm recent exposure. Chronic effects were assessed in a cross-sectional neuropsychological study in the absence of recent exposure using computerized neuropsychological tests, the General Health Questionnaire, and the subjective Memory Questionnaire. Simple correlation and multiple linear regression analyses, were used to assess relationships between the changes in total symptoms reporting from baseline to 24 h after exposure and chronic effect outcomes. There was no evidence of any association between reported symptom levels and chronic neuropsychological effects. This suggests that chronic effects of OP exposure appear to occur independently of symptoms that might immediately follow acute OP exposure. This has implications for exposure control: individuals may experience chronic effects without the benefit of earlier warning signs of toxic effects during acute exposures.

Military personnel returning from the Gulf War (GW) have reported symptoms that have not only diagnosis using known disease entities but also do not appear to occur in a predictable constellation that can be classified as a single syndrome (Persian Gulf Veterans Coordinating Board, 1995; Institute of Medicine, 1996; Iowa Persian Gulf Study Group, 1997; Proctor et al., 1998; Wolfe et al., 1998). However, prominent among complaints reported by a

high percentage of several samples of GW veterans are symptoms that suggest dysfunction in the central nervous system (CNS). These include memory loss, concentration problems, headaches, and fatigue. Freya Kame et al (2005) analyzed cross-sectional data from 18,782 white male licensed private pesticide applicators enrolled in the Agricultural Health Study in 1993–1997. Applicators provided information on lifetime pesticide use and 23 neurologic symptoms typically associated with pesticide intoxication. Among chemical classes of insecticides, associations were strongest for organophosphates and organochlorines. Associations with cumulative exposure persisted after excluding individuals who had a history of pesticide poisoning or had experienced an event involving high personal pesticide exposure. These results suggest that self-reported neurologic symptoms are associated with cumulative exposure to moderate levels of fumigants and organophosphate and organochlorine insecticides, regardless of recent exposure or history of poisoning.

2. Behavioural studies in animals

Chronic exposure of rats to one tenth of the LC50 of sarin for 30 days induced a decrease in M1 receptors in the olfactory tubercle, changes in blood and brain ChE activities and the expression of cytokines mRNA levels (Henderson et al., 2002). Guinea pigs receiving 0.3, 0.4 or 0.5×LD50 of repeated sarin injections exhibited disrupted sleep pattern in the EEG (Shih et al., 2006) and a decrease in red blood cell AChE to a low level of baseline. Obvious signs of cholinergic toxicity were observed only in animals receiving sarin. Experiments involving the application of multiple low-doses of soman induced alterations in long-term potentiation (Armstrong et al., 1997). We also reported dichlorvos administration caused a marked decrease in both the ambulatory and stereotypic components of spontaneous locomotor activity of rats. The muscle strength and coordination of the dichlorvos-treated animals was also significantly impaired. Besides, a marked deterioration in the memory function assessed in terms of the conditioned avoidance response was discernible at the end of the treatment schedule in the experimental animals (Sarin and Gill KD, 1998). In a series of experiments Gralewicz and Soćko (1997) have demonstrated that exposures to 2-chloro-1-(2,4-dichlorophenyl) vinyl diethyl phosphate (CVP) in rabbit resulted in a similar inhibition of blood AChE activity but the effect of the second exposure on body temperature and hippocampal EEG was smaller and less consistent than that of the first one. This would indicate that some permanent changes within the CNS may occur even after a single exposure to CVP. They also studied the CVP exposure in rat. One injection/day for ten days at a symptomatic (3.0 mg/kg) dose inhibiting blood and brain AChE activity by about 80%, the tolerance to CVP, assessed from the spontaneous locomotor behaviour, developed within four to five days. However, single exposure to CVP at a symptomatic (3.0 mg/kg) or subsymptomatic (1.0 mg/kg, less than 50% AChE inhibition) dose, or repeated exposure (one injection/day, for ten days) at subsymptomatic doses (1.0 mg/kg or 0.5 mg/kg) resulted in subtle changes in complex behaviours detectable after AChE activity in blood and in the brain had returned to the normal level. The changes neophobia in the open field, an increased and more persistent emotional response to a stressful stimulus, and increased EEG arousal response to an external pain signalling stimulus suggest an increased reactivity of the system or systems responsible for the induction of fear. Direct intrahypothalamic injections of CVP, unlike those of oxotremorine, a direct stimulant of cholinergic muscarinic receptors, did not induce overt changes in the animal (rabbit) behaviour and EEG. This would indicate that the changes in the CNS functions after CVP exposure may be the

consequence of increased cholinergic activity due to AChE inhibition rather than to a direct stimulation of cholinergic muscarinic receptors by CVP. The above findings provide experimental evidence that health effects of exposure to CVP, may persist after recovery of AChE activity in blood and in the brain. (Gralewicz and Soćko ,1997).

Alvin et al (2007) have demonstrated that rats when injected with CPF subcutaneously (dose range, 2.5-18.0 mg/kg) every other day over the course of 30 days, and then given a two week, CPF-free washout period, dose dependent decrements in a water maze hidden platform task and a prepulse inhibition procedure were observed during the washout period, without significant effects on open field activity, rotarod performance, grip strength, or a spontaneous novel object recognition task. After washout, levels of CPF and its metabolite 3,5,6-trichloro-2-pyridinol (TCP) were minimal in plasma and brain, however, cholinesterase inhibition was still detectible. Further, the 18.0 mg/kg dose of CPF was associated with (brain region-dependent) decreases in nerve growth factor receptors and cholinergic proteins including the vesicular acetylcholine transporter, the high affinity choline transporter, and the nicotinic acetylcholine receptor. These deficits were accompanied by decrease in anterograde and retrograde axonal transport measured in sciatic nerves exvivo. Thus, low-level (intermittent) exposure to CPF has persistent effects on neurotrophin receptors and cholinergic proteins, possibly through inhibition of fast axonal transport. Such neurochemical changes may lead to deficits in information processing and cognitive function. We report that (Binkumar et al, 2010), chronic OP (dichlorvos) exposure (2.50 mg/kg b.wt.s.c/daily for 12 weeks) can also caused nigrostriatal dopaminergic degeneration. The degenerative changes were accompanied by a loss of 60-80% of the nigral dopamine neurons and 60-70% reduction in striatal dopamine and tyrosine hydroxylase levels. Dichlorvos exposed animals also showed α-synuclein and ubiquitin positive inclusions along with swollen, dystrophic neurites and mitochondrial abnormalities like decreased complex I&IV activities, increased mitochondrial size, axonal degeneration and presence of electron dense perinuclear cytoplasmic inclusions in the substantia nigra of rats. These animals also showed evidence of oxidative stress, including increased mitochondrial ROS levels, decreased MnSOD activity and increased lipid peroxidation. Measurable impairments in neurobehavioral indices were also observed. Notable exacerbations in motor impairments, open field and catalepsy were also evident in dichlorvos exposed animals. All these findings taken together indicate that chronic dichlorvos (OP) exposure may cause nigrostaital neurodegenaration and significant behavioral impairments. Phenytoin (PHT) exposure in utero of rats demonstrate abnormal circling, decreased learning, hyperactivity, and delayed air righting reflex development. The effects of prenatal PHT on offspring learning have been found on multiple-T mazes and on spatial navigation (Morris maze). PHT-exposed offspring showed increased preweaning mortality, growth reduction, and abnormal circling. PHT noncircling offspring demonstrated impaired reference memory-based spatial learning (acquisition and reversal), but no other effects. By contrast, PHT circling offspring demonstrated not only impaired reference memory-based spatial learning, but also impaired cued platform learning, impaired spatial discrimination, and impaired working memory-based learning. These data confirm that prenatal PHT induces a specific reference memory-based spatial learning deficit even in asymptomatic (noncircling) offspring that is distinct from the impairment induced in littermates exhibiting the circling impairment. Recently we reported that chronic OP exposure (dichlorvos) may lead to significant increase in mitochondrial Ca2+ uptake.

Our results also indicated decreased mitochondrial electron transfer activities of cytochrome oxidase (complex IV) along with altered mitochondrial complex I, and complex II activity, which might have resulted from elevated mitochondrial calcium uptake. The alterations in the mitochondrial calcium uptake and mitochondrial electron transfer enzyme activities in turn might have caused an increase in malondialdehyde, protein carbonyl and 8-hydoxydeoxyguanosine formation as a result of enhanced lipid peroxidation, and as well as protein and mtDNA oxidation. All this could have been because of enhanced oxidative stress, decreased GSH levels and also decreased Mn-SOD activity in the mitochondria isolated from dichlorvos treated rat brain. Thus, chronic organophosphate exposure has the potential to disrupt cellular antioxidant defense system which in turn triggers the release of cytochrome c from mitochondria to cytosol as well as caspase-3 activation in dichlorvos treated rat brain. Low-level long-term organophosphate exposure finally resulted in oligonucleosomal DNA fragmentation, a hallmark of apoptosis. These studies provide an evidence of impaired mitochondrial bioenergetics and apoptotic neuronal degeneration after chronic low-level exposure to OPs that affect the behavioural impairement (Kaur et al., 2007). OPs can also modulate intracellular signaling pathways downstream of receptors and suggests that the diverse neurotoxic effects of many Ops may reflect their influence on multiple intracellular signaling pathways. Functional studies examining the effects of OPs on signaling events downstream of muscarinic receptor activation further support the hypothesis that OPs can interact directly with M2 receptors (Verma et al., 2008). A comparative study of paraoxon, malaoxon, and chlorpyrifos oxon in slice cultures of rat frontal cortex indicated that all three OPs inhibited cAMP formation in a concentration dependent manner (Ward and Mundy., 1996). Chlorpyrifos-oxon was also found to inhibit c-AMP synthesis in striatal dissociated cells (Huff et al., 1994).

Numerous studies have indicated that CREB is critical to several forms of use-dependent synaptic plasticity and transcription-dependent forms of memory, and evidence supports a major role for CREB in cell survival and differentiation during brain development. Since impairments of brain development and memory function are two primary neurological effects observed in laboratory studies with OPs, Schuh et al., (2002) hypothesized that the mechanisms underlying these effects may include alteration of the expression or activational status of CREB. Verma et al.,(2008) reported dichlorvos at low dose exposure, leads to reduction in the signal transduction cascade linked to receptor subtypes and adenylyl cyclase-linked signaling pathway was impaired. Finally, the phosphorylation of CREB, was significantly reduced in both low dose and high dose group animals. These reveal the significance of M2 muscarinic receptor linked adenylyl cyclase signaling pathway and phosphorylation of CREB in the development of neurobehavioral impairments after chronic low-level exposure to dichlorvos.

3. Developmental toxicity

Although some organophosphates are undergoing increasing scrutiny and restriction (U.S. Environmental Protection Agency (EPA) 2000, 2002) because of their propensity to elicit developmental neurotoxicity (Casida and Quistad 2004; Landrigan 2001; Slotkin 2004), these compounds nevertheless still comprise 50% of all insecticide use worldwide, and exposure of the human population continues to be nearly ubiquitous (Casida and Quistad 2004). Originally, it was thought that the adverse effects on brain development reflected the same

basic mechanism that underlies systemic toxicity, namely, cholinesterase inhibition and consequent cholinergic hyperstimulation (Pope 1999). However, evidence accumulating over the past decade implicates a host of other mechanisms that depend instead upon the direct targeting of events specific to the developing brain (Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 2004). Levels of pesticides detected in amniotic fluid demonstrate that the foetus has direct exposure to at least some pesticides during development (Bradman 2003). Chlorpyrifos, the most-studied organophosphate, has been shown to disrupt the basic cellular machinery that controls the patterns of neural cell maturation and the formation and activity of synapses, exclusive of the effects on cholinesterase, which are mediated instead by its metabolite, chlorpyrifos oxon (Barone et al. 2000; Casida and Quistad 2004; Gupta 2004; Pope 1999; Qiao et al. 2002, 2003; Yanai et al. 2002). These mechanisms are likely to be shared by other organophosphates, but these have not been evaluated in detail (Abu-Qare and Abou-Donia 2001; Pope 1999; Qiao et al. 2001; Slotkin 1999, 2004; Whyatt et al. 2002). Chlorpyrifos exposure during the perinatal period is known to evoke deficits in neuritic outgrowth, specifically including the targeting of cholinergic projections (Howard et al. 2005; Qiao et al. 2002, 2003; Slotkin et al. 2001). Nevertheless, (Dam et al.1999), as early as 1 day after neonatal chlorpyrifos exposure, there is a shortfall in ChAT, the constitutive marker of cholinergic projections (Dam et al.1999). The initial deficits in the development of cholinergic projections lead to the subsequent emergence of abnormalities of cholinergic innervation. Substantial deficits in cholinergic synaptic activity, and related behavioral anomalies in adolescence and adulthood (2001; Slotkin 1999,2001, 2004; Slotkin et al. 2001).

Young animals are far more susceptible than adults to organophosphate-induced growth inhibition and lethality, there is a wide range over which disparate compounds elicit such effects. For example, parathion is far more systemically toxic to newborn rats than is chlorpyrifos, in part reflecting pharmacokinetic differences centering around the ontogeny of enzymes activating the parent compounds to the corresponding oxons, compared with the enzymes that break down the oxons to inactive metabolites (Atterberry et al. 1997; Padilla et al. 2000, 2004). The maximum tolerated doses of each agent correspond closely to the relative potencies toward cholinesterase inhibition and to the rate of recovery of cholinesterase activity, thus drawing a direct mechanistic connection of cholinergic hyperstimulation to overall systemic toxicity (Pope and Chakraborti 1992; Pope et al. 1991; Tang et al. 2003). In contrast, in vitro evaluations that bypass the pharmacokinetic differences suggest that chlorpyrifos is more potent toward inhibition of cell membrane function (Barber et al. 2001) and for eliciting cytotoxicity in immature neurons and glia (Monnet-Tschudi et al. 2000), despite the fact that parathion elicits greater cholinesterase inhibition (Zurich et al. 2000); indeed, physostigmine, a nonorganophosphate cholinesterase inhibitor, is far less effective in disrupting neural cell development in vitro, even at concentrations that completely block cholinesterase (Qiao et al. 2001). Theodore et al, (2006) studied the neuritic outgrowth and cholinergic synaptic development in neonatal rats. They have given different organophosphates (chlorpyrifos, diazinon, parathion) at doses spanning the threshold for impaired growth and viability. The result indicated that Parathion (maximum tolerated dose, 0.1 mg/kg) was far more systemically toxic than was chlorpyrifos or diazinon (maximum tolerated dose, 1–5 mg/kg). Below the maximum tolerated dose, diazinon impaired neuritic outgrowth in the forebrain and brainstem, evidenced by a deficit in the ratio of membrane protein to total protein. Diazinon also

decreased choline acetyltransferase activity, whereas it did not affect hemicholinium-3 binding to the presynaptic choline transporter, an index of cholinergic neuronal activity. These results indicate a complete dichotomy between the systemic toxicity of organophosphates and their propensity to elicit developmental neurotoxicity.
Brenda Eskenazi et al (2007) investigated the relationship of prenatal and child OP urinary metabolite levels with children's neurodevelopment. Their result indicated, dialkylphosphate (DAP) levels were negatively associated with Mental Development (MDI), but child measures were positively associated. At 24 months of age, these associations reached statistical significance. Neither prenatal nor child DAPs were associated with Child Behavior Checklist (CBCL) attention problems, but both prenatal and postnatal DAPs were associated with risk of pervasive developmental disorder. Their report revealed adverse associations of prenatal DAPs with mental development and pervasive developmental problems at 24 months of age. Results should be interpreted with caution given the observed positive relationship with postnatal DAPs. Raul Harari et al (2010), studied Northern Ecuador population, where floriculture is intensive and relies on female employment, they carried out an intensive cross-sectional study to assess children's neurobehavioral functions at 6–8 years of age. They examined all 87 children attending two grades in the local public school with an expanded battery of neurobehavioral tests. Information on pesticide exposure during the index pregnancy was obtained from maternal interview. The children's current pesticide exposure was assessed from the urinary excretion of organophosphate metabolites and erythrocyte acetylcholine esterase activity. Their findings support the notion that prenatal exposure to pesticides at levels not producing adverse health outcomes in the mother can cause lasting adverse effects on brain development in children. Pesticide exposure therefore may contribute to a "silent pandemic" of developmental neurotoxicity (Raul Harari et al ,2010).

4. Role of paraoxonase in OP detoxication

In 1946, Abraham Mazur was the first to report the presence of an enzyme in animal tissue which was able to hydrolyse organophosphate compounds (Mazur,1946). This led to the initial identification of the human serum paraoxonase (PON1) enzyme in the early 1950s (Aldridge,1951a, Aldridge,1951b). PON1 was named after its ability to hydrolyse the organophosphate substrate paraoxon (paraoxonase activity, EC 3.1.8.1), which is the toxic metabolite of the insecticide parathion. Because PON1 could also hydrolyse aromatic esters, such as phenylacetate (arylesterase activity, EC 3.1.1.2), the term 'A-esterase' was introduced for the enzyme hydrolysing both compounds. This led to much discussion during the following years as to whether one enzyme or two were responsible for the paraoxonase and arylesterase activity,(La Du ,2002) but finally, conclusive evidence was delivered that both paraoxonase activity and arylesterase activity were properties of PON1. (Sorenson 1995) When Mackness and colleagues demonstrated that PON1 could prevent the accumulation of lipoperoxides in low-density lipoprotein (LDL) (Mackness,1991) thus linking PON1 to cardiovascular disease, the scientific interest in PON1 increased immensely. Despite the boom in research, to date the exact physiological function of PON1 is still unclear.
PON1 belongs to a family of serum paraoxonases, consisting of PON1, PON2 and PON3. The genes coding for these enzymes are all located next to each other on the long arm of chromosome (Primo-Parmo ,1996) (7q21.3-q22.1)7. PON1 and PON3 are expressed in the liver and excreted in the blood where they are associated with the high-density lipoprotein

(HDL) particle (Reddy, 2001). PON2 is not present in blood, but is expressed widely in a number of tissues, including the liver, lungs, brain and heart (Mochizuki 1998). Of the paraoxonase family, PON1 is the most investigated and best understood member. While it was assumed that high levels of PON1 would protect against exposure to specific OP compounds, only a single experiment that directly addressed this question had been reported prior to 1990. Main (1956) reported that injection of partially purified PON1 into rats increased their resistance to paraoxon. This observation was confirmed and extended through a series of experiments begun in costa et al laboratory in 1990. Injection of purified rabbit paraoxonase into rats increased their resistance to paraoxon exposure (Costa et al., 1990). Injection of purified rabbit PON1 into mice 4 h prior to exposure dramatically increased their resistance to chlorpyrifos oxon (Li et al., 1993). An increase in resistance to the parent compound, chlorpyrifos, was also observed (Li et al., 1995). These experiments demonstrated clearly that high levels of plasma paraoxonase could protect against exposure to chlorpyrifos oxon or chlorpyrifos. Protection was also observed when purified rabbit PON1 was injected post-exposure or 24 h prior to exposure, indicating that administration of purified or recombinant PON1 would be useful for ameliorating or even preventing adverse consequences of exposure to OP compounds. Whereas higher PON1 levels were demonstrated clearly to be protective, determining whether low levels of PON1 would result in greater sensitivity was not possible until the development of PON1knockout mice, generated by Drs. Jake Lusis, Diana Shih and co-workers (Shih et al., 1998). Knocking out the mouse PON1 gene resulted in a dramatic increase in sensitivity to chlorpyrifos oxon exposure and a modest increase in sensitivity to chlorpyrifos exposure, as assessed by measuring brain cholinesterase inhibition. Dermal exposures to levels of chlorpyrifos oxon that produced no symptoms of cholinergic effects and minimal inhibition of brain cholinesterase in wild-type mice were unexpectedly lethal to the PON1 null mice. Similar results were observed when the knockout mice were exposed to diazoxon (Li et al., 2000). Dermal exposure to 2 or 4 mg/kg diazoxon produced no measurable effect in wild-type mice, but was lethal to the PON1 knockout mice, and exposure to 1 mg/kg diazoxon had significant adverse effects in the knockout mice without measurably affecting the wild-type mice. Hemizygous mice, with only one PON1 allele, exhibited intermediate sensitivity. Exposure of the PON1 knockout mice to paraoxon, however, produced an unexpected and initially puzzling result. They were not anymore sensitive than wild-type mice to paraoxon exposure. Further experiments demonstrated that resistance of the PON1 knockout mice to diazoxon was restored by injection of purified PON1R192 or PONQ192 alloforms, with either alloform providing equivalent protection (Li et al., 2000). Resistance to chlorpyrifos oxon was also restored; however, the PON1R192 alloform provided significantly better protection that did the PON1Q192 alloform. Neither alloform provided protection against paraoxon exposure. While there was some protection afforded by PON1 against the respective parent compounds, the protective effects of PON1 were most striking with the oxonforms of chlorpyrifos and diazinon. The parent OP compound is converted to its more toxic oxon form in the liver, by cytochrome P450-mediated oxidative desulfuration, and the oxon form serves as the direct substrate for PON1. Since chlorpyrifos oxon inhibits acetylcholinesterase at least 1000 times more rapidly than chlorpyrifos (Huff et al., 1994), even a small percentage of oxon content is important with respect to an individual's PON1 status

Multiple investigators have examined the potential role of polymorphisms in veterans with unexplained illness, but the results have been mixed (Haley et al1999). Haley et al (1999)

reported that the most severely symptomatic GW veterans exhibited particularly low activity of paraoxonase (PON1) type Q, the type that would be most active in neutralizing nerve gases. Mackness et al, (2000) found that veteran's decreased capacity to metabolize OP chemicals might have contributed to their likelihood of developing GW illness. Hotoph et al (2003) found that PON1 activity, which is a major determinant of OP toxicity in human, was significantly decreased in British veterans deployed to the GW compared to nondeployed veterans. The PON1 gene presents several polymorphisms in the coding and promoter regions that affect the catalytic efficiency of the enzyme toward different substrates (the Q192R polymorphism) and its level of expression (e.g., the C-108T polymorphism). Extensive research in transgenic animal models clearly indicates that PON1 ''status'', encompassing both the Q192R polymorphism and the level of PON activity, plays a most relevant role in modulating the acute toxicity of some, but not all OPs. The important determinant is the catalytic efficiency of each PON1 allozyme toward a specific substrate; thus, in case of chlorpyrifos oxon, PON1 provides protection in vivo, and PON1R192 provides better protection than PON1Q192; in case of diazoxon, both alloforms provide the same degree of protection, while in case of paraoxon, the substrate after which the enzyme was named, PON1 does not provide any protection due to an overall low catalytic efficiency of PON1 toward this substrate. These studies in transgenic mice provide a convincing case of extrapolating the results obtained in animals to humans; however, direct and conclusive confirmation of the relevance of PON1 status in determining relative susceptibility to OP toxicity is still lacking.

5. Conclusions

This chapter covers the background information and the epidemiological evidence, on exposures of people and different experimental animals to pesticide and the consequences in regard to the neurodegeneration, neurodevelopment and neurobehavioral impairments. The fact that different studies provide equivocal results on the functions affected, together with the fact that the observed changes are usually very high, and do appear to be correlated with overt nervous system impairment, suggest that the neurobehavioural impact of low level prolonged exposure to pesticides. In addition to that pesticide exposure also affects the offspring, and consistent neurobehavioral impairments were also reported. The evidence suffers from a variety of shortcomings and sources of imprecision. These problems would tend to cause an underestimation of the true extent of the risks. The overall experimental and epidemiological evidence suggests that the substantial vulnerability of the mature and developing nervous system to low concentrations of pesticides should lead to a strengthened emphasis on protection of workers and general people who handle the pesticides that may cause harm to the nervous system. For both toxicologic and epidemiologic reasons, it is essential that the neurobehavioral potential of low-level, chronic exposure to pesticides and pesticide mixtures be ascertained. The available evidence suggests there is a high probability for subtle adverse health effects. Workers exposed to pesticides are one of the largest occupational risk groups in the world. The effects of these occupational exposures on worker's nervous systems and behaviour are just beginning to be studied. A precautionary principle in regard to neuronal toxicity should be applied in occupational health, and this issue should also attract more research, preferably with a focus on exposure assessment and valid outcome measures in prospective study designs.

PON1 status plays an important role in protecting against exposures to diazinon and chlorpyrifos, particularly to the oxon residues present in these exposures. The most important conclusion to come from studies is that to understand the role that PON1 plays in an individual's sensitivity or resistance to a given exposure or in the pharmacokinetic disposition of a specific drug, it is important to know both the levels of PON1 and it's genetic polymorphism. This too is expected to be a fruitful area of future research. In conclusion, we found that prevalence of neurologic symptoms was associated with exposure to pesticides. These associations were present in individuals with no history of pesticide poisoning or high exposure events and were independent of recent exposure. Thus, they are likely due to chronic moderate exposure. Although the neurotoxicity of high-level exposure is accepted, more attention to the risks associated with moderate low level exposure may be required. Research is needed to improve our understanding of the mechanisms involved and help in identifying the best means of protecting future generations against a silent pandemic of neurotoxicity.

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2

Dermal Exposure to Sub-Toxic Amount of Chlorpyrifos - Is It Neurotoxic?

Nilesh Kumar Mitra
International Medical University, Kuala lumpur, Malaysia

1. Introduction

Chlorpyrifos (O, O-diethyl-3, 5, 6-trichloro-2-pyridyl phosphorothioate) is an organophosphate (OP) pesticide widely used across the globe for the last 4 decades. It was registered in the United States as early as 1965. Although chlorpyrifos (CPF) has been principally used as pesticide in agriculture sector, its domestic use is found to be extensive in home-gardens as well as indoors to get rid of cockroaches, fleas, spiders and flies (Lemus & Abdelghani, 2000). It is also smeared on the body surface of the sheep and horse to eradicate lice and fleas as well as for the treatment of dog kennels. Farmers are exposed to CPF and other OP pesticides over their skin by direct contact as well as by inhalation during preparation of the spray solutions, loading of spray tanks and application of the pesticides. Acute exposure to CPF by dermal, oral and inhalation route was moderately toxic and US Environmental Protection Agency categorized it as a class II toxin (Eisler, 2000). All OP insecticides act by inhibiting the enzyme acetylcholinesterase (AChE), and thereby increase the levels of acetylcholine in the synapses. Excessive stimulation of the cholinergic post-synaptic receptors leads to cholinergic toxicity. Acute poisoning produced by accidental ingestion or inhalation of OP pesticides like chlorpyrifos causes non-lethal symptoms like nausea, vomiting, abdominal cramps, diarrhoea, excessive salivation and headache. Such poisoning may also give rise to blurred vision, muscle twitches, difficulty in breathing, random jerky movements and convulsion. Symptoms usually occur within hours of exposure and with new AChE being synthesized, after few weeks the symptoms of cholinergic toxicity disappear.

Apart from the acute cholinergic toxicity affecting the central nervous system, organophosphate pesticides also affect specific areas of the brain. These areas include the parts of the cerebral cortex which is responsible for cognition and short term-memory. Three well-designed epidemiological studies examined the patients previously poisoned by OP pesticides several years after hospitalization and found deficits in cognitive tests without any neurological abnormality. One study included 100 patients admitted to the hospital and followed nine years after the poisoning. Comparison was done with matched controls (Savage et al., 1988). Significant deficit in several cognitive tests of memory and abstraction was found among the pesticide affected patients. But neurological physical examination and electroencephalographic examination were inconclusive. A second study (Rosenstock et al., 1991 and McConnell et al., 1994) involved 36 men poisoned by OP pesticides (mainly methamidaphos). They were followed two years after hospital admission. Cognitive deficits

were observed in poisoned patients compared to the matched controls. These patients also showed significant decrease in vibrotactile sensitivity which was presumed to be an indicator of peripheral neuropathy. The third study (Steenland et al., 1994) also found deficit in sustained attention among OP pesticide affected people 7 years after the poisoning. This study involved 128 people poisoned with OP pesticides. OP pesticide induced neurotoxicity in the humans and other animals has been proposed to occur via three distinct actions: cholinergic neurotoxicity, organophosphorus ester-induced delayed neurotoxicity (OPIDN), and organophosphorus ester-induced chronic neurotoxicity (OPICN) (Abou-Donia, 2003).

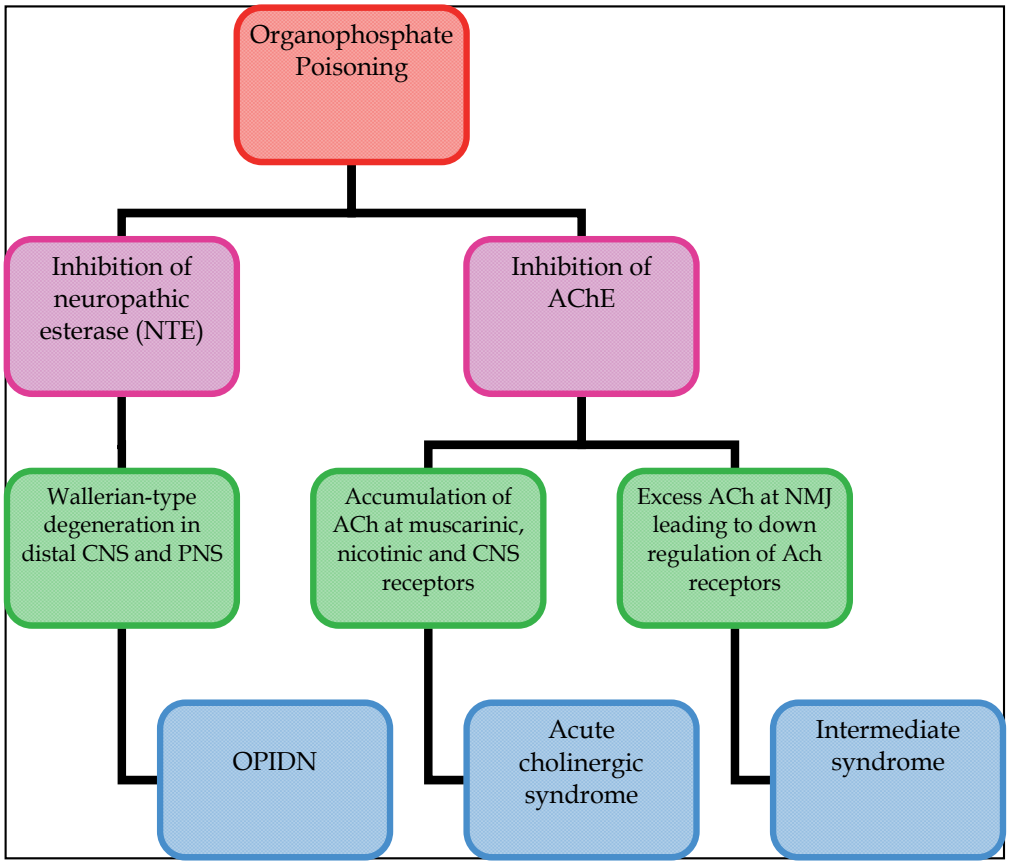


Fig. 1. Classification of organophosphate poisoning. ACh = Acetylcholine; CNS = Central nervous system; NMJ = Neuromuscular junction; OPIDN = Organophosphate-induced delayed neuropathy; PNS = Peripheral nervous system. Source: Jones & Karalliedde 2006, Davidson's Principles and Practice of Medicine, 20th edition

Although adverse effects of ingestion of CPF in sub-toxic doses by oral and inhalation methods have been proven by many studies, the general perception prevails that dermal exposure to chlorpyrifos is not as significant or as dangerous as other routes of exposure. Hence dermal exposure has not been given enough attention by the farmers and pesticide industry workers particularly in developing countries. CPF is absorbed through the skin and absorption through the skin may result in systemic intoxication. CPF and its metabolites

have been suggested to establish a reservoir and accumulate in skin resulting in longer exposure duration and more adverse long-term effects. The intensity of absorption of CPF through the skin depends on the solvent used and is usually slower than the uptake by other routes. Single dermal application of CPF diluted in ethanol for 4 hours on human volunteers was found to cause absorption of 4.3% of the applied dose and CPF was retained by the skin with mean elimination half-life of 41 hrs (Meuling et al., 2004). Application of a sub-clinical single dermal dose of CPF, 30 mg/kg, on pregnant Sprague-Dawley rats inhibited maternal and foetal brain AChE activity within 24 hours of exposure (Abu-Qare et al., 2001). The dose of a toxic material that causes death in one-half of the test population, when it is given on a short-term basis is described as its lethal dose (LD50). An acute dermal toxicity study on rats using chlorpyrifos soluble in xylene found that acute dermal LD50 for male rats was 202 mg/kg (Gaines, 1969). CPF was considered moderately toxic by oral route with an oral LD_{50} of 223 mg/kg in rats. The acceptable daily intake (ADI) for CPF by oral route as pesticide residues in food was found to be 0.003 mg/kg/body weight (Barden, 2011, ADI list, Australia). ADI is the level of intake that can be ingested daily over the life time with no appreciable health risk. Cholinesterase activity in RBC and serum has been used as a method of surveillance or biological monitoring of exposure to OP pesticides, particularly for screening of workers exposed to OP pesticides. Dermal exposure to 10 mg/kg/day of CPF in rats was found to cause RBC cholinesterase inhibition of by 16% after 4 days of application. NOEL (No observed adverse effect level) dose of dermal CPF exposure was found to be 5mg/kg/day (Donovan, 2006). The neurobehvioural, neurochemical and neurohistological studies have been done using the animal models of dermal exposure to the mixtures of OP pesticides (Abdel-Rahman et al., 2001; Abdel-Rahman et al., 2004; Abou-Donia et al., 2004). However, the morphological effect of dermal exposure to sub-toxic dose of only chlorpyrifos, the widely used pesticide in the developing world, on the central nervous system has been studied by Lim KL, Tay A, Nadarajah VD and Mitra NK (2011). Although Mitra NK et al., (2008) and Mitra NK et al., (2009) studied the neurotoxic effect of dermal application of low dose chlorpyrifos in the hippocampus and neurotoxic effect of concurrent application of stress and dermal application of low dose chlorpyrifos in the hippocampus, the doses used were $1/5^{th}$ dermal LD50 and ½ dermal LD50 of chlorpyrifos. Lim et al., (2011) had used $1/5^{th}$ dermal LD50 and $1/10^{th}$ dermal LD50 of chlorpyrifos. The methodology, results and discussion of the study by Lim et al., (2011) have been incorporated in this chapter to explain the neurotoxic effect of dermal application of sub-toxic doses of chlorpyrifos.

2. Materials and methods

2.1 Dermal application of CPF and estimation of Cholinesterase (AChE)

Commercial preparations of CPF (O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate) manufactured in Kuala Lumpur, Malaysia was used in this study. This preparation contained 38.7% W/W CPF diluted in xylene. Male Swiss albino mice (species: ICR), 60 days old (30-32 g) were used in this study. They were housed in plastic cages (six in a cage) and were exposed to natural, twelve hourly light and dark sequence. Lab chow (pellet feed) and water were given ad libitum. Animal experiments adhered to the principles stated in the guide-book of laboratory animal care and user committee of the International

Medical University and in accordance with the declaration of Helsinki. The mice were divided into 3 groups (n = 6). The experiment was conducted in two phases (one with period of experiment for 7 days and another with period of experiment for 3 weeks).
Application of CPF on the tail skin of albino mice was done in the dose regimen of 1/5th dermal LD50 and 1/10th dermal LD50 for 7 days and 3weeks (Fig.2). Surgical gauze smeared with the CPF in xylene solution (1 ml) was wrapped around the tails and a barrier of aluminium foil was applied to prevent the solution from evaporation. Daily exposure was maintained for 6 hours which was similar to the daily dermal exposure time in the agricultural workers to the pesticides.

Fig. 2. Dermal application of CPF on the tail of the mouse under occlusive bandage.

A control group was maintained with exposure to dermal application of 1 ml of xylene solvent for similar duration. Amplex Red acetylcholinesterase assay kit, an ultrasensitive method for monitoring serum AChE concentration in a fluorescence microplate reader was used. The serum samples were collected at the end of 7 days and 3 weeks in two phases of the experiment. The mean serum AChE expressed as U/ml was subjected to one way ANOVA statistical analysis followed by Post hoc LSD test.

2.2 Qualitative and quantitative studies of neurons and the glial cells in the hippocampus

The sample of forebrains collected from the groups of treated mice at the end of 7th day (phase I) and at the end of 21st day (phase II) were fixed in 10% formal saline. Hippocampal area was trimmed off by making coronal section between the optic chiasma and the infundibulum. The portion of the brain was then divided into left and right lobes by a single saggital slice. This allowed the same mouse brain to be stained by two different stains**.** The brain tissues were processed and embedded in paraffin. The left half sections (8 micron) were stained with 0.2% thionin (Nissl stain) and used for qualitative and quantitative histomorphometric study of hippocampal neurons. Right half sections (4 micron) were used for immunohistochemical stains for Glial Fibrillary Acidic Protein (GFAP). For Nissl stain, every subsequent 10th section was collected. To obtain similar sections in the right lobe, every subsequent 27th section was collected. Every 10th paraffin section (5 slides in each

animal) stained with 0.2% thionin, containing hippocampal area, was chosen from each animal in groups of treated mice and a quantitative study of the normal looking neurons was done. The slides were examined and photographed under 400X magnification with the help of a photographic camera attached to the microscope. Selection of the hippocampal area for neuronal count was done by randomly choosing two areas of CA1, one area of CA2 and two areas of CA3 parts of the hippocampus observed in a section. Image-Pro Express software was used to count neurons with prominent nucleolus within a measured rectangular area in the selected regions. Random measurements of neuronal cell diameter were also taken for each region. The absolute neuronal density (P) per unit area of section was estimated using the formula P=A x M / L+M (Aberchrombie 1946); M= Section thickness in micron (8 micron); L = Mean nuclear diameter of respective area; A = Crude neuronal count per sq.mm of section. The astrocytes with processes were stained brown with the immunohistochemical stain for GFAP filaments, particularly in stratum lacunosum-moleculare of the mouse hippocampus. The numbers of astrocytes with prominent processes were counted within a measured rectangular area. Three such areas were randomly selected in the every 27th section (3 sections in each animal). Both the mean neuronal density quantified under Nissl stain and mean astrocytic density quantified under GFAP immuno-stain expressed as values per sq mm of section , were subjected to One way ANOVA statistical analysis followed by Post hoc Bonferroni test to find out inter-group difference.

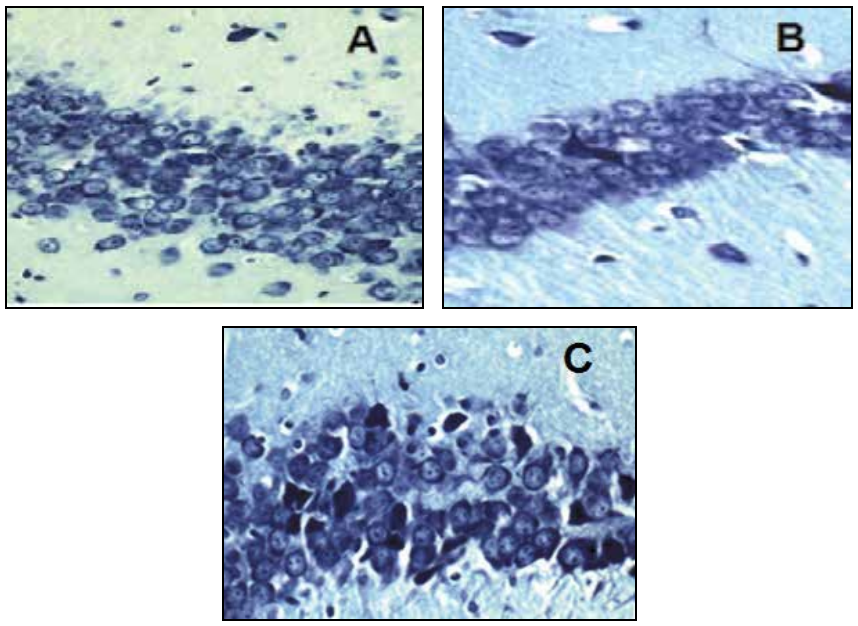
3. Results

3.1 Changes in serum cholinesterase following dermal application of CPF

Depletion of serum cholinesterase concentration in the mice group exposed to 1/5th LD50 of CPF for 3 weeks was 95.9% compared to the mean AChE level in the control group. The change was statistically significant ($p<0.05$, One way ANOVA, Post hoc LSD). On the other hand, dermal application of 1/5th LD50 of CPF for 1 week caused depletion of serum AChE by 80.2%. The change was also statistically significant ($p<0.05$). Dermal exposure to 1/10th LD50 of CPF for 3 weeks caused depletion of serum AChE by 88.3% compared to the control. The change was statistically significant ($p<0.05$). However when 1/10th LD50 of CPF was dermally applied for only 7 days, depletion of serum AChE was 30.5% and was not significant statistically.

3.2 Changes in the neuronal density of hippocampus following dermal application of CPF

The mean neuronal density per sq.mm of the section in histomorophometric study of the hippocampus was reduced by 24.7%, 18.4% and 22% compared to the control in CA1, CA2 and CA3 hippocampal areas in the group of mice exposed to 1/5th LD50 of CPF for 3 weeks. All the changes in the three areas were statistically significant ($p<0.001$, One way ANOVA, Post hoc Bonferroni). When the application was done for only 7 days, the reduction in mean neuronal density was 15.3%, 26% and 27% respectively in CA1, CA2 and CA3 hippocampal areas. The changes were statistically significant compared to the control ($p<0.05$) in CA1 and CA2 hippocampal areas. The reduction was most significant ($p<0.001$) in CA3 hippocampal area. Hence even with 7 days dermal exposure to 1/5th LD50 of CPF, the neurotoxicity in the hippocampal area was significant.



A. Control group showing prominent perinuclear nissl granules
B. Group applied with 1/10th dermal LD50 of CPF for 7 days, showing no apparent damage except few pyknosed neurons (dark coloured)
C. Group applied with 1/5th dermal LD50 of CPF for 7 days, showing many pyknosed neurons (dark coloured) [Nissl stain with Thionin, 400x, 8µ]

Fig. 3. Photomicrograph of hippocampal CA3 neurons in different groups of mice

Exposure to 1/10th LD50 of CPF for 7 days was however least toxic. It reduced the mean neuronal density by 7.6%, 13.6% and 21% in CA1, CA2 and CA3 hippocampal areas compared to the control. The change in CA3 area only was statistically significant ($p<0.05$). The observation indicated that CA3 area of the hippocampus was more susceptible to neuronal damage following dermal exposure to low dose of CPF for only 7 days (Fig. 3). Even when applied dermally for 3 weeks, the dose of 1/10 LD50 of CPF was found to be less neurotoxic. The mean neuronal density was reduced by 9%, 11% and 9.6% in CA1, CA2 and CA3 hippocampal areas in the group receiving dermal application of 1/10 LD50 of CPF for 3 weeks. One way ANOVA did not show any significant difference in the mean neuronal density in the three areas in this experimental group.

3.3 Changes in the astrocytic density in the hippocampus following dermal application of CPF

Examination of the photomicrographs revealed that following one week of application, longer and more numerous astrocytic processes were observed in the group exposed to 1/5th LD50 of CPF compared to the group exposed to 1/10th LD50 of CPF(Fig. 4) in stratum lacunosum-moleculare and stratum oriens of the hippocampus. Quantitative study showed that the mean astrocytic density per sq. mm of the section was raised in all groups receiving dermal applications of CPF for 7 days. An increase of 37.2% in mean astrocytic density was observed in the group exposed to 1/10th LD50 of CPF compared to the control, while an increase of 41% was seen in the group exposed to 1/5 LD50 of CPF. Both the changes in the

1/10th LD50 of CPF group and the 1/5 LD50 of CPF group were statistically significant ($p<0.001$, One way ANOVA, Post hoc Bonferroni). Compared to the application of CPF for 7 days, application for 3 weeks did not produce prominent visible changes in the expression of GFAP. The mean astrocytic density was increased by 9% in the mice group receiving dermal application of 1/10th LD50 of CPF for 3 weeks compared to the control. In the group receiving dermal application of 1/5th LD50 of CPF, the density of astrocytes was raised by 9.5%. One way ANOVA test did not show any significant inter-group difference in the mean astrocytic density between the control group, 1/10th LD50 of CPF group and 1/5th LD50 of CPF group in the phase II experiment (3 weeks).

7 days application	CA1	CA2	CA3
Control	881.8 (146)	710.5 (146)	640.7 (75)
CPF 1/10 LD50	814.7 (158)	613.8 (125)	504.3* (116)
CPF 1/5 LD50	768.7# (201)	578.7# (103)	483.3# (167)
3 weeks application			
Control	1098.3 (116)	642.7 (72)	639.1 (67)
CPF 1/10 LD50	998.4 (72)	571.8 (70)	577.7 (85)
CPF 1/5 LD50	826.8# (108)	524.1# (77)	496.9# (40)

Table 1. Mean (S.D) neuronal density per sq.mm of the section in different treatment groups in three hippocampal areas. # Significantly reduced in CPF 1/5 LD50 groups compared to the control group ($p<0.05$, One way ANOVA Post hoc Bonferroni) ; *Significantly reduced in CPF 1/10 LD50 groups compared to the control group ($p<0.05$, One way ANOVA Post hoc Bonferroni).

	7 days application	3 weeks application
Control	256.9 (54)	317.4 (75)
CPF 1/10 LD50	352.6# (99)	347.2 (70)
CPF 1/5 LD50	362.5# (96)	347.6 (84)

Table 2. Mean (S.D) astrocytic density per sq.mm of the section in different treatment groups stratum lacunosum-moleculare of the hippocampus. # indicates significant increase compared to the control group ($p<0.05$, One way ANOVA Post hoc Bonferroni).

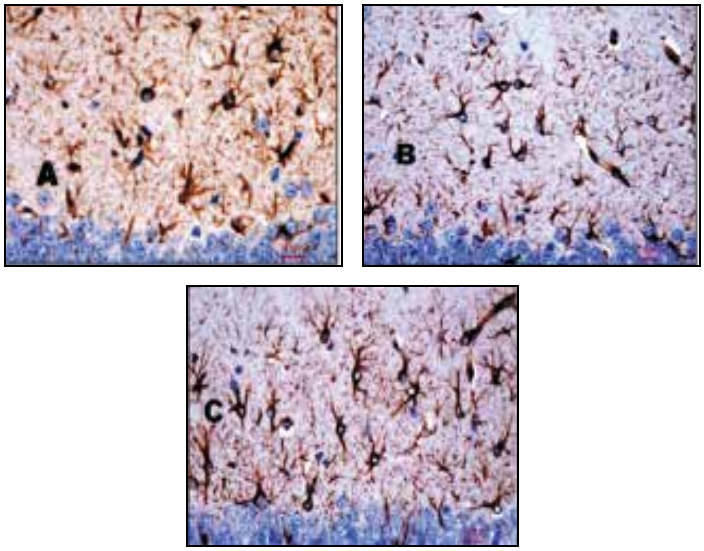


Fig. 4. Photomicrograph showing the immunohistochemical staining of GFAP expression in stratum moleculare-lacunosum of the hippocampus in groups of mice at the end of 7 days of experiment. Brown colour rounded cells with processes are the astrocytes. A-Control group; B- 1/10th LD50 of CPF group; C- 1/5th LD50 of CPF group (400X, GFAP stain, 4µ)

4. Discussion

Somewhat similar to this study, Latuszyńska et al.,(2001) used dermal application of 1/70 dermal LD50 of CPF along with 0.5 mg/ cm^2 surface area of cypermethrin and plasma cholinesterase was reduced by 81%. The reduction of plasma cholinesterase was 92% when 1/14th dermal LD50 of CPF in combination with cypermethrin 2.7 mg/cm2 was applied dermally for 1 week. This study found depletion of serum AChE by 30.5% only when 1/10 dermal LD50 of CPF was applied for 7 days. Serum AChE enzyme is produced in the liver and it is a reliable measure for detecting acute OP toxicity. Among general population, approximately 2 to 3% has a genetic variation of serum cholinesterase deficiency. Acute or chronic inflammatory conditions, malnutrition, liver disease and physiological condition like pregnancy can produce AChE deficiency. The depletion of AChE in these conditions is not as severe as observed following exposure to OP pesticides. Measuring cholinesterase activity in RBC is used as a method of surveillance for detection of exposure to OP pesticides. This is applied mainly to the workers working in pesticide industry. The role of AChE is to terminate impulse transmission at the cholinergic synapses within the nervous system by hydrolyzing acetylcholine (ACh) into choline and acetate allowing recycling of hydrolysed substrates into new neurotransmitter (Rang et al., 2001). As a consequence of the inhibition of AChE, ACh accumulation occurs at the synapses. ACh is found throughout the CNS and it is present in relatively higher concentration in the cerebral cortex, thalamus and various nuclei of the basal forebrain. As a neuromodulator, ACh has multiple effects on the CNS. Through synaptic plasticity, it plays a prominent role in learning involving the neo-cortex and hippocampus. ACh has been found to enhance the amplitude of synaptic potentials following long term potentiation in dentate gyrus, CA1 hippocampus, pyriform

cortex and neocortex. It most likely acts either by suppressing or by enhancing the current via NMDA (N-methyl-D-aspartic acid) receptors (Yu and Dayan, 2005).

In the present study on dermal application of CPF, evidence of neuronal damage was found in the stratum pyramidalis of the hippocampus. Qualitative observations of the hippocampal neurons in this study showed that following seven days of low dose CPF application (1/10th dermal LD50), no apparent damage to the neurons was visible. However at the higher dose (1/5th dermal LD50), seven days of application resulted in visible damage in the form of pyknosis. Dendritic morphology was assessed in the prefrontal cortex, CA1 area of the hippocampus and the nucleus accumbens following repeated (14 days) low dose intraperitoneal application of OP malathion (40 mg/kg BW) in mice. Dendritic length in the hippocampus and prefrontal cortex, and density of dendritic spines in all the three areas assessed were reduced (Campana et al., 2008). As part of the trisynaptic circuit, afferent inputs to the hippocampus are first sent to the dentate gyrus, which then projects to the CA3 area. The CA3 neurons then send projections to CA1. Dendrites of CA1 neurons project to the subiculum and then back to the entorhinal cortex. CA3 being an early structure in this circuit, it is the first part of the hippocampus to be affected by cholinergic overactivity. This could explain the significant ($p<0.05$) neuronal reduction observed only in CA3 hippocampal area after application of low dose CPF (1/10th dermal LD50) for seven days. This also indicated that CA3 area of the hippocampus was more susceptible to neuronal damage following dermal exposure to low dose of CPF. Agricultural workers chronically exposed to low-levels of CPF and other pesticides were found performing poorly on neurobehavioral tests (Rothlein et al., 2006). These functional deficits can be extrapolated to be caused by prolonged exposure to low dose CPF.

Following one week of CPF application at both doses (1/10th and 1/5th dermal LD50), GFAP expression as measured by astrocytic density was significantly increased compared to the control group. GFAP expression has been found to be increased following toxic insult to the CNS in many studies. A single subcutaneous injection (50 µg/kg bw, 1/2 LD50) of the cholinesterase inhibitor Sarin was found to significantly increase GFAP levels in the cerebral cortex by 269% after one hour, and to 318% after two (Damodaran et al., 2002). Qualitative examination showed that following seven days of CPF application, GFAP expression in the astrocytes was more prominent compared to the control groups. The astrocytic processes of the groups receiving CPF were longer, and greater in number. This may be attributed to the neuroprotective effect of astrocytes limiting neuronal damage. It has been suggested that the metabolites of CPF, trichloropyridinol (TCP), exert strong toxic effects on astrocytes, compromising their neuroprotective effects and thus increasing the neurotoxicity of CPF (Zurich et al., 2004). The neuroprotective effects of astrocytes have been suggested in many studies. To assess the influence of glial cells on the neurotoxicity of OPs, aggregate brain cell cultures of foetal rat telencephalon were treated with CPF and parathion for 10 days. The study by Zurich et al., found that the neurotoxicity of CPF and parathion was increased in aggregate cultures deficient in glial cells.

This study observed both neuronal damage as well as GFAP expression following low dose dermal application of CPF. It was observed that with increasing neuronal damage, GFAP expression was more and the mean astrocytic density was increased. Exposure to $1/10^{th}$ LD50 of CPF for 7 days reduced the mean neuronal density by 7.6%, 13.6% and 21% in CA1, CA2 and CA3 hippocampal areas. This group showed 37.2% increase in mean astrocytic density in stratum lacunosum-moleculare compared to the control group. In contrast, when

1/10th LD50 of CPF was applied for 21 days, a low level of neurotoxicity was produced reducing mean neuronal density by 9%, 11% and 9.6% in CA1, CA2 and CA3 hippocampal areas. This low level of neurotoxicity produced a moderate glial reaction as evidenced by 9% increase in astrocytic density compared to the control group which was not found to be statistically significant. The findings of this current study support previous suggestions that astrocytes provide neuroprotection. Although CA3 hippocampal area was found to be most susceptible out of the three main Cornu-Ammonis areas of the hippocampus towards the neurotoxic effect of low dose chlorpyrifos, the level of neurotoxicity was less (9.6% reduction in neuronal density) and insignificant when low dose (1/10 LD50) CPF was applied for 3 weeks. Comparatively when the similar dose was applied for 7 days a higher (21% reduction in neuronal density) and significant level of neurotoxicity was observed. As evidenced by the glial reaction, the level of neurotoxicity produced by application of low dose CPF for 3 weeks was less. Hence regeneration of the neurons was possible in the hippocampus Cornu-Ammonis pyramidal layer which might have been reflected in the lower levels of reduction in the neuronal density. Previous literature has suggested that neurogenesis is possible in hippocampus of adult rodents and human (Eriksson et al., 1998).

5. Conclusion

In conclusion, the study by Lim KL, Tay A, Nadarajah VD and Mitra NK (2011) has shown that the dermal application of chlorpyrifos, an organophosphate pesticide in the dose of 1/5th dermal LD50 was capable of producing significant neurotoxicity measured in the parameters of serum cholinesterase reduction, hippocampal neuronal density reduction as well as increased GFAP expression when applied for a short term period of 7 days or prolonged application period of 3 weeks. However a low dose dermal application of chlorpyrifos in the dose of 1/10th dermal LD50 produced a reduced level of neurotoxicity. Initial phase of neurotoxicity produced by a comparatively shorter duration of dermal application of low-dose chlorpyrifos stimulated significant glial reaction in the form of GFAP expression. The pesticide applicators should avoid exposure of chlorpyrifos containing pesticides to their skin to prevent neurotoxic effects of chlorpyrifos.

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3

Effect on Workers' Health Owing to Pesticides Exposure: Endocrine Target

Lidia Caporossi and Bruno Papaleo
INAIL ex-ISPESL- Department of Occupational Medicine
Italy

1.Introduction

Pesticides are used in agriculture and public health to control insects, weeds, animals, and vectors of disease. The Food and Agriculture Organization of the United Nations (FAO) defined a pesticide as 'any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm or otherwise interfering with the production, processing, storage, transport, or marketing of food, agricultural commodities, wood, wood products or animal feedstuffs, or which may be administered to animals for the control of insects, mites/spider mites or other pests in or on their bodies' (Bretveld et al., 2006).
Occupationally exposure to pesticides can occur in industry (manufacturing or formulation workers), agriculture (distribution on open fields or in closed premises, from crops), and in public health (disinfection, rodent elimination, etc.). Then, of course, some active principles are used in veterinary medicine.
In particular, for agricultural workers, we must remember that the work environment is often also the worker's home environment, since agricultural work is often done on a family or craftsman scale.
How workers are exposed may vary widely from one sector to another. An agricultural worker is likely to be exposed to numerous chemicals for short periods, and the level of exposure may vary depending on the type of crop involved, the climate or microclimate, what work is being done, the means used for distributing the chemical, and so forth.
Agricultural work in closed premises such as glasshouses or industrial plant-growing tunnels, and the formulation of commercial products (mixing the active principles with co-formulants) fall mid-way between agricultural and industrial work. Greenhouse work is done under cover with a constantly controlled microclimate, whereas industrial workers come into contact with various different products in different formulation cycles.
The integration of women into non traditional occupations raises questions concerning the impact of such jobs on women' reproductive health, moreover the number of women in the workforce is also increasing worldwide and a considerable proportion of them are of reproductive age; therefore attention is required to note reproductive dysfunction if any, due to occupational exposure (Kumar, 2004).
In literature occupational exposure to pesticides mainly could have repercussions on the reproductive system, in men and women, and on the thyroid gland. The epidemiological studies presented refer to the association between exposure in occupation of parents and the

incidence of infertility, congenital malformations, miscarriage, low birth weight, small-for-gestational-age (SGA) birth, preterm delivery and stillbirth.
The male reproductive system is vulnerable to the effects of this type of chemicals, this might be because sensitive events take place during spermatogenesis and same chemicals may affect some of these events to some extent.
The female reproductive system is also vulnerable but such data are fewer than male reproductive impairment data; this may be because male reproductive endpoints can be studied easier than the female, so that it is not easy to pinpoint which sex is more or less vulnerable to occupation related exposure. Some pesticides may interfere with the female hormonal function, which may lead to negative effects on the reproductive system through disruption of the hormonal balance necessary for proper functioning. Previous studies primarily focused on interference with the estrogen and/or androgen receptor, but the hormonal function may be disrupted in many more ways through pesticide exposure. Ovulation problems present themselves as irregular or absent menstrual periods and can be substantiated through measurement of reproductive hormones. Substances with estrogenic properties may be able to block ovulation similar to contraceptive pills.
An important question is whether occupational exposure of a parent can affect the offspring, causing malformations or reproductive system defects. There have, for instance, been reports of an increase in the risk of testicular dysgenesis sindrome, hypospadia e cryptorchidism, in the children of parents occupationally exposed to some substances (Vrijheid et al., 2003).
Beyond to the effects related to androgen and estrogen homeostasis, there is increasing evidence from animal and in vitro studies that also thyroid is vulnerable to some pesticides; the literature on thyroid-disrupting effects of individual chemicals is rapidly increasing, for some persistent compounds (Nicole-Mir, 2010); and the available evidence is much stronger and they may cause cognitive damage in humans, this effect may be mediated by induction of hypothyroidism, for other compounds is urgent to clarify their possible mechanism of interaction on this gland (Boas et al, 2006). We draw a general picture of pesticides with documented ability to interfere with the endocrine system and the impact achieved on health, with a look at prevention and protection of workers.

2. Pesticides in workplaces

As said worplaces involved in pesticides presence can be divided as agricultural and industrial one.
During occupational exposure these products are mainly absorbed by inhalation or through the skin. Absorption from the gastrointestinal tract is usually limited and is due to swallowing particles, because their size means they tend to deposit in the upper airways. On the topic of occupational exposure to these chemicals in agricultural work there is substantial agreement that the amounts inhaled – in the form of spray or vapors – are much less important than the part absorbed through the skin, except for fumigating agents which are extremely volatile. Contamination of the hands or other skin areas not covered or not otherwise properly protected by garments can account for a large proportion of what is absorbed – even exceeding half the total dose.
The risk of skin exposure to plant treatment products and pesticides has long been studied (Brouwer et al., 1992; Brouwer et al., 1992a; Muddy et al., 1990). In particular there has been close focus on the possible residual risk of handling hothouse plants and

flowers, and taking cuttings of plants that have been chemically treated is one situation that can pose problems: the risk depends largely on the volume of chemical applied and its specific characteristics, particularly its ability to penetrate the skin (Brouwer et al., 1992b; Simonelli et al., 2007).

2.1 Agriculture

The use of pesticides puts agricultural workers at particular "chemical risk". They may come into contact with various preparations at different stages of their work and in every step is needed particular attention as precaution:

- When the pesticide is purchased. Before buying supplies it is essential to establish exactly how much is needed for immediate use, so as to avoid prolonged storage, with its risk of dangerous deterioration of the product.
- Transport of the pesticide is a delicate step, which calls for special precautions; suitable vehicles must be used, and the cargo must be safely loaded and firmly fixed in place. Pesticide containers should not be placed beside the driver.
- Storage of the product. The purchaser of a pesticide is responsible for all facets of its storage and use. It should be stored in premises only used for this purpose, and kept under key. These premises should not be underground or in semi-basements, and should be dry, well-ventilated and protected from frost.
- Preparation of the mixture. All pesticides, whatever their toxicological class, must be handled with special care. Always follow the directions on the label, using the recommended doses. Mixtures should be prepared outdoors, in a position sheltered from the wind.
- Loading the mixture into the spreader machinery. Spreading or distributing: all machinery should be inspected thoroughly before use with pesticides. Is important only use the products at the doses indicated on the label, following the manufacturer's instructions. Pesticides should be used by trained personnel, with the necessary qualifications or permits, wearing appropriate protective gear. The worker does not spread pesticides if people or animals are close by, or near houses. The direction and strength of the jets should be carefully adjusted so as to avoid dispersion of the product.
- Agricultural work in the treated areas.
- Maintenance work is essential to ensure the machinery is in good working condition, so as to avoid wasteful dispersion of the pesticide. This work must be done with the utmost care and attention, by personnel wearing the necessary individual protective apparel.

The risk is greatest when handling the concentrated product: opening the packs, weighing, mixing and loading into spreaders. Various factors can influence the risk of exposure; these include the weather (temperature, wind, humidity), and technical factors such as the method of distribution, individual protective gear, and the state of maintenance of machinery.

2.2 Pesticide manufacting

Occupational exposure to pesticide is certainly a risk for workers who manufacture the active principles. In industrial processing the risk varies depending on the type of

formulation involved, meaning how the active principle is transformed for handling by the end user who normally dilutes it as directed for direct distribution on crops.
The following are some examples of formulations of plant protection products (table n. 1):

Dry powders dry formulations	The active principle is either pure or diluted in an inert powder (amorphous silicon, bentonite, colloidal clay, talc, wet colloids, etc.). These are used for dusting crops or land, foodstuffs or dry-tanning seeds.
Granulates dry formulations	The active principle is mixed in a granule of an inert substance. These are normally used as soil disinfectants or soil insecticides.
Soluble powders formulations for liquid treatments	The active principle is water-soluble. Mixed with water the powder dissolves, forming a solution in which it is uniformly, stably distributed. Some wettable powders are prepared in water-soluble packs.
Wettable powders formulations for liquid treatments	The active principle is solid, finely ground and not water-soluble. It can be suspended in water, but tends with time either to float or sink. This must be borne in mind when selecting the equipment to be used for distribution.
Emulsifiable concentrates formulations for liquid treatments	The active principle is dissolved in one or more organic solvents, giving an emulsifiable liquid that is not water-soluble. The drops remain suspended in the fluid used to distribute the product. Surfactants may be added to stabilize the formulation.
Water-based emulsions formulations for liquid treatments	The active principle is emulsified in water with specific coadjuvants to form an emulsion that remains stable for long periods (years).
Emulsifiable formulations for liquid treatments	These are stable suspensions containing fine particles of the active substance dispersed in an aqueous vehicle, with specific coadjuvants to form an emulsion that remains stable. Under this heading come formulations such as fluid and liquid pastes, concentrated suspensions, and colloid paste.
Suspension in microcapsules formulations for liquid treatments	The active principle is contained in tiny microspheres, dispersed in water with no solvents. The formulation is highly stable and the active substance is released gradually. This ensures greater, more lasting effect with less acute toxicity and phytotoxicity.
Dispersible and soluble granules formulations for liquid treatments	The active principle is finely ground with dispersing and wetting agents and prepared in microgranules that disperse or dissolve in water. They have the advantage of not creating dust and not leaving residues in the container.
Formulations for fumigation	Commercial formulations may be solid, liquid or gas. The active principles act in the gaseous form or as vapors. They are mostly used in closed premises, as insecticides or disinfectants for stocked foodstuffs, in soil or greenhouses.

Table 1. Some examples of formulations of plant protection products

The most dangerous step in the production of solid products is packing them, when workers may inhale the compounds as airborne dusts. The raw materials should ideally be mixed in closed systems. If open hoppers are used they should have hoods directly over them, connected to an aspiration system. The worker loading the product has to cut the bag and empty it, disposing of it in a container under an aspiration hood, to keep dispersion of dusts to the minimum. Hoppers loaded from big bags on special bag-emptying stands must also have their own aspiration hoods.

The use of the dangerous organochloride compounds is now forbidden in industrial processes and in agriculture, although they may arise in some settings as reaction intermediates. One example is in the production of chlorophenoxy herbicide (2,4,5 trichlorophenoxyacetic acid, sometimes called "Agent Orange") or intermediates in the synthesis of disinfectants (hexachlorophene). These processes require high pressure and temperatures, and an alkaline environment, and in these conditions dioxin, or TCDD, can easily form. In addition the reaction solvent is ethylene glycol, which can form unstable polymers that break down in a strongly exothermic reaction, raising the reactor temperature and pressure uncontrollably, with the synthesis of substantial amounts of dioxin, occasionally even blowing the safety valves (Schecter et al., 2006).

Both organic and inorganic mercury is used in the production of fungicides and this metal is well known to be neurotoxic and endocrine disrupter (Tan et al., 2009); cadmium is a frequent impurity in many phosphorus-based fertilizers and its presence could produce much more effects on endocrine system owing to its own ability to interfere as xenoestrogen (Chedrese et al., 2006; Takiguchi & Yoshihara, 2006).

3. Clinical-epidemiologic studies on workers

The toxicity of pesticides differs between the various active ingredients depending on numerous factors, first of all the lipid solubility, the acute pesticide poisonings are a frequent occurence in developing countries while they are relatively infrequent in technologically advanced countries. In general, acute poisoning has resulted from accident or lack of or improper use of protective equipment, especially for substances with high dermal toxicity, (organochlorine and organophosphorus). Various devices and systems may be involved:

- Nervous system: neurotoxic effects are prominent in many pictures of poisoning by pesticides, they may be at central or peripheral level, they are manifested in the respiratory (chest tightness, coughing, cyanosis) , the gastrointestinal (nausea , vomiting, abdominal pain) or the cardiovascular system (hypotension, bradycardia). In particular, this kind of poisoning is due to organophosphorus insecticides, carbamates and organochlorines.
- Respiratory system: it is the target organ of intoxication caused by compounds belonging to the family of dipyridilic pesticides and urea derivatives, the most toxic compound in this picture is paraquat, poisoning can cause acute severe lung disease. The symptoms of this kind of intoxication consist of burning and irritation of the throat, with the presence of necrotic and scaling in the oral mucosa, after can occur gastroenteritis, wheezing, and cyanosis. The copper sulphate used as fungicide in viticulture and fruit production can cause lung injury described as "vineyard sprayers' lung" which consist of a micronodular interstitial pulmonary fibrosis. Less

important effects may also be caused by organophosphorus and carbamate insecticides.

- Coagulation: a family of rodenticides, the coumarins, are vitamin K antagonists and exert their toxic effect on coagulation, clinical sees bruising, epistaxis, hematuria, and in severe cases internal bleeding.
- Skin: from the clinical point of view are highlighted irritant or allergic contact dermatitis with manifestations of erythema, vesicles and scaling, the substances that most commonly produce these outcomes are the dithiocarbamates thiophthalimide and organochlorine insecticides.
- Liver and kidneys: for their anatomical structure and function there are bodies particularly susceptible to the action of toxic and therefore also of pesticides.
- Reproductive system: experimental studies suggest for a lot of compounds the ability to interact with the endocrine system and reproductive capacity.
- Carcinogenic and teratogenic effects: possible carcinogenic effects were seen for some phenoxy acid substances such as herbicides, chlorophenols, arsenical compounds, triazine herbicides.

There are many reports that a high proportion of pesticides interacts with the endocrine system – and the reproductive system in particular – through various mechanisms. In an Iranian study about 50% of the products used in agriculture could potentially interfere with the endocrine system, 33% of them leading to male infertility, 8% having estrogenic activity, 4.5% antiandrogenic, and 22% thyreostatic (Ebrahimi & Shamabadi, 2007).

Herbicides, insecticides and fungicides are the main endocrine disruptor chemicals (EDCs) likely to be encountered (carbofuran, chlopyrifos, dimethoate, lindane, trillate, triflurarin2,4-D and penttachlorophenol, linuron) (Eertmans et al., 2003).

Generally the toxic action does not involve a single mechanism, and several can be needed to cause pathology. Numerous *in vivo* and *in vitro* studies documented the receptor interactions of different compounds, but transposing these findings to humans is complex and still debated. The mechanisms of action can be grouped under several broad headings,

- Direct damage to cell structures
- Interference with biochemical processes necessary for normal cell function
- Biotransformation to toxic metabolites.

This is illustrated schematically in Fig. 1.

The dose, time and duration are all important pointers to the extent of exposure, as they all influence the real absorption of the toxin, hence its potential for harm (Bretveld et al., 2006).

3.1 Effects on reproductive system and on fetus

Some pesticides may interfere with the female hormonal function, which may lead to negative effects on the reproductive system through disruption of the hormonal balance necessary for proper functioning. Previous studies primarily focused on interference with the estrogen and/or androgen receptor, but the hormonal function may be disrupted in many more ways through pesticide exposure.

Studies in women have found menstrual cycle disturbances, with significant correlations between serum levels of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) and its metabolites and polymenorrhea (missing several cycles) (Windham, 2002). A survey of 3103 women agricultural workers found that those handling and distributing pesticides – usually

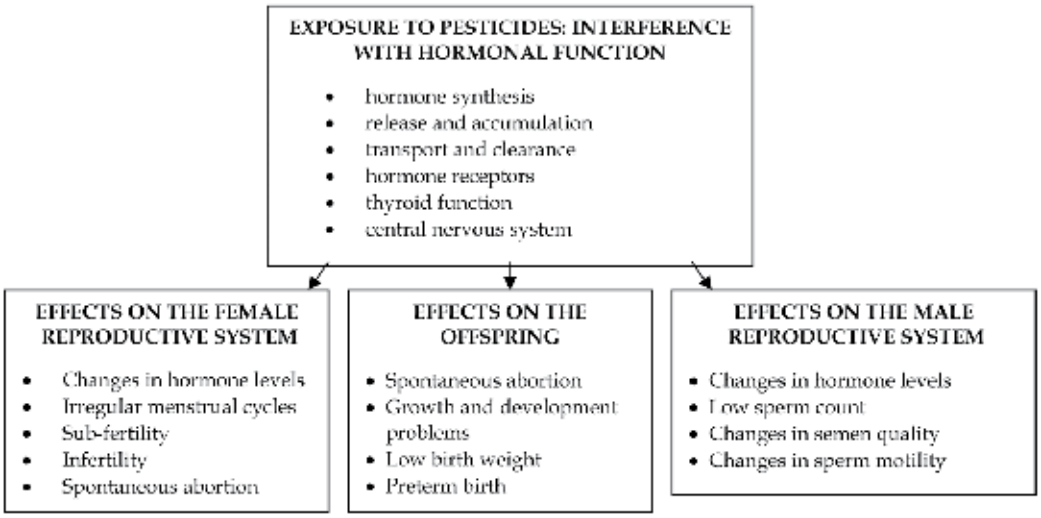

Fig. 1. Mechanisms of action of pesticides on endocrine system

mixtures – had 60-100% more episodes of amenorrhea (no menstruation) (Farr et al., 2004). Both found associations between serum levels of DDT or a metabolite of DDT and short cycles and undefined 'menstrual disturbances'. Another study found no correlations between infertility and self-reported overall pesticide exposure, working in the agricultural sector, or living on a farm during the two years before the diagnosis of infertility or the last pregnancy. The pesticides may disrupt the hormonal function of the female reproductive system and in particular the ovarian cycle (Bretveld et al., 2006).

Pesticide use may be associated with a later age at menopause.

Few other studies have examined the association between specific pesticide exposure and timing of natural menopause reported an increased hazard ratio (earlier age at menopause) for women with higher plasma levels of p,p'-1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (an isomer of DDE), a breakdown product of the pesticide DDT, using data from 1,407 women in a breast cancer case-control study. Furthermore these type of research are controversial and the results were not clear. This difference may be due to different sampling or exposure assessment strategies. The toxicology evidence, along with the two existing epidemiologic studies examining DDE and timing of menopause, it's hypothesized that use of hormonally active or ovotoxic pesticides would result in an earlier menopause in women by depleting germ cells in the ovary. However, there was no evidence in some analyses that use of pesticides led to an earlier menopause. On the contrary, taken together, use of hormonally active pesticides was associated with later age at menopause. Later age at menopause is associated with fertility in later reproductive years and possibly with an increased risk of certain reproductive cancers but a decreased risk of cardiovascular disease and overall mortality (Dalvie et al., 2004a).

A recent study reported a reduction in age-adjusted all-cause mortality by 2 percent per year increase in age at menopause. Use of pesticides was associated with a delay of 3–5 months in timing of menopause. Number of ovulatory cycles has been inversely associated with age at menopause, while cycle length has been positively associated with age at menopause. Additional analyses among younger women in the Agricultural Health Study showed that exposure to hormonally active pesticides was associated with longer menstrual cycles and

more missed menstrual periods. Pesticide exposure may lead to a later age at menopause through effects on menstrual cycles. Alternatively, hormonally active pesticides may directly affect timing of menopause through effects on follicle-stimulating hormone and luteinizing hormone. Women who use pesticides may be healthier overall than women who do not use pesticides. Among 8038 workers recruited, 62% had handled and applied various pesticide mixtures (DDT, lindane, atrazine, carbaryl, carbon tetrachloride, mancozeb and maneb, organochloride compounds, carbamates, organophosphorus compounds, phenoxy herbicides); these workers all reported a lengthening of the time of onset of the menopause, of about three to five months (Farr et al., 2006).

A Danish study of women distributing pesticides in greenhouses found a clear reduction in fertility (reduced fecundity pregnancies per month while trying to get pregnant) (Abell et al., 2000); this was also seen in Finnish workers (Taskinen et al., 1995; Sallmen et al., 2003). In the USA the incidence of lowered fertility was three times higher in women exposed to pesticides than among other women agriculture workers, and as much as nine times higher than non-agricultural workers (Fuortes et al., 1997; Smith et al., 1997). However, some of these findings have been questioned even though exposure to herbicides seems the most significant factor (Greenlee et al., 2003).

Several studies have set out to assess the effects on reproductive health by recording any abnormalities during pregnancy: spontaneous abortion, stillbirths, fetal malformations, preterm births, low birth weight. The data are not always clear or easily correlated to the real exposure conditions, for instance to DDT (Cocco et al., 2005; Dalvie et al., 2004a): in vivo and in vitro toxicological findings may be evident but epidemiological studies have not given univocal results, often because the sample had poor statistical strength or the methods were not altogether correct. Some studies found a significantly higher risk of spontaneous abortion among women directly exposed to pesticides (Arbuckle et al., 1999; Arcuckle et al., 2001; Nurminen, 1995; Petrelli et al., 2000a; Crisostomo & Molina, 2002) although it was not clear through what mechanism of interaction with the endocrine system this effect was achieved (Hanke & Jurewics, 2004). A study of exposure to phenoxy herbicides in 2000 couples found only weak correlations between spontaneous abortion and exposure up to three months before conception; however, the correlation became stronger, up to double the risk of controls, for exposure in the weeks immediately preceding conception (Arbuckle et al., 1999), the outdoor agricultural workers showed much less data (Degen & Bolt, 2000).

The data on the effect of employment in agriculture on the time to pregnancy are unequivocal, but most of them suggest that there is a relationship between the decreased fecundability ratio and pesticide exposure. Nor does the research on the sex ratio of offspring provide explicit results. The analyses indicate that parental employment in agriculture could increase the risk of congenital malformations in the offsprings, particularly such as orofacial cleft, birthmarks in the form of hemangioma as well as musculoskeletal and nervous system defects. The data on the effect of occupational exposure to pesticides on birth weight are contrasting. Although most of epidemiological studies do not reveal a significantly increased risk of SGA, a slower pace of fetal development corresponding to SGA in the population of women exposed to pyrethroids has been recently reported. There are also some indications that exposure to pesticides may contribute to stillbirth and female infertility. There are a Danish follow-up studies to examine whether exposure to pesticides during pregnancy had an adverse effect on pregnancy outcomes among gardeners and farmers. There were no significant differences in the studied pregnancy outcomes between gardeners or farmers and all other workers, except for an increased risk of very preterm birth for gardeners and a favorable birth weight for farmers (Zhu et al., 2006).

A study investigated whether the work in greenhouse during pregnancy adversely influenced infant birth weight. Work in greenhouses is performed in warm microclimate during the most time of the year, involves usually moderately intense or heavy work. The working conditions in greenhouses might involve also indirect exposure to pesticides resulting from contact with pesticide-treated flowers and vegetables. Results indicate that infants of mothers performing heavy work inside greenhouse during pregnancy had lower mean birth weight than infants of mothers working out of greenhouse. No similar effects of current exposure to pesticides was observed (Jurewicz et al., 2005).

A study of the effects of thiocarbamates and carbaryl in 3984 pregnant women in Canada found a higher risk of spontaneous abortion; the increase in risk was less evident for pre-gestational exposure to phenoxy acetic acid and triazine herbicides, and late spontaneous abortion was associated with the use of fungicides, thiocarbamates and glyphosate (Arbuckle et al., 2001). Most of these miscarriages happened in the first trimester of pregnancy. A North American study correlated the occupational use of herbicides (sulphonylurea, imidazolinone and mixtures of chlorophenoxy herbicides, sulphonylurea and benzothiodizole) with an increase in spontaneous abortions in spring, and results were similar for Ethylen-bis-dithiocarbamates fungicides, particularly maneb and mancozeb. The period of exposure to pesticides is important, as different climatic conditions may influence the level of exposure and the potential effects. There is a surprisingly significant deficit in the number of male children born to the spouses of fungicide applicators. First-trimester miscarriages occur most frequently in the spring, during the time when herbicides are applied. Use of sulfonylurea, imidizolinone containing herbicides, and the herbicide combination by Cheyenne male applicators was statistically associated with increased miscarriage risk in the spring. Limited survey data from women who are the spouses of applicators did not show major alterations of long-term endocrinologic status (menarche, menopause, endometriosis) (Garry et al., 2002).

Various studies report malformations in the neonates (limb anomalies, cleft lip/palate) (Hanke & Jurewics, 2004) but subsequent epidemiological surveys did not always confirm this (Clementi et al., 2007). An excess risk of musculoskeletal malformations was reported among children born to Finnish garden-workers (Hemminki et al., 1980)and a higher than normal risk of angioma was noted for the infants of flower-growers in a population of 8867 workers from Bogota (Restrepo et al., 1990). A Spanish study correlated exposure to pyridyl derivatives with risks for the nervous system, cleft lip/palate and multiple anomalies (Garcia et al., 1998) but no increase in risk for exposure to organophosphorus compounds, carbamates, organochlorides, fungicides and organosulphates (Garcia et al., 1999). An increased risk of cleft lip/palate was found also in Finland (Nurminen et al., 1994) and of spina bifida and hydrocephalus, particularly among mothers exposed in orchards and greenhouses, in Norway (Kristensen et al., 1997a).

A rise in fetal malformations therefore seems to be associated, more or less decisively, with handling chlorophenoxy herbicides (Schreinemachers, 2003), fungicides, trifuralin, atrazine (Garry et al., 1996), and phosphine and glyphosate-based products used for fumigation (Engel et al., 2000).

Data on low birth weight of babies born to women exposed to pesticides are also discordant. In Canada (Robert, 1988), Scotland (Saniose et al., 1991) and Norway (Kristensen et al, 1997a) the figures were the same as for controls, whereas in Indonesia (Murphy et al., 2000), Brazil (Lima et al., 1999) and Poland (Dabrowski et al., 2003; Hanke et al., 2003) birth weight was about 100 g lower than normal for women exposed in the first trimester of pregnancy, particularly to pyrethroids (Hanke et al., 2003).

Attempts to clarify the correlation between pesticide exposure and stillbirths have also found a confused picture (Bell et al., 2001). Californian studies indicated that exposure in the second trimester of pregnancy, particularly the third and fourth months, to carbamates and acetylcholinesterase inhibitors could increase the risk of stillbirth (Goulet & Theriault, 1991), but Kristensen et al. in Norway found no significant effects (Kristensen et al. 1997a; Kristensen et al. 1997b).

Organophosphorus pesticides raise a different question, as their acetylcholinesterase inhibition is associated with the possibility of interaction with hypothalamic and/or pituitary function, hence gonadal processes. Hormone assays have been used as a means of documenting these effects. A study from Pakistan (Ahmad et al., 2004) that measured acetylcholinesterase activity in women occupationally exposed to organophosphorus compounds found that half the workers had only 34.42% of the controls' activity; about 40% had 72.59% of the activity and 8% had 87.94%; the percentage of "toxicity" calculated in relation to age came to 18.42%. Excluding seven cases of acute intoxication, one third of the women reported oligomenorrhea, another third secondary amenorrhea, 16.67% early menopause and 16.67% heavy menstrual flow; there were no cases of infertility.

Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels, as well as urinary levels of FSH, LH, and E1C, a metabolite of testosterone, were measured to investigate the adverse reproductive effects of organophosphate pesticides among Chinese factory workers who were occupationally exposed to ethylparathion and methamidophos. The exposure significantly increased the serum LH. Meanwhile, the serum FSH level was slightly elevated and the serum testosterone level was decreased with increased pesticide exposure. It's possible conclude that organophosphate pesticides have a small effect on male reproductive hormones, suggestive of a secondary hormonal disturbance after testicular damage (Padungtod et al., 1998).

This was confirmed by Recio et al. (Recio et al., 2005) who found that out of 64 agricultural workers 48% had FSH outside the normal range, with values substantially higher than normal during the periods of highest pesticide use; LH was also slightly elevated, but no abnormal findings were recorded for testosterone.

Exposure to organophosphorus pesticides has been associated with changes in the chromatin structure of sperm, which may raise the proportion of cells highly susceptible to DNA denaturation (Sanchez-Pena et al., 2004); these compounds also interfere with spermatic chromosomal segregation, again raising the risk of genetic damage (such as Turner's syndrome) because of aneuploidy (Recio et al., 2001).

Significant associations have been found between serum levels of organochlorides and immune and endocrine alterations, indicative of the risk that these products interfere in the course of gestation (Gerhard et al., 1998). In 20% of women agricultural workers with repeated spontaneous abortion, serum assays found at least one polychlorinated hydrocarbon outside the reference range (Eckrich & Gerhard, 1992), and high levels of DDT and DDE in maternal blood were also associated with preterm birth and the infant's size and weight (Gerhard et al., 1999b). Another study, in women exposed to pentachlorophenol – used to protect wood – suggested a central interaction for this product, which might act in the hypothalamus or at suprahypothalamic levels, leading to ovarian and adrenal insufficiency (Gerhard et al., 1999a).

Epidemiologic studies of the negative effects on the male reproductive system have looked at semen and sex hormones when the aim was to investigate male fertility directly (Hess & Nakai, 2000; Giwercman et al., 1993; Silvestroni & Palleschi, 1999; Larsen et al., 1999) or

"man-mediated" effects, i.e. generally time to conception (Petrelli et al., 1999; De Cock et al., 1994; Curtis et al., 1999). Neither approach found univocal data, and more specific investigations are needed to see which particular type of chemical is involved and in what conditions of exposure (Petrelli & Mantovani, 2002). Experimental investigations on semen often found no real differences in morphology (Smith et al., 2004) particularly the effects of exposure to fungicides, have also not proved significant this exposure does not cause aneuploidy of the spermatozoa, unlike smoking which is a strong confounding factor (Smith et al., 2004; Harkonen et al., 1999) or sperm count (Golec et al., 2003). This illustrates the difficulties of establishing clear correlations between these biological data and potential reductions in fertility, which tend to be suggested more by studies based on "time to pregnancy" (Hanke & Jurewics, 2004; Petrelli et al., 2000b; Bonde et al., 1999).

The most significant findings come from epidemiologic studies focused on handling the most toxic products, whose biological effects are documented better, like DDT, which is still used in many developing countries to get rid of the mosquitoes that cause malaria, in fact in a study of workers using DDT to control the carriers of malaria found that clinically significant exposure was correlated with antiandrogen or estrogenic effects that might possibly cause abnormal levels of reproductive hormones, the most important finding was a positive relationship of baseline E2 and baseline testosterone with blood DDT compounds levels, especially with p'p'-DDT and -DDD. Peak post- GnRH testosterone was also positively related to p'p'-DDE.(Dalvie et al., 2004a). These studies found that 84% of workers handling these compounds had a sperm count below the levels indicated by the WHO and below Tygerberg's criterion, with 6% fulfilling Tygerberg's definition of subfertility; 10-20% complained of sexual dysfunction. Although DDT has little xenoestrogenic capacity - it is 103-106 times less potent than estradiol – its bioaccumulation may lead to estrogenic effects in certain circumstances, and at high doses it is an androgen agonist (Gray & Kelce, 1996; Toppari, 1996; Kelce, 1995).

Numerous pesticides directly harm the spermatozoon, altering the function of the Sertoli or Leydig cells, or interfering with endocrine function at various stages in hormonal regulation. These mechanisms have been amply documented *in vivo* and *in vitro* but are hard to record in epidemiologic terms (Bretveld et al. 2007) because of the specificity of occupational exposure, since the extent of exposure and its effects must be quantified exactly to clarify the negative effects on health.

More significant results come from studies aimed at detecting correlations between occupational exposure and abnormal hormone levels. In these cases even low-dose exposure can cause changes that are detectable in blood assays. Strambe et al. (Straube et al., 1999), for instance, found that acute exposure to herbicides, insecticides and fungicides in agricultural workers lowered the levels of testosterone and estradiol, while slightly raising T4 and T8 lymphocytes. Chronic exposure, on the other hand – which is closer to real working situations – led to higher testosterone levels and a higher T4:T8 ratio; in addition the LH concentration was higher than in controls. The authors concluded that the interaction involved inhibition of the aromatase system in the testosterone metabolism. Quite likely there is some interference with both the endocrine and immune systems, certainly depending on the exposure time.

Similar findings came from a study of 114 herbicide application workers who had high levels of testosterone, FSH and LH, and alterations to thyroid hormone levels too (Garry et al., 2003): in the herbicide-only applicator, significant increases in testosterone levels in fall compared to summer and also elevated levels of follicle-stimulating hormone (FSH) and

luteinizing hormone (LH) in the fall were noted. Historic fungicide use was associated with a significant alteration of the sex ratio of children borne to applicators. Lower testosterone level were associated with a shift in the sex ratio of children born to applicators. As before, among current study subjects it was noted that historic fungicide use was associated with increased numbers of girls being born.
Brazilian epidemiologic survey detected significant correlations between mortality from hormone-related tumors (ovary, prostate, testes) and at least ten years of intensive agricultural work (Koifman & Koifman, 2002).
Male reproductive toxicity involves a broad range of targets and mechanisms such as direct effects on the seminiferous epithelium and/or on Leydig and Sertoli cells supporting spermatogenesis, epididymal sperm maturation as well as endocrine disruption. Male subfertility is generally expressed as a reduced ability of the female partner to become pregnant. Male workers exposed to some pesticides have shown a decreasing number of spermatozoa and alteration of semen quality and morphology. Moreover prenatal exposure could induce testicular dysgenesis sindrome, hypospadia e cryptorchidism. (Carbone et al., 2006; Pierik et al., 2004). A father's exposure to pesticides at work predicted an adverse live-birth outcome (preterm delivery) in multivariate models(Hourani & Hilton, 2000).
Other authors find no real differences in semen quality (Larsen et al., 1999) and no correlation between exposure and reduced fertility (time to pregnancy) (Thonneau et al., 1999). Workers most likely to be exposed, besides agricultural laborers who prepare the pesticide mixtures, are those who work land or crops that have been treated, and the pilots of planes used to spray these products. Evaluation of the negative effects on greenhouse workers exposed to pesticides showed that their wives tended to have a long time to pregnancy (Petrelli et al., 2000b).

3.2 Thyroid and hormonally dependent cancers

Carcinogenesis (cancer formation) involves irreversible alteration of a stem cell, its uncontrolled proliferation and, finally, invasion of other tissues. In this sequence there are various mechanisms by which pesticides may contribute to cancer development. The most obvious mechanism is genotoxicity, direct alteration of DNA turning harmless cells into cancer cells. Even levels of exposure to organophosphates too low to significantly decrease cholinesterase levels increased chromosomal aberrations found in blood samples from farmers. Pesticides thought to cause cancer in this way include the fumigants ethylene oxide and ethylene dibromide.
Pesticides and other endocrine disrupters represent a credible "new" risk factor for hormonally dependent cancers. To date, most work has centered on breast cancer, and most studies have not shown increased risks. Additional epidemiologic investigations are warranted, but they would benefit from a better understanding of the mechanisms, dose, and co-factors involved (Muir, 2005).
An American study showed that, among women farmers, breast cancer risks were elevated for women present in fields during or shortly after pesticide application, but not among those who reported using protective clothing. Some data showed a weak association between breast cancer and farming. DDT can support the growth of estrogen-dependent tumors in rats. However, most case-control studies since 1996 have failed to confirm earlier observations of a significant positive relationship between levels of DDT and DDE (a DDT breakdown product) to breast cancer risk.

	REPRODUCTIVE TOXICITY	REFERENCES
1,1,1-TRICHLORO-2,2-BIS(P-CLOROPHENYL)ETHANE (DDT)		
Female reproductive system	Menstrual cycle disturbances, polymenorrhea, block ovulation, irregular or absent menstrual periods.	Windham, 2002 Bretveld et al., 2006
	Increases in spontaneous abortions	Arbuckle et al., 1999 Petrelli et al., 2000a Nurminen, 1995 Crisostomo & Molina, 2002
	Preterm births and low birth weight	Gerhard et al., 1999
Male reproductive system	Alterate secretion of testosterone	Dalvie et al., 2004a
	Low sperm count, subfertility, sexual dysfunction	Dalvie et al., 2004b Gray & Kelce, 1996 Toppari, 1996 Kelce, 1995
CARBAMATES		
Female reproductive system	Increases in oligomenorrhea	Farr et al., 2004
	Increases in spontaneous abortions	Arbuckle et al., 2001
	Risk of stillbirth	Goulet & Theriault, 1991
HERBICIDES		
Female reproductive system	Amenorrhea	Farr et al., 2004
	Increases in spontaneous abortions	Arbuckle et al., 1999 Garry et al., 2002 Taskinen, 1992
	Increased miscarriage risk	Garry et al., 2002
	Longer time to pregnancy	Petrelli et al., 2000b
Male reproductive system	Alterations in sex hormone output	Staube et al., 1999; Garry et al., 2003
FUMIGANTS		
Female reproductive system	Polymenorrhea	Farr et al., 2004
MIXTURES (DDT, lindane, atrazine, carbaryl, carbon tetrachloride, mancozeb, maneb, carbamates, organophosphorus compounds, phenoxy herbicides)		
Female reproductive system	Prolongation of time to menopause	Farr et al., 2004
	Reduced fertility	Abell et al., 2000 Taskinen et al., 1995 Sallmen et al., 2003 Fuortes et al., 1997 Smith et al., 1997 Greenlee et al., 2003
Male reproductive system	Alterations in sex hormone output	Staube et al., 1999; Garry et al., 2003
FUNGICIDES		
Female reproductive system	Increases in spontaneous abortions	Arbuckle et al., 2001 Garry et al., 2002
Male reproductive system	Alterations in sex hormone output	Staube et al., 1999;

		Garry et al., 2003
ORGANOPHOSPHORUS COMPOUNDS		
Female reproductive system	Oligomenorrhea, secondary amenorrhea, early menopause, heavy menstrual flow	Ahmad et al., 2004
	Alterations in sex hormone output	Padungtod et al., 1998 Recio et al., 2005
Male reproductive system	Reducing brain acetyl cholinesterase activity and monoamine levels, thus impairing hypothalamic and/or pituitary endocrine functions and gonadal process	Recio et al., 2005 Garry et al., 2003
	Decreased testosterone levels, testicular damage, sperm hyperploidy/polyploidy	Padungtod et al., 1998 Recio et al., 2001
	Alterations to chromatin structure of sperm	Shanchez-Pena et al., 2004
	Aneuploidy of sperm	Recio et al., 2001
MIXTURES (Chlorophenoxy herbicides, fungicides, trifuralin, atrazine, phosphine and glyposate-based fumigants)		
Neonatal/fetal malformations from parental exposure	Anomalies of the limbs, cleft lip/palate	Hanke & Jurewics, 2004 Nurminen et al., 1994
	Musculoskeletal malformations	Hemminki et al., 1980
	Risk of angioma	Restrepo et al., 1990
	Multiple anomalies	Garcia et al., 1998
	Spina bifida, hydrocephalus	Kristensen et al., 1997
MIXTURES (ethylparathion and methamidophos)		
Female reproductive system	Increased serum LH and FSH, decreased testosterone levels	Padungtod et al., 1998 Garry et al., 2003
MIXTURES (pesticides)		
Female reproductive system	Later age at menopause	Farr et al., 2006
Male reproductive system	Decreased number of spermatozoa, alteration of semen quality and morphology, aneuploidy of spermatozoa	Carbone et al., 2006 Pierik et al., 2004 Harkonen et al., 1999
Neonatal/fetal malformations from parental exposure	Low birth weight, small-for-gestional-age (SGA) birth, testicular dysgeneris syndrome, hypospadia and cryptorchidism	Hanke & Jurewicz., 2004 Hourani & Hilton, 2000 Jurewicz et al., 2005 Abell et al., 2000

Table 2. Summary of relation between pesticides and effects on human reproductive system

The use of Chlordane, malathion, and 2,4-D was associated with increased risk of breast cancer; risk associated with chemical use was stronger in younger women (Mills & Yang, 2005).

There is some evidence linking pesticide exposure and ovarian cancer, but findings are inconsistent. Italian research showed an association between exposure to triazine herbicides and ovarian cancer (Donna et al., 1984)

Epidemiology studies have investigated the possibility that atrazine may result in adverse effects in humans. The chloro-S-triazine herbicides (i.e., atrazine, simazine, cyanazine) constitute the largest group of herbicides. Despite their extensive usage, relatively little is known about the possible human-health effects and mechanism(s) of action of these compounds. Studies in laboratory have shown that the chlorotriazines disrupt the hormonal control of ovarian cycles. Results from these studies hypothesized that these herbicides disrupt endocrine function primarily through their action on the central nervous system. Although some studies have claimed that atrazine exposure results in an elevated risk of prostate cancer, the published literature is inconclusive with respect to human cancer incidence. But studies of prostate cancer are beginning to show consistent associations with pesticide exposure.

Case-control in Sweden and in USA studies confirm that exposures to cadmium, herbicides, and fertilizers low occupational physical activity levels have elevated prostate cancer risks. Increased risk with specific chemicals, simazine, lindane, and heptachlor suggestive increases with dichlorvos and methyl bromide (Settimi et al., 2003; Settimi et al., 2001; Sharma-Wagner et al., 2000; Mills & Yang 2003; Gammon et al., 2005).

The study among rural workers in Brazil indicated an almost two times higher probability of cancer development among rural workers, with a calculated relative risk between those exposed (agriculture workers) and the non-exposed (other occupations) of 1.6. The authors concluded that the cancers of the skin and digestive system were the most prevalent.

There are numerous reports on the possible effects of exposure to pesticides on the thyroid, in fact thyroid gland diseases (goiter, autoimmune thyroiditis, carcinoma) are associated with exposure to many chemical or physical agents (Baccarelli, 1999). Among these substances fungicides are largely studied for their impact on thyroid, particularly ethylenebis (dithiocarbamates) (EBDCs), such as maneb, zineb and mancozeb, they have been extensively used for the past 40 years; EBDCs are metabolized into ethylenethiourea (ETU), a possible human carcinogen and an antithyroid compound, so that the Environmental Protection Agency (EPA) of the United States of America has restricted their use and require workers to use protective equipment. ETU is known to cause decreases of thyroxine (T4) and increases in thyroid-stimulating hormone (TSH) in animals, researches on working population suggest that EBDCs affect the thyroid gland among heavily exposed workers even if data are yet of borderline statistical significance (Steenland et al., 1997). This trend of thyroid hormones levels is confirmed in a study on banana plantation workers, correlated with blood and urinary ETU levels, moreover was founded a higher prevalence of solitary nodules in exposed workers as detected by ultrasound (Panganiban et al., 2004). Farmers who had aerial application of fungicides to their land showed a significant shift in TSH values; subclinical hypothyroidism was noted in rural applicators (TSH values >4.5 mU/L), but not in urban control subjects.

The level of TSH was elevated also in male pesticide formulators exposed to the dust and liquid formulation of endosulfan, quinalphos, chlorpyriphos, monocrotophos, lindane, parathion, phorate, and fenvalerate as compared to a control group, but the increase was statistically insignificant (Zaidi et al., 2000).

The serum levels of thyroxine and thyroid stimulating hormone were examined in rural subjects with respect to blood levels of organochlorine pesticide, it's found that some subjects had depleted thyroxine levels in association with significantly lower organochlorine pesticide residues in blood. Sex, nutritional status, thyromegaly, or handling of pesticides in the course of work were not found to be factors contributing to depleted thyroxine levels (Srivastava et al., 1995). All informations about effect on thyroid and cancer are reported in table n. 3.

	Type of exposure	Observed effects	References
Thyroid	Pesticides	Goiter, autoimmune thyroiditis	Baccarelli, 1999
	Endosulfan, quinalphos, chlorpyriphos, monocrotophos, lindane, parathion, phorate, and fenvalerate	Significant shift in TSH values; Subclinical hypothyroidism	Zaidi et al., 2000
	Hexachlorbenzene--HCB, DDE (2,2'-2-bis(4-chlorobiphenyl)- 1,1-dichloroethylene), p,p'-DDT (2,2'-bis(4-chlorophenyl)- 1,1,1-trichloroethane) and alpha-, beta- and gamma-hexachlorcyclohexane--HCH)	Increased thyroid volume higher frequency of hypoechogenicity and frequency of positive thyroperoxidase antibodies level in blood	Panganiban et al., 2004 Srivastava et al., 1995
	Organochlorine pesticide	Depleted thyroxine levels, thyromegaly, increased TSH	Steenland et al., 1997
Cancer effects ("new" risk factor for hormonally dependent cancers)	Chlordane, malathion, and 2,4-D; DDT and DDE	Breast cancer	Mills & Yang, 2005 Donna et al., 1984
	Atrazine herbicides, and fertilizers, simazine, lindane, and heptachlor	Prostate cancer	Setimi et al., 2003 Settimi et al., 2001 Sharma-Wagner et al., 2000 Mills & Yang, 2003 Gammon et al., 2005
	Organophosphorous pesticides	Tyroid cancer	Baccarelli, 1999

Table 3. Dysfunction on thyroid gland due to pesticides in farming and hormonally dependent cancers.

4. Difficulties in epidemiological studies

There is much debate about the best methodological approach for epidemiological studies of exposure to Endocrine disruptor chemicals. Assessment of exposure to endocrine disrupters is complicated because of wide variation in endocrine disrupting potency of

substances and mechanisms by which these compounds produce their effects, and because little is known about possible interactions that may occur in exposure in many occupations. Some authors have developed a job-exposure matrix for potential endocrine disrupting chemicals, seven categories of contaminants were evaluated, for trying to obtain a more simple approach to the problem, but the job-matrix does not distinguish substances with different mechanisms or potency for endocrine disruption, nor does it incorporate any possible changes in exposure over time, and all these conditions represent great limits to its use (van Tongeren et al., 2002).

A protocol for assessing the risk of occupational exposure has been proposed, giving broad outlines, starting with risk identification and continuing through to defining dose-response relationships (Taskinen & Ahlborg, 1996). Unfortunately there is still no agreement on the validity of these questionnaires for collecting the occupational history of workers exposed to PCB. The risk of incorrect data is estimated at 13-29% and a tendency has been noted to overestimate exposure for women; for men the distribution was random (Rosenberg et al., 1987).

To overcome this problem a standardized procedure has been proposed, based on the application of two statistical tests, Gibbons' Alternative Minimum Level (AML), and a test to establish the relative standard deviation (RSD); the aim is to achieve the necessary reproducibility for data concerning biological monitoring of workers exposed to PCB. The method was validated on data collected in 1960 and gave good precision, with a low limit of detection (Willman et al., 2001).

A pivotal question is the "measure" that accurately describes exposure. This is much more difficult than checking for reproductive problems and is present as much in case-control studies in workers as in the general population. To minimize gross error it is recommended to pool biological findings with information from questionnaires, taking great care to select accurate and reliable statistical methods, based primarily on metabolic considerations (What function does the biological mechanism alter?) (Joffe, 1992).

Interpreting the results and putting them to use to prevent harm to health is a focal point, but transposition is not easy. It is also no simple matter to establish environmental and biological "cut-offs" that will adequately safeguard endocrine health (Figà-Talamanca & Giordano, 2003).

On the subjects of reproductive health, a Scandinavian group of Danish, Norwegian and Finnish researchers has proposed a classification criterion for substances causing reproductive toxicity, this takes account of epidemiological and experimental toxicology findings, to give a versatile tool for planning and verifying preventive measures in the workplace (Taskinen, 1992).

Time to pregnancy is one of the most widely used indicators and the standardized birth ratio is another – this is the ratio of observed to expected births; failure to conceive after a year of unprotected sexual relations is considered true infertility.

Epidemiological studies on occupational exposure and fertility often suffer from bias (Castilla et al., 2001). Confounding factors are important in these investigations (some are listed in Table 4).

In view of the considerable difficulties of these studies, the World Health Organization has evaluated all the data regarding the impact of work on reproductive health and the final report states that only 10% of published epidemiological studies can be considered methodologically correct.

CONFOUNDING FACTORS	
MEN	**WOMEN**
Drugs, alcohol, smoking	Drugs, alcohol, smoking
Anatomical abnormalities of the genital organs	Ovarian dysfunction
Cryptorchidism	Hyperandrogenism
Parotitis	Endometriosis
Hormonal dysfunction	Hormonal dysfunction
Diabetes mellitus	Genital infections
Varicocele	Uterine factors
Testicular trauma	Factors involving the uterine cervix
	Causes originating from a fetus

Table 4. Confounding factors in Epidemiological studies

5. Conclusions and prospects

Research in recent years has investigated the toxic effects of many compounds on the reproductive system in men and women. This is an important area because of the infinite implications of any such alteration: from the possibility of conception, through a normal pregnancy, to the birth of a healthy child. Physical health is not the only factor involved, because the emotional sphere is brought into play too. This is illustrated by the major psychological problems raised by infertility, and the tortuous diagnostic-therapeutic paths couples are willing to follow to overcome it.

One very important factor is previous environmental exposure, which largely influences the "internal" dose in the general population, especially for lipophilic contaminants that accumulate in adipose tissue and persist at length. For instance, the general population has blood levels of PCB comparable to those resulting from low-dose exposure, which makes it very complicated to check for any dose-response correlation (Hanaoka et al., 2002).

Thyroid diseases are increasing in the general population , especially among women. Although the most frequent dysfunctions have either an auto-immune or congenital etiology, the possible effects of environmental factors on the normal homeostasys should not be overlooked. The identification of the single responsible substances is complex because of its multiple target action as well as the possible interference of the complex network involving thyroid hormones their metabolism and their functions. Pesticides influence the thyroid endocrine activity trough the inhibition of thyroid peroxidase (TPO) or trough alteration of the production of thyroid hormones (T3, T4), their transport, their secretion and biosynthesis activity of this gland, and sensorial alterations especially trough the genetic adjustment of the response to T3 (Schmutzler et al., 2004; Beard & Rawlings, 1999). Among these the most significant are ethil-bi-dithio carbamate as well as some persistent chlorurate compounds.

Occupational exposure is certainly an area calling for much further research (Hoyer, 2001; Crews et al., 2000) which might take several directions:

- identification of all the chemicals that can be toxic to the endocrine system;
- description and evaluation of exposure;
- definition of the dose-response paradigm, which is a tough task on account of the widespread environmental presence of contaminants;

- identification of environmental and biological concentrations above which there is a real health risk;
- development of validated analytical methods;
- agreement on reference intervals for biological assays;
- consensus on the exact definition of "low doses";
- drafting operational protocols for epidemiological studies for the workplace;
- assessment of personal susceptibility, especially as regards sex difference.

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4

Health Problem Caused by Long-Term Organophosphorus Pesticides Exposure - Study in China

Zhi-Jun Zhou
Fudan University
China

1. Introduction

Organophosphorus pesticides (OPs), one of the most popular classes of pesticides, are widely used all over the world especially in developing countries, such as China. There are many OPs, with thousands of trade names such as dimethoate, parathion and omethoate, most of which have been used for insect control in residential and agriculture settings. The acute toxicity of OPs are believed to be due primarily to the inhibition of acetylcholinesterase (AChE) resulting in an accumulation of acetylcholine (Ach) with a sustained overstimulation of Ach receptors in the clefts of central and peripheral neuron synapses. They can cause a progression of toxic signs, including hypersecretions, convulsions, respiratory distress, coma and death. However, the heavy usage of OPs has given rise to wide public concern on their chronic toxicity. Generally, long-tem exposure to OPs can be divided into occupational exposure and non-occupational exposure. The former often involves farming population and workers employed in pesticide-related industries. And the latter is more for general population potentially exposed to OPs via a number of different routes including dietary, lifestyle or medicinal.
China is a large country with large demand of pesticides. This means that there are much more Chinese people, both occupational and non-occupational population, whose health are under the threat of OPs exposure. The presence of common and specific metabolites of OPs in urine samples taken from the general population has demonstrated the widespread exposure to OPs in China. Moreover, workers engaged in OPs production are at high risk from OPs exposure, as confirmed by higher levels of OPs metabolites in biological samples compared to those present in individuals from non-agricultural communities. Therefore, a great deal of research has been conducted by Chinese scientists to understand the adverse effects of long-term, low-level exposure to OPs in both general and occupational population.

2. OPs exposure assessment - biolocial monitoring

OPs exposure in both occupational and general population can be assessed by measurement of esterase activity and by direct measurement of urinary OPs metabolites.

2.1 Esterase activity

The activity of esterases including butyrylcholinesterase (BChE), erythrocyte acetyl cholinesterase (AChE), carboxylesterase (CarbE) and paraoxonase (PonE) can be inhibited

by OPs. However, the sensitivity of these four kinds of esterases to inhibition differs. We previously conducted a cross-sectional study among 241 workers from a pesticide plant as directly exposed group, 161 service persons in the same pesticide plant as indirectly exposed group and 150 workers without any records of pesticide exposure in another plant as control group. We measured the esterase activity of all these subjects. The results showed that the CarbE, BChE and PonE activity of subjects in exposed group was significantly lower than subjects in control group (Table 1). The inhibition of AChE activity was related to the type of workshop and work process whereas the inhibition of AChE and BChE activity does not necessarily correlate closely with exposure time and level (Table 2~4). Besides, there was a dose-response relationship between the external exposure dose and CarbE activity (Table 5).

Goup	Number	CarbE	BchE	PON
Direct Exposure	241	513.44±184.59*	39.52±17.84*	142.75±70.49*
Indirect Exposure	161	480.75±115.8#	38.67±15.34#	147.96±93.21
Control	150	615.90±149.55	44.05±12.28	167.97±112.04
p value		0.000	0.004	0.021

*: the esterase activity of subjects in exposed group are significantly lower than those in Control (p<0.01).
#: the esterase activity of subjects in Indirectly exposed group are significantly lower than those in C (p<0.01).

Table 1. Esterase activity (nmol·ml^{-1}·min^{-1}) of subjects in different groups

Esterase	Type of Workshops			*p* value
	Methamidophos (n=87)	Dimethoate (n=83)	Other OPs (n=71)	
CarbE	508.36±194.62	39.21±22.52	488.14±186.19	0.205
BChE	38.65±13.55	137.11±69.62	40.96±16.40	0.710
PonE	150.72±75.91	126.33±9.83	139.57±64.43	0.411
AChE	127.21±8.13	126.33±9.83	139.57±64.43	**0.003**

Table 2. Esterase activity (nmol·ml^{-1}·min^{-1}) of subjects in different workshops

Esterase	Type of Processes			*p* value
	Packers (n=70)	Operators (n=136)	Inspectors (n=35)	
CarbE	475.23±183.92	526.89±189.88	537.68±156.23	0.115
BChE	39.15±13.61	39.01±14.82	42.26±31.48	0.620
PonE	144.21±68.67	142.84±73.84	139.48±61.95	0.949
AChE	123.31±9.80	126.01±9.23	127.91±7.35	**0.034**

Table 3. Esterase activity (nmol·ml^{-1}·min^{-1}) of workers with different jobs in directly exposed group

Esterase	Working time (years)				*F* value	*p* value
	1~5 (n=9)	5~10 (n=48)	10~20 (n=97)	>20 (n=87)		
CarbE	531.18±283.70	448.54±154.55	509.43±195.27	551.91±167.61	3.377	0.019
BChE	43.52±19.44	41.3±26.24	40.42±15.69	37.12±13.86	0.924	0.430
PonE	146.31±79.60	135.56±67.81	137.09±66.68	152.66±75.06	0.955	0.415
AChE	127.11±7.89	124.21±9.03	125.06±9.47	126.54±9.27	0.840	0.473

Table 4. Esterase activity (nmol·ml^{-1}·min^{-1}) of workers with different working time in directly exposed group

External Exposure level (mg/m3)	Number	CarbE (nmol·ml^{-1} min^{-1})	BchE (nmol·ml^{-1} min^{-1})	PonE (nmol·ml^{-1} min^{-1})	AChE (U)
0~3	124	485.08±188.90	42.36±20.62	136.75±67.54	136.75±67.54
3~6	63	556.43±175.35	37.33±14.67	152.59±71.18	125.76±9.52
>6	54	528.44±176.70	35.56±12.78	145.04±76.04	126.72±8.77
F value		3.417	3.450	1.093	0.812
p value		**0.034**	**0.033**	0.337	0.446

Table 5. Relationship between the external exposure level and esterase activity

Similar research was done by other Chinese colleagues, for example, they (Lin et al., 2007) investigated 56 parathion exposed workers (as exposed group) and 120 non-exposed persons (as control group) and reported that there were significant differences ($p < 0.001$) of the activity of BChE, AChE, CarbE, and PonE compared with control group, but no difference ($p > 0.05$) in plasma β-glucuronidase (β-GD) activity. And the rates of abnormity (below the lower limit of activity reference range) were 37.5% and 48.2% for CarbE and BChE respectively, which were all significantly higher than that of AChE ($p < 0.001$). But there was no significant difference between PonE activity (5.4%) and AChE activity ($p > 0.05$).

2.2 Dialkylphosphate (DAP) metabolites in Urine

On the other hand, there are clear evidences from biological monitoring studies that dialkylphosphate (DAP) metabolites of OPs can be detected in urine after OPs exposure. Six common DAP metabolites, e.g, dimethylphosphate (DMP), dimethylthiophosphate (DMTP), diethylphosphate (DEP), diethylthiophosphate (DETP), diethyldithiophosphate (DEDTP), and dimethyldiithiophosphate (DMDTP) have been determined. These metabolites are non-specific to a particular organophosphate metabolism of different OPs can give rise to similar urinary metabolites. Urinary DAP metabolites reported in a number of studies are summarized in Table 6.

These metabolites in urine are useful to estimate exposure to several OPs. In the cross-sectional study mentioned above, we found that DMP and DETP concentration of workers in the directly exposed group was significantly higher than that of indirectly exposed group (Table 7). Workers in different workshops have different urinary metabolites whereas the type of job influenced the concentration of urinary metabolites (Table 8 and 9). However, we didn't find that the total exposure time will affect the urine level of DAP metabolites (Table 10).

Name	DAP metabolites	Name	DAP metabolites
Dichlorvos	DMP	Malathion	DMP, DMTP, DMDTP
Chlopyrifos	DEP, DETP	Methidathion	DMP, DMTP
Mercaptophos	DEP, DETP	Mevinphos	DMP
Diazinon	DEP, DETP	Paraoxon	DEP
Dichlofenthion	DEP	Parathion	DEP, DETP
Azinphos-methyl	DMP, DMTP, DMDTP	Methyl parathion	DMP
Dimethoate	DMP, DMTP, DMDTP	Phorate	DEDTP
Fenitrothion	DMTP	Diethquinphione	DEP, DETP
Malaoxon	DMP	Metriphonate	DMP

Table 6. Urinary DAP metabolites of different OPs

Group	Number	Median of Urinary DAP metabolites concentration (μg/gCr)				
		DMP	DEP	DETP	DMDTP	DEDTP
Directly Eexposed	161	0.01	1.06×102	9.41	2.18×102	97.48
Indirectly Exposed	122	0.00	8.24×102	8.02	2.21×102	95.10
z value		-4.839	-0.981	-2.733	-0.682	-1.165
p value		**0.000**	0.326	**0.006**	0.495	0.244

Table 7. Urinary DAP metabolites concentration of subjects in different exposed groups

DAP metabolites	Type of Workshops			*p* value
	Methamidophos (n=51)	Dimethoate (n=65)	Other OPs (n=45)	
DMP	0.00	0.00	0.00	0.137
DEP	1292	725	1471	**0.045**
DETP	8	8	8	0.394
DMDTP	342	50	480	**0.004**
DEDTP	90	88	98	**0.037**

Table 8. Urinary DAP metabolites concentration (median) of directly exposed workers in different workshops

DAP metabolites	Type of Processes			*p* value
	Packers (n=26)	Operators (n=109)	Inspectors (n=26)	
DMP	0.00	0.00	0.00	0.623
DEP	1307	1180	737	**0.016**
DETP	8	8	15	0.534
DMDTP	222	275	523	0.140
DEDTP	143	88	99	**0.008**

Table 9. Urinary DAP metabolites concentration (median) of workers with different job title in directly exposed group

DAP metabolites	Working Age Groups (years)		z value	*p* value
	≤20 (n=84)	>20 (n=77)		
DMP	0.00	0.00	-0.104	0.917
DEP	109	871	-0.338	0.698
DETP	9.41	10.9	-1.080	0.280
DMDTP	232	159	-0.688	0.491
DEDTP	110	95	-0.264	0.792

Table 10. Urinary DAP metabolites level (median) of workers with different exposure time in directly exposed group

Another study, done by our research group, investigated in detail 30 workers packaging dimethoate from a pesticide plant. Urine samples of each participant pre- and post-workshift were collected. The results showed that 100% of the workers had at least one DAP metabolite present in both pre-shift and post-shift urine samples. DMP and DMTP were the most frequent metabolites (100%) found, followed by DMDTP, DEP, DETP and finally DEDTP (Table 11). DAP metabolites with dimethyl moieties (DMP, DMTP, and DMDTP) were detected at higher concentrations than those with ethyl moieties (DEP, DETP, and DEDTP) in both time points (pre- and post- workshift). Moreover, DMP, DMTP and DMDTP concentration in the post-shift urine samples were significantly higher than that in the pre-shift urine samples (Table 12).

Groups	Detection percentage (%) of urinary DAP metabolites					
	DMP	DEP	DMTP	DMDTP	DETP	DEDTP
Pre-shift	100.0	40.0	100.0	90.0	20.0	0.0
Post-shift	100.0	53.3	100.0	96.7	26.7	6.7

Table 11. The detection percentage of urinary DAP metabolites of subjects in exposed groups

Groups	Urinary DAP metabolites concentration (μg/gCr)					
	DMP	DEP	DMTP	DMDTP	DETP	DEDTP
Pre-shift	371±1.9*	102±2.1	891±2.4*	302±2.3**	78±2.7	nd
Post-shift	741±2.1	104±1.5	1479±2.1	832±2.3	74±2.2	47±1.4

nd: not detected.
*: the urinary DAP metabolites concentration of pre-shift samples are significantly lower than those of pro-shift samples (p<0.05).
**: the urinary DAP metabolites concentration of pre-shift samples are significantly lower than those of pro-shift samples (p<0.01).

Table 12. Urinary DAP metabolites concentration (geometric mean) of subjects in exposed groups

Indeed, certain levels of DAP metabolites are also detected in non-occupationally exposed populations. We tested the urine samples of 60 college students and found that more than 86% of them had at least one type of DAP metabolites in the urine. DMDTP was the most frequent metabolite (86.7%) found, followed by DMP, DMTP, DEP, and finally DETP. And

the results showed no detectable DEDTP (Table 13). DMTP were detected at much higher concentrations than other metabolites: the geometric mean of DMTP was high as 661 μg/gCr (Table 14).

Detection	Detection percentage (%) of urinary DAP metabolites					
	DMP	DEP	DMTP	DMDTP	DETP	DEDTP
Number	51	30	48	52	18	0
Percentage (%)	85.0	50.0	80.0	86.7	30.0	0.0

Table 13. The detection percentage of urinary DAP metabolites of general population

DAP metabolites	Range of concentration	25% percentile	median	75% percentile	geometric mean	geometric standard deviation
DMP	22～1026	100	170	254	166	2.3
DEP	27～383	67	114	197	110	1.9
DMTP	109～3187	404	693	1104	661	2.1
DMDTP	24～784	68	135	219	126	2.3
DETP	24～186	37	51	91	60	2.4
DEDTP	nd	nd	Nd	nd	nd	nd

Table 14. Urinary DAP metabolites concentration (μg/gCr) of general population

3. Adverse effects caused by long-term OPs exposure

3.1 Common illness caused by long-term OPs exposure

Available evidence suggests that there is a possibility of adverse effects occurring after long-term OPs exposure although these effects may be not clearly related to the inhibition of cholinesterase. Studies on health hazards to farmers who handle, store and use OPs have documented a range of non-specific self-reported symptoms that have been attributed to chronic OPs exposure. These include burning or prickling of the skin; tingling or numbness of hands and face; muscular twitching or cramps in the face, neck, arms and legs; respiratory symptoms such as chest pain, chest stuffiness, cough, runny nose, wheezing, shortness of breath, sore throat; excessive sweating; nausea, vomiting, diarrhoea; excessive salivation; abdominal pain; lacrimation and inflammation of the eyes; difficulty in seeing; restlessness; difficulty in falling asleep; trembling of hands; and irritability.

Zhao and his colleagues use Pittsburgh Sleep Quality Index (PSQI) and Epworth Sleeping Scale (ESS) to investigate and analyze the sleeping status of 482 agricultural workers over 50 years old from 5 counties in Jiangxi province (Zhao et al., 2010). The PSQI scores of these farmers were 5.80±2.81, lower than those of general population. And the ESS scores of these farmers were 7.15±4.99, higher than those of general population. Moreover, the ESS scores of farmers who have been exposed to OPs more than 1000 days were significantly higher than other farmers (p<0.01). Zhang observed 284 occupational OPs exposed persons by dynamic ultrasonographic imaging and found a higher prevalence of fatty liver than non-exposed persons (W.P. Zhang et al., 2010).

ECG changes in workers who have been exposed to OPs were also reported. An investigation of 706 exposed workers and 707 non-exposed persons and reported that about 19.69% of the workers had abnormal ECG changes against 12.31% of the non-exposed persons (Tang et al., 2004). The abnormal ECG changes of exposed workers include sinus bradycardia, arrhythmia, incomplete right bundle branch block, and ST-T segment elevation.

Our group once analyzed a series of data of medical examination (particular ECG examination) of 87 workers exposed to three kinds of OPs and found significant differences in the prevalence of ECG abnormalities between exposed and non-exposed groups. Although the prevalence of ECG changes for exposed workers was much higher than that of prior to exposure, it did not increase with the prolongation of the exposure period. And the inhibition of AChE was not correlated to ECG disorders, which indicated that cardiac effects of OPs are not clearly related to the inhibition of AChE (Tables 15 and 16).

Groups	Number	Abnormal ECG rate	Odds ratio of prevalence	*p* value
Control	25	4.0		
Dimethoate	35	20.0	6.0	**0.07**
Methamidophos	30	13.3	3.7	0.23
Kitazin P	19	21.1	6.4	**0.09**
Totle	84	17.9	5.2	**0.07**

Table 15. ECG abnormalities of subjects in different groups

Types of ECG abnormalities		Dimethoate	Methamidophos	Kitazin P	Control
Sinus arrhythmia	Sinus tachycardia	3	4	1	0
	Sinus bradycardia	17	3	8	0
	Sinus irregularity	5	0	0	0
Ectopic arrhythmias	Premature beat	0	3	0	0
Conduction abnormalities	Right bundle branch block	4	0	0	0
Others	Low QRS wave	1	9	10	2
	Left ventricular sypervoltage	5	5	2	0
Left/right axis deviation		10	2	9	0
Total number of abnormalities		42*	26*	21*	2
Total number of subjects		410	360	145	302

*: the number of ECG abnormalities in the exposed groups are signicantly different from those of control group (p<0.01).

Table 16. Types of ECG abnormalities of subjects in different groups

Once we collected the information on OPs exposure history and signs and symptoms of the subjects through questionnaires and medical examinations among another exposed population. Then the weighting and total score of the signs and symptoms of neuromuscular system, respiratory system, circulatory system and digestive system was calculated. The results showed that the weighting and total symptom score in directly and indirectly exposed group was higher than that in control group, and there was a dose-response relationship between the internal exposure dose and digestive system score (Table 17~19). A higher percentage of abnormal hemoglobin was found in the workers in directly exposed group, in correlation with exposure time. The workers (working time 5~10 years) in directly exposed group showed a higher percentage of abnormal hemoglobin level, and there was dose-response relationship between the percentage of abnormal hemoglobin and accumulating external exposure dose (liner-liner association analysis (p<0.05) (Table 20 and 21). Besides this, some system scores and the percentage of abnormal hemoglobin were related to AChE activity regarded as an exposure dose (Table 22). There was negative correlation between the activity of AChE and signs scores according to correlation analysis. It showed a increasing trend of signs scores and percentage of abnormal hemoglobin with the decrease of AChE activity (Table 23).

Groups	Number	Symptom scores				
		neuromuscular system	respiratory system	circulatory system	digestive system	Total system scores
Directly Exposed	241	0.66±1.49	0.27±0.84	0.44±0.74	0.21±0.57	1.57±2.44
Indirectly Exposed	161	0.29±0.88	0.11±0.32	0.30±0.64	0.07±0.30	0.63±1.08
Control	150	0.03±0.16	0.05±0.22	0.06±0.27	0.03±0.22	0.16±0.54
H value		49.37	10.87	37.13	23.55	89.01
p value		0.000	0.004	0.000	0.000	0.000

Table 17. Total symptom scores of subjects in different groups

Groups	Total number	Number of person with abnormal symptoms	Number of person without abnormal symptoms	Ratio of abnormal symptoms (%)	X^2 value	p value
Directly Exposed	241	132	109	54.8	91.05	**0.000**
Indirectly Exposed	161	43	108	28.5		
Control	150	15	145	9.4		

Table 18. Ratio of abnormal symptoms of subjects in different groups

DETP (μg/gCr)	Number	Symptom scores				
		neuromuscular system	respiratory system	circulatory system	digestive system	Total system scores
0~7.5	53	0.83	0.15	0.25	0.11	1.34
7.5~15	49	0.80	0.22	0.41	0.18	1.61
>15	59	0.80	0.32	0.22	0.39	1.73
H value		1.063	2.642	2.603	6.900	3.674
p value		0.588	0.267	0.272	**0.032**	0.159

Table 19. The symptom scores are affected by internal exposure dose (urinary DETP levels)

Groups	Number	Abnormalities (%) of medical examinations					
		WBC	Hb	ECG	B ultrasonic	SBP	DBP
Directly Exposed	241	2.9	33.6	13.7	17.8	12.4	24.1
Indirectly Exposed	161	3.3	5.3	17.9	24.5	20.5	33.1
Control	150	3.1	15.6	17.5	15.1	6.3	19.4
X2		0.053	48.88	1.623	2.536	14.19	8.06
p		0.974	**0.0000**	0.444	0.111	**0.001**	**0.018**

WBC: white blood cell; Hb: hemoglobin; SBP: systolic pressure; DBP: diastolic pressure

Table 20. Medical examinations data of subjects in different groups

Rate of abnormalities (%)	Exposure time (years)				X^2 value	*p* value
	1~5 (n=9)	5~10 (n=48)	10~20 (n=97)	>20 (n=87)		
WBC	0	6.3	3.1	1.1	3.212	0.360
Hb	11.1	**47.9**	39.2	21.8	13.193	**0.004**
ECG	0	12.5	15.5	13.8	1.744	0.627
B ultrasonic	0	16.7	23.7	13.8	5.252	0.154
SBP	11.1	14.6	11.3	12.6	0.328	0.955
DBP	44.4	27.1	19.6	25.3	3.420	0.331

Table 21. Medical examinations data of workers with varied exposure time in directly exposed groups

AChE activity (U)	Number	Symptom scores				
		neuromuscular system	respiratory system	circulatory system	digestive system	Total system scores
0~120	67	1.07	0.46	0.54	0.34	2.42
120~127	54	0.59	0.15	0.39	0.20	1.33
127~134	74	0.49	0.26	0.49	0.14	1.36
>134	46	0.41	0.13	0.28	0.13	0.96
H value		10.018	16.278	3.723	11.564	8.490
p value		0.018	**0.001**	0.293	**0.009**	**0.037**

Table 22. The symptom scores were realted to the AChE activity

AChE activity (U)	Number	Abnormalities (%) of medical examinations					
		WBC	Hb	ECG	B ultrasonic	SBP	DBP
0~120	67	1.5	56.7	7.5	16.4	4.5	11.9
120~127	54	5.6	35.2	20.4	14.8	14.8	25.9
127~134	74	1.4	25.7	13.5	17.6	13.5	29.7
>134	46	4.3	10.9	15.2	23.9	19.6	30.4
X^2 value		2.724	28.840	4.330	1.591	6.398	7.813
p value		0.436	**0.000**	0.228	0.662	0.094	**0.049**
Trend X^2 value		0.157	28.051	0.878	0.959	5.164	6.330
p value		0.692	**0.000**	0.349	0.327	0.023	**0.012**

Table 23. The raiao of medical examination abnormalities were related to the AChE activity

We also compared the 686 health surveillance records in 1979 and 1995 in Shanghai Pesticide Factory to understand changes of health status among employees and evaluate the effectiveness of occupational health measures herein. We noted that less symptoms and signs score in 1995 than 1979. Higher percentage of abnormal blood pressure was found among the first year new workers. With the pass of time, the percentage of such change also increased. There were no differences of hemoglobin levels among workers who engaged in different sectors and with different working ages. ANOVA test revealed that the activity of cholinesterase in 1995 was significant higher than 1979. The job code (which dominants the magnitude of OPs exposure) was a main affecting factor to the enzyme activity. Better health status in 1995 than in 1979 was also found based upon the data of 139 workers who had received two-times examinations in 1979 and in 1995. These results confirmed that the general health status of workers exposed to pesticides was better in 1995 than in 1979 in this pesticide factory. It indicated that the occupational health measures taken during this period of time were effective.

In Shanghai Pesticide Factory, we also observed the typical tolerance phenomenon to OPs. The trend of change of ChE and clinical score among the contractor workers exposed to different levels of OPs were carefully studied. The trend of changes in blood ChE and score since starting exposure to 3 or 4 months were expressly present. We found that the ChE and score of packing workers sharply declined since the starting of exposure; there were

significant exposure-effect correlations. After withdrawing of those who were poisoned (ca. 2%) in 40-60 days, the ChE and score dropped less steep and then turn to flat. It indicated that body developed tolerance to low-level exposure to OPs in 40-60 days. High level (or higher toxicity) exposure caused poisoning in portion of the workers, but the remainders tolerated the exposure, and kept ChE and score in a steady horizon, though fluctuated and less than normal.

3.2 Neurobehavioural effects caused by long-term OPs exposure

Some, but not all, epidemiological studies demonstrated that long-term exposure to OPs may be associated with impaired neurobehavioural performance. Clinical features that have been reported include anxiety disorder, depression, psychotic symptoms, dysthymic disorder (DSM-III-R); short-term memory problems, learning disorders, attention-deficit disorders, information processing problems, eye-hand coordination problems and delayed reaction time, and autonomic dysfunction.

Zhang and his colleagues conducted a survey on a representative sample of 9811 rural residents in Zhejiang province (J.M. Zhang et al., 2009). These residents were asked about the storage of pesticides at home and about whether or not they had considered suicide within the 2 years before the interview. The Chinese version of the 12-item General Health Questionnaire (GHQ) was administered to screen for mental disorder. They found that the unadjusted odds ratio (OR) for the association between pesticide storage at home and suicidal ideation over the prior 2 years was 2.12 (95% confidence interval, CI: 1.54–2.93). After adjusting for gender, age, education, socioeconomic status, marital status, physical health, family history of suicidal behaviour, GHQ caseness and study design effects, the OR was 1.63 (95% CI: 1.13–2.35). These results indicated an association between OPs exposure and suicide ideation in rural areas of China.

3.3 Effects of long-term OPs exposure on the human reproduction

Another important feature of OPs is their endocrine disrupting effects and potential adverse impact on both male and female reproductive function. Studies carried out employing chronic exposure of animals to low doses of the OPs showed a reduction in reproductive function, both female and male. And a number of epidemiology data also demonstrated the deleterious reproductive effects of chronic exposure to OPs in occupational and/or environmental settings.

Lv and her colleagues investigated the cross-sectional association between OPs use and menstrual function among 298 women working at a OPs factory (Lv, 2004). Women were aged 21-45 years, premenopausal, not pregnant or breastfeeding, and not taking oral contraceptives. Menstrual cycle characteristics of interest included symptoms before the menstruation begins; cycle length (short cycles, long cycles, irregular cycles); missed periods (not experiencing a period for more than 6 weeks in the last 12 months); menstruation amount (large, small); and dysmenorrhea. After controlling for age, working time, and education level, the author found that women who used pesticides experienced more pre-menstruation symptoms and increased odds of irregular menstrual cycles compared with women who never used pesticides.

Zhang and her colleagues observed 601 female workers in the first production line of the pesticide factory and 873 unexposed female workers according to the reproduction occupational epidemiological method (S.H. Zhang et al., 2004). Then they reported a

significantly higher incidence of premature delivery (8.20%), post-mature delivery (7.64%), spontaneous abortion (2.83%), and pregnancy induced hypertension syndrome (6.41%) in the exposed group than the unexposed group (p=0.000, 0.003, 0.004, 0.035).

Li's investigation also showed an increased incidence of irregular menstruation, spontaneous abortion, and infertility in the OPs exposed group when compared with the control group (G.R. Li et al., 2000).

Li and Zou surveyed 161 male farmers exposed to OPs and 161 unexposed men via epidemiological questionnaires. Then these subjects received genital examinations, and their semen samples were collected for analysis. The authors found a decrease in sperm viability and percentage of sperm with forward progression, and normal sperm morphology. The semen density of farmers in the exposed group was 76.0±84.8×10^6/mL, significantly lower than those in the unexposed group (100.0±56.4×10^6/mL). Logistic regression analysis showed that chronic exposure to OPs would influence the sperm quality (W.Y. Li et al., 2004; Zou et al., 2005).

3.4 Effects of long-term OPs exposure on fetal and childhood health

Large amount of evidence have shown that fetuses can be exposed to pesticides. OPs pass through the blood–brain barrier and placenta and have also been found in amniotic fluid. In addition, the young may receive greater exposure than adults, because they eat, drink, and breathe more per unit of body weight. They are closer to the floor and surfaces where pesticides may settle, and have extensive hand-to-mouth contact. Recent studies have shown that fetuses and young children have lower than adult levels of detoxifying enzymes and their brains are developing rapidly. This suggests that the nervous system of the fetus and young children is several-fold more susceptible to potential neurotoxic effects of such low-dose OPs exposure.

Wang and his colleagues investigated the association between neurodevelopment and behavior of 301 children. Child neurodevelopment was assessed by the Gesell Development Schedule at 2 years of age. Developmental quotients (DQs) were obtained in motor, adaptive, language and social areas. They reported that geometric mean (GM) for children DAP metabolites (μg/g) were DMP: 10.38; DMTP: 6.56; DEP: 7.27; DETP: 14.26; DEDTP: 4.46 (Table 24). They found a significant correlation between DAP levels and children neurodevelopment (Table 25 and 26. They also found the DQs were higher in high dose exposure group than in the low dose exposure group. There was highly significant difference between these two groups (p=0.03) (Table 27). In addition, DAP levels were positively associated with 8-OHdG in urine (r=0.594, p=0.000) (Wang, 2009).

DAP metabolites	Detection percentage (%)	GM	Range	P25	P50	P75	P95
DMP	41.9	10.38	1.17~724.43	3.95	8.93	23.70	125.60
DMTP	36.5	6.56	0.07~478.63	2.87	5.90	13.12	58.64
DEP	71.8	7.27	0.06~169.82	3.51	7.16	14.79	54.61
DETP	69.1	14.26	1.1~977.24	5.30	12.91	37.15	128.82
DEDTP	2.7	4.46	1.07~72.44	2.46	4.45	7.69	18.36

Table 24. Creatinine-adjusted OPs urinary DAP metabolites levels among children (μg/g) (n=301)

DQ score	Mean±SD	Normal development percentage (%)	Delayed development percentage (%)
Behavioral ability	103.07±7.59	99.67	0.3
Adaptability to environment	107.03±11.87	98.67	1.3
Verbal ability	104.27±16.22	93.7	6.3
Adaptability to people	96.11±7.34	97.3	2.7

Table 25. Distribution of GSD DQ score (n=301)

DAP metabolites	Behavioral ability		Adaptability to environment		Verbal ability		Adaptability to people	
	â (95%CI)	*p*	Â (95%CI)	*p*	â (95%CI)	*p*	â (95%CI)	*p*
DMP	-0.20 (-6.88~6.35)	0.94	0.05 (-9.03~11.03)	0.85	0.02 (-13.32~13.45)	0.99	-0.25 (-9.61~3.24)	0.76
DMTP	0.12 (-2.399~6.06)	0.39	0.49 (-5.28~7.53)	0.73	-0.07 (-10.90~6.20)	0.59	-0.15 (-6.20~2.00)	0.31
DEP	-0.19 (-5.13~4.53)	0.90	-0.10 (-9.68~4.98)	0.53	-0.04 (-11.16~8.39)	0.78	-0.18 (-7.40~1.98)	0.26
DETP	-0.47 (-13.16~0.90)	0.09	-0.44 (-19.6~1.71)	0.10	-0.11 (-17.35~11.09)	0.67	-0.16 (-8.90~4.75)	0.55
DEDTP	0.13 (-1.58~7.54)	0.20	0.07 (-4.41 9.42)	0.48	0.06 (-6.12~12.34)	0.51	0.05 (-3.41~5.44)	0.65

Table 26. Adjusted coefficient (â) (95%CI) in points on the Gesell scores of children neurodevelopment for log10 unit increase in pesticide urinary metabolites (n=301)

	DQ scores	High dose group (n=212)	Low dose group (n=89)
Behavioral ability	Mean ± SD (range)	103.36±7.33 (83~125)	102.36±8.17 (90~124)
	Normal (%)	99.53%	100.00%
Adaptability to environment	Mean ± SD (range)	107.34±11.85 (83~136)	106.28±11.94 (79~135)
	Normal (%)	99.06%	97.75%
Verbal ability	Mean ± SD (range)	105.02±15.93 (66~146)	102.5±16.96 (66~138)
	Normal (%)	94.34%	92.13%
Adaptability to people	Mean ± SD (range)	96.99±7.3 (82~133)	94.02±7.02 (71~121)
	Normal (%)	98.11%	95.51%

Table 27. Gesell scores in two dose groups (n=301)

Wang also collected and analyzed urine samples of 187 pregnant women to evaluate the relationship of maternal prenatal DAP levels with birth outcomes. The results showed that GM of DAP metabolite levels (μg/g) of pregnant women were DMP: 25.75; DMTP: 11.99; DEP: 9.03; DETP: 9.45; DEDTP: 0.75. They did not found the evidence that OP pesticides at current levels adversely affect fetal development.

Luo analyzed the birth outcome data of 5571 prenatal infants in a rural area of Guangdong Province and reported that 1.13% of them were born with deformity including hydrops fetalis syndrome, neural tube defects, hydrocephalus, and congenital equinovarus. Further logistic analysis found a relationship between maternal exposure to OPs and birth defects (Luo, 2004).

3.5 Other health problems caused by long-term OPs exposure

By analyzing the death cause data of a cohort including 2270 workers employed for at least 1 year before Jan 1, 1983 and a sub-cohort of 1018 of them worked at OPs exposed workshop in a pesticide factory, we investigated the cause of death and mortality of cancer among OPs exposed workers and evaluated the relationship between long-term occupational OPs exposure and cancer occurrence. This study was followed up from Jan 1, 1983 to Dec 31, 2004. The death cause spectrum of OPs exposed workers was similar to that in reference population locally, but higher mortality of malignant tumor was found in OPs exposed workers. The SMR for all cancer, and malignant cancer were 120.2 and 119.6 respectively. SMR for malignant tumor of bladder, lung and stomach cancer were 303.7, 141.2, and 137.5 respectively ($P<0.01$). Chi-square test showed tumor mortality of exposed workers was higher than that of non-exposed workers ($P<0.01$), indicating the risk of malignant tumor death increased with exposure to OPs (Table 28 and 29).

Hong tested DNA damage in peripheral lymphocytes of workers exposed to OPs via single cell microgel electrophoresis (SCGE) and found that the cometic rate of peripheral lymphocyte among OPs exposed workers was (2.8±1.9)%, significantly higher than that in control group ($p<0.01$). The amount of T lymphocyte α-ANAE in peripheral blood among OPs exposed workers was also significantly higher than that in control group ($p<0.01$). These results suggested that chronic exposure to OPs may lead to genetic damage (Hong et al., 2002).

We studied the M_3 gene expression in peripheral blood lymphocyte of workers exposed to diamethoate and explore its role in the toxic effects of OPs. The lymphocytes in peripheral blood from 33 workers exposed to diamethoate and 15 control people were isolated and treated with saline and diamethoate in vitro, respectively. RT-PCR technique was used in determine M_3 gene expression. Basal and inducible gene expression levels were measured. The result was presented in ratio of optical density of sample mRNA and that of the reference (β-actin) as: (M_3 O.D.×353)/(248×β-actinO.D.). There (OD) no significant difference of basal gene expression level between the exposed group and control group, (1.49±0.20) versus (1.49±0.45); while the inducible gene expression level was significantly higher in exposure group to the control group, (1.92±1.07) versus (1.22±0.19). No difference was found between male and female people in both exposed and control group. The inducible gene expression level was higher in the operators than in the packers, which maybe attribute to the difference of exposure time. The inducible M3 gene expression level showed a gradient increment with the elongation of the working age: <5yr(1.69±0.95), 5～25yr (1.91±1.03), >25yr (2.09±1.25). These indicated that after long-term exposure to OPs, the basal M3 receptor gene expression level in the exposed workers did not show any difference with the control group, but the inducible gene expression level (treated with OPs in vitro) would increase and the level was related to the degree of OPs exposure.

population	Reference population		Cohort of exposed group		Expected deaths	SMR
	Death toll	Mortality	Death toll	Mortality		
All death cause	149511	819.60	263	719.19	300	87.7
All cancer	41484	227.41	100	273.46	83	120.2
Malignant tumors	41306	226.43	99	270.72	83	119.6
Nasopharyngeal cancer	519	2.85	0	0.00	1	0.0
Esophageal Cancer	2285	12.53	5	13.67	5	109.2
Gastric cancer	7258	39.79	20	54.69	15	137.5*
Intestinal cancer	3499	19.18	5	13.67	7	71.3
Liver cancer	5333	29.23	14	38.28	11	131.0
Lung cancer	10248	56.18	29	79.30	21	141.2*
Brest cancer	1229	6.74	2	5.47	2	81.2
Cervical cancer	216	1.18	0	0.00	0	0.0
Bladder cancer	657	3.60	4	10.94	1	303.7**
Leukemia	825	4.52	1	2.73	2	60.5
Benign tumors	81	0.44	1	2.73	0	615.8**
Other tumors	9334	51.17	19	51.96	19	101.5
Other diseases	108027	592.19	164	448.47	217	0.76

*: P<0.05. **: P<0.01.

Table 28. The cause of death and mortality of both OPs exposed workers and reference population

Groups	Male			Female		
	Death from tumors	Death from others	Total	Death from tumors	Death from others	Total
OPs exposed population	46	54	100	12	8	20
Reference population	36	91	127	3	13	16
Total	82	146	227	15	21	36
	X^2=7.556, p=0.006			X^2=6.223, p=0.013		

Table 29. Constituent ratio of death in OPs exposed population and reference population

4. Interaction of genetic polymorphisms and long-term OPs exposure

While this review has focused on health problems caused by long-term OPs exposure via a number of different ways including occupational, dietary, lifestyle or medicinal, it should be recognized that it is likely that polymorphisms within a variety of genes may affect susceptibility to OPs induced toxicity. Much of the work in this field has focused on OPs metabolism and detoxification pathways.

One of our studies examined whether BChE and PonE polymorphisms influenced susceptibility in OPs exposed population. We determined BChE-K, PonE-192 and PonE-55 genotypes of 75 OPs exposed workers using PCR-PFLP. And then their accumulative symptom scores and the whole blood AChE activity (mmol·h^{-1}·ml^{-1}) were measured as health index. We analyzed their health condition related to single gene site of the three gene loci to determine which kinds of genotype were susceptible. Then, we used the multiple variance analysis to see if there existed interactions among these three gene loci. Finally, we established the multi-factor linear regression equation, considering some other factors that might affect the health status such as age, gender and exposure time. The results showed that the mean AChE activities of the exposed workers with BChE-K genotype UU (61 cases), genotype UK(12 cases)and genotype KK (2 cases) were respectively 105.0±23.0, 84.4±16.4, 79.0±9.9. The accumulative symptom scores were respectively 3.7±3.8, 9.2±3.0, 12.5±0.7. The AChE activities of the exposed workers with PonE-192 genotype BB (37 cases), genotype AB (27 cases) and genotype AA (11 cases)were respectively 116.8±15.1, 91.2±15.6, 72.3±21.4. The accumulative symptom scores were respectively 2.0±3.2, 6.7±3.3, 9.7±1.8. Similarly, the AChE activities of the exposed workers with PonE-55 genotype LL (70 cases) and genotype LM (5 cases) were 102.4±23.0, 82.8±22.0. The accumulative symptom scores were 4.5±4.2, 9.2±3.6. Single variance analysis showed that the accumulative symptom scores of the individuals with abnormal homozygote of these three gene loci were the highest, which indicated that they were most susceptible to OPs exposure. Multiple variance analysis showed there were no interactions among the three gene loci. Age, gender and exposure time had no statistical significance while genotypes of the three gene loci had significant relationship to health status. In conclusion, we found that the genotypes of BChE-K, PonE-192 and PonE-55 are associated with susceptibility to OPs exposure.

Another work of our research group detected the genotypes of enzymes (PonE-192, PonE-55, BChE, P450 and NAT2) and the polymorphic distribution via 7900 genotype detecting system and CMOS Chip technique. We found that the abnormal allele frequency of PonE-192, PonE-55 and BChE was respectively 37.8%, 1.9% and 13.7% whereas the abnormal homozygote frequency of PonE-192 and BChE was 15.0% and 1.6% with no abnormal homozygote of PonE-55 (Table 30). The genotypes of all enzymes reached Hardy-Weinberg balance.

We further analyzed the effects of the genetic polymorphism of enzymes on urinary DAP metabolites, esterase activity, signs and symptoms. The results showed that the polymorphism of P450 metabolic enzymes (CYP1A2, CYP2E1) influenced the concentration of urinary DAP metabolites (DEP, DEDTP) (Table 31). The genotypes of PonE-192 and PonE-55 influenced the activity of PonE. The genotype of PonE-192*AA as well as PonE-55*ML appeared with low activity (Table 32). Lower activity of the same genotype of PonE-192 and PonE-55 (working duration less than 20 years) was found, while the BChE activity of workers more than 20 working years had the higher inhibition. We also found a relationship between PonE, BChE and exposure dose by controling the influence of genetic polymorphism (Table 33). But there was no significant relationship between genetic polymorphism and examination abnormalities of exposed workers (Table 34). The activity of PonE was lowest in the workers with genotype of PonE192*AA + PonE55*ML + BChE*KK, and the AChE activtity was lower while signs scores was higher. The genotype of PONE192*AA + PonE55*ML + BChE*KK was the most sensitive. The liner regression analysis showed the polymorphism of PonE and BChE affected the activity of AChE, indicating that the gene polymorphism influence the health effects caused by OPs exposure (Table 36).

Gene loci	Genotypes	Cases	Allele	Allele cases	Allele frequency
	Gln/Gln(AA)	32	Gln	161	0.378
PonE-192	Arg/Gln(BA)	97			
	Arg/Arg(BB)	84	Arg	265	0.622
	Met/Met(LL)	205	Met	418	0.981
PonE-55	Leu/Met(ML)	8			
	Leu/Leu(MM)	0	Leu	8	0.019
	Ala/Ala (UU)	179	Ala	416	0.863
BChE*K	Thr/ Thr (KK)	58			
	Ala/Thr (UK)	4	Thr	66	0.137
	AA	114	A	145	0.797
CYP1A1	A/G	62			
	GG	6	G	37	0.203
	GG	55	G	103	0.575
CYP1A2	G/A	95			
	AA	29	A	76	0.425
	AA	8	A	44	0.243
CYP2E1	A/T	72			
	TT	101	T	137	0.757
	GG	104	G	125	0.839
NAT2	G/A	42			
	AA	3	A	24	0.161

Table 30. The genotypes of enzymes and the polymorphic distribution

Gene loci	Genotypes	Number of people	Urinary DAP metabolites (μg/gCr)				
			DMP	DEP	DETP	DMDTP	DEDTP
	AA	114	0.00	928	9.95	252	101
CYP1A1	A/G	62	0.00	187	8.36	151	109
	GG	6	0.00	512	103.7	355	60
p value			0.142	**0.015**	0.446	0.606	0.262
	GG	55	0.00	177	9.24	355	104
CYP1A2	G/A	95	0.00	145	9.96	164	101
	AA	29	0.00	402	7.39	149	111
p value			0.988	**0.027**	0.486	0.432	0.931
	AA	8	0.00	844	9.96	245	88.7
CYP2E1	A/T	72	0.00	104	12.9	222	125
	TT	101	0.00	150	7.53	222	94.2
p value			0.189	0.527	0.195	0.795	**0.032**
	GG	104	0.00	111	9.41	169	109
NAT2	G/A	42	0.00	996	9.39	181	86
	AA	3	21.8	191	6.84	655	79.8
p value			0.079	0.920	0.414	0.419	0.164

Table 31. The influence of polymorphism of P450 metabolic enzymes on urinary DAP metabolites level

Gene loci	Genotypes	Number of people	Esterase activity			
			BChE	CarbE	PonE	AChE
BChE	UU	179	33.26±9.13	512.91±186.09	150.81±98.64	122.00±6.68
	UK	58	40.52±17.00	552.31±116.9	148.67±70.05	126.19±9.40
	KK	4	39.34±18.28	500.87±189.18	140.65±70.33	123.60±8.71
p value			0.709	0.183	0.735	0.134
PonE-192	AA	32	43.99±31.17	518.04±183.97	94.32±44.18	123.66±10.68
	AB	97	39.43±14.91	503.79±195.26	154.32±71.54	125.69±8.09
	BB	84	39.89±16.25	518.47±193.92	146.04±68.57	125.53±9.56
p value			0.475	0.924	**0.000**	0.541
PonE-55	LL	205	40.37±18.98	511.87±190.3	144.25±69.53	125.36±9.25
	LM	8	38.49±7.79	623.61±97.37	85.45±50.75	123.88±7.45
p value			0.781	0.101	**0.019**	0.653

Table 32. The influence of esterase genetic polymorphism on esterase activity

Gene loci	Genotypes	Number of people	working age		t value	*p* value
			≤20 years	≥20 years		
PonE-192	AA	32	90.53±33.21	98.11±53.86	-0.479	0.635
	AB	97	137.36±63.34	175.62±76.17	-2.701	**0.008**
	BB	84	141.09±71.92	152.32±64.49	-0.743	0.459
PonE-55	LL	205	133.27±66.14	157.73±71.55	-2.538	**0.012**
	LM	8	109.27±50.97	61.62±43.56	1.421	0.205
BChE	UU	179	42.32±21.92	35.74±11.71	2.562	**0.011**
	UK	58	39.31±15.89	41.74±18.24	-0.542	0.590
	KK	4	33.26±9.13			

Table 33. The influence of esterase genetic polymorphism on esterase activity (nmol/ml·min) of workers in different working age groups

Gene loci	Genotypes	Number of people	Abnormalities (%) of medical examinations					
			WBC	Hb	ECG	B ultrasonic	SBP	DBP
BChE*K	UU	179	2.2	32.4	14.0	19.0	12.3	24.6
	UK	58	5.2	37.9	12.1	15.5	12.1	22.4
	KK	4	0	25.0	25.0	0	25.0	25.0
p value			0.389	0.693	0.988	0.333	0.751	0.783
PonE-192	AA	32	0	28.1	15.6	18.8	12.5	28.1
	AB	97	4.1	32.0	10.3	17.5	14.4	18.6
	BB	84	3.6	35.7	17.9	15.5	9.5	26.2
p value			0.308	0.715	0.334	0.893	0.602	0.361

Table 34. The examination abnormalities of exposed workers in different genetic polymorphism

BChE*K	PonE-192	PonE-55	Number	PonE (nmol/ml·min)	AChE (U)	Symptom scores
UU	AA	LL	1	83.39	123.00	2.00
UU	BB	LL	1	144.04	131.00	0.00
KK	AA	LL	22	97.91	124.5	1.64
KK	BB	LL	59	143.67	125.85	1.27
KK	BA	LL	70	157.47	126.29	1.70
UU	BA	LL	2	187.90	117.00	1.00
KU	AA	LL	5	114.10	118.20	1.80
KU	BB	LL	23	151.87	125.00	1.39
KU	BA	LL	22	147.88	124.64	1.18
KK	**AA**	**ML**	4	**52.59**	126.00	**5.00**
KK	BA	ML	2	134.83	121.50	0.50
KU	BA	ML	2	101.80	122.00	2.00

Table 35. The relationship between multi-genetic polymorphism and esterase activity and symptom scores

5. Conclusion

We present the research results conducted in China by Chinese scientists, mostly our research group. From these, we believe that the health problem caused by OPs exposure can't be ignored, though the exposure-response was not clearly elucidated. It is good that with the economic development towards better, the working condition has been improved and workers have less exposure to OPs. The traditional types of organophosphorus pesticides with high acute toxicity, such as methamidophos, parathion; methyl parathion and phosphamidon were prohibited in China, However, long-term and low level exposure to OPs is still a serious health problem and we should pay more attention to these public problems.

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5

Pesticide Exposure of Farmworkers' Children

Paloma I. Beamer
Division of Community, Environment & Policy
Mel and Enid Zuckerman College of Public Health, University of Arizona
United States

1. Introduction

In the United States (US), most manual labor in agriculture is completed by farmworkers. Although the total number of farmworkers in the US is not well characterized, recent estimates range between 2.5-5 million (Villarejo 2003, Hansen and Donohoe 2010, McCauley et al. 2006). They are predominately Spanish-speaking immigrants with a relatively low-level of education, on average about 7 years (Table 1). Their annual family earnings are far below the 2009 US poverty threshold of $21,756 for a family of four (US Census 2009a) and approximately 83% lack health insurance (Villarejo 2003). Farmworkers are hired by agricultural corporations, contractors, and farmers to plant and tend to crops, weed, pick and pack produce. As these tasks are primarily seasonal, most farmworkers are hired on a temporary basis, and they migrate from region to region depending upon work availability. In addition to many ergonomic challenges, farmworkers face a number of occupational exposures including: dust, sun, noise, and pesticides.

Pesticides in particular present a great hazard to farmworkers because of their widespread use and inherent toxicity. Not only are farmworkers exposed to pesticides while working in

Characteristic	Farmworkers	US Average
Education		
<6th grade	25-69%	
7th-12th grade	21-70%	16%[a]
Completed high school	2-21%	84%
Preferred Language		
English	6%	80%
Spanish	78-96%	12%
Other	1-22%	8%
Family Income		
<$10,000	19-24%	5%
$10,000-<$15,000	22-45%	3%
$15,000-<$25,000	23-49%	8%
≥$25,000	2-11%	84%

Table 1. Characteristics of farmworkers and comparison with US national average (McCauley et al. 2006, US Census 2009b, Thompson et al. 2003, Eskenazi et al. 2004, Coronado et al. 2006, Quandt et al. 2004, Salvatore et al. 2008). [a]Less than high school.

the fields, they and their families may also face increased pesticide exposure due to living in proximity to where the pesticides are applied. Furthermore, children of farmworkers may be exposed to pesticides in their homes that are brought in by the farmworkers on their clothing and shoes (Fenske et al. 2000). Acute health effects of pesticides include: nausea, dizziness, vomiting, headaches, abdominal pain, dermatitis and even death (McCauley et al. 2006). Chronic health effects of pesticides are also numerous and include respiratory and memory disorders, cancer, neurological deficits, Parkinson's disease, autism, infertility, congenital birth defects, and DNA damage (Alavanja et al. 2004, Kirkhorn and Schenker 2002, Eskenazi, Bradman & Castorina 1999, Blair and Zahm 1995, Alavanja, Hoppin & Kamel 2004, Kisby et al. 2009). Babies who are exposed to pesticides prenatally are more likely to have decreased birth weight and length, as well as a smaller head size (Berkowitz et al. 2004, Whyatt et al. 2004), which may predispose them to additional health concerns.

Although farmworkers typically spend more time in active contact with crops and may therefore have greater pesticide exposures than other agricultural workers, it is difficult to study chronic health effects resulting from their pesticide exposure because they are migratory, their work is transient and they may be marginalized due to their immigration status. However, Mills and colleagues (2009), cleverly linked work history records from the main farmworker labor union (United Farmworkers) with California's Cancer Registry and pesticide use database to determine significant increases in particular types of cancer associated with exposure to specific pesticides among farmworkers. Childhood cancers have also been associated with residential and parental occupational exposures to pesticides prenatally as well as pre-conception (Daniels, Olshan & Savitz 1997, Efird et al. 2003, Shim, Mlynarek & Van Wijngaarden 2009, Wigle, Turner & Krewski 2009, Turner, Wigle & Krewski 2010).

In addition to cancer, several studies have also documented associations between pesticide exposure and neurological disorders. Priydarshi and colleagues (2000) conducted a meta-analysis of studies reporting on pesticide exposure and Parkinson's disease. They report a combined odds ratio of Parkinson's disease associated with pesticide exposure of 1.85 (95% confidence interval: 1.31-2.60) for all studies and an odds ratio of 2.16 (95% confidence interval: 1.95-2.39) for studies from the US alone. More recent studies have confirmed these findings (Elbaz and Tranchant 2007, Hancock et al. 2008). Women who live within 500 m of fields where organochlorine applications occurred during pregnancy have an odds ratio of 6.1 (95% confidence interval: 2.4-15.3) that their child will be diagnosed with autism (Roberts et al. 2007). Guilette and colleagues (1998) determined that children who lived in a high pesticide use area had decreased stamina, eye hand coordination, 30 minute memory and the ability to draw a person.

As demonstrated by the previous studies, children may have increased susceptibility to pesticide exposure as their bodies are still developing and they have reduced capabilities of detoxifying pesticides (Eskenazi, Bradman & Castorina 1999, Faustman et al. 2000). Unfortunately, not only do children have a greater susceptibility to adverse consequences of pesticide exposure than adults, they also have increased potential for exposure to pesticides. They eat more food and drink than adults on a per body weight basis. They have increased absorption across their intestines and skin and have a greater surface area to volume ratio (Arcury et al. 2007). Young children spend more time playing on the floor and have a greater frequency of mouthing objects that may have pesticide residues. In addition to being exposed to pesticides in their homes via agricultural spray drift and the take-home pathway, it is not unusual for farmworkers to take their children with them to the fields while they

work and they themselves may be employed as well (Cooper et al. 2001). Compared to other industries in the US, current labor laws allow younger children to be employed in farm work. Children can start working in farm labor at age 12 years, and by 14 years there is no limit to the number of hours they can work. During the summer approximately 25% of farmworkers are children (<18 years of age) (Moses 1989). Farm labor children are more likely to drop out of school compared to other children and they have more injuries and fatalities than adults (Wilk 1993). Furthermore, it is not unusual for farmworkers to continue working in the fields while pregnant (Goldman et al. 2004). Thus, children of farmworkers are likely to be exposed to pesticides prenatally as their mothers continue to work, in their homes via agricultural spray drift and para-occupational exposure, and as field workers themselves.

In 1989, César Chavez described how these conditions lead to an increased potential for pesticide exposures and health effects among farmworkers and their children (Chavez 1989). However, at that time very little research had been completed on these topics. In the early 1990s, it was documented that farmworkers and their children were a high-risk population for pesticide exposure and associated adverse health consequences that had not been studied enough (Moses 1989, Rust 1990). Key data needs identified were: biological and environmental monitoring, longitudinal cohort studies, and additional studies of cancer and neurological development. In the past 20 years, over 50 studies have been completed that examine pesticide exposure in farmworkers' children. A literature review has been conducted to understand if farmworker children face high pesticide exposures, how these exposures occur, if these exposures have adversely affected their health, and if interventions to reduce these exposures have been successful. From this detailed review, directions for future research are identified.

2. Methods

This literature review focuses on pesticide exposure in children of farmworkers. The search terms used for this review included: pesticides, farmworker, farm workers, children, infants, exposure, and farm labor. I used these terms to search PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), Google Scholar (http://scholar.google.com/), and ISI Web of Knowledge (http://www.isiknowledge.com/wos). I concentrated my review on studies that reported primary data collection or analysis. I restricted my review to farmworkers, agricultural workers and individuals engaged in farm labor and their children. I excluded farmers and licensed pesticide applicators. Farmworkers tend to spend more time in the fields in direct contact with pesticides, use less safety precautions and personnel protective equipment, and are more likely to track pesticides into their homes (Mills et al., 2009). I also limited myself only to studies from the United States, as conditions are likely to be very different from other parts of the world. As my focus is on understanding pesticide exposure in the children of farmworkers, I did review articles that discussed measures of farmworker contamination of the home but mostly focused my attention on studies that had direct measures of children's potential exposure.

3. Results and discussion

More than 50 articles were identified covering topics of pesticide exposure, potential health effects and interventions to reduce the exposures in farmworker children. These articles

highlight the complexity in quantifying these children's exposures and relating these exposures to adverse health effects. It is necessary to have a valid understanding of the exposures over time, a report of the diagnosis, symptom or some sort of biological marker, and methods to link the individual exposure with the health effect (McCauley et al. 2006a). In these low income, extremely mobile populations with limited health access it is extremely difficult to link the exposures with the health outcomes.

Not only is keeping track of potential health outcomes complicated, these children are exposed to multiple pesticides via multiple exposure routes and pathways. Measurements with greater specificity of these exposures that have a high degree of temporal and spatial variability are extremely expensive and time intensive, however it is not clear if measurements with less specificity can provide enough power to detect adverse health effects from chronic exposures (Hoppin et al. 2006). It is difficult to compare across studies as different media are sampled with different collection and analytical methods for different pesticides. The studies have different limits of detection, examined children of different ages, use different methods to address samples below the detection limit and report different descriptive statistics in different units. The types of pesticides used vary greatly by geographic region and crop. The amounts of pesticide used can vary greatly throughout the year, depending on the growing cycles, but also there is quite a lot of variability between years depending upon weather and pests of concern. In the US, pesticides are constantly undergoing re-registration and their permitted uses can change dramatically. For example both chlorpyrifos and diazinon were banned for residential use. In spite of all this difficulty in comparing studies and the inherent uncertainties, our knowledge about pesticide exposure in children of farmworkers has greatly increased over the last 20 years through biomonitoring and environmental media studies. Although some interventions have been moderately successful, there are still many considerations for future research.

3.1 Health effects

In spite of the many barriers to studying associations between health effects and pesticide exposures in farmworkers' children (McCauley et al. 2006a), over the last 10 years several adverse health consequences have been associated with exposures in these populations. Our understanding of adverse health effects associated with biomarker levels has come predominately from one cohort in California. In this cohort of mostly farmworker families in the Salinas Valley, increased prenatal organophosphate metabolite levels was associated with a decrease in gestation and abnormal infant reflexes (Eskenazi et al. 2004, Young et al. 2005). Prenatal and postnatal dialkyl organophosphate urinary concentrations were significantly associated with developing pervasive developmental disorder, attention problems, and attention deficit hyperactivity disorder (Eskenazi et al. 2007, Marks et al. 2010) These associations were stronger in boys, which is concerning since they may have higher exposures and biomarker levels to begin with (Arcury et al. 2007, Koch et al. 2002). PON1 enzyme levels are associated with the ability to detoxify organophosphate pesticides. Infants in this cohort had 4-fold lower PON1 levels than their mothers and there was 26-fold range among the infants their levels, indicating a large range in susceptibility due to differences in detoxifying abilities (Furlong et al. 2006). Significant differences were reported by PON1 allele in the association between persuasive disruptive disorder and maternal pesticide metabolite levels (Eskenazi et al. 2010).

Adverse effects of pesticide exposure on agricultural children's neurodevelopment has been demonstrated in other geographic regions and cohorts. Organophosphate pesticide

metabolite levels in urine was associated with deficits in speed of attention, sequencing, mental and conceptual flexibility, visual search, and concept formation among children in a rural agricultural community in Arizona (Sánchez Lizardi, O'Rourke & Morris 2008). Similarly, children of agricultural workers in Oregon and North Carolina performed poorer on measures of response speed and latency compared to the non-agricultural children (Rohlman et al. 2005).

3.2 Biomonitoring

Considering the adverse health consequences identified it is important to determine if farmworker children have high levels of pesticide exposure and what is associated with increased levels. Biomonitoring is considered to be one of the most health relevant measurements of exposure since it is the measurement of environmental contaminants in biological fluids and represents the amount of chemicals that have actually been absorbed into the body and have the potential for adverse health effects. All of the studies that have conducted biomonitoring in farmworker children have evaluated pesticide metabolites in urine. I have summarized the biomonitoring studies in Table 2. The majority of these studies were conducted in populations in Washington, Oregon and North Carolina. As evident from Table 2, these studies report different descriptive statistics from many different pesticide metabolites in different units, making it difficult to compare across studies. Furthermore, these studies mostly report the concentration of non-specific organophosphate pesticide metabolites making it impossible to elucidate which pesticides the child was exposed to or if the child was exposed to the metabolite and not the parent compound (Barr et al. 2006).

Even with all of these complexities, it is clear that farmworkers' children most likely face higher exposures as a result of their parents' employment and proximity to agricultural fields where pesticides are applied (Table 2). Several studies have reported that children of farmworkers have a greater concentration of pesticide metabolites in their urine compared to reference populations (Coronado et al. 2006, Arcury et al. 2007, Mills and Zahm 2001, Lu et al. 2000, Lambert et al. 2005). In Washington State median dimethyl organophosphate pesticide metabolite levels were 4-5 times higher in farmworker children than in reference children from the same community and not significantly different from children of pesticide applicators (Lu et al. 2000). When these metabolite levels were used to estimate dose, researchers determined that 56% of the children of either orchard applicators or field workers exceeded the US EPA chronic dietary reference dose, compared to 44% of other children whose parents did not work in agriculture (Fenske et al. 2000). While most studies have focused on non-specific organophosphate metabolites, Arcury et al. (2007) measured the urine of children 1-6 years of age in North Carolina for 14 specific pesticide metabolites. They report a median of 4 different pesticides detected in the children's urine and a maximum of 7 detects in one child. Although difficult to compare with a national reference population because of differences in age, levels were higher in general in the farmworker children for chlorpyrifos, diazinon and parathion.

There are however, results that indicate that farmworker children may not have increased exposure to all pesticides. For example, although there was a difference in dimethyl organophosphate metabolite levels between agricultural children and a reference population, there was not a significant difference for diethyl organophosphate metabolites in the same Washington populations. (Fenske et al. 2002). Follow up studies have failed to demonstrate increased exposures among farmworker children compared to suburban

Metabolite	Group	Characteristics	Units	n	Mean	Median	Max	Study
dimethyl	farmworker	California, 5-27 months old	umol/L	20		0.13	4.4	Bradman et al., 2007
		California, does not report age	ppb	9	21.99			Mills and Zahm, 2001
		Oregon, Berry Community, 2-6 years old	umol/L	52		0.23		Lambert et al., 2005
		Oregon, Cherry Community, 2-6 years old	umol/L	52		0.25		Lambert et al., 2005
		Oregon, Pear Community, 2-6 years old	umol/L	52		0.44		Lambert et al., 2005
		North Carolina, 1-6 years old	umol/L	60	0.205	0.07	2.01	Arcury et al., 2006
		Washington, 2-6 years old	umol/L	211		0.08	15.4	Curl et al., 2002
		Washington, 9 months to 6 years old	ug/mL	13	0.07	0.05	0.2	Lu et al., 2000
	reference	Washington, Urban, 2-5 years old	umol/L	96	0.19	0.11	0.93	Lu 2001
		Nationwide, 6-11 years old	umol/L	471		0.050		Barr et al., 2004
		Washington, 9 months to 6 years old	ug/mL	14	0.06	0.01	0.3	Lu et al., 2000
diethyl	farmworker	California, 5-27 months old	umol/L	20		0.001	0.21	Bradman et al., 2007
		California, does not report age	ppb	9	5.73			Mills and Zahm, 2001
		North Carolina, 1-6 years old	umol/L	59	0.06	0.04	0.52	Arcury et al., 2006
		Washington, 2-6 years old	umol/L	211		0.06	0.23	Curl et al., 2002
	reference	Washington, Urban, 2-5 years old	umol/L	96	0.05	0.04	0.31	Lu 2001
		Nationwide, 6-11 years old	umol/L	471		0.014		Barr et al., 2004

Table 2. Organophosphate pesticide metabolite levels in farmworker and reference children.

children in Seattle (Thompson et al. 2003, Lu et al. 2001, Fenske et al. 2005, Curl et al. 2002). The researchers concluded that dietary exposures may be the primary route for all children regardless of parental occupation. Koch et al. (2002) also fails to report an association with parental occupation and children's pesticide metabolite levels in an agricultural community in Washington, however, they grouped packing shed workers into the same category as truck drivers and sales people. As packing shed workers are also exposed to high levels of pesticides, this could have confounded their analysis (Calvert et al. 2008).

In one cohort in Washington State, children's urinary dialkyl organophosphate metabolite levels were significantly correlated with their parents' metabolite levels (Curl et al. 2002). In this population the geometric mean concentration of dimethyl organophosphate metabolites was 1.5-2.6 fold higher for children who lived in households with a pome (apple or pear) fruit worker than children whose parents did not work in those crops (Coronado et al. 2006). In addition, the children in this study whose parents reported thinning crops had a greater proportion of detectable residues in their urine than children of other farmworker parents (Coronado et al. 2004). However, they did not have statistically higher concentrations (Fenske et al. 2004). As 91% of the thinners worked with pome fruit, after controlling for crop there were no longer any differences between thinners and non-thinners. Similarly in another study, agricultural children from a pear community in Oregon had 1.9 and 1.8 times the dimethylthiophosphate metabolite levels in their urine compared to children from the berry and cherry communities, respectively (Lambert et al. 2005). This indicates that working with pome-fruit may pose a particular hazard to children of farmworkers.

Further analysis of biomonitoring data from this region also indicates potential exposure pathways. Children who lived within 200 ft from an orchard did have higher levels of dimethyl but not diethyl organophosphate metabolites in their urine, than those living farther away indicating that exposure may be related to proximity for certain pesticides (Fenske et al. 2002, Loewenherz et al. 1997). In addition to correlation with proximity to orchards, children's dimethyl organophosphate metabolite levels were also significantly correlated with the concentration of azinphos-methyl pesticides in their house dust in Washington State in two cohorts (Coronado et al. 2006, Lu et al. 2000, Curl et al. 2002). In another study population from California, children's diethyl organophosphate urine metabolite levels were significantly correlated with the concentration of pesticides in house dust and toy wipes (Bradman et al. 2007). The correlations with proximity and house dust concentrations demonstrate the complexity of farmworker children's exposures to pesticides, and that these children probably are exposed both from agricultural spray drift and the take-home pathway from their parents' occupation. Children's incidental ingestion exposure from house dust and mouthing other objects is directly related to their unique activity patterns as well as the pesticide loadings in their home (Beamer et al., 2009), which further complicates the elucidation of these associations. Although not among farmworkers' children, but in a rural agricultural community, loading of pesticides on children's hands was highly correlated with urinary biomarker levels and may be a better predictor than other environmental exposure measures including house dust concentration as it is a measure that integrates the children's unique activities and the pesticide levels in their homes (Shalat et al. 2003).

Additional risk factors for elevated levels of pesticides in the urine of farmworkers' children have been identified. Researchers in North Carolina and Virginia conducted a case comparison analysis. They determined that higher amounts of organophosphate pesticide

metabolites in adults and children living with a farmworker were related to households without a nuclear family structure because the additional residents tended to be additional male farmworkers that were more likely to be exposed to pesticides at work and bring them home on their clothes (Arcury et al. 2005). Also these workers tended to have to wait longer to shower and stayed in their clothes longer after work. Families who rented their home, did not have a vacuum cleaner, had a difficult to clean home, or a high percentage of carpeting flooring also had higher levels of organophosphate pesticides in their urine. Higher levels of metabolites were associated with improper handling of laundry and storage of work clothes (Arcury et al. 2005). In a larger cohort in North Carolina, the same researchers determined that children had a greater number of specific pesticide metabolites detected in their urine only if they lived in rented houses or if their mothers worked part time in farm work (Arcury et al. 2007). However, they found no predictors for non-specific organophosphate metabolite levels (Arcury et al. 2006). Similarly, in Washington State the only factor associated with increased non-specific urinary metabolite concentrations was reported organophosphate pesticide use in the garden (Fenske et al. 2002). This indicates that many of the questions or factors commonly assessed may be not be useful predictors biomarker levels, or appropriate to target in interventions aimed at reducing children's pesticide urinary levels.

As demonstrated already by this review, there are many uncertainties associated with biomonitoring of pesticide metabolites in urine. Additional uncertainties include temporal variation in urine concentration (Barr et al. 2006). Children who live in agricultural communities in Washington had higher DAP metabolites during the spraying season than during the non-spraying season (57-40% difference) (Koch et al. 2002). Season of collection also related to pesticide metabolite concentrations in North Carolina (Arcury et al. 2005). In Oregon, 3 samples were taken from each child over the course of the season and there was substantial intra-individual variability indicating that it is important to obtain multiple samples even if they are combined prior to analysis (Lambert et al. 2005). In addition to concerns about temporal variability over the course of a growing season, there is also concern of temporal variability in urine concentration over the course of a day. Some studies collected a spot (grab) sample, while others collected first morning voids or 24-hour samples and it is not clear which type of samples are most relevant for understanding pesticide exposures. Kissel and colleagues report that first morning void samples may be the best spot collections to obtain (Kissel et al. 2004). Bradman and colleagues (2007) reported that overnight and spot urine samples were significantly correlated in a population of farmworker children from California. Furthermore there may be differences in metabolic and clearance rates between people and due to differences in hydration and dietary intake throughout the day. Some studies normalize their values by the concentration of creatinine measured in the urine sample, however there has also been reported seasonal variation of creatinine levels which may add additional complexities (O'Rourke et al. 2000).

Boys have higher reported metabolite levels than girls in two studies (Arcury et al. 2007, Koch et al. 2002), and no studies report higher metabolite levels in girls. Among applicator children in Washington, younger children had higher concentrations of urine metabolites than their older siblings (Loewenherz et al. 1997). In North Carolina, differences in pesticide metabolite levels as a function of age were observed by organophosphate class. Children 3-4 years of age have higher levels of diethyl but lower levels of dimethyl metabolite levels than children 1-2 years or 5-6 years (Arcury et al. 2007, Arcury et al. 2006). It is not clear if this is

due to differences in activity patterns (Beamer et al. 2008) or physiology as metabolism, clearance and volume of distribution change with age and gender (Beamer 2007).
As demonstrated by this review of biomonitoring studies in farmworker children, it is difficult to compare values across studies even when they were analyzed by the same laboratory and/or using the same procedures. Pesticide metabolite levels will also be affected by the subject's unique activity patterns and physiology. Furthermore pesticide usage is highly variably and is a function of many variables including crop type and pest infestation levels (Fenske et al. 2005). Pesticides are also metabolized relatively rapidly by the body, and urinary metabolite levels may not be the best marker of long-term exposure.

3.3 Environmental media and personal exposure measurements

Environmental media and personal exposure measurements, especially house dust may be more persistent for rapidly metabolized pesticides and helpful in determining risk factors for chronic exposure. It is important to understand if the levels of pesticides in farmworker homes are high compared to other homes and what factors are associated with increased levels in the homes, in order to design more effective intervention strategies. However, like for biomonitoring it is complicated to compare environmental and exposure measurements across studies (Hoppin et al. 2006). Factors that can vary between studies include media sampled such as dust, soil, air, hand and surface wipes. These samples are collected with a variety of collection methods including wipes and vacuum. Different size fractions may have been analyzed for dust, soil and air concentrations. The samples may have been taken from vastly different locations in the home such as child's bedroom or the living room. Different analytical methods have been used, and different quantities of samples have been collected which can result in a wide range of detection limits. Furthermore as in the case of biomarker levels, these measurements will also be affected by site-specific factors including pesticides of interest, geographic region, crops raised, pests of concern and calendar year or season.
Non-dietary ingestion of pesticide residues contributes the most to farmworker children's aggregate organophosphate pesticide exposure (Beamer 2007, Beamer et al. 2009). Primary measures of dust ingestion exposure are house dust concentrations, hand wipes or rinses and surface wipes. Although there are several studies that have quantified pesticide levels in multiple media in farmworker homes (Lu et al. 2000, Bradman et al. 2007), levels in house dust have been reported most frequently. In general, the concentration of agricultural pesticides is higher in farmworker homes (Table 3). During the sample collection period for these studies, chlorpyrifos and diazinon were still allowed for residential purposes and the large variability observed in reference homes most likely indicates indoor applications. Furthermore, concentration of pesticides in dust from farmworker homes was associated with household pesticide use in California and Washington (Fenske et al. 2002, Bradman et al. 1997) but not in Oregon (McCauley et al. 2001, McCauley et al. 2003).
Several studies have demonstrated that proximity to fields where pesticides are applied is related to pesticide levels in house dust (Lu et al. 2000, Fenske et al. 2002, McCauley et al. 2001). In addition to higher levels of pesticides in surface wipes, Quandt et al. (2004) also reports that they detected a greater number of different pesticides in the wipes in relation to proximity. Although Fenske and colleagues (2002) did report that house dust pesticide concentration decreases with distance to field among agricultural families, this did not manifest in differences in urinary metabolite levels. Curl et al. (2002) failed to demonstrate an association between house dust levels and proximity, indicating that geographic location and local wind patterns may be important to consider.

Pesticide	Population	Location	n	Mean	Median	Max	Study
Azinphos methyl	Agricultural	Vehicle, Washington	190		0.85	38.3	Curl et al., 2002
		House, Washington	156		0.53	14.9	Curl et al., 2002
		House, Washington	13	1.47	0.75	5.3	Lu et al., 2000
		House, Washington	22	1.62	0.951	11.3	Simcox et al., 1995
		House, Oregon	26	59	5.3	16	Rothlein et al., 2006
	Reference	House, Washington	14	0.29	0.15	1.1	Lu et al., 2000
		House, Washington	11	0.33	0.283	0.82	Simcox et al., 1995
Chlorpyrifos	Agricultural	House, California	20		0.049	1.2	Bradman et al., 2007
		Vehicle, Washington	190		0.05	2.6	Curl et al., 2002
		House, Washington	156		0.03	2	Curl et al., 2002
		House, Oregon	26	0.2	0.13	1.2	Rothlein et al., 2006
		House, Washington	12	0.27	0.25	0.6	Fenske et al., 2002
		House, Washington	22	0.338	0.172	2.2	Simcox et al., 1995
	Reference	House, Washington	14	0.09	0.07	0.3	Fenske et al., 2002
		House, Massachusetts	119		ND	228	Rudel et al., 2003
		Day Care, North Carolina	4	0.107		0.3	Wison et al., 2003
		House, Maryland	126	2.38	0.355	27	Pang et al., 2002
		House, Washington	11	0.168	0.053	0.5	Simcox et al., 1995
Diazinon	Agricultural	House, California	20		0.021	0.8	Bradman et al., 2007
		Vehicle, Washington	190		0	0.8	Curl et al., 2002
		House, Washington	156		0.01	0.6	Curl et al., 2002
		House, Oregon	26	0.31	0.31	0.7	Rothlein et al., 2006
	Reference	House, Massachusetts	119		ND	51	Rudel et al., 2003
		Day Care, North Carolina	4	0.034		0.1	Wison et al., 2003
Phosmet	Agricultural	House, Washington	13	0.14	0.11	0.3	Lu et al., 2000
		Vehicle, Washington	190		0.02	34.9	Curl et al., 2002
		House, Washington	156		0.02	16.9	Curl et al., 2002
		House, Oregon	26	5.2	4.4	22	Rothlein et al., 2006
		House, Washington	22	2.54	0.519	17.1	Simcox et al., 1995
	Reference	House, Washington	14	0.09	0.09	0.2	Lu et al., 2000
		House, Washington	11	0.227	0.187	0.7	Simcox et al., 1995

Table 3. Concentration of pesticides in house dust (μg/g) in farmworker and reference populations.

There is ample evidence for the take-home exposure pathway, given that when sampled together, homes with farmworkers had more pesticides in the dust than homes with no farmworkers (Lu et al. 2000, Bradman et al. 1997, Simcox et al. 1995). In Washington State, the median house dust concentrations of dimethyl organophosphate pesticides was 7 times higher in the homes of agricultural families compared to reference families, and 10 out of 61 agricultural children had detectable pesticides on their hands while no reference children did (Lu et al. 2000). Similarly, in the agricultural households pesticides were detected on 45% of the parents work boots and 11% of the steering wheels of the family car, while no samples from reference families had any detectable levels. In another study in Washington the concentration of azinphos methyl in house dust was highly correlated with the concentration in dust from the household vehicle, indicating that the vehicle is a likely pathway for transporting pesticides from the fields to the home (Curl et al. 2002).

The contribution of the take-home pathway to household contamination may also be dependent on the number of people bringing the pesticides from the fields. In Oregon, the concentration of azinphos methyl in house dust was correlated with the number of farmworkers living in the house (McCauley et al. 2001). The composition of the household may also be a factor. Households with a non-nuclear family structure had higher levels of agricultural but not residential pesticides in wipe samples (Quandt et al. 2004). In this study the non-familial household members tended to be almost entirely additional farm laborers. Due to the number of workers in these households, they also tended to delay showering and changing out of work clothes compared to households with a nuclear family structure. McCauley and colleagues (2003) demonstrated that changing out of work clothes <2 hours from coming home from the fields was associated with significantly lower levels of azinphos methyl and total organophosphate pesticides in house dust.

Factors from the workplace environment may also relate to the potential contribution of the take-home exposure pathway. Farmworkers that reported being involved in pesticide application at work had a greater amount of pesticides in their home (Quandt et al. 2004). In Washington licensed pesticide applicators had higher levels of pesticides in their homes than even farmworkers (Fenske et al., 2005). Farmworkers who reported burning eyes, pain muscles/joints/bones, shortness of breath and blurred vision, indicating that they may be exposed to greater amounts of pesticides at work, were also more likely to have methyl-parathion, azinphos methyl, malation or phosmet detected in the dust from their vehicles or homes (Strong et al. 2004). Coronado and colleagues (2006) reports that farmworkers who worked in pome fruit had significantly higher levels of azinphos methyl in their vehicles and house dust. These studies indicate that increased exposures at the workplace likely lead to greater contamination of the farmworker homes and potentially greater exposures of their children.

Household characteristics and behaviors may also lead to increased pesticide levels in the homes. Quandt and colleagues (2004) demonstrated that a higher amount of pesticides in farmworker homes was associated with mobile houses and those who rented their home. The same researchers also report the presence of a high amount of pesticides in farmworker homes that were determined to be "difficult to clean." In a pilot study in the Central Valley of California the frequency and method of cleaning was also related to the concentration of pesticides in house dust in farmworker homes as well as the age of the farmworker home (Bradman et al., 1997).

3.4 Pesticide risk perception

Given the importance that individual and household behaviors have on the concentration of pesticides in house dust (Quandt et al., 2004; McCauley et al., 2003), it is important to understand how farmworkers perceive their risk to pesticide exposure and use of safety practices. Several studies have interviewed farmworkers about safety practices at work and in the home. These are summarized in Table 4. Although most farmworkers do launder work clothes separate from family clothes, many do not utilize safety practices that could reduce their own and their family's exposures. Cabrera and Leckie (2009) demonstrated this even in a community of farmworkers in California with a high level of understanding of the risk of pesticide exposure. Interestingly, in a study be Goldman et al. (2004), acculturation was associated with a decrease in self-protective behaviors among pregnant farmworkers. Elmore and colleagues (2001) documented that perceived lack of control and cultural health beliefs were the primary factors that decreased farmworkers' use of safety practices. In another study perceived risk of pesticide exposure had a limited relationship to safety knowledge and was also not related to safety behavior, while perceived control was strongly related to safety knowledge and safety behavior (Arcury, Quandt & Russell 2002). Interviews with mothers in farmworker households document that they experience difficulty incorporating household safety practices and behaviors in their homes because of competing responsibilities, perceived lack of control, community barriers, conflicts with their husbands' intentions and their own cultural health beliefs (Strong et al. 2009). These studies demonstrate that pesticide safety education must address issues of farmworkers' perception of control to be most effective.

Behavior	Frequency (%)
Wears protective clothing	18-67.3
Enters home with work clothing	76-98
Changes clothing when arriving home	40-66.7
Removes shoes before entering home	40-68
Launders clothes separately	56-80

Table 4. Frequency of self-protective behaviors (McCauley et al., 2001; Arcury et al., 2006; Cabrera and Leckie, 2009; McCauley et al., 2003; Thompson et al., 2003; Goldman et al., 2004).

3.5 Interventions

Given the health effects associated with chronic pesticide exposure, effective interventions are needed to reduce farmworkers' and their children's exposure to pesticides. While environmental and occupational health policies and laws are helpful in reducing these exposures, they are only useful to the extent that they can be enforced. This however, presents many challenges. For example, in 2008 there were 81,500 farms operating in the state of California (USDA 2010). During that year the California Occupational Safety and Health Administration conducted 1,113 agricultural inspections (OSHA 2011). They reported that 57% of farms were out of compliance. 251 of these inspections were as a result of an accident. At this rate a farm in California can expect a random inspection once every 95 years (81,500 farms/862 random inspections). Furthermore in 2007, California farms were in full compliance with health and safety laws in 51% of the pesticide poisoning cases indicating that the laws may not be protective enough of the workers.

Arcury and colleagues (1999) interviewed farmworkers to assess how the Environmental Protection Agency's Worker Protection Standard was being implemented. Only a third of the farmworkers reported any pesticide training. Workers with visas were more likely than those without visas to have received training. However, very few workers knew how they could be exposed to pesticides or reported using any method to protect themselves. In a follow up study, Arcury et al. (2001) continues to document that farmers did not adhere to regulations mandating training and basic sanitation facilities. However, similar to the problems in California, there are only 30 staff memebers to ensure that regulations are upheld at over 10,000 farms in North Carolina. Arcury and colleagues (2001) argue that additional regulations by themselves are not an advantageous starting point, as that creation of additional regulations will probably only make employers feel more alienated and less likely to comply. They conclude that interventions aimed at educating farmers as well as farmworkers, perhaps through cooperative extension agents, may be more effective and help reduce misunderstanding and distrust between the two groups.

Lay community health workers called "promotoras" are known to be very effective at changing health-related behaviors in low-income Latino communities. A few interventions have utilized them in an attempt to reduce pesticide exposures in the farmworker communities. Arcury and colleagues (2009) evaluated a promotora intervention in North Carolina. Promotoras conducted either a nutrition or pesticide curriculum with their clients. Participants in the pesticide curriculum were more likely to recall the promotora visit and the messages, however the only significant difference between the two groups was that they were more likely to know that pesticides may have an effect on their children's health. The intervention was not successful at increasing pesticide safety practices in the home. Researchers in New Mexico developed a comic book for promotoras to use in educating their clients about pesticides (Liebman et al. 2007). The promotoras reviewed the comic book with 273 participants. A post-intervention evaluation documented that participants successfully increased knowledge about exposure routes, children's vulnerability to pesticide exposure, the signs and symptoms of pesticide poisonings and the ways to minimize pesticide exposures in the home. However these researchers did not evaluate if this increased knowledge manifested itself in an increase of safety practices in the home.

Teran and colleagues (2008) targeted an educational intervention at a particularly vulnerable group; teenagers that engage in farm labor. They incorporated a pesticide safety curriculum into "English as a Second Language" classes at high schools in an agricultural community. Almost all of the students reported working with pesticides under the age of 16 years. The teenagers in the intervention group were less likely to report that there was not much they could do to avoid their exposures and they were also less likely to report that it was not worth trying to improve conditions. They were more likely to report that working with pesticides can cause health problems and cite more laws protecting agricultural workers. This manifested in them being more likely to wear a long sleeve shirt and a hat while working in the fields. However, they were not more likely to wash their hands before eating. This is an example of an intervention that has great potential for empowering youth, who may be more vulnerable to the risks of pesticide exposure, and potentially reducing their exposures. In the future this curriculum should be modified to result in more protective behaviors.

There are very few interventions described in the literature that compare measurements of pesticide exposure before and after an intervention. Perhaps the most ambitious and

comprehensive intervention to reduce pesticide exposure in farmworkers' children was a community-wide intervention trial called "Para Niños Saludables" (Thompson et al. 2003). The study was conducted in 24 communities in Eastern Washington, where communities as a whole were randomized into intervention and control groups. A community advisory board designed the intervention. The intervention consisted of activities that were community-wide down to individual interactions. Community wide activities consisted of hosting health fairs, festivals, and block parties, where educational materials were disseminated from booths. Schools, churches, English and citizenship classes, orchards, farms, farmworker clinics and the farmworker union were organizations targeted for intervention opportunities. Promotoras held over 1,100 small home health parties over the 2-year intervention. Additional volunteers went door-to-door or educated workers one-on-one at grocery stores and other community locations. Materials developed and approved by the community advisory board that were distributed for the intervention included educational pamphlets, child coloring books, balloons, sample packets of laundry detergents, clothes sorting bags, bins for storing boots outside, shower kits, and infant bibs with the message "Keep me pesticide free." A puppet show, local media messages, and an annual calendar design competition were used to promote pesticide protection messages. Cross sectional samples of house dust and children's urine were taking across all the communities during years 1 and 4 of the trial and assessed for pesticide levels.

This intervention did result in increased pesticide safety practices in both intervention and comparison communities over time (Strong et al. 2009). Changes were significantly greater in intervention communities for removing work shoes before entering the home and for changing out of work clothes within 1 hour of arriving home. The only specific intervention activity associated with increased precautions was participation in home health parties with promotoras, confirming this as an effective method of reaching the farmworker community. However, there were not any significant differences in pesticide metabolite levels in adults or children, or in the pesticide concentration in house dust or vehicle dust between intervention and control communities (Thompson et al. 2008). There are a number of factors that could explain the lack of ability of this trial to document a reduction in pesticide exposures. Pesticide use patterns varied dramatically between years 1 and 4. There may have been low pesticide use during the baseline year, as demonstrated by pesticide exposure levels being much lower than in other farmworker children populations from Washington State (Fenske, R.A. 2005). Also the use of certain pesticides was restricted over the trial period leading to an increase in the use of different pesticides. The community cross-sectional design of the trial may not have been powerful enough to detect small changes, and it is impossible to determine if the same farmworkers and their homes were sampled in both years. There may have been cross contamination with farmworkers from control communities participating in events at intervention communities or even moving to intervention communities, and thus changing study groups.

McCauley and colleagues (2006b) evaluated the effectiveness of a cleaning intervention in removing pesticides from farmworker homes. Conventional cleaning of linoleum floors was not effective in reducing the total amount of organophosphate pesticides, however this varied among the pesticides. Alternative cleaning products and methods should be evaluated for reducing organophosphate loading on hard floors. Steam cleaning of carpets did reduce the level of pesticides in substantially in homes with higher pesticide levels, but pesticide levels were approximately one third of baseline levels after 12 months. This

indicates that cleaning practices may need to be more intensive and on going to result in an overall decrease of pesticides from carpets.

3.6 Future directions

The review of intervention studies demonstrates that educational interventions primarily through promotoras or English classes can be effective at increasing farmworkers and their families perception of control and utilization of self-protective behaviors (Teran et al 2008, Liebman et al. 2007, Strong et al. 2009). However, to date no single intervention study has documented a decrease in actual pesticide exposures faced by farmworker children. Although the intervention designed for the "Para Niños Saludables" trial is certainly ambitious, comprehensive and admirable, there may have been some issues with the intervention design that could be examined in future studies. The intervention was conducted only over 2 years. Bradman et al. (2007) reports detection of DDT in the majority of multi-media samples taken from farmworker homes 30 years after it was banned for use in California. Arcury et al. (2007) detected pesticides in children's urine that had been banned more than 10 years prior to their birth. The results of these studies may indicate that the residence time of pesticides in farmworker homes is very long, and two years was most likely not an adequate time period to see a reduction of pesticides in house dust nor in children's urine. McCauley et al. (2006b) demonstrated how difficult it is to reduce pesticide levels in farmworker homes following an intensive cleaning operation. In order to determine the effectiveness of a behavioral intervention on reducing pesticide contamination of house dust in a reasonable time span, procedures for decontaminating farmworker homes from pesticides need to be developed, tested and implemented prior to the intervention.

The "Para Niños Saludables" intervention focused on reducing the take-home exposure pathway whereby pesticides are tracked into the homes on clothes and shoes. However, it is possible that pesticides are primarily entering the home via air infiltration of agricultural spray drift and resuspended soil. In another study it was determined that approximately 60% soil contaminants present in house dust are from air infiltration of resuspended soil rather than soil track-in on shoes (Layton and Beamer 2009). In addition to presence of farmworkers in the home, household proximity to fields where pesticides have been applied is also associated with pesticide levels in house dust (Lu et al. 2000, Fenske et al. 2002, McCauley et al. 2001). In their cleaning effectiveness study, McCauley et al. (2006b) report higher loadings of pesticides on the windows than on hard floors. This may demonstrate that the primary source of pesticides in the home in agricultural communities may be from natural ventilation through windows. Future studies should be conducted to determine the relative contributions of air infiltration and soil-track in leading to pesticide levels in house dust, in order to design interventions that are more effective in reducing children's pesticide exposure. If air infiltration is the primary pathway, regulations to reduce drift from pesticide applications will need to be reviewed and families in agricultural communities regardless of occupation will need to be educated about spray drift and how to reduce their exposures.

Most of the current biomonitoring studies of farmworker children have measured non-specific organophosphate metabolites. There is limited data on specific pesticide levels. More studies should be completed like that by Arcury et al. (2007) that will allow us to identify key pesticides that children are exposed to, in order to more effectively target future intervention strategies. As organophosphate pesticide use is decreasing and other pesticides are being used instead, it is also important to develop and use biomonitoring methods for these pesticides. We should consider examining pesticide exposures of children whose

parents are not farmworkers but may face even higher pesticide exposures. For example, in a recent analysis of the 1998-2005 US Acute Pesticide Poisonings, farmers have an incidence rate of 4.8/100,000 full time equivalents compare to 74.8 for farmworkers and 362.6 for processing/packing plant workers (Calvert et al. 2008).

4. Conclusion

In conclusion, children of farmworkers are exposed to pesticides at levels that can result in adverse health effects, particularly affecting neurodevelopment. The children's exposure levels are correlated with adults in their households, and is related to the crops and tasks that their parents engage in. There is evidence that household proximity to pesticide application and the number of household members engaged in farm work relate to pesticide contamination levels in their homes. Interventions have been conducted that are effective for increasing farmworkers self-protective behaviors and perception of control. However, they have not been successful at reducing pesticide exposure levels. Future studies should focus on effective decontamination of farmworker homes, understanding the relative contribution of air infiltration of pesticides, obtaining better exposure measurements of specific pesticides and examining exposures to children of other high-risk agricultural workers.

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Migrant Farm Workers Exposed to Pesticides in Sinaloa, Mexico

Anthon Alvarez A. and Alba D. Campaña S.
Faculty of Medicine University of Sinaloa
Mexico

1. Introduction

Mexico is one of the major importers of pesticides in the whole of Latin America. Farm workers represent one of the poorest sectors of the population in Mexico. Every harvest season, an estimated 300,000 children between the ages of 6 and 14 migrate to the northwest part of this country along with their parents, who have been contracted to work in the fields. At about age 10, half of those children begin working, and the number rises sharply as they get older. The inclusion of workers in the agricultural labor process depends on the following stages: seed, staking, weeding, cutting, harvesting and packing in addition to the application of pesticides, (the organophosphates and carbamates types are used more frequently) (Palacios et al., 2000).

Fig. 1. SINALOA STATE, *Mexico* By Thomas Nybo

Sinaloa State, Mexico is a federal entity in which there is extensive agricultural development and where production is mainly for the export market and less for the domestic one. Highly technical production processes are used which reduce cropping areas, but whose performance has increased the demand day laborers. According to (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación [SAGARPA], 2004), the agricultural area amounts to 1'266, 120 hectares (one hectare=2.471 acres) and 1,175,425 hectares are harvested.

These are large producers, who spend their production to foreign markets mainly medium and small and primarily oriented to the internal market.

According to the situational diagnoses made by the Laborers Care Program, agricultural day laborers amounted to 113 thousand people, of whom 72 000 are mostly migrants from the states of Guerrero and Oaxaca. The type of migration that occurs is primarily family and to a lesser extent, men migrating alone. Some 33.08% of the population is reported to be illiterate, of which 42.32% are male and 57.68% are women, while 45.07% are children between 6 and 14 who cannot read or write. Northern, it includes the towns of Ahome, El Fuerte, Choix, Guasave, and Angostura, where vegetables, grains and fodder are the main produce. In 2003 there was an estimated (Programa Nacional de Jornaleros Agrícolas. [PRONJAG], 2006), laborer population of 38.092 workers, of whom 31.371 were considered migrant population. A feature of this area is that the largest number of migrants are from the same state. Center Zone, corresponds to the Valley of Culiacan and municipalities, Elota, Mocorito and Navolato, producing mainly vegetables (tomato, tomato, cucumber, zucchini, eggplant), which are channeled to the export market, also corn and fodder that are destined for the domestic market. There are about 67 thousand laborers.

According to some studies (Palacios-Nava et al., 2004) on socio-demographic characteristics, the migrant population usually consists of about 75% to 25% men to women. The majority of the migrant population is young. However, this feature is accentuated in the case of women, since significant differences were found in the age distributions according to sex ($p = 0.000$). Of all women, 23% were between 8 and 14 years of age, while only 8.2% of men were found in that age. It was noted that female participation decreased as age increased, so that 76% of women were between 8 and 34, and 82% of men between 15 and 44. One of the most important migrations is to move day laborers pole to pole group of agricultural development, 91% women traveled with their families, whereas the figure for men was 65% ($p = 0.000$). There were no differences in marital status; a large percentage (58) were married, 37.7% single and the percentage of widowed or divorced was slightly higher in women than the figures for men (4.1 vs. 1.3%). According to a survey of 8 117 heads of household laborers in the main farming region, 29.3% were born in the State of Guerrero (Grammont & Lara, 2004). The migrant´s social networks play a fundamental role in availability of casual labor. Flexible production, efficiency and low cost, despite its limitations, is played over time, and according to the (Secretaria de Desarrollo Social [SEDESOL], 2004), they exceed 10 000 per season.

Significant differences were observed in place of origin ($p = 0.000$), the majority of women were from the states of Guerrero (47%), Oaxaca (25%) and Veracruz (16%), while men came mostly from Guerrero 37%), Sinaloa (24%) and Veracruz (22%). Most women traveling alone were from the state of Oaxaca.

The majority are migrants, mostly from the states of Guerrero, Oaxaca and other entities in the country. This migration has a family character. South Zone, includes the towns of Mazatlan, Rosario, and Escuinapa producing mainly chilli and mangoes. The labor force used is mostly local in character, amounting to 33 thousand people (Ramirez-Romero et al., 2006) (Table 1).

Only 1 in 10 of the child workers attends school, and even fewer finish a primary education. Migrant farm workers typically arrive in Sinaloa in September or October and work until early May. Most of the children working in the fields are not only deprived of an education but also face considerable dangers in the exposure to pesticides. (The United Nations

Migrant laborer population in the Labor Market				
State	**labor market**	**total**	**locales**	**migrants**
Baja California	Mexicali	12,000	9,600	2,400
	San Quintin	25,000	10,000	15,000
	Maneadero	1,500	450	1,050
	Ojos negros	1,800	180	1,620
	Guadalupe	300	60	240
	Eréndida SUBTOTAL ESTATAL	800 41,400	80 20,370	720 21,030
San Luis Potosí	Región Altiplano	4,699	2,098	2,601
	Zona Media	3,746	2,098	1,650
	Zona Huasteca SUBTOTAL ESTATAL	6,536 14,981	3,236 7,430	3,300 7,551
Sinaloa	Región Centro	69,685	5,085	64,600
	Región Norte	14,067	4,187	9,880
	Región Sur SUBTOTAL ESTATAL	33,685 117,437	32,165 41,437	1,520 76,0000
Sonora	Región de Cajeme	7,000	7,000	
	Región de Navojoa Huatabampo	4,000	4,000	
	Región de Guaymas-Empalme	4,000		4,000
	Región de Hermosillo	38,000		38,000
	Región de Pesquería	0		
	Región de Caborca SUBTOTAL ESTATAL	15,000 68,000	11,000	15,000 57,000

Table 1. Most representatives states of Mexico. (modified from Ramirez-Romero et al., 2006)

Children's Fund) [UNICEF], 2007.) believes that protecting children from exploitation is an integral part of ensuring their right to survival, and to a quality development.

"The fact that they're migrants means that they move from place to place, and so nobody feels completely responsible for fulfilling their basic needs and protecting their rights," says (UNICEF, 2007) Child Protection Officer Theresa Kilbane. The main toxic effects of the pesticides are derived of the inhibition of the enzyme esterase, and they could be implied in the cardiovascular alterations that are observed in the exposed organisms. The determination of the level of erythrocyte cholinesterase or plasmatic, the biological indicator used in the diagnosis of intoxications, like in the monitoring with the ends of prevention, study, or control (Table 2).

Population	Average Cholinesterase U/mL	Average Hemoglobin g/dL	Average cholinesterase-adjusted Hemoglobin U/g
Men	4.29	13.29	32.59
Women	3.98	11.66	34.63
Children	3.87	11.99	32.65
Average total	4.22	12.89	33.1

Table 2. Basal cholinesterase levels and cholinesterase adjusted to hemoglobin in agricultural laborers, Sinaloa, *Mexico.*

Inside of the parameters to considering the degree of exposure to pesticides is the minimum level of risk for oral exposure to this compounds in humans is of 0.003 mg/kg/d and the non-observed adverse effects level of 0.03 mg/kg/d (Deacon et al., 1980). However, they have been found in many foods in quantities of between 0.09 and 0.2 mg/kg/d (Bullman et al., 2002). On the other hand, the production of organic food is insufficient: To feed 9 billion people in 2050, we urgently need to adopt the most efficient farming techniques available. Today's scientific evidence demonstrates that agro-ecological methods outperform the use of chemical fertilizers in boosting food production where the hungry live -- especially in unfavorable environments. "To date, agro-ecological projects have shown an average crop yield increase of 80% in 57 developing countries, with an average increase of 116% for all African projects. Recent projects conducted in 20 African countries demonstrated a doubling of crop yields over a period of 3-10 years" (De Schutter, 2011).

The pesticides of the group of the organophosphate ones produce important functional alterations in various organs and systems of individuals who have contact with these types of substances. The effects reported more frequently are those that are related to changes in the central nervous system. Mice injected with malathion showed an decrement on dendritic morphology in neurons from the hippocampus and in the prefrontal cortex in comparison with the control group (Campaña et al., 2008). However, a percentage of patients present major complications at the level of the cardiovascular system that increase the morbidity and mortality associated with this physiological system. We know that the acute exposure to organophosphate produces bradycardia initially and then tachycardia, the latter effect dose-dependent (Aiuto et al., 1993). The mechanisms of generation of the effects of organophosphates have been associated mainly with the irreversible inhibition of acetylcholinesterase in the central and peripheral synapses whose primary function is the degradation of the acetylcholine.

The cardiovascular systems uses cholinergic processes in the sensory mechanisms, integrative and health providers and therefore are likely to be changed due to exposure to organophosphorus compounds (Ballantyne & Marrs, 1992; Gordon, 1994).

2. Antecedents

2.1 Similarities to other countries

There are many people who are involved in the migrant farm industry. Migrant farm workers are agricultural workers who move often within a yearly period for employment purposes. The families of migrant farm workers move to follow the planting. Most of these families are second and third generation migrant families. These families are usually very

poor. The workers and their families are seen in literature as an "invisible" group who are the most disadvantaged and at-risk population in the country. They are not seen, not heard, and not helped.

Children of migrant farm workers are an extremely vulnerable population of children. These children face a transient lifestyle. This lifestyle often begins at birth, and interferes with any hope for a stable education, steady friends, and familiarity with a community. Nearly every migrant child lives in poverty. It has been seen that children as young as 10 are working in the fields. It is estimated that twenty five to forty percent of the farm work is done by young children.

Migrant housing is a critical issue. Migrant workers often cannot afford to build suitable housing. Only thirty percent of the migrant population has comfortable houses. They are forced to live in dirty, overcrowded places or even worse, in tents, cars or even open fields. There are few government laws that require suitable houses, therefore, they get little help in this area. Resources in the area of housing are bare. Some states have begun to build houses and provide loans for low income residents but this does not seem to meet the needs of the migrant farm workers because they are constantly being uprooted and moved because of work situations.

There are numbers of injuries and deaths reported every year due to various accidents, such as, drowning in ditches, poor mechanical equipment, exposure to agricultural chemicals, being out in the sun and other heat related sicknesses. Children are exposed to the exact same conditions as adults, but are more vulnerable to becoming sick.

The life expectancy of migrant farm workers is 49 years compared to the national average of 73 to 75 years. There are many diseases among the migrant people like, malnutrition, maternal malnutrition, dental problems, parasitic infections, hypertension, diabetes, respiratory infections, and sexually transmitted diseases-including HIV/AIDS. There are also many mental health problems too, including, depression, anxiety, and abuse. Many of these health issues are related to not having sanitary living and working conditions. They cannot get help in this area because they cannot afford life insurance. The most commonly identified reasons for children of migrant farm workers dropping out of school include the need to work, the lack of relationships with peers at school, the need to move, getting pregnant, and or getting married. The number of children from migrant farm workers has increased, but the financing of their education has stayed the same. The families of migrant farm workers have to be independent. Families may get separated when the father leaves to find work. This leaves the mother in charge of the children. The mother usually has no money, and no way to provide for the children. Family life for migrant farm workers is extremely stressful because they face survival situations every single day. Children learn early that they are needed and they often help with the family farm work at the cost of their education. The maltreatment of children was significantly higher among migrant farm workers' families. Migrant farm workers are found to abuse their children in some states. It is more serious in some states than in others.

Migrant farm workers experience life and death situations every single day. The farm workers themselves do everything they can to support and keep a decent family. They are in great danger. But, their children are in even more danger of becoming hurt, they are innocent people that deserve a chance at the best life that they can get. They have tried to be seen, noticed and understood in the past. They have reached out for help but were rejected.

Their cries for help are unheard by the rest of the people of the world. They need to be helped. They need to be able to have a chance, a dream, a life.

The pesticide intoxication is an important cause of morbidity and mortality in the countries in development, although it has been reported that the fourth part of the pesticides that are wasted away in the entire world, is consumed by these countries. However, every year 3 million severe cases of intoxication and 22 000 deaths are recognized; (99% in the 3rd world) the collateral effects resultants of the indiscriminate use of the pesticides are disseminated in humans where these compounds alter the biochemical and physiologic functions (Banerjee, 1996, 1999; Selgrade, 1999).

The toxicity for pesticides happens mainly in underdeveloped countries, such as Mexico, and concretely in the State of Sinaloa, which is one of the States of higher agricultural activity and shown to produce the best organic corn (non transgenic) worldwide recognizable, subsequently where they are used perhaps but agro-chemicals in the country forces us to evaluate the factors of risk for the health of the residents in function of the multi-exposure.

The dangerous effects that exist for small quantities repeated over a period of time, usually years, determine the chronic effects of the compounds (Hock, 1999; Henderson et al., 2002). The chronic toxicity of a pesticide is more difficult to determine through laboratory analysis than the acute toxicity (Marrs, 2000; Varagic et al., 2001).

The effects on skin or organs and/or sensitive systems by toxins, such as: the cardiovascular system, the blood, the gastrointestinal tract and liver. The Environmental Diseases System defines cardiovascular or sanguine toxicity as the adverse effects in the cardiovascular systems and hematopoietic that are caused by the exposure to chemical substances. Specific illnesses caused by a cardiovascular or sanguine toxic include arterial hypertension, hardening of the arteries, heart arrhythmias, and factors of the coagulation and decrease of the sanguine flow toward the heart.

There are reports of data in the literature (Jeffrey, 1994; Zeimer, 1984; and Holmes., 1956) which relate to patients poisoned with organophosphate insecticides having had disorders of platelet function and blood coagulation and have suggested the importance of routine coagulation tests in these patients.

The (Environmental Protection Agency [EPA], 2007), affirms that children are more susceptible to organic damage: the reasons: first, the internal organs are still in a developmental process, for which these substances types can block the necessary blood components and vital nutrients for the cellular growth. In second place, children consume more food, in proportion with their corporal weight than the adults. This also reinforces the effect of the pesticides because exposure is more probable exposure *per orally*. Finally, the (EPA, 2007), claims that there are environments common to the children that increase their exposure to the compounds in domestic use; playing on the floor or in the grass, with pets, sucking polluted objects etc.

The question is: why are the pesticides in the foods of children? They have not taken the appropriate precautions because the children don't demand it. They are perhaps the minority group least valued at the moment in the entire world, constituting a sub-class; according to asseverations when we refer to the stratification, we locate children at the bottom in terms of power. This is especially true of the smallest children in whom the cells are in development more than in adults. In terms of power the children are one of the smaller power groups in our society in general. Therefore, the contamination by pesticides is not a priority, since the cost of fixing the problem is bigger than the cost of the health of the children, from the point of view of the industry.

The (Protection Action Network North America [PANNA], 2009), through the (Pesticide Action Network Update Service) [PANUPS] and their spokeswoman the Dr. Susan Kegley affirmed in the *Los Angeles Times* that the farmers are not making progress toward the use of less toxic alternative pesticides, which is a problem that should be pointed out.

2.2 Oxidative stress

The blood pathologies seem to be a sensitive indicator in the exposure to the pesticides as well as the oxidative stress. In a study, data suggested that oxidative stress may be involved in the effects of chlorpyrifos (an organophosphate pesticide). Significantly increased levels of malondialdehyde were found in aorta and plasma samples in rats; the nitric oxide takes place in several types of cells and it is studied well in the vascular endothelium. While this specie is not too much reactive it reactivates (scarce function oxidizer), even low physiologic concentrations (to <100 nM), it reacts quickly with oxygen to produce nitrogen dioxide (NO_2 -) that in turn can react with the nitric oxide to produce nitrogen trioxide (N_2O_3); the nitrites showed elevation in plasma, not only but also the superoxide dismutase enzyme activity in the aorta sample was statistically significant, as in plasma where its activity was elevated (Alvarez et al., 2008).

The biochemical reactions oxidize-reduction or *REDOX*, result in the formation of species reactive oxygen, such as hydrogen peroxide, radical and hydroxyl superoxide, constantly attacking the organism and occurring as a normal part of cellular metabolism (Pérez & Pérez, 2000). The increased concentrations of reactive species of oxygen contribute to the aggravation of the cell function, and has been reported to coincide with pathologies like damaging accelerated vascular (microcirculation) that accompanies the hypertensive syndrome (have been kept to speculation and without pilot bases firmly). Indirect evidence has suggested that the key word may be related to reinforcement in the production of oxygen radical (Suzuki et al., 1995). For example, nitric oxide and radical superoxide play important roles as molecules vascular signaling but, to cause peroxynitrite triggered an imbalance between these free radicals with important implications for the vascular patophysiology (Guzik et al., 2002). In the different organs several defense mechanisms exist to minimize the effect of the oxidative stress. Among them are substances of under molecular weight such as alpha-tocopherol, glutathione and ascorbic acid and mainly enzymes like superoxide dismutase (Clapés et al., 2001).

The reduction of the oxidative excessive damage is one of the mechanisms to minimize the cellular devastation (Uchiyama & Mihara, 1978). The prevention of intoxications requires the recognition of the factors that favor it, the attention to the focus of lipoperoxidation and oxidative stress, parameters that should clarify the changes in the enzymatic activities in the redox system that can happen after the exposure to xenobiotics and its relationship with alterations of arterial pressure (Kehrer, 1993).

2.3 Basic concepts of blood coagulation

In humans and other vertebrates, the blood coagulation system is a first line of defense against vascular trauma. In case of a wound (whether unintentional in accidents or intentional in surgery), blood coagulation rapidly forms a blood clot; the approximate time that it takes for skin bleeding to stop is on average 2–5 minutes if the system is functioning correctly. If there is a defect at some point of the coagulation system, bleeding may be

markedly prolonged. The vertebrate blood coagulation system consists of cellular elements (blood platelets, white cells, to some extent red cells and micro vascular remnants or micro particles) and proteins (coagulation enzymes and co-factors, and a number of anticoagulant proteins). When blood coagulation is triggered, cells and membrane remnants interact with coagulation factors assembling effective macromolecular complexes that contribute to the formation of fibrin molecules. These fibrin molecules and cells constitute the blood clot formed at the point of damage to the blood vessel. Bled of the small vessels it can be stopped by vasoconstriction and the formation of platelets, but the formation of a clot usually happens as part of the normal process of hemostasis, the factors of the clotting are component critical in the formation of a thrombus. Blood clotting is a sequential process of biochemical reactions involving plasma proteins, phospholipids, and ions of Ca. Most of the coagulation factors involved in the clotting process are designated with Roman numerals. The activated form of an enzyme factor appears with Roman numeral followed by the suffix - a, while the inactive factors are indicated by the Roman numeral alone. For example, prothrombin is designated as factor II, in any way, in this active state is: IIa, thrombin. Non-enzymatic factors do not have these designations. It is important to note that the Roman numerical designations do not indicate the sequence of reactions in the agglutination process, for example, the X factor precedes the factor II in the clotting process.

The proteins are clotting factors that have four characteristics:

1. A deficiency of factor that usually produces a tendency to bleed with the exception of factor XII, prekallikrein (Fletcher factor) and high molecular weight kininogen.
2. The chemical and physical characteristics are known factors.
3. Factor synthesis is independent of other proteins.
4. The factor can be determined in the laboratory (Table 3)

In the sense of developing an understanding of coagulation theory and the principles underlying laboratory procedures, it is useful to compare the characteristics of various clotting factors. There are three sets of factors: the group fibrinogen, prothrombin group and the control one.

Reports of data exist in the literature (Jeffrey C. Murray, 1994; M. Zeimen, 1984; and Holmes J., 1956), where they report that patients intoxicated with pesticides organophosphate presented disorder in the platelet function and the sanguine clotting for which the importance of making tests of routine clotting in this type of patients is suggested.

2.4 Altered ions in the system of clotting and hemostasis

The pesticides affect the sanguine clotting in a two-phase way. This way, it has been that the reduction of clotting is a phenomenon that is presented in the workers that manipulate or they are exposed to these compounds, due to the appearance of times of lingering prothrombin and to deficiency of the factors V and VII (Holmes, 1956). (Zeimen, 1984), studied the function of the platelets and the parameters of the sanguine clotting in nine patients intoxicated with pesticides organophosphates. In five of the nine patients there was a marked tendency to the appearance of hemorrhages. Also, the thrombolytic was irregular in all of the patients and the abnormalities of the clotting were more marked in the cases of severe intoxication. On the other hand, the continuation of the time of prothrombin has been reported, due to the transitory decrease of the activity of the factor VII, in a baby of 1, Blood samples by venous puncture were obtained; in addition, a questionnaire for labor and

Characteristic	GROUPS		
Factor	I[a]	II[b]	III[c]
Molecular weight	High	Low	¿
Plasma	Present	Present	Present
Serum	Absent	Present, except II	Present
Absortion ($BaSO_4$)	Not	Yes	nothing or partially
Destruction	Thrombin, plasmin		
Stability	Factors V and VIII unstables	Stable to the heat	Stable
Increase	inflamation, pregnancy, stress and fear, oral contraceptives	Pregnancy contraceptives orales	
Decrement		Oral anticoagulant	

aGroup I: Fibrinogen group (Factors I, V,VIII,XIII).
bGroup II: Protrombin group (Factors II, VII, IX,X).
cGroup III: Contact group (Factors XI,XII, Fletcher, factors Fitzgerald).

Table 3. Characteristics of clotting

clinical history was applied. The results indicate that the organophosphates ones prolong the time of prothrombin and diminish the percentage of retraction of the clot after the exposure to these pesticides in the migrant workers of the field. The effect with a $P \leq 0.01$ appeared, compared with its controls. Of the individually studied factors of the coagulation, factor II was of the most sensitive to these compounds, statistically very significant changes occurring ($P \leq 0.01$). Men and women of 8 to 50 years old and in voluntary form were studied randomly. Blood samples by venous puncture were obtained; (Table 4) in addition, a questionnaire for labor and clinical history was applied.

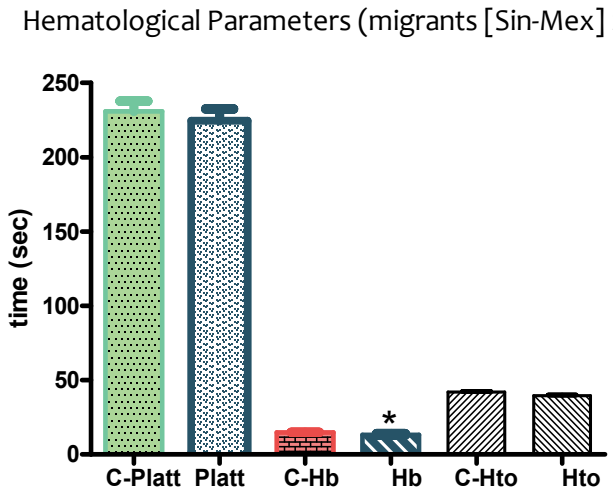
PARAMETER	METHOD
- Time of bleeding	- Ivy
-Retraction of the clot	- Platt
-Time of coagulation	- Lie-White
- Count of platelets	- Brecher
- Time of partial thromboplastin	- Langdell
- Time of prothrombin	- Quick
- Individual Activity of factors of coagulation	- Kits de Sigma Chemical
- Activity of seric pseudocholinesterase	- Merck
- Activity of Erythrocyte Acetilcholinesterase	- Magnotti

Table 4. Procedures to obtain the samples

PRAMETERS	TP	TPT	Plat	Hto	Hb	Red cel.	White cel.
Mean							
Control	12.51	31.80	215,800	41.95	14.72	4,959,000	6,556
Exposure	12.85	31.75	224,500	39.45	13.23	4,620,000	7,844
Std Deviation							
Control	0.5203	1.713	40,820	3.644	1.318	92,756	1,771
exposure	0.5796	2.121	49,970	3.967	1.384	40,522	1,775
Std Error							
Control	0.08227	0.2708	6,455	0.5762	0.2085	14,666	280.1
Exposure	0.09164	0.3354	7,900	0.6273	0.2188	6,407	0.2807
P Value P<0.0001							

Table 5. Parameters measured in migrant laborers (Sin. Mex).

Using an Analysis of variance (ANOVA), plus Dunnet's multiple comparison test, we obtained the following results: in the first case: (Figure 2.), the column C (control group) shows 14.71 of the mean of this parameter, with a standard deviation of 1.318 and standard error of 0.208 vs the column D, with a mean of 13.23, std deviation of 1.384, std error of 0.218 resulting in a significant change in the hemoglobin parameter.
In the second case (Figure 3.), our findings show contrasts as follows: the column A, control group shows 12.33 of platelets vs 14.57 of the column B seconds. The column C, control group for thromboplastin partial time 31.375 vs column D, 34.27 seconds (Table 5).



C-Platt (control platelets group) C-Hb (control hemoglobin group) C-Hto (control hematocrit group)

Fig. 2. The graphic does not show statistically significant changes in the values of platelets and hematocrit, but it shows significant change (P< 0.01) in hemoglobin value of the exposed group.

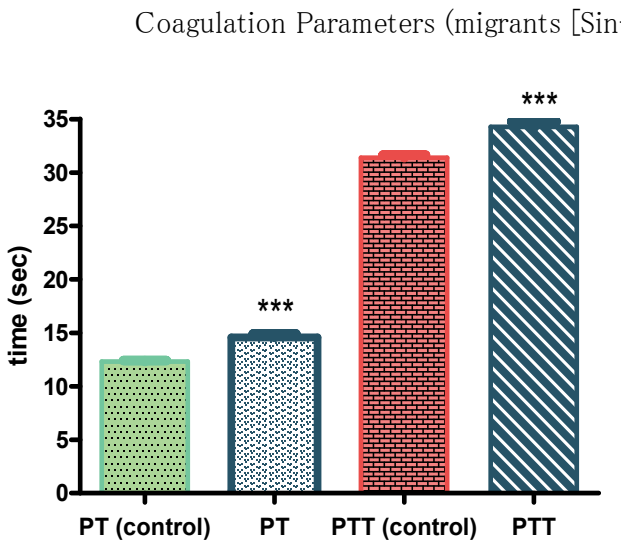


Fig. 3. Coagulation parameters obtained. The graphic shows altered coagulation times compared with the control group; being highly significant statistically ($P<0.0001$).

3. Conclusion

Navolato, Sinaloa, Mexico. This study focused on the function of platelets and the parameters of the sanguineous coagulation in migrant agricultural laborers exposed to pesticides. The aim of this study was to analyze the early effects on the sanguineous coagulation of the organophosphates ones.

Fig. 4. Pesticide fumigation in the fields of Sinaloa, Mex., (2011).

Fig. 5. Diverse agro chemical waste around habitat zone. Sinaloa, Mex., (2011)

Fig. 6. Some wild animals probably become intoxicated due to the agro chemical waste. Sinaloa, Mex., (2011)

Prothrombin (factor II) is the most sensitive parameter affected by these compounds so that we can deduce that there is a correlation with the results of the study related to individual clotting factors and the factor II presents a decrease in activity of more than 50% indicating a

selective damage by these pesticides studied on the system of coagulation and hemostasis. These findings are consistent with reports by Sweeney & Lyon, 1999, who found a selective effect of malathion on blood coagulation activity against the locomotor system. Some altered coagulation parameters could be used as early markers of exposure to organophosphate type.

Some altered coagulation parameters could be used as early markers of exposure to organophosphate pesticides. Although there are some examples of successful cooperation between government, farm owners and local communities to provide a safe environment for migrant children, more needs to be done. But many companies do not want to cooperate with these institutions to improve the living conditions of the migrant workers. There is still a long way to go.

4. Acknoledgments

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7

Work Practices, Exposure Assessment and Geographical Analysis of Pesticide Applicators in Argentina

María Josefina Lantieri[1], Mariana Butinof[1], Ricardo Fernández[4], María Inés Stimolo[2], Marcelo Blanco[3] and María del Pilar Díaz[1]
[1]*Facultad de Ciencias Médicas*
[2]*Facultad de Ciencias Económicas*
[3]*Facultad de Ciencias Agropecuarias*
[4]*Facultad de Medicina*
[1,2,3]*Universidad Nacional de Córdoba*
[4]*Universidad Católica de Córdoba*
Córdoba
Argentina

1. Introduction

1.1 Argentina

Argentina is the second largest country of South America. In its central-eastern region, there is a plain extending over more than 50 million hectares, whose high fertility and productivity provide significant comparative advantages for agriculture production (Hall et al., 1992, as cited in Manuel-Navarrete et al., 2009). This activity is one of the main axes of Argentina's economy, particularly the production of cereals and oilseeds, the primary export of Argentina, placing it among the main grain-producing countries of the world (United States Department of Agriculture, 2010).

In the history of Argentina, agriculture has been a prime contributor to economic and social development. Among the factors that explain this process are the external economic environment, the political framework, domestic economic conditions and the behaviour of production and innovation. At the same time, it is important to analyse its impact both socially and environmentally.

Several evolutionary periods can be noticed in Argentinean agriculture: a birth and expansion period (from 1862 to 1929); an agricultural recession period (from 1929 to 1950); a mechanization and modernization period (from 1950 to 1989) and the productive specialization and agriculturalization period (from 1990) (Stratta Fernández & Ríos Carmenado, 2010).

In the latter two periods, from the mid-20th century, there was an important expansion of agricultural production, marked by technological transformations. Some highlights of this period are: a) the creation of INTA (National Institute for Agricultural Technology) in 1957;

b) the total mechanization of agricultural labour; c) the introduction of improved seeds; d) the use of agrochemical products and fertilizers; and e) the popularisation of soybean cultivation (Stratta Fernández & Ríos Carmenado, 2010). There was an increase of agricultural production of almost 30%, which led not only to an expansion of the agricultural surface (17%) but also to an increase in soil productivity (Obschatko, as cited by Stratta Fernández & Ríos Carmenado, 2010).

Worldwide, the so-called "Green Revolution", with the incorporation of new machinery, the massive use of agrochemical products and fertilizers, the use of improved seeds (known hybrids in corn, sorghum and sunflower and their development in other crops such as soybean) helped to double and even triple the yields of the most important grains. In Argentina, National Institute for Agricultural Technology developed and promoted a new method of organizing production and incorporating technological changes, by means of which, between 1965 and 1985, annual grain production rose from 14 to 80 million tons (Lódola, 2008, as cited in Stratta Fernández & Ríos Carmenado, 2010). The transformations during this period tripled the value of production, doubled soil productivity, and quadrupled labour force productivity (Stratta Fernández & Ríos Carmenado, 2010).

The outstanding increase of farming activity since 1990, known as "agriculturalization", occurred as a result of this production increase and adoption of new technology. It was characterized by an increasing and continuous change in land use, including converting large stretches of forests into farmlands, so that the country has one of the highest rates of deforestation in South America (0.8%/year) (FAO, 2001, as cited by Cabido & Zak, 2010). Agricultural crops also steadily replaced stockbreeding and dairy-farming (Stratta Fernández & Ríos Carmenado, 2010).

From 1996, an extensive agricultural model developed, based on glyphosate-resistant transgenic soybean farming, no-till and the intensive use of fertilizers and pesticides. Soybeans occupied 34,700 ha in 1970, but had reached more than 18 million ha by 2010 (Sistema Integrado de Información Agropecuaria. Ministerio de Agricultura, Ganadería, Pesca y Alimentos de la República Argentina, 2011). With this, the marketing of pesticides grew strongly, from 155 million pounds in 1995 (Cámara de Sanidad Agropecuaria y Fertilizantes, 2010) up to 600 million pounds in 2007 (Secretaria de Ambiente y Desarrollo Sustentable, 2009). The growth of the sector, the rising trend in commodity prices and the continuous encroachment of the agricultural frontier into marginal areas indicate that demand for pesticides will continue its upward trend in coming years (World Bank, 2006).

1.2 Study area - Córdoba province

Córdoba province is located in the central region of Argentina (from 29º 29′ 53″ to 35º 0′ 0″ Lat. S and 61º 26′ 40″ to 65º 46′ 46″ Long. W), with much of its surface in the central-eastern Pampas. It occupies 165.321 km² and has 3, 304,825 inhabitants, with a population density of 20 inhabitants per km² and a heterogeneous population distribution: 88.7% of it is urban and only 11.3% is rural (Instituto Nacional de Estadísticas y Censos, 2011a). This means that housing areas coexist with agricultural areas without clearly defined borders, increasing the risk of non-occupational exposure to pesticides in communities adjacent to agricultural fields.

Córdoba plays an important role in the agricultural history and transformation of Argentina because of its strong livestock and agriculture sector, accounting for 48.02% of the provincial area devoted to agricultural production. Córdoba provides 90% of the total production of soybeans for export (Dirección General de Estadísticas y Censos, 2009).

The extensive crops (soybean, maize, sorghum, peanut, wheat, and sunflower) area has expanded from 3,397,050 ha in 1994/95 to 6,810,500 ha in 2009/2010 (Sistema Integrado de Información Agropecuaria, 2011). As in other provinces of Argentina, the expansion of arable lands replacing stockbreeding and dairy farming has also invaded natural ecosystem areas. About 120,000 km2 of forest in Córdoba, in the early 20th century, had been reduced to 17,000 km2 of forest and 9,600 km2 of bush by 2004 (Cabido & Zak, 2010). Between 1970 and 2000, agricultural expansion in the northern departments of Córdoba caused the loss of 10,000 km2 of dry woodland converted to annual crops, mainly soybeans (Zak et al., 2004).
About 65% of extensive crops are soybeans (Ministerio de Agricultura, Ganadería y Alimentos de la Provincia de Córdoba, 2011), representing 30% of the area cultivated with this oilseed nationally. The Argentine Agricultural Census 2002 showed that, from 1988-2002, the greatest annual increase of the crop was in Córdoba (Dirección General de Estadísticas y Censos, 2002). As expected, pesticide use has accompanied this trend.

1.3 Ecological classification of Córdoba province

In 1987, Córdoba was classified into five homogeneous ecological areas (HEAs) (Figure 1) according to soil and climatic characteristics, land use and production activities. HEA I, the so-called "North-western Extensive Livestock Area" is characterized by grass cattle; HEA II, named the "Middle Agricultural and Livestock Area" has extensive crops and beef cattle. HEA III, the "Mid-eastern Dairy Area" is the main milk shed of the province, but also has extensive crops; HEA IV, the "South-eastern Agricultural Area", is based on agriculture and, finally, HEA V, the "South-eastern Agricultural and Livestock Area", has a large area devoted to extensive crops, with a significant participation of beef cattle (Centro Regional Córdoba INTA, 1987).
Becerra et al. (2007) characterized the HEAs through a family farmers study, showing large differences related to conditions of land ownership, capitalization levels, family work and social reproduction. However, no others aspects related to labour conditions were characterized. The high diversity of production systems historically has been reduced in

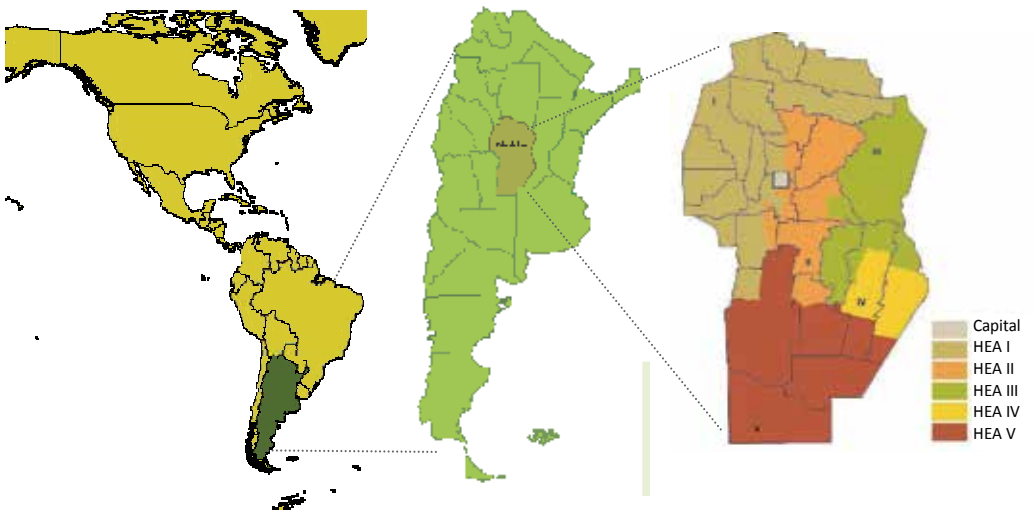

Fig. 1. Homogeneous ecological areas (HEAs) of Córdoba province

recent decades by a concentration process, expulsion of farmers and of the rural population Of 40,817 farms surveyed in 1988, there were 26,226 left in 2002, indicating a reduction of around 36%, coupled to an increase in the average size of farms (36%, from 343 ha to 467 ha in 2002) (Dirección General de Estadísticas y Censos, 2002). Both issues, the number and the size of farms, have become the emerging focus of studies on land use and access.
Even before 2001, changes had occurred: the agriculture area had grown, replacing regions devoted previously to meat and milk production (Da Veiga, 2005); nearly 90% of the increase corresponded to soybean cultivation (Martellotto et al., 2001) and cattle stock had decreased 19% between 2002 and 2009 (Calvo et al., 2009). The best lands were devoted to agriculture and the others to livestock.

1.4 Pesticides and human health

Pesticides have a strong environmental impact and produce adverse effects upon living beings, including humans, in whom acute exposure can lead to death or serious illness (WHO, 1990). The wide use of pesticides in agricultural and other settings results in continuing human exposure (Alavanja et al., 2004), shown by epidemiological studies to be associated with risks to health. Chronic exposure is most often a problem in occupational settings and can increase the risk of developmental and reproductive disorders, immune system diseases, endocrine disruption, impaired nervous-system function, and development of certain cancers (Bassil et al., 2007; Sanborn et al., 2007; WHO, 1990; Yañez et al., 2002).
The good management, use, and disposal of agrochemicals, particularly pesticides, thus becomes a global health and environmental issue in agricultural settings, particularly in developing countries, such as Argentina, where economies may be heavily dependent on agriculture (WHO, 2010).
In third world countries, pesticide poisoning is a major problem primarily due to precarious and unsafe pesticide application and handling practices. Furthermore, an enlightened and enforceable pesticide policy, legislation and regulation are scarce or absent. In Argentina, and particularly in Córdoba, legislation exists but enforcement is lacking. In effect, our previous research shows indicators of irregular or non inspection of the chemical use, at least in the studied topics of the local specific law (Lantieri et al., 2009).
Current concern in Argentina about the environmental impact and human health effects of pesticide use arises from an annual incidence of accidents/occupational diseases in agricultural activity of 94.8‰. This is one of the activities whose values were higher than the overall incidence rate for the entire national system of work risk, 56.6‰, with a mortality index of 195 fatalities per million workers covered, only surpassed by construction (229‰) (Superintendencia de Riesgo de Trabajo, 2009). It is worth pointing out that the number of acute pesticide poisonings is not a good indicator of the problem due to underreporting in rural areas (Henao et al., 1993).
In this country, and particularly in Córdoba province, despite its long agricultural history, occupational pesticide use and its health effects have not been studied in this sensitive population, until recent years.

1.5 Previous results

The only work to date is Lantieri et al. (2009), a population-based study of around 700 terrestrial pesticide applicators in Córdoba province, which assessed the current and past

use of pesticides, as well as the technologies, personal protective equipment (PPE) employed, worker practices and different social and demographic characteristics. Briefly, the main results showed that 50 per cent of the workers reside less than 500 m from the nearest cultivated area, 60 per cent of them do not use personal protective equipment and more than half spray up to 5000 ha per year. This, along with other results, would indicate that there is a risk scenario for the study population, and warrants further evaluation, particularly for the occurrence of chronic health effects.

Preliminary results showed that 40% of this sensitive population of pesticide applicators in Córdoba have had, sometimes/frequently, symptoms such as skin and eye irritation, nausea and vomiting, 35% had a medical consultation and 5.4% had been hospitalized as a result of pesticide use. Besides, unprotected workers showed differences in the frequency of symptoms such as eye irritation, headache, nervousness and depression. Many workers showed health effects related to pesticide exposure and many of the symptoms have a statistically significant association with the years of activity related to agrochemical use.

Furthermore, the exceptional increase of the cultivated area in Córdoba, particularly of glyphosate-resistant transgenic soybean, presents a particular agricultural panorama, on top of the pre-existing environmental differences defined by the homogeneous ecological areas (HEAs) classification. The adoption of new technological resources, direct sowing, complementary irrigation, use of transgenic seeds, fertilizer application and intensive pesticide usage, have given rise to new technological and economic scenarios in the HEAs (Ghida-Daza, 2009), from which we can hypothesize that new risk scenarios have also emerged in the province.

1.6 Objectives

The basic factors necessary to effectively study the association between pesticide exposure and health effects include determination of the population at risk; a valid determination of exposure; verification of diagnosis, symptom, or biological marker of a health effect among the populations being studied; methods to link individual exposure to health effects; and the ability to establish a temporal relationship between the exposure and the health effect (McCauley, 2006).

The purpose of this chapter is to provide a comprehensive characterization of terrestrial pesticide applicators in Córdoba province, with the emphasis on the assessment of occupational exposure. We provide two new methodological tools to estimate the workers' exposure level, analyze the stochastic behaviours of these measures, and supply the corresponding scales for assessing exposure risks in the regional agricultural scenarios.

2. Materials and methods

2.1 Study population and data collection

We conducted a population-based study in Córdoba province, Argentina. All the workers attending the mandatory courses provided by the Agriculture, Livestock and Food Ministry to obtain the applicator license, were asked to participate in the survey, from 2007 to 2010. After an explanation of the purpose of the study and prior informed consent, a self-administered questionnaire (adapted from Agricultural Health Study, Alavanja et al., 1996; Bonner & Alavanja, 2005) was used. Exposure determinants such as possible characteristics

that influence exposure levels, (Steward, 1999) were covered. A checklist of 49 specific pesticides in use and of forbidden or restricted-use chemicals with common and trade names was included to enhance recall. Current and historical pesticide use and its frequency, as well as social and demographic data, tasks (mixing/applying pesticides; repairing sprayer equipment), crops and area/year sprayed, personal protective equipment (PPE) used and application methods, were included, as well as pesticide prescriptions, indicated by agricultural engineers. Workers' diagnosed diseases, frequency of medical consultations and hospitalization related to adverse health events occurring during occupational exposure, and finally, questions about health of the applicator's family were included in the form.

From 1122 completed forms, a consistency analysis for various responses was carried out, leaving a sample size of 880 applicators directly exposed to pesticides (mixing/applying pesticides) for future analysis. The procedures were submitted to a regional ethics committee and all data was encrypted for legal and confidentiality purposes.

2.2 Variables

2.2.1 Social and demographical variables

Social and demographic variables included age (constructed from birth date); education level (incomplete primary school, complete primary, incomplete secondary and complete secondary, technical or university studies); marital status (married or cohabiting, and unmarried, separated, divorced or widowed); origin (country, province and department of birth; current address including province and department).

2.2.2 Exposure determinants

Exposure determinants included: personal protective equipment (PPE) used: waterproof clothing, gas mask, chemically resistant (e.g. nitrile) gloves, face shields or goggles, hat or helmet and other protective clothing (boots, apron, waterproof pants). Years mixing/applying; hectares applied in the last year; period of the year in which the applicator works, taking into account two periods (lower temperature months, from April to August; higher temperature, from September to March); applicator household proximity in meters with respect to the nearest crop, and written pesticide prescription.

2.2.3 Protection level

In order to analyze associations between some of the variables and protection habits, we constructed a new measure called protection level (Table 1), based on Dosemeci et al. (2002) scores (see adapted version in Díaz & Stimolo, *in revision*), considering firstly, three levels of protection: unprotected (0% of protection); partially protected (20% to 70%) and protected (90%); and secondly, considering as Protected those workers with 90% protection and Unprotected, the rest of the applicators (0% to 70%).

2.2.4 Geographical classification variable

As mentioned above, the regions of the province were classified through homogeneous ecological areas (Centro Regional Córdoba INTA, 1987). The notation and names of these five areas are as follows: HEA I: Northwestern Extensive Livestock Area; HEA II: Middle Agricultural and Livestock Area; HEA III: Mid-eastern Dairy Area; HEA IV: Southeastern Agricultural Area, and HEA V: Southeastern Agricultural and Livestock Area.

Scores for combinations of PPE use	Protection Categories	% of protection	Combinations of Personal Protective Equipment Used
0	Unprotected	0	Never used PPE
1	Partially Protected	20	Face shields or goggles. Other protective clothing (boot, hat)
2		30	Gas mask. Waterproof clothing.
3		40	Chemically resistant gloves.
4		50	PPE 1 y 2.
5		60	PPE 1 y 3.
6		70	PPE 2 y 3.
7	Protected	90	PPE 1, 2 y 3.

Table 1. Description of categories of protection level

2.2.5 Applicator health variables

Six variables were collected for the assessment of worker health: Symptoms: Perception of acute and sub-acute manifestations - irritative symptoms (skin and eye irritation, nausea or vomiting, chest discomfort); fatigue tiredness; nervousness or depression; headache /dizziness; Occurrence of symptoms: Never / Rarely, Sometimes / Frequently; Medical consultations related to pesticide use effects: Yes / No; and Hospitalization linked to tasks with pesticides: Yes / No.

2.3 Epidemiology and exposure assessment

Historically, the major limitation of epidemiology of pesticide effects has been inadequate exposure assessment. More accurate quantification of exposure has been the hallmark of some of the more recent epidemiological studies, and the present work gives an insight into this issue including the development of methodological techniques to assess pesticide exposure. Two indexes were created that describe intensity level and accumulated exposure to pesticides, using specific characteristics of pesticide application and of some personal habits.

2.3.1 Intensity level and cumulative exposure indexes

One of the main aims of this work is to contribute to the study of exposure to pesticides in our region. No measure has been proposed so far. We therefore created two indexes to describe applicator exposure. The Intensity Level of the pesticide Exposure (ILE) index measures instantaneous exposure intensity and has a Cronbach'α value (coefficient of reliability) equal to 0.95; the Cumulative Exposure Index (CEI) takes into account the average time of exposure, and incorporates the previous ILE information, with a similar reliability coefficient. Both indexes are based on the proposal of Dosemeci et al. (2002), carefully adapting the weighting scores procedure to our own context, using mainly local professional judgments. As mentioned, Diaz and Stimolo (*in revision*) show the selection of these scores for both indexes, although basically, these are weighted for the exposure

indicators considered most important, such as the PPE information. The ILE and CEI are presented below:

$$ILE = (mix * PPE) + (\sum_{i=1}^{n} \frac{meth * PPE}{\#meth}) + (repair * PPE) + house\_dist$$

and

$$CEI = ILE + (\sum_{i=1}^{n} \log(1 + \frac{Ha / year}{55}),$$

respectively, with *mix* representing a dichotomic response about mixing pesticides, *meth* the category of the method used with a certain PPE, *repair* the binary variable for which success is the positive response, *house_dist* the score indicating the applicator household proximity to the nearest crop, and *55* the average of ha that is treated with a single load in the crop sprayer. Both measures were evaluated for all subjects in the sample.
Using Bootstrap and Monte Carlo Resampling methods, we identified the most suitable theoretical stochastic distribution for each measure, drawing m=30,000 random samples. Thus, density models were generated (Diaz & Stimolo, *in revision*), after an appropriate goodness-of-fit analysis, and the percentiles estimated for the future use of indexes and recommendations through confidence bands. This step allowed us to estimate the cut-off points or percentiles that define the scales of pesticide exposure for the applicators. The cut-off points served as reference points for classifying subjects into, firstly, one of three categories: low, medium and high exposure, and then, one of four modalities: low, medium, medium high and high exposure. A double-blind design using the researchers as participants was implemented to check both scales. Unity minus the cross-validation error rate was reported, which showed around 80% of agreement. The most suitable density models have a Gamma distribution.

2.4 Statistical analysis for association

We used a modelling approach to verify differences between ecological areas. Assuming counts or frequencies in each category of the variables as the outcome, we fitted Poisson and Gamma generalized models to estimate the parameters (effects). Association between two or three variables was inspected through log-linear models in order to estimate the odds ratio as association measures.

3. Results

3.1 Social and demographical characteristics

The population is largely composed of Argentinean white men. Only 0.7% is foreign born, from Bolivia and Chile. Nearly all the applicators reside in Córdoba (98.3%). Generally, they are not migrant workers: 89.9% were born in Córdoba and 64% still live in the same city, while the remainder still lives within the same province. Because there were only four female applicators (0.5%), these were excluded from data analysis. The applicators are young men (34.9 y, s.d. 11.04 y) and 80% of the population are under 45; 5.9% are under 21 and 4.5% over 55 years of age. Only 25.6% have completed secondary school, technical or university studies, with 11.8% being illiterate or with incomplete primary school. 63.5% of the workers are married or cohabiting (Table 2).

Table 3 shows that 72.5% of the applicators have personally mixed or applied pesticides up to 10 years. The principal crops sprayed by the applicators surveyed were soybean (95.3%), maize (81.9%), wheat (79.5%) and alfalfa (54.8%), with the average area/year worked in the last year, 7000 ha. It should be noted that 46.5% of workers live, with their family, 500 m or less from the nearest treated crop.

Social and demographical characteristics	Number	Valid (%)[1]
Age (years)		
Mean	34.9	
Standard Deviation	11.04	
Education		
Incomplete Primary	88	11.8
Complete primary	271	36.3
Incomplete Secondary	196	26.3
Complete secondary, technical or university studies	191	25.6
Missing	134	
Marital status		
Married or cohabiting	474	63.5
Unmarried, separated, divorced or widower	273	36.5
Missing	133	
Country of origin		
Argentina	869	98.8
Bolivia	5	0.6
Chile	1	0.1
Missing	5	
Change of residence birth		
No	543	64.0
Yes	306	36.0
Missing	31	

1. Percentage considering the total of the responses

Table 2. Social and demographic characteristics of pesticide applicators. Córdoba. Argentina. 2007 – 2010

3.2 Pesticides used

The most frequently used pesticides were herbicides; glyphosate for 98.5% of the responses, 2, 4-D – 2, 4 DB for 93.5% and atrazine for 92.2%. The insecticides most commonly handled were cypermethrin (95.7%), chlorpyrifos (82.1%), endosulfan (75.6%) and dimethoate (65.8%). The less used chemicals were fungicides, pyraclostrobin + epoxiconazole (Opera) for 37.8%, azoxystrobin + ciproconazole (Amistar) for 36.7% and carbendazim + epoxiconazole (Duett) for 34.3%. Applicators have used or still use either simultaneously or serially, an average of 12 chemical products (s.d. 5), some of them having used up to 25 pesticides (range 1 to 25) (Table 4).

Work characteristics	Number	Valid %(1)
Years personally mixed/applied pesticides		
≤ 1	119	14.2
2-5	299	34.8
6-10	201	23.4
11-20	162	18.9
21-30	56	6.5
>30	18	2.1
Missing	22	
Crops sprayed		
Soybean (**Glycine max** L.)	803	95.3
Maize (**Zea mays** L.)	690	81.9
Wheat (**Triticum aestivum** L.)	670	79.5
Alfalfa (**Medicago sativa** L.)	462	54.8
Sorghum (**Sorghum vulgare** Pers.)	388	46.0
Oat (**Avena sativa** L.)	290	34.4
Peanuts (**Arachis hypogaea** L.)	221	24.9
Sunflower (**Helianthus annus** L.)	185	21.9
Average area/year applied (ha)		
Up to 5000	408	55.8
5001 to 10000	113	15.5
10001 to 15000	87	11.8
15001 to 20000	83	11.4
20001 to 25000	24	3.3
> 20000	16	2.2
Missing	149	
Applicator home location wrt the nearest crop		
< 100 m	175	25.7
101 - 200 m	56	8.2
201 - 500 m	85	12.5
501 - 1000 m	89	13.1
1001 - 1500 m	13	1.9
> 1500	262	38.5
Missing	200	

1. Percentage considering the total of the responses
wrt: with respect to

Table 3. Work characteristics among pesticide applicators, Córdoba, Argentina. 2007-2010

Current use of forbidden or restricted-use pesticides was also surveyed and the results showed, surprisingly, that applicators continue to use heptachlor (3.3%), DDT (3.2%), malathion (2.2%), parathion (2.1%), aldicarb (1.0%) and aldrin (0.5%).

Pesticides	Number	Valid % (1)
Herbicides		
Glyphosate	858	98.5
2,4 D - 2,4 DB	810	93.5
Atrazine	799	92.2
Metsulfuron	706	82.3
Dicamba	587	69.4
Acetochlor	524	60.9
Metolachlor	440	51.8
Picloram	392	46.0
Insecticides		
Cypermethrin	829	95.7
Chlorpyrifos	705	82.1
Endosulfan	638	75.6
Dimethoate	526	65.8
Deltamethrin	516	60.8
Chlorimuron	223	40.8
Methamidophos	208	26.6
Fungicides		
Pyraclostrobin + Epoxiconazole (Opera*)	299	37.8
Azoxystrobin + Ciproconazole (Amistar*)	241	36.7
Carbendazim + Epoxiconazole (Duett*)	272	34.3
Carbendazim	277	33.0
Thiram	163	19.5
Propiconazole + Difenoconazole (Taspa*)	153	19.4
Carboxin	134	16.0
Tebuconazole	90	10.7
Trifloxistrobin + Propiconazole (Poseidon*)	80	10.1
Propiconazole (Tilt*)	80	10.1
Mancozeb	52	6.3

1. Percentage considering the total of the responses.

*Trade name. The citation of trade names in this publication is not to be construed as endorsement or as approval

Table 4. Pesticides most frequently used by applicators. Córdoba, Argentina, 2007 – 2010.

3.3 Technology and personal protection

Crop sprayers with activated charcoal filter were used by 71.9% of the applicators. Backpacks were used by 36.03%. Mixing/applying with a written pesticide prescription by an agricultural engineer is less than 40% in the whole province. In Córdoba province, by law Nº 9164, chemical or biological products of agricultural use must be prescribed by an agricultural engineer.

The analysis of use of single PPE components showed that "resistant gloves" were the most used (68.9%), followed by "gas mask" (51%), "face shields or goggles" (47.4%), "other clothing" (boots, apron, waterproof pants) (36.1%), "waterproof clothing" (30.1%) and "hat

or helmet" (30.1%). Forty-four percent of workers use fabric gloves but, since we do not consider this to be effective, we do not include it in the protection level estimation. Our results indicated that 67.3% of the applicators work unprotected or partially protected (0 to 70%) and only 32.7% of them mix/apply protected 90%. We consider only the latter group of workers to be effectively protected. Finally, we found that 44.5% of the applicators live 500 m or less from the nearest treated crop.

"Protected" type Relative Risks (RR) versus "unprotected" were 0.93 (not significant) and 0.84 ($p<0.05$) in the months of lower and higher temperatures, respectively. Protection level was not associated with education ($p=0.223$). The number of hectares sprayed/year was inversely associated ($p=0.036$) with the correct use of PPE, and directly with written pesticide prescription by an agricultural engineer ($p=0.042$). Also, being married or cohabiting (RR=0.648, $p=0.025$), using a crop sprayer with activated charcoal filter ($p<0.01$), and mixing/applying under written pesticide prescription (signed by an agricultural engineer, RR=0.385, $p<0.01$) were found to be protective factors, with significant effects relative to protective behaviour.

The results in general matched those obtained by Lantieri et al. (2009). The population most exposed is young, has had several years in this activity (average up to 10 y), does not use PPE appropriately and, generally, has no suitable safety machines for this job. Moreover, in terms of the geographical areas defined by the HEAs, statistical differences between the areas were identified basically for technological variables.

3.4 Geographical analysis

Similar means of applicators' ages (34.8 y, s.d. 11.1) were found between ecological areas, except for HEA I and HEA IV, which showed significant differences, with 5.3% and 25.6% respectively, in the category of workers over 45 years old. It should be noted that the average percentage of workers over 45 is low in all the areas (19.7%). This may suggest that there is an early withdrawal from this work due to the probable deleterious effects on applicator health. As mentioned above, average years mixing/applying is also similar between areas (40% of the population mixing/applying 6 years or more); nevertheless a significant difference ($p<0.05$) was observed in the highest category (working more than 20 years) between HEA I and HEA IV (4.8% and 14.9% respectively), indicating that the applicators who have been exposed most years to pesticides belong to the South-eastern Agricultural area.

Significant differences were observed in education level between areas. In fact, HEA I had no illiterate applicators or with incomplete primary school, while in the other areas, this education level is around 10% except in HEA IV (5.6%), with a maximum of 13% in HEA III. Except for HEA I, all the areas showed a high percentage of workers in the incomplete primary school category, matching the previous report by Lantieri et al. (2009). Those authors had already noted the importance of this information, pointing out that these applicators constitute a vulnerable population group for risk assessment, in which it is necessary to implement prevention strategies. HEAI showed the highest percentage of workers with 90% protection (52.4%) and the lowest percentage of applicators with complete secondary, technical or graduate studies (11.1%). There was a weak association between protection level and areas; a tendency was found (33.8%, $p=0.101$) only between HEA I and HEA II. Only 37.3% of workers were found to be 90% protected in HEA IV, but this area had the highest percentage of applicators with complete secondary, technical or graduate studies (42.3%). Again, this matches Lantieri et al. (2009), who reported that a

higher level of general education was not associated with higher PPE use. Also, we observed that workers with a lower level of education were more efficiently protected than applicators with a high level ($p<0.05$). Similar results were also noted by other authors (MacFarlane et al., 2008).

Around 45% of the workers use a self-propelled crop sprayer with an activated charcoal filter, and 55.1% a trailed crop sprayer. No significant differences among areas were detected in the use of crop sprayers. It should be noted however, that use of the self-propelled crop sprayer with activated charcoal filter is most frequent in HEA I (63.6%), followed by HEA V (48.6%) and HEA IV (47.1%). The lowest use of the self-propelled crop sprayer with activated charcoal filter (40.1%) and the highest use of the trailed crop sprayer with activated charcoal filter (13.6%) are in HEA II. This area also had the second lowest use of 90% protection level in the province. However, it is in this region that the highest use of written pesticide prescription by an agricultural engineer (43.4%) was found. Córdoba province has Law Nº 9164 regulating the use of chemical or biological products for agricultural tasks, which requires that products must be prescribed by an agricultural engineer.

Average crop surface treated was 7400 ha, showing significant differences between HEA III (10200 ha) and HEA IV (6050 ha).

The increasing area cultivated in recent years (Secretaría de Agricultura, Ganadería y Alimentos, 2010) and the number of ha sprayed by applicators suggest an increased risk of pesticide exposure. The present geographical analysis, based on homogeneous ecological areas, adds to the knowledge of the risk scenario faced by the study population and highlights the need to study these more deeply.

Overall, these results match those already reported by Lantieri et al. (2009). The population most exposed is young, has several years in this activity (average greater than 10 y), does not use PPE appropriately and, generally, has no suitable safe machines for this job. The present work also identified statistical differences between the geographical areas defined by the HEAs, basically in technological variables.

3.5 Exposure assessment

This work provides two indexes, ILE and CEI, describing the exposure of the applicator population, proposes statistical techniques to study their stochastic behaviors and, after intensive simulation steps, supplies percentile tables for recommendation and general uses. The following paragraphs describe the main findings.

The percentile (theoretical) estimates for ILE were: p25=1.72, p70=4.83 and p99=14.07, corresponding to 21%, 51.7% and 27.3% for Low, Medium and High risk levels, respectively. The CEI estimates were: p25=20.42, p75=103.12 and p99=433.30, corresponding to 24.8%, 50.6% and 24.6% for Low, Medium and High cumulative risk levels, respectively. Appendix I, with table A, shows more details of percentiles.

In order to throw light on the middle category, we defined the scales containing four categories for both indexes. The percentile estimates followed the same tendency: ILE and CEI values were p25=1.72 and 20.42 (Low), p50=3.21 and 55.63 (Medium), p75=4.83 y 103.12 (Medium High) and p99=14.07 and 433.30 (High), respectively. Again, table B in the Appendix I gives more details.

The cut-off points were used to identify how many applicators of our sample are classified in each category. Table 5 shows the results for the classification of individuals by means of the two measures (ILE and CEI), when a) three and b) four category scales were used for the

exposure risk. Figure 2 shows a comparison between both measures with the categories of each. It should be noted that the cumulative exposure is already detected in individuals from the first category, which is reflected by the estimates that are statistically higher than those obtained for the ILE.

a) Three categories scale

Index	Category	Percentage	Index	Category	Percentage
ILE	Low	21.0	CEI	Low	24.80
	Medium	51.70		Medium	50.60
	High	27.30		High	24.60

b) Four categories scale

Index	Category	Percentage	Index	Category	Percentage
ILE	Low	21.1	CEI	Low	24.8
	Medium	28.0		Medium	27.5
	Medium High	23.6		Medium High	23.1
	High	27.3		High	24.6

Table 5. Percentage of classification of the exposure risk of the subjects belonging to study population, using the ILE and CEI measures.

3.6 Applicator health

The study population shows a high prevalence of symptoms. Applicators reported suffering, sometimes or frequently, irritative manifestations (skin and eye irritation, nausea or vomiting, chest discomfort) 47.4%, excessive fatigue tiredness 35.5%, headache 40.4%, and nervousness or depression 27.6%. At least one medical consultation and hospitalization related to occupational pesticide exposure was recorded in 35.6% and 5.4%, respectively, of workers surveyed (Table 6).

By bivariate analysis, some of these health outcomes were associated to social and demographic characteristics as well as work practices. Headache was associated to age ($p<0.05$), with more frequency in the age group of 34 to 44. Seniority in application was associated to all the symptoms studied ($p<0.05$) and to greater frequency in medical consultation ($p<0.05$).

Improper use of PPE was associated to headache and irritative symptoms ($p<0.05$), showing an increasing trend among those suffering nervousness or depression but this does not reach statistical significance.

Medical consultation was associated to seniority in application (<0.05) and is more frequent among young adults (34 to 44 years), who also suffer headache. We also found an association with marital status, with an increase among those who are married or cohabiting ($p<0.05$), and with education level ($p<0.05$). Moreover and paradoxically, medical consultation related to pesticide use has been higher among the workers who are Protected (90%).

In this population, proximity of the applicator household to the nearest treated crop was associated neither with prevalence of symptoms nor with the frequency of medical consultation or hospitalizations.

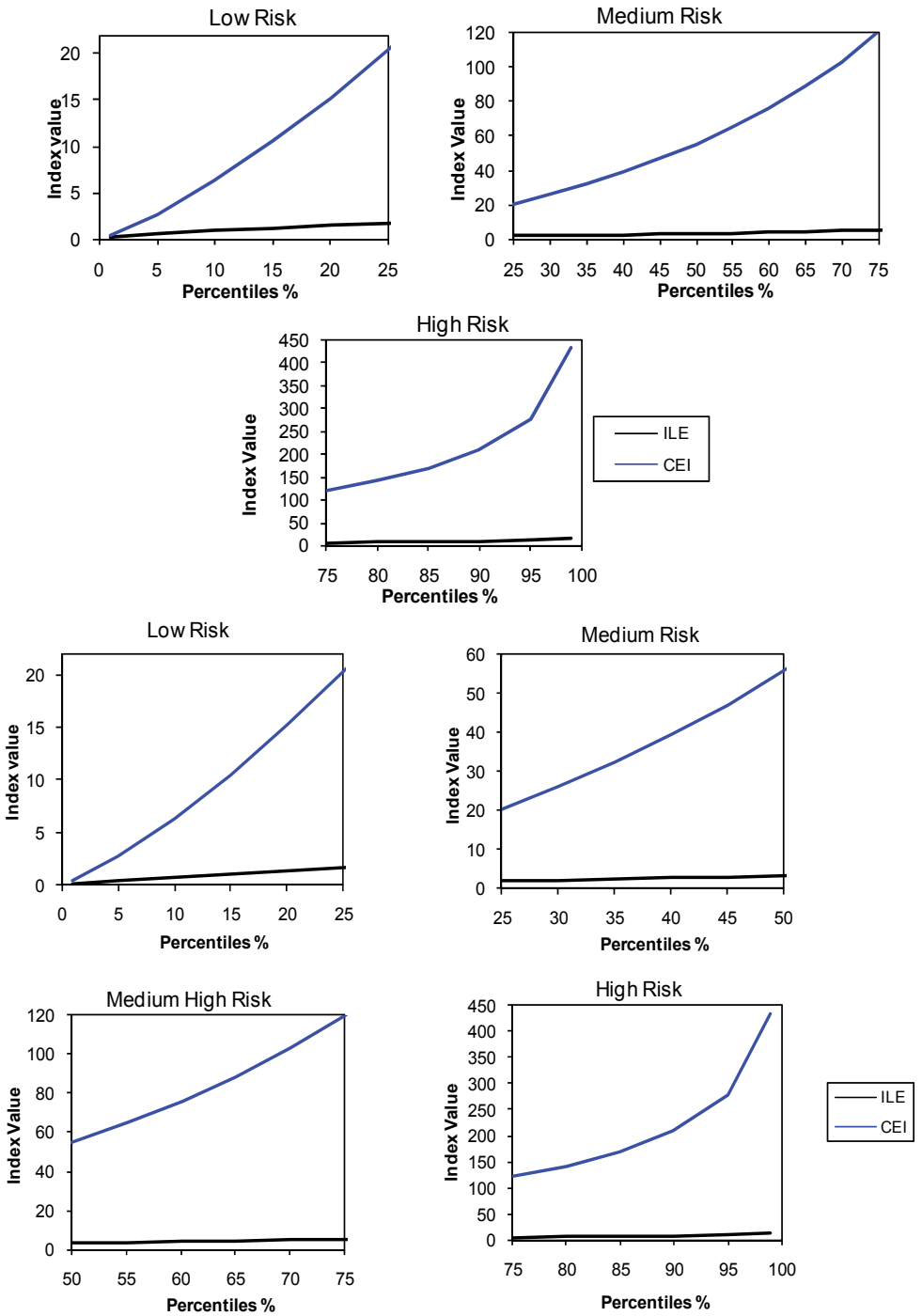

Fig. 2. Comparison between ILE and CEI behaviours, for each category: a) three category scale; b) four category scale

Symptoms	Never / Rarely[1]	Sometimes / Frequently[1]	Number
Fatigue tiredness	64.5	35.5	719
Headache	59.6	40.4	722
Nervousness or depression	72.4	27.6	648
Irritative manifestations	52.6	47.4	658
Health assistance	Never	Once or more times	Number
Hospitalization	94.6	5.4	742
Medical consultation	64.4	35.6	801

1. Percentage considering the total of the responses.

Table 6. Prevalence of symptoms, medical consultation and hospitalisation, related to occupational exposure among pesticide applicators. Córdoba, Argentina, 2007-2010.

In the exposure index (ILE and CEI), an association was found between adverse health outcomes and the highest exposure level of each index. Medical consultation at least once, for reasons related to occupational pesticide use, was associated with the highest exposure level of ILE ($p<0.05$). Using the CEI, almost all the health symptoms studied (irritative symptoms, fatigue tiredness, nervousness or depression, sometimes or frequently), as well as medical consultation at least once, were associated with the "high" category of cumulative exposure ($p<0.05$).

4. Discussion

We proposed two measures for the assessment of pesticide exposure risk. These performed well in terms of their probabilistic behaviour, which enables them to be used to draw up the percentile tables for use as a reference or recommendation and to construct two qualitative scales of exposure risk. These scales are worth testing and validating for other contexts in the future, as they are based on two robust and reliable formulations (ILE and CEI).

The main characteristics of the population most exposed is young, has several years in this activity (40% of the population have been mixing/applying for at least 6 years), does not protect itself appropriately and, generally, does not use suitable, safe machines for this job, matching previous results of Lantieri et al. (2009).

The study of pesticide applicators within HEAs highlights differences in basic characteristics of this population, such as their average age, instruction level and length of occupational exposure to pesticides. In HEA I, an area of recent agricultural development, the applicators are younger than in other areas and only 11% reached secondary school. In HEA IV, a traditional agricultural area in the province, applicators are older and at least 42% finished their high school. In terms of age and education, Lantieri et al., (2009) reported a negative statistical association in Córdoba province: on average, older pesticide applicators are those with a lower education level. The data indicate the unequal access to education between the areas of terrestrial applicators, and are valuable tools for the planning of preventive public health actions.

It is noticeable that in the newer agricultural areas, workers adopted safer labor practices in terms of the use of PPE. We did not find other statistical differences between areas in the technological variables studied; a trend to the use of more modern sprayer technology and the use of crop sprayers with activated charcoal filters, were described in traditional agricultural areas, reaching the highest proportion in the south east of the province (HEA IV). The low rate of PPE utilization found was not related to workers' educational level or to

the years personally mixing/applying pesticides. Similar results were reported by other authors, (García et al., 2002; Macfarlane et al., 2008; Schenker et al., 2002), who found that education level, professional training, risk perception, environmental safety conditions and socio-cultural background, are some of the factors associated with self-protective behavior.

The increasing cultivated area in recent years (Secretaria de Agricultura, Ganadería y Alimentos, 2010) and the number of ha sprayed by applicators suggest an increased risk of pesticide exposure. Our geographical analysis, based on homogeneous ecological areas, adds to the knowledge of the risk scenario faced by the study population and highlights the need to deepen research.

The primary goal of exposure estimation in epidemiology is to correctly rank individuals with regard to exposure level in the study population (Alavanja et al., 2004). Percentile tables for both indexes were estimated here in order to identify which population group falls into the high exposure category as well as to describe some characteristics of risk levels. The frequency distribution of each measure illustrates the empirical behaviour. Indexes were generated by adapting and using published literature and evidence of our researchers. They seem to be intuitive and simple to interpret. However, some aspects related to the possible intra-subject and inter-subject variability should be considered here.

Between subjects, within the agricultural population and temporally, exposure to specific pesticides will vary highly depending on the type of agriculture (livestock versus arable production), type of crop (vegetables, fruits, flowers), type of application method (knapsack, boom sprayer, etc.), controls installed (cabin versus no cabin), and use of personal protective devices. It is known that, given all these possible variations in determinants of agricultural exposure (and pesticides in particular), it is difficult to accurately assess the intensity, duration, and frequency of exposures (Kromhout & Heederik, 2005). Our study includes all those variables because the population is, in fact, made up of pesticide applicators in crops (mainly soybean, followed by corn). Thus, in this case, characteristics such as agriculture type or crop type would not contribute variability when estimating the level of exposure. The remaining variables already mentioned, like type of application method, controls installed and use of personal protective equipment, are covered by the index formulas.

In addition to exposure determinants, there are variations between workers and within the same worker over a day. Dermal exposure is the main route of pesticide exposure and is highly relevant in the agricultural environment, followed by the inhalable route in workers handling chemicals outdoors. Kromhout & Heederik (2005) reported that, for agricultural re-entry workers exposed to pesticides, no between-worker differences in dermal exposure were observed. One argument to explain this could be that the tasks are similar among workers, and also that everyone is exposed to the omnipresent source of exposure (dislodgeable foliar residues). However, daily within-worker concentrations varied within an average 10- to 40-fold range (Kromhout & Heederik, 2005). For this reason, estimates of these authors will be taken into account when analyzing the results of exposure levels, based on the indexes that we developed. Empirical evidence obtained in this work showed that aspects of applicator behaviour must be taken into account in order to understand this daily variability.

One important point is that a large amount of daily work (amount ha/day), coupled with a small capacity of the machine available, causes more stops for pesticides handling, and thus makes the tendency to exposure over time more likely. In other words, applicators in our population might be appropriately protected at the time of application, but not when preparing the stock for each load. Besides, when the number of hectares is increased, time is

even more restricted, and so it would not be surprising to see workers begin to perform their tasks faster and be more negligent of their protection. Finally, index values could come from people who are beginner applicators, and hence more cautious and fearful in the use of agrochemicals.

An assessment of exposures in occupational and environmental epidemiology also needs to cover the etiologically pertinent time periods. In retrospective studies, these periods lie by definition in the past of the study subjects. Ideally, the basic parameter to be estimated is the exposure intensity as a function of time. Exposure durations, average intensities, cumulated exposures, peak exposures and any other important parameters would be generated from this (Teschke et al., 2002). In our work, CEI incorporates the time by using the usual quantity of hectares that the applicator works as a surrogate variable. This proxy variable seems suitable for representing temporal information as cumulative exposure, since it is reported by the applicator considering only what is usual in his work every day.

From a public health perspective and for epidemiological purposes, estimates of actual dermal exposure and possible uptake would be a requirement to safeguard the working population from the negative health effects of dermal exposure to chemicals (Vermeulen et al., 2002). It is not possible to estimate exposure for a local situation solely on the generic exposure scenario involved. Additional measurements of dermal exposure and concomitant collection of detailed descriptive information will be necessary to evaluate potential dermal exposure (Kromhouti et al., 2004). Therefore, the present work performed a detailed and exhaustive characterization of the population of pesticide applicators.

Knowledge of ambient toxins depends on the agents and working processes used. Most epidemiological studies assessing health and environment risks have focused on the assessment of single exposures without addressing the effects of mixtures (Samet and Speizer, 1993). The synergistic effect of mixtures on the etiology of disease is of increasing concern for public health. Since there is an element of methodological uncertainty associated with determining the components of a mixture, measurements of the components most relevant to disease outcome may not be achieved (Leaderer et al., 1993). Like the present work, a few studies have dealt with multiple exposures but they had cross-sectional designs or used surrogates for exposure measurements. We believe this is a key point that must be addressed in the future in order to deepen both the design of epidemiological studies and the proper analysis of the information that is collected.

Even with the many complexities in estimating exposures, some studies have suggested that pesticides experts, industrial hygienists and crop-growing experts can identify the most important determinants of external exposures (Dosemeci et al., 2002). Our indexes were defined following this approach. As these measures constitute the first version for quantifying exposure risk in our context, we are aware that various methodological issues should be addressed before they are employed as a reference for monitoring exposure, mainly for human health uses. Therefore, we are in process of developing a series of validation studies to evaluate the effects of each exposure variable in the adapted Dosemeci algorithm, making some possible changes in the weighting procedure and then in the index formulations. Throughout these validation studies, we will monitor the most commonly used exposure scenarios observed in our population and compare the algorithm-based intensity estimates with the results for the monitoring data for that particular scenario. The scenarios could be the different homogenous ecological areas in the province. As a consequence, further refinement of the individual exposure score (value) will be carried out by using its predictive value obtained from, for example, a regression modeling based on

the exposure variables used in our algorithm and index expressions and the actual monitoring results for the given exposure scenario.

Epidemiological and health-risk assessment of potential health hazards is difficult. Our work offers a first outline about the health of a clearly susceptible population of workers. Estimating the health effects of occupational exposure to para-occupational pesticide exposure cannot be measured exclusively by traditional indicators of mortality and morbidity. The statistics on acute pesticide poisoning do not reflect the magnitude of the problem, with evident under-reporting, particularly in rural areas (Henao et al., 1993), where farm workers suffer the most severe effects, creating a greater burden of health problems (Arcury et al., 2001). The high prevalence of reported symptoms associated with pesticide exposure, as well as the medical consultation and hospital discharge rates related to their occupational exposure to pesticides, are indicators of high occupational exposure.

The association between non-proper use of PPE and headache and irritative symptoms, and between seniority in the application task and all the symptoms studied and greater frequency in medical consultation are indicators of the negative impact on the health of our workers. These issues and the hospital discharge rate reported here, fifteen times higher than that calculated for males between 15 and 64 y in the general population of the province of Córdoba[1], also present indirect evidence of intense occupational exposure.

The results obtained concerning the workers' health conditions together with the fact that those who suffer symptoms are more likely to belong to the high exposure categories of both the ILE and AEI indexes, demonstrate their applicability for monitoring in this context. Finally, we have not yet reported information about chronic disease, due to the cohort size until now.

5. Conclusion

Occupational exposure in agricultural settings in Córdoba presents a complex risk scenario in which the population is highly exposed and very vulnerable. Some causes of this are the increasing area in which pesticides are applied, the high number of active principles involved and the lack of safe working environments with training, technology and adequate health and safety measures. To this must be added the regulatory gap and the lack of enforcement.

Early involvement in the task, the proximity of applicator households to treated crops and the non-use or improper use of personal protective equipment are indicators of intense exposure and vulnerability; this is reflected in the high prevalence of symptoms and of medical consultation and hospitalization related to occupational exposure. The exposure indexes and scales developed in this work are useful tools that can be applied as part of the

[1]The hospital discharge rate was estimated with data of patient discharge (male 15 to 64 years old from Córdoba province) (Dirección de Estadísticas e Información de Salud, 2009) in the numerator and the census population for the same period (specific for sex and age group) in the denominator. The base population was adjusted with a factor of 0.458, corresponding to the proportion of the population attending public hospitals in the mentioned period, the available data on hospital discharge correspond only to official health providers (hospitals corresponding to national, provincial or municipal level) (Instituto Nacional de Estadísticas y Censos, 2011b).

assessment of occupational risks related to pesticide exposure. The goals proposed to address this complex problem are to implement epidemiological surveillance systems that allow permanent monitoring of human and environmental health, to encourage the development of alternatives to pesticide use such as integrated pest management, to encourage and strengthen citizen participation and especially that of the workers and communities most at risk, enabling their direct involvement in decision making, and improving control over compliance with existing legislation in this area and with health and safety conditions for farm workers and their families. Finally, our own challenge is to develop quality research to address the needs of this population and improve their quality of life.

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7. Apendix I

Percentiles	Intensity level		Cumulative Exposure	
	Index	Risk Levels	Index	Risk Levels
1%	0.21		0.46	
5%	0.56		2.77	
10%	0.88	LOW	6.38	LOW
15%	1.17	21%	10.55	24.8 %
20%	1.45		15.23	
25%	1.72		20.42	
30%	2.00		26.13	
35%	2.28		32.43	
40%	2.57		39.38	
45%	2.88		47.07	
50%	3.21	MEDIUM	55.63	MEDIUM
55%	3.56	51.7 %	65.24	50.6 %
60%	3.94		76.07	
65%	4.36		88.55	
70%	4.83		103.12	
75%	5.38		120.48	
80%	6.04		141.93	
85%	6.87	HIGH	169.80	HIGH
90%	8.02	27.3 %	209.36	24.6 %
95%	9.91		277.42	
99%	14.07		433.30	

Table A. ILE and CEI theoretical percentiles (three categories scale)

Percentiles	Intensity level		Cumulative Exposure	
	Index	Risk Levels	Index	Risk Levels
1%	0.21		0.46	
5%	0.56		2.77	
10%	0.88	LOW	6.38	LOW
15%	1.17	21%	10.55	24.8 %
20%	1.45		15.23	
25%	1.72		20.42	
30%	2.00		26.13	
35%	2.28	MEDIUM	32.43	MEDIUM
40%	2.57	28.0 %	39.38	27.5 %
45%	2.88		47.07	
50%	3.21		55.63	
55%	3.56	MEDIUM	65.24	MEDIUM
60%	3.94	HIGH	76.07	HIGH
65%	4.36	23.6 %	88.55	23.1 %
70%	4.83		103.12	
75%	5.38		120.48	
80%	6.04		141.93	
85%	6.87	HIGH	169.80	HIGH
90%	8.02	27.3 %	209.36	24.6 %
95%	9.91		277.42	
99%	14.07		433.30	

Table B: ILE and CEI theoretical percentiles (four categories scale)

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8

Separation of Chiral Pyrethroid Pesticides and Application in Pharmacokinetics Research and Human Exposure Assessment

Yongning Wu, Hong Miao and Sai Fan
Key Lab of Chemical Safety and Health & WHO Collaborating Center for Food Contamination Monitoring
National Institute of Nutrition and Food Safety
Chinese Center for Diesease Control and Prevention
China

1. Introduction

The pesticides are originally used to kill insects, fungi and other organisms hazarding crops to improve agricultural production. With the development of technology, people have been gradually aware of the impact of pesticides to the environment and food safety. More than 25% of over 650 existing commercial pesticides are characterized by chirality (Zheng, 2001; Williams, 1996). Of the commercial pesticides, the pyrethroid insecticides account for 25% of the sales of pesticides in the world, and most of them have chiral isomers(You et al., 2001). The chiral enantiomers of pesticides were usually not distinguished in previous studies, and as a result, the risk assessment of pyrethroid pesticides was incomplete.

With the development of stereochemistry, the chirality of compounds has aroused wide concern. The researches on medicine and pesticides have penetrated into the field of molecular stereochemistry. Especially in the field of medicine, there have been more researches on and application of the single enantiomers of chiral drugs, and the natures of the enantiomers have been well studied. However, the researches on chiral pesticides relatively lag behind. The researches of various natures of pyrethroid pesticides are usually carried out by using racemic mixture, so there are few detailed data and related researches of single chiral enantiomers. Despite the same chemical and physical properties of the enantiomers of chiral pesticides, they may have entirely different biological activities, toxicities, toxicologies and metabolic pathways in the biological systems. For instance, usually only one out of the four chiral monomers of permethrin is provided with high insecticide efficiency, and the remaining three are low efficient or even ineffective in terms of insecticidal effect. And of the eight chiral enantiomers of cypermethrin, only two monomers of cis- cypermethrin and two monomers of trans- cypermethrin have high insecticidal effect. And as a result, not only the cypermethrin with 8 chiral monomers but the beta-cypermethrin with 4 highly effective monomers has been commericially producted.

The overwhelming number of widely used pyrethroid pesticides are almost sold and used in the form of racemic mixtures. The use of low efficient or ineffective isomers of the racemic mixtures not only cannot effectively control insect pests, resulting in the waste of manpower

and material resources, but will pollute the environment, reduce the quality of agricultural products, and may lead to toxic side effects or drug induced sufferings, and thus cause serious impact on human health. It is therefore of great significance to evaluate the possible hazards and the influence of the single chiral monomers of pesticides on environment and human health by using the separation technology of chiral enantiomers.

2. Introduction of pyrethroid pesticides

The pyrethroid pesticides are primarily used in agriculture, such as controlling the cotton, vegetables and fruit-eating and leaf-eating pests, and the total sales in this area account for 95% (Katsuda, 1999). In addition, pyrethroid pesticides are also widely used as household insecticides to control mosquitoes, cockroaches and parasites in farm animals.

2.1 Production history of pyrethroid pesticides

Pyrethroid pesticides are efficient, broad-spectrum and quick neurotoxic pesticides developed on the base of the researches on the chemical structure of the natural pyrethrins. They can be divided into type Ⅰ pyrethroids without cyano-group (CN) and type Ⅱ pyrethroids containing CN by the presence of CN in the molecules. The chemical structures of common pyrethroid pesticides are shown in Figure 1.

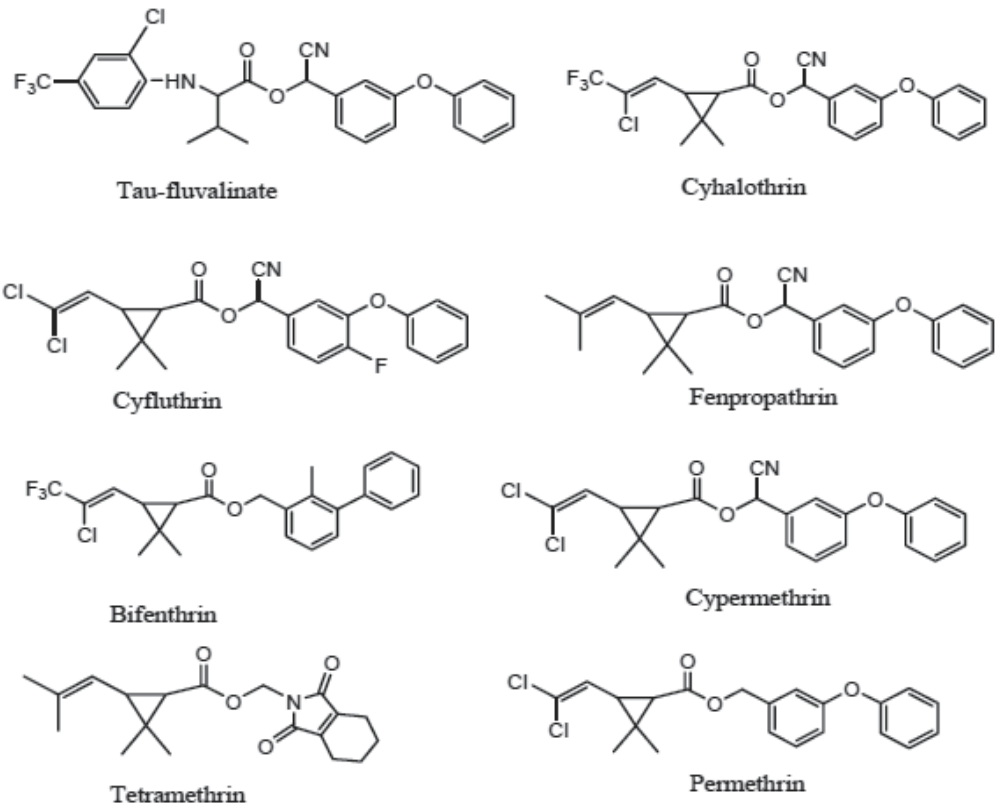

Fig. 1. Structures of pyrethroid pesticides

The researches on synthesis of pyrethroids have been carried out since the 1940s when the chemical structure of the active ingredients contained in an insecticidal plant called pyrethrum was studied and determined (Cox, 2002). In 1949, Schechter and his colleagues from the United States synthesized the first commercial pyrethrin analogues: propylene permethrin. In the 1950s to 1960s, a number of similar compounds, which were known as synthetic pyrethroids, were successfully developed. In the early 1970s, permethrin, the first pyrethroids, with light stability which can be applied to pests control in agriculture and forestry was synthesized by a team led by M. Elliottm in UK, and then popularized and applied in agriculture. Thereafter the pyrethroid pesticides have become booming. There are more than 70 pyrethroid pesticides varieties in the world including more than 20 leading ones. They have been considered the fourth largest pesticides, currently accounting for 19% of the sales of the insecticides in the world (Liu et al., 2004).

In recent years, the chiral pesticides, as a new research field, have attracted extensive attention. The commercial chiral pesticides, however, are few in number and with the majority of pyrethroid pesticides, including *beta*-cyfluthrin, *alpha*-cypermethrin (*cis*-cypermethrin), *theta*-cypermethrin (*trans*-cypermethrin), *beta*-cypermethrin, S-fenvalerate, deltamethrin, *d-trans*-allethrin, etc.. The cypermethrin series account for 26.5% of the pyrethroid pesticides. The production of single enantiomer of deltamethrin (*1R-cis-3R*) has been currently achieved commercially.

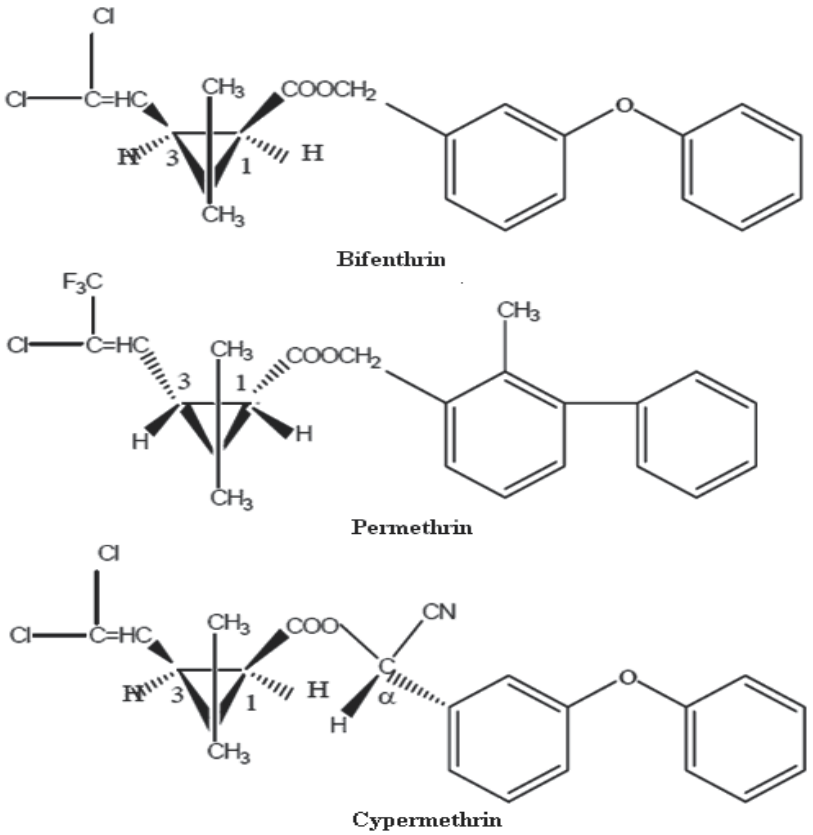

Fig. 2. Stereo-structures of bifenthrin, permethrin and cypermethrin

2.2 Chirality of pyrethroid pesticides

Chirality, a property of molecules having a non-superposable mirror image, is a term used to express the asymmetry of the molecular structure of compounds. And chirality is one of the essential attributes of the nature (Schulgasser et al., 2004). A chiral molecule is a type of molecule that lacks an internal plane of symmetry and thus has a non-superposable mirror image. The feature that is most often the cause of chirality in molecules is the presence of an asymmetric carbon atom. With the growing worldwide attention to the chirality of compounds, many countries have carried out compulsory registration of chiral pesticides, and meanwhile the data of the biological activities and degradation of the enantiomers must be declared. The pyrethroid pesticides enjoy 30% market share of pesticides in the world, most of which contain one or more chiral centers. The chirality of pyrethroid pesticides deriveds from the two chiral carbons in the traillylantimonite structure of the acid part and the α carbon atom of the alcohol part. Therefore, there may be up to 3 chiral carbon atoms in pyrethroid pesticides, and there may be 2, 4 or 8 chiral isomers (Table 2) and all the enantiomer are greatly different in their biological activities (Owen, 1975). The representative stereo-structures of pyrethroids are shown in Figure 2. The pollution of racemic mixtures has been long regarded as a single compound during the evaluation of the environmental behavior, ecological effects, potential toxicity and toxicology of the pyrethroid pesticides. And the racemic mixture of the pesticides containing two or more enantiomers have been treated as a compound without taking the feature of configuration transformation or prior metabolism of the enantiomers into account, which result in the unreliable risk assessment of pyrethroid pesticides.

2.3 Toxicological mechanism of pyrethroid pesticides

The pyrethroid pesticides, as neurotoxic pesticides, are provided with high lipophilicity, poor water-solubility, long residues and slow metabolic rate in the environment. The toxicity is mainly characterized by (i) the severe toxicity. The insecticidal activity of pyrethroids mainly resides in their action on voltage-sensitive sodium channels in the nervous system, extremely enlarging the sodium channels and leading to the hyperexcitability of the nervous system; (ii) the negative temperature dependence. The pyrethroid pesticides are of better insecticidal effect at low temperature than in high temperature; (iii) the selective toxicity. In virtue of the important role of neural sodium channels in the selective toxicity, the pyrethroid pesticides are much more toxic to insects than mammals; (iv) the varied targets. A variety of receptors, channels and enzymes are likely to be the targets of pyrethroids.

Despite the high toxicity of pyrethroid pesticides to insects, fishes, etc and low or moderate toxicity to human or mammalian, they will lead to some neurotoxic symptoms such as convulsions, tremor, ataxia, paralysis, etc. by affecting the sodium channel of human body (Albert & Pombo-Willar., 1997). Long-term exposure to pyrethroids may cause injury to the lymph nodes and spleen and the risk of canceration, and it has been found that the currently used pyrethroid pesticides, such as bifenthrin, cypermethrin, fenvalerate, etc. which have immune toxicity, will affect the function of human's immunity system. People chronically exposed to these pesticides will suffer from chronic intoxication characterized by headaches, dizziness, nausea, skin itching and other symptoms. The pyrethroid pesticides with halogen hydrocarbons and phenyl ether radical, which are provided with anti-androgen and thyroxine interferent, are considered one of chemical pesticides with environmental hormones (Hill, 1985).

pyrethroids	Number of chiral centre	Number of chiral monomers
empenthrin	3	8
fenfluthrin	2	4
fenpirithrin	3	8
furethrin	3	8
imiprothrin	2	4
prallethrin	3	8
pyresmethrin	2	4
resmethrin	2	4
tefluthrin	2	4
tetramethrin	2	4
transfluthrin	2	4
kadethrin	1	2
fenpropathrin	1	2
fenvalerate	2	4
terallethrin	1	4
protifenbute	1	2
flucythrinate	2	4
fluvalinate	2	4
brofluthrinate	2	4
flufenprox	1	2
cycloprothrin	2	4

Table 1. pyrethroids and the number of stereoisomers

2.4 Organism's resistance to pyrethroid pesticides

Although the pyrethroid pesticides have been widely used for only thirty years, the reports show that the resistance to them has covered all categories of insects. Compared with organophosphorus pesticides and carbamate pesticides, pyrethroid pesticides are much more likely to cause pests' resistance (Mahmoud et al., 1988). The domestic and foreign practices, surveys and studies have shown that, the pests in high selective environment where the pyrethroid pesticides applied in successive years or repeatedly applied in one year will be quickly provided with tolerance with high ratio and the cross-resistance in different categories. The resistance are in two main mechanisms: one is target resistance, that is, with the changes in the action target under the sequential action of pesticides, the pesticides cannot be combined themselves with targets; the other is metabolic resistance, that is, the pesticides are degraded and prevented to act on targets through the increased detoxification enzyme activity by the increase in gene expression or gene mutations. A large number of literature and reports suggest that the resistance to pyrethroid pesticides is the result from, on one hand, the changes in two amino acids found in the sodium channel gene in the resistant strains, which might be one of the main mechanisms leading to the highly resistance of housefly to the pyrethroid pesticides (Brewer & Tremble, 1994), on the other hand, the increase in the detoxification capacity of the detoxification enzymes related to the

metabolism, such as the high expression of chymotrypsin gene or the increased activity of esterase and mixed-function oxidases, which is also one of the main mechanisms of insects' resistance to pyrethroids (Josi & Reutter, 1989; Hu et al, 2008).

3. Separation of chiral monomers of pyrethroid pesticides

3.1 Detection methods of pyrethroid pesticides

The total amount of mixed modification pesticides should be determined prior to the separation of chiral monomers of pyrethroid pesticides. Currently, mainly two kinds of methods are applied to detection of pyrethroid pesticides. One is the chromatographic detection technology based on instrument method, and the other is the immunoassay technology based on the specific immune response of the antigen and antibody. The latter, including radioimmunoassay (RIA) and enzyme-linked immunoassay (EIA), is fast and sensitive. By virtue of its high specificity, it can handle a large number of samples in one application. However, due to the antibodies used in immunoassay technology are generally with high specificity and thus have poor ability of identifying other analogues, it is generally not suitable to the multi-residue detection. And furthermore, additional analytical methods such as gas/liquid chromatography coupled with mass spectrometry must be applied to the accurate quantification and confirmation after the detection by immunoassay methods.

However, the immunoaffinity chromatography (IAC), which developed from the immunological technique, provides a good solution for the purification of pyrethroid pesticides under detection in the complex sample matrix. It is a major research trend in the field of detection of pyrethroid pesticide residues by using equipment detection (Húsková et al., 2009).

3.1.1 Chromatographic detection technology

Due to the generally higher boiling point (between 130-200 ℃) and good thermal stability and weaker polarity, the gas chromatography is used as a main analytical technology. Meanwhile, the electronegative elements (halogens) in the chemical structure of pyrethroid pesticides can better respond to some selective detectors, such as electron capture detector (ECD) or negative chemical ionization (NCI) mass spectrometry detector (Wu et al., 2010). Húsková et al (Húsková et al., 2009) detected 23 endocrine interferon pesticides, including pyrethroid pesticides, in apple by using GC-MS. By comparing EI mode and NCI mode, the results showed that the NCI mode has a higher linear correlation coefficient, sensitivity and better selectivity. Wu et al(Wu et al., 2010) determined 11 pyrethroid pesticides by using liquid-liquid extraction and GC-ECD detection, and the detection limits were between 0.29 ng / L ~ 2.29 ng/L. Francesc et al (Francesc et al., 2005)determined kinds of pyrethroid pesticides in vegetable oil by gas chromatography-tandem mass spectrometry (GC-MS/MS) coupled with solid-phase extraction, and the detection limit were up to 0.3 ~ 1.4 ng/g.

However, due to the trace amount of the pesticide residues in the samples, how to minimize the loss in extraction is of great importance to the sensitivity and accuracy of the detection method. As a result, the purification technology jn the process of the sample pretreatment is crucial for the sensitivity of the detection method. Because of complex of the matrix of plant and animal tissues and the feature of pyrethroid pesticides' easily accumulating in fat tissue pose great challenges to the sample pretreatment. So the solid phase extraction (SPE), liquid-liquid extraction (LLE), gel permeation chromatography (GPC) and other complex sample

pretreatment methods are generally applied(Khay et al., 2008). Massive toxic organic solvents used in this process leads to high costs of detection and disadvantages to the environmental protection. Therefore, the IAC integrating the functions of separation, purification and concentration of sample and with high selectivity can highly purify and concentrate the specific ingredients in complex samples. So that IAC can greatly simplify the sample pretreatment process, improve the efficiency of extraction, avoid the repeated extraction and concentration, while avoiding the use of massive organic solvents and reducing costs. Therefore, as an environment friendly product, IAC is one of the major research trends in the field of sample purification.

3.1.2 Immunoassay technology

The immunoassay (IA), in spite of its specificity, sensitivity and large analysis capacity, is limited in the field of determination of pyrethroid pestices due to the difficulty in development of antibody and its limited application to single compound or compounds with similar structures. However, compared to traditional instrumental analysis methods, IA with the advantage of specificity, convenience, large analysis capacity, low costs, is becoming a hotspot in the field of rapid detection of pesticide residues. The preparation of antibodies of pyrethroids has begun in the late 1970s. Hammock et al (Keith et al., 1978) from the EPA-NERL/Human exposure research laboratory of the University of California acted as pioneer in preparing polyclonal antibody of bioallethrin in 1978. Thereafter, polyclonal antibodies of fenpropathrin, S-fenvalerate, permethrin, deltamethrin, cypermethrin and other of pyrethroid pesticides, and the class selective antibody for type Ⅰ pyrethroid pesticides and type Ⅱ pyrethroid pesticides were prepared by the team. And the corresponding ELISA methods were established (Keith et al., 1978; Shan et al., 2001; Mak et al.,2005). Skeritt et al (Skerritt et al., 1992) prepared the monoclonal antibody of phenothrin and permethrin respectively, and analyzed the residues of corresponding pyrethroids in the grain samples; the Corbel Laboratory of France prepared the monoclonal antibody which can identify deltamethrin (Queffelec et al., 1998).

Many research teams in China have also carried out the exploratory studies on the immunological analysis of pyrethroid pesticides. Zhejiang University applied for the patent by the title of *"Fenvalerate artificial antigen, antibody and its application"* in 2005 (ZL03114897.2). Yangzhou University prepared the polyclonal antibody of fenvalerate (Zhu et al., 2004). Gao Hongbin from China Agricultural University prepared the polyclonal antibody of cyhalothrin and developed the ELISA detection method (Gao et al., 2006). Li Bo et al from Nanjing Agricultural University (Li, 2007) prepared the polyclonal antibody which can identify bifenthrin and cypermethrin. Luo Ailan et al (Luo, 2004) prepared the polyclonal antibody which can identify permethrin, cypermethrin, fenpropathrin, deltamethrin and cyhalothrin in Yangzhou University and established ELISA detection technologies for the 5 pyrethroid pesticides.

Currently, the immunoaffinity chromatography (IAC), which is based on the specific combination of antigen with antibody, is promising in the analysis of pyrethroid pesticide residue as a SPE technology using the feature of antigen-antibody specific reversible binding. In virtue of its low cost and high sensitivity, more and more researchers have begun to develop the IAC technology applying in the determination of pyrethroid pesticides. And the multi-immunoaffinity of multiple antibody and class selective antibody can separate and purify multi-residue components in one application and thus provide the

immunoassay with the ability to handle determination of multi-residue, which is the development trend of affinity chromatography technology in the area of residue determination.
The author's laboratory successfully prepared polyclonal antibody and IAC column which can identify 6 pyrethroids including tau-fluvalinate, cyhalothrin, cyfluthrin, fenpropathrin, cypermethrin and deltamethrin, and the IAC columns were applied to the purification of biological samples such as pork samples (Kuang et al., 2009; Kuang et al., 2009). Based on the above researches, an Indirect competitive Enzyme-Linked Immunosorbent Assay based on monoclonal antibody for the detection of Pyrethroids' metabolite PBA was developed in our lab, and the ELISA method was successfully applied to the determination of 3-PBA in pig urine.

3.2 Methods of chiral separation of pyrethroid pesticides

The method used to obtain a single enantiomer of chiral compound can be roughly divided into synthesis and racemic mixture separation method. The synthesis method can be divided into chiral synthesis and asymmetric synthesis. Despite the ability to obtain the active single enantiomer, synthesis method is not widely applied due to its tedious synthesis process, high cost and low optical purity. The racemic mixture separation method is widely used due to its easy implementation, relatively simple operation and low cost. Over 65% of non-natural chiral pesticides are obtained by the racemic mixture separation or intermediate products. The racemic mixture separation method include crystal separation, chemical separation, biological separation, extraction separation, chiral membrane separation and chromatographic separation, in which the chromatographic separation is the most widely used one.

3.2.1 Method of chiral liquid chromatographic separation of pyrethroid pesticides

High performance liquid chromatography (HPLC), a method widely used in the separation and analysis of pesticides, can not only apply to analysis but preparation of chiral monomers of pyrethroid pesticides. The working principle of HPLC in the field of chiral separation of pyrethroid pesticides is to show the difference of physical and chemical specificity of the optical active enantiomers by the introduction of asymmetric atoms or creation of chiral environment. With this theoretical basis, the method of chiral separation of enantiomer molecules by HPLC can be divided into two categories: direct method and indirect method. The direct method falls into two methods: the method of mobile phase additives and the method of chiral stationary phase. The method of mobile phase additives is to add chiral selector into mobile phase and the chiral selector was combined with the chiral enantiomers, and then the chiral enantiomers are separated by the functions of stereo-selective attraction or repulsion of the non-chiral stationary phase. The principle of the method of chiral stationary phase separation is that the temporary compounds with different stability produced by the combination of the two enantiomers with chiral stationary phase, and the ones with poor stability will be eluted earlier when the mobile phase passes through and thereby the purpose of the separation of enantiomers is achieved. The indirect method falls into two methods as well: one is to separate the enantiomers by the derivatization of enantiomer by chiral derivatization reagent and then separate the derivative products by non-chiral stationary phase; the other is to separate the enantiomers by the derivatization of enantiomer by non-chiral derivatization reagent and then separate enantiomers by chiral stationary phase (Huhnerfuss & Shah, 2009).

Owing to the reaction of chiral derivatization, the indirect method is usually cumbersome and needing more complex aftertreatment. The method of chiral mobile phase additives is tedious as well. Furthermore, for chiral separation of pyrethroids, the molecular structure of pyrethroids lacks the functional groups which are needed for the reaction with chiral selectors, So it is frequently necessary to hydrolyze pyrethroids into acid, while, for pyrethroid pesticides containing CN, the chirality of the compound will be lost or changed with the loss of CN in the hydrolysis process. And as a result, the method of chiral mobile phase additives is not applicable to chiral separation of pyrethroids. The chiral stationary phase method is the one most widely used method in the field of chiral separation of pyrethroid pesticides by HPLC (Haginaka, 2002).

The design and development of the highly selective chiral stationary phase (SCP) are crucial for the successful chiral separation of pyrethroid pesticides by HPLC. The CSP used in chiral HPLC include CSP of cyclodextrins, proteins, crown ethers, polysaccharides, polyacrylamides, polymeric chiral surfactants, macrocyclic antibiotics and some low-molecular-weight molecules such as Pirkle type compounds. The pirkle-type CSP (also known as Pirkle type 1A), which was the pioneer CSP used in the chiral separation of pyrethroid pesticides, was first developed by Pirkle et al (Pirkle et al, 1981) in 1980. Chapman (Chapman, 1983) firstly separated the four chiral enantiomers of fenpropathrin and fenvalerate respectively by using benzeneacetic and a-[(3,5-dinitrobenzoyl amino] as CSP. Cayley et al (Cayleya & Simpson, 1986)made a systematic study in chiral separation of enantiomers of pyrethroids by using ionic bonded Pirkle type 1A CSP. The experiment results show that ionic bonded CSP is more efficient than covalent bonded CSP. Edwards et al (Edwards et al., 1987) separated 3 pair out of the 4 pair enantiomers of the cypermethrin by using cellulose derivative chiral stationary phase with hexane and isopropanol used as the mobile phase, and the two enantiomers of the alpha-cypermethrin were also well separated. Schurig et al. (Schurig et al., 1996) partly separated the two enantiomers of alpha-cypermethrin on cyclodextrins chiral stationary phase by using methanol / water as mobile phase. Yang Guosheng (Yang & Dai, 1998) separated the chiral isomers in fenpropathrin and methothrin in the mobile phase system of hexane and isopropanol by using Pirkle CSP. Wang Peng et al (Wang, 2006) made chiral separation of the enantiomers of alpha-cypermethrin and theta-cypermethrin under normal phase HPLC on the homemade chiral stationary phase by coating cellulose-tris 3, 5-dimethylphenyl carbamate (CDMPC) onto aminopropyl silica gel, and baseline separation can be achieved. Sánchez (Sánchez et al., 1998) separated bifenthrin and fenpropathrin by using Chiralspher chiral column, and he separated the enantiomers of tau-fluvalinate and permethrin on the Chiralcel OJ chiral column with hexane/ethanol as the mobile phase. This method has achieved baseline separation and was applied to the detection of residues of these pesticides in water. Faraoni (Faraoni et al., 2005) completely separated the enantiomers of fenvalerate and cyfluthrin by using non-chiral liquid phase chromatographic column coupled with chiral liquid chromatographic column (stationary phase of polysaccharide derivative and "Pirkle" column) , and this method was applied in the researches on the selective degradation of the chiral monomers of fenvalerate in soil under laboratory conditions. Bicker (Bicker et al., 2004) separated the chiral isomers of various pyrethroids by using quinine as chiral stationary phase. Li, et al. (Li et al., 2003) carried out chiral liquid chromatographic separation of fenpropathrin, beta-cypermethrin, beta-cyfluthrin and S-fenvalerate by using Chiralcel OD and Chirex 3020 chiral liquid chromatographic columns. Liu et al (Liu et al,

2005) achieved a complete separation of the enantiomers of cis-bifenthrin and permethrin on the non-chiral column coupled with Sumichiral OA-2500-I chiral analytical columns. Liu, et al. (Liu et al., 2005) separated 8 enantiomers of cyfluthrin and cypermethrin by using Chirex 00G-3019-DO chiral column respectively. The author's laboratory successfully separated isomers of cypermethrin, allethrin and cyfluthrin, and as Fig. 4 showed, 8 isomers of cypermethrin and cyfluthrin were separated, and the separation method was used to analysis the isomers of cypermethrin in pork samples (Kuang et al., 2010) The following Table 2 is made for the summary.

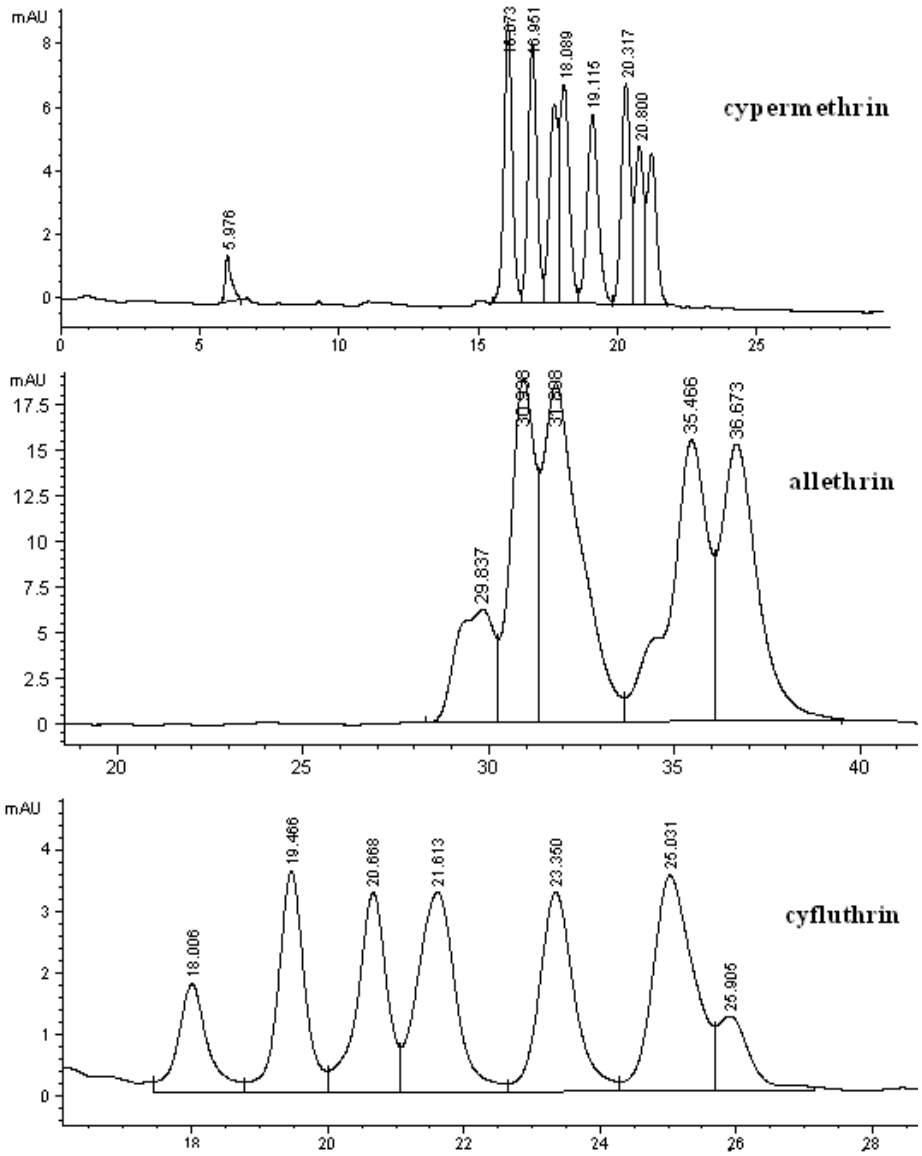

Fig. 4. Chiral separation of cypermethrin, allethrin and cyfluthrin on the CD-ph LC column

pyrethroids	Type of LC stationary phase	Separation	References
deltamethrin	N-(3,5-Dinitrobenzoyl)-(R)-(-)--phenylglycine	8 peaks	(Maguire, 1990)
cypermethrin	Daicel chiracel OD	6 peaks	(Edwards et al., 1997)
cypermethrin	Glycine Derivative I~II	8 peaks	(Naobumi et al., 1990)
phenothrin	10% QF-1 Sumipax OA-2000	6peaks	(Doi et al., 1985)
deltamethrin	RP-8/Lichrosorb Si-60	1 peaks	(Mourot et al., 1979)
cypermethrin	Pirkle type Amino column	5 peaks	(Chapman, 1983)
cyfluthrin	Dupont zorbax	5 peaks	(Tephem et al., 1990)
cypermethrin	Sumichiral OA-2500-I, Chirex DOG-3019-DO	4 peaks	(Liu et al., 2005)
cis-permethrin, trans-permethrin	Sumichiral OA-2500-I	each 2 peaks	(Liu et al., 2005)
fenvalerate	Pirkle I-A	4peaks	(Cayley & Simpson, 1986)
fenvalerate	Brush-type chiral stationary phase	4peaks	(Lee et al., 1987)
permethrin	Brush-type ligand exchange chiral stationary phase	4peaks	(Dondi et al., 1999)
tetramethrin	OA-4700 pirkle column and β-cyclodextrin	4peaks	(Deng, 2004)
prallethrin, Bioallethrin, prallethrin, cyphenothrin	Cellulose	each 2peaks	(Xu, 2003)
cis-bifenthrin (BF)，permethrin (PM)	Sumichiral OA-2500-I	BF-2peaks, PM-2peaks	(Liu et al., 2005)
cypermethrin、cyfluthrin	Chirex 00G-3019-OD column	8 peaks	(Mancini et al., 2004)
bifenthrin	OJ Daice chiral column	8 peaks	
cis-bifenthrin, trans-bifenthrin	Pirkle type Chiralcel OJ	each 4peaks	
fenvalerate	Chiralcel CD	fenvalerate 4peaks	(Li et al., 2006)
fenpropathrin，beta-cypermethrin	Chiralcel CD	each 2 peaks	
S, R-bioallethrin	β-cyclodextrin as chiral additive, C8 column	2 peaks	(Li et al., 2006)
cis-cypermethrin ，trans-cypermethrin	Cellulose-tris(3,5-dimethylphenylcarbamate)	each 2peaks	(Wang, 2006)
cypermethrin	Supelcosil LC-CN column coupled with Chiralcel OD-H	7 peaks	(Ta et al., 2006)
cis-cypermethrin	Chiralcel CD column	2 peaks	(Edwards et al., 1987)
bifenthrin and fenpropathrin	Lichrospher Si-60 column，Chriraspher column	each 2peaks	(García et al., 1996)

Table 2. Summary of LC chiral analysis of pyrethroids

3.2.2 Gas chromatographic chiral separation of pyrethroid pesticides

Gas chromatography (GC) has been widely used in the separation of various enantiomers of pyrethroid pesticides due to its advantages of lower detection limit compared with other techniques (Eljarrat et al, 2008; Hassan & Imran, 2004). The separation principle is mainly based on the hydrogen action, mating action and inclusion action of the chiral stationary phase. The commonly used chiral stationary phase of GC columns can be divided into three types: amino acid derivatives, chiral metal complexes and chiral cyclodextrins derivatives (CD) (Wang, 2006). In the Currentlly commercialed gas chiral chromatographic columns, the vast majority are produced by using derivatized cyclodextrins as the chiral stationary phase. The shortcomings of GC chiral separation are high cost and much time consumption. Liu, et al. (Liu et al., 2004; Liu et al., 2005) made chiral separation of bifenthrin, permethrin, cypermethrin and cyfluthrin by using the BGB-172 gas capillary chromatographic collum with 20% tert-butyldimethylsilyl-β-CD dissolved in 15% diphenyl and 85% dimethylpolysiloxane used as stationary phase. It was the first time to analyze the pyrethroid pesticides, cypermethrin and cyfluthrin in biological matrix by combining solid phase micro extraction (SPME) with gas chromatographic electron capture detection (ECD). And 6 peaks of the 8 chiral monomers of cypermethrin were separated on the column, and the two peaks of cis-bifenthrin reached completely baseline separation, and 3 of the 4 chiral enantiomers of permethrin can be separated. We have achieved good chiral separation of bifenthrin, permethrin and cypermethrin by using BGB-172 column based on the research work of Liu, and the chromatographyic picture of chiral separation are shown in Figure 3, and six separated peaks of theoretic eight peaks of cypermethrin were obtained from the column and baseline separation of *cis*-bifenthrin was realized. The detection of eantionmer fraction (EF) of bifenthrin or cypermethrin in tea samples was carried out and the results showed that EF value of some enantiomer of cypermethrin changed depending on the fermentation degree of tea (Kuang et al., 2010). Kutter and Class (Kutter & Class, 1992) made chiral separation of allethrin and cypermethrin by using non-chiral column DB-1701 column coupled to CDX-B chiral column with permethylated-β-cyclodextrins as stationary phase, and cis- isomers were well separated, but the trans-isomers were not well separated. Nie et al. (Nie et al., 2000) separated some enantiomers of pyrethroids containing ester by using different chiral stationary phases. Compared with single derivatized cyclodextrins CSPs, the mixed derivatized cyclodextrins CSPs are more effective in the separation of chiral enantiomers of pyrethroids. Studies have shown that some pyrethroids with thermal instability tend to be degraded during gas chromatographic analysis and lead to the transformations between enantiomers. The transformations between enantiomers of some pyrethroids even happen in organic solvents. Qin and Gan (Qin & Gan, 2007) discovered that permethrin is stable in all organic solvents, but the transformations between enantiomers of cypermethrin appear in acetone and methylene dichloride when they carried out chiral separation using gas chromatography. The transformations between enantiomers are affected by the temperature and they are affected by the water as a latent solvent when the pesticides are applied. Therefore, exposure to water or some solvent may lead to spurious results of chiral separation. In addition, for the pyrethroids with many chiral isomers, the transformations between the enantiomers may cause that of enantiomers with high biological activity into the inactive ones and lead to the product failure.

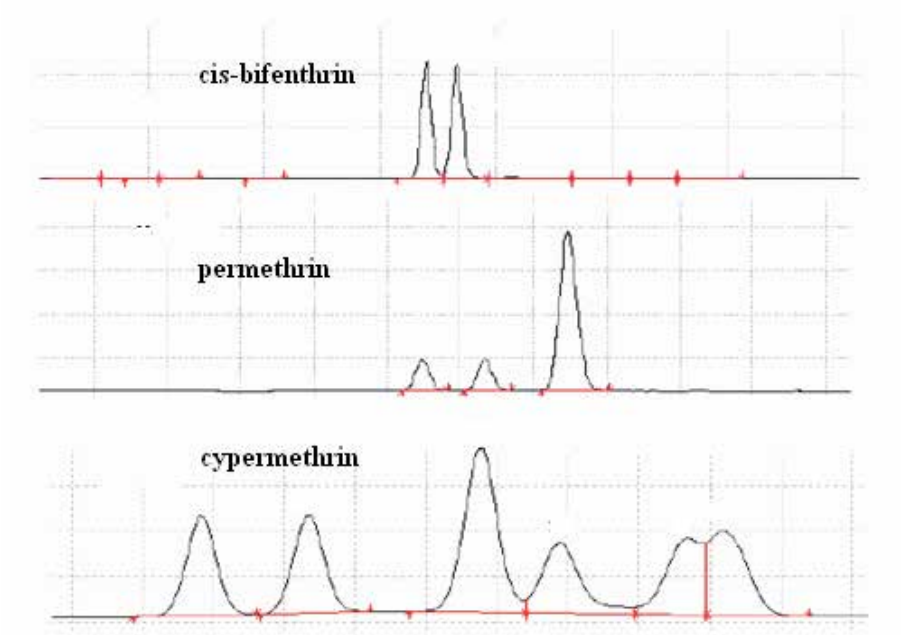

Fig. 3. Chiral GC separation of bifenthrin, permethrin and cypermethrin on BGB-172 column

Pyrethroid	Type of GC stationary phase	Detection method	Separation	References
cyfluthrin	achiral column	GC/MS	4 peaks	(Leicht et al., 1996)
phenothrin	4% DEGS	GC	4 peaks	(Doi et al., 1985)
cypermethrin(CP）	BGB-172	GC/ECD	CP-6 peaks	(Liu et al., 2004; Liu et al., 2005)
bifenthrin(BF)	BGB-172	GC/ECD	BF-2 peaks	
permethrin(PM),	BGB-172	GC/ECD	PM-3 peaks	
chrysanthemic acid	brush-type chiral stationary phase	GC-FID	2 peaks, badly separated	(Naobumi et al., 1983)
cypermethrin	permethylated-β-cyclodextrins	GC	3 peaks	(Chen, 2002)
cypermethrin	B-DEX225	GC/MS	7 peaks	(Yu, 2000)
pyrethroic acid methyl esters	cyclodextrin derivatives	GC	4 peaks	(Shi et al., 2002)

Table 3. Summary of GC chiral analysis of pyrethroids

3.2.3 Capillary electrophoresis chiral separation of pyrethroid pesticides

The capillary electrophoresis is a novel technology used in the separation of chiral pyrethroids. The commonly used chiral selectors include cyclodextrins and their derivatives, macrocyclic antibiotics, amino acid - metal complexes, chiral crown ethers, etc. 85% of the capillary electrophoresis chiral separation are currently carried out on cyclodextrins filler(Wang, 2006). Ševcík, et al. (Ševcík et al., 1997) separated cypermethrin, permethrin and fenpropathrin by using the micellar electrokinetic chromatography (MEKC). Odium dodecyl sulfatse (SDS) and cetyltrimethyl ammonium bromide (CTAB) were used as

surfactant. β-cyclodextrins, hydroxypropyl-β-cyclodextrins, dimethyl-β-cyclodextrins, γ-cyclodextrins and other chiral stationary phases were tried as chiral stationary phase. The results show that, by using γ-cyclodextrins and SDS, the two enantiomers of fenpropathrin were well separated, and 7 of 8 enantiomers of cypermethrin can be separated, and 4 enantiomers of permethrin can be separated, but the separation of the first 3 peaks is not obvious. Karcher et al. (Karcher & Rassi, 1997) taking OG (n-octyl-β-dglucoside) and OM (n-octyl-β-O-maltopyranoside) as chiral surfactants, set up the method to separate the hydrolysis products such as permethrin, cypermethrin, fenpropathrin and fenvalerate by using micellar electrokinetic capillary chromatography (MEKC). With the presence of carboxylic acid in the hydrolysis products, the separation of enantiomers will be simpler with the reduced enantiomers caused by the decreased chiral centers.

4. Selective biological toxicity of chiral monomers of pyrethroid pesticides

The chiral enantiomers of pyrethroid pesticides are characterized by certain degree of chiral selective biological toxicity. Different chiral isomer may be coupled with the same or different targets of organisms at the same or different positions in different degrees, resulting in the same or opposite efficacy or toxicity, and thus the activities of various enantiomers are quite different (Owen, 1975). In 1974, Elliottt discovered that the *cis* isomer of natural permethrin C-1 has insecticidal toxicity, and the *trans* isomer is inactive in terms of insecticidal activity (Elliott et al., 1974). At present, most attention has been focused on the pesticide of *cis*-bifenthrin (*cis*-BF) which has two enantiomers with the structures of 1S-cis-BF and 1R-cis-BF. 1R-cis-BF is more effective than 1S-cis-BF on the target organisms, while on endocrine toxicity, the estrogenic effects of 1S-cis-BF is significantly higher than 1R-cis-BF. Therefore, R-type is better than S-type in terms of insecticidal activity and otherwise in terms of toxicity. The *2S-aS* isomers of fenvalerate and cyfluthrin are more toxic to insects, while their *2R-aR* isomers have little insecticidal activity. Cypermethrin has enantiomer selective toxicity to the aquatic organism C.dubia, and 2 out of its 8 enantiomers (*1R-cis-aR* and *1R-trans-aR*) have strong toxicity to C.dubia (Liu & Gan, 2004). The insecticidal activity of deltamethrin is only associated to the dextral cis-isomers (synthesized from1*R, 3R* permethrin and *S-a*-cyanohydrin) and the other 6 stereo-isomers have been demonstrated to be inactive (Domine, 1982). The *1R-trans-aS* isomer of prallethrin has high insecticidal activity, and its enantiomer (1S-trans-αR) has only 1/200 of its activity. *S, S*-fenvalerate is the one with the highest activity in the 4 isomers of fenvalerate. According to the OECD standards, Wang Jiajia (Wang, 2008) studied the effect of beta-cypermethrin and its 4 chiral isomers on the development of zebrafish embryonic. The results showed that beta-cypermethrin has the strongest toxicity to the zebrafish embryonic at 48h. The isomer of *1R-cis-aS* is the most toxic one in the 4 chiral isomers, and then followed by *1R-trans-aS*, and the isomers of *1S-cis-aR* and *1S-trans-aR* have no teratogenic effect. In terms of cytotoxicity to SHSY5Y, the isomer of 1R-cis-αS is most toxic one.

The pyrethroid pesticides also show stereoisomeric selectivity in terms of acute toxicity to mammals. The studies show that the acute neurotoxicity of pyrethroids to mammals is associated to the three-dimensional chemical configuration of C-1 chiral center of pyrethroid pesticides. In general, the isomer with acute neurotoxicity to mammals is the same as that with insecticidal activity to the target organisms. The three-dimensional structure of the C-3 chiral centers of some pyrethroid pesticides, such as resmethrin and permanone, directly affects the acute neurotoxicity to mammals (Kolaczinski & Curtis, 2004). In a research of the

neurotoxicity of the isomers of deltamethrin to the central nervous system of rodents, it was found that the isomer of 1*R* has obvious neurotoxicity to mammals, and *1S* isomer showed no neurological toxicity even at high concentrations (Ray & Fry, 2006).
Therefore, different chiral isomers of pyrethroids have different biological activities and neurotoxicities, and thereby the residues and metabolisms in the environment and biological organisms also vary greatly. The selectivity of chiral enantiomers on neurotoxicity may be caused by the inherent structural differences of enantiomers of pyrethroids or the different metabolic rates of enantiomers in organisms. Anyway, the detailed molecular mechanism is not precisely known.

5. Pollution of pyrethroid pesticides and the degradation difference of chiral monomer in the environment

5.1 Pollution of pyrethroid pesticides in the environment

The wide application of pyrethroid pesticides in agriculture and pest control in urban areas causes their residues in the environment. According to statistics of Denmark in 2008, 89% of environmental pollution caused by the pesticides ascribed to the application of pyrethroid pesticides spray (Danish EPA, 2009). In 2009-2010, the detection results of contamination of pyrethroid pesticides in water and sediment in Spain Ebro Delta showed that 22 detected water samples contained cypermethrin residues with the maximum concentration of 57.2 ng/L, including 3 water samples containing deltamethrin residues with the maximum of 58.8 ng/L. Only the cypermethrin residues were detected in all sediment samples, and the amount was up to 71.9 ng/g. The results show that cypermethrin pollution in the water and sediment in Mingaibuluo Delta was in a serious situation (Feo et al., 2010).
More than 58,000 kg of the pyrethroid pesticides have been applied to the pest control of corn, cotton, soybean, rice, wheat and other crops in the United States since 1991 to 2000, and more than 117,000 kg active pyrethroid pesticides were applied in 2002 alone, causing a profound influence on the water quality in the United States. According to the statistics of International Emergency Economic Powers Act (IEEPA) in 2007, more than 1300 kinds of aquatic organisms were influenced by the water polluted by pesticides (Moore et al., 2009). University of California carried out a one-year follow-up detection during 2007-2008 on the water and sediment in the small streams flowing through the city of Sacramento and California, and the results showed that the detected samples had been polluted by pyrethroid pesticides in different levels, in which the residues of bifenthrin was 73 ng/L in the water, and up to 1211 ng/g in the sediments. Bifenthrin was given primary attentionin in the pyrethroid pesticides contamination in this region, followed by cypermethrin and cyfluthrin containing minor pollution (Weston et al., 2009). Environmental Protection Agency of the United States (USEPA) studied the situation of pyrethroid pesticides contamination in the air in the living environment in North Carolina and Ohio, and the results showed that pyrethroid pesticide pollution was detected in 69 of the 85 detected air samples, and the average content of pyrethroid pesticides in air dust was 100 ng/g (James et al., 2008).
Studies have reported that, in natural water systems in China, there is generally no pyrethroid pesticide residues in well water and tap water, but cypermethrin can be detected in the river water (Yu, 2008), and in urban sewage, residues of bifenthrin, cypermethrin, fenvalerate and deltamethrin and other pyrethroids at the contamination level of 0.04~1.3 μg/L were detected (Chen et al., 2005; Chen et al., 2007). The residues of pyrethroid pesticides in the water are

mainly adsorbed onto the surface of suspended particles, and then accumulated in sediments. The amount in the sediment is often hundreds or even thousands of times of that in the water (Yue, 2009). Analyzing the residue of pyrethroid pesticides in the sediment samples is an effective quantitative method to evaluate the contamination situation of pyrethroid pesticides (Weston et al., 2004). Li et al(Li et al., 2010)detected several pyrethroid pesticides in the contaminated sediments in the streams in Guangzhou by microwave extraction and gas chromatography mass spectrometry, of which the amount of beta-cypermethrin was 4.5 ng/g dry weight, permethrin 12.2 ng/g dry weight, cypermethrin 27.9 ng/g dry weight and cis-fenvalerate 2.27ng/g dry weight.

The pyrethroid pesticides in the environment can bring harm to human and food producing animals via the accumulation in plants and acqutics. As a result, in addition to the researches on the degradation of pyrethroid pesticides in animal and human, those in the environment have been also a focus at home and abroad.

5.2 Difference in degradation of chiral monomers of pyrethroid pesticides in the environment

The varied metabolism rates of chiral pesticides in the environment may mainly be caused by the differences in selective metabolisms of the bacteria in the soil. Megharaj et al. (Megharaj et al., 1989) screened 10^3 strains from the soil which can selectively degrade cypermethrin and fenvalerate and they can degrade different chiral isomers in different rates. Sakata et al. (Sakata et al., 1992) screened 10^3 strains from the soil which can selectively degrade cypermethrin and fenvalerate. Under the action of these bacteria, the iscomers of *1R-trans-aS*, *1S-cis-aS* and *1S-trans-aS* in the 8 isomers of the cypermethrin were degraded with great speed, and the remaining 5 isomers were hardly degraded; and the degradation rate of the *2R-aS* iscomer of the fenvalerate was significantly greater than the other three. The further study showed that the selectivity is caused by the interaction of the enzymes with different degradation mechanism in the degrading of bacteria, which indicated the presence of the degrading enzymes with high selectivity in the soil. Liu, et al. (Liu et al., 2005) via their researches on the environmental behavior of bifenthrin and cypermethrin, discovered that the enantiomers (-) of cis-bifenthrinand alpha-cypermethrin in the field sediments were preferentially degraded, leading to the increased proportion of their enantiomers (+), and the same degradation was observed when the sediments were cultured under the laboratory conditions.

6. Metabolism of pyrethroid pesticides in animals

6.1. Metabolism of pyrethroid pesticides in animals

Scientists have carried out more researches on the metabolism of pyrethroid pesticides in animal to further understand the potential metabolic processes of such pesticides in human body and thus find out effective ways to solve human pesticide poisoning. For example, due to the similar hydrolysis rate of pyrethroids in rat and human liver microsomes, we can speculate the potential metabolic pathway of the pesticides in human body by the researches on the rodents (Ross et al,. 2005). Mandal et al. (Mandal et al.,1992) reported that the fenvalerate could be rapidly absorbed and metabolized in goats, and the half-life of fenvalerate was close to 2h in the case of intragastric administration. Orinak (Orinak, 1993) discovered that the alpha-cypermethrin reached the peak concentration in the blood in sheep body after about 24 h. JECFA has carried out intragastric administration of ^{14}C

marked alpha-cypermethrin on rat with the dose of 2 mg/kg, and it reached the peak concentration in the blood after 3 ~ 4h, and then was rapidly metabolized in the body, and mainly excreted from the urine except for small accumulation in fat and skin tissue. In the case of the same oral dose, urinary metabolites and metabolic pathways of mice and rats and other animals were similar to those of human volunteers. The studies showed that, the main metabolic pathways of pyrethroids in mammals include cleavage and hydrolysis of ester bond followed by the hydroxylation and conjugation of cyclopropyl and phenoxyl in molecules. Due to large amounts of esterase and oxidase existing in the liver, the metabolism of pyrethroids mainly occurs in the liver of mammals and the detoxication could be reached through hydrolysis and oxidation. The biological half-life of many pyrethroids including permethrin, cypermethrin, deltamethrin, fenvalerate was 8 to 12 h (Leng et al., 1997). The bondage of 3 - phenoxybenzyl ester in the structure of pyrethroids could be hydrolyzed in the body into the same metabolites of 3-phenoxybenzoic acid (3 - PBA) which was excreted in the form of urine. In addition, *cis* and *trans*-2, 2 - (dichlorovinyl) -2,2-dimethylcyclopropane carboxylic acids (*cis*-DCCA and *trans*-DCCA) and 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA) were also the primary metabolites of pyrethroid pesticides, and can serve as the biomarkers of the assessment of human exposure to pyrethroids (Li et al., 2010). Degradation processes of some pesticides are shown in Figure 4.

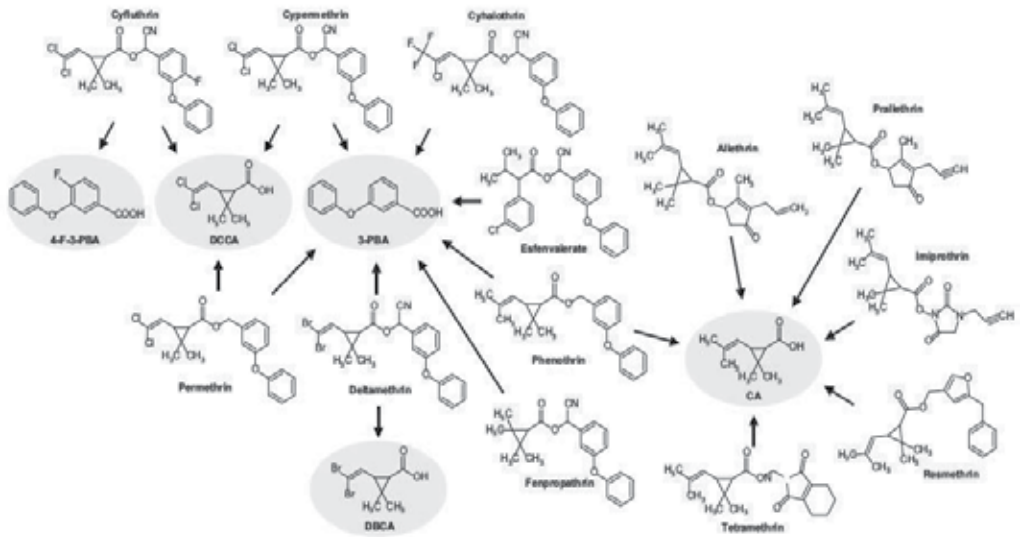

Fig. 4. Metabolic structure chart of some pyrethroids

Various carboxylesterases (CEs) contained in animal and human body, which can degrade pyrethroids, play a key role in the pesticide metabolism and detoxification. The CEs, primarily including hCE-1 and hCE-2, are of the highest activity in the liver (Testa et al., 2003). In addition to the liver, other tissues and organs are of equal importance to the metabolism of pyrethroids because of the presence of CEs in lung, small intestine, plasma and lymphocytes(James et al., 2008; Nishi et al., 2005). The human body also contains a variety of oxidative enzymes which participate in the metabolism of pyrethroids, such as dehydrogenase and cytochromes P450. The alcohol dehydrogenase and aldehyde,

dehydrogenase may participate in the oxidation processes of the phenoxybenzyl alcohol, the intermediate metabolite of permethrin, and thus the detoxication is achieved (Crow et al., 2007). But pyrethroids themselves will dampen the number and activity of the degrading enzymes. For instance, flumethrin can reduce 36% of cytochromes oxidase P450 in number in mouse liver microsomes(Nakamura et al., 2007). Eraslan et al. (Eraslan et al., 2007)treated mice with cypermethrin alone and discovered that the activity of superoxide dismutase in red blood cells was reduced in certain stages of the treatment process and the activity of catalase was significantly reduced in the whole process.

6.2 Metabolism of chiral monomers of pyrethroid pesticides in animals

The researches on the selective metabolism of pyrethroid pesticides in animals are the more in-depth section in the researches of the selective behaviors of the chiral compounds. Two factors, on one hand, the many unknown fields in the degradation process of exogenous chiral pollutants due to the complex physiological and biochemical responses in animals, on the other hand, the great concern of human on the security and the sustainable development of human body and the ecological environment, have attracted researchers to explore the activities and provide a scientifically theoretical and practical foundation for the biological safety.

In animal body, pyrethroids are metabolized by different cytochromes P450, and so different enantiomers of pyrethroids and their metabolites always show different pharmacological and metabolism features. However, owing to the complex stereostructure of the pyrethroids, which always include several chiral centers and *cis* and *trans* isomers, and the limited separation means of chiral monomers, there are few researches on the selectivity of the optical isomers of such compounds in animal body, and most of which are merely based on the selectivity of the *cis* and *trans* isomers. Takamatsu et al (Takamatsu et al., 1987), carried out oral administration on rats by marking the 4 optical isomers of fenvalerate with ^{14}C, and applied the ^{14}C monomers to the suspension of organ microsomes for cultivation in vitro as well. The results showed that the optical isomers had selectivity in different organs and the selectivity is different for the 4 optical isomers. The results of cultivation in vitro in liver microsomes were consistent with the results in vivo, suggesting that the liver was the main place of degradation. Compared with α-chiral carbon atoms, the chiral structure of 2-chiral carbon atom of fenvalerate made greater contribution to the selectivity in rats. Nishi et al. (Nishi et al., 2005)studied the hydrolysis made by carboxylesterase hCE-1 and hCE-2 to pyrethroids in human liver, and the results showed that the 2 kinds of enzymatic hydrolysis of *trans*-permethrin and *trans*-cypermethrin were faster than the corresponding *ci*- isomers. Crow et al (Crow et al, 2007) also reported that *trans*-isomers' degradation rate of pyrethroid pesticides in rat liver cells was significantly faster than *cis*-isomers. The trans-isomers had strong stereoselectivity during the hydrolysis of pyrethroids. For instance, the *trans*-isomers of permethrin were hydrolyzed faster than *cis*-isomers. The hCE-2 could hydrolyze *trans*-permethrin, but could not hydrolyze deltamethrin or resmethrin. Miyamoto et al. (Miyamoto et al., 1986) discovered that pyrethroid pesticides had no significant stereoselectivity in mouse plasma. Wang et al (Wang et al,, 2006) conducted a metabolism experiment of a pair of enantiomer of *theta (trans)*-cypermethrin in rats. The results showed that the dextro- enantiomer were degraded faster in the plasma, heart, liver and kidney. After the intravenous injection of single enantiomer in rats, the laevo-enantiomers were transformed into dextro- enantiomer.

The author's research group, by using chiral liquid chromatography, successfully separated and prepared two chiral enantiomers (*1R-3R-áS* and *1S-3S-áR*) of *alpha(cis)*-cypermethrin , and carried out animal experiment of pharmacokinetics and tissue distribution in Wu Zhishan Inbred's miniature pig by using the two enantiomers. The chiral separation was carried out in the collected blood and tissue samples. In the case of feeding single monomer to the miniature pig, all the tissues of the pig at 12h showed significant transformation among isomers of cypermethrin, and no obvious transformation was found in the two monomers in the blood sample within 2h, which is consistent with the research of Miyamoto et al. (Miyamoto et al., 1986). With the exception of the *1R-3R-áS* conformation in fat mainly transforming to its enantiomer *1S-3S-áR*, in other tissues *1R-3R-áS* transformed into the configurations of *1R-3R-áR*, *1R-3S-áS* and *1S-3R-áR*. 23.9% of *1R-3R-áS* conformation transformed into *1R-3R-áR* configuration in the liver. 1S-3S-áR configuration transformed into 1R-3S-áS or 1S-3R-áR configurations in other tissues except for in the fat, and the transformation rate was up to 35.7% at 12 h. No transformation of 1S-3S-áR configuration into its enantiomer 1R-3R-áS conformation was observed in all the tissues. On the whole, the transformations of monomers in the tissues were not so identical. The highest degree of transformation of enantiomers was observed in the liver, and it may be caused by the large amount of enzymes in the liver. The lowest degree of transformation of enantiomers was found in the fat.

The development of the researches on different metabolisms of pyrethroids in animal will provide a foundation to further understand its toxicity and residual rules, which will represent more truthfully the effects of the pollution of related pesticides on human health. All of these researches can also offer a basis to the pollution control, the guidance of rational pesticide use and the development of relevant analytical methods and maximum residue limit standards, and ultimately minimize the toxic side effects of pesticides to human health.

7. Human exposure to chiral pyrethroid pesticides and risk assessment

7.1 Introduction Total Diet Study in China

The World Health Organization (WHO), the lead of United Nations agency for health, supports total diet studies as the one of the most cost-effective means for assuring that people are not exposed to unsafe levels of toxic chemicals through food. A total diet study (TDS) enables the estimation and monitoring of dietary exposures to chemical residues, contaminants and nutrient elements. A TDS involves purchasing at the retail level foods commonly consumed by the population, preparing them as for normal consumption, homogenizing and compositing them, and finally, analyzing the foods for the chemicals of interest. Beginning 1990 the Chinese TDS has become an important tool for monitoring dietary exposures to chemicals and their associated risk to public health and such studies have been undertaken five times in China at irregular intervals.

The study design and experimental methods of the 4th Chinese TDS were similar to those used to carry out the TDS in 1990 (Chen & Gao, 1993). The food composite approach was used to study the total diet in four regions representing the average dietary patterns of different geographical areas on the mainland and covering about 50% of the Chinese population (Zhao et al., 2003). Each region comprised three provinces: North 1(N1) comprised Heilongjiang, Liaoning and Hebei; North 2 (N2) comprised Henan, Shanxi and Ningxia; South 1 (S1) comprised Jiangxi, Fujian and Shanghai; South 2 (S2) comprised Hubei, Sichuan and Guangxi. Average food consumption of a “standard” A Chinese man

(18-45 years old with average body weight of 60 kg) from 90 households (30 household per survey site) was used as the standard food consumption model for the province and the value of three provincial pooled composite was used as the food basket consumption pattern for each region. All food consumed by the standard man was aggregated into 13 food groups, namely cereals and products, Legumes, nuts, and products, potatoes and products, vegetables and products, fruits and products, meats and products, eggs and products, milk and products, aquatic foods and products, sugars, beverages and water, alcohol beverages, condiments and cooking oil. The samples were collected from local markets，grocery stores and rural households, then cooked and prepared according to local food habits of each province. The prepared foods were then blended to form the respective group composites with weights proportional to the average daily consumption for the province. These provincial composites were shipped to the National Institute of Nutrition and Food Safety in Beijing, where the composites were further mixed to form four regional basket composites according to their corresponding weight proportion in food consumption. The samples were then frozen at -30° until analysis.

7.2 Exposure and characteristics of chiral residues of pyrethroid insecticides in Chinese diet

The detection results of the composite samples in the fourth Chinese total diet study in 2007 showed that despite a certain level of contamination of pyrethroid pesticides in different parts of China, China was generally in a situation of low level of pollution. For instance, the positive rate of cypermethrin, which is of the highest frequency of detectable pyrethroid pesticides, was 27.5%, and the highest content in meat composites from Fujian Province was 95.65 μg/kg. The contribution rates of different food varied by regions in dietary exposure, the intake of meats and aquatic products were important sources of pyrethroid pesticides exposure through dietary intake. On the whole, Chinese residents' dietary exposure to pyrethroid pesticides was in a low level and far below the acceptable daily intake (ADI) recommended by JECFA. According to the exposure assessment combining with dietary consumption, the upper limit of dietary exposure in Fujian was only 1.59% of the ADI value recommended by JECFA. However, multi-residue of pyrethroid pesticides in the same composite sample was found in some areas (fox example, Hubei). Whether different pyrethroid pesticides have cooperativity is still under study, and the potential health risk caused by the diet exposure of multi-residues of pyrethroid pesticides are worthy of attention.

The results of the chiral separation of the cypermethrin positive composite samples in the total diet study showed that some chiral isomers of cypermethrin disappeared in the samples of legumes from Hubei, fruits from Henan, fruits from Guangxi, and vegetable from Heilongjiang, which may caused by the application of cypermethrin pesticide with fewer chiral monomers such as *alpha(cis)*-cypermethrin, *theta(trans)*-cypermethrin. In addition, the samples of the total diet study were cooked instant samples, while the high temperatures during cooking may be one of the causes leading to the disappearance of some isomers. Significantly enriched isomers (*1R-3S-aS* and *1S-3R-aR)* were found in animal food samples including aquatic foods from Hebei and meats from Jiangxi, and no remarkable change in the enantiomeric fraction in the meats sample from Liaoning was found. The specific reasons remains to be further studied.

The next work of our research team is to achieve information of other chiral pyrethroid pesticides in TDS samples, such as *cis*-bifenthrin, cyfluthrin, permethrin, etc.. Currently, the

5th Chinese TDS is underway, and we will apply our established method to the determination of chiral isomers in all of those TDS samples. With the combined two sets of results, more information will be acquired to assess the risk of chiral isomers on human body exposure.

8. Conclusions

The chiral separation of synthetic pyrethroids has been mainly achieved by HPLC or GC techniques. Different types of chiral columns based on cyclodextrins have been used for this purpose. Different chiral isomers of pyrethroids have different biological activities and toxicities, and thereby the residues and metabolisms in the environment and biological organisms also vary greatly.

About the different pyrethroids studied, it is necessary to bring out that only few of them have been studied in the field of chiral separation, chiral metabolism and chiral activity and toxicity perhaps due to the difficulties in the obtain of pure standards and single isomers. There is a wide range of synthetic pyrethroids yet to be studied for separating their enantiomers. With the development of technology of chiral isomers separation and preparation, the researches on the selective metabolism, residual behavior in organisms and environment will eventually get rid of the mist on the chiral isomers, and further guide the production, use and setup of regulations of chiral pesticides, and finally achieve the purpose of protect human health and environment.

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9

Biomonitoring of Contemporary Pesticides: Ethylenethiourea in Occupational Settings

M. Angela Montesano and Richard Wang
Centers for Disease Control and Prevention,
Atlanta, GA
USA

1. Introduction

Ethylenebisdithiocarbamates (EBDCs) are widely used fungicides in agriculture because of their efficacy and broad spectrum of antifungal activity. They are mainly used on fruits, ornamental plants, and vegetables (Maroni et al., 2000). EBDCs are salts of the dithiocarbamates; the salts of manganese, zinc or manganese, and zinc are the commercial products known as maneb, zineb, metiram and mancozeb (Figure 1) (Houeto et al., 1995). In dilute suspensions the compounds are not particularly stable, yielding a number of degradation products (Somerville, 1986). Ethylenethiourea (ETU, Figure 1) is present as an impurity in several EBDCs formulations, and it is formed in the presence of moisture and oxygen (Bontoyan et al., 1972; Bontoyan & Looker, 1973). ETU is important in the assessment of human health from exposure to EBDCs because it is the purported source of toxicity from EBDCs (Houeto et al., 1995) and it is a metabolic product of EBDC in mammals, plants, and other organisms (WHO, 1988). Therefore, ETU can be measured in people to assess their exposure to EBDCs.

1.1 Exposure assessment using biomonitoring

There are various methods to assess human exposure to EBDCs, such as questionnaires and environmental sampling. One approach that has attracted increased interest in recent years is biomonitoring. Biomonitoring determines a person's internal dose of a chemical by measuring the amount of the chemical or its metabolites in human tissues or fluids (e.g., blood, urine).
Biomonitoring is a powerful tool in assessing exposure to pesticides because such information can accurately and precisely identify people with significant levels of exposure to these chemicals. With this information the prevalence of exposure, the types of jobs that are most hazardous in terms of exposure and at-risk population groups can be identified. In addition, knowing the concentration of the chemical in the blood or urine can assist in the assessment of health risk from the exposure to chemicals that are known to cause disease or disorder, assist in determining the need for immediate interventions to prevent further exposure in the population, and assist in the monitoring the effectiveness of preventive measures. Biomonitoring of pesticides in occupational settings has become increasingly important in the assessment of health risk and it has become an integral part of the overall occupational health and safety strategy. Thus, biomonitoring can be used in exposure assessment and risk assessment based on known health outcomes.

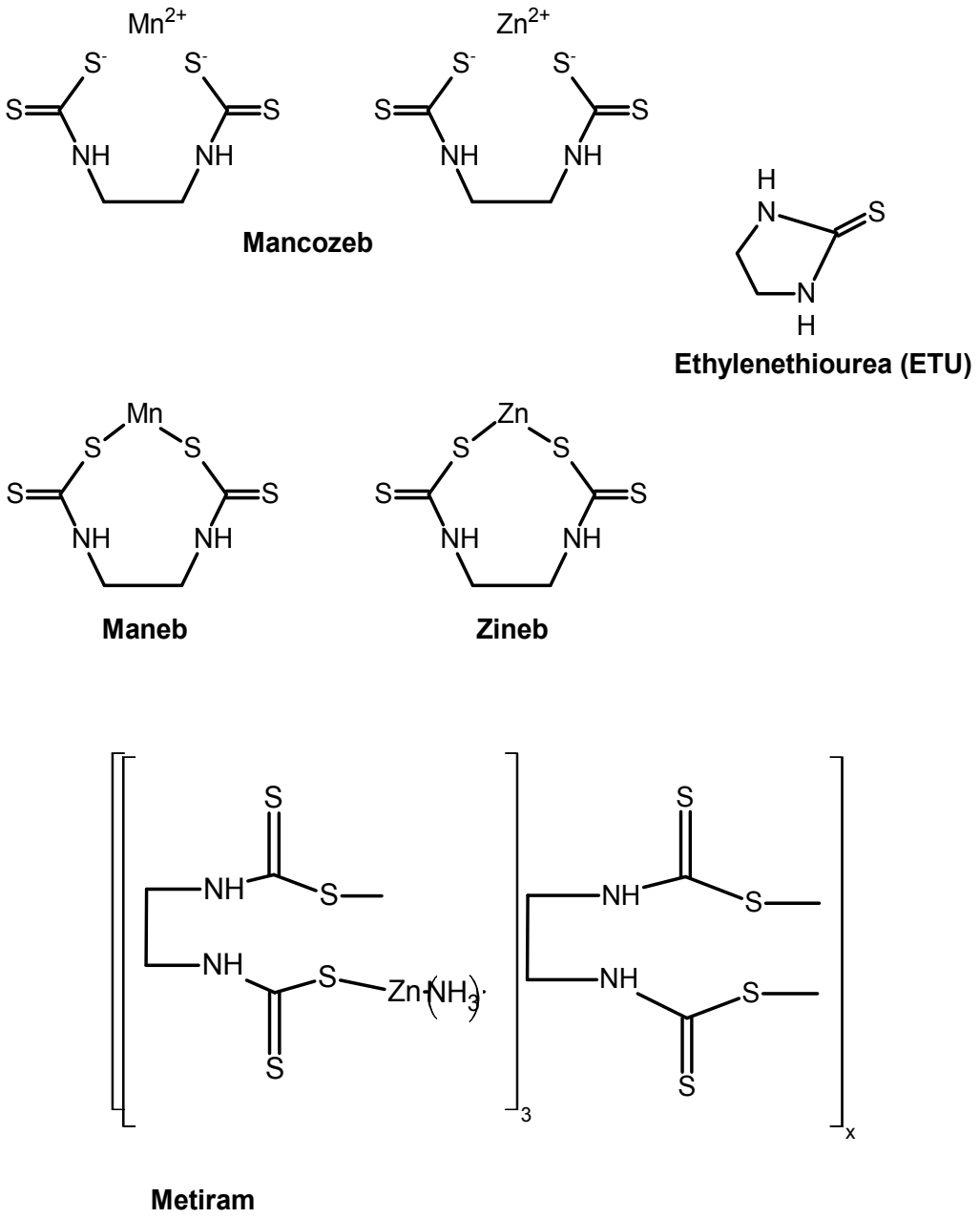

Fig. 1. Structures of the EBDCs Mancozeb, Maneb, Zineb, and Metiran and their metabolite ETU.

1.2 Populations at-risk for exposure

Farmers generally use large amounts of agrochemicals, including fertilizers and pesticides, for crop protection in order to enhance agricultural production and efficiency. For farm workers, pesticide exposure is a constant risk because of inappropriate handling of

pesticides, improper use of personal protective equipment and, also, because of inadequate information about the toxicity of the chemicals with which they are working. The improper handling of some of these chemicals without protective equipment and without using appropriate application procedures exposes farm workers to potential health effects from these chemicals. Occupational exposure to pesticides can occur in the field or in greenhouses from the direct handling of pesticides, such as in mixing or loading and during application. Workers who bring in the harvest or clean and maintain the agricultural equipment can be exposed as well. Other workers at risk for exposure to these chemicals include those who manufacture the pesticides.

Although many of the most toxic pesticides have been removed from the market or their use has been restricted in developed countries, there is still heavy use of pesticides without surveillance around the world. Doubts and uncertainties exist concerning the long-term health effects from the chronic or prolonged low-dose exposure to these newer or contemporary pesticides because of inadequate exposure and risk assessments. EBDCs are among these newer classes of pesticides: they have been on the market since the 1940's.

1.3 Toxicology

The concern for human health effects from the exposure to EBDCs largely stems from observations made in animal studies. Several long-term studies have been conducted on animals treated with ETU. The prolonged administration of ETU to animals in experimental trials has demonstrated thyroid disorders, including hypertrophy, hyperplasia, and follicular cell tumors, in rats and mice (Ulland et al., 1972; Weisburger et al., 1981; Chhabra et al., 1992; Nebbia & Fink-Gremmels, 1996). In these studies, ETU caused a general decrease in serum thyroxine (T_4) levels and increase in serum thyroid-stimulating hormone (TSH) levels, which correlated with morphological changes in the thyroid gland. These hormonal trends are attributed to ETU inhibition of thyroid peroxidase, which is responsible for the incorporation of iodine to thyroglobulin (Marinovich et al., 1997). ETU-induced thyroid tumor is attributed to an imbalance of the thyroid-pituitary axis. Besides the thyroid gland, the major site of ETU-induced carcinogenicity, the liver is also a target. Chronic ETU admistration produces hepatocellular carcinoma in mouse and rats (Innes et al., 1969; Belpoggi et al., 2002). However, ETU had mostly negative mutagenic effects in mammalian test systems (Shirasu et al., 1977; Teramoto et al., 1977) and did not induce DNA damage in cultured rat liver cells (Althaus et al., 1982). The International Agency for Research on Cancer (IARC) considers ETU as unclassifiable as a human carcinogen (IARC, 2001). The U.S. National Toxicology Program (NTP) considers ETU as reasonably anticipated being a human carcinogen (NTP, 2004).

Animal studies have demonstrated the ability of Mn-EBDC (maneb), but not manganese or EBDC alone, to selectively disrupt dopaminergic neurons in a manner similar to MPP+ (mitochondrial inhibitor, 1-methyl-4-phenylpyridinium), which can lead to Parkinson's disease (Zhang 2003). Mn-EBDC was shown to increase oxidative stress, decrease proteasomal function, and induce α-synuclein aggregation with formation of cytoplasmic inclusions in dopaminergic neural cultures (Zhou et al., 2004).

The teratogenic potential of ETU has also been investigated in animal studies. Rats treated before conception to day 15 of gestation with ETU at amounts of 10 mg or more resulted in offspring predominantly with malformation of the brain (Khera, 1973). Another study also investigated the teratogenicity of ETU in rats, mice and hamsters. ETU was teratogenic

when given orally to rats at 20-50 mg/kg body weight per day on day 6 to day 15 of gestation and to hamsters at 270-810 mg/kg body weight per day on days 6 to 13 of gestation (Teramoto et al., 1978). In addition, teratogenic studies were conducted with animals exposed to the EBDCs maneb, zineb and mancozeb. Rats exposed to maneb at a dose of 1420 mg/kg body weight, as a single dose on day 11 of gestation, developed gross malformations in all embryos (Larsson et al, 1976). Mice administered 375, 750 or 1500 mg of maneb/kg body weight on day 7 to 16 of gestation showed a decrease in fetal caudal ossification centers at all dose levels (Chernoff et al., 1979).

1.4 Toxicokinetics

EBDCs can be absorbed through the skin, the mucous membranes, and the respiratory and gastrointestinal tracts (WHO, 1988). Studies of the toxicokinetics and metabolism of EBDCs in laboratory animals have indicated that EBDCs are only partially absorbed, then rapidly metabolized and excreted with no evidence of long-term bioaccumulation (Somerville, 1986). On average, 7.5% of an EBDC dose administered is metabolized to ETU. ETU is predominantly (90%) eliminated in the urine and it has been found to have a half-life of about 28 hours in monkeys, 9 to 10 hours in rats, and 5 hours in mice (Newsome, 1974; Kato et al, 1976; Ruddick et al., 1976; Allen et al., 1978; Rose et al., 1980).

ETU is also a metabolic product of EBDC in mammals, plants, and other organisms (WHO, 1988). Therefore, ETU can be measured in human urine samples collected after EBDC occupational exposure (Kurttio et al, 1990; Colosio et al., 2002). Available data also suggest that in the general population concentrations of ETU in urine can be used as a surrogate parameter for EBDC exposure (Sciarra et al., 1994; Aprea et al., 1996; Aprea et al., 1997; Saieva et al., 2004; Colosio et al., 2002; Colosio et al., 2006).

2. Biological monitoring for ETU

Biomonitoring has been used in many exposure studies. The success of a biomonitoring study depends on getting all the parts right: These include overall study design, sample collection, analytical analysis and the interpretation of the resulting data. For biomonitoring EBDCs as well as for other target analytes in urine, the time of sampling and the great variation in the composition of spot urine samples as a result of diurnal variations can influence the results. Considerable knowledge of the toxicokinetics, i.e. the biological processing of a putative toxicant in the body, is essential for defining the time of sampling (Barr et al., 2006). After collection, the samples must be handled carefully to avoid exogenous contamination and degradation due to improper transport and storage.

Studies to evaluate the kinetics of ETU excretion were carried out through the analysis of ETU in urine of exposed workers up to 60 hours from the end of exposure. The highest rate of ETU excretion was around 15 to 22 hours after the end of exposure. Over time the excreted amount decreased, but even after 60 hours from the end of exposure, a small amount of ETU could still be detected in the urine of the exposed workers (Kurtio & Savolainen, 1990). Therefore, for workers exposed to EBDCs the ideal time for sampling is within 16 hours from the end of the work shift because of the rapid metabolism and excretion of metabolites of EBCDs including ETU (Kurtio & Savolainen, 1990; Colosio et al., 2003; Colosio et al., 2007).

Biomonitoring for assessing exposure to environmental chemicals generally requires the measurement of the relevant analytes at much lower concentrations than needed for clinical

chemistry. Specific and sensitive analytical methods are indispensable for assessing exposure in biomonitoring programs. The methods must also be reproducible and rugged. Mass spectrometric analysis, combined with gas chromatography or liquid chromatography, is the analytical method of choice in biomonitoring because of the ability to selectively quantify many environmental chemicals at very low concentrations. Now considerable mass spectrometric analysis is done using the tandem mass spectrometry (MS/MS) technique; MS/MS greatly improves selectivity of the analysis by eliminating potential interferences by other matrix (e.g., urine) components. Also, commonly used to correct for variability in sample extraction is the isotope dilution technique, which involves adding a measured amount of a stable-isotope of the target analyte to the urine sample before extraction. Quantification is done using the ratio of analyte to its isotope.

Several methods have been reported for the measurement of the metabolite ETU in urine. Table I shows the most recent analytical procedures described in the literature. Liquid-liquid extraction (LLE) into dichloromethane seems to be the most commonly used procedure for the extraction of ETU, a highly water-soluble compound. However, usually LLE requires a large volume of organic solvent. Sample preparation is expedited by lyophilizing the urine sample first and then extracting with a much smaller volume of dichloromethane than otherwise would be required. Liquid chromatography (LC), high-performance liquid chromatography (HPLC)-mass spectrometry (MS), or HPLC-MS/MS are the most commonly used analytical methods for the separation and detection of the metabolite ETU. They all use some form of liquid chromatography, but the detection systems range from simple UV or DAD (diode array detector) absorbance detection to sophisticated mass spectrometric analyses as discussed above. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces have shown efficiency in transferring the analyte from the liquid phase as it is eluted from the HPLC column into the gas phase for mass spectrometric analysis. The APCI interface has an advantage over ESI. With ESI, where the ionization occurs in the mobile phase, the sensitivity can be affected by ion suppression due the ionic concentration in the mobile phase itself.

Limits of detection (LOD) and limits of quantification (LOQ) are important parameters that define the limitations of the analytical method. The methods shown in Table I show detection values (LOD or LOQ) that range from 1 μg/L to 0.01 μg/L. It has been suggested that for monitoring the general population, the LOD must be 1 μg/L or less; higher LODs may be adequate for monitoring occupationally exposed workers (Aprea et al., 2002). Because occupationally exposed workers have different levels of exposure depending on the task performed, methods with lower LOD would be preferable if available.

During the method validation procedure it is essential to determine the LOD, LOQ, precision, accuracy of the measurements, extraction recoveries, efficiency of the derivatization reaction (if applicable), linearity, stability of the analyte in the matrix tested, and applicability to the human samples (Barr & Needham, 2002). In addition to comprehensive method validation, quality assurance/quality control (QA/QC) is a very important feature in biological monitoring. QA/QC guarantees the quality of the data by making it possible to detect systematic failures that can occur during the performance of the methods. It has two components: internal quality control, which is a set of procedures used by the staff of a laboratory to continuously confirm the reliability of the results; and external quality assessment, which is a system of checking the laboratory performance by an external agency or institution. The internal quality control programs can include repeat measurements of known biological materials to confirm the validity of an analytical run and

to measure analytical precision, proficiency testing to ensure accuracy as measured against a known reference material, and cross validation to ensure that multiple analysts and instruments obtain similar analytical values (Aprea et al., 2002; Barr & Needham, 2002; Schaller et al., 2002).

3. Review of EBDCs exposure studies in occupational settings involving the biomonitoring of the metabolite ETU in urine and its clinical relevance

3.1 Exposure levels

Knowledge of exposure levels to EBDCs in occupational settings is an important step in the process of health risk evaluation. The level of the metabolite ETU measured in urine is the most accurate indicator of EBDCs exposure. Only a few studies have been carried out measuring the workers' levels of EBDCs in occupational settings. These studies are outlined in Table II. The assessment of the workers' exposure, based on ETU concentrations in urine, has been done in Mexico in tomato growing areas (Steenland et al., 1997), in areas of floriculture in Ecuador (Colosio et al., 2003), in banana plantations in the Philippines (Panganiban et al., 2004), vineyards in Italy (Colosio et al., 2002; Corsini et al., 2005; Colosio et al., 2007; Fustinoni et al., 2008), in grains, tobacco, vegetable and cut flower areas in Thailand (Panuwet et al., 2007), potato farms in Finland, (Fustinoni et al., 2008), in the flower bulb growing industry in the Netherlands (Fustinoni et al., 2008) and in vegetable growing areas in Bulgaria (Fustinoni et al., 2008). These studies have shown that the concentration of ETU in urine of exposed workers reflects the type of activity on the farm, the use of personal protective equipment such as gloves, respirators, plastic suits and boots, and the level of mechanization and training. In studies in which one of the objectives was to determine the correlation of the type of activity to the degree of exposure, it was shown that EBDCs applicators had higher concentrations of urinary ETU after the end of the work shift than workers engaged in harvesting or maintenance of equipment.

In these EBDCs exposure studies, questionnaires were used in addition to the measurement of ETU in urine. Questionnaires are an important complement to biomonitoring to gain detailed information about the type of work and the working conditions. However, at times, biomonitoring and questionnaire data may not agree. For example, although in these studies the majority of workers reported using personal protective devices, concentrations of urinary ETU were high. Therefore, it is possible that the information reported on the questionnaire did not reflect the reality in the field. Also, in some studies, the control group showed detectable amounts of ETU in the urine. A pilot study conducted in Northern Italy with subjects not occupationally exposed to EBDCs showed that 60% of the subjects had detectable amounts of ETU in urine, with values ranging from 0.5 μg/g creatinine to 11.6 μg/g creatinine (Colosio et al., 2006). In study participants not occupationally exposed, it has been assumed that the presence of ETU results from low-level environmental exposure to EBDCs and ETU, probably by consumption of contaminated food, mainly vegetables, and drinks, such as wine (Aprea et al., 1997).

3.2 Human health effects

There have been a limited number of epidemiologic studies that investigated the health effects of EBDCs exposure in agricultural workers. Thyroid disorders are a major area of interest based on experimental animal studies demonstrating ETU-induced thyroid gland enlargement

as a consequence of impaired thyroid hormone synthesis and on an occupational health study demonstrating mild but statistically significant lower T_4 concentrations in workers with higher exposure to powdered ETU than those with lower exposure (Smith 1984).

Two cross-sectional studies, with reference groups to control for exposure to EBDCs, have been conducted to investigate the incidence of thyroid gland disorders in agricultural settings (Panganiban et al., 2004; Steenland et a., 1997; Smith 1984). Measures of thyroid hormone levels were carried out among 49 heavily exposed workers without protective equipment spraying EBDCs on tomatoes in Mexico (Steenland et al., 1997). The level of TSH was significantly higher, but within the clinical reference range, in the applicators (2.13 ± 0.15 mIU/L) compared with the control group (1.6 ± 0.19 mIU/L). There was no significant difference in T_4 concentrations between these two groups. Studies conducted with banana plantation workers in the Philippines did not find any difference in TSH and T_4 levels between workers and the control group (Panganiban et al., 2004). However, nutritional iodine intake as assessed by urinary iodine concentration was higher in the workers than in the control group. Isolated solitary thyroid nodules were found in 5 workers and 1 control participant. Also, the size of the nodule correlated well with blood ETU concentration (correlation coefficient [r^2]=0.956, p=0.001) and weakly with urinary iodine concentration (r^2=0.759, p=0.08), but not with urinary ETU concentration (r^2=0.594, p=0.213). The significance of the solitary thyroid nodules in this study of agricultural workers remains to be determined through further studies.

Case reports of workers with allergic contact dermatitis (T-cell mediated process) (Nater et al., 1979; Kleibl & Rackova, 1980; Bruze & Fregert, 1983, Campbell & Forsyth, 2003) to EBDCs and ETU have led to several epidemiologic investigations on the potential immunologic effects in workers using or manufacturing EBDCs.

A European-wide study (EUROPIT) was designed to evaluate the immune effects consequent to chronic exposure to EBDCs. This study included five fields in 4 countries: The Netherlands, Italy, Finland and Bulgaria. The total study population consisted of 248 workers exposed to EBDCs and 231 workers serving as controls because of their lack of exposure to EBDCs (Fustinoni et al., 2008; Sterenberg et al., 2008; Van Almelsvoort et al., 2008). The workers completed a self-administered questionnaire and were evaluated for their exposure to EBDCs by detection of ETU in their urine. The urinary ETU concentrations for these comparative groups in this study are shown in Table II (Fustinoni et al., 2008). Immunoglobulin, complement, mitogen-induced cytokyines, mitogen-induced proliferative response, and lymphocyte subtypes were used to investigate for alterations in functions of the immune system in these workers. The EUROPIT field study showed no association between exposure to EBCDs and an increased prevalence of allergic contact dermatitis, allergic rhinitis, asthma and asthmatic symptoms, or IgE-mediated allergic response to selected antigens in these workers (Swaen et al., 2008; Boers et al., 2008).

An increase in T-cell proliferative response to mitogens (phytohemagglutinin, anti-CD3 monoclonal antibody and phorbol myristate acetate) was observed in workers manufacturing EBDC (mancozeb) compared to controls (Colosio et al., 1996). This cellular response was similarly found in a group of vineyard workers involved in the application of mancozeb (Corsine et al., 2005). In addition, an increase in complement (C3 and C4) and IgG4 levels, and a small but statistically significant decrease in IgA levels were found in exposed workers compared to the controls (Steerenberg et al., 2008). When lymphocytes subtypes were analyzed, it was ascertained that there was an increase in CD19 cells (lymphocyte B) and a decrease in the percentage of CD25 cells (cells with IL-2 receptor)

compared to the controls (Corsine et al, 2005). Also, the number of CD8 cells (MHC class I-restricted T cells) was higher in exposed workers than in the control group (Steerenberg et al., 2008). Finally, it was also found that agricultural workers had a reduction in LPS-induced TNF-α (Corsine et al., 2005). Although the significance of these immunological data on the health of workers exposed to EBCDs is uncertain, these findings suggest that the exposure to these fungicides can modulate the immune system. The health consequence of these findings in vulnerable populations, such as agricultural workers with co-morbidities, is unknown and can be a topic for future investigation.

Method	Sample Preparation	Analytical System	Detection Limit
Aprea et al., 1993	LLE with dichloromethane	HPLC/DAD	LOQ: 0.5 µg/g creatinine
Debbart & Moore, 2002	LLE with dichloromethane	HPLC/UV detector	LOD: 0.5 µg/L
Sottani et al., 2003	LLE with dichloromethane	HPLC/ESI-MS/MS	LOD: 0.5 µg/L
Fustimoni et al., 2005	LLE with dichloromethane, BSTFA derivatization	GC/MS	LOD: 0.6 µg/L
El Balkhi et al., 2005	SPE with dichloromethane	HPLC/DAD	LOQ: 1 µg/L
Montesano et al, 2007	Lyophilization, extraction with dichloromethane	HPLC/APCI MS/MS	LOD: 0.16 µg/L
Lindh et al., 2008.	Single-step extraction/PFBBr derivatization	LC/ESI-MS/MS	LOD: 0.05 µg/L
Jones et al., 2010	LLE with dichloromethane	LC/APCI-MS	LOD: 0.25 µg/L
Jayatilaka et al., 2010	Lyophilization, 96-well-plate automated extraction with dichloromethane	HPLC/APCI-MS/MS	LOD: 0.01 µg/L

Table 1. Methods for measuring the ETU metabolite in urine

The chronic exposure of humans to the EBDC maneb or in combination with the herbicide paraquat has been linked to the neurodegenerative disorder Parkinson's disease, based on epidemiologic studies (Ferraz et al., 1988; Costello et al., 2009) and reports of Parkinsonism in workers exposed to maneb (Ferraz et al., 1988; Meco et al., 1994). Although the toxic effect have been attributed to the manganese present in this pesticide (Barbeau, 1984), an independent contribution of the Mn-EBDC complex to this disorder is plausible (Hoogenraad, 1988; Zhang, 2003). Due the limitations of these and other studies to date focusing on the health effects from the exposure to EBCDs in humans and, also, because some of the observations were subclinical or subtle, it is important to further investigate the clinical significance of occupational exposure to EBCDs.

Reference	Country/Area	Cultivation	Exposed Workers/Tasks and Controls	ETU levels in urine
Steenland et al., 1977	Mexico/Cuernavaca	Tomatoes	Applicators (n=49)	58 ± 26 µg/L[a]
			Landowners (n=14)	12 ± 3 µg/L
			Controls (n=31)	below LOD
Colosio et al., 2002	Italy/Lombardy region	Grape Vine	Workers (n=26)	
			Baseline	0.5 - 3.4 µg/g creatinine[b]
			End of Work Shift	0.5 - 95.2 µg/g creatinine
			Controls (n=13)	< 0.5 µg/g creatinine
Colosio et al., 2003	Ecuador/ around Quito	Flowers	Applicators	1.5 - 34.5 µg/g creatinine[b]
			Harvesting	0.4 - 26.4 µg/g creatinine
			Post-harvesting	0.4 - 11.1 µg/g creatinine
			Maintainers of Equipment	3.2 - 6.5 µg/g creatinine
			Controls	0.4 – 2.1 µg/g creatinine
Panganiban et al., 2004	Philippines	Banana	Directly Exposed Workers[e] (n=57)	378.34 ± 50.11 µg/L[c]
			Indirectly Exposed Workers[f] (n=3)	267.16 ± 69.9 µg/L
			Controls (n=43)	26.31 ± 6.39 µg/L
Corsini et al., 2005	Italy/Northern Italy	Grape Vine	Workers (n=13)	
			Baseline	< 0.5 µg/g creatinine[d]
			End of Work Shift	2.5 µg/g creatinine
			Control (n=13)	< 0.5 µg/g creatinine
Colosio et al., 2007	Italy/Northern Italy	Grape Vine	Workers (n=48)	
			Baseline	1.8 ± 5.3 µg/g creatinine[c]
			End of Work Shift	14.9 ± 13.0 µg/g creatinine
			Controls (n=45)	1.3 ± 1.5 µg/g creatinine
Panuwet et al., 2008	Thailand/Pong Yaeng	Vegetables	Workers (n=67)	13.2 µg/g creatinine[d]
		Flowers		
		Lychees		
	Thailand/Inthakhin	Grains	Workers (n=69)	2.2 µg/g creatinine
		Tobacco		
		Vegetables		
Fustinoni et al., 2008	Bulgaria	Vegetables	Workers (n=55),Controls (n=45)	49.6 µg/g creatinine,< 0.5[d]
	Finland	Potatoes	Workers (n=51),Controls (n=51)	7.5 µg/g creatinine, < 0.5
	Italy	Grape Vine	Workers (n=48),Controls (n=45)	11.9 µg/g creatinine, NA
	The Netherlands	Flower Bulbs	Workers (n=42)[g],Controls (n=40)	0.9 µg/g creatinine,0.9

[a]Mean ± SE
[b]Range
[c]Mean ± SD
[d]Median
[e]Directly Exposed Workers: Mixers, Applicators, Clean and Maintenance of Equipments.
[f]Indirectly Exposed Workers: Supervisors, Maintenance Crew, Research Aids.
[g]Re-entry Workers.

Table 2. Urinary concentrations of ETU in occupationally exposed workers and control groups.

4. Conclusions

It is clear that EBDCs and in particular the metabolite ETU have important toxic effects in various animal species. It is therefore desirable that further human studies should be done to better assess the exposure to EBDCs and their metabolite ETU through biomonitoring and to define these chemicals potential health risk(s). Much of the general population is potentially exposed to EBDCs, if only through the consumption of fruits and vegetables. For many agricultural workers exposure to EBDCs is chronic. Biological monitoring and the health surveillance of the workers are the basic components for risk assessment and risk management. They determine whether a relation exists between occupational exposure and disease. Unfortunately, biological monitoring and health surveillance are not currently common practices in the field. However, biological monitoring of workers exposed to EBDCs is feasible today because of advances made in analytical laboratory science. Mass spectrometric analytical methods exist that detect ETU – the commonly used marker – in human urine at very low concentrations with excellent accuracy and precision. Although these instruments are expensive and the methods are resource intensive, these concerns will likely lessen in the future because of improved technology.

Until the time when routine assessment in the field becomes feasible, additional exposure studies using biomonitoring should be undertaken to understand better the relation between health effects and levels of exposure. Information from these studies can lead to sound policy and regulatory decisions that will enhance the protection of workers and the environment.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of Centers for Disease Control and Prevention.

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10

Pesticide Residues in the Organically Produced Food

Ewa Rembiałkowska and Maciej Badowski
Warsaw University of Life Sciences
Poland

1. Introduction

A basic aim of using chemical products for plant protection (pesticides) in farming is the quantity increase and quality improvement of agricultural crops. Pesticides are destined, inter alia, to control any kinds of pests, weeds, pathogenic organisms and other factors which cause plant damage. Therefore, the use of these substances, which have biocidal effects, is deliberately introduced on crops and get through them to people. In addition, a side effect of agrochemical treatments is their movement to the various components of the natural environment.

Therefore, considering the benefits of agriculture chemization, one should also take into account, inter alia, the risk associated with the presence of pesticide residues in commercially available agri-food products. Although modern chemical plant protection products are designed in such a way as to selectively affect specific pests, pest groups, fungi, or weeds, without impacting other organisms (including humans), and the requirements on the toxicological safety placed at their registration are very stringent, there is always a risk that human exposure to pesticide residues in food products may constitute a potential danger to health.

Risk assessment for the overall population and vulnerable populations (e.g. infants, children or pregnant women) takes place both at the stage of registration of the active substance, and subsequently in the monitoring and official food control. In the first case, this process involves a thorough evaluation of the results obtained in long-term toxicological tests on animals, *in vitro* and field studies. The result of this assessment is approval (or rejection) of the proposed MRL (Maximum Residue Level), identified on the basis of field studies in line with the Good Agricultural Practice, recommended by the manufacturer. In the second case, the risk assessment is carried out based on the results of research of the market products, while it is very important to conduct research properly – from sampling, through sample analysis in an accredited laboratory, to the result along with the associated uncertainty. In both cases, the essence of the risk assessment process is to compare the estimated, potential chronic and acute exposure to a pesticide (absorbed with food) with the designated reference values which are considered safe.

A global scale of acute poisoning by plant protection chemicals is not exactly known and all data are based on estimates. In 1973, the first time the World Health Organization reported that there were 500 thousand cases of such poisoning a year. However in 2002,

the number of deaths due to acute poisoning by pesticides was estimated at about 220 thousand annually, which represents only a small percentage of the total number of such poisonings of roughly 26 million a year (Richter, 2002). The threat of the use of chemicals in agriculture is greatest in developing countries, since the awareness of the local population about the negative impact of pesticides on health is still relatively low. This is confirmed by recent research carried out in rural regions of Asia where the number of deaths due to pesticide poisoning is estimated at 300 thousand cases a year (Eddleston *et al.,* 2008).

However, not only acute poisoning is a dangerous result of the presence of pesticides in agricultural production. An equally significant threat is brought by chronic poisoning, which occurs only after a certain time, when the accumulation of substances in the body exceeds the acceptable level. Then, even low doses of pesticide absorbed by the body – when administered long enough – contribute to the highly toxic effects. Pathological changes the situation contributes to are often irreversible.

Pesticides have neurotoxic and immunotoxic effects as well as carcinogenic properties. Some of them have a chemical structure similar to human hormones, such as nonylphenol, an active substance of several pesticides. In terms of chemical structure, this compound is very similar to oestrogen, the most important female reproductive hormone. Nonylphenol may partly displace it from metabolic pathways, interfering with the reproductive cycle of females (Odum *et al.,* 1997).

A number of compounds, where it is demonstrated to affect the human endocrine system, remain in use in modern agriculture (Ansar Ahmed, 2000). These include herbicides such as atrazine, alachlor, simazine and trifluralin. Among these are the fungicides benomyl, mancozeb, maneb, metiram, viclozolin, zineb and ziram, and in the group of insecticides there are such active substances as cypermethrin, endosulfan, esfenvalerate, fenvalerate, kelthane, lindane, methomyl, permetrin (Ansar Ahmed, 2000).

It is believed that the plague of infertility, which in recent years has been recognized by WHO as a social disease (WHO, 2010), is caused, inter alia, by the presence of pesticides in agri-food production. Since the introduction of pesticides on the market, or roughly since the mid-twentieth century, we have observed a downward trend in terms of quantity and quality of the Europeans sperm. It is pointed out by Howard (2005), who as one of the reasons gives just the use of chemical pesticides, present in food. Infertility problem now concerns about 15-20% of all couples trying to conceive (WHO, 2010). The reason for such state are not only pesticides, because it probably consists of a variety of other factors, but the negative impact of pesticides is unquestionable (Howard, 2005).

In the body of every man there are certain amounts of chemical compounds that are brought by ingestion or inhalation. It concerns pesticides as well. It was proved by Gilman *et al.* (1997) by examining the contents of selected compounds in the blood plasma of women from circumpolar zone countries. It turned out that even in countries that are considered to be free from contaminants (such as Iceland and Greenland), human plasma is contaminated with compounds which are derivatives of DDT (see Figure 1 and Figure 2). This product was already withdrawn from use in the 70s of the twentieth century because of its toxic effects on human health. In the test samples there was also found the presence of other active substances of pesticides, such as chlordane, HCB and mirex, which is not irrelevant in determining the mechanism of toxicity of the mixture on the human body.

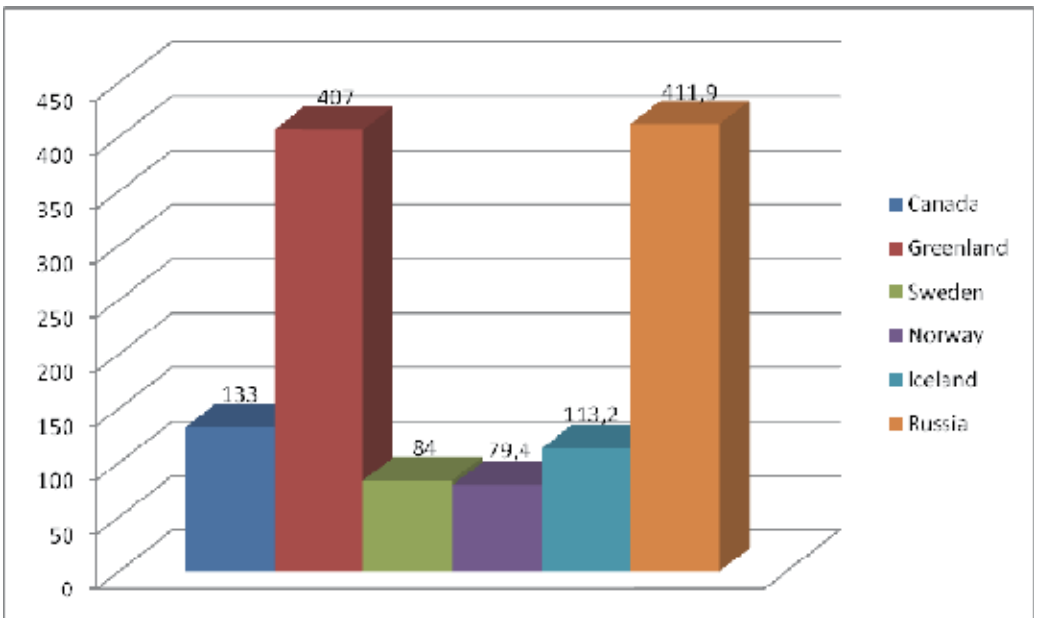

Fig. 1. The content of p,p'-DDE in the blood plasma of women (geometric mean µg/kg of fat) (Gilman *et al.*, 1997)

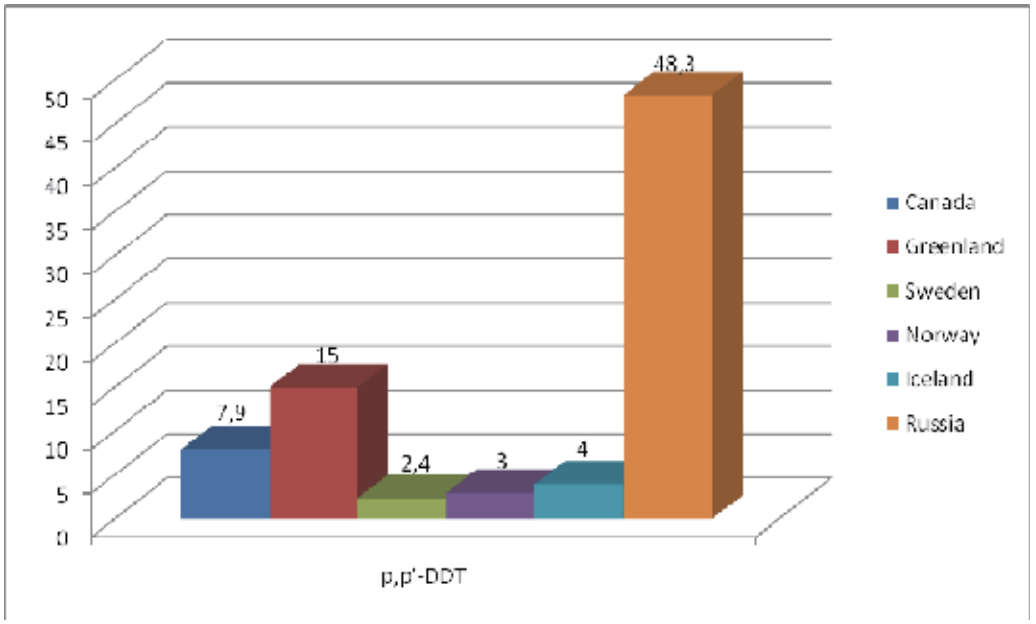

Fig. 2. The content of p,p'-DDE in the blood plasma of women (geometric mean µg/kg of fat) (Gilman *et al.*, 1997)

The exposure of children to poisoning by pesticides in fruit was the subject of the research by Pennycook *et al.* (2004) – they conducted a risk assessment of the health of British children on the basis of consumption of apples and pears. The result of their study was the number of children aged 1.5-4.5 years who are exposed to daily intake of pesticide in an amount that exceeds the maximum permissible value (ARD - acute reference dose). The analysis was carried out for the content of dithiocarbamates, phosmet, and carbendazim. Depending on harvest time and the type of tested compound, the results ranged from 10 to 226 children per day. There were therefore variable levels of pesticide residues, which sometimes reached values exceeding the ARD dose by six times.

2. Pesticides vs. organic farming

Against the background of pessimistic market research results in terms of food contamination by pesticides, a reasonable alternative appears to be the consumption of organic products. One of organic farming iron rules is to give up the use of agrochemicals, i.e. not only synthetic mineral fertilizers, but also chemical plant protection products. Such a system of agriculture is protected by law, which in addition to establishing policies requires regular inspection of the manufacturing process. In case of organic products, the European law does not define, however, other than the MRL values for conventional products and there are some products that are acceptable in organic system. They are presented in the list below (Commission Regulation (EC) No. 889/2008), and the products can be used only in case of threat to crop plant, provided that the products are used in accordance with the provisions established at Member State level.

i. Substances of crop or animal origin
   - Azadirachtin extracted from *Azadirachta indica* (Neem tree) – insecticide
   - Beeswax[1] - pruning agent
   - Gelatine – insecticide
   - Hydrolysed proteins[1] - attractant, only in authorized applications in combination with other appropriate products of this list
   - Lecithin – fungicide
   - Plant oils (e.g. mint oil, pine oil, caraway oil) - insecticide, acaricide, fungicide and sprout inhibitor
   - Pyrethrins extracted from *Chrysanthemum cinerariaefolium* – insecticide
   - Quassia extracted from *Quassiaamara* - insecticide, repellent
   - Rotenone extracted from *Derris* spp. and *Lonchocarpu* spp. and *Terphrosia* spp. – insecticide

ii. Micro-organisms used for biological pest and disease control
   - Micro-organisms (bacteria, viruses and fungi)

iii. Substances produced by micro-organisms
   - Spinosad – insecticide – only where measure are taken to minimize the risk to key parasitoids and to minimize the risk of development of resistance

iv. Substances to be used in traps and/or dispensers
   - Diammonium phosphate[1] - attractant, only in traps
   - Pheromones - attractant, sexual behavior disrupter; only in traps and dispensers
   - Pyrethroids (only deltamethrin or lambdacyhalothrin) - insecticide; only in traps with specific attractants; only against *Bactrocera oleae* and *Ceratitis capitata* Wied.

v. Preparations to be surface-spread between cultivated plants
   - Ferric phosphate (iron (III) orthophosphate) – molluscicide

vi. Other substances from traditional use in organic farming
   - Copper in the form of copper hydroxide, copper oxychloride, (tribasic) copper sulphate, cuprous oxide, copper octanoate - fungicide; up to 6 kg copper per ha per year; for perennial crops, Member States may, by derogation from the previous paragraph, provide that the 6 kg copper limit can be exceeded in a given year provided that the average quantity actually used over a 5-year period consisting of that year and of the four preceding years does not exceeded 6 kg
   - Ethylene[1] - degreening bananas, kiwis and kakis; degreening of citrus fruit only as part of a strategy for the prevention of fruit fly damage in citrus; flower induction of pineapple; sprouting inhibition in potatoes and onions
   - Fatty acid potassium salt (soft soap) – insecticide
   - Potassium aluminium (aluminium sulphate) (Kalinite)[1] - prevention of ripening of bananas
   - Lime sulphur (calcium polysulphide) - fungicide, insecticide, acaricide
   - Paraffin oil - insecticide, acaricide
   - Mineral oils - insecticide, fungicide; only in fruit trees, vines, olive trees and tropical crops (e.g. bananas)
   - Potassium permanganate - fungicide, bactericide; only in fruit trees, olive trees and vines.
   - Quartz sand[1] – repellent
   - Sulphur - fungicide, acaricide, repellent

vii. Other substances
   - Calcium hydroxide - fungicide; only in fruit trees, including nurseries, to control *Nectria galligena*
   - Potassium bicarbonate - fungicide

[1] In some countries the product is not categorized as a plant protection product.

Although at first glance the above list seems to be quite large, from a practical point of view only few of the plant toxic pesticides are currently in widespread use - pyrethrin, rotenone or neem. Pesticides containing natural toxic substances of plant origin have the capacity for rapid biodegradation. Pyrethrin in most cases decomposes in 24 hours – this time can extend up to 2 days. Rotenone is in turn more stable in field conditions, the degradation takes several days to a week. What is more, they are generally used in relatively low doses - for comparison, organophosphate insecticides, used to control virtually the same groups of insects, must be applied in amounts of 50-fold or even 100-fold. Not to mention that their biodegradation may take up to several months (Benbrook, 2004).

A very effective bioinsecticide, authorized for use in organic farming as well, is spinosad. Application of this compound involves the risk of residues of the active substance in cultivated raw materials. In 2001, one of the orchards used the pesticide for 31% of nectarines - its residues were found in 13.5% of samples tested. However, the contents were found to be very low, at levels from 0.006 to 0.029 ppm. You need to have in mind the low toxicity of spinosad for mammals, and therefore the health risk in case of such cultivated fruit is negligible (Benbrook, 2004).

The above-described biological plant protection products, authorized for use in organic farming, however, should occupy a secondary place on such farm. The idea of organic

farming is based in fact mainly on preventing the emergence of diseases and pest infestation. This can be achieved by methods that do not interfere with the natural environment and pose no threat to humans. The first way is the right crop rotation, properly matched to habitat, and economic and organizational conditions of farm (Jończyk, 2005). It allows the maintenance of high biological activity of the soil, reducing the stroke of crops by specific diseases and pests which chemical control is difficult or impossible. Frequent cultivation of the same or related plant species after each other always increases their infection by specific diseases (crop rotation diseases) which are transferred to the subsequent crop through soil and crop residues, such as stem base disease of cereals, fusariosis of many plant species, Cercospora beticola and beet necrotic yellow vein virus (BNYVV) etc., and pests, such as golden nematode and sugarbeet nematode, *Haplodiplosis equestris* etc., and their chemical control is expensive and often little effective. Furthermore, such rotation reduces intensity of onerous weed species. Each species of crop plants is accompanied by a group of weeds which the rhythm of growth and development are similar to cultivated plants. Therefore, frequent comeback of the same or related plant species to a field leads to the compensation of certain weed species.

Another way of prevention used in organic farming is the development of biodiversity on the farm. This goal is achieved through proper diversification of the landscape, which exerts a strong influence on species richness. The area surrounding the fields, woodlots and water reservoirs that are left in a position similar to the natural represent the ecological corridors and habitats for many groups of organisms, including those useful. These include species of natural enemies of pests – thanks to them the number of organisms onerous for farmers, which in a conventional farming system are controlled by pesticides, may be limited. This is demonstrated by numerous studies confirming, inter alia, a significantly higher density and more species of ground beetles (*Carabidae*) in organic farming (Kromp, 1989; Irmler, 2003).

3. The presence of pesticide residues in organic products

Each year the European Food Safety Authority (EFSA) publishes a report on monitoring of pesticide contamination in the market food in 27 European Union member states and two EFTA countries (Norway and Iceland). For several years, the report has also included the studies on organic food.

According to the report for 2007, the percentage of organic food products containing residues of pesticides at levels exceeding the MRL value was much lower than for conventional products. A similar result was obtained in 2008 (see Figure 3).

In 2007, a total of 2,980 organic products were tested. The largest group consisted of fruit and vegetables, while others included cereals and processed products. The percentage of samples containing residues of pesticides in each group is shown in Figure 4.

In 2008, there were tested 3,131 organic products in total. The largest group also represented fruit and vegetables, and other cereals and processed products included baby food as well. The percentage of samples containing residues of pesticides in each group is presented in Figure 5.

In the 90s of the twentieth century, Baker *et al.* (2002) conducted a broad study of pesticide residues in food from different sources in the U.S. These sources were classified into three groups - organic, conventional and integrated (intermediate between the other two.) The study proved conclusively that the slightest pesticide contamination occurred in organic production – in this case the percentage of such samples was more than three times lower

than in conventional production, taking into account fruit and vegetables separately as well as all fresh food. For the analysis there were used three different test programs, each of which confirmed the studied tendency. These studies concerned the years 1993-1999 and then were continued for three consecutive years by the USDA. The additional studies

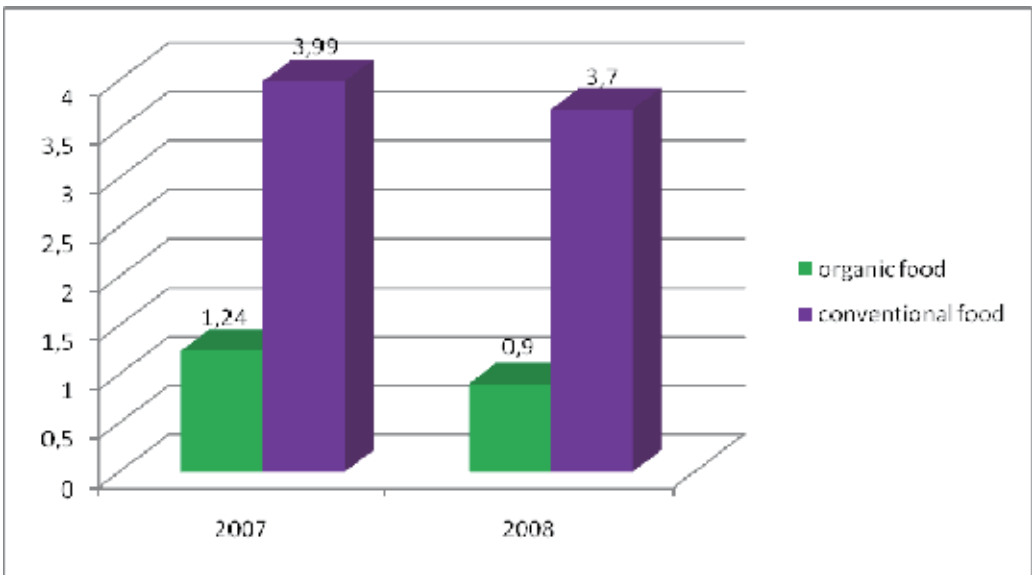

Fig. 3. Samples with pesticide residues above the MRL in European food (%) (EFSA, 2009; 2010)

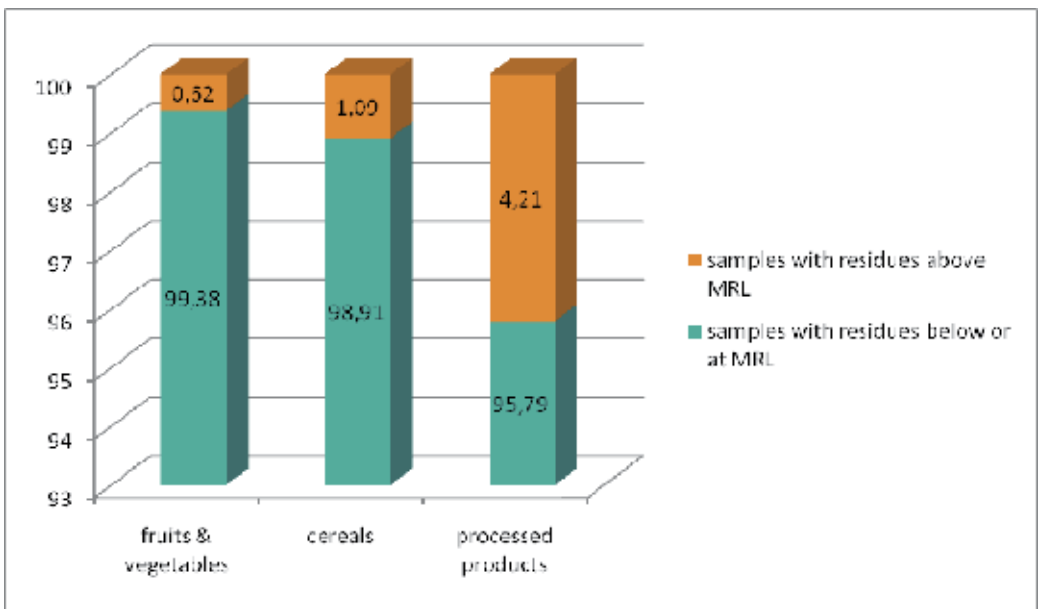

Fig. 4. The percentage of organic food samples containing residues of pesticides below and above the MRL in 2007 (EFSA, 2009)

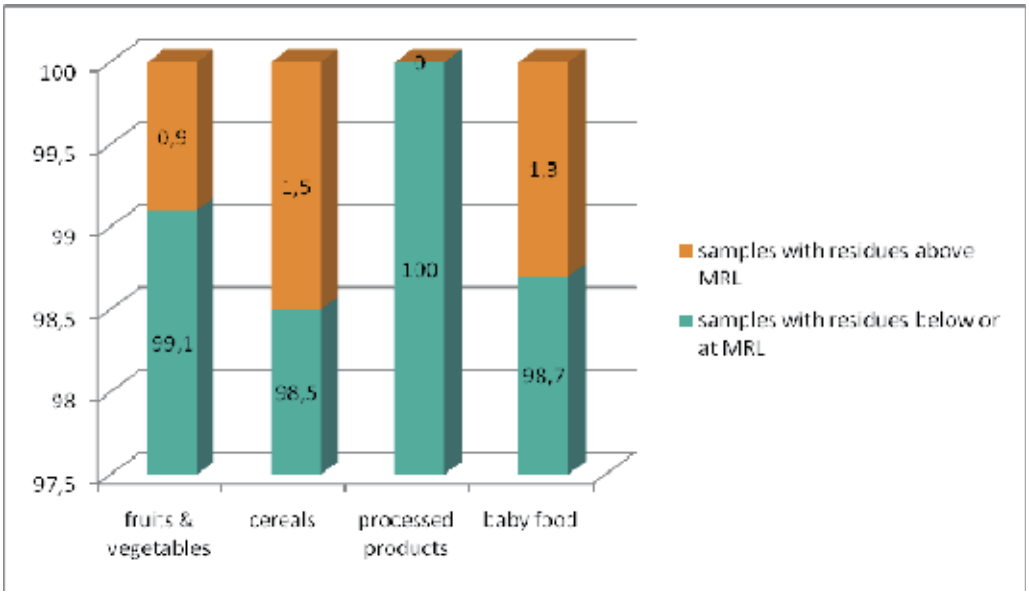

Fig. 5. The percentage of organic food samples containing residues of pesticides below and above the MRL in 2008 (EFSA, 2010)

took into account more than twice as many samples of organic fruit, which allowed the increase of statistical confidence of comparing the quality of conventional and organic products. Full results of the tests conducted are shown in Figure 6. These results do not take into account the samples with residues of organochlorine (OC) pesticides, because they are

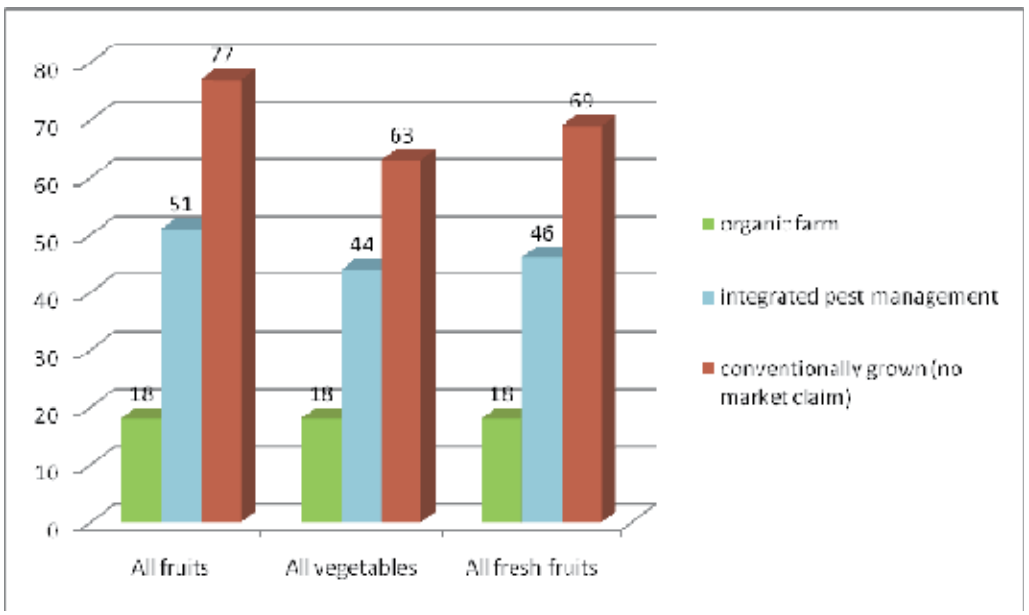

Fig. 6. Frequency of pesticide residues in fresh fruits and vegetables by market claim 1993-2002 (%) (Benbrook, 2004)

no longer allowed in any of the production systems, even conventional one. Excluding them from the analysis, there was obtained a more reliable picture of the management of plant protection products in the modern agriculture.Within 10 years, when the study was conducted, the probability of residues of at least one pesticide in conventional vegetables proved to be 3.5 times greater than for organic vegetables. Another interesting conclusion is that a consumer who buys from 1 to 2 kg of conventional peaches in the supermarket has more than 11-fold greater chance that they will get the fruit containing residues of seven pesticides at least than the chance to buy peaches completely deprived of such impurities. It is also much more possible that a person buying celeries will get raw materials containing at least five different pesticides, rather than they will buy celeries with the residue of one chemical agent at most (Benbrook, 2004).

Among conventional raw materials, the highest level of pesticide contamination was typical for such vegetables as spinach and celery, and fruit, such as apples and pears. One should keep in mind that the number of food samples from organic production was much lower (even after the addition of research in 2000-2002) than in case of the other two groups, and therefore the conclusions on the degree of exposure, depending on the species of organic fruit and vegetables, cannot be drawn.

In Sweden, there were also conducted comparative studies of three types of production (The Swedish Monitoring, 2003; 2004; 2005), and the proportions in the results were similar. However, there should be noted the scale in case of the percentage of contaminated food in the U.S. and Sweden. In the United States, pesticide residues were present much more frequently, respectively in each of the analyzed groups (see Figure 7). This difference may result from slightly different principles of organic farming and control on both continents. The approach towards agriculture in the U.S. and Europe is diverse as well. The discrepancy may stem from the difference in the functions of agriculture and rural areas, there is another

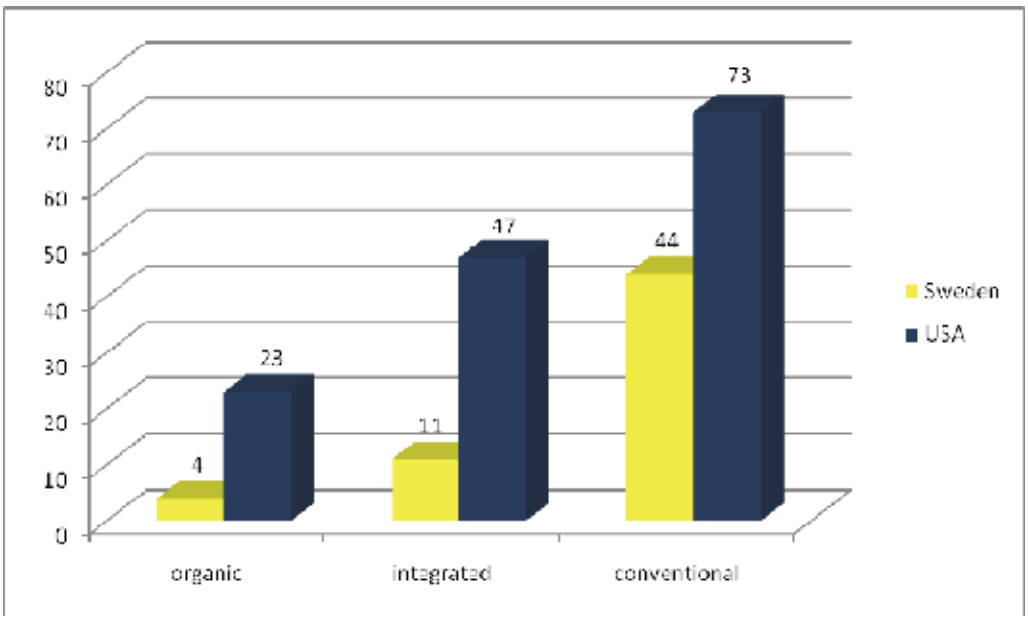

Fig. 7. The scale of contamination of agricultural crops with pesticide residues in the U.S. and Sweden (Baker *et al.*, 2002; The Swedish Monitoring, 2003; 2004; 2005)

definition of farm as well as parameters determining its performance. In Europe, a prosperous farm is considered to be one that achieves a high yield per hectare and high efficiency of livestock production. However, in the United States, a criterion for success is the profit from the dollar invested, i.e. so-called ROI (Risk on Investment). In a situation where there is a possibility of increasing the profitability of production (such as pesticide use), any fears of risk of using such solutions recede into the background. The attitude towards agricultural production, different than in Europe, contributes as well – less and less agricultural land is in private hands, and an increasingly important role in land use is played by corporations. The approach to agriculture is therefore less emotional and more economical (Nowak, 2004). Therefore, in the United States pesticides are used much more widely, which also has an impact on organic farming, since, as it is known, compounds move in the environment.

The Belgian studies conducted in 1995-2001 (AFSCA-FAVV, 2001) were of a similar nature, except that the integrated production was not taken into account. The raw materials of organic food contained pesticide residues in case of 12% of samples, while the conventional product rate reached 49%.

The UK also carried out a comparative study of the conventional market food vs. organic one. The share of samples contaminated with pesticides in different food groups is presented in Figure 8.

According to the results of these studies, the likelihood of the presence of pesticide residues in conventional infant food was 7-fold greater than in the organic products. Very large differences were also observed in case of fruit (more than 7-fold higher percentage of contaminated conventional samples compared to organic ones) and grains (there were no residues in a single organic sample, while in the conventional group the contamination comprised 42%).

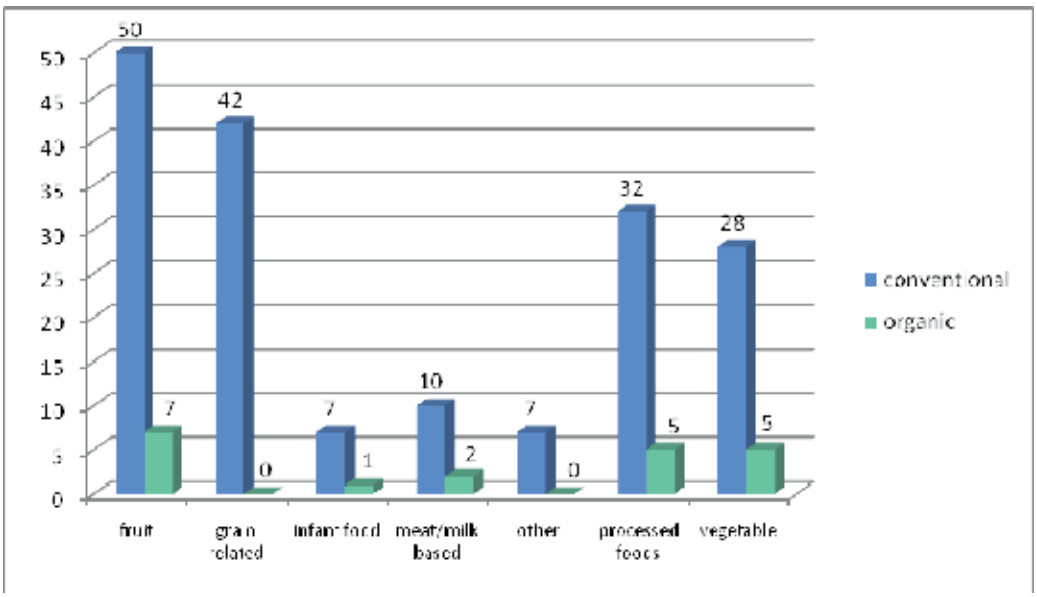

Fig. 8. Frequency of positive conventional and organic samples in foods tested by the British Pesticide Residues Committee 2001-2003 (Benbrook, 2004)

Department of Primary Industries, Victoria, Australia, supervised the monitoring of the quality of organic food in 2002-2003. The study was based on 300 samples of food, of which two-thirds were vegetables and herbs, one-third – fruit, and only 4% of the samples were cereals and oilseeds. The analytical methods allowed detecting such pesticides as organophosphate, organochloranes, triazine herbicides, carbamate insecticides, synthetic pyrethroids and a fungicide, iprodione.

Of the 300 samples tested, only two contained residues of chemical pesticides. In one of cantaloupes there was found dieldrin, banned organochlorine compound. In one of apples, however, there was present post-harvest fungicide, iprodione in trace amounts. Subsequent investigation at apple supplier's cleared this case, because the apples were stored in wooden boxes, which had previously contained fungicide treated fruit (Benbrook, 2004).

In Poland there were also conducted studies of pesticide residues in organic food. As in Sweden, samples from all three farming systems were subjected to analysis. The first such study, conducted in 2004, brought very surprising results, because among the organic raw materials there was not found any sample contaminated with pesticides. The largest percentage of raw materials containing such residues was found in integrated production (50%), leaving in this respect conventional production behind (44%). Theoretically, integrated agriculture is based on the rational use of chemical plant protection products, but so far there is no legal framework governing this sector of agriculture. Therefore, this result is possible, though it seems amazing (Gnusowski *et al*., 2005). This phenomenon can be explained by the specificity of the Polish agriculture, so-called conventional farming. Polish farmers are very diverse in terms of quantities of pesticides used: many farms use only minimal quantities or do not use them at all – this is so-called extensive farming. In contrast, some farmers belonging to minorities use large amounts of pesticides if they produce for a large city market, such as Warsaw, Krakow and Poznan. This particularly concerns the production of vegetables and fruit. If the level of pesticides in both types of households is averaged, generally the level used on conventional farms will be quite low. In Poland, an integrated method is mainly used by fruit-growers who regularly use pesticides, unlike the extensive farmers. It causes a paradoxical result of higher content of pesticides in integrated raw materials compared to conventional ones.

The results of studies conducted in 2005 and 2006 were similar, confirming the highest raw material contamination with pesticides in integrated system (Gnusowski *et al.,* 2006; 2007). Different levels of contamination in the Polish agricultural products grown by different methods are shown in Figure 9. Attention is paid to a downward tendency of conventional raw material contamination, which is not observed in the integrated production.

According to the research of the years 2004-2007, carried out in Poland by Szpyrka *et al.* (2008), the share of contaminated organic raw materials amounts to 3.6%. Later studies of 2007 (Gnusowski *et al.,* 2008) confirmed the presence of pesticide residues in 14.4% of organic samples, while in accordance with the results of recent studies (Gnusowski *et al.,* 2009), in 2008 the figure was 4.4%.

In Baden-Württemberg (Organic Monitoring, 2002-2009), for several years there was conducted monitoring of the quality of organic food available in the German market. One of the parameters were pesticide residues in fruit and vegetables. Among the conventional raw materials, 88% were contaminated with pesticide residues, and the average number of detected active substances was 3.9 per sample. In the case of organic products, pesticide residues were found in 27% of samples (typically, however, there were trace amounts,

slightly above the limit of quantification). On average, 0.5 of active substances fell to one sample of organic group. The actual results of research conducted on fruit and vegetables are presented in Figure 10.

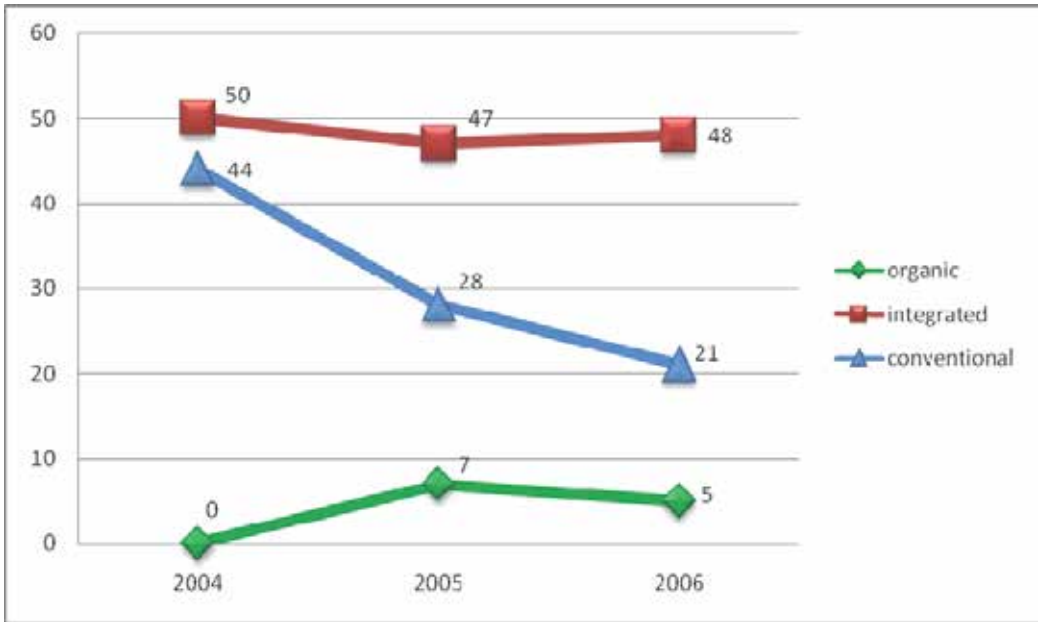

Fig. 9. The pesticide residues found in organic, integrated and conventional food in Poland (%) (Gnusowski *et al*., 2005; 2006; 2007)

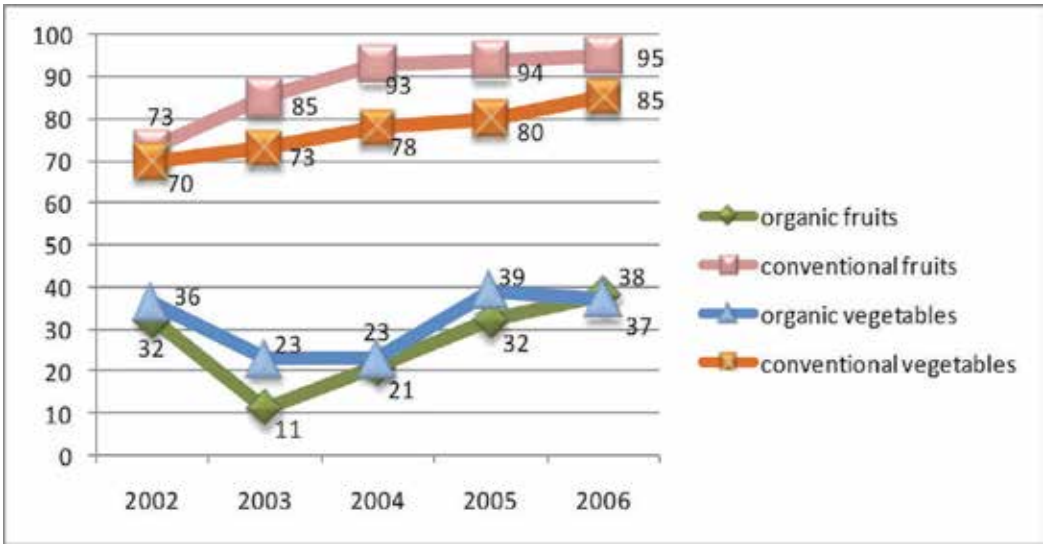

Fig. 10. The residue situation in fruits – organic vs. conventional (%) (Organic Monitoring, 2002-2006)

Figure 11 illustrates the percentage of specific pesticide contaminated raw materials, examined under monitoring in Germany. It makes one think that this percentage is very high among the conventional products. Germany is in fact a society of fairly developed ecological awareness. Relatively many contaminated samples were also found in the case of organic cultivated mushrooms and sweet peppers, which is troubling and requires further research.

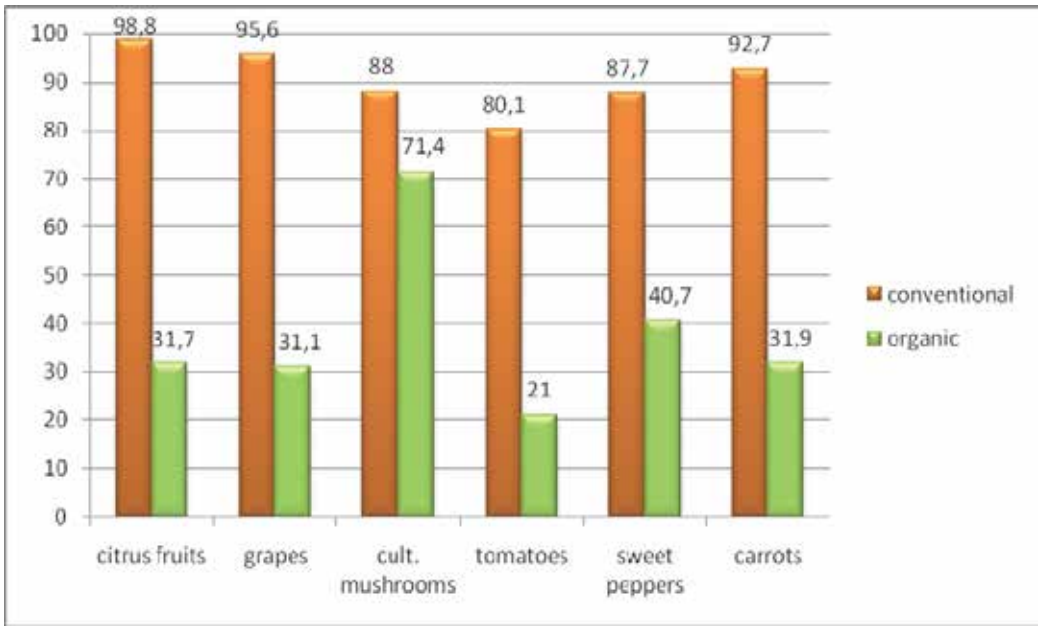

Fig. 11. The percentage of the samples of the food groups with residues – comparison between conventional and organic [%] (Organic Monitoring, 2002-2006)

The latest results of the studies in this field come from Germany (of 2009) (Organic Monitoring, 2010). The tendency towards less frequent occurrence of pesticide residues in organic raw materials remained unchanged, like the high percentage of contaminated samples of cultivated mushrooms. Detailed data are shown in Figure 12.

The above studies also brought interesting results in terms of average pesticide levels in raw materials from different production systems. It turns out that the residues of plant protection chemicals in organic products are not only much less frequent, but also in much smaller concentrations. The average levels of these contaminants in vegetables and fruit over the years 2005 – 2009, found in Germany, are presented in Figure 13.

Apart from the above research, control studies of organic raw materials tested for pesticide residues were also carried out in various other countries. The overall results of the studies are presented in Table 1.

The level of pesticide residues in plants is dependent on the species as well as the active substance of a given product. The substances used have varying toxicity, are characterized by different MRL values, and therefore, the comparison of their contents in the analyzed raw materials does not give the right conclusions. Thus, it is justified to carry out a comparative analysis of pesticide content in products where their presence has been detected. On this basis, one can assess the health risk of consumers exposed to a greater or

lower amount of toxic chemical compound. Such studies were conducted by Baker *et al.* (2002). According to their results, the greatest differences are seen between the content of

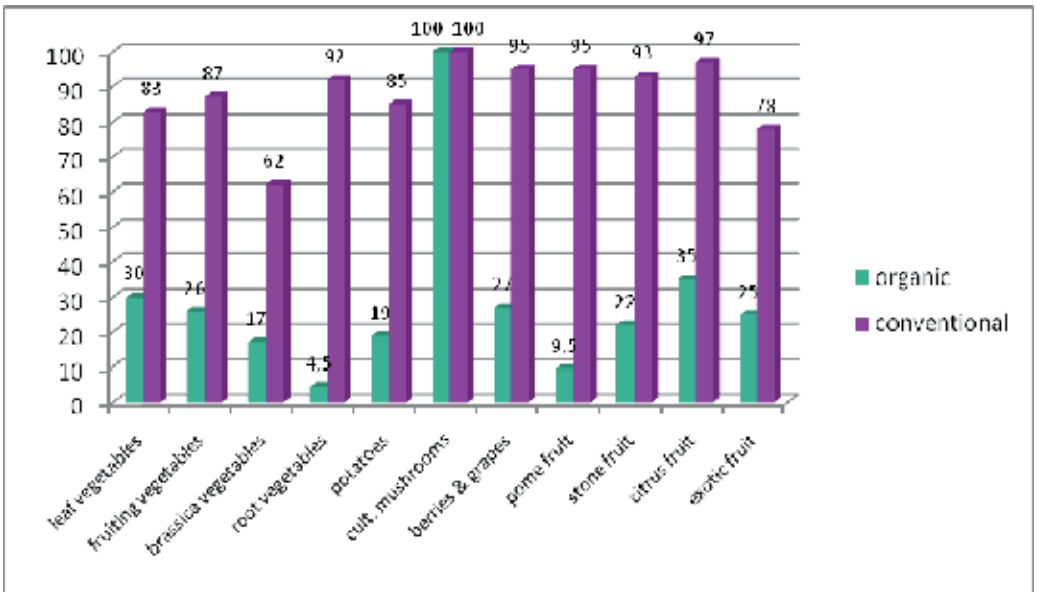

Fig. 12. The percentage of the samples of the food groups with residues – comparison between conventional and organic [%] (Organic Monitoring, 2010)

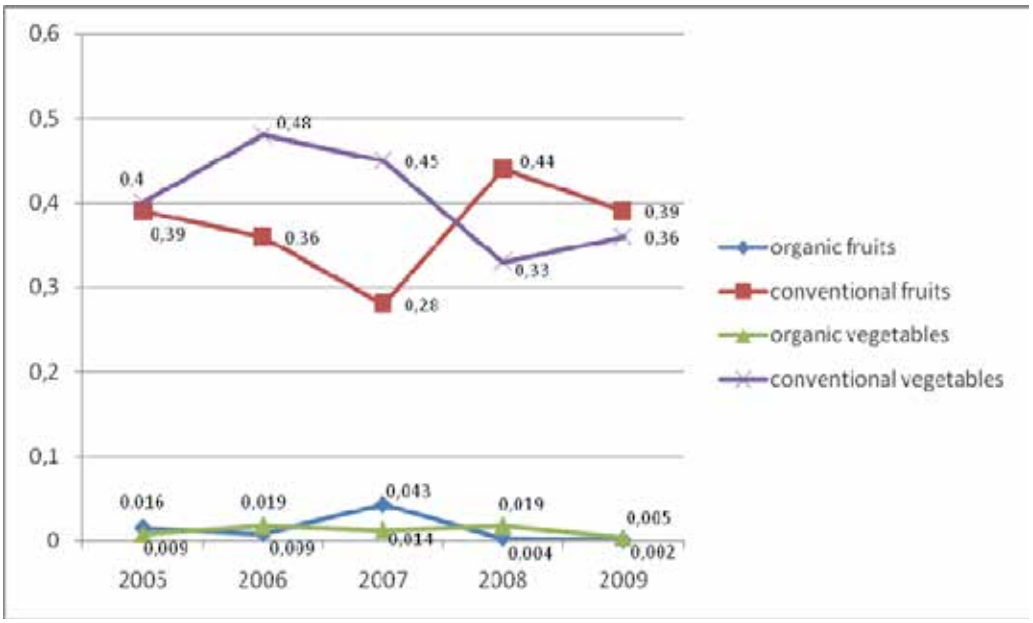

Fig. 13. The average levels of pesticide residues in conventional and organic raw materials in Germany (Organic Monitoring, 2005; 2006; 2007; 2008; 2009)

country	% of samples containing residues
The Czech Republic 2005 (Report, 2006)	14
Ireland 2004-2006 (Pesticides, 2006; 2006a; 2008)	11
Finland 2005-2007 (Pesticide, 2007; 2007a; 2008)	5
Denmark 2002-2003 (Andersen *et al.*, 2004)	3
New Zealand 2004 (Comparison, 2004)	22

Table 1. The comparison of organic crop contamination with pesticide residues

ortho-phenylphenol (commonly used fungicide) in organic and conventional pears. In case of the first ones, the level of the tested compound was more than 22-fold lower. Equally impressive results were obtained in the case of strawberries - the average level of iprodione (also a fungicide) in conventional strawberries turned out to be 7-fold higher than in organic ones. But you cannot ignore the fact that in few raw materials proportions were reversed, as was the case with pepper, celery, grapes and spinach. Ultimately, however, summing up all tested fruit and vegetables from both production systems, the average level of plant production chemicals was approximately 1.7 times higher in the conventional market raw materials (Baker *et al.*, 2002).

The same calculations were made by Benbrook (2004), who compared the contents of selected compounds that had been detected both in conventional and organic raw materials. The greatest value of the ratio of the average pesticide content in a conventional vegetable to the average level of an organic vegetable was 79.55 and concerned chlorthalonil concentration in celery. However, the average value of this ratio for the examined pesticides in the analyzed raw materials was 9.51 (Benbrook, 2004).

4. Pesticide residues in food and their contents in the human body

Undesirable effect of pesticide use in agriculture is not only the contamination of raw materials being grown, but also their movements along the food chain. The man is on its end and this is his health and life which should be a priority in risk assessment and determination of food safety. For this purpose, there are carried out studies to help answer the question of the extent to which chemicals are absorbed along with food, what their distribution in the body is and what affects their quantity.

One of the first studies concerning the relationship between the level of pesticide residues in the diet and their amount in the human body was carried by Aubert in 1987. The subject of analysis was the content of chlorinated hydrocarbons in woman's milk. Persons from whom milk samples were taken consumed organic products, but to varying degrees - their share in the diet was diverse. It was proved that organic food consumption adversely affects the content of chlorinated hydrocarbons in the milk of a tested woman. The largest quantities of pesticides were detected in people who had consumed organic products most rarely (Aubert, 1987). The observed tendency is shown in Figure 14.

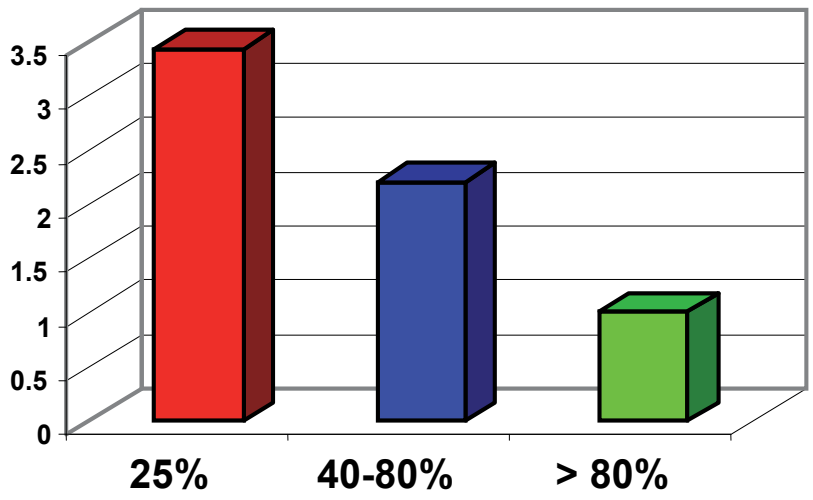

Fig. 14. Total content of chlorinated hydrocarbons in human milk (mg/kg milk fat) (Aubert, 1987)

Curl *et al.* (2003) conducted a study in the United States on children in preschool age. The degree of exposure to pesticide residue poisoning was intended to reflect the level of organophosphate insecticide metabolites, detected in the urine. The study included two groups of children - the first of them, numbering 18 persons, for three days prior to the collection of material for analysis had consumed only certified organic products. Nutrition of the second group of children was based on conventional market products. According to the results of the analyses, in the urine of organically fed children there were found smaller quantities of dimethyl metabolites - dimethylphosphate (DMP), dimethylthiophosphate (DMTP) and dimethyldithiophosphate (DMDTP) - see Figure 15. Total concentration of these metabolites was more than 8-fold lower in children on organic diet rather than children from another study group. In the case of diethyl metabolites – diethylphosphate (DEP) and diethylthiophosphate (DETP) - there were no significant differences. Moreover, the presence of DMP, DMTP, and DMDTP in urine was found significantly more often in conventionally fed children. The percentage of positive results of the analyses on organic diet children turned out to be significantly lower in case of these compounds (Curl *et al.*, 2003).

The 'cross over' type study was conducted on children of school age by Lu *et al.* (2006). The experiment consisted of three consecutive stages. The first phase lasted three days, during which children consumed conventional food. During the second stage, lasting five days, the diet was based on organic products. The third part of the study lasted seven days and returned to a conventional diet. The subject of analysis was the urine of participants and the content of organophosphate insecticide metabolites. It was observed that at the time of transition to organic food the content of MDA (malathion dicarboxylic acid, a metabolite of malathion) and TCPY (3,5,6-trichloro-2-pyridinol, a metabolite of chlorpyrifos) fell below the limit of detection and did not increase until the return of participants to a conventional diet. These active substances are ingredients of pesticides most commonly used in modern

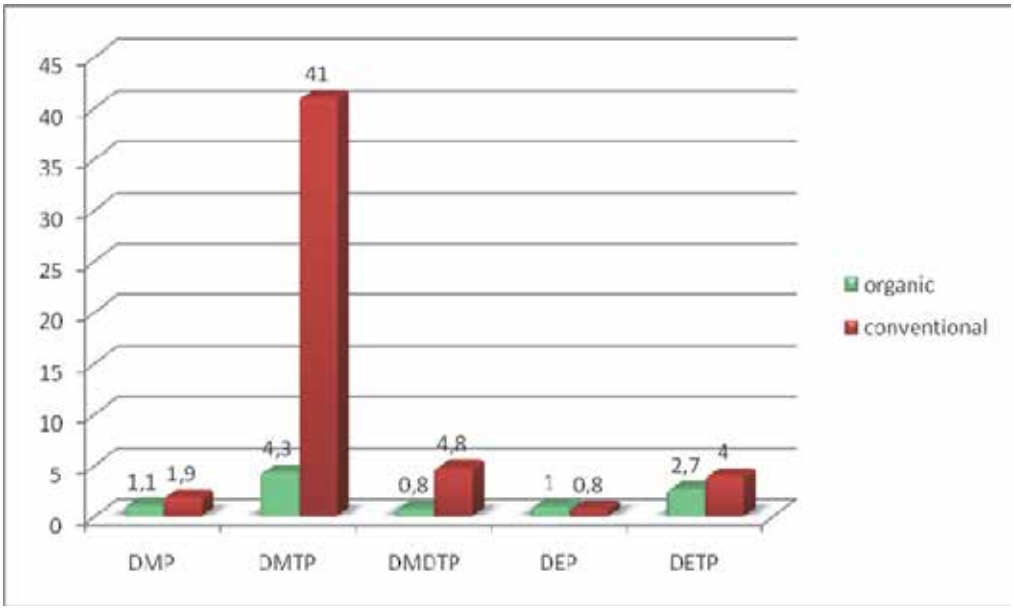

Fig. 15. Individual dialkylphosphate metabolites concentrations (µg/L) in the urine of children on organic and conventional diets (Curl *et al.*, 2003)

agriculture. As for the other metabolites of organophosphate insecticides, their concentrations were also lower during the organic diet phase, but due to the low detection rate, these results could not be considered statistically significant (Lu *et al.*, 2006). Mean levels of MDA and TCPY in subsequent stages of the study are presented in Figure 16.

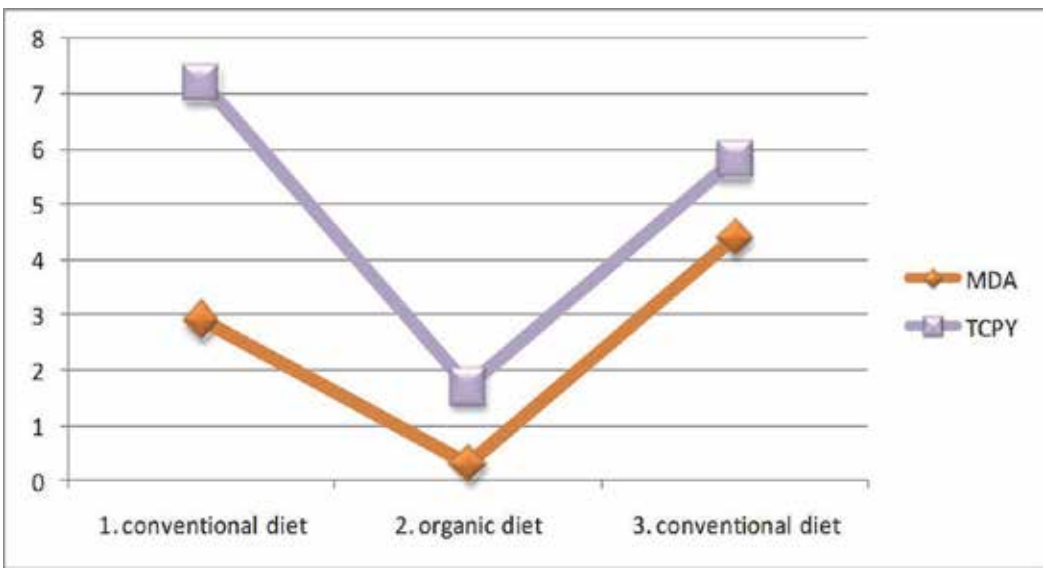

Fig. 16. Mean levels of MDA and TCPY in subsequent stages of the study (µg/L) (Lu *et al.*, 2006)

It must be added that the detection rate of the above-mentioned metabolites in urine also dropped dramatically during the second phase of the study. The percentage of samples containing MDA decreased from 60% to 22%, then back along with a conventional diet increased to the same value of 60%. For TCPY, there was a fall from 78% to 50% at the onset of the second stage, and with its completion the detection rate was 78% again.

5. Discussion

Certified organic products are created without the use of chemical plant protection products, so there is a question about the source of these few cases of pesticide contamination of such raw materials.

The answer to this question is complex. First, the pesticides are widely used around the world, having a mobile nature in agroecosystems. Organic farms do not constitute places of isolation, and are often adjacent to fields where pesticides are used. These products are often spread in the air and reach the places where plants are grown organically. Sprays applied by aircraft hit the target crop at about 25%, while the remainder of the pollutant load is dispersed to other places. When a pesticide is applied using ground-based machines, even at light wind losses amount to approx. 25% of the substance (Benbrook, 2004).

Another factor favouring the migration of chemicals is field watering, through which contaminants can be transferred along the drainage canals. Flowing water is polluted on one of conventional farms, and can get to organic farming. The growing plants absorb chemicals and that is how the contamination of organic crops happens. A similar transfer can take place by the means of dust suspended over the field as well as fog.

Some chemicals, such as organochlorine pesticides, accumulate in the soil. The products, such as chlordane, DDT, toxaphene, dieldrin, and their derivatives, are characterized by very high persistence in the soil environment - despite the fact that they have not been used for 20 years or longer, their residues can still be detected in soil profiles. In addition, some plants, such as cucumbers, melons, carrots, spinach and potatoes, have a great ability to absorb the soil-bound organochlorine compound residues. For this reason, there are cases when certification bodies or companies producing baby food require adequate testing on soils in terms of their contamination with organochlorine compounds (Benbrook, 2004).

The above-mentioned substances also often come to animal fodder and fodder concentrates, so their residues can then be detected in meat, eggs and dairy products.

So-called human factor is no less important in the quality of organic crops in terms of the presence of pesticides, which is the deliberate use of prohibited chemicals. Such practices are illegal and if proved, result in the loss of certificate. The control system is not perfect and sometimes fails to detect farmer's dishonesty.

It is consoling, however, that - as the studies adduced in this chapter prove - even if pesticide residues are found in organic raw materials, usually their content is much lower compared to conventional market products.

6. Conclusions

A number of comparative studies conducted in different countries mostly confirms that in the case of organic products there is 3-4-fold lower likelihood of the presence of pesticide residues than in conventional market products. The chance that organic raw material will include several pesticides at the same time can be up to 11-fold lower than for conventional

one. Numerous analyses have shown that the average level of organic product contamination with pesticides is 3-fold or even 10-fold lower than the average concentration of the same compound in conventional products.
Consequently, human exposure to poisoning by pesticide residues is much lower with consumption of organic fruit and vegetables.
On the conventional food market there is a group of raw materials, which is generally characterized by high contamination with pesticides, and this is often consumed by infants and children. This group includes fruit like strawberries, apples, cherries, peaches, pears and nectarines, and vegetables such as spinach, celery and sweet bell peppers. These raw materials are commonly contaminated even with several pesticides and it rarely happens that they are totally free of such residues. For this reason, consumption of these raw materials from organic sources is particularly desirable, especially for pregnant women, infants and children.
Lower exposure means lower risk of poisoning and complications associated with the accumulation of harmful compounds in the body. The spectrum of health problems that are caused by active substances contained in plant protection products is very broad: they damage the nervous system, weaken the immune system, promote the development of tumours and have mutagenic effects. Organic food, despite complete control of the production process and the prohibition of the use of pesticides, will never be 100 percent free of residues of such substances. They have already been used for decades, and conventional farming, which still dominates the world's agricultural landscape, nevertheless affects much less numerous organic crops. However, given the scale of the harmful impact of pesticides on human health, any attempt to eliminate or reduce exposure to their effects should be undertaken and supported. In this respect, organic farming is a very effective way to reduce the risks from chemicals use in agricultural production, and thus it may indirectly contribute to improving our health condition.

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Part 2

Pesticides and Human Health

11

Pesticides and Human Health

Khaled A. Osman
Alexandria University, Department of Pesticide Chemistry & Toxicology, College of Agriculture, El-Chatby, Alexandria, Egypt

1. Introduction

Chemical pesticides when used properly have been of tremendous benefit to man and his environment especially in developing countries, where they are used to eradicate insect-borne, endemic diseases, to produce adequate food and protect forests, plantation and fibers. Presently, more than 2.5 million tons of pesticides valued over US $30 billion are being used in cultivation alone all over the world. The Rachel Carson's "Silent Spring" (Carson, 1962), awakened the public to the potentially "disastrous" effect of chemical pesticides on human and the environment. "Silent Spring" heralded the start of the U.S. environment movement in which a number of biologists and ecologists all echoed the same basic view that planet earth was a finite entity and that man and the whole global biosphere were doomed unless immediate action was taken to reign-in what was considered a runaway technology. When pesticides misused or used carelessly they have caused considerable harm. The risk or hazards of using chemical pesticides have increased in recent years with the sharp rise in their consumption by agriculture, industry, householders, and government. Pesticides lead to over three million poisoning cases annually and up to 220,000 deaths, primarily in developing countries. Pesticides may present immediate danger to the user if applied improperly or without sufficient knowledge of their toxic effects. Some are highly toxic and may cause serious illness and even death if spilled on the skin, inhaled, or otherwise used carelessly. In addition, potential future hazard to human health and wildlife can be created by residues from some long-lived pesticides that may build up in the food chain and cause widespread contamination of the environment. The risk is defined as a measure of the probability that an adverse effect will occur (Wilkinson, 1986). In the case of a chemical, it is a function of the intrinsic capacity of the material to cause an adverse effect (acute toxicity, neurotoxicity, cancer, etc.) and the dose, which is usually determined by the intensity, frequency, or duration of exposure. Risk assessment is the process by which estimates of risk to humans from exposure to potentially toxic agents are extrapolated from existing data, usually generated from laboratory animals (National Research Council [NRC, 1983]). Strauss (1991) divided the risk assessment into 4 components: hazard identification, exposure assessment, dose-response assessment and risk characterization (Fig. 1).

The aim of the assessment of human exposure to pesticides is the identification of dose-effect relationships in man after both single and/or repeated exposures and also the methods for prevention of such adverse effects due to these chemicals. According to the circumstances, size of dose, and methods of assessment, human exposures might be divided

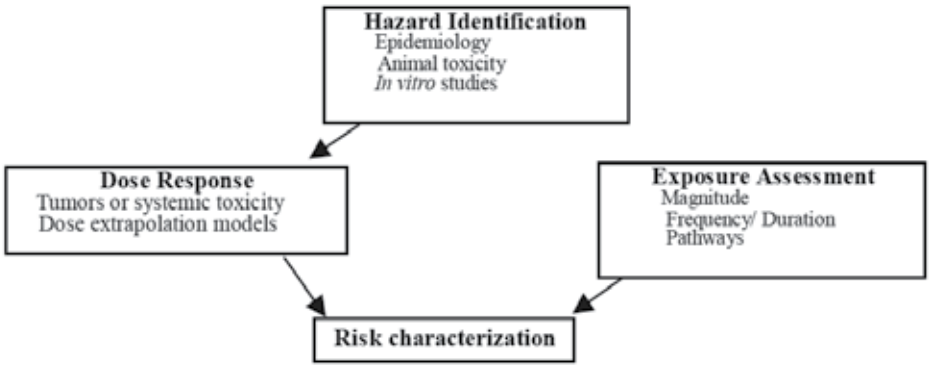

Fig. 1. Risk assessment components, adapted from Strauss, 1991.

as: a) acute/subacute poisoning (intentional, accidental, and occupational), b) long term occupational, and c) environmental exposure (via food, water etc.). The relationship among different exposures is depicted in Fig. 2.

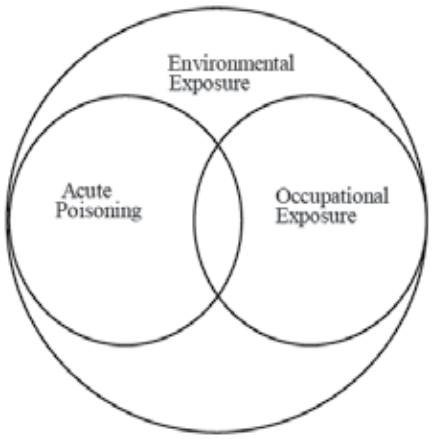

Fig. 2. The relationship among different exposure, adapted from Lotti, 1992.

It is crucial importance to understand the terminologies that describe risk assessment as following:

Risk: The probability of an adverse health effect as a result of exposure to a hazardous substance.

Risk Assessment: The use of available information to evaluate and estimate exposure to a substance and its consequent adverse health effects.

Risk Identification: The qualitative evaluation of the adverse health effects of a substance in animals or in humans.

Exposure Assessment: The evaluation of the types (routes and media), magnitudes, time, and duration of actual or anticipated exposures and of doses, when known, and when appropriate, the number of persons who are likely to be exposed.

Dose-Response Assessment: The process of estimating the relation between the dose of a substance(s) and the incidence of an adverse health effect.

Risk Characterization: The process of estimating the probable incidence of an adverse health effect of humans under various conditions of exposures, including a description of the uncertainties involved.
Risk Management: The regulatory decision that incorporates information of benefits versus risks of exposure to certain situation.
Ecological Risk Assessment: The likelihood of adverse ecological effects caused by any chemical or nonchemical stressor that can exert adverse effects on components such as individuals, population, communities or ecosystem.
No Observed Adverse Effect Level (NOAEL): The highest dose in an appropriate study that is not associated with adverse effect on the test organisms.
No Observed Adverse Effect Concentration (NOAEC): The highest concentration in an exposure media in a study that is not associated with an adverse effect on the test organisms.
Reference Dose (RfD): An estimate of the exposure that can occur continuously, on a day basis, over a prolonged period, with a reasonable expectation that no adverse effect will occur from that exposure (refer to non-cancer hazards associated with the chemical).

$$RfD = NOAL_{\text{critical effect}} / UF_{\text{interspecies}} \times UF_{\text{intraspecies}} \times MF \quad (1)$$

Where, $Uf_{\text{interspecies}}$, $UF_{\text{intraspecies}}$ and MF are safety factor for differences within species, between species and expert-derived modifying factors, respectively.
Benchmark dose (BMD): The lower 95% confidence interval on that dose level (benchmark effect, BME), or the statistical lower bound on a dose corresponding to a specific level of risk (1, 5, or 10% risk level).
Threshold limit value (TLV): The concentration of a hazardous substance to which the majority of industrial works may be repeatedly exposed every day without adverse effects.
Permissible Exposure Limit (PEL): The maximum exposure to a given chemical that an industrial worker is allowed during eight-hour workday and 40 hour workweek.
Acceptable Daily Intake (ADI): An estimate of the daily exposure that is likely to be without deleterious effects even if continued exposure occurs over a lifetime.
Maximum Residue Limit (MRL): The maximum residue level that is expected to occur in a commodity following the application of a pesticide according to good agricultural practice (GAP).
Theoretical maximum daily intake (TMDI): An estimate of dietary intake calculated using the MRL and the average daily per capita consumption of each food commodity for which an MRL has been established. The TMDI is calculated by multiplying the MRL by the average food consumption for each commodity and then summing the product:

$$TMDI = \Sigma F_i \times M_i \quad (2)$$

Because of the lack of vigorous legislation and regulations to control pesticides as well as training programs for personnel to inspect and monitor, use and to initiate training programs for pesticide consumers, the goals of this chapter is to discuss and focus on the risk assessment components and the adverse effects of pesticides.

2. Hazard identification

Hazard identification uses available data on biological end points related to chemical to determine if that chemical is likely to pose a hazard to human health. These data are also used to define the type of potential hazard, that the chemical dose induces: tumor formation, developmental effects, to act as a kidney toxicant, and so forth.

2.1 Epidemic outbreaks due to occupational and non-occupational exposure to insecticides

Toxic outbreaks or collective poisonings have resulted from misuse of almost all types of pesticides: organochlorine insecticides such as DDT, lindane, toxaphene, endrin, aldrin and dieldrin, OPs and carbamate cholinesterase (ChE) inhibitors. Such collective outbreaks may be defined as the effect, in an exposure incident, of a chemical or group of chemicals on a population in which several to many individuals are poisoned. They may occur in the general population from oral or cutaneous exposure or they may be occupational in nature, involving manufacturing workers or formulators, mixers, or applicators in agriculture and public health. While it is clear that such incidents can occur in any country, in recent years they have become less common in developed countries than in developing countries. Recently, public concern over potential adverse health effects has focused on a number of chronic end points carcinogensis, developmental and reproductive effects, immunological effects, and neurotoxicity (Hodgson & Levi, 1996). One of the most severe epidemic poisoning incidents occurred in India when lindane intended for preservation of seed grains was mixed with food grains and was consumed (Khare et al., 1977). The onset of signs of poisoning was sudden with seizures of the mixed type, i.e., grand mal, petit mal, and myoclons, predominating. The highest risk of adverse reproductive effect has been seen among male production workers at the Occidental Chemical plant in Lathrop, California, who had handled the nematicide dibromochloropropane (Babich & Davis, 1981). Also, the continued use of this pesticide in banana plantations in Costa Rica is reported to produce high rates of sterility (Thrupp, 1991). Incident cases of carcinogenic risk associated with pesticide exposure in adults of both sexes in 5 hospitals used by residents of 5 Italian rural areas were reported (Settimi et al.,1990).

2.2 Cytotoxic effects of insecticides

2.2.1 Carcinogenic effects

A number of epidemiological studies have been carried out to evaluate the association between exposure to pesticides and cancer (Settimi et al., 1990, Wolff et al., 1993 and Dewailly et al., 1994). These pesticides can play a role in the cancer process by either nongenotoxic mechanisms such as promotion, peroxisme proliferation, and hormone imbalance (Hodgson & Levi, 1996), or by affecting carcinogenic process in a variety of ways, both by altering the genome and by providing a growth advantage for neoplastic cells (Williams et al., 1992). Therefore, risk assessment model should reflect these differences in cancer mechanism. The US-Environmental Protection Agency ([U.S. EPA], 1986) has generally categorized the carcinogenic potential of a chemical based on the overall weight of evidence. The categories are as follows: Group A (Human Carcinogen), Group B (Probable Human Carcinogen), Group C (Possible Human Carcinogen), Group D (Not Classified as to Human Carcinogenicity), and Group E (Evidence of Non-carcinogenicity for Humans). DDT and its metabolite DDE, *o,p*'-DDT, an isomer of DDT, chlordecone, heptachlor, and other

pesticides which are still persistent in the environment long after being banned are considered carcinogens and involved in the causation of breast cancer as a result of estrogenic activity (xenoestrogenic substances) (McLachan et al., 1993, Stone, 1994). *p,p'*-DDT, methoxychlor and chlordecone affect estrogen production and metabolism and thus function as xenoestrogens (Davis et al., 1993). Epidemiological studies have found that breast fat and serum lipids of women with breast cancer contain significantly elevated levels of some chlorinated hydrocarbons compared with non cancer control. Therefore, tests for estrogenicity could become critical screening tools to assess the potential health consequence of new and existing pesticides. So, cancer risk assessment seeks to measure increases in the frequency of occurrence of an event in a population and to detect the occurrence of low probability events at low doses over long periods of time (Wilkinson, 1986). Also, various compounds of halogenated hydrocarbons inhibit gap junctional intercellular communication (GJIC) in normal human breast epithelial cells (HBEC) when given as a single compound or as mixtures where they can alter the post-translational level, have tumor-promoting potential in human breast tissue and exert some human health effects if they meet all the conditions to inhibit GJIC (Kang et al., 1996).

OPs react with biological molecules by means of phosphorylation of serine hydrolases (acetylcholinesterase, AChE) and of alkylation of macromolecules, DNA (World Health Organization [WHO], 1993b) which are considered to account for the acute cholinergic toxicity and initiation of the carcinogenic process, respectively. When the rate of phosphorylation is substantially higher than the rate of alkylation, *in vivo* genotoxic effects are unlikely to occur because effective doses cannot be achieved due to acute toxicity. Diazinon and dichlorvos meet these criteria, where the rate of phosphorylation of AChE being much faster than that of alkylation. On the other hand, methidathion was categorized as a group C (possible human carcinogen) depending upon evidence of increased incidence of benign and malignant hepatocellular tumors in male Chr-CD-1 mice (Quest et al., 1990).

2.2.2 Reproductive and development effects

Potential non-cancer health outcomes that may be influenced by an agent in the environment, particularly pesticides, include deleterious effects on the nervous, renal, respiratory and reproductive systems of both men and women. The mammalian development toxicity is referred to as the adverse effects initiated or evident during *in utero* development. The development toxicity includes adverse effects on the developing organism that may have resulted from exposure of either parent before conception, of the mother during prenatal development, or postnatally to the time of sexual maturation. Embryo is the most vulnerable to the initiation of major birth defects between 3 weeks and 2 months of gestation, the critical period of organogensis. The exposure to toxic chemicals during the first 2 weeks leads to fetal death, while exposure after organogensis is more likely to cause growth retardation and functional deficits (Hodgson & Levi, 1996). Also, the pesticide used by appliers and exposure of the general population of the crop-growing region of Western Minnesota are associated with increased birth anomalies (Garry et al., 1996). The primary DDT metabolite, *p,p'*-DDE interferes with the action of male sex hormones, or androgens affecting mammalian sex differentiation (Kelce et al., 1995). It is now recognized that numerous endocrine disrupting pesticides from different chemical groups have been released into the environment in large quantity since world war II and exert their action as agonistic and antagonistic receptor binding, and affect hormone synthesis, storage, release, transport, and clearance (Kavlock et al., 1996). As shown in Fig. 3,

the theoretical basis for environmental chemicals to exert hormone-like effects is relatively straight forward (McLachlan, 1993). In the simplest model, chemicals can mimic a hormone by binding to its receptor and eliciting a spectrum of biological effects. Conversely, a foreign chemical does not elicit these effects could bind a hormone receptor as inactive compound and thus block the response to the natural hormone. In both cases, the result would be an alteration in the function of the hormone system.

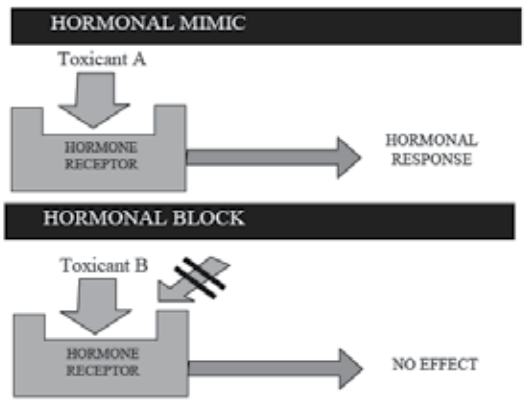

Fig. 3. Exogenous chemicals may act at hormone action site, adapted from McLachlan, 1993.

2.2.3 Neurobehavioral effects

Because of the basic similarities between mammalian and insect nervous system, insecticides (organochlorines, OPs and carbamates) are designed to attack the insect nervous system and capable of producing acute and chronic neurotoxic effects in mammals (Tanner & Longston, 1990). Both acute and chronic alterations in sensory, motor, autonomic, cognitive, and behavioral functions have been observed in people exposed occupationally to relatively high levels of insecticides. These neurobehavioral effects and chemical-induced changes in behavior may be a relatively sensitive indicator of nervous dysfunction (National Academy of Science [NAS], 1975) and can be used in neurotoxicology for neurotoxicity risk assessment (Evangelista de Duffard & Duffard, 1996). Organochlorine insecticides have effects on motor, sensory, or cognitive function that are detectable using functional indicators of neurotoxicity (Evangelista de Duffard & Duffard, 1996) to assess neurotoxicity risk.

A number of OPs cause neurotoxicity, characterized as central- peripheral distal axonopathy (Lotti, 1992 and Osman et al., 1996 and 2001). This syndrome commonly known as organophosphate induced delayed polyneuropathy (OPIDP), is totally independent of inhibition of AChE and is delayed as symptoms appear after 2-3 weeks. The mechanism of initiation of OPIDP involves the phosphorylation of a protein in the nervous system called neuropathy target esterase (NTE) and the aging of the phosphoryl enzyme complex (Johnson, 1982). The inhibition of NTE activity in human lymphocytes has been shown to predict the onset of OPIDP in a patient poisoned with chlorpyrifos (Lotti et al., 1986). Preliminary studies in Central America suggested that mild or subclinical cases of OPIDP after severe cholinergic poisoning with methamidophos may be much more common than previously suspected (WHO, 1993a). In humans, OPIDP also has been reported to occur after poisoning with merphos, mipafox, leptophos,

trichlorphon and trichlornate (Lotti et al., 1984). One of the main tasks of toxicology and risk assessment is to determine, through experiments with animals and documentation of adverse effects following accidental exposure of human, safe limits of exposure to toxic chemicals. Since new pesticides are being released into the environment, it is essential to use rapid and sensitive toxicological screening procedures for these and already existing pesticides. Once behavioral neurotoxic effects have been identified, it is important to improve the understanding of the mechanism of neurotoxicity at the neurochemical, neurophysiological, cellular, and molecular levels of analysis (Evangelista de Duffard & Duffard, 1996). Neurotoxicity risk assessment will be improved by a more complete understanding of the interrelationships between the various levels of nervous system. Neurobehavioral toxicology contributes directly to this issue by systemically assessing the threshold and magnitude of exposure beyond which normal processes are significantly affected.

2.2.4 Immunotoxic effects

The exposure of humans to some insecticides alters immune phenotypes or function and potential disease susceptibility (WHO, 1990). Aplastic anemia has been described as idiosyncratic immunologic response to exposure to organochlorine pesticides (Hayes & Laws, 1991). Also, allergic responses, especially allergic dermatitis, can be seen with many classes of pesticides (Hogan, 1990). Individuals consuming ground water contaminated with low levels of aldicarb in Wisconsin were reported to have abnormalities in T-cell subset in women with otherwise intact immune systems and they are potentially at risk for immunologic damage (Fiore et al., 1986). It has been argued that decrease immune surveillance resulting from inhibition of monocyte esterases by chronic OPs exposure may result in the development of lymphoma (Newcombe, 1992) and the exposure to chlorpyrifos during a development period is known to produce deficits in immune competence (Navarro et al., 2001).

3. Dose-response assessment

3.1 The basic elements of dose- response assessment

In the dose-response assessment, data from human and animal studies are used to estimate the amount of chemical that is expected to produce a given effect in humans. In this step it is generally necessary to apply mathematical models to calculate a quantitive risk estimate usable for low-dose exposure. Dose-response assessment can be viewed as three critical steps identification of the effect (and related exposure level) of most concern, a characterization of the uncertainty present in the database, and an estimate of the exposure level presumed to be free of risk to the human conceptus (Kavlock & Setzer, 1996). In the first step, data from exposed experimental species, as well as any epidemiological information, is examined for the highest dose level that is without a significant adverse effect (the no observed adverse effect level, NOAL). In the second step, the adequacy, relevance, and uncertainties in extrapolating the NOAEL from the experimental species to the target species are estimated. In the final step, the critical NOAEL (The lowest NOAEL in the database on a particular chemical) is divided by the product of uncertainty factors (UF), as well as any expert-derived modifying factors (MF) to obtain the reference dose (RfD) or reference concentration (RfC) for an inhaled chemical. The current methods for estimating human health risks from exposure to threshold-acting toxicant in water or food, such as those established by U.S. EPA, (1980). These methods generally estimate a single, constant

daily intake rate that is low enough to be considered safe or acceptable. This intake rate is termed the acceptable daily intake (ADI, expressed in milligrams per kilogram). Another avenue to improve the dose-response component of the risk assessment process is to better use data generated from standardized testing procedures, independent of knowledge of toxicokinetic or toxicodynamic factors that may be used to adjust the magnitude of the uncertainty factors. This can be done by using benchmark dose (BMD) approach (Crump, 1984). In the BMD approach, a particular effect level is chosen and the dose inducing that response is calculated using a statistical model (Fig. 4). The BMD is then defined as the lower of 95% confidence interval on that dose level (benchmark effect, BME).

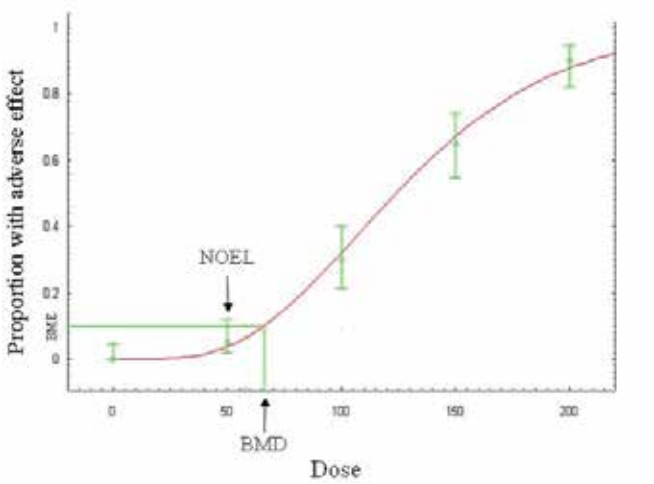

Fig. 4. Benchmark dose calculation, adapted from Crump, 1984.

The dose-response curve (Fig. 5) can take an anomalous form because the effective dose is not directly proportional to administered dose (O'Flaherty, 1986). This is particularly true at the high or maximum tolerated doses that are characteristic of toxicity and carcinogenicity studies. Also, among the many reasons why administered and effective dose may not be directly proportional to each other are capacity limited systemic or first pass elimination, rate limiting availability of cofactors, shifts in tissue distribution caused by saturation of binding sites, especially in plasma and alterations in blood flow rates to critical tissues.

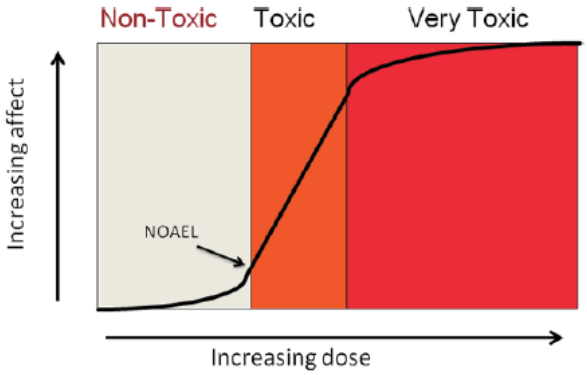

Fig. 5. The dose-response curve, adapted from O'Flaherty, 1986.

3.2 Toxicity test in animals: extrapolating to human risks

The process of assessing risks based on animal experiments involves extrapolations from high doses to low doses, between dose route and exposure scenarios, and between various animal species (Clewell & Anderson, 1985). Risk assessment oriented-research paradigm in toxicology centers on the relationships linking exposure tissue dose, initial tissue interaction, and toxic response, together with the strategies for extrapolating from observed responses in animals to expected response incidence in humans exposed at very much lower concentration as shown in Fig. 6.

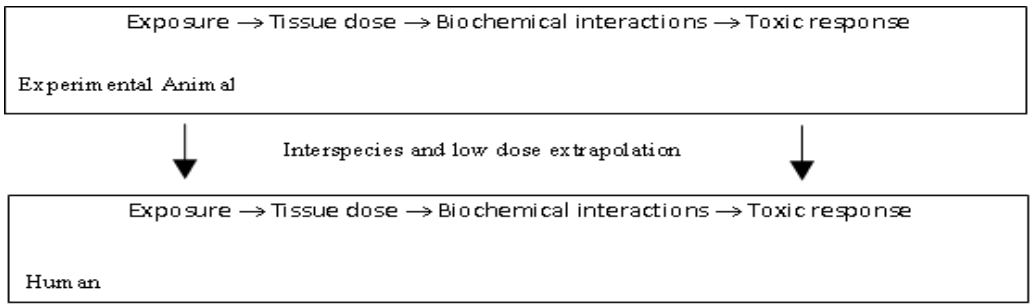

Fig. 6. The individual processes involved in the expression of toxic responses in a risk assessment context. Adapted from Andersen et al., 1995.

The dose-effect and dose-response relationship in occupational neurotoxicology are rarely studied by means of biochemical methods. Some biochemical methods are however available to extrapolate from animal to man and used in monitoring human exposures and can be framed in three categories: exploring the delivery of chemicals to the site of action, the modifications of the molecular target induced by chemicals, and the biochemical consequence of these modifications. The possibility of measuring adducts in hemoglobin will enable an estimation of the dose of electrophiles closer to the target and to extrapolate dose more precisely across species (Lotti et al., 1989). Once the number of adducts and their persistence in man, after exposure to a given chemical are known, it will be possible to compare them with data from animals dosed with a known amount of electrophilic chemicals and therefore to extrapolate toxicity more precisely. There are number of OPs that cause OPIDP when the threshold of inhibition of NTE reach 70-80% (Johnson, 1982). For an OP the use of the therapeutic index (TI) is useful to predict the safety. The TI is defined as the ratio of the median toxic dose (TD_{50}) over the median effective dose (ED_{50}). For maximal safety, the TD_{50} must occur at a dose far as possible above the LD_{50}. That is, the dose-response curve for the toxic effect should be as far to the right of the dose-response curve for effect dose as possible (Klassen, 1986).

4. Exposure assessment

The exposure assessment seeks to determine the extent to which a population is exposed to the material. Exposure assessment uses available data relevant to population exposure, such as emission data, measurement of the material in the environmental media, and biomarker information. Fate and transport of the material in the environment, routes of exposure and

pharmacokinetics of the chemical in the body may be considered in the exposure assessment.

4.1 Dietary exposures to insecticides

The consumption of foods and water containing environmental contaminants is a potentially significant source of human exposure to numerous pesticides. So, it is important to understand the magnitude, sources, and variability of dietary exposures to environmental contaminants experienced by members of the population, the precision of dietary exposure estimates possible from existing data, and the prospect of using dietary exposures in epidemiologic studies designed to characterize the human health effects of specific insecticides or classes of insecticides (Berry, 1992). Total exposure assessment from dietary and other sources is used in evaluating risk and for comparison with recommended allowable daily intake. Food and water-borne residues are the most important sources of exposure to the general population. In many countries including Argentina, Panama, Brazil, Costa Rica, Guatemala, El-Salvador, Mexico and India, nursing infants potentially ingested organohalogens at a ratio many times that of ADI as estimated by Food and Agriculture Organization/World Health Organization [FAO/WHO], 1988). A high proportion of DDE in Indian buffalo's milk that might reflect the presence of ages residues of DDT, whereas that of TDE would indicate contamination of more recent origin (Kapoor & Kalra, 1993). Therefore, animals yielding milk contaminated with high levels of DDE will require fairly long holding period than those are able to yield milk of acceptable quality after the elimination of the potent source of contamination.

Data from toxicological investigations are a substantial part of the assessment of a pesticide. The toxicological studies should identify possible adverse health effects of the compound and establish the dose at which such effects are likely to occur, and particularly identify a dose level where adverse effects are absent. The majority of the toxicological data used in the risk assessment are generated in studies performed according to internationally accepted standards (guidelines), which for each type of study state the minimum requirements for an acceptable performance. In addition, regulatory agencies also require that, to be used in decision making, the investigations are performed according to Good Laboratory Practice (GLP) involving quality control and quality assurance. The different types of toxicological investigations required for the evaluation of a pesticide involve studies on acute effects, including effects on skin and mucous membranes, and more important long-term studies on chronic effects, including carcinogenicity following repeated daily exposure. Studies of effects on reproduction over a minimum of two generations are also required, as well as special studies on teratogenicity and embryo/fetotoxicity, and on effects on the genetic materials. In addition, studies on absorption, biotransformation, distribution, and excretion are also required. In these studies, effects on macromolecules, such as DNA, enzymes, and other biochemical parameters are often included. The toxicological data that are used in risk assessment are usually generated from animal experiments and *in vitro* investigations. When human data are available, for example from occupational or accidental exposure, these of course are considered highly valuable. From the toxicological data a NOEL or NOAEL is identified as the highest daily dose level that does not produce observable effects or adverse effects in the most sensitive animal species. In establishing the ADI for humans the NOEL is reduced by a safety factor, which take into account the uncertainties of the results of the investigations, the extrapolation from animals to humans, and the variations in sensitivity and life-style within the human population. When the toxicological background material is

considered sufficient, a safety factor of 100 is normally used (a factor of 10 for differences between species and 10 for differences within species (Fig. 7). Additional safety factors are occasionally used, for example, when the biological effect is considered to particularly serious or when uncertainty exists in the evaluation of the consequences of a finding. Safety factors of 1000 or even higher have occasionally been used, when a clear NOAEL cannot be established on the basis of available data, lowest observed adverse effect level (LOAEL) is sometimes identified and used to establish an ADI. On the other hand, MRL is based on field spraying trials and subsequent determinations of residues. These investigations include different methods of application, including those using the highest dosages and used in such a way that the lowest possible amount of residue is produced. The MRL is never established at a level higher than needed even if the established ADI value would allow a higher residue content. In practice, this means that the intake of most pesticides by the general population is well below the ADI. In the evaluation of the health risk, the possible total intake of the pesticide is calculated as if the concentration in all the food in which it can be present is at the MRL for each single food item. This mean that exceeding the MRL in one single sample does not automatically result in exceeding the ADI, as many samples normally are without detectable residues.

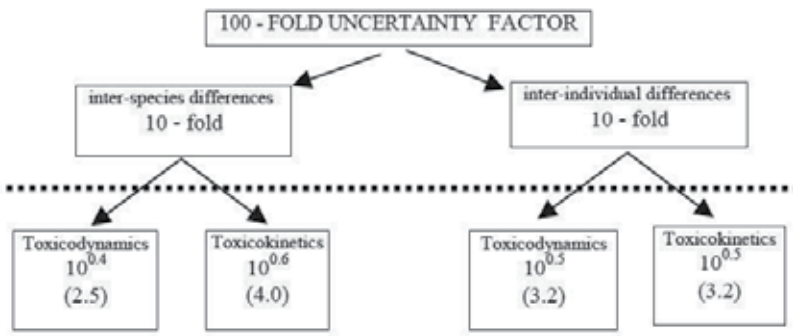

Fig. 7. Subdivision of the 100 -fold uncertainty factor, adapted from Walton et al., 2001.

4.2 Biomarkers in risk assessment

The field of biomarkers has been the object of increased interest in the past few years. The term biomarker is used to mean biological biochemical/molecular markers, which can be measured by chemical, biochemical or molecular biological techniques (Costa, 1996). Biomarkers are usually divided in three categories : biomarkers of exposure, of effect and of susceptibility (NRC, 1987). Additional subdivisions of and overlaps between different types of biomarkers should also be considered. For example, certain biomarkers of exposure, e.g. DNA adduct, may be also considered as biomarker of effect. Fig. (8) shows the fate and reactions of a xenobiotic in the human body and the types of tests available to investigate human exposure (Aldridge, 1986). Once a chemical is absorbed and distributed through the plasma pool, it attaches itself to the molecular target either directly or after metabolic activation, then a cascade of biochemical and physiological changes occurs, which triggers the morphological, clinical expression of toxicity. Evaluations of human exposure might be performed at any stage of this process but their significant is obviously different.

The best available tools are in the area of biomarkers of exposure is the of measurement of neurotoxic chemicals and their metabolites in biological fluid which provide useful and

reliable indicators of exposure (Henderson et al., 1987, Costa, 1996). An ideal biomarker of exposure is chemical specific, detectable in trace quantities, inexpensive and quantitatively relatable to prior exposures. Also, the binding of a toxicant to hemoglobin is considered a good biomarkers to measure cumulative internal dose due to repeated exposures, because red blood cells are long-lived (approximately 4 months in humans), while adducts to albumin reflect more recent exposure because albumin has a shorter lifetime in blood (20-25 days) (Henderson et al., 1987). Therefore hemoglobin adducts could be used as a biomonitoring technique for evaluating residues in food for specific pesticides which form arylamine-hemoglobin adducts (Sabbioni & Neuman, 1990).

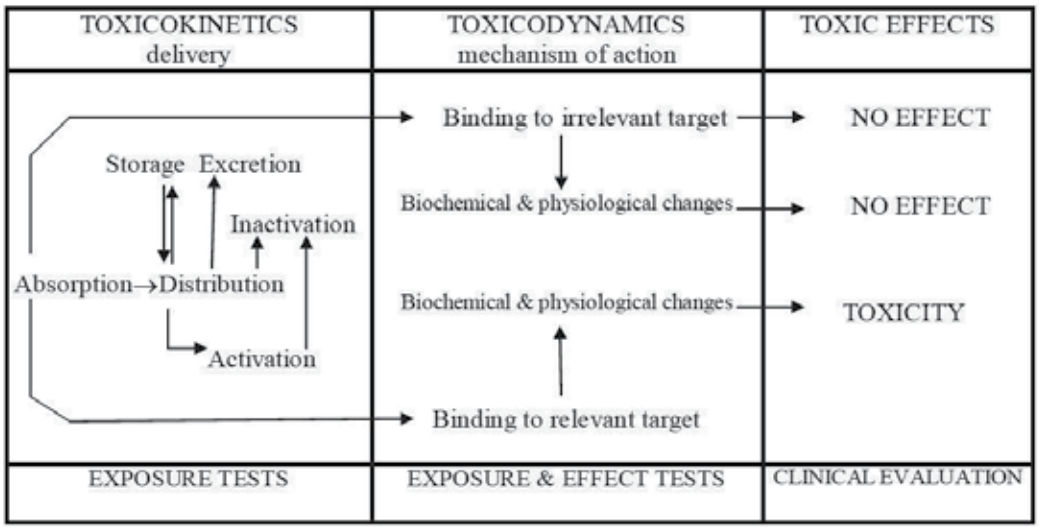

Fig. 8. Fate and reaction of a xenobiotic in human body, adapted from Aldridge, 1986.

Biomarkers of effect should reflect early biochemical modifications that precede structural or functional damage. Thus, knowledge of the mechanism(s) that led to ultimate toxicity is necessary to development specific and useful biomarkers. Such markers should identify early and reversible biochemical events that may also be predictive of later responses (Silbergeld, 1993). The oldest and probably still the best example of the application of such strategy to neurotoxic compounds is represented by the measurement of red blood cell AChE following exposure to OPs. Erythrocyte AChE, in particular, was found to be better correlated with brain or diaphragm activity than plasma ChE (Padilla et al., 1994). OPs such leptophos, EPN, cyanofenophos, trichloronate and salithion proved to cause irreversible ataxia in chicken, mice and sheep and their AChE inhibition stands for their acute toxicity, while NTE inhibition is responsible for their paralytic ataxia and can be used as a standard screening method for delayed neuropathy (El-Sebae et al., 1981). The precise measurement of ChE (erythrocyte or plasma) can be useful as a measurement for low level exposure to OPs in epidemiological research (Richter et al., 1986, Dyer et al., 2001). Recent studies conducted have shown that lower group mean plasma and erythrocyte ChE level in populations living adjacent to cotton fields which are sprayed regularly with OPs (Richter et al., 1986). Also, urinary alkylphosphates are sensitive indicators of OPs exposure, and have been shown to correlate with symptoms.

A comparative inhibition of rodent and human erythrocyte AChE by two anticholinesterase carbamates, carbaryl and carbofuran was evaluated by using Michaelis constant (K_m), maximum velocity (V_{max}), concentration of pesticide required to inhibit 50% of the enzyme activity (IC_{50}) and bimolecular rate constant (K_i) (Rao et al., 1994). Although there are inherent differences in kinetics of substrate hydrolysis between rodent and human erythrocyte AChE, the kinetics of inhibition *in vitro* by carbofuran and carbaryl as estimated by the comparative of ki are quite similar between species and may be useful in human risk assessment. Also, optical sensors can be used for detection anticholinesterases by immobilizing fluorescein isothiocyanate (FIC)-tagged ell organ AChE on quartz fibers and monitoring enzyme activity (Rogers et al., 1991). These biosensors detect concentrations of the carbamate insecticides such bendiocarb and methomyl and the OPs echothiophate and paraoxon in the nanomolar to micromolar range. On the other hand, malathion, parathion, and dicrotophos were not detected even at millimolar concentrations, but, longer exposure or prior modification of these compounds (i.e. to malaoxon, paraoxon) may increase the biosensors detection limits. These AChE biosensors are fast, sensitive, reusable, easy to operate and portable, so it show potential adaptability to field use. So, the measurement of blood AChE activity remains an excellent biomarker for exposure and effect of OPs exposure under both acute and chronic conditions.

The measurement of lymphocyte NTE has been suggested as a potential biomarker to monitor for OPIDP (El-Sebae et al., 1981, Bertoncin et al., 1985, Lotti et al., 1986 and Sigolaeva et al.,1999). The sensitivity of human lymphocyte NTE to several OP inhibitors is similar to that of the nervous system enzyme. Best example is its application in humans in an attempted suicide with chlorpyrifos in which, based on 60% inhibition of lymphocyte NTE, it was correctly predicted that a neuropathy would develop well after recovery from acute cholinergic poisoning had occurred (Lotti et al., 1986). Also, in order to assess the risk of OPIDP from exposure, it is useful to determine the relative potency of the oxon analogue for inhibition of NTE versus AChE (Lotti & Johnson, 1978) as well as using of the k_i values in preference to fixed-time I_{50} when making assessment of the neuropathic risk of OPs (Richardson et al., 1993). Also, pesticides may induce oxidative stress leading to generation of reactive oxygen species (ROS) and/or free radicals which are well known to be deleterious to many biological molecules and to produce a broad range of deleterious effects (Osman, 1999, Osman et al., 2000, Salama et al., 2001). The measurement of alteration in antioxidants or oxygen free radical scavenging enzymes can be used as biomarkers for exposure and effects.

4.3 Toxicokinetics of insecticides related to risk assessment

Tests exploring the toxicokinetics include measurements of the chemical or its metabolites in body fluids. Virtually all pesticide exposure can be assessed in this way, only depending on availability of analytical procedures. Furthermore, the understanding of the mechanism of action and the availability of a biomarker effect allows studies on quantitive relationships between the concentration of the compound or its metabolites in the body fluids and their effect on the target. Most OPs are activated to their corresponding oxygen analog by an oxidative desulfuration reaction, which is catalyzed by cytochrome P450 (Vasilic et al., 1987). Upon phosphorylation of AChE, a portion of the molecule, the leaving group, is released and excreted. Both the parent compound and the oxon can undergo a series of detoxication reactions that are mediated by various A-esterases (paraoxonase, carboxyesterase), by P450, and by glutathione transeferases. The leaving group, *p*-

nitrophenol in the case of parathion, which is also generated by hydrolytic cleavage, and alkylphosphates are excreted in the urine and can be quantified as an index of OPs exposure (Richter et al., 1986). For occupational exposures, pesticides are unusual in that dermal residues are often the most important source of systemic absorption. In general, respiratory exposure in the occupational setting is much less than dermal exposure, with the exception of exposure to aerosols, powders or dust, concentrated vapors, work in enclosed spaces, or pesticides which are gaseous at room temperature or on contact with water (especially the fumigants). Dermal absorption of drift may contribute to community exposures, and for evaluation of community exposure, monitoring of respirable residues is important in the research setting (WHO, 1993a). In agricultural communities little studies have been done to evaluate routes of exposure to communities exposed through skin and by aerosol inhalation to drift from adjacent fields, although some studies suggest significant systemic absorption of pesticides, resulting in ChE depression, among persons in such communities under selected conditions (Richter et al., 1986). The provision of dermal absorption data is required for the registration of agrochemicals, particularly in USA (Scott et al., 1992). Sharp (1987), and Maddy (1990) reported that exposure of users of pesticides containing active ingredients which have the potential of causing adverse effects, especially chronic effects, has to be accurately measured in order to make meaningful risk assessment and risks mitigation determinations. Analysis of residues on cloth pads that had been worn on various parts of the body may provide an overestimate exposure than in the case when such studies are done on humans or other primates.

Nutley & Cocker (1993) analyzed over 400 urine samples obtained from 140 workers with potential occupational exposure to OPs during various agricultural activities, sheep dipping or pesticide formulation. The measurement of dialkyl phosphate metabolites in urine provides a sensitive biological monitoring method suitable for use in the assessment of occupational exposure to many OPs. Metabolites were detected in people with exposure to OPs at levels below those that cause a decrease in ChE activity. OPs exposure among children living in two Seattle metropolitan communities were assessed by measuring urinary metabolites, and identified possible exposure risk factors through a potential interview. Concentrations of dialkyl phosphate compounds, the common metabolites of OPs, were significantly higher in children whose parents reported to pesticide use in the garden (Lu et al., 2001). Therefore, OP pesticides use should be avoided in areas where children are likely to play and the measurement of OP metabolites in postpartum meconium and/or urinary dialkyl phosphate metabolites is useful for monitoring exposure to OPs and is capable of detecting low levels of exposure not detected by depression of ChE activity. Moreover, the presence of DDE levels in organisms is a good biological indicator of chronic exposure to DDT (Woodruff et al., 1994). Also, V_{max} and K_m values for animal and human would be used to develop a physiological based pharmacokinetic (PB-PK)/physiologically based pharmacodynamic (PB-PD) model to predict the fate and toxicity of pesticides in animals and man (Knaak et al., 1993).

Although it is intuitive that pesticides which bioaccumulate and biomagnify are of special concern to those species that consume them, the relative contribution of these processes to toxicity is dependent on trophic level in the food web, life stage, physiological conditions favoring lipid mobilization (e.g. pregnancy, lactation), and reproductive strategy. DDT is one of best known organochlorine insecticides which accumulated in the food chain and known to be transferred from mother to offspring via milk (Wooley & Talens, 1971). The levels determined in human milk are more than 10 times higher than those in cow's milk

(Jensen, 1983). Also Due to the lipophilic nature of DDT and its principle metabolite, DDE, these compounds have been found in diverse human samples of serum, adipose tissue, and breast milk (Woodruff et al., 1994). The half-life of DDT in human adipose tissue is approximately 7.5 years, while the amount of serum DDT varied according to the levels of lipid circulating in the blood. The ratio between the levels of DDT in adipose tissue and blood was 300 to 1.

5. Risk characterization

Risk characterization is the last step of the risk assessment process. This step evaluates assessments of human health and ecological effects, identifies human sub-populations or ecological species potentially at risk, and delineates areas of uncertainty, limitations, and assumptions made in the risk assessment.

5.1 Effect of insecticides on non-target organisms

Pesticides occupy a rather unique position among the many chemicals that man encounters daily, as they are deliberately added to the environment. Ideally their injurious action would be highly specific for undesirable target pests. However, most of pesticides are not highly selective to many nontarget species, including humans, and other desirable forms of life that coinhabit the environment. The ecological risk assessment evaluates the likelihood of adverse ecological effects caused by any chemical, physical or biological entity (including pesticides) that induce adverse effects on the components (individuals, population, communities or ecosystem) (Norton et al., 1992, EPA, 1995). Pesticides may affect the non-target organisms by direct contact or through translocation from the sites of application through the various media. The extent to which translocation within the environment occurs will depend to a large degree on the physicochemical properties of the pesticides (Murphy, 1986).

Factors that affecting the risk assessment of pesticides include the application rate and time, sorption processes in the soil, uptake by crops, volatilization, biotic and abiotic transformation, mineralization, factors influencing the biodegradation of active ingredient in soils, mobility and leaching, and drinking water quality aspects (Pawlizki, 1991). The "Framework for Ecological Risk Assessment" which is developed for risk assessment of ecological effects is illustrated in Fig. 9 (U.S. EPA, 1995). This framework is conceptually similar to the approach used for human health risk assessment, but it is distinctive in its emphasis in two areas. First, ecological risk assessment can consider effects beyond those on individuals of a single species and may examine a population community, or ecosystem. Second, there is no single set of ecological values to be protected that can be generally applied.

The nature of environment exposures to chemicals and the population variability in response make it difficult to determine population risk from traditional epidemiological studies (Spear, 1991). This has given rise to attempt to predict risk from environmental transformation and transport, exposure mechanisms, and biological response probabilities. For readily degradable chemicals emitted at intervals, the ecological risk can be related to the time taken for the chemical to fall to a level causing no effect on most individuals, e.g. 95% of the species (Straalen et al., 1992). The deleterious effects of endocrine-disrupting chemicals in the environment on the reproductive success of wildlife population have been documented (Colborn et al., 1993). These deleterious health effects have been observed in the presence of numerous man-made chemicals. DDT applied in a mosquito control

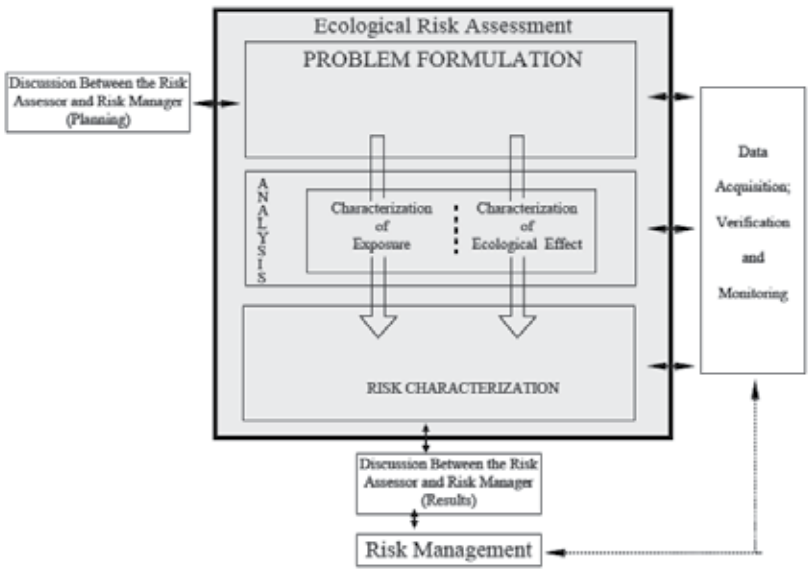

Fig. 9. Framework for ecological risk assessment, adapted from U.S. EPA, 1995.

program in a tropical or subtropical area may ultimately have adverse effects on species in Arctic regions (Murphy, 1986). Such small quantities that may be present in mud and surface waters are taken up by plankton and other food sources for phytophagous fish. The phytophagous fish are eaten by carnivorous fish. These fish may migrate and be ingested by birds in Arctic climates, such as falcons and eagles, in sufficient quantities to contribute doses of the insecticide or its metabolites that can affect avian reproduction. The reproductive parameters such as thinned eggshells and lowered the thickness index of American kestrels (*Faclo sparverius*) after exposure to kelthane at dietary concentration have been affected (Clark et al., 1990). Also, insecticides may affect bird populations by reducing the insect prey base available to the birds, while herbicides affect various bird populations through a variety of pathways, including a direct reduction of the food base of granivorous species, reducing invertebrate abundance by removing the plants that invertebrates depend on as food or habitat, and reducing nesting cover (O'Conner, 1992).

5.2 Endogenous and exogenous factors affecting human risk

For human health, a number of factors contribute to a wide range of risks, including endogenous factors such as genetic predisposition, age (embryo, fetus and children) and gender and exogenous factors which include diet, disease conditions, climate and past exposures. Endocrine disruption of the developing brain can permanently alter the behavior, whereas similar exposures of fully differentiated brain could be without effect (Kavlock et al., 1996). There are specific critical periods of sensitivity to endocrine disruption which vary for different organs and species. The unique changes in physiology during development may increase sensitivity to endocrine-disrupting agents. Also, adult males and females are affected by endocrine disruptors, and the physiologic states in the adult (e.g., early pregnancy) may enhance susceptibility. Infants and children are growing and developing. Their metabolic rates are more rapid than those of adults. Children generally receive greater dietary exposure in milligrams per kilogram of body weight (mg/kg bw) of

pesticides than adult, due to higher food intake rates (Whyatt and Nicholson, 1991). Also, there are differences in their ability to activate, detoxify, and excrete xenobiotic compounds. Both irritant and allergic inflammatory reactions are weaker in older patients (Harvell & Maibach, 1994). It is not clear why this occurs, but it could be due to an age-related decrease in percutaneous absorption or an age-related difference in the inflammatory cascade. Newborns, on the other hand, especially preterm neonates, have immature epidermal barriers, which can lead to potential problems with percutaneous absorption of toxins (Kravchenko, I., Maibach, 2003). All these differences can affect the toxicity of pesticide to infants and children, and for these reason the toxicity of pesticides is frequently differed in children and adults. The quantitive differences between children and adults are usually less than a factor of approximately 10-fold (NAS, 1993).

Many OPs appear to be better inhibitors of ChE, suggesting that this enzyme may be a more sensitive indicator of exposure. However, this is not true for all OPs. Furthermore, plasma ChE activity displays a higher variability because it can be affected by other exogenus agents (e.g., drugs) or physiological and pathological conditions (e.g., pregnancy or liver damage) (Chatonnet and Lockridge, 1989). In addition, genetic variants of human serum ChE exist (Lockridge, 1990). Individuals with atypical ChE, which occurs in homozygous form in 1 out of 3500 Caucasian and consists of a single amino acid substitution in position 70 (glycine instead of aspartic acid), have an abnormal response to muscle relaxant succinylcholine. It is known that genetic difference in detoxification enzymes and non-specific binding account for some of the inter-individual variation in susceptibility to anti-ChEs (Mutch et al., 1992). Esterases which hydrolyze OPs are called A-esterases and might be involved in their detoxification. Subjects with low serum A-esterase activity are more susceptible to the toxic effects of OPs (Brealy et al., 1980).

Specific factors that may contribute to excess cancer incidence among farmers include prolonged occupational exposure to sunlight, diet, contaminated drinking water, and occupational exposure to a variety of potential hazardous chemicals and biological agents (Blair et al., 1992). The exposure to these nonchemical factors could also adversely affect the nervous system resulting in effects similar to those produced by endocrine disruptors (Kavlock et al., 1996). Also, seasonal and regional variability of food consumption rates, possibly due to availability and prices and residue levels may be important contributors to interindividual variation of dietary exposures (Maclntosh et al., 1996). An increase in DDT accumulation was found in tropical areas and/or regions with greater agriculture activity (Lopez-Carrillo et al., 1996).

5.3 Exposure to mixtures

Humans are more likely to be exposed to chemical mixtures than to a single chemical under most environmental and occupational conditions. Many chemicals of the mixtures modify stratum corneum lipid fluidity as precutaneous absorption enhancers, and the skin barrier absorption rate which increase or inhibit the formation of more readily absorbable toxic residues and metabolites. When conducting risk assessments of chemical mixtures, the assessor must also consider factors that influence toxicity (i.e., chemical interactions). A toxicological interaction is a circumstance in which exposure to two or more chemicals results in qualitatively or quantitatively altered biological response relative to that predicted from the actions of a single chemical (NRC, 1980). The study of combined action or interaction of chemicals involves the challenges of how to characterize antagonistic,

additive, or synergistic action. It is therefore of crucial importance to understand the terminology that describe combined interaction of agents in terms of the mechanisms of action (Groyen et al., 1999).

Toxicokinetics and toxicodynamics can be altered in particular circumstances of exposure to mixtures (Lotti, 1987). For instance, as it often occurs in practical conditions of exposure to impurities and mixtures of pesticides, the toxicokinetics might be deeply influenced and the net toxicological effect remarkably changed. Examples include inhibition of the detoxification mechanisms as in the case of impurities of OPs or as in the case of mixtures of pyrethroids and OPs, and also accelerated biotransformation as in the case of liver enzyme induced by certain chlorinated pesticides. On the other hand, toxicodynamics might also be influenced by the competition for the target as in the case of some carbamates which prevent the delayed neuropathy caused by some OPs (Johnson, 1982). The acceleration of parathion metabolism in the gastrointestinal (GI) tract of lindane-pretreated rats could have been due to either a prolonged residence time of parathion or increase GI nitroreductase activity or both (Chadwick et al., 1990). The increased in nitroreductase activity may account for lindane-parathion interaction and could influence the metabolism, toxicity and risk assessment of many other environmental nitrocompounds that become toxic, mutagenic or carcinogenic upon reduction of their nitro-groups. Also, the chemical components in topically exposed mixtures may have significant effects on the prepenetration fate, penetration/distribution pattern, metabolism and precutaneous profile of parathion/drug in the mixture (Qiao et al., 1996). Therefore, multiple level interactive effects on parathion absorption must be considered into any effort to identify critical mechanisms that affect assessment of topically exposed mixtures.

Specific chemicals which individually did not inhibit GJIC at a given concentration, could, when mixed with other chemicals, which also did not inhibit GJIC at a certain concentration, may interact to inhibit GJIC (Kang et al., 1996). Also, because humans are exposed to many of this kind of estrogenlike chemicals in their food supply and because several combinations of these chemicals in mixtures can affect GJIC when no chemical single could. It is assumed that these chemicals might be tumor promoters of human breast cancers and thus could be entertained as possible contributors to the multistage nature of human carcinogenesis. Therefore, consideration of the adverse effects caused by exposure to mixtures must an integral part of protecting human health.

6. Conclusion

Risk assessment is a multidisciplinary task related to toxicology, analytical chemistry, biochemistry, molecular biology, health disciplines, politics, etc. The four key aspects of risk assessment are; hazard identification, dose response, exposure assessment and risk characterization. They are all driven by dynamics based on intake, absorption and effect.

Important advances have recently occurred in analytical methodologies that allow the determination of biomarkers of internal dose as part of national monitoring programmes. Once the risk assessment process is completed for a given pesticide, regulators begin the risk management step; to decide how much exposure will be allowed and, if necessary, establish risk reduction options to ensure that with reasonable certainty, a pesticide will not be harmful to humans.These risk assessments support two major types of regulatory decisions (Dearfield & Moore, 2005), the approval and registration of pesticides and the setting of standards for acceptable exposure levels in air, water and food. Using the conclusions of the

risk assessment, regulators can approve a pesticide as proposed, or adopt protective measures to limit its exposure (occupational or non-occupational). Decisions taken by regulators have important economical consequences for the industry that manufactures pesticides, the farmers, the workers and the consumers. Such consequences, justify the need for regulators to have well supported data related to the four steps of the risk assessment process. Risk assessment requires a robust analytical basis; studies addressed either to hazard identification or exposure estimation. Both have to be conducted under good laboratory practice (GLP) certification (OCDE, 1997).

There is a principle difference between pesticides and other environmental chemicals. Pesticides are approved by the authorities for use in the production of food crops. This means that in a number of cases residues are accepted in food. However, the pesticide residues in foods are very closely regulated. This, of course, does not imply that pesticide residues are accepted in other media such as drinking water and air. Approval by regulatory agencies of a pesticide demands that the health risk associated with exposure to the compound be evaluated by the regulating authorities. The joint Meeting of the FAO panel of Experts on Pesticide Residues in food and the Environment and the WHO Expert Group on Pesticide Residues (JMPR) normally convene annually, and the FAO Panel of Expert is responsible for reviewing pesticide use pattern (GLP) data on the chemistry and composition of pesticides, methods of analyzing pesticide residues, and for estimating MRLs that might occur following the use of a pesticide according to GAP. The WHO Expert Group is responsible for reviewing toxicological and related data on the pesticides and, where possible, for estimating ADI's for humans.

Risks can be minimized once the hazard and the routes of exposure to the hazard are understood. Therefore, pesticides must undergo a rigorous regulatory procedure designed to determine the hazard level of a product and assess the risks associated with that product before gaining approval and being placed on the market. There is also a need to force the authorities to give more emphasis on the health of the humans and treat the issue on a priority basis. Also, careful and prospective health studies are required to determine the actual exposure levels and the precise pesticides to which people are exposed. Without further studies, we cannot determine if these low-level chronic exposures can result in either short or long term health effects. In fact, what needs to be done immediately is to appraise the adverse health effects caused by the pesticides and if they are found to be dangerous beyond a certain level, restrictions should be imposed on their use as well as exposure to human health.

In conclusion, there is an urgent need to initiate necessary action to restrict sales of many of the most toxic pesticides, improving facilities for disposal of hazardous wastes, creating more awareness among the farmers and authorities in enforcing and ensuring the use of protective gear while handling pesticides. Farmers needs to be encouraged to reduce, if not eliminate the use of pesticides, with the introduction of incentives to the farmers to help them shift from synthetic pesticides to bio-pesticides and organic farming. Also there is a need to draw the focus of all individuals, families, communities and nations on this crucial issue and raise the demand for lesser exposure of the pesticides to humans and animals with a view to their well being. For general people, to reduce the risk from pesticides they have to eat organically and ecologically grown food, wash and peel vegetables and fruit, grow their own food, avoid fatty foods or trim fat from meat as persistent pesticides are stored in fatty tissue, cook vegetables rather than eat them raw all the time, cook meat and chicken

thoroughly, garden in a non-chemical way without pesticides, avoid using chemical and pesticide based head lice shampoos, keep away from areas that have been freshly sprayed with pesticides and if their job involve exposure to pesticides they should wear proper protective clothing.

7. References

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12

Pesticides and Human Health

Alewu B. and Nosiri C.
Shehu Idris College of Health Science and Technology, Makarfi, Nigeria

1. Introduction

Pesticides are those chemicals that are used to destroy unwanted forms of life or organisms. They include insecticides, rodenticides, herbicides, fungicides, fumigants etc. They are expected to have a selective action or toxicity to animals and man. This has made the government of many countries to introduce legislation which prevent the use of the more dangerous and persistent chemicals as pesticides and to demand withdrawal from the market any licensed chemical found to be harmful to animals or man.

In 1977, it was estimated that every year 20,000 fatalities result from the use of pesticides. Most of these occurred in developing countries (Forget, 1991). The 1981 estimate by OXFAM gave a figure of 40,000 fatalities from about two million cases of poison per year (Akubue, 1997).

Pesticides are those substances which, on entering the body by whatever route, e.g. ingestion, inhalation, or absorption through intact skin, produce harmful effects. The effect may be in the form of damage to the tissues or as a disturbance of the functioning of the body.

According to the project of cooperative extension officers of cornel, University, Michigan state university, the science of toxicology is based on the principle that there is a relationship between a toxic reaction (response) and the amount of the poison received (the dose). An important assumption in this relationship is that there is almost always a dose below which no response occurs or can be measured. A second assumption is that once a maximum response is reached any further increases in the dose will not result in any increased effect. In a particular instance, a dose – response relationship does not hold true in regard to true allergic relations. Allergic reactions are special kind of changes in the immune system; they are not really toxic responses. The difference between allergies and toxic reactions is that a toxic effect is directly, the result of the toxic chemical acting on the cells. Allergic responses are the result of a chemical stimulating the body to release natural chemicals which are in turn directly responsible for the effects seen. Thus in allergic reaction, the chemical acts as a trigger, not as bullet.

For all types of toxicity of chemicals to humans , knowing the dose response relationship is a necessary part of understanding the cause and effect relationship between chemical exposure and illness. As Paracelsus in the 16th century once wrote, “The right dose differentiates a poison from a remedy”. Note that the toxicity of a chemical is an inherent quality of the chemical and cannot be changed without changing the chemical to another form. The toxic effects on humans are related to the amount of exposure.

1.1 Measure of exposure

Exposure to poisons can be intentional or unintentional. The effects vary with the amount of exposure. Contamination of food or water with varying doses of chemicals can be obtained each time when contaminated food and drink are taken. Some commonly used measures for expressing levels of contaminants are;

Parts per million (PPM), which in metric equivalent is in milligrams per kilogram (mg/kg). This is approximately the amount in the water as one teaspoon per 1,000 gallons.

Parts per billion (PPB), which in metric equivalent is in micrograms per kilogram (μg/kg). This is approximately the amount in water as one teaspoon per 1,000,000 gallons.

However, as an example, individual's sensitivity to alcohol varies, as do individual sensitivity to other poisons. In testing the effects of poison to human health, lower animals are used.

In one particular measure of effects e.g. ED_{50} which means effective dose for 50 percent of animal tested. The ED_{50} of any poison varies depending on the effect measured. In general, the less severe the effect measure, the lower the ED_{50} for that particular effect. Obviously, poisons are not tested in humans in such a fashion. Instead animals are used to predict the toxicity that may occur in humans.

One of the most commonly used measures of toxicity is the LD_{50}. The LD_{50} (the lethal dose for 50 percent of the animals tested) of a poison is usually expressed in milligrams of chemical per kilogram of body weight (mg/kg). A chemical with a small LD50 (like 5mg/kg) is highly toxic. A chemical with a large LD_{50} (1,000 to 5,000 mg/kg) is practically non-toxic. The LD_{50} says nothing about non-lethal effects though. A chemical may have a large LD_{50}, but may produce illness at very small exposure levels. It is incorrect to say that chemicals with small LD_{50}'s are more dangerous than chemicals with large LD_{50}'s. They are simply more toxic. The danger or risk of adverse effect of chemicals is mostly determined by how they are used, not by the inherent toxicity of the chemical itself.

The LD_{50} of different poisons may easily be compared; however, it is always necessary to know which species was used for the tests, the age, sex and how the poison was administered (the route of exposure), since the LD_{50} of a poison may vary considerably based on these factors. Some pesticides (poisons) may be extremely toxic if swallowed (oral exposure) and not very toxic at all if splashed on the skin (dermal exposure). If the oral LD_{50} of a poison were 10mg/kg, 50 percent of the animals that swallowed 10mg/kg would be expected to die and 50 percent to live.

The potency of a pesticide is a measure of its strength compared to other poisons. The more potent the poison, the less it takes to kill; the less potent the pesticide, the more it take to kill. The potencies of pesticides are often compared using signal words or categories as 'Danger-poison' (skull and cross bones) – highly toxic.

Moderately toxic – WARNING

Slightly toxic – CAUTION

Practically non – toxic – none required.

Toxicity assessment is quite complex, many factors can affect the result of toxicity tests. Some of these factors include variables like temperature food, light, and stressful environmental conditions. Others factors related to the animals itself include age sex, health, and hormonal status.

The NOEL (No Observable Effect Level) is the highest dose or exposure level of a poison that produces no noticeable toxic effect on animals.

In toxicology, residue tolerance levels of poisons that are permitted in food or in drinking water for instance, are usually set from 100 to 1,000 times less than the NOEL to produce a wide margin of safety for humans.

The TLV (Threshold Limit Value) for a chemical is the airborne concentration of the chemical (expressed in PPM) that produces no adverse effects in workers exposed for eight hours per day, five days per week. The TLV is usually set to prevent minor toxic effects like skin or eye irritation.

Very often, people compared poisons based on their LD_{50}s and use the decisions about the safety of a chemical based on this number. This is over simplified approach to comparing chemical because the LD_{50}'s and base decisions about the safety of a chemical based on this number. This is an over simplified approach to comparing chemicals because the LD_{50} is simply one point on the dose-response curve that reflects the potential of the compound to cause death. What is more important in assessing chemical safety is the threshold dose, and the slope of the dose-response curve, shows how fast the response increases as the dose increases. It is quite possible that a chemical will produce a very undesirable toxic effect (such as reproductive toxicity or birth defects) at doses which cause no death at all.

A true assessment of chemical toxicity involves comparisons of numerous dose-response curves covering many different types of toxic effects. The determination of which pesticides will be restricted use pesticides involves this approach. Some restricted use pesticides have very large $_{LD50s}$ (low acute oral toxicity) however, they may be very strong skin or eye irritants and thus require special handling.

Although there is no direct extrapolation of animal studies to man, the knowledge gained from dose-response studies in animals is used to set standards for human exposure and the amount of chemical residue that is allowed in the environment. As mentioned previously, numerous dose-response relationships must be determined, in many different species. Without this information, it is impossible to accurately predict the health risks associated with chemical exposure. With adequate information, we can make informed decisions about chemical exposure and work to minimize the risk to human health and the environment.

Pesticides differ from any other chemical substances because they are deliberately spread into the environment. As a consequence, a great part of the human population may be exposed either in the general environment or in the working setting. The occupational exposure involving the manufacturing and the use of pesticides, takes place mainly through dermal or respiratory route. While the environmental exposure, involving general population is mainly due to the ingestion of the contaminated foods and water. The environmental and occupational exposure determines the detrimental effect that this exposure could have on reproductive function. In women, if primordial follicles are destroyed extensively, they cannot be regenerated. This can cause premature ovarian failure and early menopause.

Note the information contained in this topic is not a substitute for pesticide labels. The trade names used here are for convenience only, no endorsement of products is intended nor criticism of unnamed products implied.

In California, suspected pesticide-related illness and suspected work related illnesses and injuries are reportable conditions. In 1998, the occupational Health branch of the California department of health services (CDHS) received a report from California department of pesticide regulation (CDPR) of a pesticide exposure incident in fresno county involving 34 farm workers. CDHS investigated this incident by reviewing medical records of the 34

workers and interviewing 29. The finding indicated that the workers became ill after early re-entry into a cotton field that had been sprayed with a cholinesterase inhibiting carbamate pesticide.

1.2 Prenatal exposure

The study of the effects of low level exposure to environmental neurotoxic agents has been one of the most important aspects of environmental research. The developing organism may be more susceptible to many of these chemicals than the adults. This susceptibity varies during different stages of development, but severe behavioural neurochemical, and neurophysiological abnormalities can be measured in adults that were exposed during neuronal development. Apparently, one of the problems of toxicity testing is to correlate these behavioral alterations with the anatomical changes induced by the neonatal exposure.
The large scale pollution of our environment by chemicals released from industrial and other sources has resulted in many well-publicized episodes of mass poisonings of populations in modern world. While these episodes have highlighted the consequences of unchecked industrial growth, they have not shed light on what may be a much more urgent problem facing mankind: the problem of low level exposure to a variety of chemicals throughout the life cycle. This problem is of completely different nature from the problem of acute mass poisoning in that it not only exposes all age groups but also exposes these groups to several chemicals simultaneously whose individual toxicities even today are largely unknown. The realization that biological systems and ecosystem may not be able to cope with the continuously rising tide of chemical pollution has led government agencies to pass legislation before toxicity thresholds had been adequate determined. In fact, it has resulted in a dilemma for the toxicologist in that more is known about the toxicity of many of the chemicals from cases of human poisoning than is known from animal studies. The production of a new untested chemicals to the already existing pool at an ever-increasing rate also seem to the possibility of careful dose-response studies on identified toxic agents because of the pressure to respond to today's emergency rather than yesterday's head line.
In response to this emergency, biologists have tried to develop rapid screening tests that would serve as early indicators of toxicity. Such tests show a great deal of promise in the screening of potential carcinogens and of neurotoxic substances.
The minimata episode in the 1950's in Japan has made it clear that pregnant women who are exposed to low concentrations of pesticides (e.g methimercury) may experience few symptoms and yet can give birth to severely retarded children. This effect has since been documented in animal studies and has been shown to be due to the increased fetal brain concentration of mercury (null et al; 1973). As was stated earlier, the purpose of many animal studies, therefore, has been to define the threshold level of exposure to mercury below which no neurological abnormalities will be observed in the offspring.

2. Principles of biological tests for toxicity

Toxicology has been defined as the study of the effects of chemical agents on biological material with special emphasis on harmful effects. It basically involves an understanding of all effects of essentially all chemicals on all types of living matter. There is ample evidence to indicate that every chemical is capable, under some conditions of producing some type of effect on every biological tissue. Toxicologic tests are therefore the tests that define the conditions that must be present when a biological cell is affected by a given chemical entity,

and the nature of the effect which is produced. As far as the conditions that must be present are concerned, they may vary from being practically unattainable under ordinary circumstances to being so readily attained that simple exposure of living tissue to certain chemical produces destruction of the cells. As far as the nature of any effect of a chemical on living tissue is concerned, effects may be of such minor significance that the tissue is able to carry on its ordinary function in a normal manner so that it is only under conditions of stress or critical tests that a chemical induced effect is even detectable. Effects may result from small amounts of some chemicals and required to produce any positive findings. Generally it is a simple matter to separate those relatively few chemicals that in small amounts produce prompt effects that are distinctly harmful to living cells from those that are practically harmless when exposure is over a short period of time, but it becomes difficult to demonstrate that small amounts of some compounds do not produce some types of toxicity when animals are exposed over a long period of time.

Most of the biological methods which have been developed in toxicology are the result of the practical need to obtain as much information as possible about the effects of chemical in so far as they may be pertinent to man's continued physical well-being. The continuing economic progress of the human race has been accompanied by a continuing increase in the numbers of chemical entities to which man is either in intentionally or unintentionally exposed. A person may be exposed through direct industrial of domestic occupational contact, through contact with the clothes or devices he wears, the food, he used and drugs he consumes and the atmosphere he inhales. It is necessary not only to understand the toxicities that can occur but also to obtain assurance that exposure of man to large number of chemical entities will mot lead to obvious direct or insidious indirect detrimental effects. Consequently , it is essential that chemicals which are to be intentionally administered to man, such as food additives, food substitutes or drugs, it is necessary to obtain as much toxicity data as is economical possible.

Because of the moral ethical and legal restrictions regarding the use of humans for experimental purposes in order to acquire toxicological data, only limited amounts of such data are available. Information regarding the effects of chemicals on human is obtained only after a chemical is used by human or from limited types of experimental procedures that may be conducted on humans. Biological methods in toxicology therefore generally involve the use of expendable species of animals on the hypothesis that toxicity studies in suitable species have an extrapolative value for man.

Several of the procedures involved in testing for toxicity involve the use of non-mammalian species and even cell cultures. It would be of great advantage to be able to utilize such species as bacteria, neurospora, daphnia, drosophila, the various echinoderms or fish for evaluation of toxicity because of the economic advantage and abundance of such populations of living cells. Furthermore some of these species lend themselves to accurate and simple procedures such as those that make use of their accurately defined and measurable genetic characteristics, reproductive processes and enzymatic performance. The main drawbacks associated with the use of such species are the dissimilarities in translocation barriers as compared to man and differences in or the lack of biotransformation mechanisms that are present in man. These factors preclude extrapolation of the data obtained on most non-mammalian species to man. Never the less such tests serve the purpose of alerting the investigator to potential toxic hazards which can then be further studied in mammalian species.

However when any chemical is used in massive quantities such as in agriculture and becomes available in the general environment, it is necessary to evaluate the toxicity of that agent in many species which may directly or indirectly influence the overall welfare of man. It should be recognized that there are many variations in both short and long term chemical-induced toxicity between various mammalian species of animals, however careful complete evaluation of the effects of chemicals on animals have been shown to be the most rational, acceptable and successful means of determining most types of toxicity for purposes of extrapolation to man. The principal exception is the rather unsuccessful evaluation of immunogenic types of toxicity. It is interesting to know that many workers before the 19th century described the actions of poisons and their antidotes, these studies seemed to lack the scientific approach. The first to undertake scientific studies on the harmful effects of chemicals on biological systems was M.J.B. Orfilia (1787-1853) a Spaniard at the University of Paris (USA today, 1989). He is regard as the father of modern toxicology and was the first to introduce quantization in the study of the actions of chemicals on animals and to consider toxicology as a separate discipline from pharmacology. Orfilia was the author of the first book on harmful effects of chemicals (in 1815). He not only studied and reported on the effect of chemical but also on the treatment of poisoning due to such chemicals.

Toxicology as a science has its basis in the science of chemistry and biochemistry and is dependent on the knowledge of physiology. Pathology is often regarded as part of toxicology because the effect of the chemical on the biological system may appear as macro- or microscopical deviations of the normal cell or organ.

Toxicology is an offshoot of, and closely related to pharmacology because a pharmacologist attempts to understand the beneficial effects of the chemical when used therapeutically as well as its harmful or adverse effects. There are three main divisions of toxicology namely, economic, forensic and environmental toxicology, each with its own specialist toxicologist.

2.1 Economic toxicology

This concerns the harmful effect of chemicals, administered to man or animals in order to produce a specific effect. This includes drugs which are administered to modify physiological functions or eliminate some bacteria/parasitic organisms in the body. The study of drug toxicity (including tests for toxicity) is a major area of economic toxicology. It includes studies on the safety of food additives and cosmetics. Some chemicals have selective action on biological organisms and are used by man to eliminate pests and insects (as pesticides and insecticides) which become the uneconomic species. The human which is protected from the effect of the pest and insects becomes the economic species. The effect of the chemical on both economic and uneconomic species also forms a part of economic toxicology.

2.2 Forensic toxicology

This concerns the medical and legal aspects of the harmful effects of chemical on animals, including man. The medical aspects refer to the diagnosis and treatment of the effects of the chemical and the harmful effects produced by it. In forensic toxicology, an attempt is made to identify the chemical in the tissue by chemical analysis and in case of death, to establish the cause or circumstances of death. Both intentional and accidental exposures to the chemical are of interest to forensic toxicologists. They develop methods for the management

and treatment of acute and chronic poisoning including the use of antidotes. These are specifically referred to as clinical toxicologists.

2.3 Environmental toxicology

This deals with the harmful effects to man and animals of chemicals that are present as contaminants of the environment. The chemical may be present in the air, water, soil or food. In the urban centers, because of the industrial activities, the environment may be polluted by particles or by gasses. Environmental toxicology is concerned with hazardous substances in the air, water or soil, the disposal of industrial waste and the protection of the environment and peoples either at home or at manufacturing sites from industrial emissions. The environmental toxicologist deals with the evaluation of the effects of and the establishment of the limits of safety of exposure to these chemicals i.e. estimating the health risks of a particular chemical.

3. Factors that affect the toxicity of chemicals

Among the factors that affect the toxicity of a chemical are the chemical, biological, genetic factors and the route of administration or exposure to the chemicals.

3.1 Chemical factors

The chemical structure determines the ability of the chemical to interact with specific receptors responsible for the observed effects. Biotransformation mechanism is dependent on the structure and may produce a metabolite that is more toxic or less toxic than the parent chemical. It may influence the excretion process of the chemical or its metabolite. It is known that some chemicals do induce or inhibit metabolizing enzymes and in this way modify their activities and those of other chemicals metabolized by the same enzymes.

3.2 Biological factors

Here the factors include biotransformation and elimination mechanisms, plasma protein binding, storage of the biological membrane through which it passes. Each of this will influence the toxicity. Age, sex, nutritional status of the individual and species of aminal also play roles too.

3.3 Genetic factors

Genetic differences may have a great effect on toxicity. For example, some individuals are genetically deficient in blood psendocholinesterase enzymes. Thus succinylcholine (a muscle relaxant) normally hydrolyzed by psendocholinesterase, will induce prolonged muscle relaxation in a person genetically deficient in the enzyme contrary to expectation.

The anti-cholinesterase's used in therapeutics' are generally those which reversibly inactivate cholinesterase for a few hours. Insecticides of the carbamate type act by reversible inhibition of cholinesterase but organophosphorous insecticides inhibit the enzyme almost or completely irreversible so that recovery depends on formation of fresh enzymes. This process may take weeks although clinical recovery is usually evident in days. Cases of poisoning are usually, after agricultural, industrial or transport accidents. Substances of this type have also been studied for use in war (nerve gas). The prominence of individual effects varies with different agents, e.g. sweating and salivation are not usual in dyflos poisoning.

A typical case of poisoning by cutaneous absorption, will, perhaps after a delay, develop headache confusion, anorexia and a sense of unreality. The patient is often giddy, apprehensive and restless. Conspicuous salivation, rhinorrhoea and sweating follow, with respiratory wheeze and dyspnoea indicating the onset of broncho constriction and excessive bronchial secretion. Miosis may occur and cause the headache, but it is not invariable nor is it an index of severity, for it may be due to a local effect of the poison entering via the conjunctiva. Vomiting and cramping abdominal pains may lead to diarrhoea and tenesmus, and there may also be urinary incontinence. Muscle twitching typically begins in the eyelids, tongue and face, then extends to the neck and limbs and is accompanied by severe weakness. Progressive respiratory difficulty leads to convulsions and coma. Death is due to a combination of the actions in the central nervous system, to paralysis of the respiratory muscles by peripheral neuromuscular block, and to excessive bronchial secretions causing respiratory failure. At autopsy, ideal intrsusception are commonly found.

4. Route of administration or entry into the body

Chemicals may enter the body through inhalation, by contact with the skin and by oral route. In addition, drugs may be administered by porenteral routes. The toxicity of a chemical may be many times greater through one route than through the other. A typical example is curare which is not absorbed orally and hence induces no toxicity. Its toxicity manifests itself when administered parenterally.

5. Toxicological effects

The toxicological actions of a chemical or materials may induce acute or chronic toxic effects or poisoning. Acute effects arise form an exposure to a chemical or an over dosage of drugs and the poisoning may be accidental, suicidal or homicidal. This is a toxicological emergency and demands emergency management, care and treatment.

Chronic poisoning is caused by ingestion of or exposure to the chemical over a period. In certain cases of occupational poisoning, ingestion of polluted water or inhalation of insecticides by farm workers results in chronic poisoning. It takes times to manifest itself and an equally long time to treat.

However chronic poisoning may produce an acute toxic effect which will necessitate toxicological emergency action and a chronic management of the patient until the body load is reduced or poison eliminated form the body.

6. The environment

The human environment contains many chemicals that are toxic to man and animals. It is one of the sources of health hazard and is responsible for various acute toxicities and many chronic illnesses. The environmental chemicals may be present in the atmosphere as air pollutants, in soil or water including the under ground water as contaminants and in food as residue or contaminants.

The sources of environmental hazardous chemicals are two, namely, natural sources and man-made sources. For the sake of this topic, emphasis is laid on man-made source.

6.1 Man-made sources

These arise from human activities and include chemicals that reach the atmosphere as a result of industrial activities. There are many chemicals that are present or are used in work places and therefore constitute occupational hazard. Many industrial activities pollute the atmosphere with particulate matters and gasses like carbonmonoxide, sulphurdioxide, hydrocarbon e.t.c. Some factory smoke stacks release particles which are deposited onto vegetables crops that are consumed by man.

Some potential toxic chemicals are normally found in the home as drugs, as pesticides. These are responsible for accidental poisoning among children. Studies in developed countries have shown that poisoning is the second or third most important cause of fatal accidents in the home (Backett, 1965). Pesticides used by farmers for enhanced food production may appear in the food and the same applies to food preservatives. In Nigeria, some farmers and traders used pesticides to preserve grains like beans, maize, rice, etc, in spite of the fact that pesticides are not approved for the preservation of grains. Through this source, pesticides are consumed by the unsuspecting public with possible acute or often chronic consequences. The pesticides used in the farm may be carried by rain, run-off to contaminate the streams and rivers in countries without the necessary controls.

There are many activities that routinely release hazardous chemicals to the environment. The petrol station attendant inhales benzene as petrol is put in the car and benzene is also released to the air. The exhaust fumes form motor vehicles release a number of chemicals including particulate matters in to the atmosphere. Such substance in the air create problems in the some developed countries like the USA to control and set limits of emission from motor vehicles for permit to ply the road.

The Natural Resources Defense Council (NRDC) of the USA estimated in 1989 that industry is pumping more than 361 million pounds of cancer producing chemicals into the air yearly (USA Today, 1989).

Over the years, human beings have tended to depend on some sudden and unexpected episodes to realize the dangers of environmental pollution from man-made sources. There are many instances which point to the fact that pollution can cause serious illness or death. In 1952 in London, a dense fog (SMOG) due to environmental pollution settles over the city for 4 days. This resulted in about 3,500 to 4,000 deaths in greater London alone (Klassen, 1990). Ten years later a similar episode occurred in London and caused many deaths particularly amongst the elderly and children (Akubue, 1997).

In the Meuse valley in Belgium in 1930, a heavy fog associated with very stable air mass caused severe respiratory symptoms and death, the death rate in the community during the period was 10 times more than normal. The air pollution was said to have come from the industrial plants in the neighbor hood,

In June, 1996 in Santiago, Chile, there was part of SMOG as air pollution reached an alarming level. There were many deaths from respiratory diseases. The city authorities ordered 300,000 cars off the roads in an effort to improve the quanlity of air.

There are also many instances of major accidental release of toxic chemicals in to the atmosphere.

In 1976, there was a massive exposure of the city and people of seveso, in Italy to 2, 3, 7, 8-tetra chlorobenzodioxin (TCDD) (Ottobin, 1991). This was as a result of an explosion in a manufacturing plant. Many people particularly children suffered chlodacne and thousands of animals like chicken, birds, dogs and horse died a few days after the explosion. It is believed that dioxins contaminants of herbicide, agents' orange, were responsible for many

health problems of Vietnam Veterans (Ottoni, 1991). Dioxins of which TCDDTS, the most toxic and most persistent poison, are by-product contaminants of many chemicals reactions and are formed during the manufacture of chemicals like trichlorophenols and the combustion of waste materials. Studies have shown that if TCDD is not a cancer inducer, it is a promoter. In Nigeria, there is no doubt that TCDD is formed as a by-product of chemical processes being carried out in the industries and as a product of combustion of waste materials. Hence, TCDD is in the air around us. The question that needs to be answered is, what have we done to bring its level to the bear rest minimum?

An explosion at an industrial plant in Bhopal, India in 1985 released into the environment methyl-isocyanate and this was responsible for the death of about 1,500 people (Keritage, 1992). This would not have happened if adequate safety measures were in place in the industry. Other toxic chemicals worthy of note are discussed below.

6.1.1 Dichlorodiphenyltrichloroethane

DDT is an organochlorine insecticides (also referred to as halobenzene derivative) which was widely used in agriculture and in malaria control. It is highly soluble in fats and poorly soluble in water. In the body it is stored in the fat depot. It is only slowly eliminated from the body. DDT is usually used as solution in organic solvents, especially kerosene. It is established that DDT increases the incidence of liver cancer in mice (Innes, 1969) there is yet no evidence for such an effect in humans. However, its use has been limited or withdrawn in many countries because of uncertainly of the effect of prolonged exposure and storage in man, beside, some insects developed resistance to it.

6.1.1.1 Symptoms

Symptoms of acute poisoning include vomiting tremor, and convulsion. There is anesthesia of the tongue, lips and face with marked apprehension and excitement. Diarrhoea may occur.

A study indicated that carbonate pesticides namely aldicarb, aldicarb sulfoxide, baygon, penthiocarb, carbofuran, 3-hydrocarbofuram, carbaryl, desmedipham, methiocab, methomyl, thiodicarb, oxamyl, and propham was made in ground and surface water from an agricultural zone of the Yaqui valley located in northwest Mexico. From the result of trace determinations made by liquid chromatograph (LC) with post-Column fluorescence detection, it showed that the level of contamination with methiocarb was about 5.4 mg/l in a ground water sample and that for 3-hydroxycarbofuran was 18mg/l in a surface water sample (Garcia-de-Llasera, 2001).

Carbofuran was estimated for an Acceptable Daily intake (ADI) in 1978 and 1979 and a temporary ADI for man was estimated to be 0-0.003mg/kg body weight (FAO/WHO, 1977; FAO.1980). The available data reflected that carbofuran is a highly toxic carbonate ester whose metabolic profile has been well defined. Carbofuran is a potent, reversible cholinesterase inhibitor. Chlolinesterase inhibition and acute toxic signs of poisoning are subject to rapid spontaneous reversal and recovery. The measurement and evaluation of cholinesterase depression induced by carbofuran, because of the rapid reversibility, is difficult and required substantial care.

Similarly, in a group of mice (100 male and 100 females, Charles River CD-1mice/group were fed carbofuran in the diet of dosage levels of 0, 20, 125, or 500 mg/kg for two years. It was reported that a localized hair loss and reddening of the ear(s) frequently followed by

scabbing or sloughing of portions of the ears was noted with greater frequency in the treated mice. (Pesticide residues in food: 1950 evaluation)
In a similar study using fenamiphos, a carbonate pesticide like carbofuran, exposure of rats to technical grade fenamiphos (purity, 92.2%) diluted with a 1:1 mixture of ethanol and polyethylene glycol 400 for aerosolozation in a dynamic flow inhalation chamber at doses of 0,0.03,0.25, or 3.5 mg/l for 6hrs per day, five days per week for three weeks showed a significant decrease in (48-7.9%) in plasma cholinesterase activity and a slight decrease (9-18%) in erythrocyte acetyl cholinesterase activity in animals of each sex at 3.5µg/l (Thysen, 1979b)

6.1.2 Benzene Hexachloride and Lindane

Benzene hexachloride (BHC) is a mixture of eight isomers with gamma isomer (Lindane) being the most toxic and active but most rapidly excreted. The toxic effects of Lindane resemble those of DDT. It is a CNS stimulant and a potent inducer of hepatic microsomal enzymes. BHC is said to cause aplastic anaemia (Akubue, 1997)

6.1.3 Polycyclic chlorinated substance chlorinated cyclodienes

The substances are many with varying toxicities. Examples are aldrin, dieldrin, heptachlor, Endosulfan and chlordane. They stimulate the CNS and induce convulsion. Before this, there may be headache with nausea, dizziness, vomiting and mild chronic jerking, and tremor ataxia. The CNS stimulation may be followed by depression which may end in respiratory failure. The insecticides have potentials as carcinogens and cause haematoma in mice. Hence their use in some countries (e.g. USA) is banned.

6.1.4 Organophosphorous insecticide

Organophosphorous insecticides are extensively used in agriculture. Through they do not persist in the environment; they can cause serious toxic effect in man, being potent and irreversible inhibitors of cholinesterase. The symptoms of poisoning are due to muscarinic, nicotinic and CNS effects.
In acute (mild to moderate) poisoning from ingestion or inhalation, the following symptoms can be as observed (headache, dizziness, tremor of the tongue and eyelids, miosis and impaired vision. These symptoms are followed by nausea, vomiting, salivation, tearing, abdominal cramps, and sweating, slow, pulse and muscle fasciculation. In severe poisoning, diarrhoea, pinpoint pupils not reactive to light, respiratory difficulty, cyanosis, convulsion, coma and heart block may be observed.
Chronic poisoning may occur and inhibition of cholinesterase can persist for up to 6 weeks. Delayed neuropathy occasionally develops after poisoning.

7. Rodenticides

Many drugs had been used to kill small animal like rats and mice. Amongst these are fluoroacetate, x-naphthylthiourea(Antu) and pindone (warfarin- like anticoagulant).

7.1 Fluoroacetate sodium

Fluoro acetate sodium is extremely toxic to rodents and to man and other animals. Fluoroacetate is present in the plant known as *Dichapetalum cymosum* which grows in

Nigeria and is used as rat poison. Fluoroacetate is too toxic for use in the home. It has been withdrawn from the market in some countries but fluoro actamide, which has the same toxicity, is still on the market.

The most prominent effects of a cute poisoning in man from ingestion or inalation of fluoroacetate are vomiting and convulsions. It induces irregular heart beat, exhaustion and coma. Death is usually due to respiratory failure.

7.2 Pindone

This is a coumarin anticoagulant with actions similar to warfarin which is also used as rodenticide. In large doses, they can cause vascular collapse.

8. Herbicides

Herbicides are now used extensively to destroy noxious weeds. The most common ones are the following;

- 2, 4-dichlorophenoxyacetic acid (2, 4-D)
- 2, 4, 5- trichlorophenoxyacetic acid (2, 4, 5-T)
- Dinitrophenols
- Paraquat.

8.1 Chlorophenoxy compounds (2, 4-D and 2, 4, 5,-T)

Chlorophenoxy compounds are used to control broad-leaf weeds. They rarely cause toxicity in man though contact dermatitis is known to occur. The effect is due to contaminant called TCDD(2,3,7,8- tetrachlorodibenzo-p-dioxin).

8.2 Dinitrophenols

Dinitrophenols are also used extensively in weed control. The toxic effect is due to the uncoupling of oxidative phosphorylation. Thus the metabolic rate is increased with a subsequent rise in body temperature. Other symptoms of acute poisoning include nausea, restlessness, sweating tachycardia, fever rapid respiration, fever and cyanosis.

9. Fungicide

Most fungicides that are used to control fungal diseases on seeds and plants are not particularly toxic except the mercurials. However, some do produce toxic effects in man. A good example is seen in carbamates e.g aldicarb, aminocarb, carbofuran, propoxur and bendiocarb. Health hazards to man are mainly as a result of occupational exposure.

9.1 Dithiocarbamates

Both dimethyldithiocarbamate and ethylenebisdithiocarbamate have low acute toxicity. However, thay may have teratogenic as well as carcinogenic effect and can produce disulfiram-like effects when alcohol is ingested. It is known that in humans, in the environment and during cooking of contaminated food, the ethylenebisdithiocarbamate is broken down to form ethylenethiourea, which is carcinogenic and teratogenic.

Other chemicals used as fungicides include hexachlorobenzene and penta chlophenol. The toxic effects of pentacholophenol resemble those of nitrophenols which increase the metabolic rate by uncoupling oxidative phosphorylation.

10. Fumigants

Funigants are used in gaseous form as pesticides to reach areas that are inaccessible. Examples include cynide, crabondisulphide, carbon tetrachloride, thylene oxide, methylbromide and phosphine.

10.1 Cyanide

Cyanide as hydrogen gas, is used as a fumigant in ships and buildings. It is also used in industry in chemical synthesis and electroplating and as household pesticides and rodenticide.
Large doses of hydrogen cyanide can cause rapid respiration, convulsion. Loss of consciousness and death within 5minutes: lower doses produce headache, staggering gait, dilated pupils, palpitation, unconsciousness, violent convulsion and death. Combustion of plastic materials releases hydrogen cycanide. This was the cause of death of 303 pilgrims in 1980 in Riyadh, Saudi Arabia (Wager, 1983).
Cyanide in the body complexes with cytochrome oxidase and is responsible for cellular oxygen transport. In this way it interferes with oxygen uptake by the body cells.

10.2 Methyl bromide

Methyl bromide is a colourless gas used as a fumigant for soil, stored dried food stuffs and for disinfection of fresh fruits and vegetables.
Methyl bromide causes headache, blurred vision, weakness, oliguria, anuria, confusion, drowsiness, convulsion, and coma. There may be circulatory collapse or respiratory failure. With low doses, the symptoms may take 12 to 24 hours to appear.

10.3 Phosphine (PH_3)

This is used to fumigate grains. It is slowly released from aluminum phosphine tablets in the presence of atmospheric moisture. Poisoning by inhalation may cause a fall in blood pressure, pulmonary oedema, collapse, convulsion and coma. The first sign of chronic poisoning is toothache followed by swelling of the jaw and necrosis of the mandible (phossy jaw). There may be anaemia and spontaneous fractures.

11. Carcinogens

Chemical substances have been associated with cancer in man long before it was demonstrated experimentally in animals. For example the high incidence of cancer of the scrotum was reported amongst chimney workers in 1775 (Wolf, 1953). This was thought to be due to exposure to soot. Cancer was also recognized as an industrial hazard among coal tar workers. It is now generally accepted thatmany human cancers are directly and indirectly due to environmental factors which induces exposure to chemicals and ionizing radiations (Tomatis, 1976)
Chemicals known to be responsible for certain human and animal cancer are referred to as carcinogens. Some of these chemicals produce the same type of cancer in animals and in man but with some other chemicals there is species variation for example, 2- methylamine causes bladder cancer in man and dogs (WHO, 1972). Occupational exposure to chemicals is known to cause cancer mostly that of the skin, bladder, lungs and sinuses (WHO, 1972).
Cancer develops slowly and there may be along latent period (15-40 years) before the cancer is detected. It can occur at the site of application or at a site far away from the site of contact.

Certain carcinogenic agents are not themselves carcinogenic until metabolized to an active product which is called proximate carcinogen.
It is also well established that chemical like polycyclic hydrocarbons e.g. 3.4- benpyrene present in coal tar) and 3, 4-benzphenanthrene azo dyes e.g. Dimethylnitrosamine are potent carcinogens. Thus a very wide range of chemical structures can induce carcinogenicity. This has made determination of the mechanism of induction rather difficult. It is believed that most organic carcinogens react covalently with macromolecules in the tissue. Others are converted to reactive metabolites which contain an electrophilic atom. It is this reactive (elctrophilic) group in the carcinogen or its metabolite that reacts with any nucleophilis atom of the target macro molecules of the cell. The nucleophilic site is on the DNA, RNA, and/ or protein. Carcinogens can react covalently with DNA in vivo directly or after conversion to the reactive group.
The problem of carcinogens is very great in developing countries where control measures and legislation are not available to protect the public from the industrial hazard of these chemicals and from the use of such chemicals in foodstuffs, cosmetic and agriculture. Many local industries do not even try to protect their workers and the workers, out of ignorance do not protect themselves. It may be realized that even when chemical is declared non-carcinogenic in other developed country, because of our genetic make-up and because of other promoting factors which may be presented in our environment may be carcinogenic in our surroundings. It is therefore imperative that all chemicals in use for whatever purpose should be monitored for possible carcinogenic effects (Akubue, 1997)

12. Chemical mutagen

A change in the hereditary constitution (otherwise called genotype) of the individual is known as mutation. This may be produced by chemicals and also by radiation and hence called mutagens. Mutation can occur spontaneously by unknown mechanisms.
Mutagens act by altering the genetic make-up of the individual and this can be handed down to the offspring during cell division. This means the new cell will have heritable characteristics. The mutation may occur as a result of alteration of one or more nucleotide or changes in number as structure of chromosomes.
In general, two kinds of mutation can be recognized: Chromosome and gene mutation. A chromosome mutation may be of several types e.g. Deletion, duplication inversion, translocation (Roberts, 1982).
However, on some occasions, two homologous chromosomes, instead of separating during cell division, called meiosis, go off into the same gamete. This phenomenon is known as non-disjunction and it results in half the gametes having two of the chromosomes whilst the other half has none. The fusion of the first kind of gamete with a normal gamete of the other sex will give an individual with three such chromosomes i.e. the normal pair plus an extra one. This condition is called trisomy.
Quite how important non-disjunction has been in generating useful genetic novelty is uncertain, but there is no doubt it can have profound effect on an organism's development. For example, mongolism (better called Down's syndrome after the clinician who first described it) is now known to be caused by the presence o f an extra chromosome in the cells, chromosome number 21 to be precise. This is one of the smallest of all the human chromosomes, and yet its presence plays havoc with the individual's normal development. Sufferers, form Down`s syndrome, if they survive at all, have a characteristically slit-eyed

appearance, reduced resistance to infection and are always mentally deficient, all because of one extra chromosome.
The short description above is a tip of an iceberg of the several havoc pesticides can cause to the body.
A gene mutation arises as a result of a chemical change in an individual gene. An alteration in the sequence of nucleotides in that part of a DNA molecule corresponding to a single gene will change the amino acids making up a protein, and this can have far- reaching consequences on the development of an organisms.
Drastic effects can some times be produced by a seemingly trivial change in the nucleotide sequence of a gene. This can be seen, for example in the formation of hemoglobin. In the inherited disease known as sickle cell anaemia, the red blood cells, normally biconcave discs, are sickle-shaped and the victims suffers all the symptoms of extreme oxygen shortage, weakness, emaciation, kidney and heart failure.
The anemia is not caused by a distorted shape of the red blood cells as such, but by the fact that they contain abnormal haemoglobin, haemoglobin S, which inefficient at carrying oxygen. Gene mutation can be by deletion, inversion substitution.
Note that although the addition of whole chromosome as seen above may have been disastrous more often than it has been helpful, it is highly likely that the addition of new genes within individual chromosomes (insertion) has been extremely important in promoting evolutionary novelty. In general terms it is important to appreciate that variation is not always disadvantageous (Roberts, 1982)
Where the action of the mutagen is in the human germ cells (i.e. spermatozoa or ova), the offspring will carry the mutant genes in its cells. This may result in death of the zygote) or abnormal offspring. The effect of the mutation appears only in the offspring and may take a few generations before it manifest itself.
Mutation may also affect somatic cells. The effect is not passed on to future generations but may be responsible for carcinogenesis. For example, the alteration in genetic material may cause cell division in cells that normally do not divide during adult life. Such a division will eventually lead to cancer.
Though it is not known how many gene mutations in man can be attributed to chemicals it is acknowledged that many chemicals can cause gene mutation in micro-organisms and in insects. Also many chemicals can cause chromosome aberrations in man and can interact with nucleic acids. This may be a pointer to the fact that there are many chemical mutagens in our environment.
Among the chemicals shown to be mutagenic in mammalian cells are ethyleneoxide, Ziridine, aminobiphenyl, dimethylsulphate, benzidine e.t.c (Fischbein, 1979)

13. Teratogens

Teratogens are agents that cause abnormalities of foetal development. They usually interfere with the development of the foetus at a dose that produce no serious toxic effect on the mother or impairment of placental function. However, in high doses most of them will cause foetal death followed by abortion or resorption of the foetus.
One of the earliest reports of environmental factors being responsible for the birth of malformed babies came form Australia 1940 (Gregg, 1941). Mothers who suffered form the mild virus disease (rubella) during the first trimester of pregnancy gave birth to blind or deaf children. It was found that if the infection occurred after the third mouth of pregnancy,

no abnormality developed. This was followed about twenty years later by the thalidomide disaster of the early sixties when a seemingly non-toxic drug produced teratogenic effect. Some environmental pollutions, like chemical defoliants, 2, 4, 5-T (2, 4, 5-trichlorophenoxyacetic acid), are teratogenic in animals. Their effects in man are not yet fully established.

Evidence for the teratogenic effect in man of organophosphorous insecticides is still inconclusive though such an effect in animal is well established. Lead which is an environmental pollutant may be teratogenic (Scalon, 1972).

From the above account it is obvious that a lot needs to be known about chemical teratogens. It is not unlikely that most of the congenital malformations encountered in a community may have been due to environmental chemicals (Akubue, 1997). As teratogenic effect cannot be reversed or treated, the only right approach is to take necessary steps to prevent it. This is best achieved by avoiding occupational exposure to environmental chemical during pregnancy, particularly during the first trimester.

Developed countries do monitor the activities of industrial companies with regard to pollution and waste disposal. In 1992, Dexter corporation in the USA was fined thirteen million dollars (US$ 13 million for activities in its manufacturing plant in Windsor Locks Connecticut, USA, (EPA, 1990) with regard to illegally disposing of carbondisulphide and discharging hazardous waste and waste water into a river.

The above instances of the effect of environmental pollution and the action taken by a government environmental protection agency against a manufacturing company are presented to draw the attention of all sundry to the time bomb we are setting on. Nigeria and all developing countries must learn from the mistakes of the developed countries. We try to industrialize, we must not repeat the mistakes which other countries made in the past and paid heavily for them.

Apparently, in developing countries like Nigeria where increasing use of pesticides is absolutely vital for it purpose as to protection of economically important crops such as tobacco, cotton, rice and so on with little attention paid to its deleterious effects (Carlson et al., 1998 Roex et al., 2001) and for feeding increasing large populations adequately, some recommendation have made by Iyaniwura, (1991b) in order to maximize the benefits at the least risk to human population. They include;

The increasing need for extension workers to embark on the training of farmers in the choice of pesticides, storage, applications, technique and the use and disposal of spent container become very important in this area.

The need for the development of preventive measures, diagnostic tests and treatment facilities in pesticide poisoning will go along way in helping farmers.

That training of physicians and health workers in high risk areas should includes instruction on the diagnosis and treatment of pesticide poisoning.

Trained scientist specialize in the treatment of victims of pesticide poisoning should be made available to health officials and hospital at those locations where pesticides are being used.

It is worthy of note here that regulations or restrictions of use and production of the chemical itself need to be enforced to avoid damage to man, animals, insects or wild life generally. In this regulations, various tests are performed including; chemical characteristics, toxicological characteristics physiological and biochemical, behavioral, environmental, ecological and tolerance assessment.

Pollution cause immediate health effects, in many cases, the effect may be delayed for 5 to 10 years or more. The greatest danger is that the effect may be unnoticed until irreversible

damage is done. This is worrying in our circumstances. In developing countries specifically, Nigeria, an average Nigerian does not feel threatened by any chemical or any operation that has no acute toxic effect. It is known that the health effect may appear long after the person has left the area of exposure. By this time, the health effect may not be associated with the exposure.

Some of the effects that are known to be induced by pesticides in humans includes cancer (various types) infertility, liver damage, kidney damage, premature death, hyperactivity in children, bronchitis, defective sight and blindness, birth defects, blood diseases, nervous system damage, heart defects and sudden death.

These illnesses are very devastating with high morbidity and mortality rates but as they are preventable, they are best prevented. The cost of prevention is next to nothing compared with the cost of treating the ailments or managing the in curable ones.

14. Biopesticides and human health

People have been using biotechnology for millennia. This technology is based on the use of microorganisms, which e.g. ferment the sugar in barley to alcohol during beer production. Other examples of everyday products that undergo biotechnological processing are cheese, yogurt, vinegar, wine, yeast, and sourdough. Without knowledge of the exact backgrounds, our ancestors used these methods to discover and improve a range of applications that made their life easier. Genetic engineering is a modern subspecialty of biotechnology. It is concerned with the targeted modification of the genetic material of bacteria or plants, for example to stimulate them to biosynthesize desired products. Today genetic engineering is primarily used in the field of medicine, but is also applied in industry and agriculture.

Biotechnology is the science that modifies the genetic composition of plants, animals and microorganisms. It is used to incorporate genetic material from one living organism to another. Biological pesticides are produced through the use of biotechnology by harnessing the pest-fighting abilities of existing plants and microbes. When these products have unique biological properties, they also pose unique regulatory challenges. In addressing these challenges, the Environmental Protection Agency (EPA), the U.S. Department of Agriculture (USDA), and the Food and Drug Administration (FDA) have shared responsibility for regulating agricultural biotechnology in the United States. For instance, EPA regulates pesticides created through biotechnology as a part of its regulatory jurisdiction over all pesticides marketed and used in the United States. As such, EPA has tailored its basic regulatory framework to fit the distinctive characteristics of these genetically engineered biological pesticides (EPA, 2003). In theory, biotechnology could be used to prevent pest problems and thus reduce the need for pest management and pesticide use. Since the beginning of agriculture, plant breeders have developed crop varieties that were resistant to or tolerant of particular pests. For example, cotton varieties with long, twisted bracts (called frego bracts) around the bolls are resistant to boll weevil damage and solid stemmed wheat varieties are not damaged by the wheat stem sawfly (Cox, 1993).

The tools of biotechnology could be used to make plant breeding easier and quicker. Genetically engineered crop plants will be in farmers' hands in the next few years. We are therefore at a critical point where we need to evaluate this new technology and the impact it will have to our agricultural systems and human health. What problems will the new technology bring? However, genetic engineering has the tendency to move agriculture in

the opposite direction, towards maintaining or increasing present pesticide use patterns. The total world production of biopesticides is over 3,000 tons/yr, which is increasing at a rapid rate. India has a vast potential for biopesticides as it utilizes more than 100,000 tons (1992-93) of pesticides annually. Most (80%) of the pesticides are used on cotton (45%), rice (30%) and vegetables (5%), the remaining crops receiving only 20% as share.

14.1 Benefits of biopesticides

International organisations and bodies such as the United Nations Food and Agriculture Organisation (FAO), the World Health Organisation (WHO) and the International Council for Science, as well as a number of national food safety authorities and medical associations, have all positively commented on the safety and/or benefits of agricultural biotechnology. There are direct environmental benefits which arise from the different management techniques plant biotechnology makes possible. For example, herbicide-tolerant crops facilitate the use of no-till agriculture, which reduces both soil erosion and energy inputs. At the same time, soil organic matter is maximized, which reduces agriculture's contribution to global emissions of greenhouse gases, linked to climate change. Pest-resistance reduces the need for spraying, with consequent benefits to non-target organisms and overall biodiversity. Also, by making farming more efficient on limited land area, plant biotechnology contributes significantly to preventing habitat destruction – the biggest single threat to biodiversity.

Organic food sales are increasing because the public is willing to pay more for pesticide-free vegetables, fruits and dairy products. Biotech based pesticides therefore are becoming viable alternatives to chemical pesticides. For instance a biocidal product of plant origin that can combat insects as well as fungus and bacteria would find ready acceptance in the hand of farmers for the control of diseases and pests. It could be grown by farmers themselves and the seeds can be turned to organic pesticides. Is it the spraying of yeast formulation on fruits before they are transported to selling centres to keep the fruits fresh? Biotech pesticides or biopesticides tend to harness nature for solving health problems of agricultural crops. These substances used in controlling pests in crops are ground water and environment friendly. They are used in small quantities. Crop yield are not affected by them. As they are of natural origin, they mutate hence their use result in substantial labor cost savings.

It is important to know the mode of action of biopesticide as compared to traditional pesticide prior to using it. A biopesticide cannot be considered as non toxic because it is natural in origin, but can generally be considered only as less toxic to humans.

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13

A Forensic View of Pesticide Poisonings in Brazil

Bruno Sabino, Hannah Rozenbaum and Adriana Oliveira
Instituto de Criminalística Carlos Éboli [ICCE]
Brazil

1. Introduction

Pesticides are substances extensively used in the country or in town for preventing, destroying, repelling or mitigating any pest. These groups of substances include different classes of compounds that present different types of toxicity. Although there are benefits to the use of pesticides, there are also drawbacks, such as potential toxicity to humans and other animals. According to the Stockholm Convention on Persistent Organic Pollutants, 10 of the 12 most dangerous and persistent organic chemicals are pesticides.

Because of their potential toxicity, these substances are commonly used as poisons in homicides, homicides attempts, suicides and also in cases of crime against animals. In forensic laboratories, foodstuffs and drinks used to cause the poisoning are usually analyzed to determine the presence of any potential toxic substance in the material. In this chapter we will explore the forensic aspects evolving the use of pesticides in crimes against life.

Primarily, we will present a database of the types of pesticides found in our forensic laboratories. We will analyze these data to create a pesticide poisoning profile in Brazil, determining the classes of pesticides used by region; the types of matrices used to cause the poisoning, and the incidence of death associated to the poisoning.

After tracing the main types of pesticides used in suspected cases of poisoning analyzed in forensic laboratories, we will be able to discuss the acute toxicity of the different types of pesticides currently associated with poisonings. We can use the literature to preview the acute effects of the ingestion of the foodstuffs and the drinks contaminated with the pesticides. Besides, we can determine the potential of causing death due to the ingestion of the contaminated material.

Other important aspect to be discussed in this chapter is the legal regulation of pesticides trade in Brazil. The Brazilian law is restrictive in the regulation of pesticides use and sale. The permission of pesticides use is specific for the culture exploited and for the class of pesticide released. Besides, the purchase of these compounds, in regard to the most toxic ones, are restricted to the agronomist engineer and requires the presentation of prescription. The legal aspects will alert us about the importance of the fiscalization to inhibit the indiscriminate use of these substances in crimes.

Finally, we will detail the forensic analytical chemistry of pesticides in diverse matrices currently found in forensic institutes. In Brazilian Institutes, the techniques used in forensic chemistry in pesticides analysis most often includes the simple Thin Layer Chromatography

(TLC) with confirmation by using a Gas Chromatography coupled to Mass Spectrometry (GC-MS) technique. In some Brazilian advanced Institutes and in developed countries, other sophisticated methods can be used, such as: Liquid Chromatography coupled to Mass Spectrometry (LC-MS), and Gas Chromatography coupled to Mass Spectrometry in tandem (CG-MS-MS). Alternatively, our group has recently published a simple liquid–liquid extraction step followed by an enzymatic analysis using a freeze-dried preparation of the enzyme acetylcholinesterase obtained from rat brain to detect the presence of aldicarb and other cholinesterase inhibitors in meat products for forensic purposes. We will also present some confirmatory techniques that can be used in a portable presentation, like Fourier Transform Infrared Spectroscopy (FTIR), and Raman Spectroscopy. We will discuss the different types of sample preparation to obtain the best clean-up of the sample. Besides, we will list and discuss the main advantages and disadvantages of the principal instrumental methods used to detect and quantify the pesticides in foodstuffs and drinks matrices.
In conclusion, this chapter will discuss the main aspects of forensic toxicology involving the pesticides and intends to be an alert for other countries that faces the growing problem of pesticides poisoning.

2. Pesticide poisoning around the world

The first global estimates of the extent of pesticide poisoning were published in 1990 by the World Health Organisation (WHO) (WHO, 1990). Based on extrapolations from limited data, it was estimated that 3 million cases of pesticide poisonings annually occurred worldwide, with 220,000 deaths; the majority of which are intentional (Konradsen, van der Hoek et al., 2003).
The WHO estimates, based on data from 2001, that 849,000 people die globally from self-harm each year (WHO, 2002). How many of these cases are a result of poisoning with pesticides is not known. However, poisoning is the most common form of fatal self-harm, such as suicides, in rural Asia, accounting for over 60% of all deaths (Somasundaram & Rajadurai, 1995; Phillips, Li et al., 2002; Joseph, Abraham et al., 2003) and is of far greater importance than hanging and other physical forms of self-harm. Furthermore, a review of poisoning studies reveals that pesticides are the most common way of self-poisoning in many rural areas and are associated with a high mortality rate (Eddleston, 2000). A recent national survey, in the year 2000, in Bangladesh showed that 14% of all deaths (3971 of 28,998) of women between 10 and 50 years of age were due to self-poisoning; the majority of which used pesticides (Yusuf, Akhter et al., 2000). The problem is particularly severe in Sri Lanka (Berger, 1988; Van der Hoek, Konradsen et al., 1998), where pesticide poisoning was the commonest cause of hospital death in six rural districts in 1995 (Sri_Lanka, 1995). In many countries, the widespread availability of acutely toxic pesticides used in agriculture has made the selection of pesticides as the agents of choice for self-harm well known to both healthcare workers and public-health authorities (Nalin, 1973; Kasilo, Hobane et al., 1991; Daisley & Hutchinson, 1998).
A retrospective study, covering the period from January 2000 to December 2005, based on autopsies samples sent to the Laboratory of Forensic Toxicology in the National Institute of Health in Morocco shows that, from total of 3104 analyses performed in the laboratory, 130 cases (4.19%) were related to fatal pesticide poisoning (El Cadi, Mezzane et al., 2008).
In the Morroco survey, fatal pesticide intoxications were classified as suicide in 23.1% of cases, uncertain in 75% of cases, and accidental in only 1.5% of fatal poisonings. The average

of age in all cases was 28±15 years old. The highest frequency of pesticide poisoning (40.8%) was found for those 20-39 years old. The difference between genders in fatal pesticide poisoning was small: 51% male and 48% female. This difference may be explained by the predominance of suicides for females. Another study confirms this result (Abdullat, Hadidi et al., 2006). The Samples were sent for toxicological analysis from the majority of Moroccan cities, including urban areas and rural regions; however, the best represented city was Rabat with 55 cases (42.3%). This representation may be explained by the time of storage of samples from other cities. The data show that insecticides were the most frequent cause of fatal pesticide poisoning (75.2%), followed by aluminium phosphide (21.5%). Among insecticides, organophosphorus compounds were the most frequent (55.4%), followed by carbamates (15.4%) and organochlorine (4.6%). One case of pyrethroid and one case of coumarinic anticoagulant were found (El Cadi, Mezzane et al., 2008).

The incidence of pesticide poisoning in Morocco in the period between 2000 and 2005 was 4.19%, which is compared to other series (Casey & Vale, 1994; Vougiouklakis, Boumba et al., 2006; Soltaninejad, Faryadi et al., 2007). However, there is a lack of systematic study and declaration in Morocco, especially in rural areas. This lack makes the discussion of the results difficult and may underestimate the number of fatal pesticide poisonings. The data show that pesticides are commonly used for suicide. This result is in accordance with previous studies (Eddleston & Phillips, 2004; Nesime, Lokman et al., 2004; Vougiouklakis, Boumba et al., 2005). In fact, in Sri Lanka, 90% of suicides are due to deliberate pesticide ingestion (Manuel, Gunnell et al., 2008), and the WHO reports that pesticides are now the most common method of suicide worldwide (Testud & Grillet, 2007).

In Coimbra, Portugal, a three-year retrospective study was performed between January 2000 and December 2002 in the Forensic Toxicology Laboratory, which received 639 blood samples for pesticide-analysis. In 2000, out of a total of 149 analysis requests, 30 cases were positive, 63.3% from male individuals and 36.7% from female individuals. In 2001, the analysis requests increased (240), as did the number of positive cases (43), 74.4% from male individuals and 25.6% from female individuals. In 2002, the total cases analysed increased to 250, with 38 positive results (73.6% from male individuals and 26.4% from female individuals) (Teixeira, Proenca et al., 2004).

According to this study, among the pesticides, organophosphorus insecticides still constitute the most important class detected in forensic intoxications, representing 63% of the total positive cases, followed by herbicides, with 33% of the positive results. Quinalphos is the most common organophosphorus insecticide, which was present in 32 of the 111 positive cases, followed by the herbicide paraquat, which was detected in 31 cases (Teixeira, Proenca et al., 2004).

2.1 Pesticide poisoning in Brazil

In the 2009 census, the population of Brazil reached 194,400,000, the 5th-largest country in population in the world. Brazil is administratively divided into 26 Federal States and one Federal District. Rio de Janeiro State, with about 16,000,000 people (2010), represents almost 8% of the national population and 0.5% of the national territory area.

In 2005, Brazil was 6th in the world in the rate of homicide of the entire population, counting 25.8 homicides per hundred-thousand inhabitants. In 1997, Rio de Janeiro had the highest homicide index in the country (66.3), and in 2007, it had the 4th-highest index of 52,2, much higher than the national index and higher than that of the country with the highest index, El Salvador, with a 50,1 index (Waiselfisz, 1998).

In 2003, Brazil created a computerised on-line system to transfer in real time the organophosphorus- and carbamates-intoxication information from Legal Medical Institutes (IML, acronym from the Portuguese, Institutos Médicos Legais), known as the National System of Toxico-Pharmacology Information (SINITOX, acronym from Portuguese, Sistema Nacional de Informações Fármaco-Toxicológicas) (SINITOX, 2008).
Table 1 shows the total number of general pesticide-intoxication cases in Brazil, according to SINITOX, involving pesticides used to protect agriculture and horticultural corps against damage, veterinary pesticides, veterinary products and rodenticides (SINITOX, 2008):

Year	Number of Cases
2004	13,942
2005	14,648
2006	15,907
2007	15,377
2008	10,914

Table 1. Number of intoxication cases involving general pesticides in Brazil. Source: (SINITOX, 2008).

The circumstances of these intoxications include individual accident, group accident, environmental accident, occupational accident, therapeutic use, improper medical prescription, self-administration mistake, self-medication, abuse, food ingestion, abortion attempts, suicide attempts or suicide, violence, homicide and ignored cases. The cases with forensic linkage include abortion attempts, suicide attempts or suicide, violence and homicide. Figure 1 shows the percent index with forensic linkage in relation to the total number of cases in Brazil (Table 1) (SINITOX, 2008).
In Rio de Janeiro State, the cases with forensic linkage show the following trend (Figure 2).
The overall death rate per the population involving such occurrences is less than 2%, but this index may be underestimated because the deaths without medical assistance when the body is not sent for a health service might not be reported. In fact, the poisoning rates in Brazil are highly underestimated due to under-reporting. According to the Brazilian Ministry of Health, for each reported poisoning, another 50 were not reported. The reasons for not reporting include lack of access to medical care in poor areas, the lack of a precise diagnosis due to the symptoms of pesticide poisoning being similar to the other health problems and the lack of an efficient information transfer system from the hospitals to the National Centre of Toxicological Vigilance, despite the reporting being mandatory (SINITOX, 2008).
In the state of Mato Grosso do Sul, the Brazilian state with the 8th-highest agricultural production (Rebelo, Vasconcelos et al., 2010), 1,355 cases of voluntary or involuntary poisoning were reported to the Integrated Centre of Toxicological Vigilance of the State Health Department from 1992 to 2002, with an average of 123.2 poisonings/year, 176 of which lead to death. The case fatality rate (CFR) was 3 times higher than that of the average states in Brazil (Recena, Pires et al., 2006).
Some studies have shown an association between exposure to pesticides and symptoms of depression, an important factor often linked to suicides and attempts. In Luz, a micro-region of the state of Minas Gerais, which is the 6th-highest agricultural producer in Brazil, a study

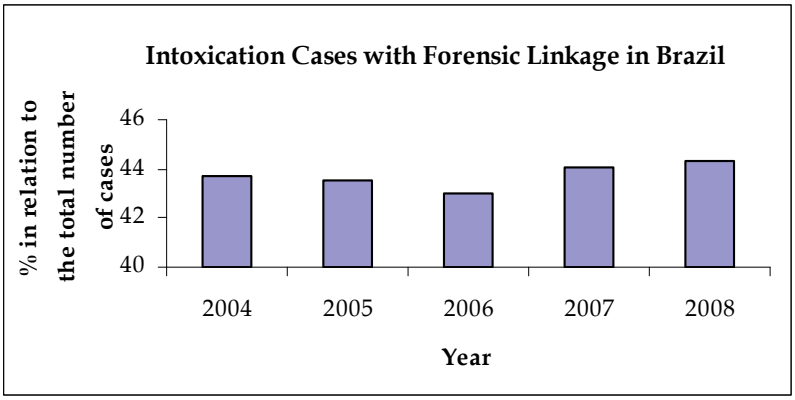

Fig. 1. Intoxication Cases with Forensic Linkage involving pesticides in Brazil

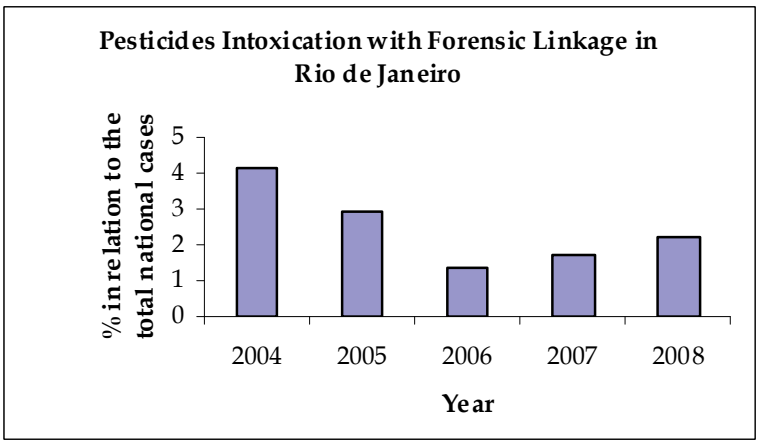

Fig. 2. Trend of cases involving pesticides with forensic linkage in state of Rio de Janeiro

investigated the relationship between poisoning cases and pesticides (Meyer, Resende et al., 2007). From 2000 to 2004, 19 suicides occurred in the region (22.6/100,000 inhab/year), 18 involved male rural workers, and 57.9% by ingestion of pesticides. A group of 50 residents was interviewed and gave biological samples for analysis. The results show that 98% of the residents handled pesticides regularly, 72% had never used any protection equipment, 56% had never read information sheets, and 40% presented poisoning values in the analysis results. The number of suicide incidence was more than twice the highest state average in Brazil, and the number of pesticide-poisoning cases was high compared to SINITOX data (Meyer, Resende et al., 2007).

In Rio de Janeiro State, in the case of humans violent deaths and homicides attempts, and crimes against animals and/or to the environment, when the crime is registered to a police department, the victims, in death cases, are sent to the Legal Medical Institute to perform an autopsy and toxicological analysis. The materials associated to the poisoning collected at the crime scene are sent to the Criminalistic Institute for identification and characterisation.

Rio de Janeiro is not considered to be a typical agricultural state in Brazil and is not among the top ten highest agricultural producers in the country. Despite this fact, the number of intoxication cases involving the use of pesticides is extremely high. Table 2 shows the number of materials examined in the Rio de Janeiro Criminalistic Institute, related to diverse crimes, where the presence of pesticides in the period of 2003 until 2010 was found using numerous analytical methods discussed later in this chapter (data not published):

Year	Identified Materials
2003	77
2004	76
2005	77
2006	71
2007	69
2008	81
2009	78
2010	89

Table 2. Number of materials associated to the poisoning collected at the crime scene that resulted positive in general pesticides analysis in state of Rio de Janeiro.

The slight increase in the number of cases identified in the last three years may be related to the diversification of the analytical techniques used, including enzymatic methods and instrumental analysis.

According to the data available at the Chemical Sector of the Rio de Janeiro Criminalistic Institute and the Rio de Janeiro Police State, out of the 89 investigated cases with positive pesticide identifications in 2010, 10 are related to the irregular commercialisation of the pesticide, 17 are related to crime against animals generally causing domestic-animal deaths, 30 are from materials used in completed suicides, 3 in suicide attempts, 18 are related to homicide attempts, 2 cases are related to the use of pesticide in homicide followed by suicide, and 5 cases have not be determined by the legal guidelines. In the related cases, aldicarb, from carbamate chemical group, was identified 77 times. Other carbamates detected include carbofuran (five times), carbaryl (Rebelo, Vasconcelos et al.) and iprovalicarb (Rebelo, Vasconcelos et al.). Other pesticides identified were second-generation coumarinic derivatives (six cases), picloran (two cases), cipermetrin (two cases), deltametrin (one case), and DDVP (one case). In two cases, the association of two pesticides among the 89 investigated cases with positive pesticide identifications was identified. Aldicarb was present in 78.6% of all cases, and at least one of the compounds identified was from the carbamate class in 85.4% of all cases (data not published).

3. Toxicity of carbamates

The numerous cases of pesticide poisoning all over the world and in Brazil, especially involving the use of the carbamate compound aldicarb, described in the previously sections, is consistent with the acute toxicity of this substance. In order to understand the lethal capacity of this pesticide we will briefly describe the well known toxicology of the

carbamates. Then we will describe some studies with human volunteers to highlight the carbamate amount required to cause human death by oral ingestion.

3.1 Carbamates toxicology

The primary mode of aldicarb toxicity is cholinesterase inhibition. Carbamate insecticides are known to directly affect the enzyme acetylcholinesterase (AChE), which is associated with the outer surface of membranes. This results in a buildup of acetylcholine (ACh), which acts on the plasma membrane to produce the primary expression of neurotoxicity (Blum & Manzo, 1985). It is commonly accepted that carbamates interfere with the ability of AChE to break down the chemical transmitter ACh at synaptic and myoneural junctions, although the precise biochemical mechanism for this interaction remains an object of discussion. It is known, however, that the same mechanism of action is evident in both target and nontarget organisms. Aldicarb and other carbamate insecticides further cause depression of other cholinesterases ("pseudocholinesterases") in the red blood cells and plasma of humans and other vetebrate species, but the degree of inhibition necessary to produce adverse effects in exposed subjects is speculative and the subject of current research (Risher, Mink et al., 1987).
The relationship for carbamates between intoxication symptoms and cholinesterase activity was first described in a series of experiments in which brain and plasma ChE activities were determined in rats after propoxur was administered intramuscularly at different dosages. The animals were killed at the moment the first symptoms appeared or at a given time after injection. At dosages that did not produce any noticeable symptoms (0.25-1.0 mg/kg), the activities of both brain and plasma ChE were reduced by varying amounts, down to about 60% of the normal level. The dose at which a very slight tremor occurred (2.0 mg/kg) reduced the brain and plasma ChE activities to 470% and 49% of the normal levels, respectively; the animals was killed immediately after this symptom was observed. At higher dosages (10.0 and 50.0 mg/kg) the degrees of inhibition of both brain and plasma cholinesterase closely followed the severity of the symptoms that were produced, with the brain ChE usually showing 5-15% greater inhibition than the plasma ChE (Vandekar, Plestina et al., 1971).
Various cholinesterases have also been identified in the brain, liver, pancreas, intestine, heart, and skeletal muscle of mammals and may be distinguished from one another and from AChE ("true cholinesterases") by substrate and inhibitor specificity. Erythrocyte AChE is a more appropriate indicator of the level of AChE in the central nervous system (CNS) than plasma AChE. Blood ChE generally becomes markedly depressed prior to the onset of cholinergic symptoms, and symptoms do not usually appear until the cholinesterase level reaches 25% of the pre-exposure value. A decrease of 60% in RBC AChE level warrants removal from the source of exposure (Blum & Manzo, 1985).
The relationship between brain cholinesterase inhibition and carbamates toxicity has also been studied. In another series of experiments, the relationship between the brain and plasma cholinesterase activities and the degree of symptoms was studied during the infusion of propoxur into the jugular vein of rats (Plestina, unpublished data). Different rates of infusion, ranging from 0.04 to 0.80 i.v.-LD50/hour, were used. At given time intervals, the animals were sacrificed and their brain and plasma ChE levels were determined spectrophotometrically (Ellman, Courtney et al., 1961) and the analytical procedure was completed within 12 min after the animals had been decapitated. Three main symptoms (tremor, muscle fasciculations, and salivation) were regularly recorded and were arbitrarily classified according to 5 degrees of intensity. These results show good correlation

between the activities of the two enzymes and good agreement between the degree of enzyme depression and the intensity of symptoms, the onset of symptoms being recorded only after the brain ChE activity dropped to about 50% of the normal value. It may be noted that, during the second hour of infusion, a steady state was reached regarding enzyme inhibition and symptom severity (Vandekar, Plestina et al., 1971).

Signs and symptoms of aldicarb intoxication are typically cholinergic and may be ameliorated by the administration of atropine sulphate. Because AChE is present in substantial excess at cholinergic synapses, 60% to 90% of the enzyme must be inhibited before the onset of cholinergic dysfunction (35). Symptoms of AChE inhibition and subsequent accumulation of ACh in nervous tissue and effector organs mimic the muscarinic, nicotinic, and CNS actions of ACh and may be categorised as follows (Blum & Manzo, 1985):

1. Muscarinic Signs. The stimulation of muscarinic receptors (found primarily in the smooth muscle, the heart, and exocrine glands) results in the following symptoms:
   a. tightness in the chest and wheezing due to bronchoconstriction;
   b. increased bronchial secretions, salivation, lacrimation, and sweating;
   c. increased gastrointestinal tone, with consequent development of nausea, vomiting, abdominal cramps, diarrhea, and involuntary defecation;
   d. frequent contraction of the smooth muscle of the bladder, resulting in involuntary urination;
   e. bradycardia that can progress to heart blockage;
   f. constriction of the pupils.
2. Nicotinic Signs. The accumulation of Ach at the endings of motor nerves to skeletal muscle and autonomic ganglia results in the following symptoms (Blum & Manzo, 1985):
   a. Muscular effects, including easy fatigability and mild weakness, followed by involuntary twitching and cramps. Weakness affects the muscles involved in respiration and contributes to dyspnea, hypoxemia, and cyanosis.
   b. Nicotinic actions at autonomic ganglia may, in severe intoxication, mask some of the muscarinic effects. Thus, tachycardia caused by stimulation of sympathetic ganglia may override the usual bradycardia due to muscarinic action on the heart. Elevation of blood pressure and hyperglycemia also reflect nicotinic action at sympathetic ganglia.

Without going into a lengthy discussion of the morphology and function of the mammalian neuromuscular system, the transmission of electrical impulses between nerves and at myoneural junctions generally occurs through the release of chemical transmitters that bind with specific receptors on the postsynaptic terminal or motor end plate, respectively. As the chemical transmitter, ACh in certain nerve synapses and at neuromuscular junctions binds to the receptor sites; an esterase (AChE) rapidly hydrolyses the ACh into acetyl and choline fractions so that the stimulated nerves or muscles are not continually excited. Essentially, aldicarb and other cholinesterase inhibitors in some way prevent the breakdown of ACh and the subsequent return to a more normal or resting state for the nerve and/or muscle cells (Risher, Mink et al., 1987).

3.1 Studies of carbamates toxicity in volunteers

In order to evaluate the toxicity of carbamates in humans, a 42-year-old male volunteer (90 kg body weight) ingested 1.5 mg of propoxur per kg of body weight about 2 hours after his

usual "continental" breakfast. The lowest erythrocyte cholinesterase level (27.0 %Y of normal) was observed 15 min after ingestion. No signs were observed at that time, but moderate discomfort, described as "pressure in the head", was present. Blurred vision and nausea developed 3 min later. Twenty minutes after ingestion, the subject was pale and his face was sweating; his pulse rate was 140/min (before ingestion it was 76/min) and his blood pressure was 175/95 mm Hg (before ingestion it was 135/90 mm Hg). Within the next 10 min, pronounced nausea, with repeated vomiting and profuse sweating, developed. These symptoms lasted, with no change in intensity, from about the 30th minute until about the 45th minute, and during this period, erythrocyte cholinesterase activity recovered from a level of 50.4 % to one of 55.5 % of its normal value. One hour after ingestion, the subject was feeling better and his sweating was less pronounced, but he still felt nauseated and tired. His pulse and blood pressure were found to be normal 10 min later, and 2 hr after ingestion, he was feeling well and he had a complete lunch and dinner without discomfort. The rapid disappearance of symptoms was consistent with the further rapid recovery of erythrocyte cholinesterase activity (Vandekar, Plestina et al., 1971).

Studies examining the acute effects of aldicarb administered orally to human volunteers show the same pattern of rapid acetylcholinesterase inhibition and rapid recovery seen in experimental animal models (NN Hamada, unpublished data). In human subjects, following two preliminary analyses of blood acetylcholinesterase activity, groups consisting of four adult male volunteers each were given aqueous solutions of aldicarb at acute oral doses of 0.025, 0.05, or 0.1 mg/kg. In a similar trial, two subjects were given doses of 0.05 or 0.26 mg/kg (Baron & Merriam, 1988; WHO, 1991; FAO/WHO, 1993). In both trials, individuals were monitored prior to aldicarb exposure and served as their own controls. Observation for signs of poisoning and measurements of whole-blood acetylcholinesterase activity was made for 6 hr following treatment (Baron, 1994).

Acute cholinergic signs and symptoms of overexposure were only observed in subjects exposed to a dose of 0.1 mg/kg or higher. Clinical signs of overexposure were not noted at doses of 0.05 mg/kg or lower. By 6 hr after administration, acetylcholinesterase activity had returned to normal, and clinical cholinergic signs and symptoms had disappeared without medical treatment. A dose-related depression from pre-trial values of whole blood acetylcholinesterase was observed in all individuals, mostly for 1 to 2 hr after exposure (Baron, 1994).

In an experimental study with human subjects, three groups of four adult males, all in good health, were administered single oral doses of aldicarb (analytical grade, 99.2% pure) in water solutions corresponding to 0.1, 0.05, or 0.025 mg insecticide/kg body weight. Blood cholinesterase levels were monitored both before and after dosing, and the symptoms resulting from the treatment were observed by physicians. Blood samples were collected from all subjects at 18 hr and 1 hr before ingestion of the aldicarb and at 1, 2, 4, and 6 hr after dosing. A maximum dose of 0.1 mg/kg body weight was selected based upon the 0.1 mg/kg body weight no-effect level determined in the 2-year rat feeding study of Weil and Carpenter, while the other dosages selected for this experiment were one-half and one-fourth of the rat NOEL. Subjects receiving the 0.1 mg/kg dose manifested a variety of cholinergic symptoms including malaise, weakness in the arms and legs, pupils that were contracted and nonreactive to light, epigastric cramping pain, sweating of hands and forehead, air hunger, frequent yawning, salivation, slurred speech, nausea, and vomiting. The aldicarb-induced cholinesterase depression was reported to be rapidly reversible, and

by 6 hr after administration, all symptoms had disappeared and the subjects reportedly felt normal again (Risher, Mink et al., 1987).
According to the data above, the mean dose of aldicarb responsible for acute intoxication in humans is about 0.1 mg/kg. The only commercial product containing aldicarb in Brazil is Temik15®, which has a declared percentage of 15% of aldicarb in its composition. Each Temik15® grain has a mean weight around 0.3 mg, which gives an aldicarb mean mass of 45 µg per grain. Thus, the minimum number of Temik15® grains necessary to cause an acute intoxication in humans, and possible the human death, is about 140 grains. Our casuistic at Carlos Éboli Criminalistic Institute includes the analysis of foodstuffs used to cause death (homicide or suicide) containing significantly more than 140 grains of Temik15®.

4. Legal regulation

Several countries use the legal regulation of pesticides trading and utilization in order to control and prevent the acute deaths associated with the most toxic compounds described above. In this section we will discuss the effects of the legal regulation of pesticides in some countries and the perspectives for the future application of pesticides in agriculture. We will also explore the current situation of pesticides use in Brazil and the impacts of the legal regulation of these compounds in our country.
Eddleston et al. (2002) discussed four different avenues with potential for reducing the use and availability of pesticides important for acute poisonings: voluntary guidelines, safe-use initiatives, and international policy instruments; changes in farming practice, namely integrated pest management (IPM) and plant biotechnology; direct restrictions of pesticide use; and the introduction of a minimum pesticide list (Eddleston, Karalliedde et al., 2002).
In the early 1980s, debate about the effects of uncontrolled pesticide use on health in the developing world grew worldwide. International organisations, national governments, and industry all responded to these concerns with a series of non-binding proposals (Konradsen, van der Hoek et al., 2003).
The major response was the production of the International Code of Conduct on the Distribution and Use of Pesticides in 1985 by the Food and Agriculture Organisation (FAO) of the United Nations (FAO, 2002). In November 2002, FAO adopted a revised Code of Conduct incorporating concerns and experiences generated since the drafting of the previous version. The Code attempts to rationalise the use of pesticides and reduce the health and environmental risks associated with pesticides establishing:
(...) voluntary standards of conduct for all public and private entities engaged in or associated with the distribution and use of pesticides, particularly where there is inadequate or no national legislation to regulate pesticides. (Article 1.1)
In particular, it wished to ensure that the benefits derived from the use of pesticides be achieved without significant adverse effects on people or environment (Article 1.3).
The new version of the Code of Conduct adapts a 'life-cycle' concept to address all stages from product development to the final disposal of containers and products. Manufacturers are requested to supply only pesticides of adequate quality, packaged and labelled as appropriate for each specific market, and to retain an interest in the product as far as the ultimate consumer, keeping track of uses and the occurrence of problems requiring changes in labelling, directions for use, packaging, formulation or product availability. In particular, the Code states that pesticides whose handling and application require the use of personal protective equipment that is uncomfortable, expensive or not readily available should be

avoided, especially in the case of small-scale users in tropical climates (Article 3.5) (Konradsen, van der Hoek et al., 2003).

The Code further stipulates that highly toxic and hazardous products (such as WHO Classes Ia and Ib) may be prohibited for importation, sale and purchase if other control measures or good marketing practices are insufficient to ensure that the product can be handled with acceptable risk to the user (Article 7.5). Many countries do not enforce these standards, and if the Code were to be followed, the use of Class I pesticides would be prohibited in many developing countries. This issue relates especially to occupational exposure because the required safety equipment is expensive and cumbersome in the tropics and almost never worn (Konradsen, van der Hoek et al., 2003).

National governments are called upon in the Code of Conduct to have the overall responsibility to regulate the availability, distribution and use of pesticides in their countries and should ensure the allocation of adequate resources for the mandate (Article 3.1).

In spite of international efforts to support developing countries in achieving the capacity to implement and supervise the Code of Conduct, many developing countries still do not have this necessary oversight capacity (Konradsen, van der Hoek et al., 2003).

In the second global survey finalised in October 1994 to assess the state of implementation of the Code of Conduct, it was concluded that, although progress had been made towards compliance with various provisions of the Code, there is a continuing need by governments for assistance, especially in the Asia and Pacific region. More than half the national agencies responding to the questionnaire indicated a need for technical assistance and increased government support to strengthen their national capacities and infrastructures necessary to effectively operate their pesticide-control schemes (FAO, 1996).

The crucial role of national-government capacity in enforcing the Code was explicitly acknowledged by the then-Director of the FAO in his introduction to the Code in 1985 (FAO, 1990):

In the absence of effective pesticide registration processes and of a governmental infrastructure for controlling the availability of pesticides, some countries importing pesticides must heavily rely on the pesticide industry to promote the safe and proper distribution and use of pesticides. In these circumstances, foreign manufacturers, exporters and importers, (...), must accept a share of the responsibility for safety and efficiency in distribution and use.

Unfortunately, despite increasing support to improve the capacity of national agencies since the mid-1980s, policing of the Code is still so severely hampered by a lack of resources and political will that there is still no effective mechanism to enforce it or publicise violations. Furthermore, the Code of Conduct does not give direct attention to the issue of self-harm with pesticides and therefore fails to provide policy guidelines or assign responsibilities on this complex issue. Also, the revised Code does not directly call for an elimination of the most hazardous pesticides and that adherence to the great majority of the articles in the Code is voluntary will likely reduce its overall effect on the number of deaths from acute poisoning (Konradsen, van der Hoek et al., 2003). Recently, an updated version of this Code was published, reinforcing these concepts (FAO, 2010).

Piola and colleagues recently showed that a national ban on the organophosphate parathion reduced the number of deaths reported to their poison centre in Rosario, Argentina (Piola & Prada, 1999; Piola, Prada et al., 2001). Between 1977–1985 and 1990–1994, 21 lethal pesticide-poisoning cases were reported to the centre, including 15 adult cases of self-poisoning and 4 accidental cases in children, 17 of which were due to parathion. Due to the high number of

deaths occurring nationally with this pesticide, it was banned throughout Argentina in 1994. The last death from parathion in Rosario was reported in 1995. There was a marked fall in the number of deaths due to poisoning from 16 in the first half of the decade to 4 in the second half of the decade (Piola, Prada et al., 2001).

Parathion was also banned in Jordan in 1981 after studies showed that it was responsible for >90% of deaths from pesticides in the country. The total number of poisoning deaths undergoing autopsy in Amman fell from 58 in 1978 and 49 in 1980 to 28 in 1982 and 10 in 1984 (Konradsen, van der Hoek et al., 2003).

Paraquat was introduced to Samoa in 1974. Soon after, public-health officials noticed a growing epidemic of self-poisoning. The total suicide rate increased from 10/100,000 in 1974 to 28/100,000 in 1978 to 50/100,000 in 1982. Because of this epidemic, a community-based campaign was set up to reduce its use for self-harm. At the same time, however, imports fell temporarily due to financial problems. The suicide rate fell rapidly, mirroring the fall in imports, to 15/100,000 within 2 years. Interestingly, the suicide rate between 1984 and 1988, although much reduced at 15–20/100,000, is still more than 80% due to paraquat and has continued to rise. Suicide with this pesticide had become the method of choice. The pesticide was never banned and remains the cause of around 80% of all self-harm deaths (Zinn, 1995). Banning paraquat is still the subject of active debate in Samoa today (Konradsen, van der Hoek et al., 2003).

Since the late 1980s, the Sri Lankan government took an active role in determining which pesticides can be used in the country. By the mid-1990s, all Class I pesticides were banned in Sri Lanka. As a result, the number of deaths due to metamidaphos and other Class I organophosphorus fell dramatically, as documented for one district hospital. Unfortunately, another highly toxic (although Class II) compound, the organochlorine endosulfan, then replaced the Class I organophosphorus in agricultural practice. The number of self-poisoning deaths rose as endosulfan became more popular. Endosulfan was therefore banned in 1998 and deaths fell from 50 to 3 in the same district hospital over the next 3 years. No single compound has since taken its place, but there is currently an increase in the importance of WHO Class II organophosphorus, such as dimethoate and fenthion. Sri Lanka is attempting to shift to less toxic pesticides in the hope that this shift will reduce the number of deaths from deliberate self-poisoning. Thus far, these attempts have been complicated by the replacement pesticides also being sufficiently toxic to cause deaths from self-poisoning. Compared to the early 1990s, there has been little overall effect from switching from one form to another. Future attempts to ban pesticides must carefully predict the likely consequences of switching to another pesticide in agricultural and self-harming practice (Roberts, Karunarathna et al., 2003).

An increased use of pesticides in the Philippines during the 1970s coincided with a 27% increase in mortality from non-traumatic causes among economically active men. The incidence in men between the age of 15 and 34 of stroke—a condition rare in this age group but that can be confused with some types of acute pesticide poisoning—also rose during this period but then fell by more than 60% in the two years following a ban on endrin (Loevinsohn, 1987).

A recent Chinese study concluded that a major component of preventive efforts to reduce acute poisoning in rural areas will be restricting the availability of pesticides. The authors indicate that the availability of potent pesticides in the homes of most residents makes this type of poisoning the preferred method of self-harm (Phillips, Li et al., 2002). This study also supports the idea that not all people who die following acts of self-harm actually wish to die

(Eddleston, 2000). In a district in Sri Lanka, peaks of fatal self-harm poisoning coincided with ploughing seasons. This trend was not because people were more determined in their self-harm attempts in this season but because it was the time when farmers use paraquat (Hettiarachchi & Kodithuwakku, 1989). Furthermore, the often impulsive behaviour linked with the ingestion of pesticides and the influence of alcohol during the events makes it important to restrict pesticide availability in homes (Konradsen, van der Hoek et al., 2003).
Overall, these studies suggest that limiting the availability of toxic pesticides will reduce the number of deaths from poisoning and the number of deaths from self-harm. A similar reduction in self-harm deaths has occurred in the UK, Japan, Australia and India following the replacement of barbiturates with benzodiazepines as the most common sedative prescription (Singh, Singhi et al., 1997). Restrictions on availability are currently being adopted by the Ministers of Health of eight Central American and Caribbean countries. These countries have agreed to ban the 12 most problematic pesticides in their region together with a list of 115 pesticides that are restricted in at least one of these countries. The organizers of this approach—the PlagSalud initiative—hope to reduce pesticide poisoning in Central America by 50% by restricting the most toxic pesticides (Wesseling, Aragon et al., 2001). No results are yet available.

4.1 Legal regulation In Brazil

In 2008, Brazil reached the alarming position of the greatest pesticide end user in the world. The National Union of Industries for Agricultural Defensive Products (Sindag, acronym from Portuguese) survey showed that pesticides sales totaled $7.125 billion USD, when the country with the second-highest index, the United States, had $6.6 billion USD in pesticide sales. It is important to highlight the marked increment in Brazilian rural production and export rate and, consequently, the use of agricultural products. Brazil is also the third largest exporting country in the world, losing only to United States and to the European Union, but Brazil was in the sixth place in 2000. The agricultural-exporting mean rate between the years of 2000 and 2008 has grown by 18% compared to 10% for the European Union and 8% for the United States (Rebelo, Vasconcelos et al., 2010).
There are currently about 600 active compounds used in the formulation of pesticides, legally registered all over the world for use as agricultural chemical defence. Of these compounds, about 350 are part of the composition of 98% of the most-used pesticides and 80% are commonly used in the agriculture of Latin American countries, such as Brazil.
The top ten most-used pesticides in Brazil are responsible for 76,45% of all commercialised pesticides. These compounds are as follows (in decreasing order): glyphosate and salts, cypermethrin, mineral oil, vegetal oil, sulphur, 2,4-dichlorophenoxyacetic acid, atrazine, metamidophos, acephate and carbendanzim. In a class scale, organochlorines are the most-used compounds, followed by organophosphorus, carbamates, and pyrethroids (Rebelo, Vasconcelos et al., 2010).
In Brazil, the 1989 Pesticide Law regulates the research, experimentation, production, packing, labeling, transport, storage, commercialization, final destination of packing, register, classification, control and supervision of pesticides and related compounds. Despite the constant updates of the monographs of pesticides compounds, several substances banned in the European Union and in United States are still used in Brazilian agriculture. Metamidophos, for example, will be totally prohibited in Brazilian farms from June 2012, according to Sanitary National Agency (ANVISA, acronym in Portuguese).

Aldicarb, used as insecticide, acaricide or nematicide in potato, coffee, sugar cane, and citrus cultures, is considered extremely toxic. This substance is produced in only one industry in the United States. The use of this substance as a pesticide is prohibited in more than 60 countries, including members of the European Union. In the state of California in the United States, close to 2000 people showed intoxication symptoms after eating contaminated fruit in 1985. Despite this great toxic potential, the deadline for use in the USA is August 2018, according to the factory producer. In Latin America, this substance is legally used in Brazil, Chile, Argentina, Peru and Colombia, among other countries. This pesticide is also used in Australia and in South Africa; in the latter, there are several problems related to misuse, as in Brazil. The formulated aldicarb product to Brazil is special and includes the addition of a distressingly agent, denatonuim benzoate, to avoid accidental ingestion by humans and/or animals. This product arrives in Brazil, and a sole importer in São Paulo State receives and distributes it to a few allowed resales in three states, only to registered and certified farmers. Aldicarb is one of the five most-used pesticides in the state of Bahia.

Several banned pesticides in European Union and in United States are still commonly used in Brazilian crops. The regulatory agencies are working very slow in the law revision of the real necessity of using these pesticides. In 2008, 14 substances were sent to be evaluated; from this group, eight (methyl parathion, lacotophen, phorate, carbofuran, abamectin, tyran, paraquat and glyphosate) are still waiting for government decision (February of 2011). Cyhexatin, used in citrus, has been banned since 2011. In a revision published in 2010, trichlorfon was banned in Brazil. In addition, the commercialisation of all the formulated products with endosulfan will be cancelled from 2013 by manufacturer solicitation, and the use and application of phosmet products were reassessed. A 2011 publication has mandated the banning of methamidophos starting in June 2012.

Regarding aldicarb, the deadline of its use is 2018, according to information from the manufacturer. In Brazil, in relation to intoxications, the main problem is the irregular commercialisation of aldicarb-based products in small packaging containing about five grams of the product for use as a rodenticide. Nevertheless, this substance has one common fault as a rodenticide; it produces symptoms very rapidly. Rodent behaviour is such that an individual encountering a new food for the first time will normally test feed and may not take a substantial quantity for many hours or even days (Hadler & Buckle, 1992). If the bait causes distressing symptoms during the test-feeding period, the rodent is intelligent enough to recognise cause and effect and becomes “poison shy” or 'bait shy.' However, it is very common to find the irregular street trade of this product in big urban centres, far from agricultural regions. To the facility of obtaining this product in small doses, it is usual the association of aldicarb in crime scenes, generally in the typical grains of the commercial product and in the package used in irregular trade.

Brazilian legislation imposes that subdivision and packaging of pesticides are restricted to the manufacturer or to the handler, supervised by the producer, and used in conditions and places pre-authorised by the government. In addition, pesticides can only be sold directly to the final user and with the legally licensed professional prescription submission. This irregular trade also disrespects the Environmental Law that considers the production, processing, packaging, import, export, trade, gift, transport, maintenance, keeping, storing or use of substances that are dangerous or harmful to human health or the environment to be a crime, in disagreement with specific legislation.

5. Forensic analysis of pesticides

As forensic laboratories work for the Justice, their responsibility is very peculiar. Analysis certifies the eventual correlation between illness and toxicity, and in medico-legal cases, the presence of a poison furnishes the first and foremost evidence. Whichever is the cause of poisoning (therapeutic, industrial or criminal), even if damage is not voluntary, common law imposes at least compensation for the ill effects that follow intoxication. This aspect, which is of great importance, shows the permanence of the relationship between toxicology and forensic medicine, which requires an exceptional security of the methods used in the laboratory and extreme prudence in statement and interpretation of results.

The wide variety of matrices and pesticides associated with homicides and suicides, as described above, requires the development of detection and quantification techniques with adequate precision and accuracy of pesticides residues in different matrices, such as water, soil, foodstuffs and biological matrices.

The main pesticides associated with intoxications and the instrumental methods used in forensic analysis of these compounds in different matrices are organochlorines, organophosphorus, and carbamates.

Organochlorine pesticides are composed by carbon and chlorine atoms in several isomers conformations from hexachlorocyclohexane and from cyclodienes. The main representative substances of this group are cyclohexanes (BHC, DDT, lindane, pentachlorophenol) and cyclodienes (aldrin, endrin, endosulphan). The organophosphorus have, with no exception, a central pentavalent phosphorus atom linked to an oxygen or sulphur atom by a double covalent linkage and can be represented by the compounds Malathion, Parathion, and others. Finally, the carbamate pesticides are compounds derivated from carbamic acid, and their main substances analysed in toxicological laboratories are aldicarb, carbofuran and carbaril (Figure 3).

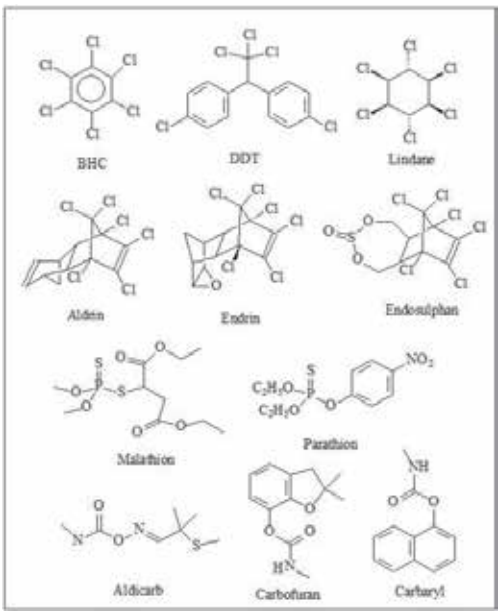

Fig. 3. Chemical structures of some pesticides

In the last few years, several studies have been published based on the development of more precise, cheaper and faster analytical procedures. Analytical chemistry has witness a significant improvement with the establishment of new methodologies by the use of more sensible instrumental techniques that use small quantities of samples, such as mass spectrometry (MS), Fourier Transform Infrared Spectroscopy (FTIR) and Ultraviolet-Visible Spectrometry (UV-Vis) and Raman Spectrometry (RAMAN).

Pesticide analysis in different matrices is traditionally accomplished using chromatographic techniques and liquid and gas chromatography in particular. These techniques are used because of their capacities to separate the compounds present in a sample and to permit the identification and quantification of these compounds using specific detector systems. The identification and quantification of pesticides can be accomplished by coupling the detectors, such as electron capture (Suchan, 2004), fluorescence (Nedelkoska, 2004), UV-VIS (Nedelkoska, 2004), flame ionisation (Engelmann, 2003) and MS, with the chromatographic systems.

For gas-chromatography (GC) separation, a great variety of stationary phases have been used in capillary columns. Most research groups have only used one column for the same class of pesticides. However, some authors have reported the use of two columns with different polarities and different geometric parameters under the same or different chromatographic conditions to confirm the peak identification. Different columns have also been used to compare mass spectrometry detectors to classical detectors for analysing residues from the same class of pesticides, even more so when compounds from different pesticide classes were analysed in one run in gas chromatography coupled to mass spectrometry (CG-MS). GC is combined with different types of detection methods, mainly depending on the class of pesticides to be detected. Electron-capture detection (ECD) is often employed for organochlorine and pyrethroid analyses. Electrolytic-conductivity detection after GC separation has also been proposed for the detection of several pesticides residues, including organochlorines, pyrethroids, triazines and carbamates. Both flame photometric detection (FPD) with a phosphorus filter and nitrogen-phosphorus detection (NPD) have been used for organophosphorus detection. Besides these conventional element-specific detection methods, GC use with mass spectrometric detectors, including single-quadrupole, ion-trap, and triple-quadrupole mass spectrometers, has been adapted to the analysis of pesticides. The use of MS has the advantages over conventional element-specific detectors of being able to determine pesticides from different classes (organochlorines, organophosphorus, pyrethroids, triazines and carbamates) in the same acquisition run (LeDoux, 2011).

Parveen et al. monitored pesticide residues belonging to different pesticide classes, such as organochlorines, organophosphorus, pyrethroids and carbamates, in 206 vegetables samples from Karachi, Pakistan using HPLC and GC-FID (Parveen, Khuhro et al., 2005).

Pesticide residues of organochlorines, organophosphorus, pyrethroids and carbamates groups in fruits and vegetables were monitored in Sao Paulo City, Brazil by Gebara et al. (Gebara, Ciscato et al., 2005). A total of 2223 samples comprising 700 vegetables and 1523 fruits collected from general stores and wholesale shops were analysed for 100 pesticide residues of insecticides and fungicides with GC equipped with different detectors (ECD, NPD, FPD).

Fresh foodstuffs from El Ejido, Almeria, Spain were collected from September 2001 to July 2002 to monitor 81 multiclass pesticides (organophosphorus, organochlorines and pyrimidine) in approximately 4000 vegetable samples by Arrebola et al. (Arrebola, 2003).

The analysis was conducted through single injection using gas-chromatography chemical ionisation and electron ionisation tandem mass spectrometry (GC–MS–MS).The vegetable samples were extracted in dichloromethane for multi-pesticide residues of dichlorvos, methamidophos, mevinphos, acephate, omethoate, lindane, diazinon, disulfoto, parathionmethyl, chlorpyrifos, malthion, fenthion, dicofol, ethion, endosulfan-I, II, permethrin, cyfluthrin and deltamethrin and were analysed with GC–MS–MS.

Liquid chromatography has been used for the analysis of polar and/or non-volatile and/or thermally labile pesticides for which GC conditions were not suitable, mainly carbamates and triazines. Various stationary phases have been tried for the separation of pesticides (Pacáková, 1996), but reverse phase is generally preferred (LeDoux, 2011). Liquid chromatography has also been combined with conventional detectors, such as fluorescence or UV detectors, to identify and quantify pesticides. The first has been used for carbamates analyses and the second for triazines analyses. Liquid chromatography coupled with a diode-array detector (LC-DAD) has been used in triazine and carbamate analyses (Baranowska, 2005; Baranowska, 2006). Recently, liquid chromatography has been coupled with different kinds of mass spectrometric detectors, including single-quadrupole, ion-trap, tandem-MS, and time-of-flight MS (TOF-MS), to determine pesticides in aqueous and solid environmental samples and in foods of vegetable origin (Pico, Blasco et al., 2004; Hercegová, 2007; LeDoux, 2011).

Recently, the number of compounds that are only amenable to liquid-chromatographic techniques has increased in relation to those amenable to the previously widely used gas-chromatographic techniques. For this reason, liquid-chromatography mass spectrometry (LC-MS/MS) detection systems are increasingly commonly used (Sannino, 2004; Soler, 2004; Liu, 2005; Soler, 2005; Soler, 2005; Soler, 2007; Kmellár, 2008). B. Kmellar et al. developed a sensitive multi-residue pesticide method for the determination of 160 multi-class pesticides in different kinds of vegetables using an LC-MS/MS system (Kmellár, 2008).

Liu et al. determined carbamates and organophosphorus in 25 samples, including vegetables and fruits, using LC-MS. All samples were collected from local markets and supermarkets in China (Liu, 2005).

Special care must be taken when using the techniques mentioned above that the preparation, extraction and clean-up of the samples are performed well, especially the most complex steps, to reduce the matrix effects. Even with the advent of advanced hyphenated techniques based on mass spectrometry, some complex fatty matrices usually require extensive sample extraction and purification (Gilbert-López, 2009). There are diverse methods of extraction described in the literature, such as solid-liquid extraction, liquid-liquid extraction, supercritical-fluid extraction, Soxhlet extraction and microwave-assisted extraction. The best method will depend on the sample matrix to be analysed. Current methods involve the use of one or more of the techniques for the sample-extraction steps. Several solid-liquid extraction and liquid-liquid extraction protocols have been standardised for extracting 23 organochlorines and 22 organophosphorus residues from the fatty foods of animal origin (milk and milk products, meat and meat products, fish and seafood, eggs) (European_Committee_of_Standardization, 1996).

In GC-MS analysis of carbamate, organophosphorus and organochlorine pesticides, the use of solvent extraction is recommended, such as chloroform or dichloromethane. In HPLC analysis, the recommended solvents are acetonitrile or methanol, and for thin layer chromatography, acetone (Passagli, 2009).

Moreover, co-extracted matrix constituents, especially lipids, can interfere with target-compound identification. Several approaches have been attempted to eliminate co-extraction interference from extracts, including freezing centrifugation, liquid-liquid partitioning, gel-permeation chromatography (GPC), solid-phase extraction (SPE), solid-phase micro-extraction (SPME) matrix solid-phase dispersion (MSPD), etc. Out of all of these techniques, the most commonly applied approach for pesticides extraction in fatty vegetable matrices so far is liquid partitioning with organic solvents followed by a clean-up with SPE or GPC (Gilbert-López, 2009).

Cases involving acute fatalities due to ingestion of organophosphorus pesticides, such as chlorpyrifos, diazinon, malathion and parathion, are presented by Mee-Jung Park et al. In this work, SPE and GC-MS were used for the analysis of organophosphorus in post-mortem blood (Park 2009).

Traditional chromatographic methods are effective for the environmental analysis of pesticides but have limitations and require adequate monitoring. Enzymatic methods have been used for many years as an alternative method of the detection of pesticides. The main enzymes used are acetylcholinesterase, butyrylcholinesterase, alkaline phosphatase, organophosphorus hydrolase and tyrosinase. The enzymatic methods are based on the proportional activation or inhibition of the enzyme with the concentration of the pesticide. Research on enzymatic methods of detection and some of the problems and challenges associated with these methods are extensively discussed in a review written by Van Dyk et al. These methods can serve as a tool for screening large samples and can be followed up with the more traditional chromatographic methods of analysis (Dyk, 2011).

Our group in Brazil developed a simple and low-cost methodology based on the inhibition of a stable preparation of the enzyme acetylcholinesterase obtained from rat brain specially adapted for forensic purposes. The method proved to be precise and accurate, detecting as little as 40 μg/kg of the pesticide aldicarb in meat samples (Sabino, Torraca et al., 2010). The technique comprises an initial extraction step with the solvent methylene chloride followed by a colourimetric acetylcholinesterase assay. This method is rapid and cheap, demanding only basic laboratory equipment and glassware. Although the method was validated for use with meats samples, it can be easily adapted for other matrices. Taking into account that all other carbamates are also potent enzyme inhibitors but that aldicarb is the contaminant most frequently found in Brazil, the results of contaminated forensic samples were expressed in aldicarb equivalents. This method could also be adapted to detect thionophosphate insecticides (Cunha Bastos V, 1991; de Lima, Bastos Neto Jda et al., 1996; Sabino, Torraca et al., 2010).

5.1 Forensic analysis of pesticides in Brazil

In our routine at the Chemistry Division of Carlos Éboli Rio de Janeiro Criminalistic Institute (SPQ-ICCE-RJ, acronym in Portuguese), we seldom receive food contaminated with pesticides, especially Temik15®, used in homicide and suicide intoxications. In such cases, the use of a TLC technique is not always capable of revealing the presence of aldicarb due to the lipids presents in food that hinder the chromatographic separation of the compounds. In these cases, the use of the enzymatic methodology described above was applied and showed good results (data not published).

Vibrational spectrometry provides useful, well-established analytical techniques for quantitative determinations of major and minor components from solid, liquid and gaseous

samples. Applications of Fourier-transform infrared (FTIR) and FT-Raman spectrometry can be found in a variety of fields, from pharmaceuticals to paint solvents, and the references found in the literature related to different procedures based on the use of both techniques have grown dramatically in recent years. This effect is probably due to the improvements in FT-based equipment and the decreasing acquisition and maintenance cost, together with the advances in automation that enhance data acquisition. Armenta et al. showed the applicability of vibrational spectrometry, basically FTIR and FT-Raman, for the analysis of pesticides in agrochemical formulations. The main advantage that FT-Raman presents over FTIR spectrometry is the very weak Raman spectra of glass, water and plastic packaging, which allow direct analysis of samples inside glass bottles or plastic bags without opening the package, thus minimising the risk of contamination. In this article, it is clearly shown that these techniques enable fast, non-destructive, precise and accurate measurements, and thus vibrational spectrometry is a promising tool for analysis in the agrochemical-industry samples (Armenta, 2005).

In Brazil, thin-layer chromatography (TLC) is still a common technique used in pesticide identification in forensic toxicological and chemistry laboratories. It is widely used in laboratories throughout the world for food analysis and quality control. Numerous applications of TLC have been reported in the areas of food-composition intentional additives, adulterants, contaminants, and decomposition involving determinations of compound classes. This old technique consists of the separation of compounds in a mixture by differential migration through an adsorbent layer withheld in a plane surface plate. It is also routinely used in many laboratories in the chemical/pharmaceutical and related industries for both qualitative and semi-quantitative work. It is a simple, fast and economic technique. There is considerable literature describing pesticide analysis by TLC with environmental and food monitoring. Marcos P. work describes the use of this technique in carbamate (aldicarb, carbofuran, carbaryl and propoxur) pesticide identifications by applying a mixture of hexane/ethyl acetate (6:4) (Passagli, 2009).

In Brazilian forensic laboratories, especially outside the big urban centres, there is an enormous lack of instrumental methodologies. In these laboratories, the main technique available to the forensic scientist to determine the presence of a pesticide in a material is TLC. The major disadvantage of TLC is its limited sensitivity when compared to other instrumental techniques (GC-MS and LC-MS).

General reviews of pesticide TLC analysis, including some information on the analysis of foods, crops, and other agricultural samples, have been published (Chen & Wang, 1996; Torres, Pico et al., 1996; Sherma, 2000). Diverse papers describing the analysis of synthetic pyrethroids and their metabolites using thin-layer chromatography have been reported: cypermethrin in soil and in animals, deltamethrin in animals (Ruzo, Engel et al., 1979), fenpropathrin in water, soil and plants, fenvalerate in plants, permethrin in insects and plants, permethrin and cypermethrin in soil and plants (Chen & Wang, 1996). Carbofuran and its environmental by-products, hydroxycarbofuran and 3-ketocarbofuran, were analysed using high-performance TLC. Carbaryl, aldicarb, oxamyl, butocarboxim and butoxycarboxim and several other pesticides in drinking water were detected at picogram levels by coupling TLC with an enzymic inhibition test designed for cholinesterase-inhibiting insecticides (Yang, Goldsmith et al., 1996).

Currently, gas chromatography is widespread and extremely valuable in routine analysis in Brazilian forensic laboratories. The ability to couple this technique to mass spectrometry has enhanced its use in drug analysis, pesticide identification in foodstuffs and environmental

samples, and perfume control quality, among others. There are several works describing pesticide identifications using gas chromatography coupled to mass spectrometry (GC-MS). Marcos P. work uses this technique in the identification of some organophosphorus pesticides (chlorpyriphos, armitraz and diazinon) and carbamate pesticides (aldicarb, carbophuran, carbaryl and propoxur) (Passagli, 2009).

Another important technique used in Brazilian forensic toxicological and chemistry laboratories is high-efficiency liquid chromatography (HPLC). This technique is an imperative tool used in forensic laboratories in Brazil and in other countries around the world. It is very common to see the use of this type of equipment in forensic television programs, such as CSI, CSI Miami, Crossing Jordan, and Law and Order, to obtain criminal evidence. The application of this technique is not limited to forensic chemistry but is also used in biochemistry, environmental sciences, pharmacological chemistry, and toxicology. HPLC uses a liquid mobile phase and a finely divided stationary phase in the chromatography system. The main detectors coupled to HPLC are based in the absorption of ultraviolet or visible radiation. Marcos P. Work uses this technique in carbamate-pesticide (aldicarb, carbophuran, carbaryl and propoxur) and in coumarinic-rodenticide (cumatetralil, bromadiolone, brodifacoum and difethialone) identifications, which are the most common compounds used in suicide attempts (Passagli, 2009). A few toxicological laboratories in Brazil have a mass spectrometer coupled to HPLC equipment, but there is a growing number of laboratories using this association due to its great sensibility and to the preparation facility of aqueous samples compared to GC-MS.

The previously cited techniques could also be used in association with other techniques, such as spectroscopic techniques. Recently, SPQ-ICCE-RJ have acquired FTIR equipments. The infrared absorption technique is able to detect microscope residues without sample damage, a substantial forensic advantage allowing future sample re-analysis. The interaction of infrared light with the substance alters the vibrations of atoms, giving important information that permits the identification of the compound. The FTIR spectrometer can be used in diverse matrices (solids, liquids and pastes) and has a coupled library that can be used in the compounds identifications. The FTIR applications are very diverse and have a huge contribution in several pesticide-identification occurrences.

5.1.1 Forensic cases in Brazil

Some suicide and homicide attempts are described next, including photos of materials collected in police occurrences, which were identified as pesticides using the instrumental techniques cited above. The use of the distressingly agent, denatonium benzoate, in the formulation of Temik15® to avoid accidental ingestion may explain why the number of completed homicides using this product is low.

The use of crossing techniques has permitted the unequivocal identifications of the substances, enhancing the results' reliability. About ten years ago, SPQ-ICCE-RJ only used TLC comparative with standard solutions of the pesticides to identify the presence of a pesticide in a sample. With the introduction of other techniques (FTIR and GC-MS), other compounds that were not previously detected have been identified, increasing the toxic-substance positive-identification index and the number of cases resolved. Recently, homicide attempts using juice contaminated with cypermethrin and water contaminated with chlorpyrifos were solved using the GC-MS technique.

The use of FTIR techniques in forensic science in association with other analytical techniques or as a stand-alone method, increases the quality of the results available to assist with the solving of police cases and, consequently, improves justice.

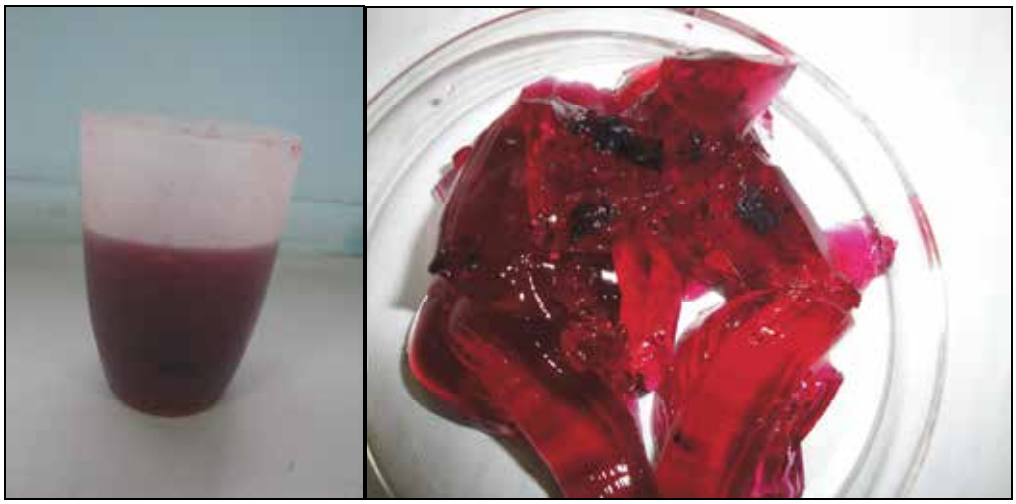

Fig. 4. Homicide attempt. Glass with jelly found in the victim's refrigerator possibly left by his cousin. The grey-coloured grains permeated into the food. The use of TLC, GC-MS, and FTIR instrumental techniques permitted the identification of aldicarb and carbophuran in the grains found in the food.

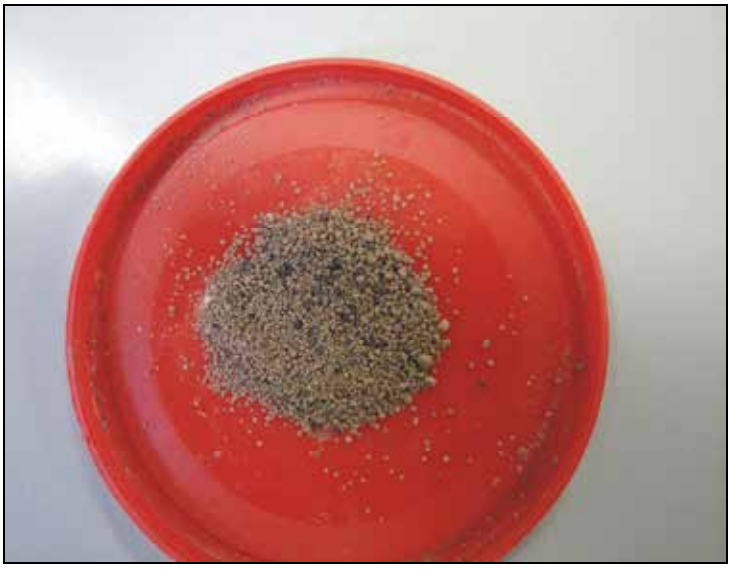

Fig. 5. Homicide attempt against a child possibly accomplished by the stepmother. A tin containing a mixture of chocolate powder and grey-coloured grains. The use of TLC and FTIR instrumental techniques permitted the identification of aldicarb in the grains found in the food.

Fig. 6. Homicide Attempt. Mixture of coffee and typical rose grains. The use of TLC permitted the identification of a coumarin compound used in rodenticide formulations.

Fig. 7. Suicide. Food (pasta) found next to the corpse. On the surface of the food, grey-coloured grains were identified. The use of TLC and FTIR instrumental techniques permitted the identification of aldicarb in the grains found in the food.

6. Conclusions

This study has clearly shown that pesticides, specially carbamates, are extensively used in Brazil and other countries for self-poisoning and homicides attempts, and contributes to the population patterns of morbidity and mortality in these developing nations. These results point to the need of a deep investigation of poisonings in these areas and for the implementation of strategies that would minimize the number of cases related to intentional and unintentional pesticide poisoning.

In our text we have presented several examples where government restriction to the most hazardous pesticides were of great importance in reducing the number of poisoning cases and deaths related. Government actions, such as educational and preventive programs, stronger regulation and a more efficient information system are essential to access and avoid intentional poisoning involving the use of pesticides. Moreover, the adequate control of pesticides trade must be effective to prevent the free access of the population to these toxic compounds.

Action has to be effective at least in the areas of pesticide policy, pesticide information, mental healthcare, clinical management of pesticide intoxication and occupational safety. The role of forensic sciences in this context is crucial to identify the compounds involved in the poisoning and characterize the criminal intent in an homicide attempt, for example. Besides, the results obtained in forensic laboratories could be used to generate a valuable database of the most common used pesticides for poisoning. Such database would help the activities of the regulatory agencies concerning the compounds most used in crimes against life. However, taken into account the complex chemical structures and wide variety of pesticides and matrices used in human poisoning, the Criminalistic Institutes must have adequate and validated techniques and advanced analytical equipments to be able to perform the chemical analysis of these samples.

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14

Characteristics and Trends with Respect to Unintentional Pesticide Poisoning Mortality and Hospitalization in Taiwan, 1999-2008

Wu-Chien Chien[1], Ching-Huang Lai[1], Jouni J.K. Jaakkola[2], Lu Pai[3], Senyeong Kao[1], Jin-Ding Lin[1] and Yu-Chen Hung[1]
[1]*School of Public Health, National Defense Medical Center, Taiwan*
[2]*Center for Environmental and Respiratory Health Research, Institute of Health Sciences, University of Oulu, Oulu,*
[3]*Institute of Injury Prevention & Control, Taipei Medical University, Taiwan,*
[1,3]*ROC*
[2]*Finland*

1. Introduction

Pesticides are a group of chemicals widely used in agriculture to control insects, microorganisms, fungi, weeds, and other pests. Control of these pests serves to increase crop yield and decrease manual labour.

In 2000 and 2001, over 5 billion kilograms of pesticides were used annually throughout the world [1, 2]. Between 1999 and 2008, 9.6 million kilograms of pesticides were used annually in Taiwan. This was responsible for 0.8% of the total pesticide use in the world [2]. Efforts should be made to assess the risks of pesticides in the general population in terms of the extent and types of exposure.

A number of approaches have been taken by researchers to acquire information on pesticide poisoning in Taiwan. Pesticide exposure in Taiwan was recently evaluated using the nationwide registry maintained by the Network of Taiwan's Poison Control Centers (PCC) [3]. Over the course of eight years (1985-1993), 23,436 telephone calls concerning human poisoning exposure were recorded. The most frequent cause for poisoning exposure was pesticides (29.3%). Another study reported that there were 4,799 organophosphate pesticide (OP) exposures from July 1985 through December 2006 during a 21.5 year [4]. These studies were based on information collected in telephone interviews on poisoning exposures. The source population was poorly specified and therefore these figures could not be used for estimating rates of poisoning.

We used the nationwide population-based registry to assess the occurrence of pesticide poisoning episode in Taiwan between 1999 and 2008. To our knowledge, this is the largest and most complete nationwide population-based study to examine the characteristics and trends of unintentional pesticide poisoning mortality and hospitalization in Taiwan by sex, age, and cause between 1999 and 2008.

2. Materials and methods

2.1 Database

Data were collected from the official Vital Statistics System for the period 1999 to 2008. This system collects data from all administrative divisions in Taiwan.

According to law, each division officer must report death certificate to the Health Department of the Executive Yuan. Death certificate includes demographic factors, date and site of death, and cause of death (according to the International Classification of Diseases, 9th Revision, Clinical Modifications). The coding of injury death did not change from 1999 to 2008. Injuries were classified by intent and mechanism. Unintentional injuries included MVI (E810-E825), poisoning (E850-E869), falls (E880-E888), fire and flames (E890-E899), drowning (E910), suffocation (E911-E913), and other. Mortality rates were age- adjusted to each year's standard population. Annual population estimates were taken from the Statistical Yearbook of the Ministry of Interior. The data was then age-adjusted to the Year 2000 Standard Population of World Health Organization (WHO).

In this study, we used the Health Insurance Database from 1999 to 2008 released by the Taiwan National Health Research Institute (NHRI) in 2009 to investigate the trend of hospitalization due to pesticide poisoning.

Taiwan inaugurated its National Health Insurance (NHI) program in 1995 to finance healthcare for all citizens of Taiwan. There are currently 23.03 million enrolees covered by the program, representing over 99% of the island's population. The National Health Insurance Research Database 2009 (NHIRD) contains all the medical claims data as well as a registry of the 23.03 million enrolees covered by the NHI. The NHIRD, nationwide population-based dataset, provides an excellent opportunity to examine the trend of hospitalization due to unintentional pesticide poisoning.

This study was exempt from full review by the Institutional Review Board, as the dataset used consisted of de-identified secondary data released to the public for research purposes.

2.2 Statistical analysis

We calculated mortality and hospitalization rate using the number of poisoning as the numerator and the denominator was based on the mid-year population in the "population by age report" provided by the Ministry of the Interior. Data were age-adjusted by the direct method to the 2000 world standard population from the WHO. Afterwards, we calculated ten-year trends in unintentional poisoning mortality and hospitalization rates and categorized them by gender, age, and type of pesticide.

Six age groups on the Department of Health's classification were used: infants and toddlers aged 0-4, child aged 5-14, young adults aged 15-24, mature adults aged 25-44, middle-aged adults aged 45-64, and elderly aged 65 or more. With regards to NHI hospitalization data, since Medical facilities are required to file NHI claims on a monthly basis and the same episode may be reported several times if the hospitalization extends over different months. This was taking into account by accepting a hospitalization with a given ID and hospitalization date only once. All the data were analyzed by SPSS 18.0 software. Simple linear regression was used to test the trends of injury mortality rates. The dependent variable in the regression equation was the mortality rate or hospitalization rate, and the independent variable was the year.

3. Result

3.1 Mortality rate and trends

During the 10-year period from 1999 to 2008, unintentional pesticide poisoning accounted for 594 deaths in Taiwan, representing a mortality rate of 0.2405 per 100,000 person-years (Table 1). Males, the dominant group, accounted for 77% (457/594) of pesticide poisoning deaths (Table 1). Men demonstrated a mortality rate 3.4 times higher than women (0.3708 versus 0.1097 per 100,000 person-years). Decreasing trends in unintentional pesticide poisoning death rates after 1999 were seen for both genders (Figure 1).

	Age groups	0-4		5-14		15-24		25-44		45-64		65+		Overall	
		No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate
Mortality rate	Total	1	0.0075	0	0	27	0.0750	164	0.2169	206	0.4431	196	0.9324	594	0.2405
	Male	1	0.0144	0	0	24	0.1291	134	0.3499	170	0.7417	128	1.2113	457	0.3708
	Female	0	0	0	0	3	0.0176	30	0.0804	036	0.1516	68	0.6510	137	0.1097
		No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate
Hospitalization rate	Total	200	1.3394	53	0.1701	241	0.6801	1588	2.0840	2527	5.4376	1709	8.2515	6328	2.6193
	Male	131	1.6294	33	0.2029	188	0.9943	1186	3.0792	1878	8.2369	1249	12.0132	4666	3.8545
	Female	69	1.0228	20	0.1345	63	0.3475	402	1.0624	648	2.7095	460	4.4500	1662	1.3813

Table 1. Numbers of deaths and age and sex standardized mortality rates (per 100,000 person-years) from unintentional pesticide poisoning, Taiwan, 1999-2008

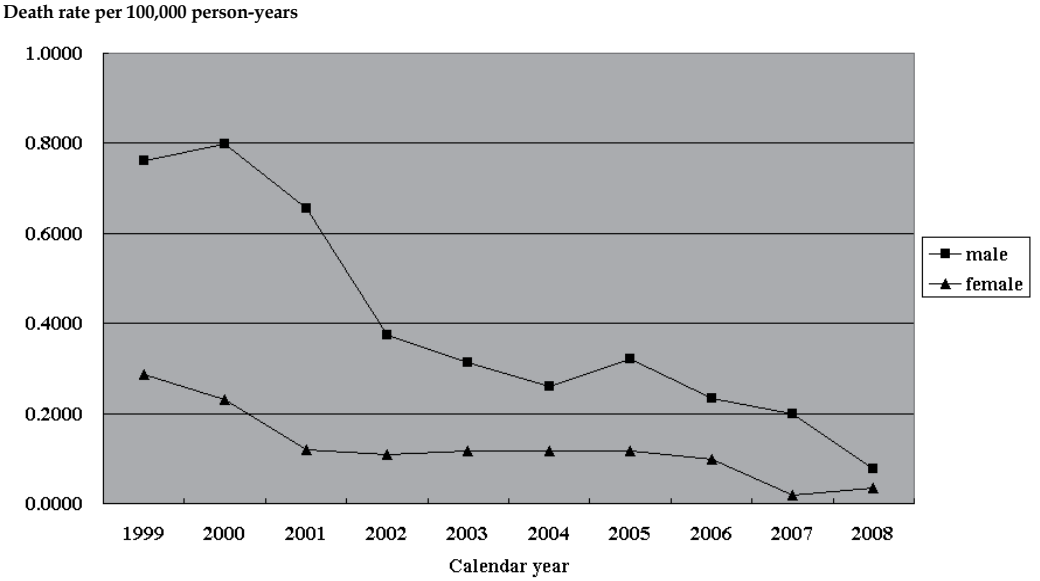

Fig. 1. Unintentional pesticide poisoning mortality rate (per 100,000 person-years) by gender, Taiwan, 1999-2008.

With regards to age, adult aging from 45 to 64 (0.44/100,000 PY or 10^{-5} PY) and elderly aging above 65 (0.93 x 10^{-5} PY) consistently had higher mortality rates than other age groups. The

unintentional pesticide poisoning mortality rate declined, mainly because of a decrease in the mortality rate of adults aging 45 to 64 and elderly aging 65 and above. The population aged 65 or more and 45-64 demonstrated a declining trend in mortality, with a drop of 90.1% and 87.8%, respectively ($p<0.001$) (Figure 2).

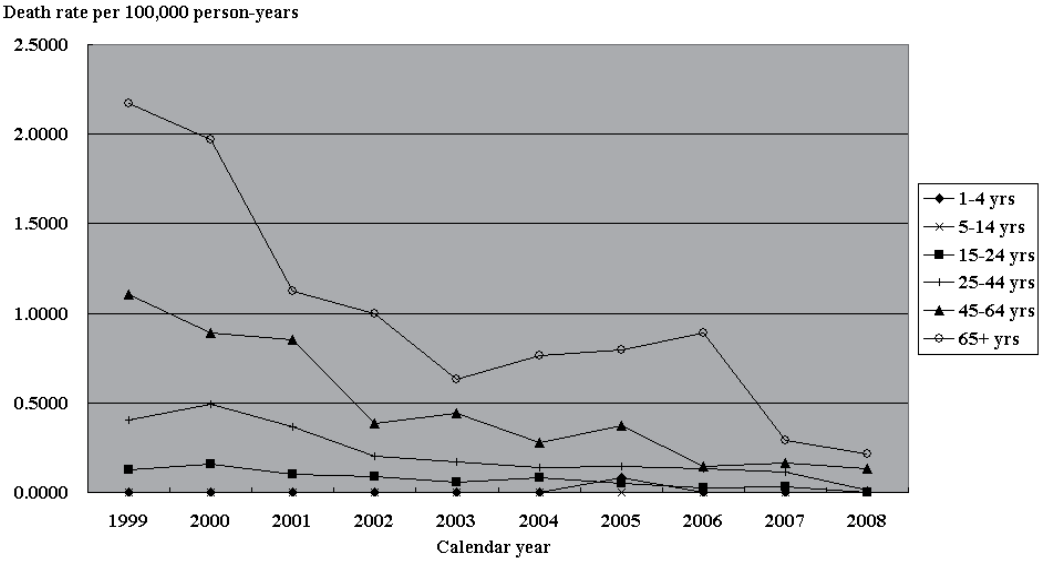

Fig. 2. Unintentional pesticide poisoning mortality rate (per 100,000 person-years) by age group, Taiwan 1999-2008.

3.2 Hospitalizations and trends

Between 1999 and 2008, there were 6,328 hospitalizations in Taiwan caused by unintentional pesticide poisoning, representing a hospitalization rate of 2.6193 per 100,000 person-years (Table 1). The hospitalization rate decreased by about 60%; this change was statistically significant ($p<0.001$).

The results showed that males were at higher risk of hospitalization as well (3.8545 per 100,000 person-years versus 1.3813 per 100,000 person-years). Decreasing trends in hospitalization rate due to unintentional pesticides poisoning after 1999 were seen in both genders (Figure 3). In terms of age, elderly aging above 65 had the highest hospitalization rate (8.2515 per 100,000 person-years).

3.3 Pesticides responsible for hospitalization

The Vital Statistics System applies a three-digit E863 code for unintentional poisoning by agriculture and horticultural chemical and pharmaceutical preparations, which restricts further analysis of different types of pesticides associated with unintentional poisoning deaths. However we were able to retrieve information on pesticide types most often responsible for poisoning from the hospitalization data.

Table 2 presents the hospitalization for major types of pesticide poisoning including "Organophosphate insecticides" (hospitalization rate: 1.2247 per 100,000 person-years), "unspecified insecticides" (hospitalization rate: 0.5152 per 100,000 person-years), "herbicides" (hospitalization rate: 0.4495 per 100,000 person-years) and "unspecified agricultural and horticultural chemical and pharmaceutical preparations other than plant

foods and fertilizers" (hospitalization rate: 0.2117 per 100,000 person-years). The unintentional pesticide poisoning hospitalization rate declined significant over time (trend test $p<0.001$), mainly because of a decrease in organophosphate insecticides and herbicide hospitalization for the adult and elderly hospitalization rate. (Figure 4 and Figure 5)

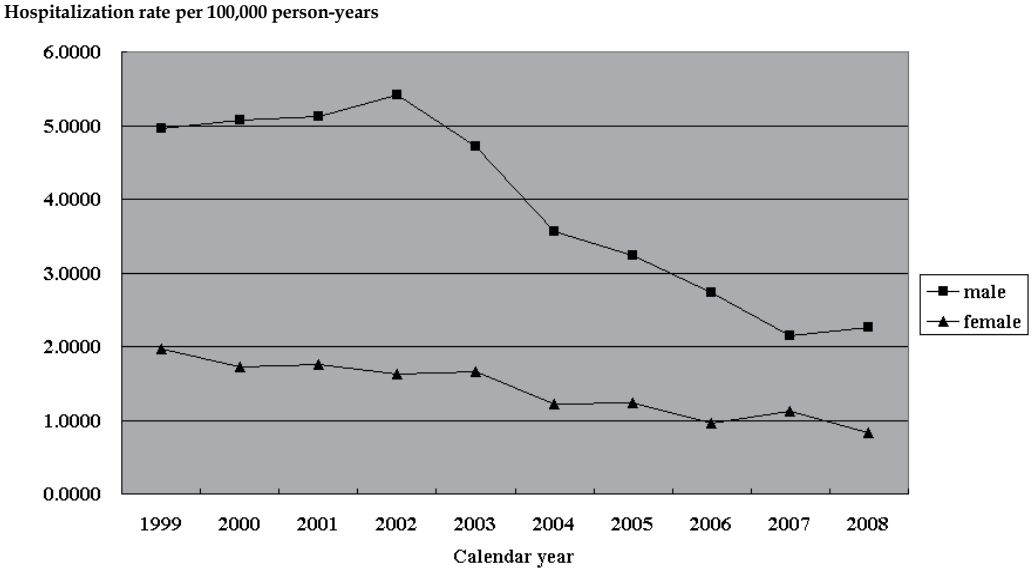

Fig. 3. Unintentional pesticide poisoning hospitalization rate (per 100,000 person-years) by gender, Taiwan, 1999-2008.

3.4 Occupations of the unintentional pesticide poisoning deaths and hospitalizations

The Vital Statistics System categorizes farm workers, forest workers, fishermen, and ranch workers into one occupational group. This group accounted for 98 deaths (16.5%). The 151 affected handicap, long-term sick, or old fragile health group (25.4%) represented a large proportion of unintentional pesticide poisoning deaths. Most of the 6328 episodes of unintentional pesticide poisoning hospitalization were among farm workers (N=3007, 47.5%) (data not shown).

4. Discussions

The results from our nation-wide analysis indicate that both mortality and hospitalization rate due to unintentional pesticide poisoning was decreasing after 1999 both in men and women. The results also show that men were at higher risk of mortality and hospitalization. The mortality and hospitalization rates were the highest among elderly above 65 years of age. "Organophosphate insecticides", "unspecified insecticides", "herbicides" and "unspecified agricultural and horticultural chemical and pharmaceutical preparations other than plant foods and fertilizer" were the major types of pesticide poisoning.

4.1 Unintentional pesticide poisoning mortality and hospitalization by gender

Calvert et al. [5] identified acute pesticide poisoning cases in agricultural workers from 1998 to 2005 from the Sentinel Event Notification System for Occupational Risks-Pesticides (SENSOR-Pesticides) program in the US. The authors found that acute pesticide poisoning

Category	Hospitalization			Hospitalization rate per 100,000 person years		
	Total (%)	Male (%)	Female (%)	Total	Male	Female
Accidental poisoning by insecticides of organochlorine compounds (E863.0)	27 (0.43)	22 (0.47)	5 (0.30)	0.0116	0.0192	0.0039
Accidental poisoning by insecticides of organophosphorus compounds (E863.1)	2975 (47.01)	2263 (48.50)	712 (42.84)	1.2247	1.8640	0.5858
Accidental poisoning by carbamates (E863.2)	170 (2.69)	135 (2.89)	35 (2.11)	0.0715	0.1143	0.0282
Accidental poisoning by mixtures of insecticides (E863.3)	97 (1.53)	70 (1.50)	27 (1.62)	0.0410	0.0597	0.0221
Accidental poisoning by other and unspecified insecticides (E863.4)	1197 (18.92)	842 (18.05)	355 (21.36)	0.5152	0.7230	0.3059
Accidental poisoning by herbicides (E863.5)	1103 (17.43)	830 (17.79)	273 (16.43)	0.4495	0.6763	0.2205
Accidental poisoning by fungicides (E863.6)	9 (0.14)	7 (0.15)	2 (0.12)	0.0040	0.0061	0.0016
Accidental poisoning by rodenticides (E863.7)	238 (3.76)	130 (2.79)	108 (6.50)	0.1054	0.1126	0.0983
Accidental poisoning by fumigants (E863.8)	4 (0.06)	2 (0.04)	2 (0.12)	0.0016	0.0015	0.0017
Accidental poisoning by other and unspecified agricultural and horticultural chemical and pharmaceutical preparations other than plant foods and fertilizers (E863.9)	508 (8.03)	365 (7.82)	143 (8.60)	0.2117	0.3034	0.1197
Overall	6328 (100.00)	4666 (100.00)	1662 (100.00)	2.6193	3.8545	1.3813

Table 2. Annual hospitalizations and hospitalization rate per 100,000 person-years due to unintentional pesticide poisoning

rate was almost two fold higher in female agriculture workers compared to males. In a hospital-based study at emergency departments (ED) in two medical centres in southwest Taiwan, 1512 poisoning cases were enrolled [6]. The authors estimated 4.2 poisonings per 1000 ED visits between January 2001 and December 2002. The female to male ratio of poisoning-related emergency department visits was 1.2. Overall, 66.1% of the poisoning exposures involved suicidal intent. Pesticide poisoning accounted for 14.5%. Based upon the above data, it was estimated that unintentional pesticide poisoning accounted for 0.21 cases of poisoning per 1000 ED visits. Our study showed that mortality as well as hospitalization rates were 3.4 and 2.8 times higher in male than female, respectively. Possible explanations for the differences in risk of unintentional pesticide poisoning in gender are the risk of exposure, rate of ascertainment or susceptibility.

4.2 Unintentional pesticide poisoning mortality and hospitalization by age

Lee et al. [6] reported that age greater or equal to 61 years was a significant predictor for poisoning-related fatalities (OR 4.3, 95% CI 2.6-7.2). The unintentional pesticide poisoning mortality rate increased with age, presumably due to the combination of low education and economic levels and higher fatality in the elderly [7].

4.3 Insecticide information on the pesticides responsible for pesticide poisoning

Calvert et al. [5] showed that insecticides alone or in combination with other pesticides were implicated in more than half of the pesticide poisoning cases (N=1,761, 54%). Cholinesterase inhibitors (organophosphates and N-methyl carbamates) were prominent among the insecticides (N=892, 51%), particularly chlorpyrifos (N=190), methamidophos (N=130), dimethoate (N=84), malathion (N=78), and diazinon (N=70).

In the report of Lin et al. that the top five compounds in the organophosphate poisoning, based on the Network of Taiwan's Poison Control Centers (PCC), were from mevinphos (18.4%), chlorpyrifos (17.6%), methamidophos (8%), dimethoate (5.2%), and fenitrothion (4.9%) [4]., whereas the most fatal compounds were mevinphos (138 deaths/524 poisoning), methamidophos (68/524), dimethoate (33/524), chlorpyrifos (30/524), and parathion (25/524). With the development of agriculture, more and more victims were exposed to new types of insecticides and herbicides, such as mevinphos, methamidophos, dimethoate, chlorpyrifos, parathion, and paraquat. Paraquat is one of the most commonly used herbicides in Taiwan and has been the most common lethal agent of poisoning for a long time. Some measures have to be taken by authorities, including banning some of the most toxic pesticides such as WHO Pesticide Hazard Class I organophosphates and dimethyl organophosphates, and promoting less use of pesticides.

4.4 Occupations of the affected unintentional pesticide poisoning

Pesticide poisoning is a typical occupational disease among agricultural workers. Our study found agricultural workers were at greater risk of pesticide poisoning hospitalization than non-agricultural workers. A variety of work-related factors are related to unintentional pesticide poisoning such as pesticide usage, pesticide application days, hazardous practices, and poor hygiene[8].

4.5 Factors that contributed to unintentional pesticide poisoning

The most common job category related to pesticide exposure was pesticide handlers 33% (N=1,068), and they were performing routine work without pesticide application 67% (N=2,135). The most common factors contributing to pesticide exposure were off-target drift, early reentry into a recently treated area, and use in conflict with the label [5].

4.6 Injury pyramid of unintentional pesticide poisoning

An injury pyramid is often used graphically to depict the relative effect of fatal and nonfatal injuries from top to bottom [9]. The variant pyramid size and shape represents the magnitude and nature of the injury cause. Analyzing the size and shape of each injury pyramid is useful in the assessment of the relative frequency and lethality of the injury mechanism and intent. In our study, unintentional pesticide poisonings was responsible for 594 deaths and 6,328 hospitalizations in Taiwan during the 10-year period from 1999 to 2008. The type of injury pyramid can be categorized as the classically shaped pyramid because of the low case-fatality ratio. Previous studies showed that the poisoning-related fatality rate was in the range of 4.3% to 5.7% in Taiwan based on hospital-based data [6].

5. Limitations

Several limitations need to be considered in the interpretation of our findings. First, hospitalization data has over-presentation of severe poisoning and symptoms, because of both self-selection to the hospital policlinics and admission to the hospital care. The quality

of diagnostic practice and criteria for hospitalization may vary and there is a possibility of under-reporting of pesticide poisoning in Taiwan.

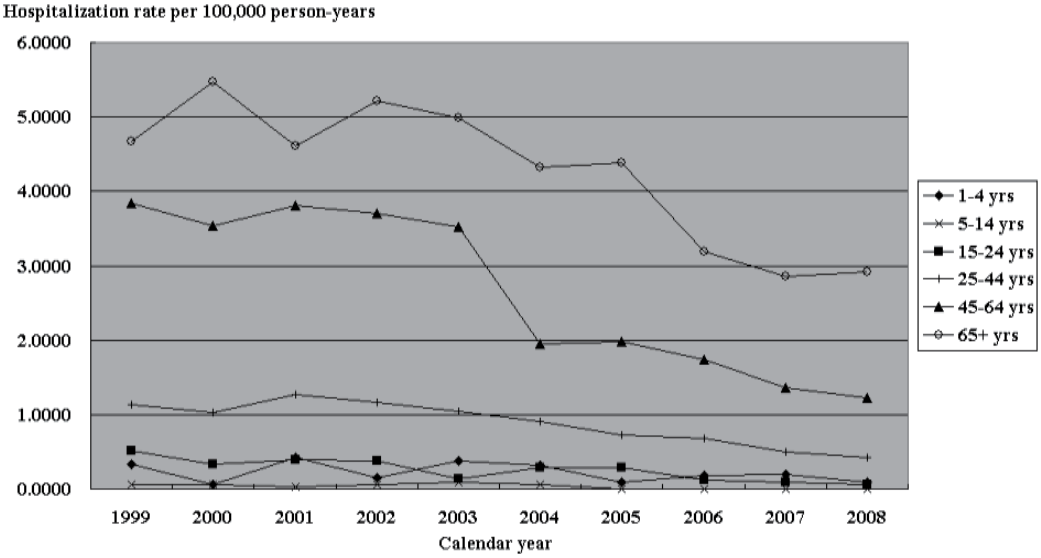

Fig. 4. Unintentional organophosphate insecticides poisoning hospitalization rate (per 100,000 person-years) by age group, Taiwan, 1999-2008.

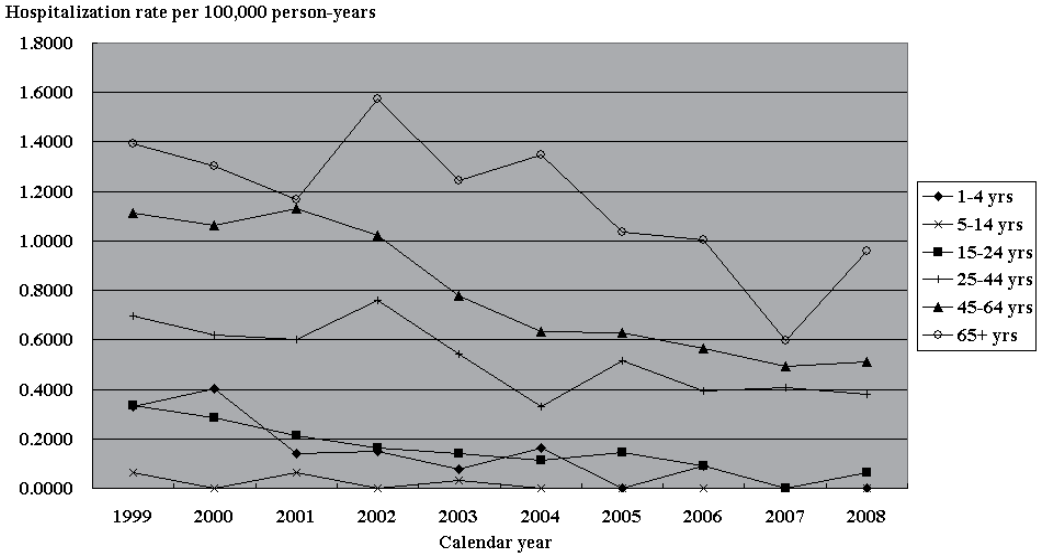

Fig. 5. Unintentional herbicide poisoning hospitalization rate (per 100,000 person-years) by age group, Taiwan, 1999-2008.

Second, the comorbidity diagnoses, which rely totally on claims data reported by physicians or hospitals, may be less accurate than if all individuals were assessed through a single standardized procedure [10]. Health professionals may not receive much training in environmental toxicology or pesticide poisoning. The signs and symptoms of pesticide

poisoning often resemble those of more common conditions, which may be diagnosed preferentially. The NHIRD used discharge diagnoses provided by treating physicians; no standardized criteria are used to define hospitalization cases. This increases the probability for case misclassification. The NHIRD, designed as an administrative dataset, does not include some important individual characteristics for further analyses such as smoking, alcohol consumption, all of which may contribute to death or hospitalization. The mortality and hospitalization rates may be underestimations, because of possible under-reporting of cases to the databases in the analyses. Administrative databases are known to be subject to possible undercoding and overcoding errors[11]. The difficulty of receiving reimbursement through workers' compensation may also bias health care providers diagnosis and reporting of episodes of unintentional pesticide poisoning. The health care professionals may fear that their patients may be subject to retaliation.

6. Strengths of this study

Litchfield [12] categorized studies on acute pesticide poisoning in agriculture into three categories: clinical case reports, descriptive epidemiology studies, and cross-sectional studies. Several studies conducted in China, India, and Taiwan was based on hospital-based case reports [3, 4, 6, 13, 14]. They were insufficient of information on the source population for estimation of poisoning rates. A particular strength of this study is the use of two nationwide population-based data sets, allowing us to trace medical services received by all patients after poisoning. Using the same ICD-9-CM codes over the study period would retain the internal validity of the temporal trend analyses. To our knowledge the present study is the most complete nationwide population-based study conducted to assess the risk of pesticide poisoning and evaluate time trends for pesticide poisoning.

7. Conclusion

Men were higher risk of mortality and hospitalization rates from unintentional pesticide poisoning. Although the mortality and hospitalization rates from unintentional pesticide poisoning have declined, development of prevention programs to reduce the "organophosphate insecticides", "unspecified insecticides", "herbicides" and "unspecified agricultural and horticultural chemical and pharmaceutical preparations other than plant foods and fertilizers" poisoning rates remain important in the future. There is relatively little known about the health effects of chronic pesticide exposure [15, 16]. Surveillance of pesticide poisoning is important for development of effective policies, practices and regulations for prevention of hazardous pesticide exposures and poisoning. Currently no authority in Taiwan is in charge of pesticide poison surveillance.

8. Acknowledgements

This study is based in part on data from the National Health Insurance Research Database provided by the Bureau of National Health Insurance, Department of Health and managed by National Health Research Institutes. The interpretation and conclusions contained herein do not represent those of the Bureau of National Health Insurance, Department of Health or National Health Research Institutes.

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15

Pathology of Endosulfan

Ozlem Ozmen
University of Mehmet Akif Ersoy
Turkey

1. Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro- 6,9-methano-2,4,3-benzo-o-dioxa-thiepin-3-oxide) is a chlorinated cyclodiene insecticide which acts as a contact poison in a wide variety of insects and mites (Naqvi and Vaishnavi, 1993). Endosulfan was first registered for use in the USA in 1954 to control agricultural insects and mite pests. Due to its toxic effect, the World Health Organization (WHO) has classified Endosulfan as a moderately hazardous Class II pesticide (WHO, 2002). Endosulfan is a persistent organic pollutant. The half-life of endosulfan in water varies from 3 to 7 days to about 5 months, depending on the dissolved oxygen, turbidity, pH and other contaminants in the water. This insecticide is a mixture of two stereoisomers, namely α- and β- endosulfan (Hayes and Laws, 1991), in a ratio of 7:3. It has been used worldwide in agriculture, viticulture and horticulture (Hack et al., 1995; Oktay et al., 2003; Mor and Ozmen, 2003; Yavuz et al., 2007).
Endosulfan can cause toxic effects in almost all tissues of both humans and animals, including the liver, lung, central nervous system, genital system, pancreas etc. (Howard, 1991, Mor and Ozmen, 2003; Kalender et al., 2004b; Hatipoglu et al., 2009). It is also effect blood biochemistry and hematological values (Hatipoglu et al., 2009). Endosulfan is a contact hepatotoxin that is readily absorbed into an organism through its stomach, lung and even through the skin (Howard, 1991).
The primary purpose of this chapter is to provide pathological findings in endosulfan toxicity in animals and human. It contains descriptions and evaluations of pathological studies about endosulfan. Gross and histopathological lesions are described in different kind of animals and human in experimental and natural toxication cases.

2. Nervous system toxicity

Clinical signs, such as depression, inappatence and slight nervous symptoms such as teeth grinding and hyperexcitability reported in the rabbits suffer from subacute endosulfan toxication (Mor and Ozmen, 2010a; Mor and Ozmen, 2010b). In acute toxication by endosulfan in cattle cause rapid and difficult breathing, foamy exudates in mouth, tremors, exophtalmos, coma and death (Mor and Ozmen, 2003). At the gross examination of the brains, marked hyperemia at the meningeal vessels and slight hemorrhages in brains and cerebellums in rabbits suffer from endosulfan poisoning were reported. The occurring of findings is prominent in rabbits that had shown clinical nervous symptoms (Mor and Ozmen, 2010b).

Histopathology of the central nervous system (CNS) lesions are commonly included hemorrhages, marked edema with enlargement of Virchow Robin spaces, degenerations, slight perivascular cuffing and slight gliosis in the rabbits. Immunohistochemistry of the CNS were revealed a strong apoptotic activity in neurons and microglial cells in rabbits in subacute endosulfan toxicity (Mor and Ozmen, 2010b). The main biochemical changes of CNS lesions revealed decreases in serum and tissue acetylcholinesterase activity and are commonly reported in the endosulfan treated animals (Gupta, 1976; Jia and Misra, 2007; Mor and Ozmen, 2010b).

Excitations are the primary CNS symptom in human. Convulsions and seizures can occur suddenly after a massive overdose. Convulsions usually accompanied by confusion, incoordination, excitability, or, in some instances, coma. Syncope may be the earliest sign of endosulfan toxicity (Moon and Chun, 2009).

Endosulfan can cause (lipid peroxidation) LPO was also increased in brain and it is the most sensitive organ to oxidative damage (Ballesteros et al., 2009). Endosulfan is also decreased mitogen activated protein kinase activity (MAPK), gap junctional communication (GJIC) and connexin 43 in neuronal stem cells (Kang et al., 2011). Endosulfan had cytotoxic effects on rat glial and neuronal cell cultures as well as on human glial and neuronal cells in an in vitro study in tissue cultures (Chan et al., 2006)

3. Hepatic toxicity

The mainly effected organ in endosulfan toxicity is liver. Swollen and pale livers commonly seen in this toxicity at the gross examination even in subacute poisoning (Mor and Ozmen,

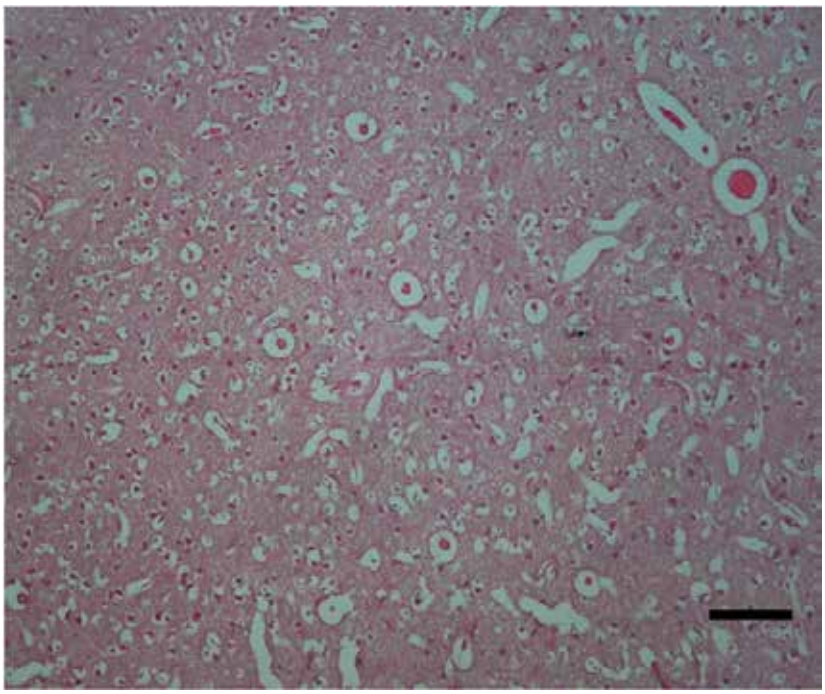

Fig. 1. Marked edema, with enlargement of Virchow Robin spaces, in a rabbit suffer from endosulfan toxication, HE, Bar= 200 μm.

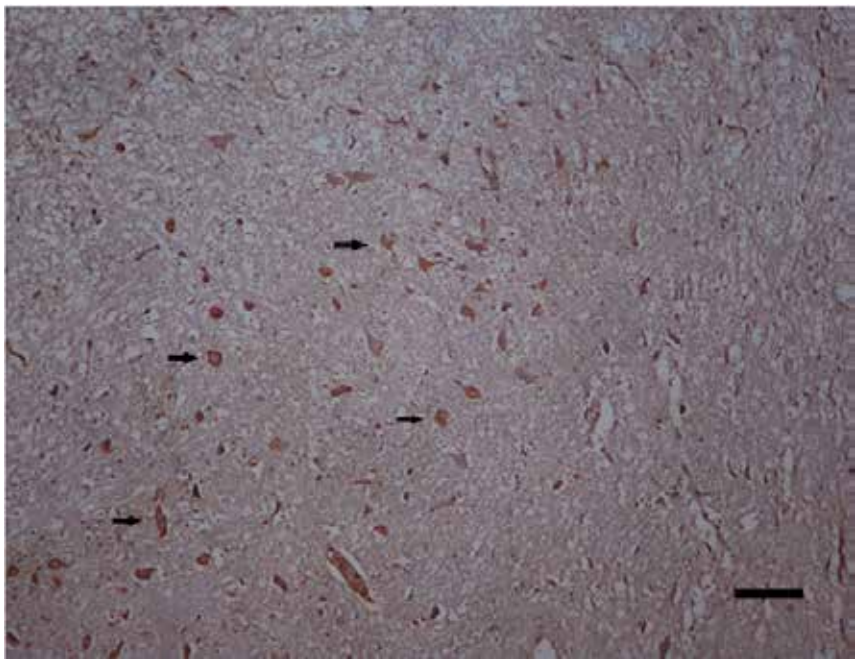

Fig. 2. Caspase-3 positive reaction in neurons (arrows) in brain in a rabbit treated with endosulfan. ABP method, with DAB, Harris hematoxylin counterstain, Bar= 200µm.

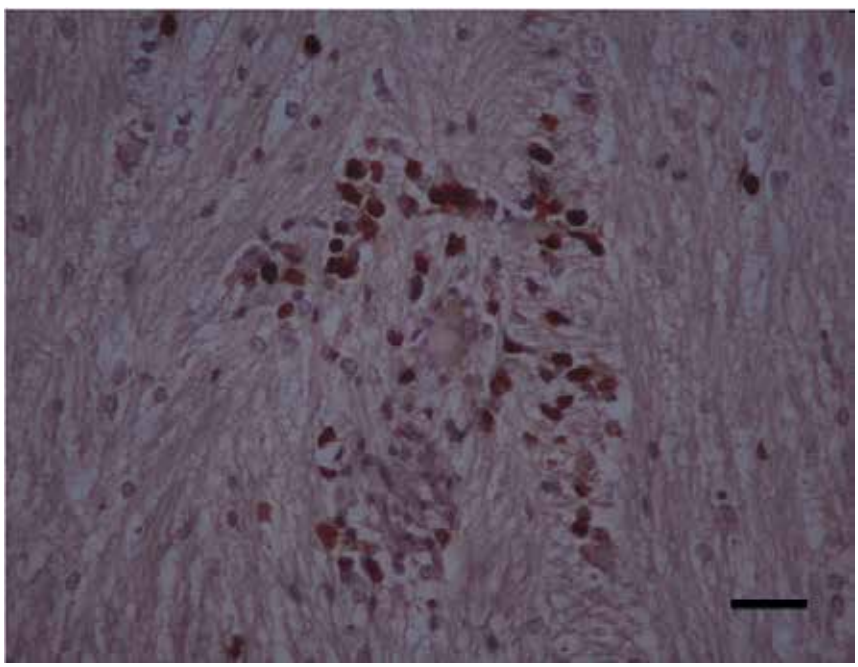

Fig. 3. Caspase-3 positive reaction in microglial cells in a rabbit suffer from endosulfan poisoning. ABP method, with DAB, Harris hematoxylin counterstain, Bar= 100µm.

2003; Mor and Ozmen, 2010a). At necropsy, hemorrhages can be seen in livers in acute poisoning in cattle (Mor and Ozmen, 2003). Liver histology of rabbits suffers from endosulfan toxication characterized by loss of radial cellular arrangement, hypertrophy of hepatocytes, significant increase of Kupffer cells, circulatory disturbances, focal necrosis, fatty degeneration, nuclear pyknosis, narrowing of sinusoids and bile duct hyperplasia. Hemorrhages and infiltration of inflammatory cells that localized around the central vein and portal space can be seen. Interlobular mononuclear inflammatory cells among vacuolated hepatic cells and dilated congested sinusoids are reported. Apoptotic activity in liver cells increased in livers by endosufan exposure (Mor and Ozmen, 2010a). Liver enzyme levels are elevated in endosulfan toxicity (Khan et al., 2010). Endosulfan can also cause catalase (CAT) inhibition and increase of LPO levels in liver (Ballesteros et al., 2009).

Histopathological examinations of liver tissues of long term (180 days) exposure of endosulfan shows chronic toxic hepatitis in liver in mice. There is portal mononuclear inflammatory infiltration and some eosinophyl leucocytes, lobulary inflammation and liver cell necrosis. Generally, any neoplastic and dysplastic changes have not been observed in liver. Histopathological examinations of liver tissues of short term (90 days) exposure show some regenerative findings with mild hepatitis. Hepatocytes had more than one nucleus, nuclear hyperchromasy and minimal microvesiculary fatty degeneration. In addition, crude glycogen granules in hepatocytes also are reported (Kurutas and Doran, 2001). Microscopical hepatic lesions of endosulfan poisoning are more severe in diabetic or protein malnourished rats (Benjamin et al., 2006).

4. Nephrotoxicity

Kidney changes in endosulfan poisoning are dose dependent. Tubular dilation, hydropic degeneration in tubular epithelium, hemorrhage in the cortical and medulla part of the kidney were reported (Kayhan et al., 2009). The effect of the endosulfan is mainly on the proximal convoluted tubule cells (Powers et al 1978; Caglar et al., 2003; Benjamin et al., 2006). Mitochondrial degeneration, lipofuscin granules and membranous structures in cytoplasm of proximal convoluted tubule cells were reported in mice suffer from endosulfan toxication (Caglar et al., 2003). While degenerative changes have been observed in proximal or distal convoluted tubules; glomerular tuft and Bowman's capsule are generally normal in mild endosulfan poisoning in rats. Lesions occur more severe in diabetic and malnourished rats and became worse related the duration of the toxication. In severely poisoned rats complete necrosis of tubular epithelium and hemorrhages in glomeruli are prominent. Increased Bowman's spaces commonly have been seen in severely affected rats (Benjamin et al., 2006). There is an increase in the cytoplasmic density of some of the distal convoluted tubule cells are generally observed. Extenstion in the length of some cells, and cytoplasmic bulges toward the lumen from the apical cytoplasm are reported. Ultrastructurally fusion in pedicels and focal thickening at glomerular basal membrane were also reported in some glomeruli (Caglar et al., 2003).

Renal calcium deposits may be seen in endosulfan toxication in males. The toxic nephropathy observed in animals was characterized as degenerative changes in the proximal convoluted tubules at the junction of the cortex and medulla, and associated cloudy swelling, fatty degeneration, and necrosis of the tubular epithelium (Powers et al 1978). Glucose-6-phosphate dehydrogenase (G6PD), Catalase (CAT), Superoxide Dismutase

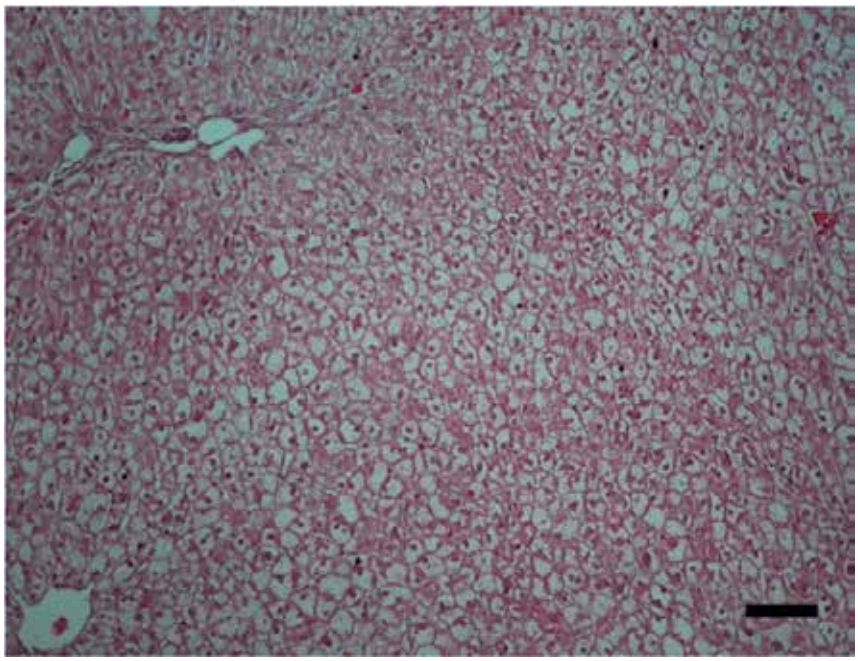

Fig. 4. Severe lipidosis and pycnosis at the nucleus of hepatocytes in a rabbit suffer from endosulfan poisoning, HE, Bar= 200 µm.

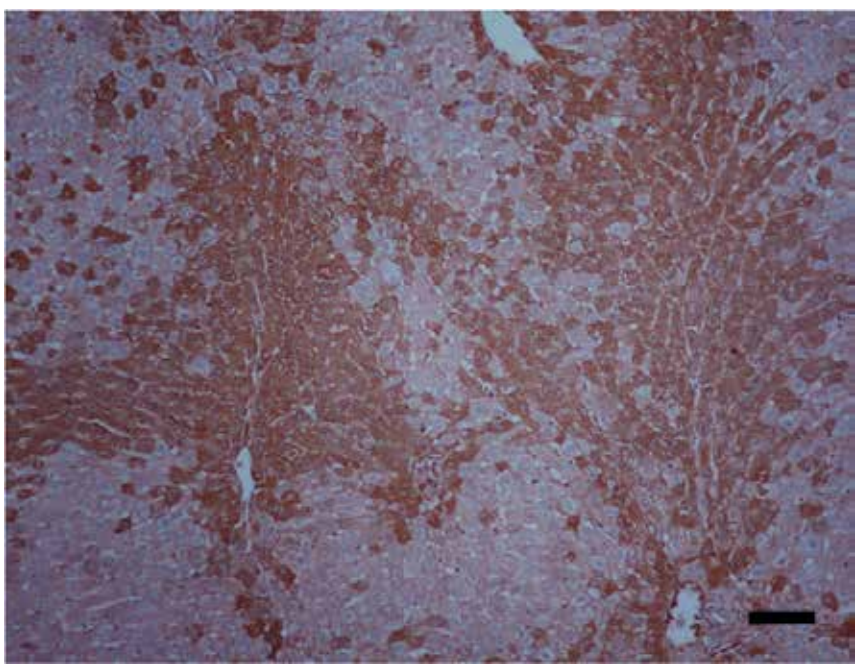

Fig. 5. Severe caspase-3 immunoreaction indicating apoptosis in hepatocytes and sinus endothelial cells in a rabbit suffer from endosulfan poisoning, ABP method, with DAB, Harris hematoxylin counter stain, Bar= 200µm.

(SOD), GSH (gulutatione) and malondi-aldehid (MDA) activities increased in the endosulfan - treated group kidney tissues. Degeneration and necrosis in kidneys may be thought that oxidative stress may play a role to the mediator in changing configuration of cell membrane and seem to account for the morphologic alteration of kidney (Caglar et al., 2003).

5. Reproductive toxicity

Endosulfan toxicity commonly studied especially in males. Numerous studies have consistently demonstrated that endosulfan behaves physiologically as an anti-androgen (Wilson and LeBlanc, 1998). The effects of endosulfan are most pronounced in immature animals whose reproductive systems and brains are still developing (Sinha et al.1995; Sinha et al.1997). Studies showed that toxicity can cause morphological and functional changes in male reproductive system. The main problems are decreased spermatozoon count and testosterone inhibition (Khan and Sinha, 1996; Esin, 2008; Hatipoglu et al., 2009). In mice, endosulfan reduces overall sperm count and increases the prevalence of malformed sperm (Khan and Sinha., 1996). Histologically, numerous seminiferous tubules show significant decrease to complete spermatogenesis at puberty. This finding can cause the decrease in daily sperm production observed in the endosulfan-exposed male rats (Dalsenter et al., 1999). Degenerative areas in testis and decreased number of spermatozoon in seminiferous tubules are apparent in subacute poisoning in male rabbits (Khan and Sinha, 1996; Esin, 2008; Hatipoglu et al., 2009). Significant decreases in the mean spermatozoon counts and spermatozoon with abnormal head number (twinheaded) is reported (Khan and Sinha, 1996). A significant elevation in the activities of the enzymes LDH (lactate dehydrogenase), GGT (gamma glutamyl transpeptidase) and G6PDH (glucose-6-phosphate dehydrogenase is also observed (Sinha et al., 1997).

Estrogenic effects of endosulfan were conducted an in vivo study of by Raizado et al (1991). A dose related increase in testicular atrophy occurred in treated male rats, characterized by degeneration and necrosis of the germinal cells lining the seminiferous tubules, multinucleated cells (fusion bodies), and calcium deposition resulting in aspermatogenesis. No treatment related effects were noted on the reproductive organs in female rats (Powers et al 1978).

Male Wistar prepubertal rats that treated by endosulfan, statistically significant decreases reported in body, testes, epididymal, ventral prostate and seminal vesicle weights compared to controls (Chitra et al 1999).

Developmental/reproductive toxicity or endocrine disruption occurs only at doses causing neurotoxicity. Toxicity to the fetus or young animals is not more severe than that shown by adults (Silva and Gammon, 2009).

6. Endocrine toxicity

Endosulfan poisoning can cause histological pancreas lesions. The serum amylase levels are generally normal, whereas the lipase and glucose levels are increased. The histopathological examinations of the pancreases are indicated that single-cell necrosis and degenerative changes had occurred in the pancreatic cells; especially in the beta cells, in rabbits suffer from endosulfan poisoning. Immunohistochemistry of the pancreatic tissues revealed a

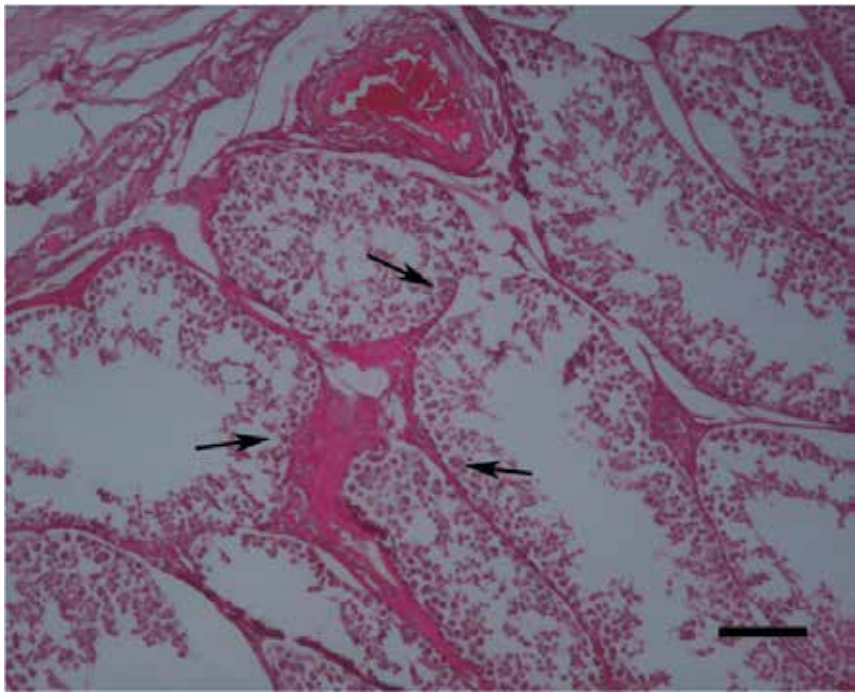

Fig. 6. Degenerative and necrotic seminiferus tubules, completely absence of spermatozoon and decreased Sertoli cells (arrows), HE, bar= 200µm.

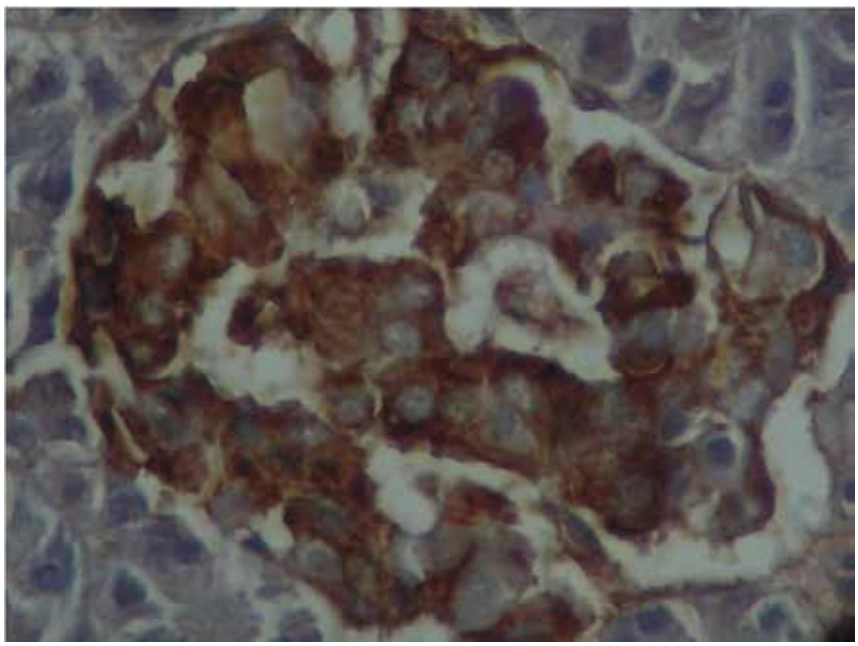

Fig. 7. Strong insulin expression in normal pancreas of a rabbit, ABP method, with DAB, Harris hematoxylin counter stain, Bar= 100µm.

marked reduction in concentration and distribution of insulin, proinsulin, and amylin. The number of the endocrine cells in pancreas in endosulfan treated rabbits is significantly decreased (Ozmen et al., 2010). In electron microscopy studies, swelling of mitochondria, vacuoles in cytoplasm, dissolution of mitochondrial matrix, picnotic nucleus in β cells in Langerhans islet reported after endosulfan treatment (Kalender et al., 2004a)

Endosulfan poisoning may affect reproductive endocrine hormones. Recent information indicates that endosulfan mimics non-uterotrophic E(2) actions, strengthening the hypothesis that endosulfan is a widespread xenoestrogen (Varayoud et al., 2008), acts via a membrane version of the estrogen receptor-α on pituitary cells and can provoke Ca++ influx via L-type channels, leading to prolactin (PRL) secretion (Watson et al., 2007), and alters circulating levels of prolactin, luteinizing hormone, growth hormone, and thyroid stimulating hormone (Caride et al 2010).

In addition to inducing cell proliferation, endosulfan induced proliferation of the progesterone receptor, another oestrogen-mimicking effect (Soto et al 1995). Parathyroid hyperplasia occurred in treated males, as did medial calcification of the aorta and medial calcification of the mesenteric artery, and calcium deposits in the stomach (Powers et al 1978). The adrenals of rabbits given a single dermal dose of 100 mg/kg of endosulfan exhibited microscopic changes, including swollen cells with foamy cytoplasm and eccentric nuclei (Gupta and Chandra, 1975).

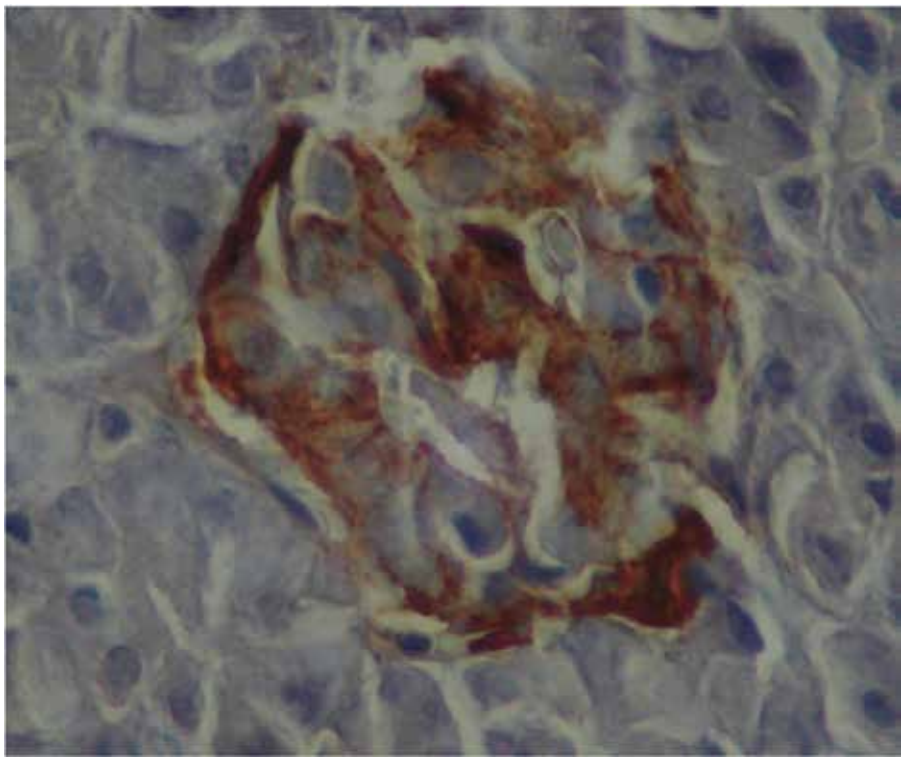

Fig. 8. Marked decreasing in insulin expressed cells in a rabbit suffers from endosulfan toxication, ABP method, with DAB, Harris hematoxylin counter stain, Bar= 100µm.

7. Breast toxicity

Microscopic examinations of breast tissues of long term and short term exposure showed lymphocytic infiltration in stroma of breast tissue. There is no neoplastic and dysplastic changes in breast in endosulfan administration reported (Kurutas and Doran, 2001).

8. Muscle toxicity

Muscle necroses are reported in rats severely toxicated and under health stress (Benjamin et al., 2006). Endosulfan can cause inhibitory effect on on skeletal muscle MDH of the freshwater catfish Clarias batrachus (Misra and Shuckla, 2003).

9. Genotoxicity

Genotoxicity in tests for gene mutation, chromosomal aberration and DNA damage are reported in endosulfan toxicity (Silva and Beauvais, 2010).

10. Cardiotoxicity

Endosulfan poisoning caused the hypotension and the abnormalities on electrocardiogram at presentation. Over half of the patients developed complications, such as rhabdomyolysis, hepatic toxicity, and hypotension (Moon and Chun, 2009). Glutathionperoxidase (Gpx), Catalase (CAT) and Superoxide Dismutase (SOD) activities increased in the endosulfan - treated group heart tissues (Jalili et al., 2007). The hearts generally indicated severe

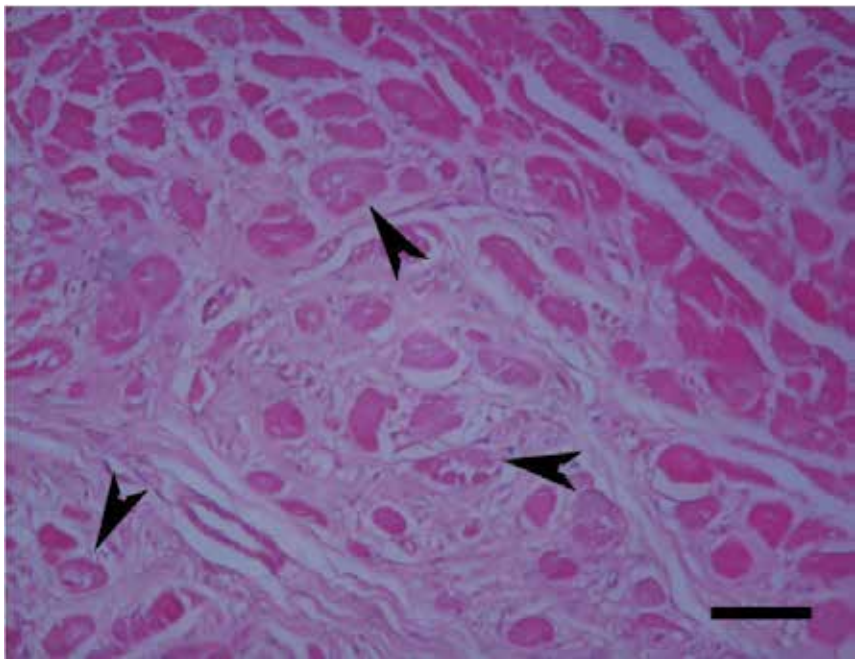

Fig. 9. Severe degeneration at the myocardial cells (arrows) in a rabbit suffer from subacute endosulfan poisoning, HE, Bar= 50 μm.

congestion, hemorrhages, with interstitial edema. In some places diapedesis of leukocytes may be seen. Different degrees of degeneration can be seen in myocardium, granular appearance with picnotic nuclei may observe in some myofibrils. Thickening of wall of arteries were reported (Jalili et al.2007). In electron microscopic investigations cytoplasmic edema and swelling and vacuolization of mitochondria of myocardial cells in endosulfan toxicity may observed (Kalender et al., 2004a). Significantly decreased were reported in serum calcium levels of endosulfan treated rats. No calcification was observed in heart muscle tissues of the rats (Ozmen and Elcuman, 1998).

11. Other organ toxicity

Marked and extensive hemorrhages can be seen in the spleen in protein malnourished and diabetic rats in endosulfan poisoning (Benjamin et al., 2006). Lungs are commonly affected especially in acute poisoning (Mor and Ozmen, 2003). Lungs are generally edematous and hemorrhagic (Mor and Ozmen, 2003; Hatipoglu et al., 2009; Fazekas, et al., 2010). Subacute toxicity may affect almost all organs (Mor and Ozmen, 2010a). Gastro intestinal system commonly affected by poisoning especially toxication occurs by oral route. Hemorrhages in all part of the system can be seen (Mor and Ozmen, 2003; Hatipoglu et al., 2009).

Although endosulfan is a poisonous component ameliorative effect of some anti-oxidants like as vitamin C or E also reported (Muruguesan et al., 2005; Hatipoglu et al., 2009; Ozmen et al., 2010; Mor and Ozmen, 2010a; Mor and Ozmen, 2010b).

12. Human toxicity

The major symptoms of acute endosulfan intoxication in human are nausea, vomiting, gagging, diarrhea, agitation, writhing, loss of consciousness, cyanosis, dyspnea, foaming at the mouth, noisy breathing, headache, and dizziness. Ingestion of endosulfan can also cause restlessness, irritability, vertigo, muscle twitching, confusion, stupor, coma, abnormal blood and urine chemistry. Patients may be asymptomatic but fatality can occur due to usually pulmonary, renal or cardiovascular disorders. Severe metabolic acidosis with high anion gap reported human suffer from endosulfan poisoning (Terziev et al., 1974; Blanco-Coronado et al., 1992; Segasothy and Pang. 1992). Residues of endosulfan have been detected in multiple human tissues including blood, fetal placenta, breast milk, and mammary adipose tissue (Hernandez et al., 2002; Cerrillo et al., 2005).

Acute accidental or intentional ingestion of large amounts of endosulfan resulted in death in humans. Autopsies revealed edema and congestion of the brain and lungs, hemorrhage of the medullary layer of the kidneys, acute lung emphysema, and chromatolysis of the neurons (Terziev et al., 1974). Dark-red/purple body and cyanotic face is also reported Autopsy revealed edematous lungs (Demeter and Heyndrickx, 1978). Acute renal failure, disseminated intravascular coagulation, thrombi in the pulmonary arteries and aorta, and cardiogenic shock may be seen. Postmortem finding included bilateral pleural effusions, hyaline membranes, microatelectasia, polymorphonuclear lymphocytes and red cells in the alveoli, and interstitial fibrosis also reported (Blanco-Coronado et al., 1992). Cardio-respiratory arrest was described in endosulfan poisoning in human (Lo et al., 1995; Yildiz et al., 2008).

In humans, endosulfan exposure has been associated with congenital defects, developmental delays, and death (ATSDR, 2000).

13. Toxicity in chicken

Hyperexcitability, tremors, vocalization, violent beating of wings, ataxia and convulsions leading to death were reported in endosulfan treated chicken. Pathologically, liver, kidney and gall bladder enlargement, small spleen or splenomegaly was reported. Histopathologically lymphoid depletion in spleen and bursa and hyperamia can be seen in kidney. Congestion, focal neuronal degenerative changes, meningeal thickening and focal areas of gliosis were reported in the brain (Selvaraj et al., 2000).

14. Toxicity in birds

Endosulfan is immunosuppressive in bird species (Bhattacharya et al., 1993; Kurkure et al., 1993; Khurana and Chauhan, 1998; Garg et al., 2004). Exposure of chicken eggs to extremely low doses of endosulfan results in adverse effects on the liver and brain enzymes decreased DNA and RNA in the brain, and immunosuppression (Pushpanjali et al., 2005). Exposure of chickens to sublethal doses of endosulfan has adverse effects on metabolism (Garg et al., 2004).

15. Aquatic toxicology

Numerous studies available about endosulfan toxicity in aquatic species especially fish. The sensitivity of aquatic animals to endosulfan has been well described (Naqvi and Vaishnavi, 1993; Pandey et al., 2001; Dorval and Hontela, 2003; Dorval et al., 2003; Matthiessen, 1981; Wan et al., 2005). Toxicity is primarily mediated by inhibition of important ion transport proteins in a variety of tissues (Naqvi and Vaishnavi, 1993), and endosulfan exposure may also induce oxidative stress (Pandey et al., 2001; Dorval and Hontela, 2003; Dorval et al., 2003). The toxicity of waterborne endosulfan is such that levels in agricultural run-off may exceed the median lethal concentration for many of the inhabitants of contaminated waterways (Matthiessen, 1981; Wan et al., 2005). Endosulfan is toxic to aquatic organisms and has been shown to damage the gills, liver and kidneys of fish (Altinok and Capkin, 2007). Even low environmental concentrations of endosulfan can have potentially harmful effects on exposed animals (Brunelli et al., 2009). Endosulfan can cause suppression growth and reproductive activity in zebrafish (Balasubramani and Pandian, 2008).

The hepatic lesions in fish suffer from endosulfan toxicity are characterized by generalized toxic necrosis, focal necrosis, and subcapsular oedema, reduction in melanomacrophage, entres and perivascular haemopoietic tissue, and toxic accumulations of lipid are also reported. Focal necrosis is often seen in the hepatic tissue surrounding bile ducts. In brain, endosulfan-related changes are included encephalitis, meningitis and oedema, with an associated inflammatory infiltrate of eosinophilic granule cells. Severe focal encephalitis and intracerebral haemorrhage can be seen. In later stages, substantial glial scarring which probably resulted from the earlier encephalitis are reported. The pathological changes in brain show that endosulfan has neurotoxic effects in fish. The brain lesions are probably sufficient to cause behavioral changes. Fish become temporarily hyperactive and uncoordinated (Matthiessen and Roberts, 1982).

Generally the tubular structure did not alter in the livers containing low doses residues of endosulfan. Sinusoids are dilated in most of the treated fish. Dark, atrophied hepatocytes with pyknotic nuclei usually present. Vacuolization in cytoplasm of hepatocytes can be seen. Lysis of cell membranes in liver containing endosulfan resulted in the loss of celularity in some livers. Numerous hepatocytes become shrunken and dark; their nuclei characterized by bizarre shape, condensation of chromatin, and smaller size. In many hepatocytes, the previous compartmentation in the areas of high metabolic activity and storage is lost. This is at least partly a consequence of proliferation of RER. At the microscopical observations, vacuolation can be observed, due to the presence of dilated RER, which often filled whole cytoplasm. Concentric membranous bodies of RER found in some hepatocytes. Myelinated bodies can be presented in the cytoplasm of hepatocytes in the livers of treated fish. They may be presented in the mitochondria. Fibrous material and myelinated bodies observed in the secondary lysosomes. Regression of hepatocyte microvilli in the space of Disse and bile canaliculi have been commonly seen in the livers of treated fish. Bile canaliculi may be dilated. The percentage of hepatocytes with proliferated and dilated RER is significantly greater in fish containing residues of endosulfan (Nowak, 1996).

Histological lesions in gills are seen in liver, spleen, and trunk kidney of rainbow trout exposed to endosulfan. The endosulfan poisoning can cause primarily of epithelial lifting of the outer layer of the lamellar epithelium with the space under the epithelium filled with eosinophilic material of gill filaments of rainbow trout (Altinok and Capkin, 2007).

Endosulfan exposure can cause enteropathology with vacuoles in the villi tips, and led to loss of integrity of the epithelium. In severe cases, vacuolated epithelium, fusion, and complete loss of integrity of areas of villi may be seen. In the very severe cases some necrosis and loss of epithelium integrity on the tips of intestinal villi are reported. In the liver the primary effects are glycogen depletion and lipidosis (Glower et al., 2007). One of the important toxic causes of the fish is endosulfan (Ton et al., 2000).

Hyperplasia usually present as an increased number of epithelial cells at the distal or basal portions. Endosulfan exposed fish gills are also caused hypertrophy of epithelial cells on the lamellae, fusion of two or more lamellae, and epithelial necrosis (Altinok and Capkin, 2007).

Morphological and ultra structural analysis of the hepatic cells from the fish exposed to endosulfan revealed depletion on the concentration of liver glycogen and an apparent proliferation of the endoplasmic reticulum. In studies by electron microscopy, with rainbow trout are observed an increase in the size of the cell nucleus and depletion in the concentration of hepatic glycogen. They also observed an increase in the hepatocytes volume and diameter and a proliferation of the endoplasmic reticulum, a possible indication of mixed-function oxygenase (MFO) induction in different species of fishes (Salvo et al., 2007).

The trunk kidney of fish exposed to endosulfan had enlarged sinusoids within an apparently decreased amount of hematopoietic tissue. Some nephrons can occlude glomerular capillaries and separation of the renal tubular epithelium from the surrounding connective tissue. Necrosis usually present in hematopoietic tissue, glomerular cells and tubular cells. Glomeruli may contain eosinophilic exudate. The liver has a low number of necrotic hepatocytes and enlarged hepatic perisinusoidal areas containing eosinophilic material. Vacuolar dystrophy of hepatocytes and hypertrophy of hepatocytes also observed.

Melanomacrophage centers (MMC) are scattered throughout spleen. Exudate and necrosis in the splenic white pulp can observe (Altinok and Capkin, 2007).

16. Conclusion

Endosulfan can cause toxic effects in all tissue but some protective agents like as vitamin C and E may have ameliorative affect in this toxicity both human and animals.

17. References

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Pesticides and Parkinson's Disease

Arthur G. Fitzmaurice and Jeff M. Bronstein
David Geffen School of Medicine at UCLA
United States of America

1. Introduction

1.1 Clinical and pathological aspects of Parkinson's disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder, affecting millions of people worldwide (Dorsey, Constantinescu et al. 2007). While some cases of familial PD have been reported, the etiology of most cases is still unknown. Significant progress in understanding the pathophysiology of PD has been made from genetic and epidemiologic studies that have implicated defects in a few key biological processes as potential final common pathological pathways.

PD is a progressive motor disorder characterized by death of dopaminergic neurons in the region of the brain called the substantia nigra pars compacta although other areas of the central and peripheral nervous system are involved (Braak, Del Tredici et al. 2003). The loss of dopaminergic neurons in PD leads to motor symptoms that include akinesia (inability to initiate movement), bradykinesia (slowness of movement), resting tremor, and balance problems. Non-motor symptoms can include cognitive impairments, mood disturbances, sleep dysfunction, gastrointestinal problems, and dysautonomia. PD is a progressive disorder and despite several effective therapies that treat many of the symptoms, there are no treatments that alter disease progression. Uncovering the causes of PD is likely necessary to find effective disease modifying therapies.

The pathological hallmark of PD is the presence of Lewy bodies, which are cytosolic inclusions with several molecular components although α-synuclein (α-syn) is the predominant protein (Spillantini, Schmidt et al. 1997). Lewy bodies also contain ubiquitin, a polypeptide that targets proteins to the ubiquitin proteasome system (UPS) for degradation.

1.2 Genes versus environment

Despite the elucidation of approximately 18 genes in familial PD and the identification of multiple risk factor genes using genome wide association studies on thousands of patients, only a small fraction of PD risk has been accounted for (Hardy 2010). Thus, environmental factors almost certainly play a major role in the pathogenesis of PD.

One of the first important clues that the environment may contribute to the pathogenesis of PD came in 1982 from the observation that a street drug contained a contaminant called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which caused almost overnight a clinical syndrome resembling PD. It was subsequently found that MPTP killed dopaminergic neurons by being converted enzymatically to MPP+, specifically entering dopamine neurons via the dopamine transporter, and inhibiting complex I in the

mitochondrial respiratory chain. Notably, the chemical structure of the MPTP metabolite MPP+ is similar to paraquat, a commonly used pesticide. These and other observations led to a series of epidemiologic studies probing pesticides as potential contributors to the etiology of PD.

Although genetics hasn't found the cause of 95% of PD cases, the identification of specific genes and their functions have provided important clues into pathological processes that appear to be involved in non-genetic forms of PD. For example, mutations in the α-syn gene led to the finding that α-syn is the major component of Lewy bodies. Mutations in other genes have identified dysfunction of protein degradation (the UPS and autophagy) as possibly being involved in the pathogenesis of PD. Since other PD genes are involved in mitochondrial function and MPTP inhibits oxidative respiration, mitochondrial dysfunction also has been implicated in the pathogenesis of PD. We believe that environmental toxins may increase the risk of PD by causing dysfunction in these cellular processes. Here, we will review the evidence that pesticides are associated with the development of PD and the mechanisms by which they might act.

2. Pathophysiology of Parkinson's disease

2.1 Lewy bodies and α-synuclein homeostasis

Lewy bodies are the pathological hallmark of PD and the major component of these intracytosolic inclusions is α-syn (Spillantini, Schmidt et al. 1997). α-Syn exists in multiple forms including soluble monomers, oligomers and fibrils. The multimeric forms appear to be the toxic species and their formation is dependent on several factors including amino acid substitutions due to mutations in its gene, α-syn concentration, and the presence of dopamine and dopamine adducts (Li, Lin et al. 2005; Mazzulli, Armakola et al. 2007; Burke, Kumar et al. 2008). Exogenous factors such as pesticides have also been reported to increase α-syn aggregation. Given that α-syn aggregation appears central to the pathogenesis of PD and pesticides appear to promote this process via a variety of mechanisms, we will briefly discuss α-syn homeostasis.

2.1.1 α-Synuclein

α-Syn is a predominantly neuronal protein that was first implicated in the development of Alzheimer's disease. The identification of three mutations—A53T, A30P, and G188A—in its gene in a few families with dominantly-inherited PD led to the finding that fibrillar α-syn is the major component of Lewy bodies not only in these patients but also in sporadic PD (Nussbaum and Polymeropoulos 1997; Spillantini, Schmidt et al. 1997; Kruger, Kuhn et al. 1998; Trojanowski, Goedert et al. 1998; Giasson, Jakes et al. 2000). Overexpression of normal α-syn by gene multiplication causes fairly typical PD (Farrer, Kachergus et al. 2004), and people who have an α-syn promoter that confers a higher level of expression are at higher risk of developing PD (Pals, Lincoln et al. 2004; Mueller, Fuchs et al. 2005). Thus, increased levels of normal α-syn increases one's risk of getting PD and if it is high enough, it causes it. Importantly with respect to this review, certain pesticides can cause α-syn levels to increase providing a theoretical mechanism to contribute to PD (see below for individual pesticides). Furthermore, pesticides can directly increase the rate of α-syn fibril formation adding another method they can contribute to the pathogenesis of PD (Uversky, Li et al. 2001).

2.1.2 Ubiquitin-proteasome system dysfunction in Parkinson's disease

α-Syn concentrations are determined by the relative amount of its expression and degradation, and the higher the concentration, the more likely it is to form aggregates. Both the ubiquitin-proteasome system (UPS) and autophagy have been shown to degrade α-syn. The soluble form appears to be degraded by the UPS while the lysosomal pathway appears to degrade aggregated forms of the protein (Liu, Corboy et al. 2003; Cuervo, Stefanis et al. 2004; Zhang, Tang et al. 2008; Mak, McCormack et al. 2010). The UPS is a highly regulated ATP-dependent degradative multi-subunit pathway that helps clear the cell of damaged, misfolded or otherwise unneeded proteins. Proteins are targeted to the UPS by ubiquitin-activating enzymes (E1), ubiqutin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3). Once polyubiquitinated, proteins are recognized by the 19S regulatory complex of the 26S proteasome and translocated to the 20S complex for degradation. Finally, ubiqutin is recycled via thiol proteases called deubiquitinating enzymes, which fall into the ubiquitin carboxyl-terminal hydrolase (UCH) or ubiquitin-specific processing protease (UBP) families (Goldberg 2003).

Three known genetic causes of PD involve aspects of UPS function. Parkin gene mutations cause autosomal recessive PD and is an E3 ubiquitin ligase necessary for targeting proteins for degradation. UCH-L1 gene mutations cause autosomal dominant (AD) PD and UCH-L1 is necessary for the recycling of ubiquitin. Finally, α-syn is a substrate for the UPS and mutations and duplication of its gene cause AD PD. There is also evidence that UPS dysfunction is involved in sporadic PD. Reduced UPS activity has been found in brains of PD patients (McNaught and Jenner 2001) and some investigators have found that administration of UPS inhibitors to rodents can recreate some of the features of PD although these models remain controversial (Bove, Zhou et al. 2006; Kordower, Kanaan et al. 2006; Manning-Bog, Reaney et al. 2006; McNaught and Olanow 2006; Schapira, Cleeter et al. 2006; Zeng, Bukhatwa et al. 2006). Finally, we have found that several commonly used pesticides inhibit the UPS and are associated with an increased risk of developing PD (Wang, Li et al. 2006; Chou, Maidment et al. 2008).

2.1.3 Autophagy and Parkinson's disease

Autophagy is a cellular process that involves protein and organelle degradation. Dysfunction of autophagy has long been known to be involved in disease but only recently has been implicated in the pathogenesis of PD. Gaucher's disease is an autosomal recessive lysosomal storage disease caused by mutations in its gene that lead to dysfunction of autophagy and are associated with a marked increased risk of developing typical PD with Lewy bodies (Aharon-Peretz, Rosenbaum et al. 2004; Neumann, Bras et al. 2009; Sun, Liou et al. 2010). Another autosomal recessive Parkinsonian disorder (PARK9) is caused by a mutation in another lysosomal gene, ATP13A2 (Ramirez, Heimbach et al. 2006). PINK1 has also been shown to be a modifier of autophagy and mutations in its gene cause PD with Lewy bodies (PARK6) (Narendra, Jin et al. 2010; Samaranch, Lorenzo-Betancor et al. 2010). Additional evidence for a role of autophagy in PD comes from studies of sporadic PD brains where increased numbers of autophasomes have been described (Anglade, Vyas et al. 1997).

α-Syn clearance is likely carried out by both the UPS and autophagy. Large aggregates of α-syn proteins are likely degraded by macroautophagy but soluble α-syn can undergo degradation via an alternate lysosomal pathway, chaperone-mediated autophagy (CMA)

(Massey, Zhang et al. 2006). α-Syn has also been found to inhibit lysosomal macroautopaghy and oligomers are resistant to CMA adding further support for a possible role of protein degradation dysfunction in the pathogenesis of PD (Martinez-Vicente, Talloczy et al. 2008).

2.2 Mitochondrial dysfunction and oxidative stress

The role of mitochondrial dysfunction in the pathophysiology of PD was first suggested by the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin selective for nigral dopaminergic neurons, acts through inhibition of complex I of the electron transport chain. MPTP is converted by monoamine oxidase (MAO-B) to its toxic metabolite 1-methyl-4-phenylpyridinium (MPP+), which is rapidly concentrated by dopaminergic neurons into the mitochondria and produces cell death (Langston, Ballard et al. 1983; Chiba, Trevor et al. 1985; Javitch, D'Amato et al. 1985; Gainetdinov, Fumagalli et al. 1998). This discovery led to the findings that complex I activity is reduced not only in brains of PD patients but also in peripheral mitochondria (Schapira, Cooper et al. 1990; Haas, Nasirian et al. 1995). Furthermore, mutations in some genes that code for mitochondrial associated proteins can cause PD (e.g. DJ1 and PINK1) and chronic systemic administration of complex I inhibitor (rotenone) in rodents reproduces many of the clinical and pathological aspects of PD (Betarbet, Sherer et al. 2000).

It is still unclear what are the downstream targets of mitochondrial dysfunction. ATP depletion is not necessary in the rotenone rodent model for its toxicity but the generation of reactive oxygen species (ROS) appears to be essential. ROS are known to oxidize DNA, lipids and proteins to cause cellular damage. Interestingly, ROS from complex I inhibition leads to UPS inhibition (Chou, Li et al. 2010). Furthermore, the formation of ROS from complex I inhibition likely contributes to the Lewy-like bodies observed in the rotenone model (Betarbet, Canet-Aviles et al. 2006).

2.2.1 Aldehyde dehydrogenase (ALDH) inhibition

Another form of mitochondrial dysfunction implicated in PD involves the inhibition of aldehyde dehydrogenase 2 (ALDH2), a mitochondrial ALDH. This enzyme is responsible for the detoxification of aldehydes that could otherwise modify proteins. For example, the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) is detoxified by ALDH2 and increased HNE has been reported in post mortem PD brains as adducts (Yoritaka, Hattori et al. 1996) and as a component of Lewy bodies (Castellani, Perry et al. 2002). Furthermore, HNE has been shown to prevent α-syn fibrillation and form α-syn oligomers, which are toxic to primary mesencephalic cultures (Qin, Hu et al. 2007). Another ALDH2 substrate, the dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL), has also been reported to induce α-syn aggregation and be toxic to dopaminergic neurons (Burke, Kumar et al. 2008). ALDH involvement in the pathogenesis of PD is not yet well established but preliminary *in vitro* and epidemiology studies have implicated this enzyme as a possible mediator of some pesticides' toxicity (see benomyl below).

2.3 Altered dopamine homeostasis

Conventional wisdom in the pathophysiology of PD is that dopaminergic neurons are selectively vulnerable, although more recent evidence suggests that neuronal loss is more widespread. One hypothesis for this possible vulnerability is via the metabolism of dopamine itself (Hastings 2009).

Dopamine and its metabolites are toxic and dopamine adducts have been shown to stabilize α-syn oligomers. DOPAL, a substrate for ALDH2, is particularly toxic. Interestingly, DOPAL is formed by the enzyme MAO-B and blocking this enzyme with specific drugs appears to alter the progression of PD (Olanow, Rascol et al. 2009). Thus, alterations in levels of dopamine or its metabolites might contribute to neuronal loss. Increased levels of VMAT2, a vesicular transporter that lowers cytosolic dopamine levels, lowers the risk of developing PD (Glatt, Wahner et al. 2006). Further support for altered dopamine homeostasis in PD comes from a recent report that polymorphisms in the dopamine transporter (DAT) gene in combination with pesticide exposure also increases the risk of PD (Ritz, Manthripragada et al. 2009).

Taken together, dysfunction of several cellular processes appears to contribute to the pathogenesis of PD. Aggregation of α-syn (oligomerization and possibly fibril formation) is the leading candidate for the final common pathway for neurons to die in PD. There is evidence that pesticides cause dysfunction in many of these processes providing potential mechanisms for their toxicity (Figure 1).

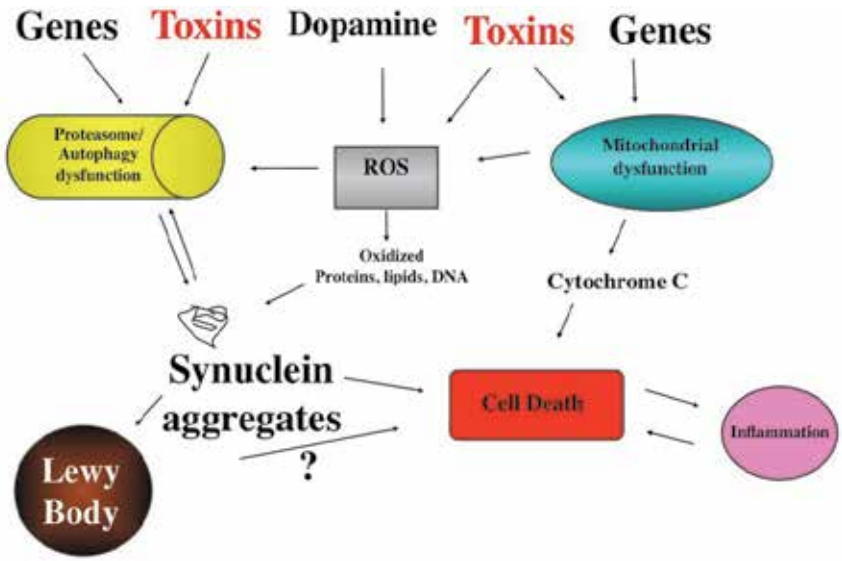

Fig. 1. Proposed pathophysiology of Parkinson's disease.

3. Epidemiology of Parkinson's disease

3.1 Environment and Parkinson's disease

Over the past two decades, several epidemiologic studies have identified a number of environmental factors that are associated with an altered risk of developing PD. Smoking tobacco is almost universally found to be associated with a lower risk of developing the disease (Ritz, Ascherio et al. 2007). Caffeine and alcohol consumption have also been associated with a reduced risk of PD (Hellenbrand, Seidler et al. 1996). Since all of these addictive behaviors are associated with reduced incidence, it has been proposed that they may be surrogate markers for a common behavioral phenotype of pre-clinical PD patients rather than these exposures all being protective. The use of nonsteroidal anti-inflammatory

drugs has also been found to reduce the risk of PD suggesting inflammation may be somehow involved in its pathogenesis (Wahner, Bronstein et al. 2007).

A number of studies have found strong associations between an increased risk of PD and rural living, well-water consumption, farm occupations, and pesticide exposure. These reports have been reviewed extensively by others so we will not review all the studies here (Le Couteur, McLean et al. 1999; Di Monte 2003; Alavanja, Hoppin et al. 2004; Kamel and Hoppin 2004; Li, Mink et al. 2005; Brown, Rumsby et al. 2006). The association with pesticide exposure has been the most provocative association with developing PD to date although almost all of these reports were based on self-reporting pesticide exposure (i.e. potential recall bias) and the diagnosis of PD was not confirmed (Gartner, Battistutta et al. 2005). Despite these weaknesses, a meta-analysis of case–control studies obtained a combined odds ratio (OR) for PD risk of 1.94 (95% CI, 1.49–2.53) (Priyadarshi, Khuder et al. 2000). Subsequent studies reported OR of up to 7.0 (Brown, Rumsby et al. 2006).

Recently, the issue of potential recall bias was mitigated by determining pesticide exposure in a prospective manner. Petrovitch et al reported an increased risk of developing PD in Japanese-American men who worked on a plantation and were exposed to pesticides (Petrovitch, Ross et al. 2002). Similarly, Ascherio et al found a 70% increased risk of developing PD in those who reported significant pesticide exposure (Ascherio, Chen et al. 2006). These reports add support for a true association between pesticides and PD but still are limited in that they did not identify individual toxins and dose response relationships could not be determined.

3.2 Specific pesticides as risk factors

There are a few ongoing studies that address both the issue of recall bias and are identifying specific pesticides that confer an altered risk of developing PD. The Agricultural Health Study (AHS) is a prospective study, including 84,740 private pesticide applicators (mostly farmers) and their spouses recruited in 1993-97 in Iowa and North Carolina. Pesticide exposure was self-reported but felt to be reliable. The diagnosis of PD was also self-reported but later confirmed by direct examination. The first report from this study found an association between PD with increasing lifetime days of use of any pesticide but no specific pesticide could be definitely implicated due to lack of statistical power (Kamel, Tanner et al. 2007). Recently, the investigators reported that PD was associated with rotenone (OR 2.5, 95% CI 1.3, 4.7) and paraquat use (OR 2.5, 95% CI 1.4, 4.7) (Tanner, Kamel et al. 2011). The strength of this study is that it is prospective, the diagnosis was confirmed by examination, and specific toxins were identified. The primary weakness of this study is that they have only 110 cases limiting their power to test a number of pesticides individually and in combinations. The small number of cases also limits their ability to test gene-environment interactions. One additional limitation was that quantitation of pesticide exposure, types of exposure and length of exposures were self-reported. Despite these shortcomings, this study adds strong epidemiological evidence that pesticides are associated with an increased risk of developing PD, especially for rotenone and paraquat.

Ritz and colleagues at UCLA have taken another approach to identifying specific pesticides that are associated with an altered risk of PD. We took advantage of the California Pesticide Use Reporting database and Geographic Information System land-use maps to estimate historical exposure. All commercial pesticide applications have been recorded by compound, quantity, and specific location since 1974. Thus, individual subject exposures

can be approximated by using their residential and occupational addresses for the past 37 years. In this Parkinson's Environment Gene (PEG) study, neurologists specializing in movement disorders went into the field to confirm the diagnosis in over 350 incident PD cases in the central California valley where pesticides are applied liberally and the risk of PD appears to be increased (Ritz and Yu 2000; Kang, Bronstein et al. 2005). A similar number of age and sex matched control subjects were also recruited from the same communities. In addition to several lifestyle and medical assessments, DNA and serum samples were also obtained.

Individual pesticides were investigated in the PEG study based on previous reports implicating the agents as possibly involved in the pathogenesis of PD based on previous epidemiologic and/or laboratory studies. Maneb and paraquat were investigated because administration of pesticides to rodents produces a nice model of PD (see below). Estimates for maneb and paraquat exposures incurred between 1974 and 1999 were generated based on their residence. Exposure to both pesticides within 500m of their homes increased PD risk by 75% (95% CI 1.13, 2.73). Subjects aged ≤ 60 yo were at much higher risk of developing PD when exposed to either maneb or paraquat alone (odds ratio (OR) = 2.27, 95% CI: 0.91, 5.70) or to both pesticides in combination (OR = 4.17, 95% CI: 1.15, 15.16) (Costello, Cockburn et al. 2009). PEG investigators have found similar associations with organophospate pesticides—diazinon (OR 1.73, CI 1.23, 2.45) and chlorpyrifos (OR 1.50, CI 1.04, 2.18) (Manthripragada, Costello et al. 2010)—and ziram (OR 3.01, CI 1.69, 5.38). In subjects ≤ 60 yo, exposure to both ziram and paraquat had a 6-fold increase in risk of PD (CI 1.94, 18.33) (Wang, Costello et al. 2011). It is important to note that all estimates of exposures were not dependent on subject recall for total exposure or duration of exposure. Recent exposure to pesticides (1990 to 1999) was not generally associated with an increased risk of PD consistent with the theory that PD pathology likely starts several years before it manifests itself clinically.

The population is exposed to pesticides in a variety of ways, not just inhalation from spraying and crop dusters. Gatto et al. looked at five pesticides that were likely to be detected in well water (Gatto, Cockburn et al. 2009). Although local well water was not analyzed, these pesticides were identified based on their solubility, half-lives, and adsorptive properties. These included organophosphates (diazinon, dimethoate, chlorpyrifos), a carbamate (methomyl), and a sulfite ester (propargite). Excluding those who did not consume well water, potential inhalation and ingestion of each pesticide was associated with 23-57% increased risk of PD. Consuming well water potentiated this effect to a 41-75% increased risk. Up to a two-fold increase was observed for those who consumed water with the highest potential contamination of at least one of these pesticides. Finally, those with PD were found to have consumed well water an average of 4.3 years longer than controls. Because PEG has enrolled over 350 cases, we have statistical power to test gene-environment interactions. Not surprisingly, the risk of developing PD in pesticide-exposed subjects is clearly altered based on the subject's genetic background (see below).

3.3 Gene-environment interactions

Gene–environment interaction analyses for pesticides and PD have been rare due to small sample size and difficulty obtaining exposure data (Deng, Newman et al. 2004; Elbaz, Levecque et al. 2004; Kelada, Checkoway et al. 2006; Hancock, Martin et al. 2008). Elbaz et al found that pesticides had a modest effect in subjects who were not CYP2D6 poor

metabolizers, had an increased effect in poor metabolizers (approximately twofold), but poor metabolizers were not at increased PD risk in the absence of pesticide exposure (Elbaz, Levecque et al. 2004). Hancook et al found a gene-environment association in PD for pesticides and nitric oxide synthase 1 polymorphisms (Hancock, Martin et al. 2008). Kelada et al described a very modest risk of developing PD with specific dopamine transporter (DAT) alleles but a 5.7 fold increase (CI 1.73-18.53) in developing PD in subjects with occupational exposure to pesticides. These studies added proof of concept that the effect of environmental exposures on the risk of developing PD is at least partially dependent on one's genetic background (Kelada, Checkoway et al. 2006). Unfortunately, exposure assessments were very limited in all of these studies and individual toxins could not be determined.

Gene-environment analysis in Ritz's PEG study has only recently begun but has already revealed intriguing results. We replicated the DAT polymorphism's interaction with pesticide exposure described by Kelada et al for at least maneb and paraquat (Ritz, Manthripragada et al. 2009). Unexposed subjects with more susceptibility alleles had a 30% increased risk of developing PD whereas exposed subjects had an almost five-fold increased risk (OR = 4.53; 95% CI, 1.70-12.1). Importantly, there was a gene dose effect as well. In a similar manner, variations in PON1, the gene that encodes Paraoxonase 1 that metabolizes chlorpyrifos and diazinon, potentiated the increased PD risks associated with these organophosphates (Manthripragada, Costello et al. 2010).

For example, diazinon was associated with a 73% increased risk of PD (CI 1.23, 2.45) but the risk increases to 267% (CI 1.09, 6.55) in individuals who carry PON1 risk alleles. Variations in the dinucleotide repeat sequence (REP1) within the α-syn promoter appear to alter the risk to paraquat exposure (Gatto et al., 2010). Finally, we have preliminary evidence that variations in ALDH2 gene potentiate the increased risks associated with dithiocarbamates and other pesticides that inhibit ALDH activity (Fitzmaurice, Rhodes et al. 2010).

Clearly, the number of potential gene-environment interactions is enormous but we have clear proof of concept that these interactions need to be considered to truly understand environmental risks in PD. It will take very large sample sizes and good exposure analysis to obtain a better understanding of the many potential interactions that confer the bulk of PD risk factors. Alternatively, a candidate gene approach coupled with a better understanding of the pharmacokinetics and toxicity of specific pesticides may allow us to test gene-environment interactions using smaller sample sizes.

4. From association to causality - do pesticides cause PD and if so, how?

Epidemiological studies have clearly established the association between pesticide exposure and the development of PD. The possibility that this association represents causality has been strengthened by recent studies that addressed the problem of recall bias and have demonstrated a dose-effect relationship. Now that some individual pesticides have been implicated, mechanistic studies could be pursued. These studies are reviewed within the context of our current understanding of the pathophysiology of PD.

4.1 Rotenone

Rotenone is produced naturally in roots of certain plant species such as the jicama vine. It is a widely used domestic garden pesticide and because it is degraded by the sun in a matter of days, users tend to spray rotenone frequently. Rotenone is also a well-

characterized, high-affinity, specific inhibitor of complex I of the mitochondrial respiratory electron transport chain. Low complex I activity had been reported to be associated with PD both in brain and peripheral mitochondria but it wasn't known whether this is causal or a surrogate marker for something else. To further investigate this, Greenamyre and colleagues chronically administered the complex I inhibitor, rotenone, systemically into rodents. Some of these rats developed selective dopaminergic neuronal death as well as many of the motor features of PD. Importantly, neurons developed intracytoplasmic inclusions that were found to contain α-syn (Betarbet, Sherer et al. 2000). α-Syn pathology in the gastro-intestinal tract has also been described in the rotenone model similar to that seen in PD (Drolet, Cannon et al. 2009). Even small amounts of rotenone delivered intragastrically reproduces many of the same features described in rats given rotenone subcutaneously but in this model, the various stages of PD are reproduced in a progressive manner (Pan-Montojo, Anichtchik et al. 2010).

The mechanisms of rotenone toxicity are not completely clear but likely are more dependent on oxidative stress than energy failure (Sherer, Betarbet et al. 2002). The downstream targets of rotenone-induced oxidative damage are likely vast but the UPS appears to be one of them (Betarbet, Canet-Aviles et al. 2006; Wang, Li et al. 2006; Chou, Li et al. 2010).

Until recently, there have not been convincing epidemiologic reports linking rotenone exposure to PD. Dhillon et al reported an over 10 fold increase in risk although this study was limited because exposures were self-reported (Dhillon, Tarbutton et al. 2008). The Agricultural Health Study did find a 2.5 fold increased risk with prospective questionnaires adding further support for rotenone as a PD risk factor (Tanner, Kamel et al. 2011). Furthermore, many organic farmers in the 1970s used rotenone as a natural pesticide and a number of them have developed PD at a young age although scientific confirmation of these anecdotal reports is lacking. Other pesticides that are complex I inhibitors are used even less frequently than rotenone so little is known about associations with PD although one would predict a similar effect.

4.2 Paraquat

One of the first pesticides investigated for its potential link to PD was paraquat due to its structural similarity to MPTP, the drug that caused acute Parkinsonism in drug addicts. MPTP kills dopaminergic neurons by being metabolized to MPP+ by MAO-B, entering dopamine cells via the dopamine transporter and then inhibiting complex I in the mitochondrial respiratory chain. Paraquat is ubiquitously used as an herbicide to control weed growth and exposure to paraquat is associated with an increased risk of PD (Hertzman, Wiens et al. 1990; Semchuk, Love et al. 1992; Liou, Tsai et al. 1997).

Additional support for paraquat increasing the risk of PD comes from animal studies. Mice infused with paraquat for three consecutive weeks exhibit dopamine cell loss and cytosolic α-syn aggregates (Brooks, Chadwick et al. 1999; Manning-Bog, McCormack et al. 2002; McCormack, Thiruchelvam et al. 2002). The mechanism by which paraquat causes dopamine cell death is not clear. Since it is structurally very similar to MPTP, it was presumed that paraquat acted in a similar manner. Surprisingly, unlike MPP+, paraquat is not a substrate for the dopamine transporter and does not inhibit complex I except at very high concentrations (Richardson et al 2005). Paraquat toxicity does appear to be dependent on increasing oxidative stress and its action as a redox-cycler appears likely involved in its toxicity (McCormack, Atienza et al. 2005).

4.3 Dithiocarbamates (maneb and ziram)

Dithiocarbamates (DTCs) are a class of some of the most commonly used organic fungicides. They are classified into 2 groups based on whether there is a carbonyl (group 1) or hydrogen on the nitrogen carbamate. Most DTCs are complexed with metals including zinc (e.g. ziram and zineb), iron (e.g. ferbam) and manganese (e.g. maneb). DTCs first became relevant to PD researchers in 1985 when Corsini et al found that diethyldithiocarbamate pretreatment enhanced MPTP toxicity in mice (Corsini, Pintus et al. 1985). They proposed that diethyldithiocarbamate would potentiate MPTP toxicity by inhibiting superoxide dismutase since they believed at that time that MPTP acted primarily as a redox cycler. Thiruchelvam et al. later reported that maneb potentiated the toxicity of paraquat preferentially in the nigrostriatal dopaminergic system (Thiruchelvam, Brockel et al. 2000; Thiruchelvam, McCormack et al. 2003). Furthermore, maneb and paraquat exposure was found to exacerbate α-synucleinopathy in A53T transgenic mice (Norris, Uryu et al. 2007).

The animal models using maneb and paraquat were intriguing but it was only recently that an association between maneb and paraquat exposures and PD were reported (Costello, Cockburn et al. 2009). Similar to the animals studies, residential exposure to maneb and paraquat exposure together is associated with a 114% increased risk of newly diagnosed PD. Furthermore, the risk of PD was increased to 317% for cases ≤ 60 yo. Neither pesticide alone was associated with PD but there were few subjects with maneb only exposure so that the true effect for maneb alone could not be assessed. When both occupational and residential exposures are taken into account, subjects exposed to maneb and paraquat alone had a 126% and 50% increase in risk of developing PD respectively but for exposure to maneb and paraquat together, the risk increased to 8.75x (CI 2.3-33.2) in the younger group (Wang, Costello et al. 2011). These epidemiologic data taken together with the animal data are quite compelling that these pesticides truly increase the risk of PD.

As mentioned above, DTCs are a large group of fungicides with similar structures. We identified another DTC, ziram, in an unbiased screen to identify pesticides that inhibit the proteasome (Wang, Li et al. 2006). Maneb and some other DTCs were also found to inhibit the UPS but at higher concentrations (Chou, Maidment et al. 2008). Ziram selectively killed dopaminergic neurons in primary cultures and increased α-syn levels in the remaining neurons. Systemic administration of ziram alone into mice caused progressive motor dysfunction and dopaminergic neuronal damage (Chou et al 2008). Furthermore, subjects exposed to ziram alone had a 201% (CI 1.69, 5.38) increase of risk of developing PD and a 598% (CI 1.95, 18.3) increased risk when exposed with paraquat in subjects ≤ 60 yo (Wang, Costello et al. 2011). These data add further support for the role of DTCs as a causal risk factor for PD.

It is still not completely clear how DTCs act biologically. We have found that they do not increase oxidative stress and therefore are unlikely acting through the mitochondrial respiratory chain (Wang, Li et al. 2006). DTCs clearly inhibit the UPS and their potency depends on whether they contain a tertiary or a secondary amino group. Ziram was studied extensively given its high potency to inhibit the UPS and we found that it acts by interfering with the ubiquitin E1 ligase with an IC_{50} of 161 nM (Chou, Maidment et al. 2008). Zhou et al reported that maneb also inhibited the UPS but at higher concentrations (IC_{50} of approx. 6 μM) and increased protein carbonyls suggesting increased oxidative stress (Zhou, Shie et al. 2004). We also found that maneb inhibits the UPS at much higher concentrations than ziram but we did not find evidence of oxidative stress. Differences may very well be due to differences in the techniques used since we used an *in vitro* 26S UPS assay and DCF

fluorescence to detect ROS and Zhou et al used an *in vitro* 20S UPS assay and protein carbonyl immunohistochemistry for detection of oxidative stress. Recently, we have found that both maneb and ziram inhibit ALDH2 at environmentally-relevant concentrations, adding another potential mechanism of toxicity, especially to dopaminergic neurons (Fitzmaurice, Rhodes et al. 2010). Since ziram does not contain manganese, it is very unlikely that it is the manganese in maneb that confers its toxicity as some have suggested.

4.4 Benomyl

Another important fungicide implicated in PD pathogenesis is the benzimidazole compound benomyl. It was developed as a microtubule inhibitor and is sprayed on fruits, nuts, and leaves to prevent fungal growth. Preliminary findings from the PEG study revealed benomyl exposure increased PD risk by 138% (CI 1.33, 4.27) (Fitzmaurice, Rhodes et al. 2010).

Benomyl metabolizes spontaneously into another fungicide (carbendazim) and enzymatically into several thiocarbmate compounds. We have shown that benomyl and carbendazim are UPS inhibitors, although they are not as potent as ziram (Wang, Li et al. 2006; Fitzmaurice, Ackerman et al. 2010). Furthermore, benomyl has also been reported to inhibit mitochondrial ALDH (Staub, Quistad et al. 1998). Although these studies focused on hepatic ALDH, we recently reported that benomyl exposure reduced ALDH2 activity *ex vivo* in rat neuronal suspensions (Fitzmaurice, Ackerman et al. 2010). We have also found that exposure to benomyl or one of its ALDH2-inhibiting metabolites (S-methyl-N-butylthiocarbamate, or MBT) causes dopaminergic neuronal death *in vitro*, while the UPS-inhibiting metabolite (carbendazim) does not. These findings, combined with the observation that DTCs also inhibit ALDH2, suggest that ALDH2 inhibition may be an important mechanism in pesticide toxicity with respect to PD.

The toxicity of ALDH2 inhibition is likely due to the accumulation of toxic aldehydes. We would predict that ALDH2 inhibition would lead to increased levels of DOPAL and HNE adducts and preliminary studies in primary cultures support this hypothesis. Furthermore, the loss of dopaminergic neurons due to benomyl was attenuated by co-treatment with the MAO-B inhibitor pargyline which decreases DOPAL formation (Fitzmaurice, Ackerman et al. 2010). Since DOPAL and HNE accumulation have been reported to induce α-syn aggregation (Burke, Kumar et al. 2008), these findings support ALDH2 inhibition as an important mediator of pesticide toxicity in PD.

5. Summary

The causes of PD are not completely understood but both genetic and epidemiologic studies suggest that dysfunction of one or more biological processes lead to α-syn aggregation and neuronal death. Epidemiologic studies have clearly shown PD to be associated with pesticide exposure and specific pesticides conferring at least some of this increased risk have recently been identified. The fact that administration of pesticides to animals recapitulates many of the behavioral and pathological features of PD provides evidence that the associations found in epidemiologic studies are causal. Elucidating the mechanisms of pesticide toxicity in mammals not only strengthens the hypothesis that exposure to these toxins can increase the risk of developing PD, but also furthers our understanding of the pathophysiology of the disease in general. It is clear that the list of pesticides discussed in this chapter is not complete and that pesticides are not the only environmental toxins that

alter the risk of PD, but the preponderance of evidence taken together supports an important role of pesticides in the pathogenesis of PD. A better understanding of these issues will take us one step closer to a cure.

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Dithiocarbamate Toxicity - An Appraisal

Narayan C. Rath, Komal S. Rasaputra, Rohana Liyanage,
Gerry R. Huff and William E. Huff
United States Department of Agriculture/Agricultural Research Service,
Department of Poultry Science,
Department of Chemistry and Biochemistry,
University of Arkansas,
Fayetteville,
USA

1. Introduction

Dithiocarbamates (DTC) are organosulfur compounds represented by a general structure (R1R2)N-(C=S)-SX, where R can be substituted by an alkyl, alkylene, aryl, or similar other group, and X usually by a metal ion (Edwards, 1991; Kamrin, 1997; US EPA, 2001). Discovered in the 1930s, the DTC were first introduced as fungicides for commercial applications during World War II (Ware & Whitacre, 2004). Besides their wide use as fungicides for treatment of crops, vegetables, seeds, and ornamental plants, they are also used as accelerators in the rubber industry, animal repellants, and biocides in many household products (Edwards et al., 1991; Kamrin, 1997). Figure 1 shows examples of some common DTC pesticides. Thiram, disulfiram, ziram, and ferbam are analogous dialkyl DTC with differences in their R groups and the later two containing different metal ions between their S atoms. Pyrrolidinedithiocarbamate (PDTC) is a monomeric DTC which contains a five member ring attached to its N atom. It is a metabolic inhibitor used in cell physiological studies (Schreck et al., 1992; Cvek and Dvorak, 2007). In ethylene-bis-dithiocarbamates (EBDTC), the R groups of two DTC molecules form an ethylene bridge. The EBDTC are regarded as polymeric DTC because their metal ions can bind several molecules to form polymeric complexes. Some examples of EBDTC are zineb, maneb, and mancozeb which are used in preharvest agricultural applications. The DTC anions are highly reactive which can conjugate with other molecules containing SH groups and form metal chelates. The multisite interactions of DTC give them advantage to influence the biological activities of different proteins, enzymes, and exert toxic effects. Some of those modes of action of DTC compounds have been exploited for their use in clinical applications (Morrison et al., 2010). However, the extensive use of these chemicals in agriculture has raised concern for their effects as occupational and ecotoxicological hazards. Several reviews on the biological and toxicological effects of DTC summarize many studies in the field (Edwards et al., 1991; US EPA, 2001; Cvek and Dvorak, 2007). The objective of this review is to highlight some of the recent findings on the effects of dialkyl DTC and EBDTC with emphasis on studies of the avian system which has not been a focus of earlier literature.

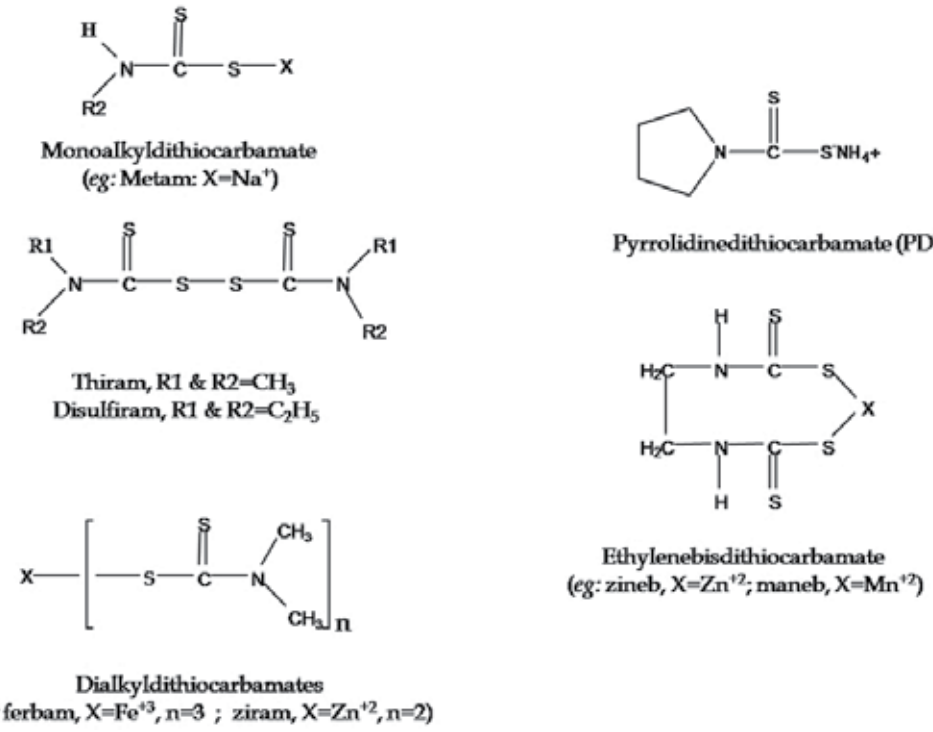

Fig. 1. Structures of some representative dithiocarbamates, R1, R2= alkyl (CH_3, C_2H_5)
X= metal ion (Na^+, Mn^{+2}, Zn^{+2}, Fe^{+3})

2. Metabolism and the toxic effects

The toxicological effects of DTC can occur from their absorption through skin exposure, ingestion, and inhalation. The lipophilic nature of DTC makes them suitable for their passage across the cell membrane. Metabolic studies with representative DTC (ex. thiram, disulfiram, mancozeb) have shown that these chemicals undergo detoxification through S-glucoronidation or biodegrade to different metabolites such as carbon disulfide (CS_2), thiourea, alkylamines, ethyleneamines, and several other biotransformation products (Edwards et al., 1991; US EPA, 2001). CS_2 is a general neuropathic agent and ethylenethiourea (ETU) which is a metabolite of EBDTC, has antithyroid and carcinogenic effects (Edwards et al., 1991; DeCaprio et al., 1992; Houeto et al., 1995; US EPA, 2001). Under physiological conditions most dialkyl DTC can reoxidize to form thiuram disulfide (Burkitt et al., 1998). Thus, the toxic effects of DTC can be due to the whole molecule and their decomposition products such as CS_2 and ETU. The intact DTC molecules exhibit both pro oxidant and antioxidant activities (Nobel et al., 1995; Liu et al., 1996; Orrenius et al., 1996; Burkitt et al., 1998; Wild & Mulcahy, 1999; Cereser et al., 2001). Whereas the disulfide bridges and the metal complexes contribute to their prooxidant effects, the SH contributes to their antioxidant effects (Orrenius et al., 1996; Elskens & Penninckx, 1997). Tissue or organ specific toxic effects of these chemicals may be due to the differential competency of their intracellular passage and binding to crucial structural and functional entities of the cells eventually leading to the metabolic disruptions, pathological changes, and cell death.

3. Molecular and cellular effects

The DTC compounds can form mixed disulfides with other molecules containing SH functions such as proteins, peptides and enzymes modulating their biological activities. The covalent modification of cysteine residues in the active sites can affect enzyme activities. As antioxidants, they react with hydroxyl radicals, peroxides, and superoxide ions, and inhibit their oxidative potential (Nobel et al., 1995; Liu et al., 1996). As prooxidants, DTC increase Cu catalyzed reactive oxygen species (ROS) formation and change the balance of reduced glutathione (GSH) to its oxidized form (GSSG) in favor of the later (Burkitt et al., 1998). GSH is a sulfohydryl containing tripeptide critical for protecting cells against oxidative stress. It is a major antioxidant in the body and an important regulator of cell proliferation, gene transcription, and apoptosis (Rana et al., 2002; Biswas & Rahman, 2009). GSH is necessary for detoxification of xenobiotics, carcinogens, and maintenance of immunity. Accumulation of oxidized form of glutathione (GSSG) leads to the activation of transcription factor nuclear factor kappa B (NF-kB) stimulating stress and inflammatory response, and cell survival (Dellhale et al., 2004). The conversion of GSSG to GSH, catalyzed by glutathione reductase, is inhibited by DTC which also inactivate several different transcription factors principally, the NF-kB and hypoxia inducible factor (Haddad, 2002 & 2003; Biswas & Rahman, 2009). PDTC is a popular inhibitor of transcription factor NF-kB which modulates the expression of many enzymes and proteins including nitric oxide synthase, heat shock protein 70 (HSP70), and induces endoplasmic reticulum stress (Schreck et al., 1992; Cvek & Dvorak, 2007; Chen et al., 2010; Cotogni et al., 2010). Prevention of binding of NF-kB to DNA induces apoptosis. DTC inhibit proteosome dependent protein degradation (Wang et al, 2006, 2011; Lovborg et al, 2006; Daniel et al, 2007; Chou et al, 2008) and promote peptide amidation (Mains et al., 1986). Thiram increases oxidative stress and induces formation of lipid peroxides, protein carbonyls, and stimulates changes in membrane potential of cells leading to ion influx inducing cell death (Erl et al., 2000; Sook Han et al., 2003; Grosicka et al., 2005). Similarly, disulfiram also induces oxidative stress that changes mitochondrial permeability leading to mitochondrial injury (Balakirev and Zimmer, 2001). A number of enzymes are inhibited by DTC which include cyclooxygenase, (Lee et al., 2002), heme oxygenase (Kushida et al, 2002), cytochrome P450, superoxide dismutase, glutathione reductase, and caspase (Dalvi et al., 2002; Cvek & Dvorak, 2007; Seefeldt et al., 2009). The superoxide dismutase inhibitory activity of thiram and disulfiram is implicated in their anti-angiogenic effects (Marikovsky et al., 2002; Shian et al., 2003). The aldehyde dehydrogenase inhibitory activity of disulfiram is the basis of its therapeutic efficacy against alcoholism (Edwards et al., 1991; Cvek & Dvorak, 2007). Disulfiram also suppresses matrix metalloproteinase (MMP) expression in osteosarcoma cells through modulation of NF-kB and activator protein-1, and possibly its metal chelating properties (Cho et al., 2007).

3.1 Neuropathic effects

Peripheral neuropathy induced by DTC is a major toxic effect which has been reported in humans and animals (Frisoni & Di Monda, 1989). Many DTC pesticides including several dialkyl dithiocarbamates and EBDTC are implicated in inducing Parkinson's-like neuropathy. The ability of DTC to inhibit acetylcholine esterase, an enzyme responsible for degradation of the neurotransmitter acetylcholine, was considered to cause neuropathy (Edwards et al., 1991), but later studies did not substantiate this mode of action. However, Viviani et al., (2008) using adrenomedullary PC12 cells showed propineb, an EBTC, to induce acetylcholine release which is mediated through depolymerization of cytoskeletal

actins. Since most DTC compounds can metabolize to CS_2, their neuropathic effects were thought to be mediated by this metabolite alone. Johnson et al. (1998) showed cross linking of neurofilament proteins induced by CS_2 as a mechanism for its axonopathic and neurotoxic effects. However, metabolic studies with different DTC have not supported the role of CS_2 as the sole mechanism for their neurotoxic effects (SAP report, 2001). Stimulation of non selective cation channels by thiram, ziram, and maneb cause the influx of Ca^{++} and Cu^{++} into mitochondria increasing oxidative stress which induce apoptosis of PC12 cells and dopaminergic neuronal damage (Sook Han et al., 2003; Barlow et al., 2005). DTC metal complexes induce dopamine oxidation and produce intraneuronal oxidative stress leading to neuronal damage (Fitsanakis et al., 2002). Since DTC chelate heavy metals such as Cu, Zn, and Fe, leading to their intraneuronal accumulations, these metals have been implicated in promoting lipid peroxidation, oxidative stress, and enzyme inhibitions causing neurotoxic effects (Nobel et al., 1995; Valentine et al., 2009; Viquez et al, 2009; Viola-Rhenals et al., 2007). Increased production of reactive oxygen species by the actions of mancozeb and zineb is also implicated in their neuronal toxicities (Domico et al., 2007). Maneb, an EBDTC containing Mn^{+2}, was found to induce nitric oxide production, lipid peroxidation, and cause Parkinson's like disease syndrome in mice (Gupta et al., 2010). Mancozeb, thiram, and disulfiram cause membrane potential changes and impair ATP dependent glutamate uptake into the synaptic vesicles and prevent binding of glutamate to its receptors resulting in excitotoxic effects in the brain (Nagendra et al., 1997; Vaccari et al., 1999). Ubiquitin proteosome pathway maintains the balance of cellular proteins through their degradation since abnormal accumulation of protein can interfere with cell functions (Myung et al, 2001). Both disulfiram and ziram inhibit ubiquitin proteosomal pathways causing dopaminergic cell damage (Lovborg et al., 2006; Chou et al., 2008). Disulfiram also reduces the activity of brain enzyme peptidoglycine-5 hydroxylating monooxygenase and alpha-melanocyte stimulating hormones affecting behavioral changes in rats (Rahman et al., 1997).

3.2 Reproductive and endocrine disruptive effects

Chemicals which interfere with endocrine functions altering the synthesis, metabolism, and secretion of hormones, or their target organ effects, are called endocrine disruptors (Diamanti-Kandarakis et al., 2009). There are several reports suggesting the endocrine disruptive actions of DTC. Studies by Stoker et al (1993; 2003) showed that thiram induces ovulatory delay and affects fecundity in rats. Some of these effects of DTC are related to the interference of enzymes involved in the synthesis of catecholamines which regulate neuroendocrine functions (Stoker et al., 1993; Goldman et al., 1994). Thiram inhibits spermatogenesis in rats (Mishra et al., 1998). Mancozeb affects ovarian function and disrupts the estrous cycle, inducing infertility in rats (Cooper et al., 1999; Cecconi et al., 2007). The hypothyroid and antithyroid effects of zineb and mancozeb are associated with their metabolite ethinylthiourea (ETU) (Houeto et al., 1995; US EPA, 2001; Panganiban et al., 2004; Axelstad et al, 2011). Both thiram and disulfiram inhibit 11β hydroxyl steroid dehydrogenase 2, an enzyme that catalyzes conversion of hormonally active glucocorticoids, cortisol and corticosterone, to their inactive metabolites, and interfere with binding to their receptors (Atansov et al., 2003; Garbrecht et al., 2006).

3.3 Immunomodulatory effects

The immunomodulatory effects of DTC can be largely related to their ability to prevent activation of transcription factors and other signaling mechanisms. Lipopolysaccharide

induced tumor necrosis factor alpha production by promyelocytic THP-1 cells is inhibited by mancozeb (Corsini et al., 2006). Ziram interferes with the lytic function of natural killer cells through modification of their cell surface proteins such as CD16 which is necessary for their binding to target cells (Taylor & Whalen, 2009) and potentiates Concanavalin A induced interferon-γ and interleukin-6 production by the vascular lymph node cells (De Jong et al., 2002). In U937 lymphoma cells, ziram produces its toxic effects by activating intracellular caspase-3 enzyme and mitochondrial cytochrome c release which lead to their apoptosis (Li et al., 2010). However, with respect to cellular immunity, studies have shown DTC induce activation of T cells, natural killer (NK) cells, and increase immunoglobulin secretion by B cells (Corsini et al., 2006, 2008). Thiram induces lymphocyte sensitization, hypersensitivity, and allergic dermatitis (Saunders & Watkins, 2001). Although the mechanism of allergic dermatitis induced by thiram is not well understood, the involvement of T cells is likely. DTC can act as haptens which on conjugating to proteins may induce allergic hypersensitivity. Cytofluorometric study by Lombardi et al. (1991) showed increased splenic population of T cytotoxic/suppressor cells induced by dimethyl and diethyl DTC. The effects of different DTC on immunity needs better understanding.

3.4 Carcinogenic and teratogenic effects

The EBDTC in general, are considered to be carcinogenic because of their metabolite ETU that produces thyroid and pituitary tumors (Houeto et al., 1995). Steenland et al. (1997) showed the genotoxic effects of mancozeb indicated by increased chromosomal translocations and sister chromatid exchange in the blood cells of workers exposed to it. In vitro studies with zineb on human lymphocytes and CHO cells showed it to induce DNA strand breaks suggesting its carcinogenic potential in the event that the affected cells survive and propagate (Soloneski et al., 2002; 2003; Gonzalez et al., 2003). Calviello et al. (2006) showed DNA single strand breaks in rat fibroblasts exposed to mancozeb. DNA breaks and chromosomal aberration induced by thiram in CHO cells was reported by Mosseso et al. (1994), but in vivo tests employing different doses of ferbam, which is similar to thiram, showed no significant induction of aneuploidy (Shanthi & Krishnamoorthy, 2002). Although the recovery of DTC damaged cells and their survival is important for carcinogenicity, there is meagre evidence in its favor (Hasegawa et al., 1988). Studies on the effects of DTC on developing rat embryos show that these agents induce cleft palate, wavy rib formation, and long bone distortions (Roll, 1971). Several recent studies have shown sodium metam, thiram, and disulfiram caused notochord distortions, and craniofacial abnormalities in zebra fish embryos (Haendel et al., 2004; Tilton, et al., 2006; Teraoka et al., 2006; van Boxtel et al., 2010). Some effects of these chemicals on craniofacial malformation are attributed to their down-regulating effects on genes related to transforming growth factor beta-1 (TGF-β1) which plays an important role in skeletal morphogenesis. Inhibition of lysyl oxidase, a Cu^{++} dependent enzyme essential for collagen cross linking, by the chelating actions of DTC is also suggested as another possible mechanism in the induction of craniofacial abnormalities (van Boxtel et al., 2010).

4. DTC effects on avian systems

The major bulk of research on DTC has been carried out using mammalian models or cells. But their effect on avian growth plate cartilage is noteworthy because in relatively small doses and short exposure time, certain DTC can induce cartilage defects in growing birds

which render them lame (Vargas et al., 1983). The teratogenic and embryo toxic effects of DTC on avian system was recognized as early as 1955 when researchers noticed exposure to thiram caused leg problems in poultry (Waibel et al., 1955). These effects of thiram also were observed in later years (Page, 1975; Guitart et al., 1996). With the identification of tibial dyschondroplasia (TD), a defect of endochondral bone formation in young poultry by Leach & Nesheim (1965), correlations showed that DTC caused poultry leg problems (Vargas et al., 1983). Subsequent studies by different investigators showed that both dimethyl and diethyl DTC caused TD in post hatch poultry (Veltmann et al., 1985; Edwards, 1987; Orth & Cook, 1994; Rath et al., 2004). With TD, the proximal growth plates of the tibia and tibio-tarsal bones fail to ossify leading to the retention of unresolved cartilage. Feeding post hatch chickens diets containing thiram 50-100 mg /kg feed for a day or two is sufficient to induce TD (Rath et al., 2004; 2005; 2007b) (Figure 2). The incidence and severity of the disease is related to the age of the chicks; during the early phase of growth when the bones are fast growing, the effects are more severe. Subsequent studies showed that the dimeric and trimeric analogs such as thiram, disulfiram, ferbam, and ziram would induce this defect, where as the monomeric DTC such as potassium dimethyl dithiocarbamate, sodium metam, or PDTC were ineffective or less potent in similar concentrations (Rath et al., 2004; 2007b). The induction of TD by thiram is dose dependent. Feeding a thiram containing diet was more effective in inducing tibial dyschondroplasia than their subcutaneous administration (unpublished observation). Thiram reduces feed intake resulting in body weight loss but it does not stop longitudinal growth of bones. Whether, feed intake is affected through the influence of thiram on hypothalamic mechanisms is not known but based on its demonstrated neuroendocrine effects (Stoker et al., 1993), it may be a possibility. Thiram causes an elevation in serum corticosterone level (Rath et al., 2004) which can be related to its inhibitory effect on 11β-hydroxysteroid dehydrogenase-2 that mediates the conversion of corticosterone to its inactive metabolite 11-hydroxycorticosterone (Atanasov et al., 2003). Endochondral bone formation is a complex process which involves an orderly transition of cartilage from proliferative to hypertrophic state when they undergo chondrolytic degeneration and replaced by osteoblast (Reddi & Anderson, 1976; Burdan et al., 2009). Angiogenesis and neovascularization of growth plate is essential for bone formation. Thiram exerts a high level of toxicity on endothelial cells inducing death of capillary vessels in the growth plate and interferes with the hypertrophic process resulting in premature death of chondrocytes. Apoptosis of growth plate chondrocytes and blood vessels are evident by histochemical staining and the assessment of DNA fragmentation (Rath et al., 2005) (Figure 3). Treatment with thiram reduces the concentrations of enzymes and proteins associated with bone development which may be related to the cell death in growth plate (Rath et al., 2005). Both in chickens and turkeys, thiram interferes with growth plate modeling and angiogenesis by interfering with matrix metalloproteinases (MMP) production (Hasky-Negev et al., 2008; Dan et al., 2009). Vascular endothelial growth factor (VEGF) is a regulator of angiogenesis that acts through its receptors. Thiram down-regulates the expression of genes for VEGF receptor and Bcl-2, an antiapoptotic protein, in the growth plate (Rath et al., 2007a). Tian et al. (2009) showed the down-regulation of matrilin, and MMP-13 genes in growth plates of chickens that were treated with thiram. Expressions of these genes are important in growth plate maturation. Consistent with literature on the action of DTC in different systems, there was also a decrease in glutathione levels in growth plate cartilage of thiram-treated chickens (Rath et al., 2005). Comparative proteomics of growth plate tissue extracts showed decreases in several proteins in thiram-fed chickens most of which were

associated with energy metabolism, signal transduction, and secretory functions (Rasaputra et al., 2010). Down-regulation of those proteins may be responsible for chondrocyte death. The differential effect of thiram on hypertrophic chondrocytes may be related to the developmental transition of cells when they become prone to the toxic effects of DTC. Hypertrophy of chondrocytes is necessary for lengthening of bone which results in a significant change in cell volume (Farnum et al., 2002). Increases in cell volume occur from increased protein synthesis and influx of inorganic solutes, and osmolytes. The latter processes are affected by the changes in membrane permeability and increased ion channel activities. Recently, Bush

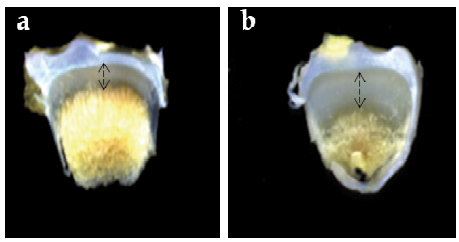

Fig. 2. Proximal tibial growth plates (arrow) of 14 day-old chickens fed either (a) a control diet or (b) a diet containing 100 mg thiram/kg feed for 48 hours between days 8 and 9 showing tibial dyschondroplasia evident by an irregular broadening of growth plate.

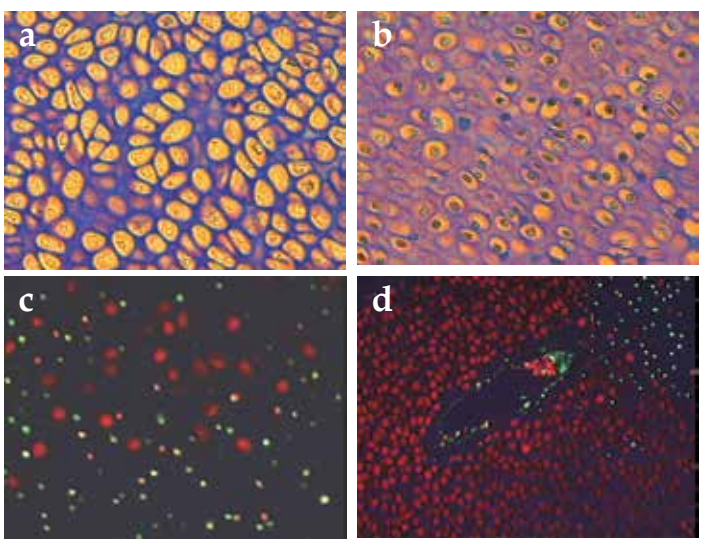

Fig. 3. Histology and histochemistry of hypertrophic zone chondrocytes of (a) normal tibial growth plate (b) thiram-induced dyschondroplastic growth plate with diminished chondrocyte volumes, pyknotic nuclei, and matrix rarefaction. (c&d) Dyschondroplastic zone chondrocytes showing terminal deoxynucleotidyl transferase mediated fluorescein dUTP nick end labeling (TUNEL) of apoptotic cells with yellow to green fluorescence and healthy chondrocytes with red fluorescence due to propidium iodide staining. (d) A dead capillary vessel surrounded by both healthy and apoptotic chondrocytes (adapted from Rath et al., 2005).

et al. (2010) showed an increased expression of Na^+ K^+ Cl^- cotransporter protein (NKCC) in hypertrophic chondrocytes. Pucci et al. (2007) also observed changes in mitochondrial membrane potentials of hypertrophic chondrocytes that permeate influx of cationic molecules. It is possible that changes in chondrocyte membrane permeability during hypertrophy facilitate higher influx of thiram into the cells inducing metabolic inhibitions, oxidative stress, and apoptosis. Thiram also can inhibit other molecular changes associated with the ossification process. Using microarray analysis of chicken growth plate, Horvat-Gordon et al. (2010) showed high expression of several genes associated with angiogenesis and oxido-reductive metabolism in hypertrophic chondrocytes. The proteins encoded by these genes such as the transferrin, matrix metalloproteinases, aldehyde dehydrogenase, lysyl oxidase, and superoxide dismutase contain metal ions that are prone to chelation by DTC which can modulate their activities and cause metabolic dysregulations. Marikovsky et al. (2002) have shown that both thiram and disulfiram interfere with angiogenesis through inhibition of superoxide dismutase.

4.1 Effects on chondrocyte culture

Proteomics is a powerful tool to identify biomarkers and understand the mechanisms of action of toxicants (Kennedy, 2002). To find whether thiram induces peptide and protein changes, the growth plate chondrocytes in culture were treated with sub lethal concentrations of thiram for 48 h. The viability of the cells were determined by monitoring the release of lactate dehydrogenase (LDH) into the culture medium as an indicator of cell damage (Rath et al., 1995) which showed no significant change at 48 h. The peptide profiles of these chondrocyte extracts were examined by means of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) in the *m/z* range of 1,000 to 7,000, and compared between control and thiram treated cells. Differential expression of the peptides was determined using statistical algorithms and principal component analysis by the use of ClinproTool™ software (Bruker Daltonics, Germany). Comparing approximately 50 spectral peaks, 4 showed quantitative differences in thiram treated chondrocytes with 2 peptides corresponding to *m/z* 3004.5 and 3310, elevated, and 2 corresponding to *m/z* 1778.9, 2556.3, decreased (Figures 4 & 5) (Rasaputra et al., unpublished). Although the functional significance of the changes in these peptides is currently unknown such information can be useful to identify toxicity associated peptide biomarkers. Similarly, comparing the protein profiles of control and thiram treated chondrocytes by two dimensional gel electrophoresis, several proteins were found to be decreased by thiram treatment, particularly a heat shock protein HSP70 was significantly down-regulated (Rasaputra et al., unpublished). HSP70 is necessary for protein folding and protects the cells from oxidative stress and apoptosis (Beere et al., 2000; Mosser et al., 2000; Guzhova & Margulis, 2006). Its chondroprotective effect has been shown in mammalian models (Otsuka et al., 1996; Etienne et al., 2008). It is possible that the decrement in the levels of HSP70 contributes to the loss of chondrocyte viability. In conclusion, the effect of DTC on growth plate development provides a good experimental model to study the toxicology of these compounds in skeletal system.

5. Conclusion

From the preceding discussion, it is evident that the DTC modify cellular metabolism by their direct interactions with different molecules such as signaling proteins, peptides, and

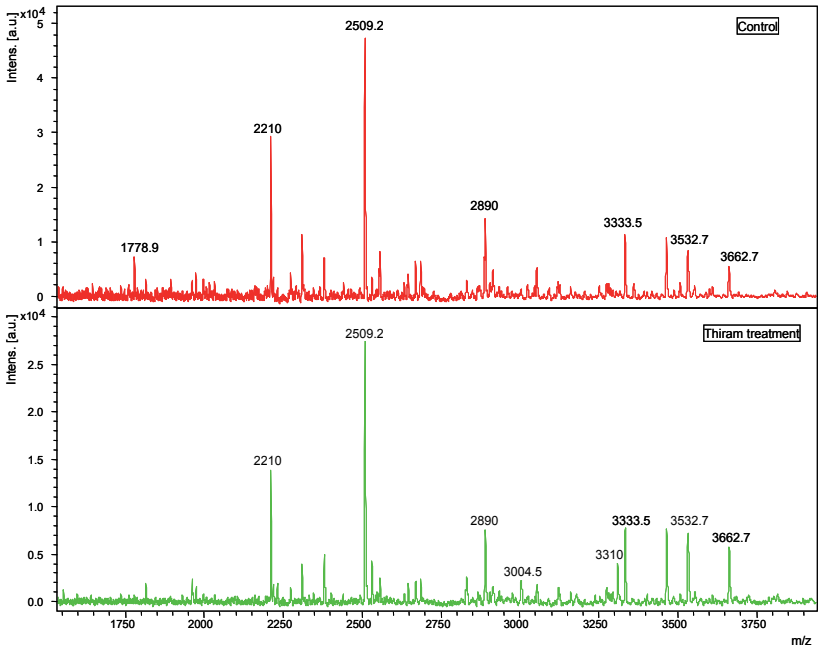

Fig. 4. The MALDI-TOF mass spectral profiles of control and thiram treated chondrocyte extracts showing peptide peaks in the *m/z* 1500-4000.

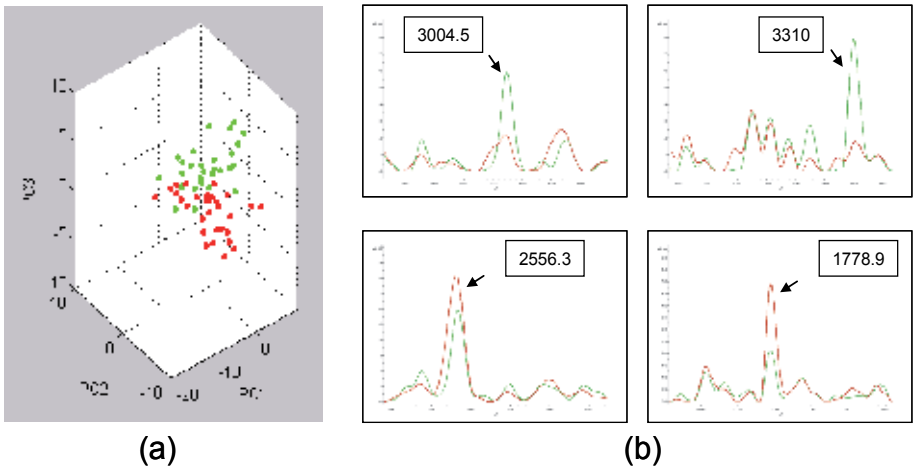

Fig. 5. (a) Principal component analysis of mass spectrum showing similarities and differences in peptide profiles of control (red) and thiram treated chondrocytes (green), and (b) profiles of the differentially expressed peptides ($P \leq 0.001$).

enzymes, and influence the oxido-reductive metabolism of the cells. Their metal chelating properties additionally, contribute to their prooxidative effects. The cells exposed to DTC experience increased oxidative stress and metabolic dysregulations leading to tissue damage, and apoptosis. The disparate vulnerability of tissues to the toxic effects of different DTC may be due to the differences in their membrane permeability and cellular constituents

interacting with these chemicals. Dividing and differentiating cells may be more susceptible to the toxic effects of DTC. Although some of their metabolites such as carbon disulfide and ethinylurea contribute to certain organ specific pathologies, it is most likely that the whole molecules are responsible for their acute toxicities. There is little evidence of the ecotoxicological hazards of these chemicals. High propensity of dithiocarbamates to modulate signal transduction mechanisms, provide the promise for their usefulness in various pharmaceutical applications.

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18

Progress in Antidotes (Acetylcholinesterase Reactivators) Against Organophosphorus Pesticides

Kamil Musilek[1,3,4], Ondrej Holas[2], Anna Horova[1], Miroslav Pohanka[1,4,], Jana Zdarova-Karasova[1,4], Daniel Jun[1,4] and Kamil Kuca[1,3,4]
[1]*University of Defence, Faculty of Military Health Sciences, Hradec Kralove*
[2]*Charles University in Prague, Faculty of Pharmacy in Hradec Kralove, Hradec Kralove*
[3]*University of Jan Evangelista Purkynje, Faculty of Science, Usti nad Labem*
[4]*University Hospital, Hradec Kralove*
Czech Republic

1. Introduction

The use of pesticides allows human to stabilize and increase agricultural production (Wang 2009). Among various types of pesticides, the organophosphorus pesticides (OPP) are targeted to the insect elimination (Fukuto 1990). They were developed as esters of phosphonic or phosphoric acid or their thio-analogues e.g. paraoxon, chlorpyriphos, diazinon, dimethoate (Figure 1).

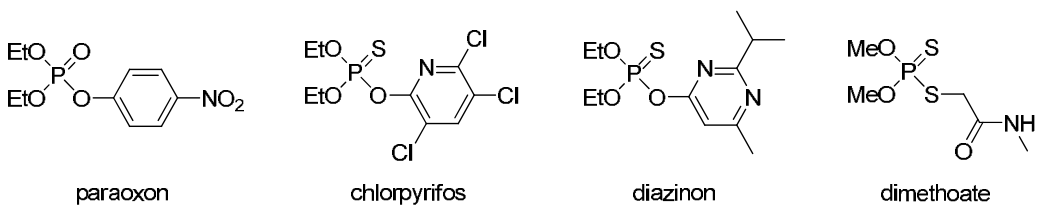


Fig. 1. Organophosphorus insecticides.

Their mechanism of action consists in the irreversible inhibition of cholinesterases in the insect body, namely acetylcholinesterase (AChE; EC 3.1.1.7) or butyrylcholinesterase (BChE; EC 3.1.1.8) (Marrs 1993). The cholinesterases irreversible inhibition is based on formation of covalent bond between OPP and serine moiety in the AChE active site. The AChE is responsible for termination of neuronal transmission via degradation of acetylcholine in the synaptic cleft. This irreversible AChE inhibition causes the accumulation of acetylcholine in the synaptic cleft and thus permanent activation of cholinergic (muscarinic or nicotinic) receptors (Bajgar 2004). The disrupted neuronal transmission causes the insect death (Brooks 1986).

However, the OPP are not selective for insect species, but they have same mechanism of action for the warm-blooded organism (Figure 2) including human (Bajgar 2004). Thus, the

human may be also easily intoxicated by OPP. Consequently, human AChE (hAChE) is irreversibly inhibited in Ser203 and cannot fulfil its natural function (Marrs 1993). The acetylcholine accumulation and consequent overstimulation of receptors leading to cholinergic crisis is common feature for such intoxication. The muscarinic (e.g. lacrimation, salivation, miosis), nicotinic (e.g. neuromuscular blockade) or central (e.g. breath depression) symptoms can be observed (Bajgar 2004). If the OPP intoxication remains untreated, the organism dies.

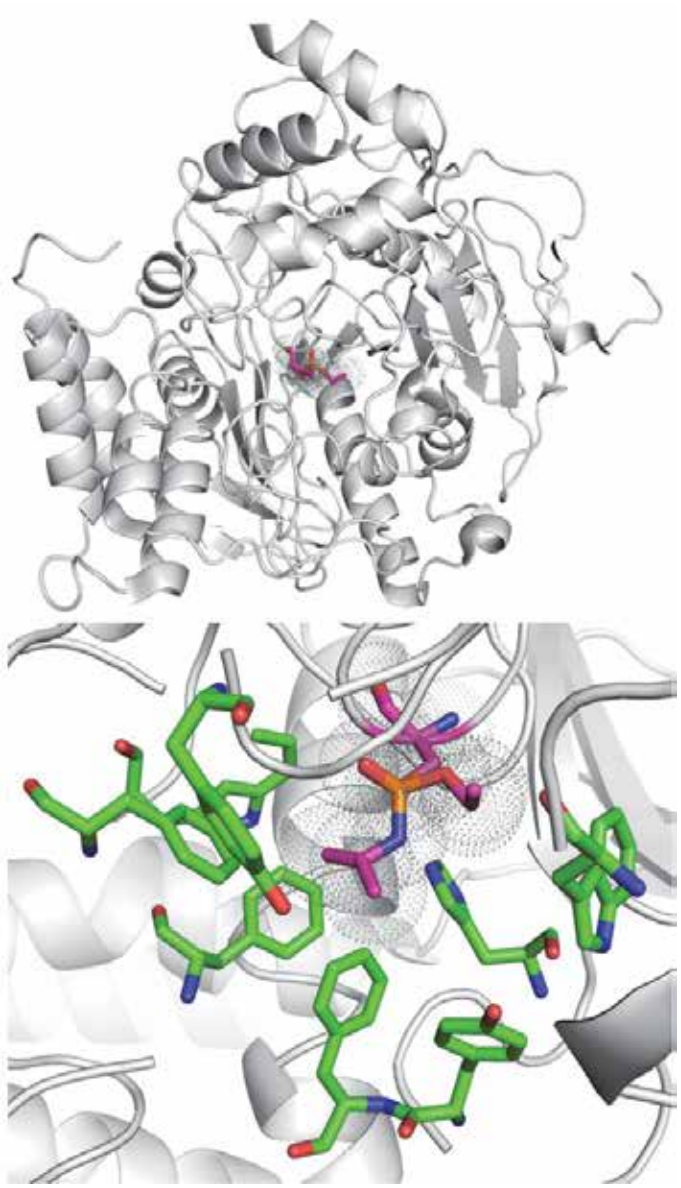


Fig. 2. Mice AChE inhibited by fenamiphos (in magenta; whole enzyme –left; active site – right; 2wu3.pdb) (Hornberg 2010).

The OPP intoxications of human are relatively widespread. They are usually arising from careless manipulation with OPP or the suicidal use of some OPP (Eddleston 2002). The terrorist misuse of the OPP should also not be underestimated from the point of view of food or water supplies contamination (Satoh 2000). The OPP intoxications were estimated to be annually responsible for 200 000 deaths that represent only about 15-30 % of all OPP intoxication (Eddleston 2008).

The general treatment of OPP intoxication has several necessary steps. The non-pharmacologic treatment is focused on resuscitation, oxygen supply or decontamination depending on the OPP entrance to the human body (e.g. skin, eye, gastric decontamination) (Eddleston 2008). The pharmacologic treatment consists in the administration of the symptomatic and causal drugs. The parasympatolytics (usually atropine; Figure 2) are used as the symptomatic treatment that is able to decrease the effects of the accumulated acetylcholine on the cholinergic receptors (Robenshtok 2002). Similarly, the anticonvulsives (usually diazepam; Figure 2) are used as the symptomatic treatment to decrease the neuromuscular seizures (Marrs 2003). Differently from symptomatic drugs, the AChE reactivators were developed as the causal treatment to cleave to OPP moiety from AChE serine active site and to reactivate its native function (Bajgar 2007).

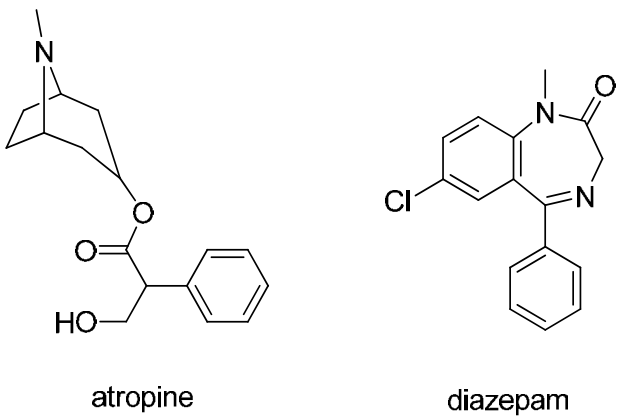


Fig. 3. Drugs used for symptomatic treatment of the OPP intoxication.

The mechanism of AChE reactivation consists in the nucleophilic attack of the reactivator towards the OPP moiety (Marrs 1993). This attack is provided by hydroxyiminomethyl (oxime) moiety. The covalent bond between OPP and AChE serine is cleaved, the complex of reactivator-OPP (phosphorylated reactivator) is formed and the AChE is reactivated (Figure 4) (Eyer 2003). If the reactivation is successful, the AChE function is fully restored. However, the "aging" process may also take place (Mason 1993). In this case, the OPP-AChE complex is degraded and further coordinated within the cholinesterase active site. Such "aged" OPP-AChE complex cannot be reactivated by known oxime reactivators (Worek 2007). The aging process is well known for highly toxic nerve agents (e.g. sarin, soman, tabun, VX), but it is also known for some OP insecticides (e.g. dimethoate, fenamiphos) (Hornberg 2010). For the aging reasons, the acute OPP intoxication should be rapidly treated by causal drugs (oxime reactivators) (Bajgar 2007).

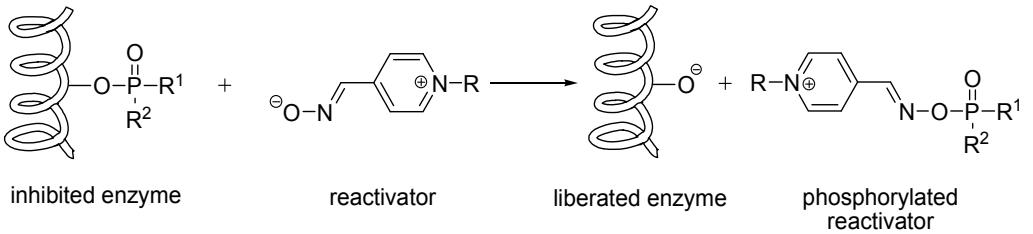


Fig. 4. Cholinesterase reactivation by oxime reactivator.

The oxime reactivators were developed since 1950´s. The original idea of cholinesterase reactivation came from reactivation activity of hydroxylamine analogues (Wilson 1953, Wilson 1955a-b). However, the better results were obtained from quaternary heteroaromatic compounds with oxime moiety. The pralidoxime (2-hydroxyiminomethyl-1-methylpyridinium chloride) was the first clinically used AChE reactivator (Figure 5) (Wilson 1955c, Namba 1958). Further, the bisquaternary compounds with one or two oxime moieties were developed – e.g. trimedoxime (1,1'-trimethylene-bis-(4-hydroxyiminomethylpyridinium) dichloride; Poziomek 1958), methoxime (1,1'-methylene-bis-(4-hydroxyiminomethylpyridinium) dichloride; Hobbiger 1960), obidoxime (1,1'-oxydimethylene-bis-(4-hydroxyiminomethylpyridinium) dichloride; Luettringhaus 1964), asoxime (HI-6; 1,1'-oxydimethylene-(2-hydroxyiminomethylpyridinium)-(4'-carbamoylpyridinium) dichloride; Hagedorn 1969; Figure 5).

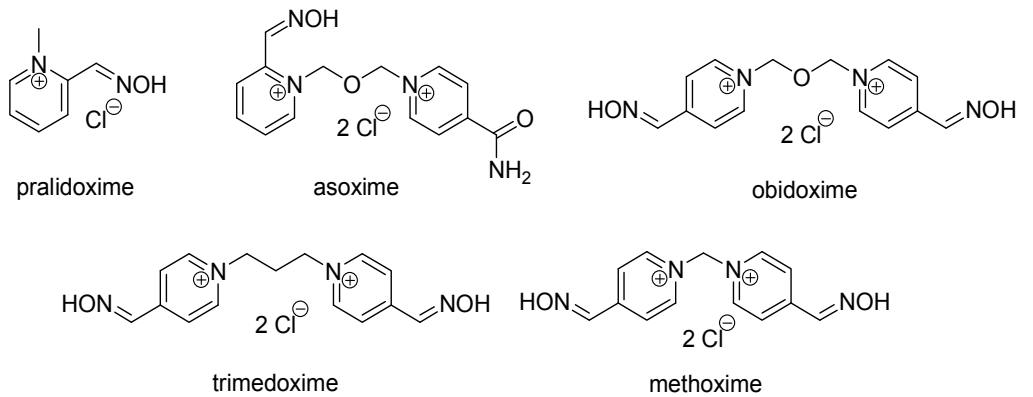


Fig. 5. Commercially available cholinesterase reactivators.

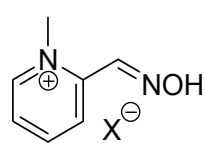
2. Commercially available acetylcholinesterase reactivators

The commercially available reactivators (pralidoxime, methoxime, trimedoxime, obidoxime, asoxime) were developed in the second half of the 20th century and more or less successfully used against intoxication by organophosphorus compounds. However, these reactivators were primarily aimed to diminish the intoxications by highly toxic nerve agents (Musilek 2011a). Thus, their use against OPP intoxications was usually made as a side process in the development of nerve agent antidotes. Though the commercially available reactivators were not directly pointed to OPP intoxication, some of them manifested satisfactory results in reactivation OPP inhibited cholinesterases.

2.1 Pralidoxime

The pralidoxime (Figure 6) was firstly described in 1955 and it was the first AChE reactivator available for clinical practice (Wilson 1955c, Namba 1958). Since 1950's, this drug was introduced globally and it remains in the standard treatment of OPP intoxication in many countries. However, the pralidoxime reactivation of OPP inhibited AChE was found to be debatable for many reason (Eddleston 2009). Whilst the reactivator concentration attainable in human blood after i.m. or i.v. administration was formerly suggested to be maximally 100 μM (Tattersall 1993), the *in vitro* studies reported limited pralidoxime reactivation of some OPP-inhibited (paraoxon, methylparaoxon, lephtophos-oxon, dichlorvos, methamidophos) hAChE (Table 1; Jun 2010, Jun 2011). Though pralidoxime presented some *in vitro* reactivation ability at 100 μM, it had limited reactivation at 10 μM that is more probably presented in human body after i.v. or i.m. administration of its suitable dose. Moreover, some published studies presented very high doses of pralidoxime *in vitro* (up to 700 μM), but did not consider attainable plasma concentration or possible adverse effects (Rios 2005). From *in vitro* evaluation point of view, pralidoxime seems not to be valuable reactivator for OPP intoxication compared to other commercially available compounds.

The *in vivo* animal studies concerned to pralidoxime also suggested its limited reactivation of OPP intoxicated animals. These findings were confirmed for e.g. paraoxon (Petroianu 2006a), methylparaoxon (Petroianu 2007a) or dichlorvos (Khan 1988). The pralidoxime was also determined with intermediate acute toxicity among standard five reactivators for mice and rats (Table 2; Musilek 2007a, Musilek 2010). Furthermore, many human studies with pralidoxime treatment of OPP intoxications are available, because pralidoxime chloride or dimethansulfonate is globally the most used cholinesterase reactivator and usually the antidote of the first choice. However, the pralidoxime was introduced to clinical practice without relevant clinical studies (Eddleston 2008). Thus, some randomised and double blind placebo controlled trials were made in the last two decades (Johnson 1996, Cherian 1997, Eddleston 2002). However, the opinion on pralidoxime effectiveness or ineffectiveness during OPP poisoning treatment had varied among such trials from the point of e.g. OPP type, OPP dose, delay before treatment, pralidoxime dosage (Buckley 2005, Eddleston 2008). Thus, the randomised controlled trial was performed (Eddleston 2009). Though patients with relatively low-dose occupational poisoning by diethyl OPPs showed clinically improvement after low-dose pralidoxime administration, the use of WHO recommended high pralidoxime doses did not improved survival of the OPP self-poisoned patients. Summarizing the *in vitro*, *in vivo* and human data, the use of pralidoxime remains questionable issue and it does not seem to be relevant drug of OPP poisoning treatment.



X = Cl, CH_3SO_3

pralidoxime

Fig. 6. Pralidoxime salts used against OPP intoxication.

	Reactivation±SD (%)									
Reactivator	**pralidoxime**		**methoxime**		**asoxime**		**trimedoxime**		**obidoxime**	
Reactivator concentration/ OPP (Reference)	100 μM	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM	10 μM
paraoxon (Musilek 2011b)	10.7±0.3	2.1±0.1	16.1±0.5	1.8±0.3	6.2±0.6	1.7±0.1	44.3±0.6	22.5±1.3	59.7±1.0	22.4±0.4
methylparaoxon (Musilek 2011b)	30.2±0.3	22.4±0.7	14.2±0.1	14.3±0.2	13.6±0.2	17.9±0.4	51.4±0.9	59.5±0.7	61.7±0.3	45.3±0.9
leptophos-oxon (Jun 2010)	13.3±0.9	4.1±1.3	52.7±0.5	12.0±0.9	32.8±8.0	11.6±0.4	51.3±0.5	26.4±2.7	50.3±0.9	31.5±0
dichlorvos (Jun 2011)	2.6±0.6	0.2±0.6	0	0	0	0.6±1.1	0	0	2.0±1.2	3.3±2.3
methamidophos (Jun 2011)	53.4±3.1	53.8±22.6	61.7±2.4	68.1±11.4	37.4±12.3	75.2±14.6	9.4±7.5	53.1±10.9	45.0±0.5	93.5±3.9

Table 1. *In vitro* reactivation of human OPP inhibited AChE by commercially available oximes.

2.2 Methoxime and asoxime

The methoxime and lately asoxime (HI-6; Figure 7) were firstly described in 1960's (Hobbiger 1960, Hagedorn 1969). Both compounds were found to be very effective in case of nerve agent inhibited cholinesterases (Kassa 2002). Notably, the asoxime was found to be one of the most broad spectrum reactivators of nerve agent inhibited AChE up-to-date (Jokanovic 2008). However, asoxime was also found to be poor reactivator of dimethyl or diethyl OPP inhibited hAChE *in vitro*, if compared to other commercial reactivators (Table 1; Musilek 2011b, Jun 2010, Jun 2011). Similarly, the methoxime presented low reactivation ability for dimethyl or diethyl OPP inhibited hAChE, especially at human attainable concentration 10 μM. Thus, both compounds represent AChE reactivators with improved ability against nerve agents, but reduced for OPPs *in vitro*.

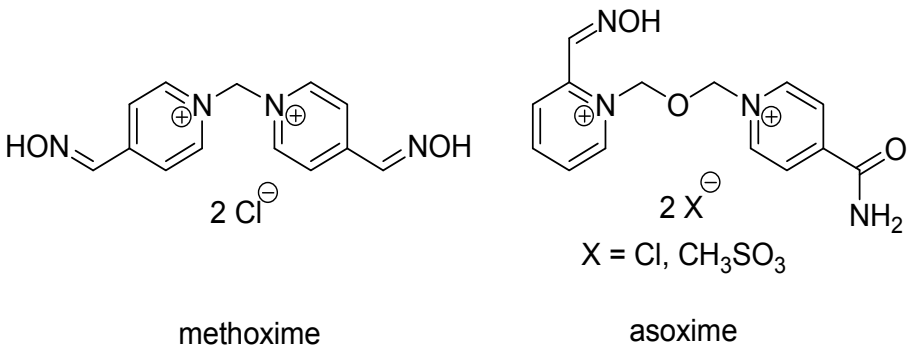


Fig. 7. Methoxime and asoxime salts available for organophosphorus intoxication treatment.

Reactivator/ Acute toxicity (Reference)	**pralidoxime**	**methoxime**	**asoxime**	**trimedoxime**	**obidoxime**
LD_{50} mice (mg/kg) (Musilek 2010)	263.6 (253.7-273.8)	641.8 (590.5-716.0)	671.3 (627.4-718.3)	149.3 (124.1–184.5)	188.4 (156.3–208.0)
LD_{50} rat (mg/kg) (Musilek 2007a)	377.5 (325.7-437.4)	441.8 (384.6-518.4)	781.3 (738.4-826.6)	150.5 (142.1-159.4)	211.07 (176.4-252.6)

Table 2. Acute toxicity of commercially available reactivators in mice and rat after i.m. administration.

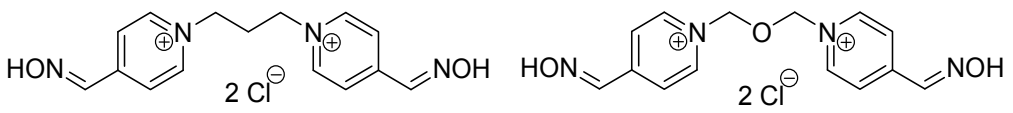
The *in vivo* animal data available for OPP reactivation by both compounds are very limited. Their acute toxicity for mice and rats was found very low among commercially available reactivators (Table 2; Musilek 2007a, Musilek 2010). The methoxime was suggested to be better AChE reactivator than pralidoxime for rats intoxicated by paraoxon (Petroianu 2006a). For methylparaoxon intoxicated rats, methoxime resulted as better reactivator than pralidoxime or obidoxime, but worse reactivator than trimedoxime (Petroianu 2007a). The asoxime use for *in vivo* animal model intoxicated by OPP was not found. Similarly, no relevant data of methoxime or asoxime use for human intoxicated by OPP were found. The explanation probably consists in poor *in vitro* reactivation of OPP by methoxime and asoxime that presumed their poor reactivation ability *in vivo* and the possible use of other potent reactivators. Though both compounds were found less toxic in comparison with other standard reactivators, they do not seem to be relevant drugs for OPP poisoning treatment, when only *in vitro*, limited *in vivo* animal data and no human data are available.

2.3 Trimedoxime and obidoxime

Trimedoxime and obidoxime were developed as bisquaternary bis-oximes with the aim to improve reactivation ability of pralidoxime (Poziomek 1958, Luettringhaus 1964). Both of them were successfully used against nerve agent inhibited AChE and belong to standards on the field (Antonijevic 2007). Their reactivation ability against OPP inhibited hAChE *in vitro* was found quite similar with slightly better results in case of the obidoxime. They were able to effectively reverse the dimethyl or diethyl OPP exposure *in vitro* at human attainable concentration 10 μM (Table 1; Musilek 2011b, Jun 2010, Jun 2011). Though their reactivation ability for dichlorvos inhibited hAChE remained poor, they resulted as the best hAChE reactivators of OPPs among five commercial standards *in vitro*.

The *in vivo* animal toxicity for rat and mice (Table 2; Musilek 2007a, Musilek 2010) assumes both trimedoxime and obidoxime as relatively toxic compounds among standard five oximes, when trimedoxime is the most toxic one. Plausibly, these finding may explain trimedoxime underutilization during OPP-animal studies, where relevant literature data were not found (Lorke 2009). On the other hand, the less toxic obidoxime was several times used for animals exposed to OPPs. The *in vivo* efficacy of obidoxime in rats exposed to paraoxon was found superior to pralidoxime (Nurulain 2009). The older study in parathion poisoned dogs suggested that obidoxime is able to reverse parathion inhibited AChE in blood and some brain areas (Kewitz 1980).

The human data for OPP poisoned patients with trimedoxime treatment are again not known. However, one study suggested that unintentional application of trimedoxime and



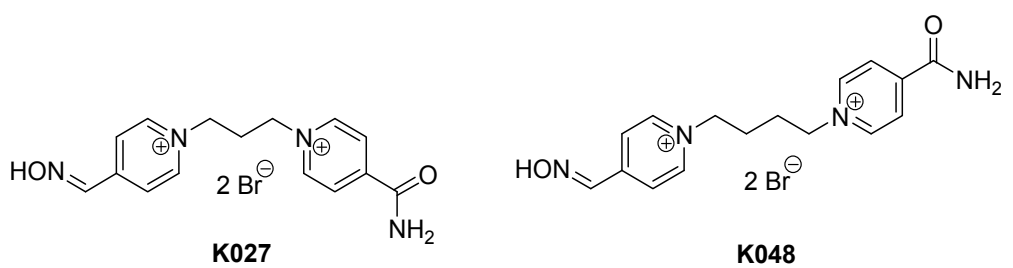
trimedoxime obidoxime

Fig. 8. Trimedoxime and obidoxime.

atropine combination from auto-injector to healthy adults causes only very mild adverse effects (Bentur 2006). More interestingly, similar study determined unintentional application of trimedoxime-atropine auto-injector to children in adult relevant doses, where no adverse effects related to trimedoxime were found (Kozer 2005). Both findings presume the safe human use of trimedoxime in human relevant doses. The obidoxime treatment of OPP poisoned patients was better reported. The combined obidoxime-atropine treatment was effective in patients poisoned by smaller doses of parathion, while the poisoning by the high dose of parathion was not successfully reactivated until parathion levels declined (Thiermann 1997). In the same study, obidoxime was reported as ineffective for oxydemetonmethyl poisoning, but the time elapsed between ingestion and oxime therapy was longer than one day (Thiermann 1997). The enzyme-based assay for quantification of paraoxon in blood of parathion poisoned patients confirmed significant obidoxime reactivation of low plasma paraoxon concentration, whilst diethylphosphoryloxime formation during obidoxime-induced reactivation did not markedly contribute to the re-inhibition of AChE (Eyer 1998). Though obidoxime presented some increased animal toxicity, it seems to be convenient oxime for treatment of human OPP poisoning from the standard five AChE reactivators in human relevant doses.

3. Upcoming acetylcholinesterase reactivators

There were many attempts to develop potent AChE reactivators for treatment of OPP poisoning (Musilek 2011a). Besides the oximes developed against nerve agents (Musilek 2007b), there were over 300 oximes prepared and tested. In the last decade, some of them presented very promising results against OPP exposure. Namely, some mono-oximes from K-compound series such as K027 (1,1'-trimethylene-(4-hydroxyiminomethylpyridinium)-(4-carbamoylpyridinium) dibromide; Kuca 2003a; Figure9) and K048 (1,1'-tetramethylene-(4-hydroxyiminomethylpyridinium)-(4-carbamoylpyridinium) dibromide; Kuca 2003b; Figure 9) were highlighted against some OPP poisoning *in vitro* and *in vivo*.



K027 **K048**

Fig. 9. Novel AChE reactivators developed for treatment of OPP poisoning.

Both compounds showed some reactivation of dimethyl- and diethyl-phosphorylated human AChE *in vitro* (Table 3; Musilek 2011b). The oxime K027 resulted better than K048 at both used concentration for paraoxon inhibited hAChE and almost comparable with the best commercial oxime against OPP (obidoxime) at human attainable concentration 10 μM. On the other hand, obidoxime was found superior to K027 or K048 for methylparaoxon inhibited hAChE *in vitro*. Though the obidoxime was again superior to K027 or K048 for leptophos-oxon inhibited hAChE at human attainable concentration 10 μM, the results of obidoxime and K027 reactivation at higher concentration (100 μM) were found quite similar.

	Reactivation±SD (%)					
Reactivator	**K027**		**K048**		**obidoxime**	
Reactivator concentration/OPP (Reference)	100 μM	10 μM	100 μM	10 μM	100 μM	10 μM
paraoxon (Musilek 2011b)	48.0±0.5	20.8±1.0	25.7±0.7	12.5±0.2	59.7±1.0	22.4±0.4
methylparaoxon (Musilek 2011b)	55.6±0.7	33.9±0.3	54.4±0.9	29.1±0.4	61.7±0.3	45.3±0.9
leptophos-oxon (Jun 2010)	49.3±0.5	16.4±0.9	26.1±0.4	6.6±0.4	50.3±0.9	31.5±0

Table 3. *In vitro* reactivation of human OPP inhibited AChE by promising upcoming oximes.

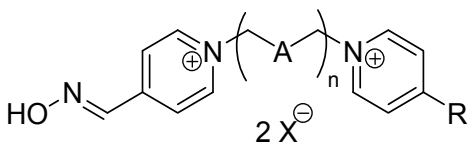
The *in vivo* animal data of K027 and K048 showed some interesting findings. Firstly, their acute toxicity was found lower than toxicity of trimedoxime or obidoxime in mice and rats (Table 4; Calic 2006, Lorke 2008, Kovarik 2009, Musilek 2010). Whereas reactivator K048 was only slightly less toxic than obidoxime, compound K027 was found to be less or comparable toxic with methoxime or asoxime that are the least toxic commercial reactivators (Table 2). The low acute toxicity of K027 might allow its higher dosage in comparison with obidoxime. Secondly, the experiments with rats exposed to paraoxon and methylparaoxon showed that both K027 and K048 provided statistically significant protection against chosen OPPs *in vivo* (Petroianu 2007a-b). Unfortunately, there are no available data for other animal species (e.g. guinea-pigs, pigs, dogs, monkeys) that might confirm/disprove published findings and predict reactivation effect of K027 or K048 in human (Worek 2011). Nevertheless, oxime K027 presented up-to-date very promising results in reactivation of some OPPs that are comparable or better than the best commercially available compound (obidoxime) together with K027 decreased animal toxicity. For these reasons, further experiments are necessary and might reveal K027 valuable properties in reactivation of OPP inhibited AChE.

Reactivator/Acute toxicity (Reference)	**K027**	**K048**	**Obidoxime**
LD_{50} mice (mg/kg) (Calic 2006, Musilek 2010)	672.8 (599.0–755.3) i.p.	224.9 (154.2–328.0) i.p.	188.4 (156.3–208.0) i.m.
LD_{50} rat (mg/kg) (Musilek 2007a, Lorke 2008, Kovarik 2009)	612.0 i.p.	238.3 (199.7-284.3) i.p.	211.07 (176.4-252.6) i.m.

Table 4. Acute toxicity of promising upcoming reactivators in mice and rat.

4. Structure activity relationship of AChE reactivators for OPP intoxication

From the point of view of medicinal chemistry, some trends based on structure activity relationship (SAR) may be considered for reactivators of OPP inhibited AChE (Figure 10; Musilek 2011a). Concerning the functional group, the oxime moiety remains essential for the activity of the reactivator (Kuca 2006). Its position on the heteroaromatic ring influences the reactivation ability. The 4-position of oxime moiety is preferred for OPPs reactivation,



A: $(CH_2)_{3\text{-}4}$; CH_2OCH_2; ???
R: CH=NOH; $CONH_2$; ???

Fig. 10. Structural model suitable for reactivation of OPP inhibited AChE.

instead of the 2-position or 3-position (De Jong 1981). This finding is affected by pKa, where the 3-positioned oxime has a high value, and also by steric hindrance of the reactivator molecule (Cabal 1998). The increased quantity of the oxime moieties in the molecule of AChE reactivator is not essential for reactivation and it usually increases toxicity (Musilek 2007a). The mono-oxime compounds (K027, K048) showed similar or higher reactivation ability compared to bis-oximes (trimedoxime, obidoxime) and presented a lower animal toxicity (Lorke 2009).

Additionally, bisquaternary compounds were found to be superior to monoquaternary compounds (Kuca 2006). Apparently, cation-π or π-π interactions with AChE aromatic residues (His, Phe, Trp, Tyr) are responsible for these findings (Musilek 2010, Musilek 2011b). Among various used heteroaromatic moieties, the pyridinium compounds were the most often utilized. Other moieties (e.g. 5-membered rings) did not show satisfactory reactivation which might be caused by inappropriate pKa values or steric hindrance within the enzyme active site (Cabal 1998).

Concerning the connecting linker at bisquaternary compounds, it has a significant effect on reactivation capability and toxicity. The length and constitution of the linker are the most important factors. For OPPs, alkylene linkage from 3 to 5 equivalents of C-C bond was found to be optimal for reactivation (Kuca 2003a-b), whereas the animal toxicity was not affected by this type of linkage (Petroianu 2006b). The addition of a double bond or an aromatic moiety (source of π-electrons) increased the reactivation ability, but it also increased reactivator toxicity (Musilek 2005, Musilek 2006, Musilek 2007c-e, Musilek 2010).

Concerning the non-oxime part of the molecule, various functional groups may be introduced to increase the reactivation ability as was found beneficially with the use of 3- or 4-carbamoyl, methylcarbonyl or isoquinolinium moieties (Musilek 2007a, Musilek 2007e, Musilek 2008). Indeed from a toxicity point of view, the carbamoyl, carboxyl and methylcarbonyl moieties were found to be very promising candidates (Kassa 2008, Kassa 2009, Berend 2008).

5. Conclusion

The organophosphorus pesticides (OPPs) are heterogeneous group of organophosphorus compounds. Their biological activity manifests as inhibition of cholinesterases and so ranks them as life endangering agents. The necessary treatment of OPP exposure contains parasympatholytics (e.g. atropine), oxime reactivator and anticonvulsive drug (e.g. diazepam) (Bajgar 2007). The causal treatment of organophosphorus intoxication (oxime reactivator) varies globally among five commercial compounds. Recently, the most important oximes in case of OPP intoxication are pralidoxime and obidoxime. Although

pralidoxime was the first oxime available for OPP treatment and it is currently the most frequently used, its ability to reactivate AChE inhibited by various OPPs, is rather poor (Buckley 2011). Consequently, bisquaternary compounds have been found to be more effective. Surprisingly, asoxime developed for nerve agent intoxication, showed in the case of OPPs intoxication, little or no reactivation capability (Stojiljkovic 2006). On the other hand, the trimedoxime and obidoxime were found to be very good for the treatment of OPP intoxication. Specifically, obidoxime should be the first choice compound in combination with atropine and diazepam for a positive clinical outcome (Stojiljkovic 2006).
Since the first use of pralidoxime against OPP intoxication, over 300 different oximes have been synthesized and evaluated (Musilek 2011a). From these, there are some very promising novel reactivators produced in the last decade. Though some of them were originally developed for nerve agent poisoning, they showed increased reactivation ability against various types of OPPs. Notably, compound K027 showed an increased reactivation capability (dimethoxy- and diethoxy- OPPs) with decreased toxicity, as compared to commercial compounds both using *in vitro* and *in vivo* animal models (Petroianu 2006a-b, Petroianu 2007a-c). These findings make compound K027 the lead compound for further studies and development.

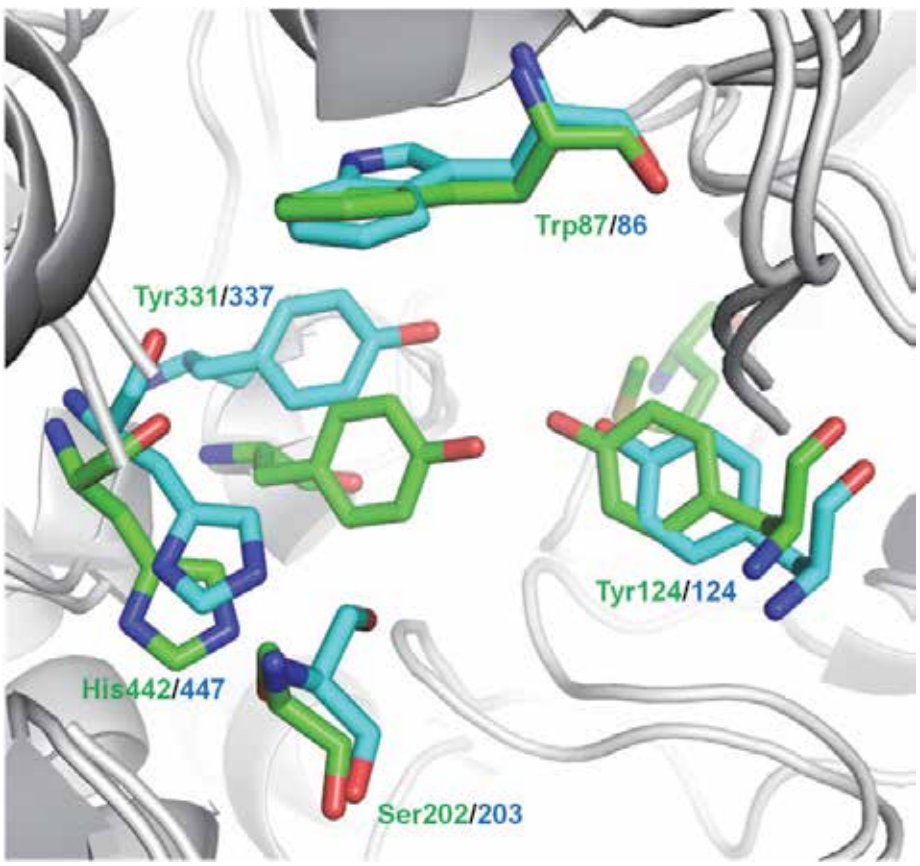


Fig. 11. Structural differences between aphid/human AChE (green/blue; 2hcp/1b41.pdb) AChE (Kryger 2000, Pang 2007).

Additionally, molecular modelling has become an important technique for understanding the mechanisms of OPP action in the last decade. Namely, OPP inhibit the AChE active site differently than the nerve agents. This experience will most probably be used for the future design of new antidotal compounds. Additionally, safer OPPs more specific for insect parasites may be constructed based on the differences between insect and human AChE (Figure 11; Pang 2009a-b).

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Laboratory Tests with Androgenic and Anti-Androgenic Pesticides – Comparative Studies on Endocrine Modulation in the Reproductive System of Invertebrates and Vertebrates

Watermann, B.T., Gnass, K., Kolodzey, H. and Thomsen, A.E.
LimnoMar, Laboratory for Aquatic Research
Germany

1. Introduction

The acute toxicity and sublethal effects of pesticides are well established and published in a wealth of literature (Ecobichon, 2001). The endocrine potential of technical and agriculture biocides (pesticides) is less documented but gained considerable attention due to the fact that their impact on endocrine modulation was observed at much lower concentrations than observed for the induction of acute toxic effects. The first studies published on the endocrine potential of pesticides were *in vitro* or laboratory experiments with human breast cancer carcinoma cells (MCF-7) or hamster ovary cells (CHO K1) (Table 1 and 2). In ecotoxicology, terrestrial wildlife populations like birds or cats were primarily investigated. Aquatic species and invertebrates were largely ignored. Furthermore, the mode of action of pesticides at extremely low concentrations has not satisfactory be elucidated and validated. Regarding wildlife with numerous phyla and taxa, the mode of action has to be defined in each phylum as the receptors present in the mammalian kingdom may not be present in other phyla. Most of the results presented in this chapter have been gathered in the framework of the EU-Project COMPRENDO focussing on the understanding of the action androgenic and anti-androgenic compounds used as technical or agricultural biocides (Schulte-Oehlmann *et al.* 2006). Several compounds with these potentials have been selected to expose a broad spectrum of phyla from invertebrates to vertebrates.

Tributyltin compounds which were developed as molluscicides found their most wide spread application as antifouling biocide. Most authors link the androgenic potency of tributyltin oxide (TBT) to the inhibition of aromatase activity which was first detected in molluscs (Bettin *et al.* 1996). In addition, several other hypotheses of the mode of action of TBT can be found in the literature: inhibition of testosterone excretion, modulation of testosterone levels and effects on the release of neuropeptides (Oehlmann *et al.* 2007). Triphenyltin used as pesticides in potato culture can act as aromatase inhibitor (Schulte-Oehlmann *et al.*, 2000).

The systemic fungicide FEN is a potential androgen as it acts as aromatase inhibitor (Hirsch *et al.*1986, 1987), however, estrogenic activity was demonstrated as well (Andersen *et al.* 2002).

Compound	Estrogenic	Anti-estrogenic	Active substance Compound/metabolites
Captan		Androgen receptor binding	Compound
Chlordecon	Estrogen receptor binding	Androgen receptor binding	Compound
Chlorpyrifos	Estrogen receptor interaction		Compound
Deltamethrin	Estrogen receptor interaction		Compound
Dicofol	Estrogen receptor binding	Androgen receptor binding	Compound
Dieldrin	Estrogen receptor binding	Androgen receptor binding	Compound
Fenarimol	Receptor binding	Androgen receptor binding	Compound
Fenitothrion		Androgen receptor interaction	Compound
Iprodion	Aromatase stimulation		Compound
Methiocarb		Androgen receptor interaction	Compound
Methomyl	Aromatase stimulation		Compound
Methoxychlor	Estrogen receptor binding	Androgen receptor binding	Compound
Myclobutanil	Estrogen receptor binding	Androgen receptor binding	Compound
Nitrofen	Estrogen receptor binding	Receptor interaction	Compound
Primicarb	Aromatase stimulation		Compound
Prochloraz	Aromatase stimulation		Compound
Propamocarb	Aromatase stimulation		Compound
Tolclofos-methyl	Estrogen receptor interaction		Compound
Triadimefon		Receptor interaction	Compound
Tribenuron-methyl	Estrogen receptor interaction		Compound

Andersen *et al*. 2002, Okubo *et al*. 2004

Table 1. Mode of endocrine modulation *in vitro* of selected pesticides with of estrogenic or anti-estrogenic potential

Compound	**Androgenic**	**Anti-androgenic**	**Active substance Compound/metabolites**
DDE		Receptor Inhibition	Compound
Dichlorvos		Receptor Interaction	Compound
Endosulfan	Aromatase inhibition		Compound
Fenarimol (FEN)	Aromatase inhibition	Receptor Inhibition	Compound
Prochloraz	Aromatase inhibition		Compound
Tributyltin-oxide (TBT)	Aromatase inhibition		Compound
Triphenyltin-oxide (TPT)	Aromatase inhibtion		Compound
Vinclozolin (VIN)		Receptor Inhibition	M1/M2

Andersen *et al.* 2002, Körner *et al*. 2004, Okubo *et al.* 2004

Table 2. Mode of endocrine modulation *in vitro* of selected pesticides with androgenic or anti-androgenic potential

Vinclozolin is applied a as a non-systemic fungicide on fruit and vegetables where it prevents spore germination (US National Library of Medicine. 2006, Szeto *et al.* 1989) and was one of the first chemicals reported to be an anti-androgen (Gray *et al.* 1994). VIN itself has a poor affinity to the mammalian androgen receptor, however, in vivo it is hydrolyzed to two open-ringed metabolites, M1 (2-(3,5-dichlorophenyl)-carbamoyloxy-2-methyl-3-butenoic acid) and M2 (3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide) which act as androgen receptor antagonists by preventing transcription of androgen dependent genes (Kelce *et al.* 1994, Wong *et al*. 1995, Andersen *et al*. 2002).

The compound pp'-DDE (1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene) is the major metabolite of pp'-DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) which is still used in some African countries to control malaria transmitting mosquitoes (Nyarango *et al.* 2006). Pp'-DDE is even more persistent than DDT ($T_{½}$ = 2-20 years) and very bioaccumulative (log K_{ow} = 5.8). The anti-androgenic action of pp'-DDE was first reported by Kelce *et al.* (1995).

Methylurea-based compounds like diuron and linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) have been applied as herbicides to control a variety of annual weeds, was shown to be a weak competitive androgen receptor antagonist in vitro (Cook *et al.* 1993). They induced a positive response in the immature and adult rat Hershberger assay (Lambright *et al.* 2000), and suppressed androgen-dependent gene expression (McIntyre *et al.* 2000). It is relatively water soluble with a low potential for bioaccumulation (log K_{ow} 3.2) and listed as a possible human carcinogen (US National Library of Medicine 2006).

2. Endocrine modulation of pesticides with androgenic potential

2.1 Triphenyltin compounds

Laboratory experiments with TPT at concentrations of 100 – 500 ng/L revealed in two echinoderm species the potency to alter different reproductive parameters, such as gonad maturation and oocyte/egg development. Particularly, in both the sea urchin (*Paracentrotus lividus*) and the crinoid (*Antedon mediterranea*), TPT appeared to promote spermatogenesis and to inhibit oogenesis by stimulating the phagocytosis activity. In addition, TPT resulted to be an inhibitor of echinoderm oocyte development, as in both species cited it caused a significant size reduction. The androgenic activity of TPT observed on the reproductive endpoints was confirmed by the direct steroid level measurements carried out in parallel in the same exposed specimens. In fact, in both species the compound caused a significant increase of testosterone levels and a decrease of ethinylestradiol (Sugni *et al.* 2010).

TPT induced a concentration dependent decrease of P450-aromatase which was statistically significant at the highest TPT concentration tested (225 ng/L). Additionally, increased metabolism of testosterone to form dihydrotestosterone (DHT) and 5-androstane-3,17-diol was observed, suggesting increased 5-reductase activity in the gonads of TPT-exposed individuals (Lavado *et al.* 2006). At 100, 225 and 500 ng/L TPT females of *Paracentrotus lividus* displayed an increased percentage of oocytes with vacuolated ooplasm, up to 50% at the highest concentration. In parallel, the proliferation activity in the female gonad decreased (di Benedetto, 2003).

In crustacea the aromatase inhibitor TPT caused stimulating effects in the male reproductive system of *Acartia tonsa* at concentrations of 4.5 and 11 ng TPT-Sn/L, whereas at 28 ng TPT-Sn/L inhibiting effects were observed in the female gonad (Watermann *et al.* 2011a). In *A. tonsa,* adverse effects of TPT on oogenesis on the level number of oogonia, degeneration of previtellogenic and vitellogenic oocytes, yolk synthesis and maturation were evident at the two lowest exposure concentrations of 1.4 and 3.5 ng TPT-Sn/L. In contrast, at 22 ng TPT-Sn/L, the perinuclear sites of yolk formation were more prominent but irregular in shape. Thus, in males TPT exerted stimulation of the gonad at the lowest concentrations and an atrophic effect at the highest concentration. In *A. tonsa*, the apoptotic index of oogonia and oocytes was elevated compared to the control at all exposure concentrations. These observations indicate that degeneration and loss of oocytes were primarily due to apoptosis as it is known at deprivation of estrogens by aromatase inhibitors in mammals (Thiantanawat *et al.* 2003). In males of *A. tonsa* exposed to TPT, no disrupting effects on spermatogenesis were observed. In contrast, the proliferating activity of the gonad appeared more active in exposed groups than in the control. The latter was not quantifiable. However, in the accessory sexual glands like the spermatophore, the wall displayed irregular formation or hypertrophy along with reduced core secretions (Watermann *et al.* cit. op.).

The effects of TPT on molluscan species have been reported on a variety of species. In *Marisa cornuarietis* TPT induced several alterations in the female and male reproductive system. In males exposed to 30 ng/L TPT, in 40% of specimens the prostate was hypertrophic while the gonad was in a maturing or ripe stage. In males exposed to 250 ng/L TPT the gonad was ripe or spawned and no more spermatogenesis was present. In females exposed to 250 ng/L TPT the gonad contained in 50% of specimens singular atrophic oocytes. In 30% of specimens the albumen/capsule gland was transformed to a prostate. In females exposed to

500 ng/L TPT the size and number of follicles were reduced to 27.3% in relation to the control. Arrest of oogenesis was present in 72.7% of females. Oogonia were prematurely released from the follicle epithelium and floated in the follicle lumen. In 45% of females the albumen/capsule gland was transformed to a prostate gland. Morphological investigations in this species revealed after exposure to TPT concentrations in the range cited above the induction of imposex (Oehlmann *et al.* 2007).

In other molluscan species similar observations were published. In the females of the abalone *Haliotis gigantea*, it was a strong masculinizator agent, promoting spermatogenic processes within the ovary (Horiguchi *et al.* 2002), and in *Hinia reticulata* this compound induced ovary atrophy (Schulte-Oehlmann *et al.* 2000). In *Haliotis madama* a decrease in population size was registered following a displacement of male and female reproductive cycle in organisms exposed to TPT (Horiguchi *et al.* 2000). The interaction of TPT with the lipid metabolisms of the ramshorn snail *Marisa cornuarietis* at environmentally relevant concentrations of 30, 125, 500 ng/L as Sn in a semi-static water regime for 7 days was studied by Lyssimachou *et al.* (2009). Percentage of lipids and total fatty acid content decreased significantly in TPT-exposed females while the activity of peroxisomal acyl-CoA oxidase, involved in fatty acid catabolism, increased. In addition, fatty acid profiles (carbon chain length and unsaturation degree) were significantly altered in exposed females but not in males.

In fish laboratory experiments with a triazine pesticide revealed first indications of endocrine modulation on mature male Atlantic salmon (*Salmo salar*). Short term exposure to 2.0 – 20 µg/L atrazine impaired the mating abilities of male salmon (Moore & Waring, 1998). The most striking effect of TPT was observed in the female gonad of *Pimephales promelas*. The percentage of atretic oocytes in relation to the total number of oocytes was slightly decreased at 10, 32, and 100 ng/L, whereas at 1000 ng/L a significant increase could be observed (Figure 1). In female fish exposed to TPT a characteristic alteration of the shape of oocytes was encountered, described as pre-atretic oocytes progressing to atretic oocytes. The percentage of pre-atretic oocytes was significantly increased at 32 ng/L TPT in relation to the control. In the male liver the composition of stored material changed clearly in males into the direction of the dominant storage of fat instead of glycogen. Dependent on the selected concentration, TPT may act as an androgen and as an anti-androgen. In females of fathead minnow at concentrations of 10 – 100 ng/L TPT the percentage of atretic oocyte was decreased, at 1,000 ng/L it was significantly elevated (Figure 2). At all TPT concentrations elevated percentages of oocytes displayed indented chorion, indicating a pre-atretic stage, most pronounced at 32 ng/L. In parallel, the thickness of the chorion was very heterogenous leading to some oocytes with very thin chorion wall, most pronounced at 32 ng/L. On the other hand the percentage of postovulatory bodies was reduced at all TPT concentrations.

In another freshwater species *Rutilus rutilus* TPT induced in the female gonad an increased prevalence of atretic oocytes in relation to the control. Whereas the prevalence of atretic oocytes in the control was 10 – 12.5%, it increased to 33% at a concentration of 500ng/L (van Ballegoy & Watermann, unpublished).

Androgenic effects of TPT on amphibian species, e.g. the tadpole *Xenopus laevis* were observed in laboratory experiments. After exposure to concentrations of 0.04, 0.2, 0.4, 2.0, and 3.9 µg/L TPT males of this species exhibited a stimulation of spermatogenesis which was most pronounced in animals exposed to 0.04 µg/L TPT. The number of spermatocysts

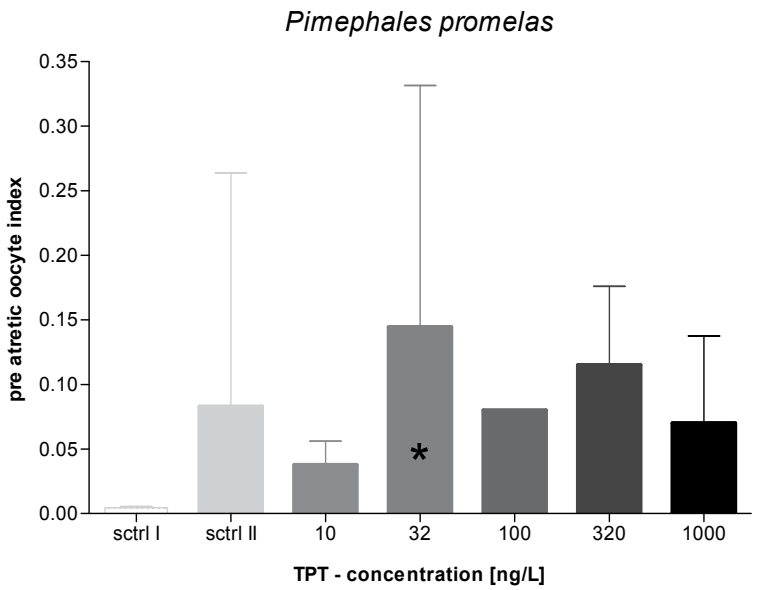

Fig. 1. *Pimephales promelas* exposed to triphenyltin, pre atreric index = number of pre atretic oocytes/total number of oocytes, 3 to 6 animals in one group, median & interquartile range, statistics: Kruskall Wallis with Dunn's Multiple Comparison Test, significant difference between solvent control I and exposure of 32 ng/L, level of significance P<0.05

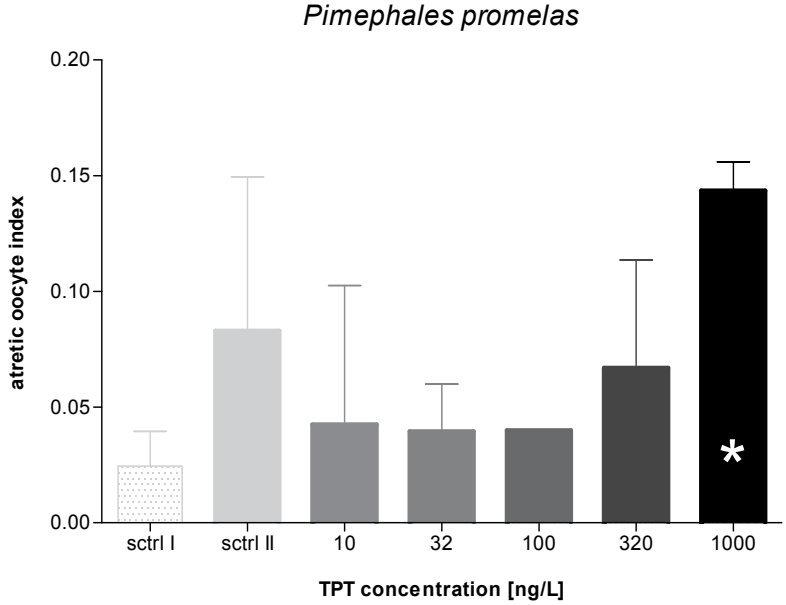

Fig. 2. *Pimephales promelas* exposed to triphenyltin, atretic index = number of tretic oocytes/total number of oocytes, 3 to 6 animals in one group, median & interquartile range, statistics: Kruskall Wallis with Dunn's Multiple Comparison Test, significant difference between solvent control I and exposure of 1000 ng/L, level of significance P<0.05

per gonad was elevated in relation to the control. In females exposed to the same range of concentrations of TPT, the number of follicles per animal was decreased in relation to the controls. The percentage of specimens with singular follicles was elevated with percentages of 12.5 - 25% at all concentrations. On the other hand, the size of the ovarian cavities increased with increasing concentrations (unpublished results).
In laboratory experiments with mammals, organotin compounds like TBT and TPT and pharmaceutical non-steroidal aromatase inhibitors like letrozole caused species-, developmental- and dose-dependent androgenic or anti-androgenic effects (Junker *et al.* 1994; Yu *et al.* 2004). In a recent laboratory study, TPT induced anti-androgenic effects in pubertal male rats. Administration of 2, 6, and 12 mg/kg/day decreased testis weight; epididymis and prostate weights were reduced at 6 and 12 mg/kg/day, and seminal vesicle weights at 6 mg/kg/day (Grote *et al.* 2004). In rats the histopathological effects of 2 or 6 mg TPT/kg b.w. on the reproductive tissue of female pubertal rats as part of a comprehensive pubertal assay. At both dose levels an increase in the number of all follicle stages was observed. Furthermore, exposure to 2 mg TPT/ kg b.w. led to a significant reduction in the diameter of tertiary follicles. A significant increase in the atretic index was observed in tertiary and pre-ovulatory follicles after exposure to 6 mg TPT (Watermann *et al.* 2009).

2.2 Tributlytin compounds

Publications on endocrine modulation by tributyltin compounds in echinoderms are rare. Probably the first report on endocrine effects of ingested TBT in echinoderms was the study of Mercier *et al.* (1994). Alterations in the female gonad of starfish (*Leptasterias polaris*) occurred at concentrations of TBT of 0.26 µg/g wet weight. Mature oocytes were smaller and the gonad possessed a thinner epithelium than in the control animals. Girard *et al.* (1997) reported on inhibition of sea urchin egg cleavage after exposure to TBT concentrations of 50 – 100 nM. In the Bay of Brest (France) the arrest and delay in embryonic development in sea urchin (*Sphaerechinus granularis*) was suspected to be linked with dumped pesticides and TBT (Quiniou *et al.* 1999).
In crustacea the first reports on endocrine effects of TBT were published by Johansen & Møhlenberg (1987). At concentrations of 10, 50 and 100 ng/L TBT the egg production of the copepod *Acartia tonsa* was reduced at 18, 19 and 37%. The authors pointed out that the selected concentrations were lower than found in Danish coastal waters. A study of Kusk & Petersen (1997) on acute and chronic toxicity of TBT in Acartia revealed inhibition of the developmental rate of larvae at 1 ng/L TBT.
The endocrine modulation of TBT in molluscs comprises a huge body of literature and was one of the first and intensively studied endocrine effects in invertebrates. More than 150 molluscan species worldwide were affected by the endocrine modulation induced by TBT. Deriving from laboratory experiments and in situ studies it was evident that the endocrine effects could be induced at extremely low concentration of 0.1 ng/L TBT (Oehlmann *et al.* 2007; Shi *et al.* 2005). In most species TBT is able to induce a phenomenon called imposex, the appearance of male sexual characteristics in females. In other species TBT can induce intersex phenomena which mean the presence of females with a female gonad and a progressive reduction of female sexual accessory organs, leading to a transformation into male accessory organs like prostate or rudimentary penis (Bauer *et al.* 1995, Watermann *et al.* 2008).

Molluscs can conjugate a variety of steroids to form fatty acid esters. The freshwater ramshorn snail *Marisa cornuarietis* was used to investigate sex differences in endogenous levels of esterified steroids. Testosterone and estradiol were mainly found in the esterified form in the digestive gland/gonad complex of *M. cornuarietis*, and males had higher levels of esterified steroids than females (4–10-fold). Exposure to TBT led to a decrease in both esterified testosterone (60–85%) and estradiol (16–53%) in females after 100 days exposure, but had no effect on the hormonal level in males (Janer et al. 2006). In contrast, histological investigations in male *Marisa cornuarietis* exposed to 60 and 250 ng/l TBT displayed disturbed spermatogenesis. Spermatogonia detached prematurely from the germinal epithelium in the tubules, spermatocytes and spermatids showed degenerative changes, whereas in singular tubules multinucleated giant cells of fused spermatids were visible. The prostate of males displayed at all concentrations enlarged, hypertrophied, and vacuolated gland cells. The surface of the penis sheath exhibited a low degree of invaginations and reduced numbers of mucous cells. In the gonad of females exposed to 30, 60, 125, 250 and 500 ng/L TBT several alterations in oogenesis were present. In 25 – 60% of females the oogonia detached prematurely of the follicle epithelium, floating in the lumen of the follicles. The albumen/capsule gland of up to 90% of females was transformed to prostate gland. The length of the vagina was reduced in 30 -50% of females in association with a reduced invagination of the vaginal epithelium.

In the amphibian species *Xenopus laevis* an advanced development and differentiation of the gonad was visible at a concentration of 33 ng TBT/L in relation to the control. At a concentration of 326 ng TBT/L approximately 30% of males straight tubules had increased in number and size compared to the control. In exposed females several indications of dedifferentiation and regression of the gonad could be observed. The percentage of animals with singular follicles increased slightly in relation to the control from a concentration of 160 – 3,255 ng/L TBT with 22.2% to 37.5%. The percentage of specimens with numerous follicles decreased from 80% at 16 ng TBT/L to 62.5% at 3,255 ng TBT/L. The size of the ovarian cavities decreased with increasing concentration of TBT. The resorption of oogonia was elevated at the lower concentrations between 3 and 33 ng TBT/L with prevalences of 37.5% and 31.6% respectively.

2.3 Fenarimol

A restricted number of laboratory studies were performed with echinoderms which elucidated a weak sensitivity to FEN. In females of *Paracentrotus lividus* at concentrations of 30 and 300 ng/L in the female gonad 50% and 18.2% respectively of animals exhibited enlarged, atrophic oocytes with vacuolated ooplasm. FEN was more effective on *A. mediterranea* specimens, altering both the maturative stage and reduced the oocyte size. In the crinoid species FEN seems to behave in agreement with its putative role of androgenic compounds, promoting male maturation, inhibiting oogenesis processes as well as inducing the production of smaller size oocytes. This FEN androgenic activity was partially confirmed by steroid measurement with an increase of testosterone in this specie. However in both the echinoderms also a marked estrogenic effect was detected, particularly in the crinoid, where an up to 10-fold E2 level increase was registered (Sugni *et al.* 2010).

In the crustacean copepod *Acartia tonsa* FEN caused disturbances of oogenesis on the level of oocyte differentiation and meiosis were observed at all concentrations (2.8 – 105 µg/L FEN).

However, the yolk production was not affected at 2.8 - 42 µg/L, but reduced at 105 µg/L. At 7.0 µg/L FEN. In exposed males, no alteration of spermatogenesis occurred, but the proliferation activity in the gonad was much more pronounced than in the control. In *Daphnia magna* FEN caused disturbances of the embryonic development and synergistic effects with testosterone (Mu and LeBlanc, 2005) as well as reduced fecundity (LeBlanc 2007).

In the molluscan species *Marisa cornuarietis,* males exposed to 100, 300 and 1000 ng/l FEN spermatogenesis was disturbed or even arrested in up to 75% of males. In numerous tubules aggregations of clumbed chromatin could be observed in spermatocytes and spermatids, indicating degenerating processes. The penis glands displayed degeneration of gland cells in association with an extreme dilatation and ramification of the gland ducts. In contrast, males exposed to 3000ng/l FEN displayed a normal gonad with active spermatogenesis. The female gonad displayed exposed to concentrations of 100, 300 and 1000 ng/L FEN disturbance and arrest in oogenesis with enlarged follicles. The follicle epithelium contained high amounts of lipo-pigments. In up to 75% of animals prematurely detached and degenerating oogonia were observed in the follicle lumen. The vagina of all specimens was reduced in length and the invagination of the vaginal epithelium was reduced. In contrast females exposed to 3000ng/l FEN displayed a normal gonad with active oogenesis. Exposure of Marisa cornuarietis lead to imposex induction (Oehlmann *et al.* 2007). Exposure to FEN and MT did not alter levels of esterified steroids in males or in females, although exposed females developed imposex after 150 days exposure.

In fish fenarimol induced in the female gonad of *Rutilus rutilus* elevated percentages of atresia in relation to the control (10 – 12%) with 16.7% (0.3 µg/L), 14.3% (1.7 µg/L) and 25% (3.3 µg/L). In the male gonad of this fish species a hypertrophy was observed with prevalence of 55.6% (1.7 µg/L) and 57.1% (3.3 µg/L).

Tadpoles exposed to concentrations of 3.312, 33.12, 165.6, 331.2 µg/L FEN showed a stimulation of spermatogenesis and disturbance of oogenesis. All males investigated displayed an early stage of gametogenesis with spermatocysts filled with spermatogonia, spermatocytes and early spermatids. In females the number of follicles decreased with increasing concentration of FEN. The percentage of singular follicles per specimen increased in relation to the control with 40% to 72.7 - 60% at concentrations of 165.6 - 331.2 µg/L FEN. In parallel the percentage of specimens with numerous follicles decreased in relation to the control with 60% down to 27 - 40% at concentrations of 165.6 - 331.2 µg/L FEN. The frequency of large ovarian cavities increased with increasing concentration compared to the control with a percentage of 20%, up to 63.6 - 50% at concentrations of 165.6 - 331.2 µg/L FEN. Correspondingly, the percentage of small ovarian follicles decreased with increasing concentrations.

In mammals FEN is acting as a potent inhibitor of the aromatase activity in the brain and ovary of rats, and in mammalian cell culture assays (Hirsch *et al.* 1987; Andersen *et al.* 2002). On the other hand, FEN binds both to the estrogen and androgen receptor in estrogen sensitive human breast cancer MCF-7 cells and is able to induce cell proliferation (Okubo *et al.* 2004). FEN prevents the increase of the number of nuclear estrogen receptors in the brain of male rats during the early postnatal period (Hirsch et al. 1987). This mechanism was supposed to cause a dose-dependent decrease in fertility in male rats (Hirsch *et al.* 1986).

3. Endocrine modulation of pesticides with anti-androgen potential

3.1 p,p'-DDE

In echinoderms several anti-androgenic effects were observed in a restricted number of species. In *Paracentrotus lividus* after exposure to 100, 500 and 2,500 ng/L DDE the density of ripe sperm in the tubules was reduced up to 67% of males at the highest concentration. DDE inhibited spermatogenesis and reduced the egg size in female *P. lividus* but enhanced the egg size in A*nterdon mediterranea* (Lavado *et al.* 2010). In experiments on echinoderm regenerative response DDE interfered with fundamental processes of developmental physiology via endocrine modulation of *A. mediterranea* (Sugni *et al.* 2008).

In the crustacean *A. tonsa* exposed to ≥0.55 ng/L p,p-DDE induced severe feminizing alterations in the male gonad and accessory organs. Spermatogenesis was impaired at all stages with increased rates of apoptosis. At 0.5 ng/L the gonad structure was altered in 20% of male *A. tonsa.* Primary and secondary spermatocytes displayed a pale cytoplasm and apoptotic cell death. The frequency of meiotic figures decreased. This type of alteration was even stronger in males exposed to 3.5 ng/L as the majority of visible spermatogonia, spermatocytes, and spermatids showed apoptotic figures. In the centre of the gonad a remarkable intercellular space occurred due to the lack of spermatocytes in late meiosis. Males exposed to the highest applied concentration of 22 ng/L p,p'-DDE exhibited a gonad with dominating spermatocytes devoid of spermatogonia and spermatids. The spermatocytes either displayed apoptotic figures or were necrotic. At concentrations of 0.0014 to 0.0088 µg/L p,p'-DDE the wall of the spermatophore was irregular in shape in 30% of males. Number and density of core secretions of the spermatophore were clearly reduced at a concentration of 0.0014 µg/L p,p'-DDE. In p,p-DDE-exposed females, an intensification of oogenesis with enhancement of oogonia proliferation and yolk synthesis was observed at ≥0.55 ng/L p,p-DDE.

In the female gonad at DDE concentrations of 1.4 – 8.8 ng/L, a striking feature was the increase in number and size of pre-vitellogenic and vitellogenic oocytes as well as a prominent yolk synthesis. Moreover, in 15% of females exposed to 0.5 ng/L DDE tightly packed oogonia and oocytes were encountered. The perinuclear sites of yolk synthesis were abundant and enlarged in relation to the control. Moreover, a few number of oogonia displayed apoptotic figures at 0.5 ng/L. Females exposed to 1.4 ng/L showed a more pronounced yolk synthesis with extended perinuclear sites of yolk formation compared to those exposed to 0.5 ng/L. The yolk masses were irregular formed in vitellogenic oocytes. The proliferation index of the female gonad increased in relation to the control after exposure to 0.5 and 3.5 ng/L, it was significantly elevated at 8.8 ng/L and decreased again at 1.4 ng/L (Figure 4) (Watermann *et al.* 2011b).

Apart of the modulating effects on the reproductive system, DDE was shown to act as an anti-ecdysteroid *in vitro* in crustaceans and aquatic insects (Dinan *et al.* 2001; Soin & Smagghe, 2007)) and is thus a potential endocrine disrupter in arthropods.

In the fish species *Pimephales promelas* disturbance in the spermatogenesis was noticed with increasing concentrations (10, 100, 1000, 10000 ng/L p,p'-DDE). Primarily, degenerative spermatocytes occurred to a higher degree in the tubules, leading to focal empty spaces in the tubule periphery. In the female gonad increasing DDE concentrations led to a heterogenous formation of the chorion leading to a certain percentage of oocytes with a thin chorion wall. In parallel the percentage of fat vacuoles in relation to yolk vacuoles increased with increasing concentration of DDE, right from 10 ng/L. The number of atretic oocytes was clearly elevated at 10,000 ng/L p,p'-DDE (Figure 3).

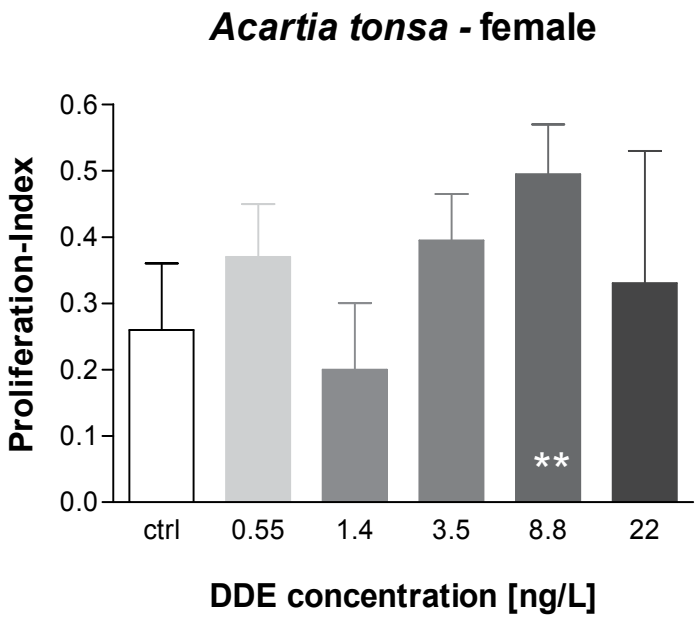

Fig. 3. *Acartia tonsa* exposed to DDE, Proliferation-Index = No. of proliferating oogonia / Total No. of oogonia, Statistics: Kruskall-Wallis test with Dunn's Multiple Comparison test, significant difference between control and exposure of 8.8 ng/L, level of significance $P<0.01$, no statistics with exposure of 22 ng/L as N was too small

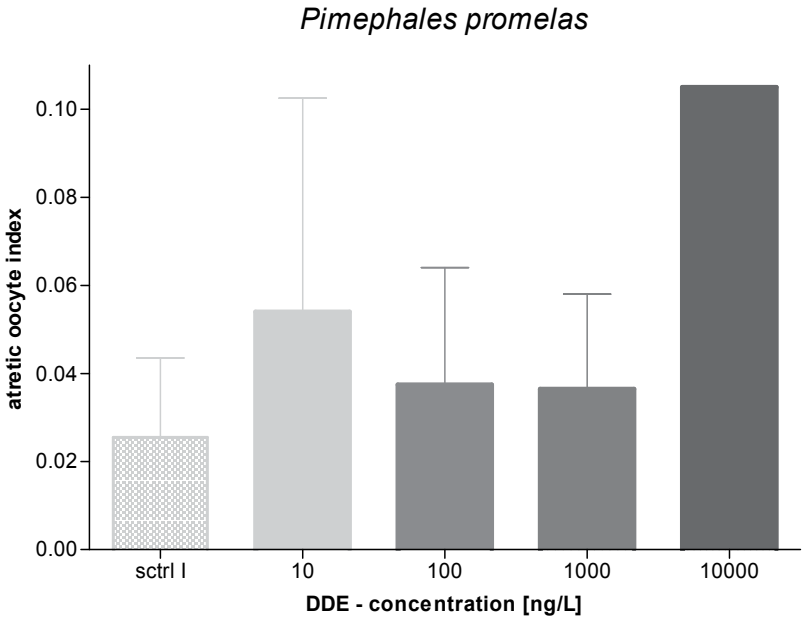

Fig. 4. Pimephales promelas exposed to DDE, atretic index = number of atretic oocytes/total number of oocytes, 2 to 5 animals in one group, median and interquartille range, no comparitive statistics, N too small

In males disturbance of spermatogenesis reflected in lack of cysts was noticed in all concentrations. The most pronouncecd effects are described for the testis displaying disturbance of spermatogenesis, but as elevated rates of atresia which cannot be sufficiently explained. From DDT-polluted rivers in the North of India combined with laboratory experiments Singh et al (2008) observed decreased sperm motility in catfish and *Heteropneustes fossilis*. In females of Pimephales a reduced chorion thickness, an elevated rate of atresia, and reduced percentage of postovulatory bodies was observed at 10,000 ng/L only.

In mammals p,p'-DDE has little ability to bind to the oestrogen receptor but inhibits androgen binding to the androgen receptor, androgen induced transcriptional activity, as well as androgen action in developing, pubertal and adult male rats (Kelce et al. 1995). After administration of 100 mg/kg/day DDE Sprague Dawley rats displayed hypospadiasis and increased numbers of retained nipples (Gray et al. 1999b). After administration of 750 and 1000 mg/kg/day DDE rats developed testicular changes, which were characterized by disorganization of the testis and loss of germ cells within a few, randomly distributed tubules. Effects on germ cells were more apparent in the epididymis, which contained increased numbers of sloughed, round germ cells within epididymal tubules of rats exposed 1000 mg/kg/day DDE (O'Connor et al. 2002). Shi et al. (2009) reported on apoptosis induced by p,p'-DDE in Sertoli cells of rat via a FasL-dependent pathway.

3.2 Vinclozolin

No investigations have been carried out with echinoderms up to now. In the crustacean copepod *Acartia tonsa* the response of the male gonad to ≥0.10 mg/L VIN exposure was heterogeneous; some areas in the gonad were stimulated, whereas others displayed a disturbed spermatogenesis. Multiple spermatocytes exhibited a diffuse and slightly vacuolated cyctoplasm at concentrations ≥0.10 mg/L. In addition, the spermatophore formation was affected leading to deformations. In female VIN exposed *A. tonsa* no statistically significant effects were observed (Watermann *et al.* 2011b).

In the cladoceran *Daphnia magna* VIN reduced the number of neonate males at 1 mg/L (Haeba *et al.* 2008). Interestingly, this sex ratio modulation in a crustacean corresponds to the anti-androgenic action of VIN in vertebrates.

In selected molluscan species (*Marias cornuarietis, Nucella lapillus* and *Hinia reticulate*a) exposure for 5 months with concentrations of vinclozolin between 30 ng/L and 1000 ng/L induced in males a reduction of the penis and prostate length (Tillmann *et al.* 2001; Oehlmann *et al*. 2007).

First reports on the anti-androgenic effects in fish were published by Makynen et al. (2000). They reported on slight increase of estradiol in the serum of male fish and gonad atrophy in female fish after exposure of 200 – 700 µg/L VIN. They suspected as active agents not the compound but the metabolites M1 and M2 (Makynen et al. 2000). Some years later Kiparissis et al. (2003) observed intersex in Japanese medaka after exposure of 5000 µg/L VIN. In *Rutilus rutilus* vinclozolin induced elevated levels of atresia in the female gonad. Whereas in the control group atresia was observed in 10 – 12% of females, the levels increased to 16.7% at 1.4 µg/L and to 28.6% at 2.86 µg/L. In the male gonad atrophy was present with prevalence of 50% at 1.4 µg/L and 44.4% at 2.86/L.

In mammals VIN has been found to induce significant feminization of male rats by altering the development of the reproductive tract, causing low sperm count, hypospadiasis, and other deformations (Kelce *et al.* 1994, Gray *et al.* 1999a). In laboratory experiments with rats

vinclozolin combined with five other anti-androgens markedly increased the frequencies of hypospadias in rat (Christiansen *et al*. 2008). In a similar study with a mixture of seven anti-androgens reproductive malformations in rats were more frequent than after exposure to vinclozolin alone (Rider et al. 2008).

4. Conclusions

Most data presented here were related to echinoderms, crustaceans, molluscs as invertebrates, and amphibian, fish as vertebrates. Comparison with data on studies with mammals show some striking similarities on the morphological and histological effect level. Focussing on the endocrine modulation of the reproductive system of the species under investigation, quite heterogenous structures of gonads, varying existence and structure of accessory organs, different regulatory pathways mediated by different hormone-types had to be taken into account. Nevertheless, some cell and tissue differentiation and reproduction features found in spermatogenesis and oogenesis display common structures and principles. It is not surprising that echinoderms may posses control mechanisms of physiological processes, in terms of molecules and actions, rather similar to those of vertebrates. Available data suggest that sex steroids (progestins, androgens and estrogens) have a role in regulating reproduction and other physiological processes in echinoderms. Past and recent investigations have identified vertebrate type steroids, i.e. progesterone, testosterone and 17b-estradiol in several echinoderm species (see for literature Sugni *et al.* 2007).
In crustaceans steroids with structural similarities to those of vertebrates could be identified (Fingerman *et al.* 1993, Lafont & Mathieu, 2007, LeBlanc, 2007).
Prosobranch molluscs provide strong evidence for EDC-related effects on development, fecundity and reproduction in invertebrates. The case of imposex as a result of exposure to TBT (and for some species after exposure to TPT) is the clearly dominant example of population-level EDC effects in wildlife. However, other xeno-androgens acting as aromatase inhibitors or AR agonists have been shown to cause almost identical effects at extremely low concentrations. These examples support the hypothesis that a modulation of vertebrate-type steroid levels in prosobranchs plays a key role in imposex development, although an involvement of neuropeptides cannot be ruled out. Furthermore, reproduction and sexual development of prosobranchs are also affected by xeno-estrogens such as Bisphenol A, Octylphenol, and estradiol at environmentally relevant exposure levels reflecting their high susceptibility to EDCs in general (Oehlmann *et al.* 2007).
Danish studies have indicated increased occurrence of cryptochordism in sons of female gardeners (Weidner et al. 1998) and reduced fecundability in female greenhouse workers (Abell *et al.* 2000). On the other hand surveillance studies of young men in Northern Europe showed that in relation to sperm counts from the 1940s with averages higher than 100mill/mL, this number went down to 40 mill/mL (Andersson *et al*. 2008). When this decline is continuing, the authors assume and increased number of infertile couples and lower fertility rates in the future. One of the most discussed factor is the multiple exposure to pesticides with dominant estrogenic or anti-androgenic properties. (Orton et al. 2011)

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The introduction of the synthetic organochlorine, organophosphate, carbamate and pyrethroid pesticides by 1950's marked the beginning of the modern pesticides era and a new stage in the agriculture development. Evolved from the chemicals designed originally as warfare agents, the synthetic pesticides demonstrated a high effectiveness in preventing, destroying or controlling any pest. Therefore, their application in the agriculture practices made it possible enhancing crops and livestock's yields and obtaining higher-quality products, to satisfy the food demand of the continuously rising world's population. Nevertheless, the increase of the pesticide use estimated to 2.5 million tons annually worldwide since 1950., created a number of public and environment concerns.

This book, organized in two sections, addresses the various aspects of the pesticides exposure and the related health effects. It offers a large amount of practical information to the professionals interested in pesticides issues.

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