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Genotoxicity

A Predictable Risk to Our Actual World

Edited by Marcelo L. Larramendy and Sonia Soloneski

GENOTOXICITY - A PREDICTABLE RISK TO OUR ACTUAL WORLD

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and **Sonia Soloneski**

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Meet the editors

Marcelo L. Larramendy, Ph.D., serves as a professor of Molecular Cell Biology at the School of Natural Sciences and Museum (National University of La Plata (UNLP), Argentina). He was appointed the senior researcher of the National Scientific and Technological Research Council of Argentina and is a former member of the Executive Committee of the Latin American Association of Environmental Mutagenesis, Teratogenesis and Carcinogenesis. He is the author of more than 450 contributions, including scientific publications, research communications and conferences worldwide, and a recipient of several national and international awards. Prof. Larramendy is a regular lecturer at the international A. Hollaender Courses organized by the International Academy for Engineering and Media Science (IAEMS) and former guest scientist at the National Institutes of Health (USA) and the University of Helsinki (Finland). He is an expert in genetic toxicology and is, or has been, a referee for more than 20 international scientific journals. In 2015, he became a member of the International Panel of Experts at the International Agency for Research on Cancer (IARC, WHO, Lyon, France) for the evaluation of DDT, 2,4-D and lindane. Presently, Prof. Larramendy is the head of the Laboratory of Molecular Cytogenetics and Genotoxicology at UNLP.

Sonia Soloneski is a PhD in Natural Sciences and professor assistant of Molecular Cell Biology at the Faculty of Natural Sciences and Museum of La Plata, National University of La Plata, Argentina. She became a member of the National Scientific and Technological Research Council (CONICET) of Argentina in the field of genetic toxicology. Presently, she is a member of the Latin American Association of Environmental Mutagenesis, Teratogenesis and Carcinogenesis (ALAMCTA), the Argentinean Society of Toxicology (ATA) and the Argentinean Society of Genetics (SAG). She has authored more than 350 scientific publications in the field, including scientific publications in research papers, reviewed journals and conferences worldwide. She is a referent for issues related to the fields of genetic toxicology, mutagenesis and ecotoxicology.

Contents

Preface

In the actual world, living species today are inevitably exposed to numerous chemical, physical and biological agents. These entities are called xenobiotics, a term derived from the Greek words ξένος (xenos) = foreigner, stranger and βίος (bios, vios) = life, plus the Greek suffix for adjectives -τικός, -ή, -ό (tic). They are typically human-made and, in most cases, are necessary to preserve and improve the quality of both human and environmental health. It is estimated that humans are exposed to between one and three million different xenobiotics throughout their life cycle. These agents frequently have unexpected consequences to living organisms, however. Our understanding of how these agents can cause genetic alterations to DNA and their role in the different biological systems continues to be an area of intense interest in sectors including health, disease, pharmaceutical, environment, industry, agriculture and food, among others.

The large number of untested environmental agents makes it vital that priorities are established for evaluating DNA damage inducers. Although the genotoxicity of many xenobiotics is well known even today, interest in understanding the mechanisms of action in many xenobiotics is being continuously stimulated by their continued re-evaluation. The purpose of genotoxicity is to determine whether the substance, product or environmental agent can cause genetic alterations in somatic and/or germline cells and then to establish the potential risk of these xenobiotics for humans and environmental organisms. Whereas DNA damage in somatic cells has been associated with degenerative conditions such as ageing and cancer, it is related in germinal cells to an increased risk of infertility, spontaneous abortion or genetic and chromosomal defects in offspring that result in genetic diseases. In general terms, most of these xenobiotics are metabolised in the body to produce less toxicity. After being metabolised, however, most can be chemically reactive, leading to toxicity, with the potential to become more toxic and, in some cases, they are carcinogenic. Carcinogenicity from various environmental causes is a major public concern because cancer is the leading cause of death in many places worldwide.

Without knowledge of the genotoxic properties of the many xenobiotics, the evaluation of responses in living organisms, including humans, is difficult, and consequently the regulation of environmental genotoxicants is a complex and hard process. Accurate identification of the different classes of environmental genotoxicants would permit regulatory international scientific agencies to use this information in a variety of legislative decisions to establish priorities of public and scientific concern.

Although a vast literature is available on genotoxicology, this book contains important investigations into the diverse chemical hazards encountered in both anthropogenic and natu-

ral environments and provides valuable information about the genotoxicity of several xenobiotics that can negatively affect the health of humans and ecosystems.

Organised into six chapters, this volume begins with an overview of the current advantages and disadvantages of the use of cytotoxicity and its viability in *in vitro* bioassays for toxicity screening. Readers are introduced to this subject to help them select the most suitable cytotoxicity and viability tests for study. The second chapter provides genotoxic information on assessment by *in vitro* comet assay, and by cell-free DNA quantification damage methodologies and their correlation with the enhanced inflammatory state observed in end-stage renal disease patients. The third chapter describes a study employing the retinal cells of the zebrafish *Danio rerio* as an *in vivo* model to characterise the toxic effect exerted by the synthetic pyrethroid insecticide cypermethrin, using histology, immunofluorescence, comet assay, enzyme activity and gene expression studies. The fourth chapter is an update regarding the use of the quantitative structure–activity relationships (QSAR) model, which applies multivariate statistical methods to correlate structural or molecular descriptors of known carcinogen compounds in the assessment of the carcinogenic risk of environmental chemicals. The fifth chapter is an interesting discussion about the role of extremely low-frequency electromagnetic fields (EMF) on human genotoxicity, focusing on the interaction of very low and extremely low EMF frequencies on living systems. Finally, this book includes a chapter discussing the effects of the environmental exposure of children and pregnant women to agrochemicals, stressing their genotoxic potential in these particular human populations.

The editors of *Genotoxicology - A Predictable Risk to Our Actual World* are enormously grateful to all the contributing authors for sharing their knowledge and insights in this book project. They have made an extensive effort to gather the information included in every chapter. This book is designed to provide an overview of the different genotoxicants and their effects on living organisms, including humans. The contributions made by the specialists in this field of research are gratefully acknowledged. We hope that the information presented in this book will meet the expectations and needs of all those interested in the different aspects of the genotoxicity field. The publication of this book is of great importance to those scientists, pharmacologists, physicians and veterinarians, as well as engineers, teachers, graduate students and administrators of environmental programmes, who make use of these investigations to understand both the basic and applied genotoxic aspects of known and new xenobiotics and to guide them in their future investigations.

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Chapter 1

In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages

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Additional information is available at the end of the chapter

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Abstract

Cytotoxicity is one of the most important indicators for biological evaluation *in vitro* studies. *In vitro*, chemicals such as drugs and pesticides have different cytotoxicity mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors etc. In order to determine the cell death caused by these damages, there is a need for cheap, reliable and reproducible short-term cytotoxicity and cell viability assays. Cytotoxicity and cell viability assays are based on various cell functions. A broad spectrum of cytotoxicity assays is currently used in the fields of toxicology and pharmacology. There are different classifications for these assays: (i) dye exclusion assays; (ii) colorimetric assays; (iii) fluorometric assays; and (iv) luminometric assays. Choosing the appropriate method among these assays is important for obtaining accurate and reliable results. When selecting the cytotoxicity and cell viability assays to be used in the study, different parameters have to be considered such as the availability in the laboratory where the study is to be performed, test compounds, detection mechanism, specificity, and sensitivity. In this chapter, information will be given about in vitro cytotoxicity and viability assays, these assays will be classified and their advantages and disadvantages will be emphasized. The aim of this chapter is to guide the researcher interested in this subject to select the appropriate assay for their study.

Keywords: cell viability, cytotoxicity, *in vitro* assays, advantages, disadvantages

1. Introduction

Viability levels and/or proliferation rates of cells are good indicators of cell health. Physical and chemical agents can affect cell health and metabolism. These agents may cause toxicity on cells via different mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors, inhibition of polydeoxynucleotide

elongation, and enzymatic reactions [1]. In order to determine the cell death caused by these mechanisms, there is a need for cheap, reliable and reproducible short-term cytotoxicity and cell viability assays.

In vitro cell viability and cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and for drug screening. Application of these assays has been of increasing interest over recent years. Currently, these assays are also used in oncological researches to evaluate both compound toxicity and tumor cell growth inhibition during drug development. Because, they are rapid, inexpensive and do not require the use of animals. Furthermore, they are useful for testing large number of samples. Cell viability and cytotoxicity assays are based on various cell functions such as cell membrane permeability, enzyme activity, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity [1].

In vitro cytotoxicity and/or cell viability assays have some advantages, such as speed, reduced cost and potential for automation, and tests using human cells may be more relevant than some in vivo animal tests. However, they have some disadvantages because they are not technically advanced enough yet, to replace animal tests [2].

It is important to know how many viable cells are remaining and/or how many cells are dead at the end of the experiment. A broad spectrum of cytotoxicity and cell viability assays is currently used in the fields of toxicology and pharmacology. The choice of assay method is crucial in the assessment of the interaction type [3].

2. Classification of cytotoxicity and cell viability assays

Although there are different classifications for cytotoxicity and cell viability assays, in this chapter, these assays are classified according to measurement types of end points (color changes, fluorescence, luminescent etc.).

1. Dye exclusion: Trypan blue, eosin, Congo red, erythrosine B assays.
2. Colorimetric assays: MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and crystal violet assay.
3. Fluorometric assays: alamarBlue assay and CFDA-AM assay.
4. Luminometric assays: ATP assay and real-time viability assay.

2.1. Dye exclusion assays

The proportion of viable cells in a cell population can be estimated in various methods. The simplest and widely used one of the methods is dye exclusion method. In dye exclusion method, viable cells exclude dyes, but dead cells not exclude them. Although the staining procedure is quite simple, experimental procedure of large number of samples is difficult and time consuming [4]. Determination of membrane integrity is possible via dye exclusion

method. A variety of such dyes have been employed, including eosin, Congo red, erythrosine B, and trypan blue [5, 6]. Of the dyes listed, trypan blue has been used the most extensively [7–10].

If dye exclusion assays are used, following factors must be considered (i) lethally damaged cells by cytotoxic agents may require several days to lose their membrane integrity, (ii) the surviving cells may continue to proliferate during this time, and (iii) some lethally damaged cells are not appear to be stained with dye at the end of the culture period, because they may undergo an early disintegration. Factors (ii) and (iii) may cause an underestimate of cell death when the results of the assay are based on percent viability expression [11–13].

Dye exclusion assays have unique advantages for chemosensitivity testing. They are comparatively simple, require small numbers of cells, are rapid, and are capable of detecting cell kill in nondividing cell populations. Further investigations into the possible role of these assays in chemosensitivity testing are warranted [11]. However, none of these dyes is recommended for use on monolayer cell cultures but rather they are intended for cells in suspension; thus monolayer cells must first trypsinized [6].

2.1.1. Trypan blue dye exclusion assay

This dye exclusion assay is used to determine the number of viable and/or dead cells in a cell suspension. Trypan blue is a large negatively charged molecule. Trypan blue dye exclusion assay is based on the principle that live cells possess intact cell membranes that exclude this dye, whereas dead cells do not. In this assay, adherent or nonadherent cells are incubated with serial dilutions of test compounds for various times. After the compound treatment, cells are washed and suspended. Cell suspension is mixed with dye and then visually examined to determine whether cells take up or exclude dye. Viable cells will have a clear cytoplasm, whereas dead cells will have a blue cytoplasm [14, 15]. Number of viable and/or dead cells per unit volume is determined by light microscopy as a percentage of untreated control cells [15, 16].

Advantages: This method is simple, inexpensive, and a good indicator of membrane integrity [17], and dead cells are colored blue within seconds of exposure to the dye [18].

Disadvantages: Cell counting is generally done using a hemacytometer [19]. Therefore, counting errors (~10%) could be occurred. Counting errors have been attributed to poor dispersion of cells, cell loss during cell dispersion, inaccurate dilution of cells, improper filling of the chamber and presence of air bubbles in the chamber [17].

While the staining procedure is quite simple, it is difficult to process large number of samples concurrently, particularly where the exact timing of progressive cytotoxic effects is required [4]. Furthermore, trypan blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions. Therefore, it is not sufficiently sensitive to use for in vitro cytotoxicity testing. Another disadvantage of trypan blue is toxic side effect of this dye on mammalian cells [20].

2.1.2. Erythrosine B dye exclusion assay

Erythrosine B, also known as erythrosine or Red No. 3, is primarily used as food coloring agent [20, 21]. Erythrosine B has already been introduced as a vital dye for counting viable cells. Principle of this dye exclusion assay is similar to trypan blue dye exclusion assay principle. Although erythrosine B is an alternative bio-safe vital dye for cell counting; it is not widely used to count viable or dead cells.

Advantages: It has benefits such as low cost, versatility, and bio-safety [20].

Disadvantages: Its procedure is time-consuming and labor-intensive. Moreover, potential disadvantages include contamination of reusable cell counting chamber, variations of hemocytometer filling rates, and inter-user variations [20].

2.2. Colorimetric assays

Principle of colorimetric assays is the measurement of a biochemical marker to evaluate metabolic activity of the cells. Reagents used in colorimetric assays develop a color in response to the viability of cells, allowing the colorimetric measurement of cell viability via spectrophotometer. Colorimetric assays are applicable for adherent or suspended cell lines, easy to perform, and comparably economical [22, 23]. Commercial kits of colorimetric assays are available from several companies and generally experimental procedures of these assays are available in kit packages.

2.2.1. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is one of the most commonly used colorimeteric assay to assess cytotoxicity or cell viability [24]. This assay determines principally cell viability through determination of mitochondrial function of cells by measuring activity of mitochondrial enzymes such as succinate dehydrogenase [18]. In this assay, MTT is reduced to a purple formazan by NADH. This product can be quantified by light absorbance at a specific wavelength.

Advantages: This method is far superior to the previously mentioned dye exclusion methods because it is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests [18, 25].

Disadvantages: MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore, prior to measuring the absorbance, an organic solvent such as dimethyl sulfoxide (DMSO) or isopropanol is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error [18, 26].

Additional control experiments should be conducted to reduce false-positive or false-negative results that caused by background interference due to inclusion of particles. This interference could lead to an overestimation of the cell viability. This can often be controlled by subtraction of the background absorbance of the cells in the presence of the particles, but without the assay reagents [18, 26].

2.2.2. MTS assay

The MTS assay (5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt assay) is a colorimetric assay. This assay is based on the conversion of a tetrazolium salt into a colored formazan by mitochondrial activity of living cells. The amount of produced formazan is depend on the viable cell number in culture and can be measured with spectrophotometer at 492 nm.

Advantages: Previous studies suggest that the MTS in vitro cytotoxicity assay combines all features of a good measurement system in terms of ease of use, precision, and rapid indication of toxicity [27, 28]. MTS assay is a rapid, sensitive, economic, and specific in vitro cytotoxicity assay. Performance of this assay is very competitive to other toxicological tests. This assay provides ideal properties for cytotoxicity measurement because it is easy to use, rapid, reliable, and inexpensive. Therefore, it can be used for onsite toxicological assessments [27, 29–31].

Disadvantages: The level of absorbance measured at 492 nm is influenced by the incubation time, cell type, and cell number. The proportion of MTS detection reagents to cells in culture also influences the measured absorbance level. Previous studies suggested a linear relationship between incubation time and absorbance for short incubation times up to 5 hours [29, 32, 33]. Therefore, proper incubation times for this assay are 1–3 hours.

2.2.3. XTT assay

A colorimetric method based on the tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium, monosodium salt) was first described by Scudiero et al. [34]. While MTT produced a water-insoluble formazan compound which required dissolving the dye in order to measure its absorbance, the XTT produces a water-soluble dye. The procedure of XTT is simply for measuring proliferation and is therefore an excellent solution for quantitating cells and determining their viability. XTT is used to assay cell proliferation as response to different growth factors. It is also used for assaying cytotoxicity.

This assay is based on the ability reduction of the tetrazolium salt XTT to orange-colored formazan compounds by metabolic active cells. Orange-colored formazan is water soluble and its intensity can be measured with a spectrophotometer. There is a linear relationship between the intensity of the formazan and the number of viable cells. The use of multiwell plates and a spectrophotometer (or ELISA reader) allows for study with a large number of samples and obtaining results easily and rapidly. The procedure of this assay includes cell cultivation in a 96-well plate, adding the XTT reagent and incubation for 2–24 hours. During the incubation time, an orange color is formed and the intensity of color can be measured with a spectrophotometer [34, 35].

Advantages: XTT assay is speed, sensitive, easy to use, and safe method. It has high sensitivity and accuracy [35].

Disadvantages: XTT assay performance depends on reductive capacity of viable cells with the mitochondrial dehydrogenase activity. Therefore, changes of reductive capacity of viable cells resulting from enzymatic regulation, pH, cellular ion concentration (e.g., sodium, calcium, potassium), cell cycle variation, or other environmental factors may affect the final absorbance reading [34, 35].

2.2.4. WST-1 assay

WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium monosodium salt) cell proliferation assay is a simple, colorimetric assay designed to measure the relative proliferation rates of cells in culture. The principle of this assay is based on the conversion of the tetrazolium salt WST-1 into a highly water-soluble formazan by mitochondrial dehydrogenase enzymes in the presence of intermediate electron acceptor, such as mPMS (1-methoxy-5-methyl-phenazinium methyl sulfate) [36]. The water-soluble salt is released into the cell culture medium. Within incubation period, the reaction produces a color change which is directly proportional to the amount of mitochondrial dehydrogenase in cell culture and thus, the assay measures the metabolic activity of cells.

To perform the assay, the WST-1 reagent that is ready-to-use is added directly into the media of cells cultured in multiwell plates. The cultures are then given 30 minutes–4 hours to reduce the reagent into the dye form. The plate is then immediately read at 450 nm with a reference reading at 630 nm [37].

Advantages: It is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity tests. Furthermore, phenol red indicators in cell culture medium do not interfere with the dye reaction. Because the colored dye which produced at the end of experiment is water-soluble, it is not required a solvent and additional incubation time [37].

Disadvantages: The standard incubation time of WST-1 time is 2 h. Whether one-time addition of WST-1 can reflect the effect of the testing agents at different time points on the trend of relative cell viability is still unclear [37].

2.2.5. WST-8 assay

WST-8 assay is a colorimetric assay for the determination of viable cell numbers and can be used for cell proliferation assays as well as cytotoxicity assays. WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium, monosodium salt), a highly stable and water-soluble WST, is utilized in Cell Counting Kit-8 (CCK-8). It is more sensitive than WST-1 particularly at neutral pH [37]. Because of the electron mediator, 1-methoxy PMS in this kit is highly stable, and CCK-8 is stable for at least 6 months at the room temperature and for 1 year at 0–5°C. Since WST-8, WST-8 formazan, and 1-methoxy PMS have no cytotoxicity on cells in the culture media, same cells from the previous assay may be used for additional experiments.

Advantages: WST-8 is not cell permeable, which results in low cytotoxicity. Therefore, after the assay, it is possible to continue further experiments using the same cells. Furthermore, it produces the water-soluble formazan upon cellular reduction, which would provide an additional advantage to the method by allowing a simpler assay procedure and not required an extra step to dissolve the formazan [28].

Disadvantages: An important consideration is that reduction of assay substrates is impacted by changes in intracellular metabolic activity that has no direct effect on overall cell viability [15].

2.2.6. LDH (lactate dehydrogenase) assay

LDH (lactate dehydrogenase) cytotoxicity assay is a colorimetric method of assaying cellular cytotoxicity. LDH Cytotoxicity Assay Kit can be used with different cell types not only for assaying cell-mediated cytotoxicity but also for assessment of cytotoxicity mediated by toxic chemicals and other test compounds. The assay measures the stable, cytosolic, lactate dehydrogenase (LDH) enzyme quantitatively. This enzyme releases from damaged cells. LDH is an enzyme that is normally found within the cell cytoplasm. When cell viability reduced leakiness of the plasma membrane increase and therefore LDH enzyme is released into the cell culture medium. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt (iodonitrotetrazolium (INT)) into a red color formazan by diaphorase. In the first step, LDH catalyze conversion of lactate to pyruvate and thus NAD is reduced to $NADH/H^+$. In a second step, catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to red formazan [38, 39].

The LDH activity is determined as NADH oxidation or INT reduction over a defined time period. The resulting red formazan absorbs maximally at 492 nm and can be measured quantitatively at 490 nm.

The detergent Triton X-100 is commonly used as positive control in the LDH assay to determine the maximum LDH release from the cells. In addition, well-known membranolytic particles such as crystalline silica can be used as a positive control in LDH assay [40].

Advantages: Reliability, speed, and simple evaluation are some of characteristics of this assay. Because, the loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage [38, 41].

Disadvantages: The major limitation of this assay is that serum and some other compounds have inherent LDH activity. For example, the fetal calf serum has extremely high background readings. Therefore, this assay is limited to serum-free or low-serum conditions, limiting the assay culture period (depending on your cells' tolerance to low serum) and reducing the scope of the assay as it can no longer allow determination of cell death caused under normal growth conditions (i.e. in 10% fetal calf serum). At a minimum, you should always first test the assay with an unused aliquot of the media you intend to use and compare the reading to that from media lacking supplements (e.g. straight DMEM) [42].

2.2.7. SRB (Sulforhodamine B) assay

SRB (Sulforhodamine B) assay is a rapid and sensitive colorimetric method for measuring the drug-induced cytotoxicity in both attached and suspension cell cultures. This assay as first described by Skehan and colleagues was developed for use in the disease-orientated, large-scale anticancer drug discovery program of the National Cancer Institute (NCI) that was launched in 1985. SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under mildly acidic conditions, SRB binds to protein basic amino acid residues in TCA-fixed (trichloroacetic acid) cells to provide a sensitive index of cellular protein. SRB assay is also used to evaluate colony formation and colony extinction [43].

Advantages: The SRB assay is simple, fast, and sensitive. It provided good linearity with cell number, permitted the use of saturating dye concentrations, is less sensitive to environmental fluctuations, is independent of intermediary metabolism, and provided a fixed end point that is not require a time-sensitive measurement of initial reaction velocity [43]. Reproducibility of this assay is high.

Disadvantages: It is important to obtain and maintain a homogeneous cell suspension. Cellular clumps/aggregates should be avoided for high assay performance.

2.2.8. NRU (neutral red uptake) assay

The neutral red uptake (NRU) assay is also one of the most used colorimetric cytotoxicity/ cell viability assay. This assay was developed by Borenfreund and Puerner [44]. This assay was based on the ability of viable cells to take up the supravital dye neutral red. This weakly cationic dye penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes. The dye is then extracted from the viable cells using an acidified ethanol solution and the absorbance of the dye is measured using spectrophotometer.

Neutral red uptake depends on the capacity of cells to maintain pH gradients through the ATP production. At physiological pH, net charge of the dye is zero. This charge enables the dye to penetrate the cell membranes. Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus, the dye becomes charged and is retained inside the lysosomes. When the cell dies or pH gradient is reduced, the dye cannot be retained. In addition, the uptake of neutral red by viable cells can be modified by alterations in cell surface or lysosomal membranes. Thus, it is possible to distinguish between viable, damaged, or dead cells [44]. Lysosomal uptake of neutral red dye is a highly sensitive indicator of cell viability. The assay can quantitate cell viability and measure cell replication, cytostatic effects or cytotoxic effects depending on the seeding density [45]. Absorbance is measured at 540 nm in multiwell plate reader spectrophotometer.

Advantages: NRR assay is a good marker of lysosomal damage. Also, speed and simple evaluation are some advantages of this assay.

Disadvantages: It has been reported that the NRR assay is either minimally or not at all affected by natural factors, such as temperature and salinity, but is mainly influenced by pollutants [46].

2.2.9. CVS assay (crystal violet assay)

Adherent cells detach from cell culture plates during cell death. This feature can be used for the indirect assessment of cell death and to determine differences in proliferation rate upon stimulation with cytotoxic agents. One simple method to detect maintained adherence of cells is crystal violet assay. In this assay, crystal violet dye binds to proteins and DNA of viable cells, and thus, attached cells are stained with this dye. Cells lose their adherence during cell death and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. Crystal violet assay is a quick and reliable screening method that is suitable for the examination of the impact of chemotherapeutics or other compounds on cell survival and growth inhibition [47].

Advantages: Crystal violet staining is a quick and versatile assay for screening cell viability under diverse stimulation conditions [48]. However, it is potentially compromised by proliferative responses that occur at the same time as cell death responses. Therefore, chemical inhibitors of caspases and/or of necroptosis may be incorporated into the assay [49, 50]. Alternatively, molecular studies (e.g., overexpression or knockdown) can be performed to more specifically address the nature of cell death [51].

Disadvantages: Crystal violet assay is insensitive to changes in cell metabolic activity. Therefore, this assay is not appropriate for studies used cell metabolism affected compounds. While crystal violet assay is suitable for the examination of the impact of chemotherapeutics or other compounds on cell survival and growth inhibition, it is not able to measure cell proliferation rate [51].

2.3. Fluorometric assays

Fluorometric assays of cell viability and cytotoxicity are easy to perform with the use of a fluorescence microscope, fluorometer, fluorescence microplate reader or flow cytometer, and they offer many advantages over traditional dye exclusion and colorimetric assays. Fluorometric assays are also applicable for adherent or suspended cell lines and easy to use. These assays are more sensitive than colorimetric assays [52–54]. Commercial kits of fluorometric assays are available from several companies and generally experimental procedures of these assays are available in kit packages.

2.3.1. alamarBlue (AB) assay

alamarBlue assay is also known as resazurin reduction assay. The alamarBlue assay is based on the conversion of the blue nonfluorescent dye resazurin, which is converted to the pink fluorescent resorufin by mitochondrial and other enzymes such as diaphorases [53].

Resazurin is a phenoxazin-3-one dye and cell permeable redox indicator that can be used to monitor viable cell number with protocols similar to those utilizing the tetrazolium compounds [55]. It is known to act as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor [52]. It is a nontoxic and cell permeable compound. Color of this compound is blue and it is nonfluorescent. After entering cells, resazurin is reduced to resorufin. Resorufin is red in color and highly fluorescent compound. Viable cells convert continuously resazurin to resofurin, increasing overall fluorescence and color of the cell culture medium. The quantity of produced resofurin is related to the number of viable cells. Ratio of viable cells can be quantified using a microplate reader fluorometer equipped with a 560 nm excitation/590 nm emission filter set. Resofurin can also be measured by absorbance changes, but absorbance detection is not often used because absorbance detection is less sensitive than fluorescence measurement.

The incubation period required to generate a sufficient fluorescent signal above background is usually about 1–4 hours, depending on metabolic activity of the cells, the cell density per well and other conditions such as the culture medium type [54].

Advantages: alamarBlue (resazurin reduction) assay is relatively inexpensive and more sensitive than tetrazolium assays. Also, it can be multiplexed with other methods such as measuring caspase activity to gather more information about the cytotoxicity mechanism.

Disadvantages: Fluorescent interference from test compounds and the often overlooked direct toxic effects on the cells are possible [54].

2.3.2. CFDA-AM assay

CFDA-AM (5-carboxyfluorescein diacetate, acetoxymethyl ester) is another fluorogenic dye that is used for cytotoxicity determination. It is indicator for plasma membrane integrity. The dye CFDA-AM is nontoxic esterase substrate that can be converted by nonspecific esterases of viable cells from a membrane permeable, nonpolar, nonfluorescent substance to polar, fluorescent dye, carboxyfluorescein (CF). The conversion of CFDA-AM to CF by the cells indicates the integrity of plasma membrane, since only an intact membrane can maintain the cytoplasmic milieu which is needed to support esterase activity [56].

Advantages: CFDA-AM and alamarBlue assays were shown to be applicable in parallel on the same set of the cells, since both are nontoxic to cells, require similar incubation times, and can be detected at different wavelengths without interferences [56–58].

Disadvantages: Fluorescent interference from test compounds is possible.

2.3.3. Protease viability marker assay (GF-AFC assay)

Measurement of a conserved and constitutive protease enzyme activity of viable cells is used as a good indicator of cell viability. A cell permeable fluorogenic protease substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) has been recently developed to selectively detect protease activity that is restricted to viable cells [59]. The GF-AFC substrate can penetrate viable cells. In these cells, cytoplasmic aminopeptidase activity removes the gly and phe amino acids to release aminofluorocoumarin (AFC) and produce a fluorescent signal proportional to the number of viable cells [54].

When cells die, this protease activity is rapidly loss. Therefore, this protease activity is a selective marker of the viable cell population. The signal generated from this assay approach has been shown to correlate well with other established methods of determining cell viability such as an ATP assay [54].

Advantages: It is relatively nontoxic to cells in culture. Also, in opposite to exposure of cells to tetrazolium, long-term exposure of the GF-AFC substrate cells results in little change in viability of cells. This assay is suitable for multiplexing with other assays, because at the end of the assay, cell population remains viable and can be used for subsequent assays. Furthermore, the incubation time is much shorter (30 min-1 hour) compared to 1–4 hours required for the tetrazolium assays [54].

Disadvantages: Fluorescent interference from test compounds is possible.

2.4. Luminometric assays

Luminometric assays provide fast and simple determination of cell proliferation and cytotoxicity in mammalian cells. These assays can be performed in a convenient 96-well and 384-well microtiter plate format and detection by luminometric microplate reader [54, 60, 61]. A remarkable feature of the luminometric assays is the persistent and stable glow-type signal produced after reagent addition. This attribute can be harnessed to produce both viability and cytotoxicity values from the same well [59]. Commercial kits of luminometric assays are available from several companies and generally experimental procedures of these assays are available in kit packages.

2.4.1. ATP assay

ATP (adenosine tri-phosphate) represents the most important chemical energy reservoir in cells and is used for biological synthesis, signaling, transport, and movement processes. Therefore, cellular ATP is one of the most sensitive end points in measuring cell viability [62]. When cells damaged lethally and lose membrane integrity, they lose the ability to synthetize ATP and the ATP level of cells decreases dramatically [54, 63]. The ATP assay is based on the reaction of luciferin to oxyluciferin. Enzyme luciferase catalyzes this reaction in the presence of Mg^{2+} ions and ATP yielding a luminescent signal. There is a linear relationship between the intensity of luminescent signal and ATP concentration [61] or cell number [64].

The ATP assay chemistry can typically detect fewer than 10 cells per well, and therefore, it has been widely used 1536-well plate format.

Advantages: ATP assay is the fastest cell viability assay to use, the most sensitive, and is less prone to artifacts than other viability assays. The luminescent signal reaches steady state and stabilizes within 10 min after addition of reagent. It does not have an incubation step for conversion of substrate into colored compound. This also eliminates a plate handling step [54].

Disadvantages: The ATP assay sensitivity is usually limited by reproducibility of pipetting replicate samples rather than a result of the assay chemistry [54].

2.4.2. Real-time viability assay

Recently, a new approach is developed to measure viable cell number in real time [60]. In this assay, an engineered luciferase derived from a marine shrimp and a small molecule prosubstrate is used. The pro-substrate and luciferase are added directly to the cell culture medium as a reagent. The pro-substrate is not a substrate of luciferase. Viable cells with an active metabolism reduce the pro-substrate into a substrate, which used by luciferase, to generate a luminescent signal. The assay can be performed in two formats: continuous read and endpoint measurement. In the continuous read format, the luminescent signal can be repeatedly recorded from the sample wells over an extended period to measure the number of cells in "real time" [54, 60].

Advantages: This assay is the only assay which allows to real-time measurement of cell viability/cytotoxicity. The rapid decrease in luminescent signal following cell death enables multiplexing this assay with other luminescent assays that contain a lysis step that will kill cells. The decrease in luminescence following cell death is important to eliminate interference with subsequent luminescent assays [54, 60].

Disadvantages: A limitation of the real time assay results from the eventual depletion of pro-substrate by metabolically active cells. Generally, the luminescent signal generated correlates with the number of metabolically active cells. However, the length of the time the luminescent signal will be linear with cell number will depend on the number of cells per well and their metabolic activity. Therefore, it is recommended that the maximum incubation time to maintain linearity should be empirically determined for each cell type and seeding density [54, 60].

3. Conclusions

A broad spectrum of cytotoxicity and cell viability assays is currently used in the fields of toxicology and pharmacology. An ideal assay for *in vitro* viability and/or cytotoxicity determination should be a rapid, safe, reliable, efficient, and time- and cost-effective. It should not interfere with test compound. The choice of assay method is crucial in the assessment of the interaction type. The assay may change the interpretation of the compound interaction. Therefore, the assay method should be chosen with caution, considering the mechanism of action of the test compound [3]. Tissue or cell type used in the study also affects the performance of cytotoxicity and/or cell viability assays. Therefore, before choosing an assay for study, different methods should be tried and compared. If it is possible, more than one assay should be used to determine cytotoxicity and/or cell viability in *in vitro* studies. Thus, reliability of the obtained results would increase.

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Chapter 2

DNA Damage in End-Stage Renal Disease Patients. Assessment by *In Vitro* Comet Assay and by Cell-Free DNA Quantification

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Additional information is available at the end of the chapter

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Abstract

Inflammation is a common feature in end stage renal disease (ESRD) that might contribute to increase DNA damage. ESRD patients present increased circulating cell-free DNA (cfDNA) and different types of DNA injury. The underlying inflammatory process in ESRD may be associated with increased genomic damage and cfDNA contributing to further enhance inflammation. We analyzed the degree of genomic damage in ESRD patients under hemodialysis therapy, using the comet assay and cfDNA quantification. ESRD patients presented significantly higher C-reactive protein (CRP) and cell damaged DNA. The cfDNA correlated with age and inflammatory stage. Nine out of 39 patients died during the one year follow-up period and presented significantly higher cfDNA, than those who persisted alive. At lower CRP values, the increased DNA damage is still within the cell, and at higher CRP the damaged DNA is released in to plasma. The higher degree of genomic damage in ESRD might be a consequence of inflammation and aging, and may contribute to increase cancer and cardiovascular mortality risk. Our data suggest that the comet assay is more sensitive for low-grade inflammatory conditions, while cfDNA appears as a good biomarker for more severe inflammatory conditions, and as a biomarker for the outcome of ESRD patients.

Keywords: chronic kidney disease, end-stage renal disease, inflammation, genomic damage, comet assay, cell-free DNA

1. Introduction

Kidneys are important in homeostasis, ensuring the excretion of toxic substances and regulating blood volume, blood pressure, concentration of electrolytes, plasma osmolarity and the

acid/base balance. The kidneys also have endocrine functions, producing hormones, such as erythropoietin and calcitriol.

Chronic kidney disease (CKD) is characterized by a decline in kidney function and/or altered renal structure, leading to a gradual to permanent loss of kidney function over time. End-stage renal disease (ESRD), the worst stage of chronic kidney disease (CKD), requires dialysis to prevent accumulation of toxins, excessive water and electrolytes, or kidney transplantation [1].

Inflammation is a common feature in CKD, especially enhanced in ESRD patients on hemodialysis (HD). This chronic inflammatory state seems to contribute to aggravate kidney dysfunction and favor the occurrence of comorbidities and the risk of mortality [2, 3].

Chromosomal abnormalities, reduced DNA repair and DNA lesions have been reported in CKD patients [4]; increased levels of circulating cell-free DNA (cfDNA) [5], DNA-histone complexes [6] and different types of DNA injury [4, 7, 8] were also reported. The DNA-histone complexes have been proposed as markers of cardiovascular (CV) events in CKD and ESRD patients [6]. These genetic changes may explain, at least in part, the increased risk of cancer in these patients. In ESRD patients the HD treatment seems to contribute *per se* to enhance inflammation and, thus, it may also favor genetic damage and the associated complications [9, 10].

2. Chronic kidney disease and inflammation

CKD is associated with high mortality rates and its prevalence is increasing worldwide. The five clinical stages of CKD are based on the values of glomerular filtration rate and albuminuria (**Table 1**). At stages 1 and 2, patients are usually asymptomatic, presenting kidney damage and/or loss of kidney function. Stages 3 and 4 are associated with deterioration of renal function, from mild to severe dysfunction. In stage 5 (or ESRD), loss of kidney function is irreversible and the patients need renal replacement therapy [1, 11, 12]. Given the increasing prevalence of ESRD patients on HD treatment, CKD is a major health public problem, with significant socio-economic consequences and a considerable impact on functional status and quality of life of patients [13].

Diabetes *mellitus* and arterial hypertension are the two most common causes of CKD [15, 16]. Other possible causes, although less common, include glomerulonephritis, nephrolithiasis, pyelonephritis and polycystic kidney disease [17, 18].

Regardless of technologic improvements in dialysis, ESRD is associated with substantial morbidity and mortality risks [19]. Actually, the improvements in dialysis procedures and in membrane flux, with higher clearance of small solutes, do not necessarily improve patient's survival [20, 21].

In CKD patients, the CV disease (CVD) events are the most frequent causes of death [22], while infections and malignancies are the most common non-cardiovascular causes, particularly in ESRD patients on HD. The high incidence of CVD in CKD patients has been

		Albuminuria (mg/g)		
CKD stages	**GFR (ml/min/1.73 m²)**	**<30**	**30–300**	**>300**
1	≥90	LR	MIR	HR
2	60–89	LR	MIR	HR
3a	45–59	MIR	HR	VHR
3b	30–44	HR	VHR	VHR
4	15–29	VHR	VHR	VHR
5	<15	VHR	VHR	VHR

LR, low risk; MIR, moderately increased risk; HR, high risk; VHR, very high risk.

Table 1. Prognosis of chronic kidney disease (CKD), according to glomerular filtration rate (GFR) and albuminuria (adapted from Ref. [14]).

associated with the high prevalence of traditional and non-traditional CV risk factors. Diabetes mellitus, arterial hypertension, dyslipidemia, obesity, sedentarism, smoking habits and age are important traditional CVD risk factors. Non-traditional CVD risk factors in CKD patients are more specifically related to the disease itself and/or to dialysis associated complications (e.g., inflammation, anemia, oxidative stress, hyperphosphatemia, left ventricular hypertrophy, endothelial dysfunction, insulin resistance and high levels of lipoprotein(a)). Comorbidities, such as infection, inflammation, oxidative stress, iron deficiency, anemia, vascular calcification, uremia and volume overload, are associated with a poor outcome and increased mortality risk in patients undergoing HD [3, 23–25]. Associations of these risk factors in CKD patients seem to represent a cumulative and additive risk for CV events. Actually, it has been difficult to find a biomarker or a panel of biomarkers that allows the evaluation/prognostic of the clinical condition. This is particularly complex for ESRD patients in HD, as they present several processes associated with renal tissue damage and, thus, some markers of renal injury may become relevant.

Inflammation, a hallmark of CKD, is triggered by harmful stimuli, able to activate polymorphonuclear cells and monocytes, which produce several inflammatory cytokines, reactive oxygen metabolites and proteases that can amplify the inflammatory response to a systemic level, by inducing the activation of other inflammatory cells and the production of other cytokines and of several acute-phase proteins. It seems that the persistent inflammation in CKD triggers self-enhancement of the inflammatory cascade and exacerbates wasting and vascular calcification, amplifying the risk for poor outcome [26]. Actually, inflammation is a morbidity and mortality risk factor for CKD patients. In ESRD patients on HD treatment, the chronic inflammatory state is especially enhanced, as well as vascular calcification, endothelial dysfunction and wasting [27, 28]. Thus, several biomarkers of inflammation have been largely studied as predictive markers of CVD risk and mortality in CKD patients.

The inflammatory biomarkers, C-reactive protein (CRP), interleukin (IL)-6 and tumor necrosis factor (TNF)-α, have been reported to be enhanced in CKD [2, 29]. According to Chronic

Renal Insufficiency Cohort (CRIC) study, the inflammatory biomarkers IL-1β, IL-1 receptor antagonist, IL-6, TNF-α, CRP and fibrinogen, are correlated negatively with markers of kidney function, and positively with albuminuria [30]. A cytokine and a T cell imbalance have been also reported in ESRD [31]. CRP measurement was reported as a good predictor of mortality in HD patients [32], while IL-6 was considered a predictor of all-cause and CVD mortality [33, 34]. In a recent study by our team we found that CRP was an independent risk factor for mortality in HD patients [3].

There are other factors that may contribute to the persistence of inflammation in CKD patients, besides the pro-inflammatory factors released along the inflammatory response. The impairment in immune response, involving neutrophils and T cells, favors the risk of infection [35]. In HD patients, infections, such as catheter-related bloodstream infections and access site infections, as well as thrombotic events, are common and enhance inflammation [36]. An increase in pro-inflammatory cytokines alongside with a reduction in their clearance also favors the pro-inflammatory state. Inadequate antioxidant defenses to face the enhanced production of reactive oxygen species (ROS) may favor the inflammatory milieu. Retention of uremic solutes, such as guanidines, interferes with monocyte/macrophage inflammatory activity, which may favor CVD and infection [37]. Obesity increases the risk for kidney disease in the general population [38] and is associated with an altered production of adipokines and a low-grade inflammatory state. For instance, hyperleptinemia has been associated with several CVD risk factors, namely, inflammation, insulin resistance, protein energy wasting and with progression of CKD [39]. Adiponectin, an anti-inflammatory adipokine that is usually reduced in obesity, is increased in CKD patients, probably due to the development of adiponectin resistance, and has been associated with increased mortality risk [40]. In HD patients the overproduction of pro-inflammatory cytokines, the enhancement in phagocyte oxidative burst, activation of NADPH oxidase and the removal of antioxidants by the dialysis procedure [41], produce an additional inflammatory stimuli.

Malnutrition and protein-energy wasting, common in CKD, may also contribute to the inflammatory condition of CKD patients [42]. Mineral and bone disorders, comorbidities associated with CKD, are also linked to the inflammatory process [42].

The close relationship between inflammation and anemia, a common complication of CKD, is well known. Anemia mainly results from a reduced production of erythropoietin (EPO) by the failing kidneys. The increase of the inflammatory cytokine IL-6 in CKD patients leads to an increase in the production of hepcidin that is able to induce the development of a functional iron deficiency. Hepcidin inhibits iron absorption by the enterocytes, and the mobilization of iron stores, from the macrophages of the reticuloendothelial system, compromising iron availability for erythropoiesis. The increase of hepcidin often leads to a functional iron deficiency in CKD patients. Iron deficiency, either absolute or functional, can contribute to the development or worsening of anemia in CKD patients [3, 43]. Inflammation is enhanced in patients who develop resistance to recombinant human EPO (rhEPO) therapy; however, the mechanisms responsible for the development of the hyporesponse to rhEPO are not fully understood [5, 43].

Inflammation is also common to other inflammatory conditions, such as aging, obesity, diabetes *mellitus* and CVD. Thus, the coexistence of these diseases with CKD may further enhance inflammation, contributing and/or aggravating the inflammatory-associated complications, namely the risk for CV events [44]. Indeed, several pro-inflammatory cytokines that are enhanced in CKD present proatherogenic properties, such as up-regulation of adhesion molecules, enhancement of endothelial dysfunction, promotion of vascular calcification and insulin resistance, and oxidative stress generation [31].

3. Inflammation and DNA damage

More recently, inflammation and inflammatory conditions, including CKD, have been associated to DNA damage. The positive correlation between the levels of DNA damage and the mortality risk in CKD patients suggests that genomic damage can be valuable for prognosis in these patients [8].

The chronic inflammatory state in CKD patients favor genomic damage, which may be induced by inflammatory products and mediators, as well as by external environmental factors, as those associated to the HD procedure [45]. Unrepaired or incorrectly repaired nuclear or mitochondrial DNA damage leads to cell cycle arrest and apoptosis or to mutations. Mutations include intra- or interstrand cross-links, cross-links between DNA bases and proteins, single-strand breaks (SSB), double-strand breaks (DSB) and oxidized DNA bases. DNA repair capacity is essential to correct DNA damage, reduce the genomic damage and, therefore, to reduce cancer risk that appears to be higher in CKD [4].

The genomic damage can be detected by sensitive biomarkers, like unscheduled DNA synthesis (UDS), sister-chromatid exchange (SCE), mitotic index, telomere length, mitochondrial DNA, micronucleus (MN) assay, comet assay fluorescence *in situ* hybridization (FISH) with DNA or with protein (Immuno-FISH), comparative genomic hybridization (CGH); array-comparative genomic hybridization (array-CGH), spectral karyotyping (SKY), G-banding and flow cytometry [4, 8, 46–48]. These approaches can be used for the identification of genomic lesions, susceptibility to environmental genotoxins and inadequate DNA repair in CKD and HD patients [46].

3.1. Comet assay

The comet assay or single cell gel electrophoresis (SCGE), introduced in 1984, is a sensitive and simple technique for detecting DNA damage at the level of a single cell, under neutral or alkaline conditions; this test can be complemented with the use of repair enzymes. This assay is useful for measuring SSB, DSB and alkaline labile sites (ALS) in cells and is dependent on the ability of breaks to relax DNA supercoiling linked to the nuclear matrix [49–51]. Concisely, the comet assay requires a suspension of cells embedded in low melting agarose, cellular lyses (to remove plasmatic membranes, cytosol, nucleoplasm and proteins), DNA denaturation (release of histones from DNA), and electrophoresis at neutral or alkaline

conditions, where DNA moves to the anode, in a way that is dependent on the number of lesions in the nucleoid, forming a comet. The neutral method (pH = 8.4) only detects DSB, while the alkaline method (pH > 13), with higher sensitivity, identifies both SSB and DSB [51]. For this procedure, it is important to optimize agarose concentration (0.6–0.8%), alkaline unwinding time (40 minutes) and electrophoresis conditions (time, voltage and current, usually 1.15 V/cm), to achieve reliable data on the degree of DNA damage [52]. After electrophoresis, samples are neutralized, stained with a DNA-binding fluorescence dye and analyzed by fluorescence microscopy [49–51, 53, 54]. The comet is composed by a head that contains the undamaged DNA of the nucleus, and by a comet tail, which includes SSB and DSB [49, 50]. The number of DNA breaks is shown by the intensity and length of the tail to the head of the comet [49]. The percentage of DNA in the tail (%T), the tail length and tail moment, provided by an adequate software, measures the DNA damage. The tail moment represents the product of %T and tail length [55, 56].

The scoring systems for the comet assay can use a computer-based image system (semi-automated or automated) coupled to a microscope, and the results are expressed in arbitrary units (AU). Using this visual scoring system, a total of 100 comets per 2 replicate gels are observed, and each comet is assigned to 1 of 5 classes, according to the tail and head intensity. In class 0, there is no DNA in the tail (undamaged DNA); and from class 1 to class 4 (severe damage), the increase of DNA in the tail is proportional to DNA damage. The average extension of DNA migration is calculated by assigning numerical values to each migration class. The comet scoring into classes should be randomly performed in the gel, avoiding edges and areas/cells close to bubbles or artifacts of the gel; ideally, the same operator should perform all scorings. For each sample, the score is calculated applying the following formula: (percentage of cells in class 0 × 0) + (percentage of cells in class 1 × 1) + (percentage of cells in class 2 × 2) + (percentage of cells in class 3 × 3) + (percentage of cells in class 4 × 4) [57, 58]. Afterwards, DNA damage is calculated in arbitrary units (AU) using the formula:

$$AU = \frac{[((0 \times N0) + (1 \times N1) + (2 \times N2) + (3 \times N3) + (4 \times N4)) \times 100}{\text{number of analyzed comets}]} \quad (1)$$

where N0, N1, N2, N3 and N4 are the numbers of comets in classes 0, 1, 2, 3 and 4, respectively. The values of DNA damage reported in AU may be transformed into estimated percentage of DNA in the tail (E%T), using: E%T = (AU/5) + 10, that converts the visual score to a pseudo-percentage score, ps (ps = vs/5 + 10) in a scale range limited to 10–90% [59]; or the conversion curve E%T = (AU − 25.87)/4.46 [60].

More recently, several methodological modifications of the comet assay were developed to detect and quantify DNA damage. The OpenComet, an automated software tool, allows the quantitative measurement of SSB, DSB, ALS and DNA crosslinks with high accuracy and reproducibility, with the advantage of a shorter analysis time [61]. The CometQ is an innovative, fully automated tool to analyze the images of comet assay with high accuracy, sensitivity and good predictive positive value [62]. The high throughput comet (HT-COMET) assay provides

accuracy, efficiency and gives DNA damage profile that allows the determination of the proportion of highly damaged cells [63]. The Comet-FISH measures the percentage of DNA lesions or DNA modifications in the comet tail, which can be enzymatically or chemically converted into strand breaks, providing a way to study the molecular mechanisms of different repair pathways and the screening of drugs, as potential specific inhibitors for repair pathways [64]. Comparing with the MN assay, the comet assay allows the study of non-proliferating cells and does not need to use cell cultures [4]. However, the assay of MN, also known as Howell-Jolly bodies, is recognized as robust, sensitive, fast and reliable method, which analyses cytogenetic damage, namely, chromosomal breaks (clastogenesis), disruptions of mitotic apparatus with chromosomal losses (aneugenesis) and amplifications [51, 53].

3.2. Cell-free DNA

Human plasma contains cell free nucleic acids, including genomic DNA, mitochondrial DNA, mRNAs and miRNAS, all with different functions [65]. DNA is released following cell damage and is raised in several clinical conditions, such as diabetes, trauma, cancer, systemic lupus erythematosus, age-associated inflammation and inflammation-associated diseases [65, 66].

In diabetes *mellitus*, one of the most common causes of CKD, cfDNA levels were reported to be increased, both in patients with and without microvascular complications, though higher in those with microvascular disturbances [67]. It was hypothesized that in diabetes, the reactive oxygen and nitrogen species cause DNA strand-breakage, which may activate the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1) [68, 69]. The activation of PARP induces depletion of DNA, reducing glycolysis, electron transport and ATP formation; moreover, it inhibits the synthesis of glyceraldehyde 3-phophate by poly-ADP-ribosylation dehydrogenase. All these mechanisms seem to lead to acute endothelial dysfunction, favoring the development of diabetic complications [67].

As referred, inflammation is a hallmark of CKD and is particularly enhanced in ESRD patients under HD. The underlying inflammatory process might contribute to increase DNA damage [66]. In ESRD patients on HD, the cellular necrosis and apoptosis occurring along the HD process [70], the enhanced production of ROS and toxins, such as advanced glycation end products derived from oxidative peroxidation [71, 72], may contribute to a higher rise in cfDNA levels. Modifications in DNA repair mechanisms may also favor the increase of DNA damage [8]. Epigenetic variations, including DNA methylation patterns, histone modifications, chromatin remodeling, microRNAs and long non-coding RNAs, can change the flow of gene expression, acting as genotoxic modifiers by promoting DNA damage and chromosome abnormalities [51].

The traditional method for DNA quantification is the ultraviolet absorbance spectroscopy assay, which is not applicable to biological samples. In this case, after DNA extraction from the biological fluid, cfDNA can be quantified, using specific dyes, by colorimetry or emission fluorometry; however, these methods are complex and expensive. Goldshtein et al. [73] developed a simple, inexpensive and accurate test for cfDNA evaluation that does not require prior

processing of samples. Briefly, SYBR® Gold stain is diluted in dimethyl sulfoxide (1:1000 dilution) and phosphate buffer (1:8 dilution); the biological fluid (serum, whole blood, urine or supernatant of cell cultures) is mixed with SYBR® Gold solution (final stain dilution: 1:10,000) and cfDNA fluorescence is measured with a fluorimeter (emission wavelength 535 nm, excitation wavelength of 488 nm). Czeiger et al. applied this method to a study using an animal model and patients with colorectal cancer; and found that mice inoculated with patient's cancer cells, presented a positive correlation between cfDNA and tumor size [74]; comparing cfDNA levels between controls and preoperative patients, cfDNA levels were higher in patients; 1 year after, the levels of cfDNA were higher in patients who remained with the disease or died, as compared with those without disease; in accordance, the authors proposed that in colorectal cancer patients the levels of cfDNA had a prognostic value, for death and for the outcome of the disease [74].

4. DNA damage in ESRD patients

Our team has been interested in studying DNA damage and its correlation with the enhanced inflammatory state observed in different inflammatory conditions, as in ESRD under HD treatment and in psoriasis *vulgaris*; in both these clinical conditions we found that cfDNA levels were increased and correlated with inflammatory markers, as IL-6 and CRP in ESRD [66]; and, in psoriasis, cfDNA levels were correlated positively with IL-6, suggesting a linkage with psoriasis severity [75].

In a more recent work, we analyzed the degree of genomic damage in ESRD patients under HD therapy for more than 1 year, using two different approaches, the alkaline *in vitro* comet assay and the cfDNA quantification (according to Goldshtein et al. method [73]), in order to evaluate DNA damage within the cell and the circulating free DNA, respectively. We studied 39 ESRD patients (24 males and 15 females with a median age of 68, [58–77] interquartile ranges) that were under HD therapeutic, 2–3 times per week, 3–5 hours each HD session, for a median time of 67, [40–94] months; high-flux polysulfone FX-class dialyser of Frenesius (Bad Hamburg, Germany) was used for the HD procedure. The main causes of renal failure were diabetic nephropathy (n = 12), hypertensive nephrosclerosis (n = 11), pyelonephritis (n = 5), IgA nephropathy (n = 4), polycystic kidney disease (n = 3), other diseases (n = 2) and of uncertain etiology (n = 2). Besides rhEPO therapy, patients were under iron and folate prophylactic therapies, in accordance to the recommendations of "KDIGO Clinical Practice Guideline for Anemia in Chronic Kidney Disease" [76], to avoid nutrient erythropoietic deficiencies. A group of 15 healthy volunteers, 2 males and 13 females, with normal hematological and biochemical values, without history of renal or inflammatory diseases, was also studied. This control group was matched as far as possible for age, once the age of HD patients is usually high. ESRD patients and controls were matched for body mass index, but not for gender (**Table 2**).

We found that ESRD patients presented significantly lower values of erythrocytes, hemoglobin concentration and hematocrit; the erythrocytes were less hemoglobinized, as showed by the significantly lower value of mean cell hemoglobin concentration; however, iron stores were increased, as ferritin was significantly increased (about sixfold the control

	Controls (*n* = 15)	**ERSD patients (*n* = 39)**	***P*-value**
Sociodemographic data			
Age (years)	52 [40–55]	68 [58–77]	0.001
Gender [(M/F); *n* (%)]	2 (13%)/13 (87%)	24 (62%)/15 (38%)	0.002
BMI (kg/m^2)	22.9 [20.6–27.2]	25.2 [21.5–27.8]	0.329
Hematologic data			
RBC (×10^{12}/l)	4.60 [4.20–5.00]	3.70 [3.50–3.90]	<0.001
Ht (%)	40.3 ± 4.7	35.8 ± 4.1	0.001
Hb (g/dl)	13.6 ± 1.5	11.8 ± 1.5	<0.001
MCV (fl)	89.0 [84.0–94.0]	96.9 [95.2–99.2]	<0.001
MCH (pg)	30.7 [28.1–31.6]	31.9 [30.8–32.6]	0.006
MCHC (g/dl)	33.7 ± 1.1	32.8 ± 1.1	0.008
WBC (×10^9/l)	7.3 [5.4–8.1]	5.8 [5.1–7.7]	0.164
Biochemical data			
Iron (μg/dl)	69.5 [64.5–110.8]	65.0 [56.0–87.0]	0.299
Ferritin (μg/dl)	68 [15–137]	461 [351–680]	<0.001
Transferrin (mg/dl)	307 [238–338]	173 [158–194]	<0.001
Transferrin saturation (%)	20.5 [15.9–26.7]	27.8 [22.2–42.5]	0.008
CRP (mg/l)	0.7 [0.6–0.4]	2.9 [1.7–12.5]	0.017
Cell-free DNA (ng/ml)	116 [90–267]	371 [217–563]	0.002*

BMI, body mass index; CRP, C-reactive protein; F, female; Ht, hematocrit; Hb, hemoglobin; M, male; WBC, white blood cell; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RBC, red blood cell. $P < 0.05$ was accepted as statistically significant.

Results are presented as mean ± standard deviation or as median [interquartile range]; differences between groups were tested using chi-squared test and Fisher's exact test for categorical variables; for continuous variables, the unpaired Student's t-test or the Mann-Whitney U test were used, according to the distribution of the variable.

*Loss of significance after statistical adjustment for age (analysis of covariance (ANCOVA)).

Table 2. Sociodemographic data, hematologic, biochemical, and cell-free DNA values in end-stage renal disease (ERSD) patients and controls.

value). These findings suggest a functional iron deficiency that seems to be linked to the high inflammatory state observed in ERSD patients, with significantly higher CRP values (**Table 2**). Considering that inflammation regulates iron absorption and iron availability for hemoglobin synthesis, the enhanced inflammatory state in ESRD patients contributes to worsening of anemia and to the reduction in erythrocyte hemoglobinization.

We used the comet assay to evaluate DNA structural damage in blood cells from controls and ESRD patients on HD. The distribution of comets was obtained by visual scoring into five classes (**Figure 1**), based on the length of migration and/or in the relative proportion of DNA in the head and in the tail [57, 58].

Figure 1. Comet images of lymphocytes from end-stage renal disease (ERSD) patients showing different migration patterns, according to the levels of DNA damage, from class 0 (undamaged) to class 4 (severe damage).

DNA damage presented as AU, for controls and patients, are displayed in **Figure 2**. We found that DNA damage (AU) was significantly higher in ESRD patients (71 [36–127]), when compared to controls (34 [0–61]). A significant increase was also observed for %T in ESRD patients, when compared to controls. We found a %T of 24.2 [17.2–35.4] and 16.8 [10.0–22.2] (expressed in pseudo percentage), and 10.12 [2.27–22.71] and 1.82 [−5.80–7.88] (using the conversion curve) for patients and controls, respectively (**Figure 3**). The conversion curve provides a better fitting between %T and AU [55, 60] and showed negative values of %T for AU below 26, indicating that %T was zero; above 400 AU, the %T was 84%, in accordance with others [55, 60]. Our data is in accordance with other studies reporting that the levels of DNA breaks and oxidative DNA lesions, measured by the comet assay, are higher in dialysis patients then in controls [77].

We also found that %T was negatively correlated (Spearman's rank correlation) with CRP ($r = -0.368$; $P = 0.021$) and ferritin ($r = -0.404$; $P = 0.011$), in ESRD patients; no significant correlations were found between DNA lesions and the rhEPO dose used to treat anemia ($r = 0.171$; $P = 0.306$), or the time of HD treatment ($r = -0.186$; $P = 0.256$). In a cross-sectional study, the oxidative DNA lesions found in dialysis patients were inversely correlated with the duration of the dialysis sessions [77, 78].

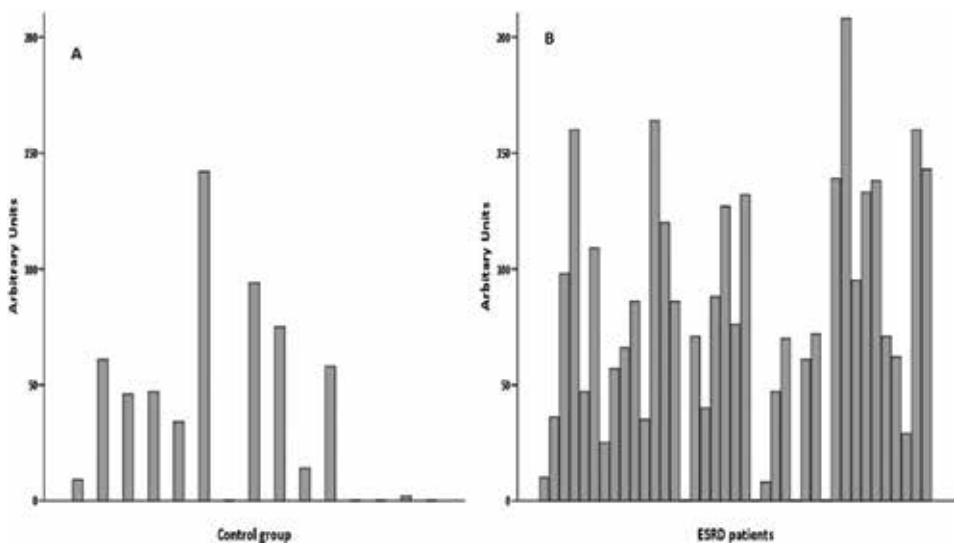

Figure 2. DNA damage, presented in arbitrary units, for each of the 15 healthy controls (A) and for the 39 ESRD patients (B).

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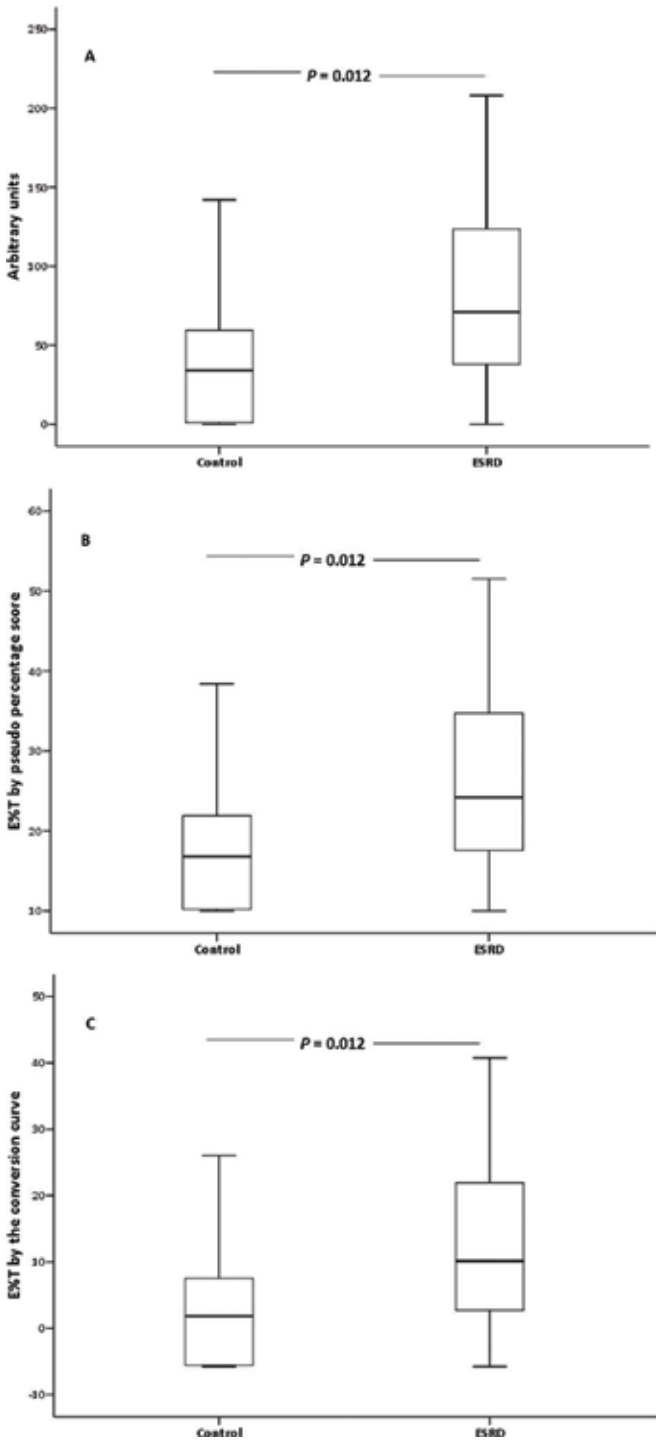

Figure 3. (A) Mean values in arbitrary units and the estimated percentage of DNA in the tail (E%T) calculated using two equations: (B) the pseudo percentage score (ps = vs/5 + 10) and (C) the conversion curve = (AU − 25.87)/4.46, in controls and end-stage renal disease (ESRD) patients (differences between groups were tested using Mann-Whitney U test).

We did not find significant differences in DNA damage (comet tail length or tail intensity) for diabetics and nondiabetic ESRD patients, as reported by Ersson et al. [77]; however, our findings are in accordance with Mamur et al., reporting no difference in comet tail length or tail intensity between diabetic and non-diabetic ESRD patients on HD [78].

Our data suggest that long-term dialysis treatment or diabetes *mellitus* do not affect DNA damage, however there are still few studies and controversial data. Ersson et al. reported lower levels of DNA damage in salivary gland tissues of ESRD patients, as compared to controls, suggesting that ESRD might affect DNA in different ways, in peripheral tissues and in blood mononuclear cells [77].

Concerning cell-free DNA, we found that ESRD patients had a significantly higher value, compared to control; however, after statistical adjustment for age, the significance was lost (**Table 2**). Cell-free DNA correlated (Spearman's rank correlation) significantly and positively with age in both groups ($r = 0.342$, $P = 0.033$; $r = 0.589$, $P = 0.021$; in patients and controls, respectively), and with CRP in ESRD patients ($r = 0.483$; $P = 0.002$). Our results are in accordance with others reporting increased levels of cfDNA in hemodialyzed patients [79].

To study the predictive risk of mortality associated with DNA damage, we recorded the number of deaths that occurred along 1 year after the analytical study of the 39 ESRD patients; 9 out of the 39 ESRD patients died. We compared the analytical data from ESRD patients who were alive and from the patients who died in the 1 year follow-up period (the Mann-Whitney U test was used). The latter patients presented significantly higher ($P = 0.006$) cfDNA values (713 [415–809] ng/ml) than those who were still alive (337 [192–484] ng/ml). A trend towards ($P = 0.149$) higher CRP levels (7.5 [1.6–45.7] mg/l) in those who died, compared to those who were still alive (2.7 [1.7–9.3] mg/l), was also found.

The differences in DNA damage, observed between controls and ERSD patients, could be higher, if we were able to gather a gender matched population. It is known that DNA lesions are higher in women, both in healthy [43, 80–82] and in pathological conditions [83].

Divergent results have been reported for the levels of DNA damage and the time of dialysis treatment. Some studies showed a reduction of DNA damage on long-term maintenance HD [84, 85], while others showed an increase [8, 86, 87]. Recently, it was reported that online hemodiafiltration (OL-HDF) reduced the levels of DNA damage in these patients, as this approach provides a reduction of inflammation and oxidative stress [10]. In fact, a reduction of binucleated cells with micronuclei in patients that changed from low-flux HD to post-dilution OL-HDF, as well as an increase in plasma antioxidant capacity, were shown [88]. Both single high-flux HD and OL-HDF remove circulating mitochondrial DNA, a pro-inflammatory agent, which has been correlated with the chronic inflammatory grade of hemodialyzed patients [89]. Moreover, OL-HDF procedure has been associated with lower levels of the inflammatory markers, IL-6 and CRP, and with an improvement on endothelial (dys)function, in ESRD patients [90, 91]. Aberrant DNA hypermethylation has been also observed in dialysis patients and associated with the inflammatory state and with the

dialysis technique; patients under OL-HDF showed lower DNA methylation patterns than patients under HD, although higher than controls, suggesting a reduction in DNA hypermethylation, with decreasing inflammation [92].

Dietary supplementation with folic acid [87, 93], vitamins A, B and B12 [93], zinc [94] and selenium [87] may also contribute to reduce/avoid genomic damage, once nutritional supplementation has antioxidant effects, prevents cancer, increases DNA repair capacity, and improves CV and all-cause mortality rates [87].

The inverse correlation that we observed between %T and CRP in ESRD patients, suggests that as CRP (inflammation) levels increase, the damage in DNA also increases; however, it seems that for lower CRP values the damaged DNA is still within the cell, while at higher CRP values the increasing damaged DNA is released into plasma.

The increase of cfDNA in ESRD patients was also reported by others [5, 65, 66, 70, 79]. The slightly lower cfDNA values found in our study, compared with those found by others in HD patients [5], may be related with time of sample collection, as the levels of cfDNA increase during and after HD, returning to pre-HD levels half an hour post-HD [95].

We should notice that our study has some limitations, namely, the small sample size, the lack of age and gender matched controls. Thus, further studies in larger populations are needed to strengthen the value of cfDNA as a biomarker of inflammation and poor outcome in ESRD patients. A recent study showed that circulating free DNA, by favoring calcium phosphate precipitation and crystallization, may be involved in arterial calcification [96], a common feature in ESRD patients under HD. Thus, cfDNA, appears to be a biomarker for CVD risk, and a direct contributor for CV events, the main cause of death in ERSD patients.

5. Conclusions remarks

ESRD is characterized by a low-grade chronic inflammatory state, which favors the development of comorbidities. Genetic damage has been reported in ESRD patients, especially in those under HD. The higher degree of genomic damage in ESRD patients might be a consequence of inflammation and aging, and may contribute to increase the risk for cancer and cardiovascular mortality. Several associations with DNA damage (evaluated by cfDNA and comet assay) have been reported and support this hypothesis; however, data is limited and controversial (**Table 3**).

Our studies showed that cell damaged DNA is increased in ESRD patients, and suggest that at lower CRP values the damaged DNA remains within the cell, while at higher CRP values damaged DNA is released into plasma and may contribute to further enhance inflammation in ESRD patients and increase mortality risk. Actually, we found that ESRD patients who died within the one year follow-up period of the study, presented higher circulating damaged

Comet assay		
Positive association	**Negative association**	**No association**
Male gender [9]	CRP*	Gender [78]
Diabetes [9]	Ferritin*	Diabetes [78]*
Mortality [8, 97]	Dialysis sessions duration [77]	Duration of HD [78]*
Frequency of micronuclei [98]		Ferritin [78]
BMI > 25 kg/m^2 [78]		Age [78]
Intact PTH > 300 pg/ml [78]		Hb [78]
Leptin [99]		Hypertension [78]
Treatment modality [9]		rhEPO dose*

cfDNA	
Positive association	**No association**
Age*	TNF-α [70]
CRP [66]*	IL-10 [70]
IL-6 [66, 70]	Dialysis duration [100]
All-cause mortality (post-dialysis) [5]	WBC count (before HD) [100]
Last 3-month mean: SBP, WBC, serum albumin, Cr, normalized protein catabolic rate [101]	Length of the HD session [95]
In HD diabetic patients: SBP, Hb A1c, and serum albumin [101]	

BMI, body mass index; Cr, creatinine; CRP, C-reactive protein; Hb, hemoglobin; IL, interleukin; PTH, parathyroid hormone; rhEPO, recombinant human erythropoietin; SBP, systolic blood pressure; TNF, tumor necrosis factor; WBC, white blood cell.

*According to our data.

Table 3. Associations reported for comet assay and cell-free (cf) DNA on hemodialysis (HD) patients [96–101].

DNA and inflammation. Moreover, our data suggest that the comet assay is more sensitive for low grade inflammatory conditions, while cfDNA appears as a good biomarker for more severe inflammatory conditions, as well as a biomarker for the outcome of ESRD patients. In summary, the genomic damage in ESRD patients seems to result, at least in part, from inflammation and aging, and may contribute to increase the risk for cancer and CV mortality.

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Chapter 3

Genotoxicity Induced by Cypermethrin in the Zebrafish Retina

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Additional information is available at the end of the chapter

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Abstract

Cypermethrin (Cyp), is one of the most common contaminants in freshwater aquatic systems. We evaluated its possible genotoxic effect and oxidative stress in retinal cells of adult zebrafish exposed to 0.3 μg/L and 0.6 μg/L Cyp. Both the histological and immunofluorescence (IF) techniques showed the presence of apoptotic cells in the zebrafish retina after 9 days of treatment with 0.6 μg/L Cyp. Thus, histone γ-H2AX, a double-stranded DNA damage marker, was immunodetected in both the outer and inner nuclear layer after exposure to 0.6 μg/L Cyp for 12 days, while the anti-caspase-3 apoptotic antibody was detected in the outer nuclear layer. Compared with the morphological evidence, the damage index (DI) showed significant differences with 0.3 μg/L from day 9, while with 0.6 μg/L all the stages evaluated showed very significant differences. According to these results, it was verified that the activities of superoxide dismutase (SOD) and catalase (CAT) increased significantly after exposure to 0.6 μg/L Cyp. The same treatment caused a significant positive regulation of the mRNA levels of both genes. These results indicate that Cyp causes DNA damage and oxidative stress. This pyrethroid also has the potential to induce apoptosis in the cells of the retina.

Keywords: genotoxicity, cypermethrin, zebrafish, retina

1. Introduction

Pyrethroid insecticides are potent neurotoxicants for various insect and vertebrate groups. Pyrethroids exhibit a typical toxic action pattern of a strongly excitatory effect on the nervous system [1]. The main target sites for pyrethroids are the voltage-dependent sodium

channels of neuronal membranes [2–4]. Based on different behavioral, neurophysiological, and biochemical profiles, two classes of pyrethroids have been identified. The type I are associated with hyperexcitation and weak tremors, while type II, who have a cyano group, are associated with a more complex syndrome, including clonic seizures [1, 5]. Type II pyrethroids are extensively applied to control pests in residential and agricultural environments [6].

Cypermethrin, ((RS)-α-cyano-3-phenoxybenzyl (1RS) cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate), is a type II synthetic pyrethroid insecticide that has high insecticidal activity, relatively low toxicity in birds and mammals, and adequate air and light stability [7, 8]. This compound has a wide range of uses in numerous crops such as cotton, cereals, vegetables, and fruit trees, where it controls many insects including lepidopterans, coleopterans, and hemipterans. It is also used in food storage sites, to control flies and fleas in domestic farms and extensively for the control of cockroaches, mosquitoes, and lice [9].

It presents a moderate persistence in the environment and its residues are not usually accumulated in a significant way. In soil, it has a half-life between 4 and 56 days, being more persistent in anaerobic environments. Under aerobic conditions, it is moderately sensitive to light and undergoes microbial degradation. In water, it is stable in neutral or acidic conditions, with a half-life that exceeds 50 days and a photolysis resistance greater than 100 days. In surface water bodies, the Cyp concentration decreases rapidly by adsorption to the sediment, suspended particles, and plants.

Despite the beneficial roles, Cyp enters the brain, accumulates in a significant amount, and exerts neurotoxicity on non-target organisms [10]. In rodents, Cyp induces nigrostriatal dopaminergic neurotoxicity and, if co-administered, increases the neurodegenerative potential of other toxic chemical substances [11, 12]. Pyrethroids also induce neurotoxic chronic effects in humans [13]. In aquatic organisms, previous studies by our group allowed to determine the effect of Cyp on the tadpole's brain of two South American anurans species [14–16]. In these studies, developed under laboratory conditions using sublethal and acute Cyp doses, both their survival rate and altered brain morphology were analyzed. In the studies using prometamorphic *Bufo arenarum* larvae, high mortality rates (~65–70%) were observed in animals exposed to 39 and 156 μg/L Cyp per 96 h [16]. In this species, the LC_{50} at 96 h was 110 μg/L, so the sensitivity of this species coincides with that previously reported for *Physalaemus biligonigerus* [14]. At the histological and ultrastructural levels, the most affected telencephalon regions were marginal and intermediate layers, surrounding the brain ventricles. TUNEL and DNA fragmentation assays confirmed the time and dose-dependent increase in the number of apoptotic telencephalic cells, as well as the DNA damage of the immature brain cells of *B. arenarum* tadpoles. However, the CNS of vertebrates is immensely complex and we were not able to determine the possible apoptotic mechanisms triggered by Cyp. For this reason, many studies focused to understand the processes triggered by drugs and neurotoxicant substances that can act on the CNS are conducted by studying a relatively simple and

accessible region of the CNS, such as the retina. In the present study, we investigate, in an *in vivo* model such as adult zebrafish, the possible link between oxidative stress and apoptosis in the relatively simple neuronal system of zebrafish retina.

Zebrafish is one of the most widely used vertebrates as a model organism for genetic studies [17, 18] and environmental toxicology [19–22]. In addition, it has been used in studies of ecological monitoring of the environment and during evaluations of multiple pollutants such as organic, endocrine disruptors, and heavy metals [23–25].

Environment pollution, especially aquatic, is a serious problem all over the world. Not only does it affect the survival and reproduction of aquatic organisms but it has a negative impact on human health, fundamentally via bioconcentration processes. Sensitivity to different pollutants makes zebrafish an ideal model organism for environmental monitoring. Characteristic changes in morphology, gene expression, behavior, and physiology can be observed as biological indicators. The International Organization for Standardization first published the zebrafish toxicity test in 1984. Thereafter, several countries promulgated their own toxicity testing standards through their use. Many environmental pollutants, including pyrethroids, interfere with the functions of the endocrine system, affect development, produce DNA damage, and induce oxidative stress in zebrafish [26–29].

This *in vivo* study was performed to characterize Cyp dose- and time-dependent toxicity using histology, immunofluorescence (IF) and comet assay (CA) studies, superoxide dismutase (SOD) and catalase (CAT) enzyme activity as well as *sod* and *cat* gene expression (**Figure 1**).

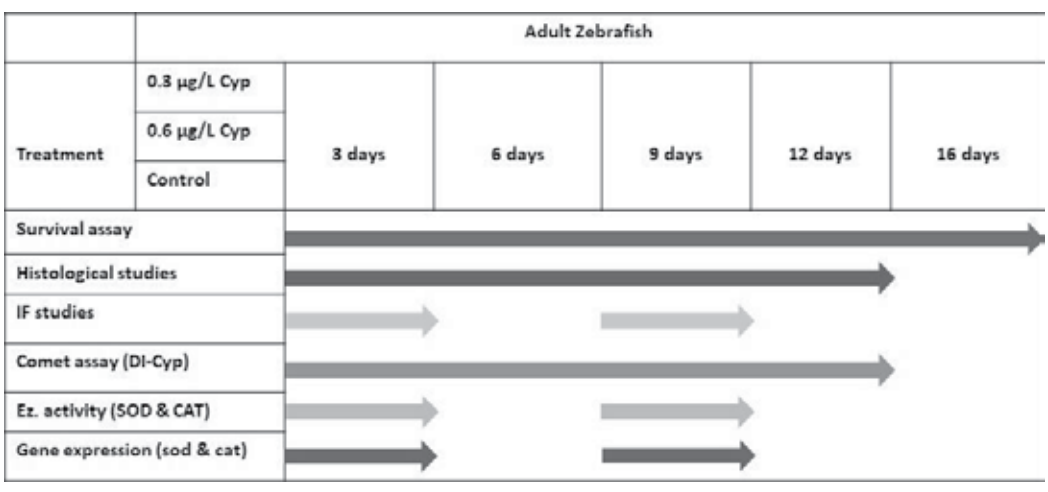

Figure 1. Summary of experimental procedures and techniques used during this work.

2. *In vivo* cypermethrin bioassay

Based on reported concentration of Cyp in agroecological aquatic systems [27, 30], adult fish kept in aquarium according to [31] were treated with 0.3 and 0.6 μg/L Cyp solutions. Each treatment was carried out by triplicate, with their respective control, for 3, 6, 9, 12, and 16 days, with daily changes of the solution. All fish exposed to 0.3 μg/L Cyp survived for the first 12 days, while the survival rate was 80% at the end of the bioassay. Regarding the fish group exposed to 0.6 μg/L Cyp, the survival rate at the 12 days of exposure was 100%; from day 13, the percentage was 80%, decreasing to 50% on day 15 to finally reach 30% at the end of the bioassay. Based on the survival rate with both Cyp concentrations, it was decided to perform all the remaining *in vivo* studies with both concentrations, using 12 days as maximum exposure time.

3. Histological and immunofluorescence analysis

Eyes of control and *in vivo* exposed animals were processed according to Casco et al. [16]. The cured blocks were cross-sectioned at 0.5 μm of thickness using an ultramicrotome. Semithin sections were toluidine blue stained and recorded by the charge-coupled device (CCD) camera, coupled to an Olympus BX50 microscope at 40 and 100×. In the histological study, just after 9 days of treatment, zebrafish exposed to 0.6 μg/L Cyp show significant changes in retinal cell layers. Apoptotic-like cells could be viewed mostly in the photoreceptor cell layer and, to a lesser extent, in the inner nuclear layer (**Figure 2**).

Unlike what was reported for the amphibian larvae brain [14–16], in the present work the major changes in the retinal cell layers were triggered with Cyp concentrations much lower than those found in surface water or runoff from agricultural practices in Argentina [32] and during relatively short exposure periods.

In addition to the possible effect on alterations in nerve impulse transmission by the modification of voltage-dependent sodium channel behavior, more recently the studies by Mun et al. [11] determined that Cyp causes oxidative stress neurotoxicity in rats. These effects are associated with an increased production of reactive oxygen species (ROS). As has been proven in numerous studies, oxidative damage of DNA is an inevitable consequence of cellular metabolism, but toxic insults increase the propensity to raise the levels of ROS. These interact with DNA, leading to serious consequences for cells [33]. Based on this background, it was hypothesized that retinas of zebrafish exposed for 12 days at 0.6 μg/L Cyp undergo a series of alterations that together lead to modifications in the synaptic connections of their neurons and in the formation of apoptotic figures in the inner and outer nuclear layer cells. To corroborate the histological observations about the increase of apoptotic bodies on the fish retinal cells layers submitted for prolonged periods (12 days) to the highest concentration of Cyp (0.6 μg/L), an IF study was carried out using microscopy confocal laser techniques.

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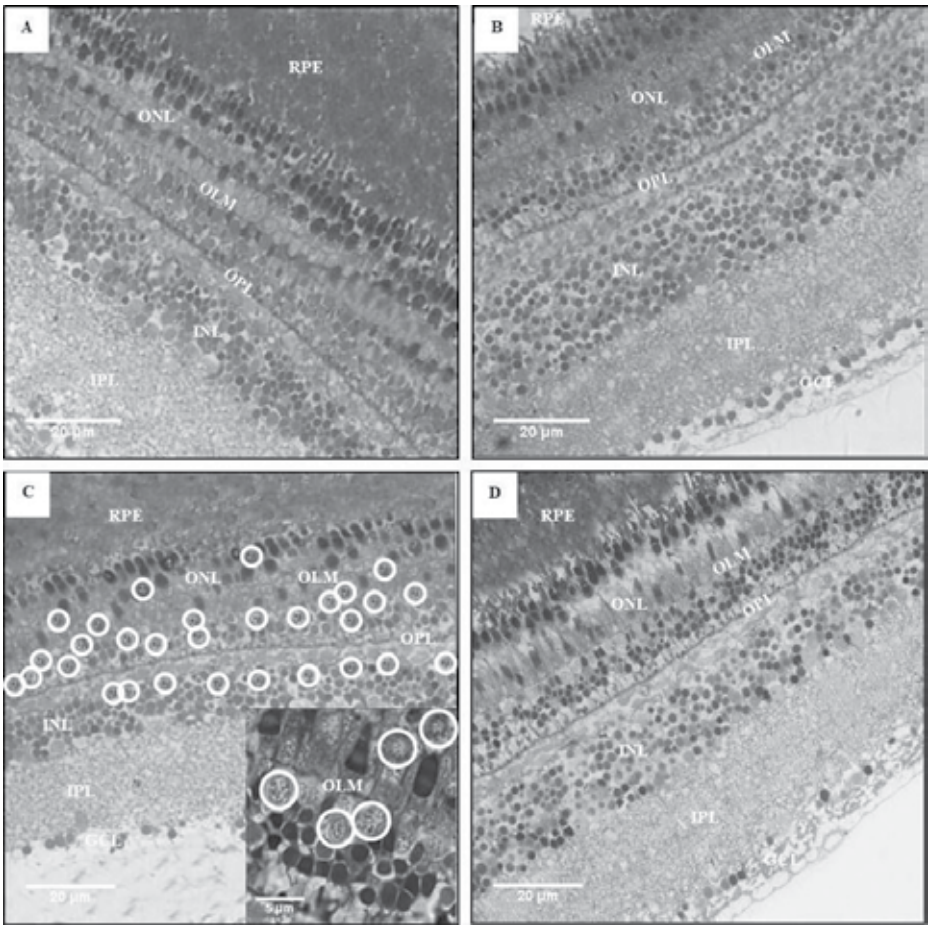

Figure 2. Retinal histological sections of zebrafish exposed to 0.6 µg/L Cyp. No significant morphological changes were observed during the first 9 days of exposure (A) in relation to the control of the zebrafish (B). After 12 days of exposure, the retinas of zebrafish showed apoptotic nuclei of the photoreceptor layers (C), in comparison with the control (D), the white's circle show apoptotic figures. Inset shows more precisely the nuclei morphology. ONL: external nuclear layer; OLM: external limiting membrane; OPL: external plexiform layer; INL: internal nuclear layer; IPL: internal plexiform layer; GCL: Ganglion Cell Layer.

The immunofluorescence (IF) reactions were done by using the anti-γ-H2AX antibody to identify cells with double strand DNA damage [34] and simultaneously the anti-caspase-3 antibody, since caspase-3 has been identified as a key executor and one of the most important downstream players in apoptosis pathways [35]. In this case, adult fish eyes were fixed in 4% paraformaldehyde, optimal cutting temperature (OCT)-embedded and cryo-sectioned at 10-µm thickness. The primary antibodies used were anti-γ-H2AX (1/300) (Abcam, Cambridge, Ab11174) and anti-caspase-3 (Abcam, Cambridge, UK, ab2302) (1/300) and proceeded according to Izaguirre et al. [14]. Photomicrography was performed with a laser confocal system Zeiss, LSM800 (Carl Zeiss AG). Acquisitions of z-stacks were performed with

Fiji software. In this study, coinciding with the histological evidences, after 12 days of exposure, the anti-γ-H2AX immunoreactivity was found on both the outer and the inner nuclear layer cells (**Figure 3A** and **B**), while the caspase-3 immunoreactivity was only detected at the outer nuclear layer (**Figure 4A** and **B**).

These results are consistent with the Rogakou et al. [34] works, who showed that the phosphorylation of histone H2AX is an early response to DNA fragmentation, prior to activation of the protease caspase-3. According to our studies, the rapid activation of γ-H2AX compared

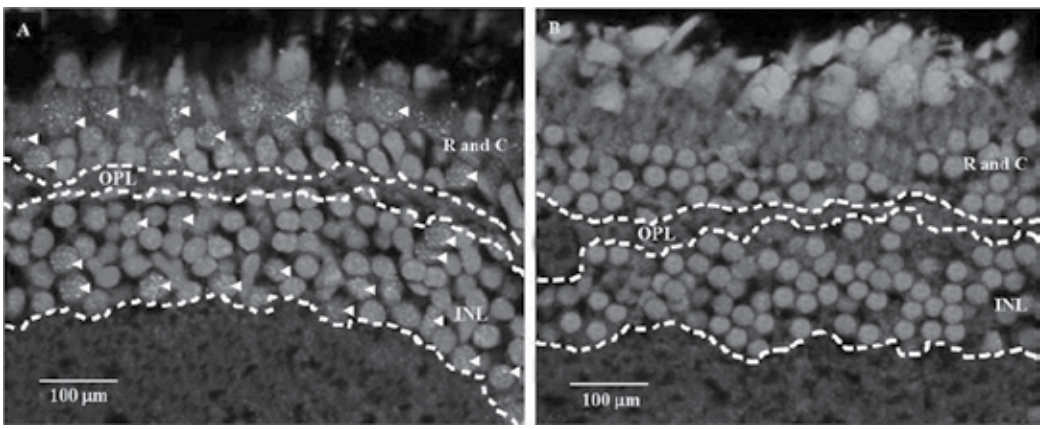

Figure 3. Immunofluorescence labeling of zebrafish retinal cells exposed to 0.6 µg/L Cyp for 12 days using anti γ-H2AX antibody. (A) Retinas of exposed fish showed immunoreactivity at the level of outer nuclear layer and the inner nuclear layer (arrowheads). (B) Retinal cells of the control animals are devoid of immunoreactivity for γ-H2AX. R and C: Rods and Cones; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer.

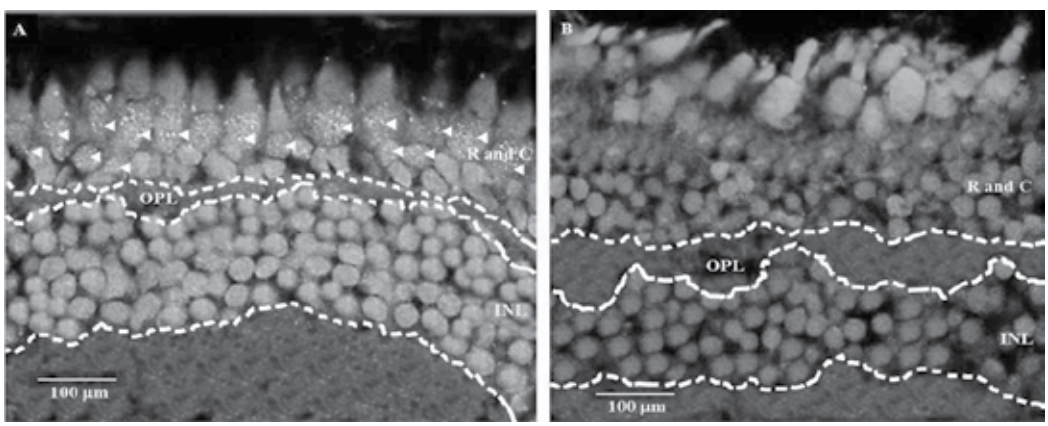

Figure 4. Immunofluorescence labeling of zebrafish retinal cells exposed to 0.6 µg/L Cyp for 12 days using anti caspase-3 antibody. (A) Retinas of exposed fish showed immunoreactivity at the level of outer nuclear layer (arrowhead). (B) Retinal cells of the control animals are devoid of immunoreactivity for caspase-3. R and C: Rods and Cones; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer.

to caspase-3 suggests that the double-stranded DNA breakdown may represent an early event in the apoptotic pathway if the DNA molecule is not repaired [36] and could explain the differential response of the retinal cells found in our experiments.

Similar results were also found in other species. For example, Patel et al. [37] reported that Cyp induced DNA damage in vital mice organs such as the brain, liver, and kidney, of animals intraperitoneally injected with 12.5, 25, 50, 100, and 200 mg/kg body weight of Cyp, for 5 consecutive days. Mukhopadhyay et al. [38] have found similar results, revealing a significant dose-dependent increase in DNA damage in the cells of brain ganglia and anterior midgut of *Drosophila melanogaster* exposed to low concentrations of Cyp (0.0004, 0.0008, 0.002, 0.2, and 0.5 ppm). In fish, Poletta et al. [39] found a significant increase in DNA damage of epithelial gill cells of *P. lineatus* after *in vivo* acute exposure (96 h) to 0.15 and 0.3 μg/L Cyp compared to controls.

Therefore, cells of the zebrafish retina also prove to be sensitive to DNA damage produced by 0.3 and 0.6 μg/L Cyp with similar behavior. In addition, we can see that, from the 9 days of exposure, the increase in DNA damage is very significant compared with the control and with the other exposure groups. Such high level of damage detected indicates that it is very sensitive to pyrethroids for long-term exposure.

In several studies, it was verified that histone γ-H2AX is an indicator sensitive to the breakdown of double-stranded DNA induced by chemical agents [40, 41]. Recently, using combination of IF, flow cytometry, and Western blot, Huang et al. [42] demonstrated that the expression of γ-H2AX in a murine macrophages cell line, exposed for 48 h at different Cyp concentrations, is significantly induced following a dose-dependent model. In these studies, Cyp was found to reduce cell viability and induce apoptotic processes. In the same study, it was shown that Cyp also increases the production of reactive oxygen species (ROS) and causes DNA damage in a dose-dependent manner. On the other hand, arrest in the G1 phase of the cell cycle induced by this pyrethroid is associated with increased expression of wild-type p21 and p53 as well as the down-regulation of cyclins D1 and E as well as protein kinase CDK4. Additionally, these studies demonstrated that Cyp treatment activates MAPK-signaling pathways by inducing the c-Jun (JNK) N-terminal kinases, the phosphorylation of extracellularly regulated protein kinases, ERK 1/2, and increased levels of the cleaved poly ADP-ribose polymerase (PARP). In the same study, pretreatment with the antioxidant N-acetylcysteine (NAC) effectively abolishes both Cyp-induced cell cytotoxicity, such as G1 cell cycle arrest, DNA damage, PARP activity, JNK activation, and ERK 1/2. In addition, specific inhibitors of JNK (SP600125) and ERK 1/2 (PD98059) reverse the phosphorylation of both molecules and attenuate apoptosis. Taken together, these data suggest that Cyp causes immune cell death through the induction of cell cycle arrest and JNK/ERK-mediated ROS-regulated apoptosis.

Of great interest is the recent finding that both in rat retinal ganglion cell enriched cultures and in ischemia/reperfusion mouse visual axis retinal injury models, JNK inhibitors (SP600125 and TAT-JNK-III) both *in vivo* and *in vitro* caused dose-dependent and significant protection in both models [43]. In this study, it was shown that in cell cultures both JNK inhibitors provoke significant and dose-dependent protection against glutamate excitotoxicity and death of retinal ganglion cells induced by the removal of trophic factors from the culture medium. In

addition, I/R injury showed both the thinning of the entire retina, as well as the internal plexiform and nuclear laminae, as well as a significant decrease in the number of ganglion cells. As indicated earlier, similar results were obtained in our *in vivo* assays of the animals treated with 0.6 μg/L Cyp for 12 days.

Regarding the immunopositive signal of caspase-3 observed in photoreceptors of zebrafish, it suggests that these cells have entered an irreversible pathway of cellular apoptosis due to the increase in the ROS production, leading to DNA rupture and therefore the expression of proteins involved in the cellular apoptosis pathway is increased. The results obtained here are consistent with previous reports showing the effect of Cyp on different cell types, which exhibit alterations in different molecules involved in the cellular apoptosis pathway. Thus, Jin et al. [44] show that exposure of zebrafish embryos to 3 and 10 μg/L Cyp for 3 days induces apoptosis and immunotoxicity, confirming an increase in the activity of caspase-3 and -9 after exposure. This same group, by analyzing messenger RNA levels of different genes related to programmed cell death (p53, Apaf-1 and caspase-3), reported that Cyp induces oxidative stress, DNA damage, and apoptosis, showing that they are significantly increased, whereas the ratio between Bcl-2/Bax genes decreases significantly after exposure to 1 and 3 μg/L Cyp for 8 days. More recently, Raszewski et al. [45, 46] demonstrate that Cyp exposure induces dose- and time-dependent apoptosis in the SH-SY5Y undifferentiated human brain cell line. This work demonstrates that Cyp causes increases in caspase-3 activity, while a decrease in Bcl-2 and Bax concomitantly occurs. However, unlike Kim et al. [43] studies, in the experiments performed with the SH-SY5Y cell line, the application of signal transduction inhibitors SP600125 (from JNK), PD98059 (from ERK 1.2), SL-327 (from MEK1 and MEK2), and SB202190 (from p38 MAPK) failed to attenuate the effect of the pesticide in the cultures of this neural cell type.

From the analysis of the results of the *in vivo* model described here, the existence of at least two groups of retinal cells with a differential sensitivity to Cyp exposure could be postulated. Thus, while photoreceptors would appear to be prone to oxidative DNA damage by excessive ROS generation and would respond by increasing the expression of proteins that lead to cell death by apoptosis (immunopositive for γ-H2AX and caspase-3), the horizontal, bipolar, and amacrine cells would be sensitive to DNA damage by oxidative stress (immunopositive for γ-H2AX) but, at the time of the test, it is not verified that they enter the apoptosis pathway (immunonegative for caspase-3).

A possible alternative explanation (although not exclusive) for the differential behavior of outer and inner nuclear layer cells, to the cytotoxic effect of Cyp, could be due to the tissular regeneration processes in the retina of bone fish [47]. It is known that these are due to the process of proliferation of the ring of embryonic neuroepithelial cells, known as the germinal peripheral zone. Bernardos et al. [48] studies allowed to demonstrate that Müller cells (MC) are responsible ones for these processes. This cell population is located mainly in the inner nuclear layer. Its proliferation rate increases in response to lesions, maintaining the pool of undifferentiated cells and undergoing differentiation processes, to form neuronal progenitor cells [49].

Based on the regenerative capacity of these cells in teleost fish, and given the possible molecular mechanisms involved in the genotoxicity induced by Cyp, the results would suggest that

although this pyrethroid causes a generalized DNA damage in all neural cells of the retina, it could be as much a selective mechanism of activation of the apoptotic route, as a differential process of regeneration in the different cellular plates. Thus, because the MCs are in the inner nuclear layer, the replacement of the horizontal, amacrine, and bipolar cells could be faster than the rest of the cell types.

4. Comet assay

Based on the results obtained in the histological and IF studies, and since the most solid hypothesis suggests that the effect of Cyp on retinal cells could be mediated by the generation of oxidative stress, the studies were deepened, combining the CA techniques, to determine the damage index DNA, and activity of the antioxidant enzymes SOD and CAT as a measure of the oxidative stress caused by exposure to Cyp. These studies were completed with the analysis of *sod* and *cat* gene expression.

Cell viability was determined before the application of the CA by fluorescent DNA-binding dyes. A total of 100 cells were counted per sample and the percentage of viable cells was determined [50]. The CA was performed as described for Singh et al. [51] with modifications required by retinal cells of zebrafish: DNA unwinding during 10 min and electrophoresis at 300 mA, 0.7 V/cm, during 10 min too. Cells were visually classified into five classes according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4), resulting in a single DNA damage score (damage index, DI = $n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4$), where n_1, n_2, n_3, and n_4 are the number of cells in each class of damage, respectively [52].

In the CA of retinal cells, with the concentration of 0.3 μg/L Cyp, it is verified that until the 9 day of the bioassay, there are no significant differences in relation to their controls; from day 9 onwards, there are statistically very significant differences ($p < 0.01$) regarding both the controls and the previous stages (**Figure 5**).

The bioassays performed with 0.6 μg/L Cyp showed DI with statistically very significant differences ($p < 0.01$) both with respect to their controls and between the different stages evaluated in the bioassay (**Figure 6**).

As previously stated, there are many reports postulating that pyrethroids in general and Cyp in particular are oxidative stress triggers; however, direct genotoxic mechanisms cannot be ruled out. Thus, spectral studies of calf thymus DNA demonstrated a bathochromic shift in UV absorption spectra, revealing that Cyp could bind with DNA. According to these studies, the recognition and reaction of Cyp with DNA is attributed to the vibratory modes of the active site and it is postulated that the possible mechanism of this interaction would be responsible for the chromosomal aberrations verified in the bioassay [52]. The molecular mechanisms responsible for the genotoxicity of pyrethroids are not clearly understood yet; however, chromosomal aberrations, sister chromatid exchange, and micronuclei formation observed in plants and animals clearly indicate that these compounds interact with DNA and induce their damage.

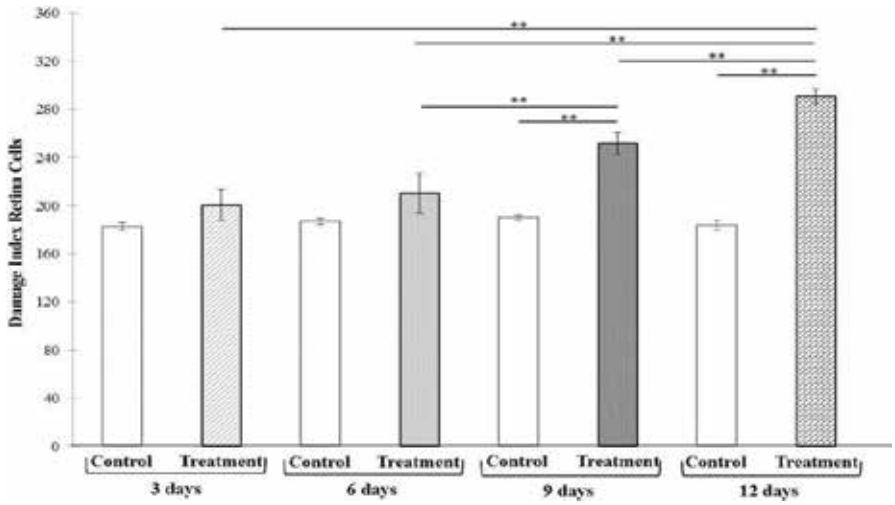

Figure 5. Bar graphic showing damage index values of DNA in retinal cells of zebrafish exposed to 0.3 μg/L of Cyp. Statistically significant differences are indicated by asterisks (**$p < 0.01$).

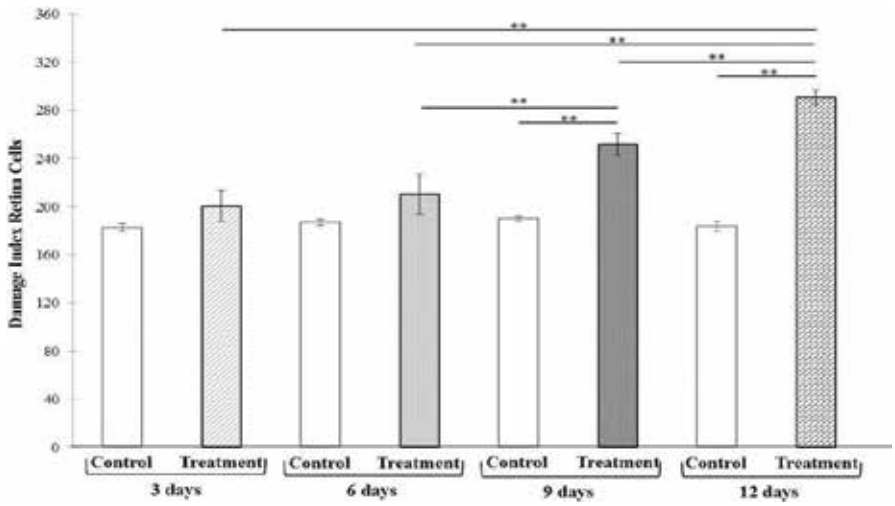

Figure 6. Bar graphic showing damage index values of DNA in retinal cells of zebrafish exposed to 0.6 μg/L of Cyp. Statistically significant differences are indicated by asterisks (**$p < 0.01$).

In addition to the direct interaction damages, those caused by the generation of free radicals as determined by the Giray et al. [53] works could be added in which rats treated with Cyp showed increases in lipid peroxidation at the cerebral and hepatic levels. Because of its small size ($V = 536.40$ Å^3) and hydrophobicity, the molecule of Cyp can easily cross cell membranes, reach, and interact with DNA through its acid moieties. To understand this interaction, the molecule can be divided into two parts (**Figure 7**). Region 1 would constitute the "active site" of the molecule. It contains highly electronegative atoms such as chlorine, active ester, and dimethyl groups. The crystallographic data demonstrate that this portion offers a relatively

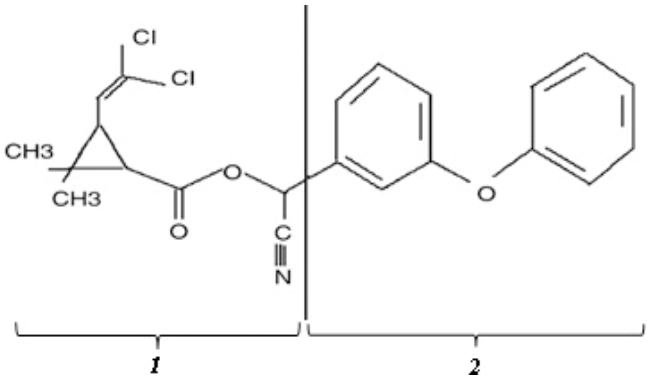

Figure 7. Cypermethrin structure showing regions (1) postulated as responsible for the interaction with DNA and (2) stabilizing support region. Adapted from [54].

flexible structure [52] which would allow the cyano, carbonyl, methyl, and chloride groups to be located nearby and interact with the DNA. On the other hand, region 2 constitutes the comparatively rigid part of the molecule providing support for the stabilization of such interaction (**Figure 7**). The molecular vibration of the atoms that involve the active region 1 would polarize the DNA molecule, promoting the binding. The binding of Cyp to DNA through polarization can lead to the destabilization of the DNA structure and the duplex unwinding, inducing chromosomal damage. Such binding was demonstrated in UV absorption studies.

Thus, the results of the CA studies as well as the morphological and IF studies allow to postulate that Cyp (or possibly its metabolites) could directly interact with the DNA molecule causing the duplex rupture.

5. Enzyme activity and gene expression of SOD and CAT

For these determinations, retinas were homogenized and then centrifuged. SOD activity was spectrophotometrically measured at 550 nm [55]. The activity of SOD was calculated according to the standard curve of SOD and expressed as U/mg protein [56]. On the other hand, CAT activity was determined by recording the absorbance of the generated stable chromophore at 405 nm due to H_2O_2 consumption according to Aebi's method [57]. In these studies, we found that the activity of SOD (**Figure 8**) and CAT (**Figure 9**) increases when retinal cells were exposed to 0.6 μg/L Cyp. Like other organisms, fish can neutralize the elevated ROS levels in their systems, with protective ROS-scavenging enzymes such as SOD and CAT. Thus, it is possible that an increase in the activity of these enzymes contributes to the elimination from the cells of ROS induced by Cyp exposure.

Then, we examined the representative genes that encode proteins that are used to combat oxidative stress (such as SOD and CAT) to determine whether these might serve as molecular endpoints for Cyp exposure in zebrafish. The total RNAs of retinas from adult fish treated with Cyp 0.6 μg/L for 3 and 12 days were isolated and processed according to Jin et al. [26]. Reverse transcriptase (RT) products were used directly for the polymerase chain reaction

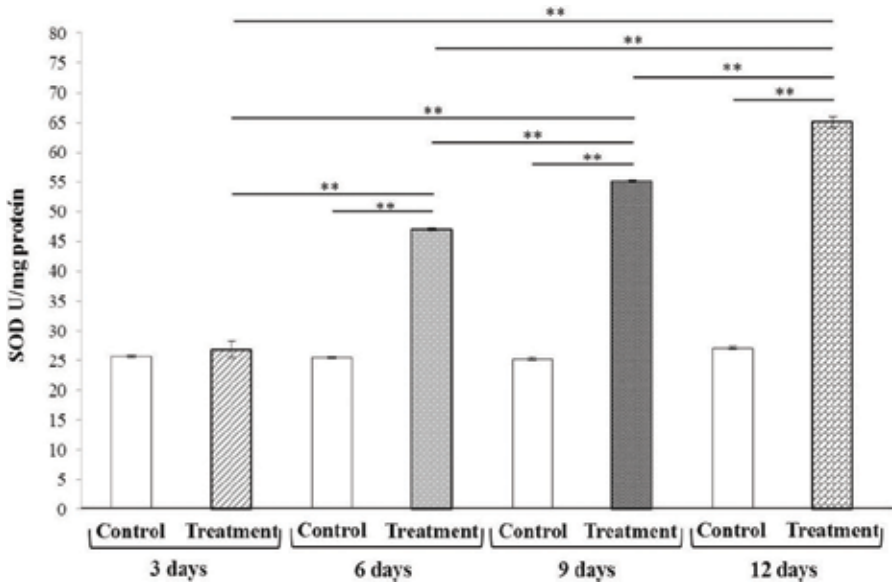

Figure 8. Bar graphic showing the values of SOD activity in the retinal cells of the zebrafish exposed to 0.6 μg/L Cyp. Statistically significant differences are indicated by asterisks (**$p < 0.01$).

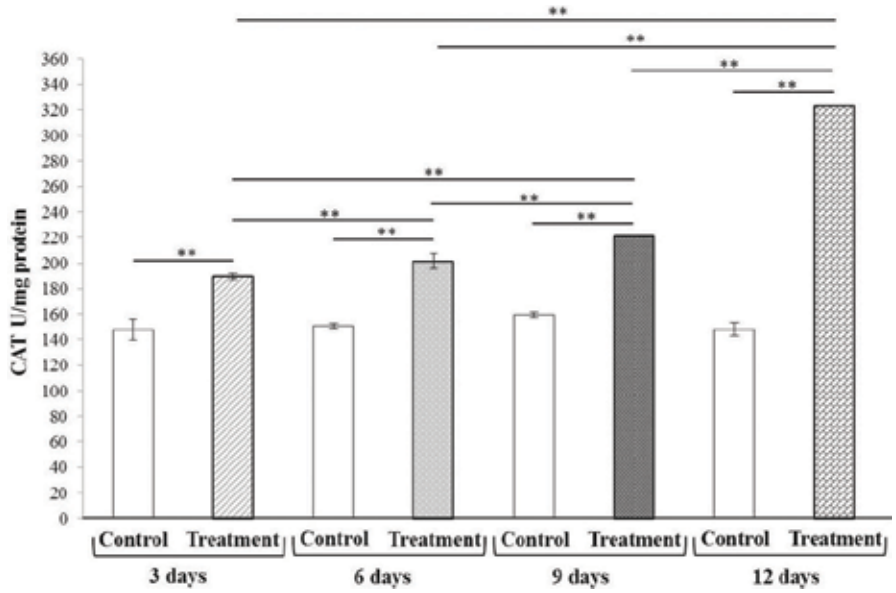

Figure 9. Bar graphic showing the values of CAT activity in the retinal cells of the zebrafish exposed to 0.6 μg/L Cyp. Statistically significant differences are indicated by asterisks (**$p < 0.01$).

(PCR). These were performed using an Ivema T-18 thermo-cycler (Llavallol, BA, Argentina) using the following program: denaturation for 10 min at 95°C, followed by 30 cycles of 1 min at 95°C, 90 s at 50°C and 90 s at 72°C. Oligonucleotide primers were used to detect the gene

expression of β-actin, SOD (AY N° 195,857), and CAT (AF N° 170,069). As housekeeping gene, β-actin transcripts were used for data standardization. We found that the expression levels of the mRNA of SOD and CAT enzymes had a significant increase in the retinal cells of the zebrafish after 12 days of exposure to the highest concentration of Cyp (0.6 μg/L) (**Figure 10**), which is consistent with the findings in the evaluation of enzyme activity. Therefore, we suggest that the increased levels of mRNA and its close relationship with the increase in enzyme activity are related to the removal mechanisms of ROS.

The toxicity of pesticides in fish is related to an increased production of ROS, leading to oxidative damage [58]. Fish, like many other vertebrates, try to reduce the damage caused by oxidative stress by using an antioxidant defense system. The first line of defense consists of antioxidant molecules, such as glutathione, vitamin C and E, and carotenoids [59]. Antioxidant enzymes include another defense mechanism, including SOD, CAT, glutathione peroxidase (GPx), and glutathione S-transferase (GST) [60–62]. Several studies have shown that synthetic pyrethroids are extremely toxic to fish and aquatic invertebrates [63–65]. The antioxidant defense system of living organisms can be analyzed by the activity and gene expression of enzymatic antioxidants, such as SOD, CAT, and GPx [66].

Our results demonstrate that, in zebrafish retinas, the CAT enzyme is more sensitive and would respond more rapidly than SOD as a defense mechanism to the increase in the production of ROS to avoid the possible oxidative effect caused by Cyp.

Accordingly, several studies have shown that Cyp produces an imbalance in the production of ROS and consequently an alteration in the activity of the antioxidant enzymes SOD and CAT in various species and organs as a defense mechanism to oxidative stress. Kale et al. [66] reported that erythrocytes of rats exposed to 2500 mg/kg of Cyp showed an increase in the

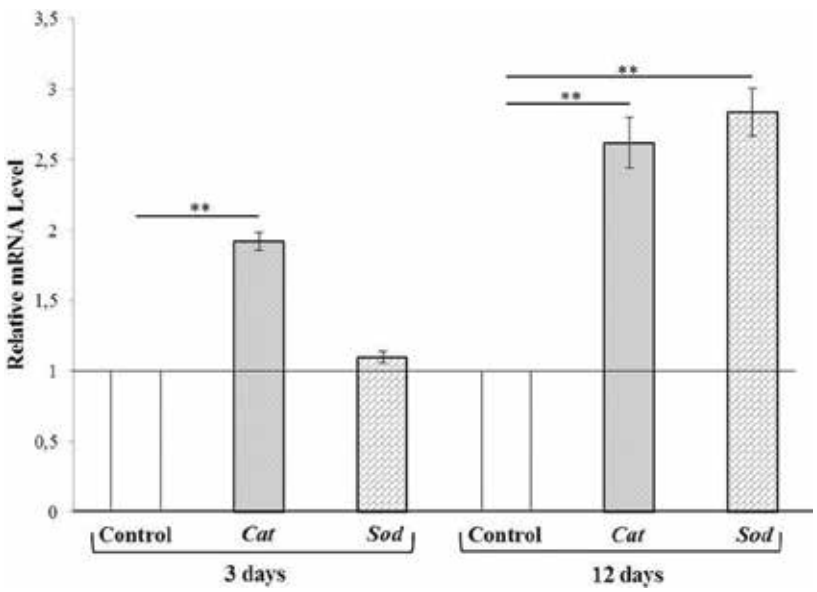

Figure 10. Bar graphic depicting the gene expression levels of *cat* and *sod* in the retina of the zebrafish exposed to 0.6 μg/L Cyp. Statistically significant differences are indicated by asterisks (**$p < 0.01$).

activity of the antioxidant enzymes SOD and CAT because of the increase in ROS production. In addition, they demonstrate that the gradual decrease in the activity of both enzymes, after a period, is due to a decrease in the production of formed ROS. More recently, studies by Yonar et al. [58] indicate that Cyp induces an increase in the enzymatic activity of SOD and CAT in blood, liver, kidney, and gills of *Cyprinus carpio*, showing that pesticides can induce oxidative stress, leading to the generation of free radicals and causing peroxidation of lipids. Increased lipid peroxidation and ROS production may affect the activity of antioxidant enzymes, which are sensitive indicators of increased oxidative stress.

In addition, in the present study, genes encoding stress-sensitive SOD and CAT enzymes were used as molecular biomarkers of the bioassay for stress evaluation by Cyp and to complement the morphological, immunofluorescence, and EC studies. Similar studies have been performed to evaluate the behavior of other species using real-time PCR techniques as well as RNA microarrays [67–69]. At present, there are few studies evaluating the gene expression of SOD and CAT in zebrafish retina in response to agents that promote oxidative stress [70] and no studies have been reported relating stress oxidative stress caused by Cyp, and the gene expression of antioxidant enzymes in the retina. Induction of CAT mRNA, SOD, and GPx expression was recently demonstrated by Jin et al. [27] (4 days) or low concentrations (1 μg/L) for prolonged exposures (8 days), the use of high concentrations of Cyp (3 μg/L) for moderate exposures (4 days) or low concentrations (1 μg/L). In this work, it is proposed that the levels of expression promoted by the pyrethroid are the product of increased oxidative stress. In the present study, doses are much lower than those used by Jin et al. [27], obtaining greater discrimination of the behavior of two of the enzymes of response to oxidative stress. Thus, mRNA levels of the SOD enzyme in retinas of zebrafish treated with 0.6 μg/L for 3 days did not cause significant increases, whereas those corresponding to CAT resulted in very significant levels of expression. When the transcript levels of both genes were evaluated toward the end of the bioassay (12 days), both genes responded with very significant levels of expression.

In conclusion, the RT-*sq*PCR assay adopted in this work is an effective technique for the rapid determination of the oxidative effect in zebu fish exposed to Cyp. It is suggested that the significant increase in the levels of gene expression and enzymatic activity of SOD and CAT would be closely related to the mechanisms that would be put in place to decrease or eliminate the oxidative stress caused by the high production of ROS and thus avoid oxidative damage from pyrethroid exposure.

6. Conclusions

This study demonstrates that Cyp generates a variety of detrimental effects in retinal cells of zebrafish over a relatively short time of exposure and at concentrations much lower than those found in runoff and surface water in natural environments. Effects observed include apoptosis, DNA damage, increased expression of γ-H2AX and caspase-3 genes, and alterations in antioxidant enzyme activities as well as in the expression of their respective genes. Our results not only provide important information to fully understand the potential mechanisms

of Cyp-induced neurotoxicity but also suggest that zebrafish can serve as an ideal model for studying developmental toxicity of environmental contaminants.

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Chapter 4

Assessment of Potential Carcinogenicity by Quantitative Structure-Activity Relationship (QSAR)

Davor Zeljezic

Additional information is available at the end of the chapter

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Abstract

Already in 1978, Elisabeth C. Miller and James A. Miller came with a presumption that electrophilic molecules are predicted to be carcinogens. It is because DNA molecule is reached in nucleophilic centres that may covalently bind to such substances. Rules deduced by Millers are even nowadays irrefutable, and they are used as the basis of testing of the substance for its carcinogenicity potential. Toxicological discipline that emerged from Millers' research is based on dependence of chemical structure of the substance and their biological activity. Even further, there are strict regularities between molecular structures and activities. The tool used in assessment of biological activity of a substance is known as SAR, an abbreviation from structure–activity relationship. Besides electrophilic centres, in assessment of carcinogenic potential of a substance, the SAR also encounters chemical surrounding (neighbouring functional groups), size of the substance, its lipophilicity, number and position of aryl rings, substitutions of hydrogens, epoxides in aliphatic moieties or rings, resonance stabilisation, etc. To these days, SAR has been upgraded to quantitative SAR (QSAR) which applies multivariate statistical methods quantitatively comparing detected characteristics of "alerts" with biological activity of known carcinogens. Nowadays, chemical industry developing novel active substances is unthinkable without application of QSAR.

Keywords: structure–activity relationship, mutagenicity, carcinogenicity, QSAR, molecule descriptors

1. Introduction

Already in the 1940s, for the first time, Auerbach and Robson have reported that a chemical agent may induce mutations which are the main driving event in the process of carcinogenesis [1].

They studied the effect of exposure to nervous poison gas yperite (mustard gas (bis [2-chloroethyl] sulphide)) first used in World War I by the German army. They came to this conclusion by exposing the vine flies to components of mustard gas.

Several years later, in 1947, Beerenblum and Schubick [2] published the results of the study confirming that chemicals may induce mice skin carcinoma, thus being able to act as carcinogens.

Following these pioneer reports, even pore evidences have been gathered indicating that chemical agents, as it had been first acknowledged for ionising radiation, may interact with human genome by changing nitrogenous bases and inducing mutations. It was considered that mechanism by which DNA bases are changed is covalent binding of small functional group from the chemical substance or by binding the entire substance to the base. In that way position of polar functional groups (e.g. hydrogens bound to highly electronegative atom) in nitrogenous bases is changed. Such covalently modified bases have changed the ability of forming hydrogen bonds. Thus, in the process of DNA replication, instead of binding complementary nitrogenous base, they will form hydrogen bonds in a way that change the genetic code and produce mutations. Besides changing the way of base hydrogen binding, chemicals may affect DNA replication fork in a way which may lead to insertion or deletion of bases due to structural changes of DNA caused by covalent binding of bulky substances recognised as forming of bulky DNA adducts [3].

Based on all gathered knowledge regarding the interaction of chemical substances and DNA, changing in the ability of hydrogen binding of nitrogenous bases, in 1978 Miller concluded that majority of electrophilic molecules are predicted to be initiators of carcinogenicity due to their affinity to covalently bind to nucleophilic centres in DNA [4]. The conclusion was based on the researches that Miller spouses have been conducted from 1951. Finally, in 1983 Millers and the associates have concluded that there is a strong correlation and regularity between chemical structure of the substance and its biological activity (potentially direct carcinogenicity) and pathway of its metabolic transformation (potentially activation and indirect carcinogenicity) [5].

Based on their findings, a novel chapter in predicting biological activity of chemical substances, besides, in regard to their carcinogenic activity, has been initiated. The principle in assessing the mutagenic and carcinogenic potential is based on comparing the structure, with special concern regarding functional groups of the chemical, with already evaluated substances with known outcome. It is entirely a theoretically approach-based principle that relies on the database for chemicals that have been previously tested in vitro or in vivo or were proven to be biologically (in)active in other ways. Since it is based on relation of the molecular structure of substance of interest and reordered activities in read-across approach considering relevant molecules containing corresponding functional groups, the assessment approach was named as the structure–activity relationship approach or, as abbreviated, SAR [6]. The SAR identifies potential electrophilic centres in the substance of the interest by comparing them to those that have the potential to attack and bind nucleophilic centres in DNA. It also identifies structural moieties and fragments which may contribute to DNA covalent binding. These electrophilic centres are assigned as alerts.

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2. Electrophilic centres

2.1. Types of electrophilic centres

There are two types of electrophilic centres that may interact with nitrogenous bases in DNA and change their ability to form hydrogen bonds with other, not any more, complementary bases. First of them are natively present in the chemical's structure and do not require any metabolic change. The second group of potential electrophilic centres is functional groups that require metabolic activation to be transformed into electrophilic centres [7].

2.1.1. Direct-acting electrophilic species

Direct-acting electrophilic species in terms of the ability to attack nucleophiles in DNA in minority is concerning yet identified carcinogens. Molecules bearing these functional groups do not require metabolic activation to be able to interact with nitrogenous bases and induce mutations. There are four major classes of electrophilic species that may directly bind to DNA (**Figure 1**).

Concerning the formaldehyde ion mode of action, its DNA-binding activity is more complex than just transferring the functional group or entirely binding nitrogenous bases (**Figure 2**). In **Figure 2**, R^1 stands for primary nitrogenous base attacked by the carbon atom as electrophilic centre of formaldehyde ion. In this way formaldehyde binds DNA by a peptide bound. However, in the second step, the carbon atom attacks exocyclic amino group of the complementary nitrogenous base and forms a covalent bond. By acting as such, two complementary DNA strands become covalently bound, instead by hydrogen bonds, which hinder the gene transcription and DNA replication resulting in base insertions or deletions. Such agents that form covalent bonds between complementary DNA strands are considered as cross-linking agents.

2.1.2. Indirect-acting electrophilic species

Indirect-acting electrophilic species are those potentially electrophilic groups that require a metabolic transformation to be activated and able to interact with a DNA [7].

Carbonyls and carboxylates, in the course of metabolic activation, expose carbon atoms, like in case of formaldehyde ion, which becomes electrophilic and is able to attack nucleophilic centres in nitrogenous bases (**Figure 3**). Further, cyclophosphamide, an antineoplastic drug, by metabolic activation and P450 oxidase activity dissociates to phosphoramide mustard and acrolein that belongs to carboxylates. Although majority of acrolein is excreted in urine as mercapturic acid following its conjugation with glutathione, small ratio of acrolein will form epoxide glycine aldehyde and reacts with guanine in DNA, which results in the changed base. Such formed chimeric structure hinders its ability to form hydrogen bonds with cytosine, as the complementary base, in the course of DNA replication, thus inducing changes in newly synthesised DNA strand (**Figure 3**).

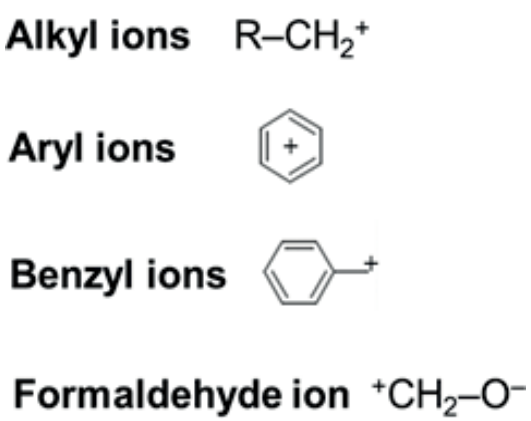

Figure 1. Direct-acting electrophilic species known to be able to act as carcinogens.

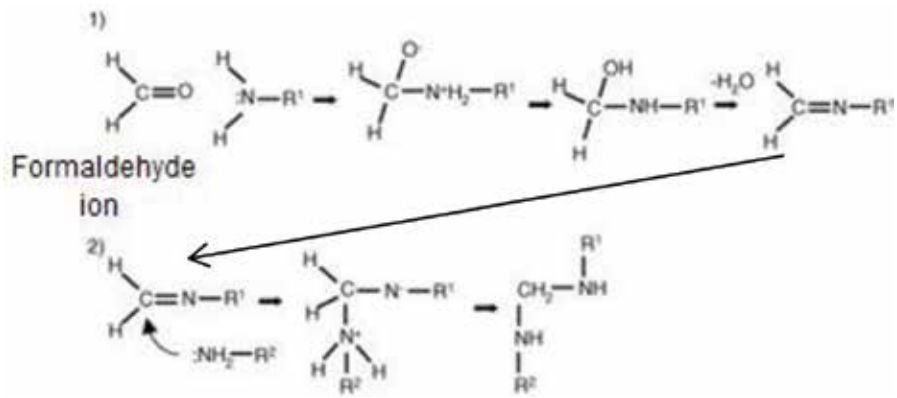

Figure 2. Formaldehyde ion mode of mutagenic action.

As far as acyl halides are concerned as potential carcinogens, it has to be notified that an electrophilic centre to be formed, halide atom, should dissociate in the process of metabolic activation. In this reaction cytochrome P450 is involved, and in the course of this reaction, the carbon atom becomes available to attack nucleophilic centre in DNA.

As an example of carbenes registered as potential carcinogens, carbon tetrachloride has been present. As shown in **Figure 4**, it can form tricyclic structure with nitrogenous base. It is highly reactive and may open attacking nitrogenous bases in DNA. Its use has been associated to refrigerant agents.

Considering the nitrogen groups as potential electrophilic centres, there are several possible examples (**Figure 5**).

Again, cyclophosphamide as antineoplastic drug by metabolic activation forms aziridinium which is energetically instable, and the ring opens easily. Opened ring interacts

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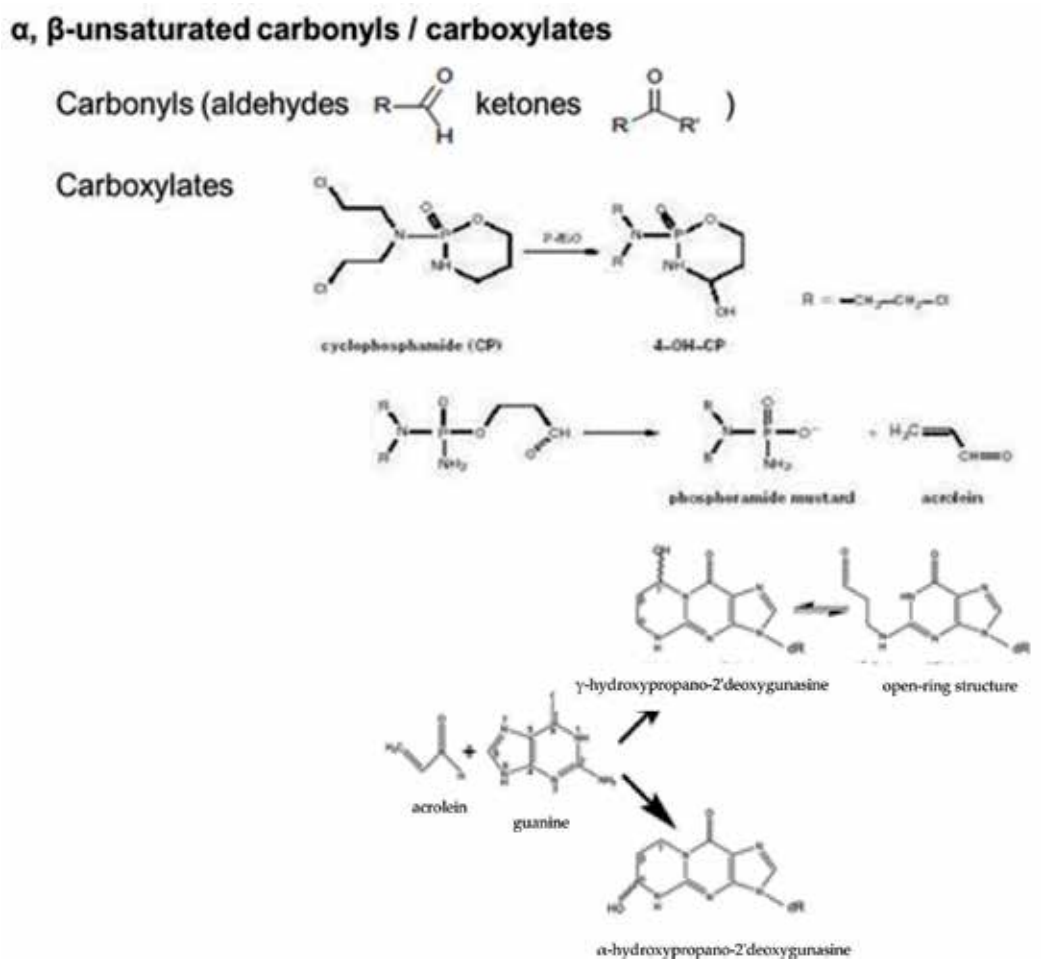

Figure 3. Interactions of carbonyls, carboxylates and carboxylates with DNA.

with nitrogenous bases in DNA modifying their ability to pair complementary bases (**Figure 5**). In more details reaction of aziridinium ion and nitrogenous base is presented in **Figure 6**.

The consequence of such interaction of aziridinium ion to DNA cross-linking is formed. After opening the aziridinium ring, carbon atom reacts with intra-ring nitrogen atom. Nevertheless, chlorine atom from the second moiety dissociates, and formed ion interacts with intra-base nitrogen of complementary nitrogenous base forming cross-links. The same interaction is presented in **Figure 7**.

Nitrogen radicals are formed by metabolic activation either. They may interact with DNA in several ways. One of them is presented in **Figure 8**, concerning mutagenic activity of benzidine.

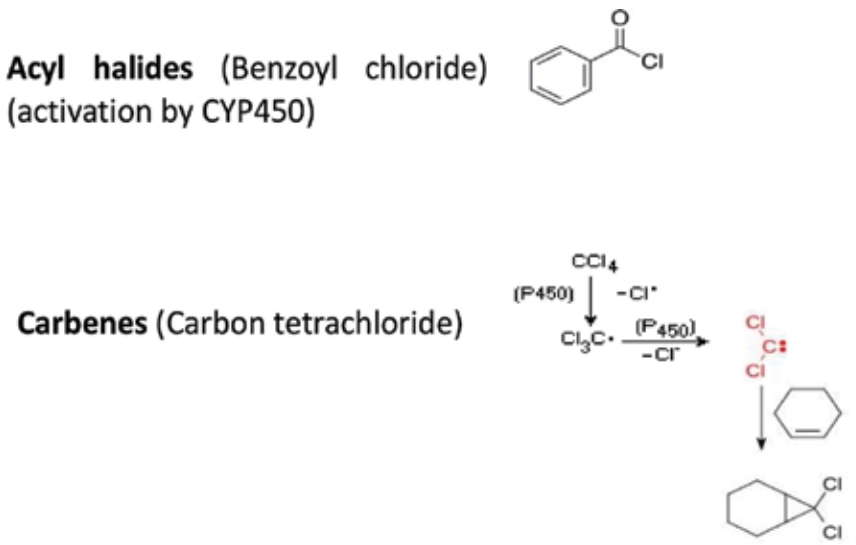

Figure 4. Acyl halides and carbenes as metabolically induced electrophilic species.

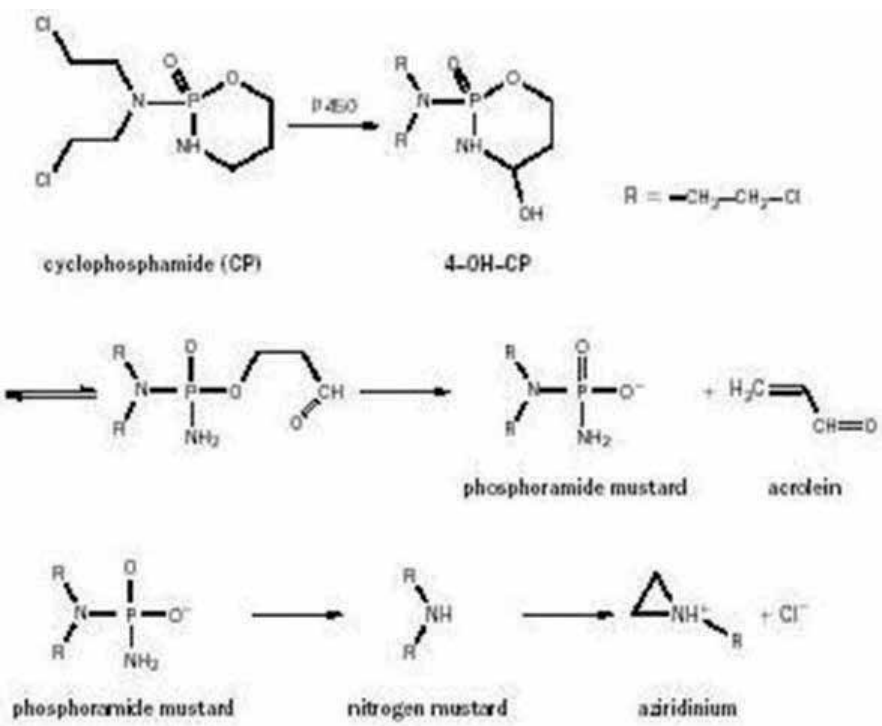

Figure 5. Aziridinium as metabolically induced electrophilic site.

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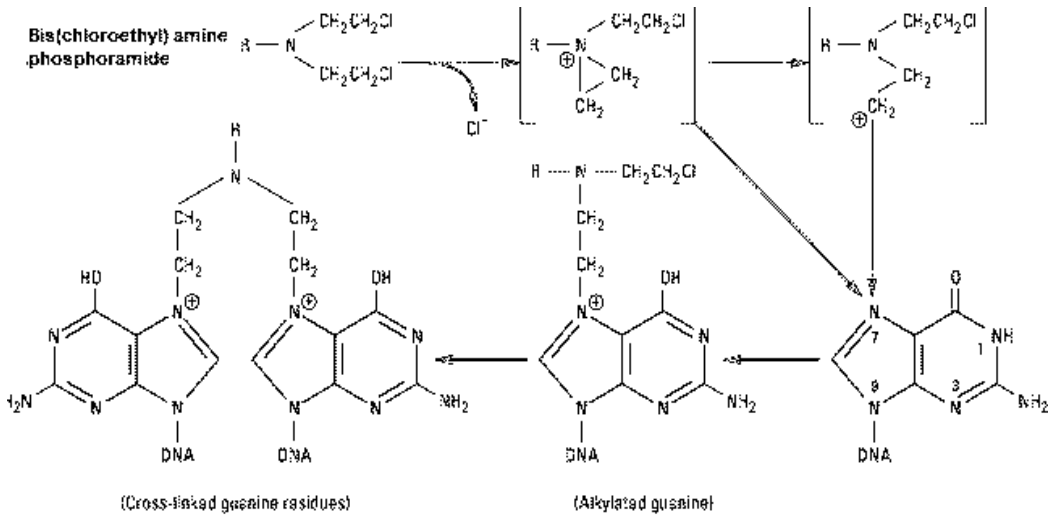

Figure 6. Yperite interaction with nitrogenous bases in DNA.

Benzidine is used as the solvent in dye production. By activity of N-acetyltransferase in the presence of acetyl coenzyme A, it is transduced to the form containing two acetyl functional groups. Acetyl groups easily dissociate leaving two nitrogen radicals which may interact with N1 or C2 of guanine in DNA and inducing irregular base pairing.

Phenylamine is another example of the substance that exhibits nitrogen radical several rounds of metabolic changes as shown in **Figure 9**, which interacts with the genome.

Metabolic transformation and toxicokinetics of phenylamine (arylamine) are rather complex processes as it can be seen in **Figure 9**. Phenylamine is used as the manufacture of precursors to polyurethane and other industrial chemicals. In the liver phenylamine is oxidised to hydroxylamine which enters the bloodstream to be excreted by the urinary tract. In urinary bladder epithelium, it may follow several pathways. In the first one, it is N-acetylated to form N-arylacetamide. This product is further activated by N-acetyltransferase 1 or 2 resulting in formation of acetylated derivate. Acetyl group easily dissociates under conditions present in bladder epithelium, leaving nitrenium ion being able to covalently bind DNA and induce mutation centres. Other scenarios foresee formation of sulfonoxy ester or nitrenium ion, both of which are chemically instable and bind covalently to nitrogenous bases in DNA resulting in DNA mutations [7].

Peroxy radicals (R–O–O ·) belong to reactive oxygen species (ROS) and are characterised with extremely long half-life (in order of seconds) compared to other ROS [8]. They are spontaneously formed by the process of autoxidation mostly of unsaturated fatty acids in the food, when hydrogen atom is removed and the rest of the molecule interacts with molecular oxygen producing the peroxy radical. It predominantly interacts with thymine forming highly mutagenic 5-(hydroperoxymethyl)-2′-deoxyuridine, 5-formyl-2′-deoxyuridine and 5-(hydroxymethyl)-2′-deoxyuridine [8, 9].

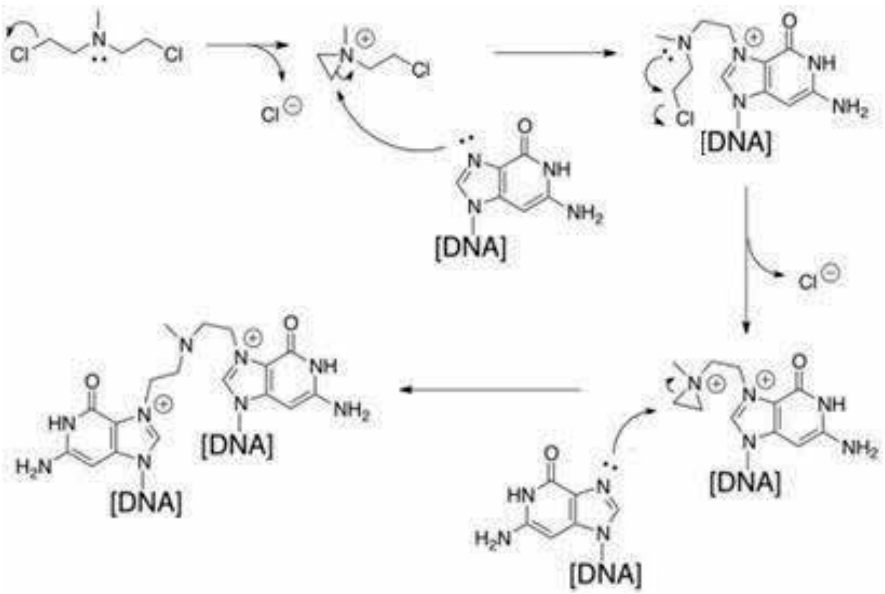

Figure 7. Yperite interaction and genotoxic mode of action.

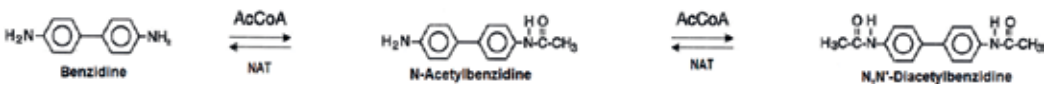

Figure 8. Activation of benzidine into DNA-binding substance.

Epoxides present the next electrophilic oxygen-containing group that requires metabolic processes to be formed. They are cyclic ethers in the form of equilateral triangles with one of the atoms being oxygen. Their high ring strain makes them highly reactive. In attacking nitrogenous bases in DNA, the ring opens, and carbon atom reacts with nucleophilic centre in the base.

Examples of epoxides together with their reaction with DNA are shown in **Figure 10**.

Aflatoxin B_1 is a mycotoxin predominantly produced by the fungus *Aspergillus flavus*. It is wildly present in food and feed, especially in areas with warm and humid climate. It can be found in grain, various nuts and wine. It is suspected to be a potent hepatocarcinogen, although lately there is a strong indication that its carcinogenic effect is potentiated by coinfection with the virus of hepatitis B. Nevertheless, aflatoxin forms epoxide, and after the opening of ring, it forms DNA adducts by binding to N7 of guanine. N7 of guanine is a preferential site for adduct formation, especially those which formation is mediated by epoxide ring openings. Another such examples are vinyl chloride which is raw a material in production of plastic polymer polyvinyl chloride. Benzo(a)pyrene belongs to a wide group for chemical names like polycyclic aromatic hydrocarbons (PAHs). Benzo(a)pyrene is a class 1 carcinogen to humans according to the International Agency for Research on Cancer (IARC). It is a constituent of chimney soot, coal tar and exhaustion gases especially those from diesel engines, cigarette smoke and every

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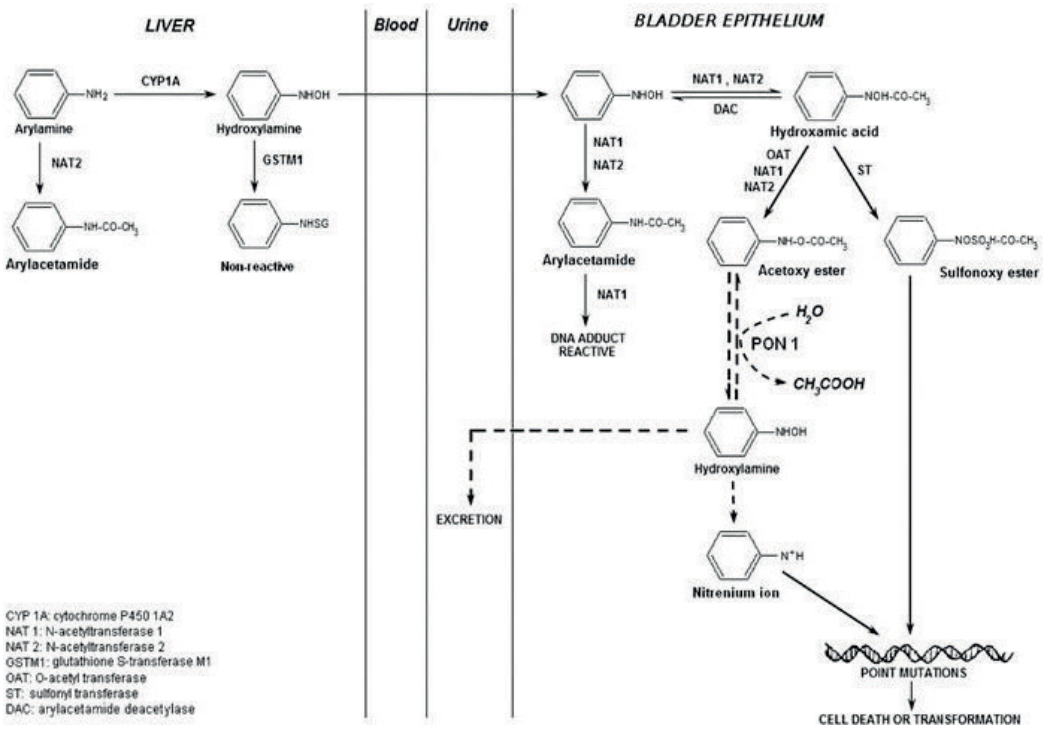

Figure 9. Mutagenic mode of action of phenylamine (arylamine).

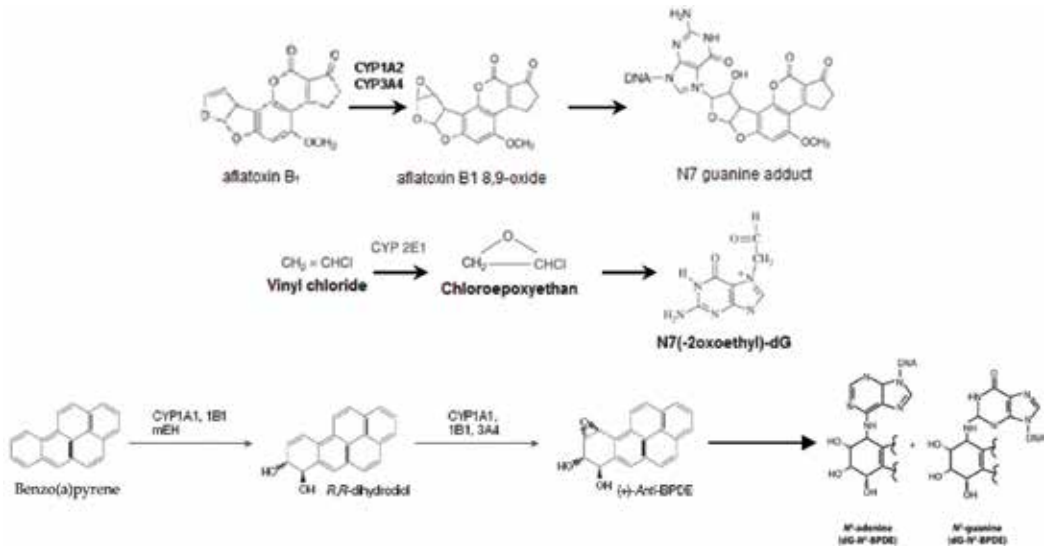

Figure 10. Examples of molecules that by metabolic oxygenation form epoxides. In further steps epoxide rings open and DNA adducts are formed.

smoke originating from combustion of the organic matter. As with previous two chemicals, it is capable to form bulky DNA adducts by binding N7 of guanine. There are many other chemicals being able to via epoxide intermediate bind to DNA by forming bulky adducts. To those substances belongs bisphenol A, also identified as endocrine disruptor exhibiting its hormone poisonous activity by DNA binding. But also to via epoxide formation large group of resin monomers used in endodontic materials such as BisGMA, TEGDMA and UDMA (**Figure 11**) are activated [7].

As it was shown in the examples given in **Figure 10**, single epoxide may lead to single DNA adduct formation. Speaking of resin monomers, they are capable of forming two distant epoxides in the same molecule, thus being able to covalently bind two guanosines. Thus, they may form cross-links by covalently linking guanines in complementary DNA strands or form dimers by binding two guanines in the same DNA strand.

A last group of electrophilic centres that require metabolic transformation of precursor molecule to be formed are sulfonium ions. It is a species containing sulphur atom that has an octet of electrons but bears a formal charge of +1. It can be present in two different structures: open and ring (**Figure 12**).

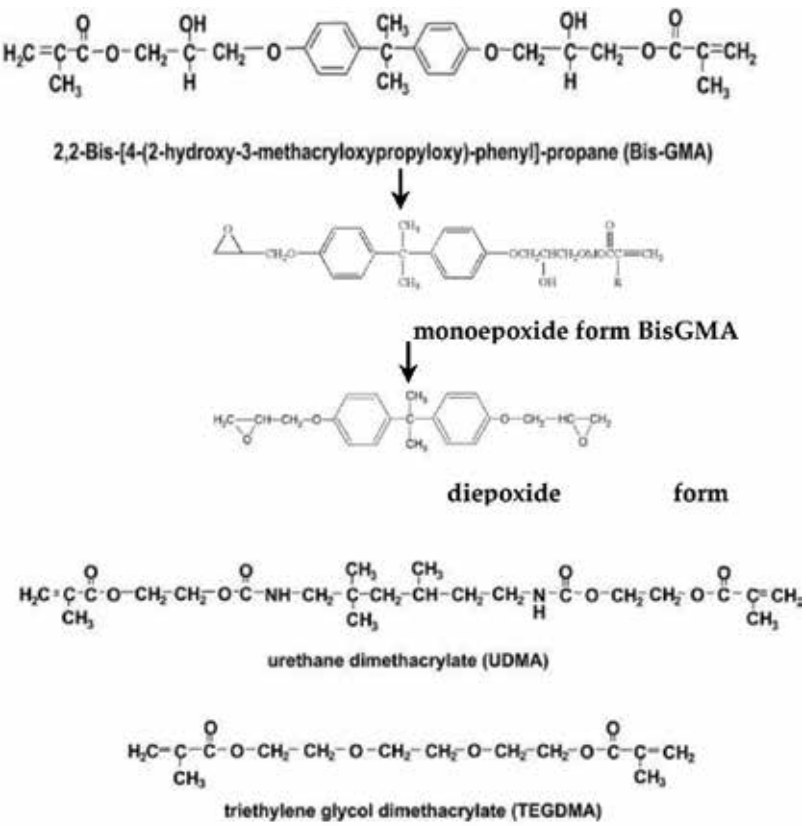

Figure 11. Resin monomers used in endodontic materials and process of forming diepoxide of the molecules.

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Figure 12. Forms of sulfonium ions. In the open form, H atoms and methyl group may be replaced by any organic moiety.

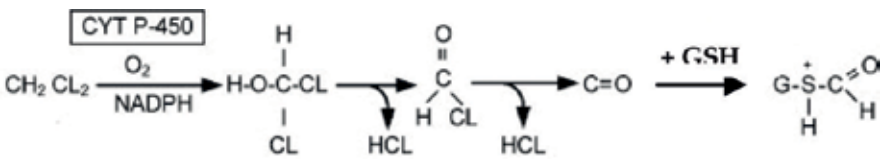

Figure 13. Formation of sulfonium ion by metabolic transformation of dichloromethane.

Formation of sulfonium ion is most frequently preceded by the second step of metabolic transformation, chelation with glutathione. Chelation produces sulfonium ion which, due to its electrophilic characteristics, may attack nucleophilic centres in DNA (**Figure 13**). One of examples for chemicals that form sulfonium ion is dichloromethane [10]. The substance is used as the leaching agent not only in the industry but also in production of decaffeinated coffee and tea. Except via sulfonium ion, it has been proved that in mice degradation of dichloromethane goes down to formaldehyde which induces mutations by forming protein-DNA cross-links [11]. Some other chemicals known to interact with nitrogenous bases through sulfonium forms are shown in **Figure 14**. Dibromoethane, for instance, is used as fungicide, insecticide and precursor in the production of insect repellents. Tetrachloro-1,4-benzoquinone is a fungicide, while hydroquinone is applied in the skin whitening cosmetics.

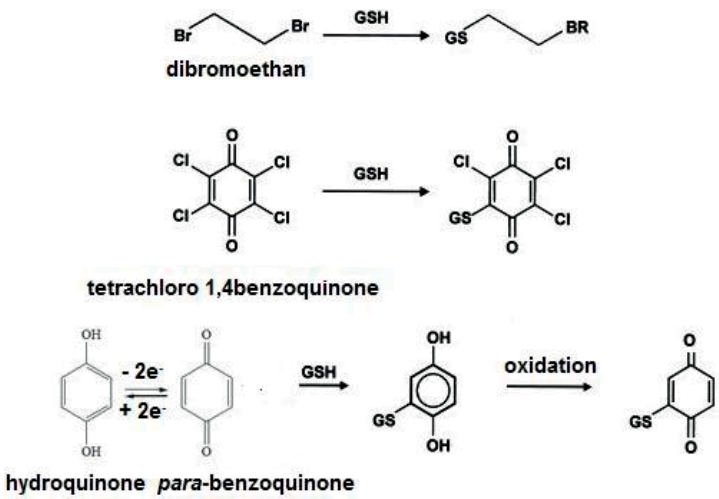

Figure 14. Examples of substances that form sulfonium ion as the result of glutathione binding in process of metabolic transformation.

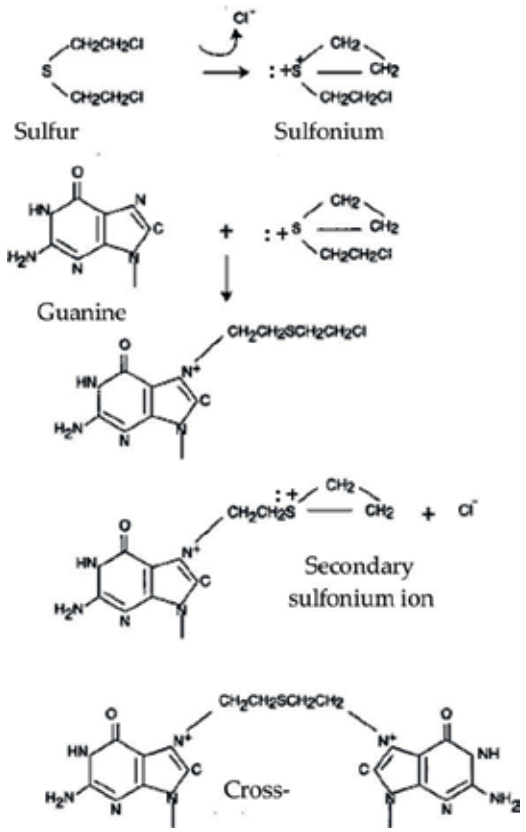

Figure 15. Formation of circular form of sulfonium ion and its interaction with DNA.

Earlier, we have talked by mustard gas, which in its initial form contains nitrogen atom that binds two chloroethyl moieties and attacks DNA via aziridinium ion formation. However, other form of mustard gas, sulphur mustard, contains sulphur instead nitrogen and forms circular form of sulfonium ion which, after opening, attaches to guanine. As in the case of mustard, sulphur mustard is capable of forming two rings in succession, thus covalently binding complementary DNA strands and acting as cross-linking agent (**Figure 15**; [12]).

3. Nucleophilic centres in DNA

Each of four nitrogenous bases in DNA that form genetic code poses specific nitrogen and oxygen atoms that are capable of donating an electron pair to an electrophilic centre to form a covalent bond. In this way nitrogenous bases are structurally changed. Their ability to form hydrogen bonds with complementary bases is also altered, and, if not repaired, after the DNA replication, new synthesised DNA strand will contain a base with which damaged one can bind. In this way, a mutation is formed and fixed and will result in the change of the genetic code.

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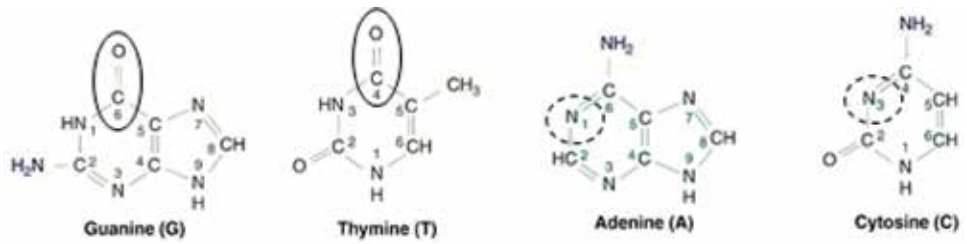

Figure 16. Nucleophilic sites in nitrogenous bases which are predominantly prone to alkylations. Solid-lined ellipses indicate the most frequent atoms being subjected to alkylation, while dash-lined circles roundless frequently alkylated atoms.

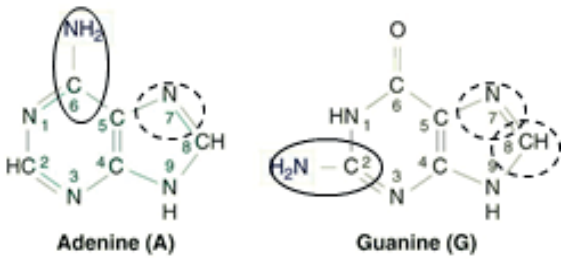

Figure 17. Nucleophilic sites in nitrogenous bases which are predominantly prone to formation of DNA adducts. Solid-lined ellipses indicate the most frequent atoms being subjected to binding of bulky molecules, while dash-lined circles round less frequently bound atoms.

Due to steric properties of DNA bases and DNA itself, there is a regularity which nucleophilic sites are available for binding of bulky molecules and adduct formation and which are predominantly alkylation sites (**Figures 16** and **17**).

Primary cites for transferring an alkyl functional group from mutagenic substances are exocyclic oxygen bound to C6 atom of guanine or C4 atom of thymine. Less frequently alkylation occurs at N1 atom adenine or N3 atom of cytosine.

Exocyclic nitrogen bound to C6 in adenine and C2 in guanine is a primary site of DNA adduct formation. Nevertheless, as shown in examples of electrophilic centres, many bulky adducts prefer N7 atom of guanine or adenine. Besides C8 atom of guanine is also prone to bounding of bulky molecules, but that site is primary nucleophile for oxidative changes of base by ROS.

4. Characteristics that mediate electrophilic centre activity

Over the time even more substances have been analysed for SAR, and all the data helped the database which rely on the entire approach used in SAR to identify potential carcinogens. Broadening the database and expanding it with data on other molecular characteristics than

solely functional groups lead additional knowledge regarding dependence of chemical structure and biological activity to be acquired. It has been learned that other features such as the presence and distribution of other functional groups that are even not electrophilic centres, planarity of the molecule, distribution of aromatic rings, points in the molecules where epoxides are formed, and many other characteristics influence activation of electrophilic centre, its stability (half-life) and activity. Thus, all of them should be considered in prediction of possible mutagenic and carcinogenic potential of the substance of interest.

Size of the molecule is one of its characteristics that matters in prediction of its reactivity. Molecules with molecular weight beyond 1000 are not likely to be absorbed and enter the bloodstream. Even in the event of such scenario, it is highly unlikely that they will be able to enter the cell and be approached by the active sites of enzymes needed for metabolic transformation. Last but not least, such bulky molecules will not trespass the nuclear envelope in order to get into interaction with DNA.

Highly hydrophilic substances will be hardly absorbed in the organism, either. Even so, they are rapidly excreted which mitigates their DNA damaging activity. On the other site, highly lipophilic chemicals will not effectively dissolve in blood plasma or cytoplasm, which are aqueous media, which, again, hinders them to reach and damage genetic material. For an effective mutagen, a balance between its lipophilicity and hydrophilicity is needed.

Regarding the polycyclic aromatic substances such as PAHs, dioxins (tetrachlorodibenzo-p-dioxin (TCDD)), aflatoxin B1, etc., planarity in structure is an important element which determines their mutagenic potential. Thus, molecules of planar in structure, with less than four aromatic rings connected in line and molecular size 100–150 Å, are potent mutagens (**Figure 18**).

Two PAHs with six benzene rings may be demonstrated as an example to that rule. Benzo(a) pyrene has six benzene rings which are not all linearly bound. Pentacene also consists of six benzene rings, but they are all in a single line. Alignment of rings gives more plenary structure to benzo(a)pyrene over pentacene, which results in benzo(a)pyrene being a potent carcinogen and pentacene an inert molecule in terms of mutagenicity (**Figure 19**).

The second example when difference in planarity significantly alters mutagenic potential of the molecules is 2-aminobiphenyl and 4-aminobiphenyl. They are both products contained in cigarette smoke. However, 4-aminobiphenyl is planar, and amino group can be N-hydroxylated forming the product than attacks neutrophilic centre on C8 of guanine and forms DNA adduct.

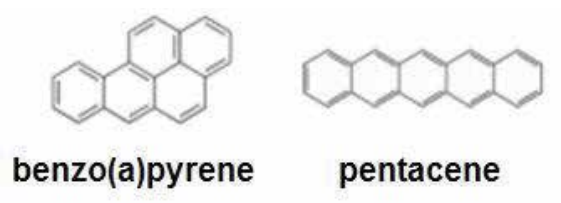

Figure 18. Example of two molecules with an equal number of benzene rings. However, opposite to pentacene, benzo(a) pyrene has less than four rings linearly aligned and exhibits potent mutagenicity.

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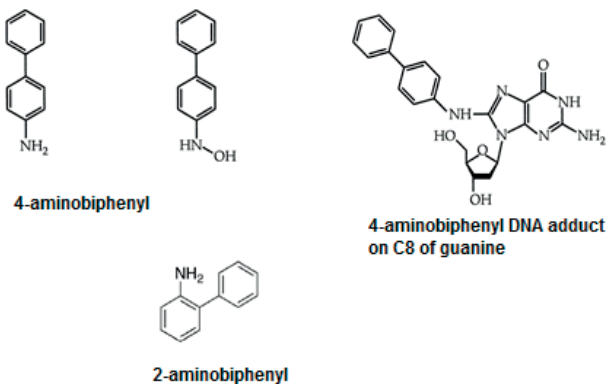

Figure 19. Significance of planarity in DNA binding on example of aminophenyls.

Opposite, 2-aminobiphenyl has lost its planarity, amino group is not achievable for metabolic transformation and the substance remains inactive in terms of carcinogenicity.

The effect of hydrogen substitution may also exert effects on mutagenicity of the substance of interest. It has been proven that substitution of a chloro, methoxy or methyl group in ortho-position to amino group of phenylamine enhances its mutagenic potency (**Figure 20**). It is because ortho-substituted phenylamine forms adducts with DNA that are more efficient in affecting hydrogen bonding with substitute instead of complementary base, thus having higher mutagenic potential.

However, in other cases when mutagenic potency is not determined at the level of already formed DNA adduct and its ability to bind non-complementary nitrogenous base, but earlier at the stage of metabolic transformation, the size of substituent is a critical factor affecting its later activity. For instance, bulky substituents sterically hinder N-hydroxylation or N-acetylation of neighbouring amino group. This may prevent its activation, for instance, later formation of nitrogen radical, as we talked earlier when discussing mechanisms of metabolic activation of electrophilic centres.

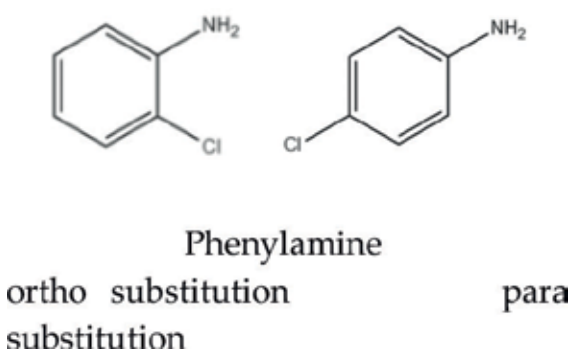

Figure 20. Ortho- and para-substitution of phenylamine as a cause of differences in mutagenic potency.

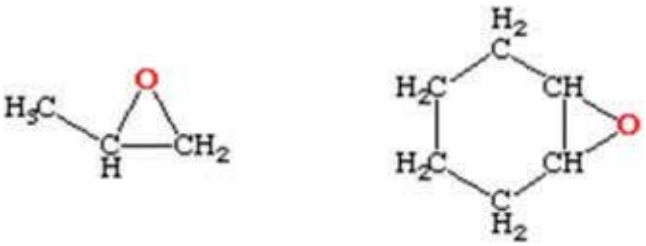

Figure 21. More reactive epoxide on aliphatic chain and less active one on cycloaliphatic ring.

Flexibility in the structure of molecule strongly affects several potential electrophilic centres that are formed by opening of the triangle structures (e.g. epoxides and sulfonium ions). Cycloaliphatic rings are rigid in comparison to aliphatic moieties. Thus, epoxide formed on the ring will be less active than the one formed on the chain structure which will more easily open (**Figure 21**).

Earlier, when discussing epoxides formed in resin monomers such as BisGMA, TEGDMA and UDMA, we already mentioned that as more potentially electrophilic centres are present in the molecule, the higher carcinogenic potential is (**Figure 11**). Distance between multiple electrophilic centres additionally contributes to reactivity of the substance.

Finally, resonance stabilisation of a metabolically formed electrophilic centre prolongs its half-life. Most electrophiles are readily hydrolysed or neutralised by antioxidant molecules in the cell (e.g. glutathione). Resonance stabilisation remains electrophile reactive providing them better chances to reach genetic material and interact with DNA. It is achieved by the presence of conjugated double bonds, aryl moiety, aromatic rings and structures that allow electrophilic centres to remain silent in cyclic form until they reach the target molecule [7].

5. QSAR as the method of choice

Predicting mutagenic and carcinogenic activity is a quite complex task. It demands not only the knowledge of chemical structure of substance of interest together with all functional groups being acknowledged but also its physical and steric properties, as well as metabolic pathways of the substance in organism. Thus, for efficient assessment, a vast database is crucial, which is obtained by assembling all available knowledge gathered by analysing and collecting data obtained for as large number of substances as possible experimentally, empirically, from results of epidemiological studies and case reports.

By using previously described SAR regularities in prediction of carcinogenicity of the substance of interest, quantitative structure–activity relationship (QSAR) was developed as a relevant non-experimental tool. It is considered that the first QSAR, at its simplified basic level, was Mills equation which predicted melting and boiling points of chemicals based on the number of carbon atoms in the chain [13]. Other pioneers in QSAR methodology are Overtone [14] and Mayer [15] who deduced that that the toxicity of organic chemicals to

aquatic species is proportional with their partition coefficient. This research directly bound chemical structure and biological activity of chemicals in living organisms. However, Hansch [16] who is considered to be the father of QSAR publishes research showing that a range of biological activities could be modelled mathematically using simple physicochemical properties. Following the entrance of the substance into the organism, the chemical is subjected to the absorption, metabolism, excretion, metabolic changes and transport to nucleus where it can react with nitrogenous bases of DNA. As discussed earlier, predictive modelling studies aiming to exploring all such attributes that affect the activity, property and toxicity of chemicals are bases for developing the database that will be used in QSAR.

How does exactly the QSAR function? QSAR grew out of physical organic chemistry based on studies to show how differential reaction rates of chemical reactions depend on the differences in molecular structure. In classical means QSAR model was developed on the basis of comparison of so-called descriptors from the tested substance of interest with dataset obtained during the years of empirical testing of substances of known structural, steric and physical characteristics [17]. In the contest of QSAR, molecular descriptors are characteristics regarding specific information about a substance of interest [18]. These are its chemical structure and properties, steric properties, physical properties and, for substances already present in database, biological/toxicological activity. For the purpose of QSAR, descriptors from qualitative are transferred to the numerical or quantitative representations of chemical by using suitable algorithms. Thus, QSAR is a simple mathematical model that can correlate chemistry with the properties of substance of interest using various computationally or experimentally derived quantitative parameters known as descriptors [17]. They are used as independent variables for mutagenicity prediction model development [19]. The selection of relevant descriptors is a well-known issue in QSR, and its reliability depends on the quantity of substances that have been evaluated for all screened molecular descriptors and entered the referent database [17].

Regarding the database in relation to which descriptors are evaluated, it gathers the knowledge regarding physicochemical (hydrophobic, steric or electronic), structural (based on frequency of occurrence of a substructure) and molecular structure, relations between functional groups, their mutual influence and influence of entire chemical structure of the molecule, possible pathways and products of their metabolic activation and others (as spoken earlier) that may affect the biological properties of detected functional groups.

Based on above-discussed conditions for electrophilic centre to affect DNA, descriptors may be classified into several groups: substituent constants; whole molecular, topological and structural descriptors, indicator variables; thermodynamical descriptors; and electronic and spatial parameters.

Substituent constants are basically physicochemical descriptors mediated by differences in molecular structure of the molecule. Whole molecular descriptors represent expansions of the substituent constant approach and represent features other than functional groups. For instance, they are lipo−/hydrophilic ratio, dissociation constants, van der Waals volume, etc. Topological descriptors represent the position of the individual atoms and the bonded connections between them. Structural descriptors refer to content of functional groups [19]. Indicator variables are used for comparison of two molecules by utilising all other independent

variables. It can be employed only when the two sets of compounds are identical in every respect. Electronic parameters describe electronic aspects of both the entire molecule and its specific parts, such as atoms, bonds and molecular fragments. Spatial parameters reflect spatial arrangement of the molecules and the surface occupied by the molecules.

Several prerequisites are necessary for model development in classical QSAR: the compounds to be studied should be closely related congeners, thereby increasing the probability of having the same mechanism of action; the biological activity data to be used in modelling should be accurate and measured under uniform conditions; and the activity parameter must be intrinsically additive [20]. Thus, in certain restricted way, it may be indicated that QSAR relies on read-across approach, since it evaluates the substance of interest based accordingly on substances that have been evaluated and for which a structure-action relationship has been identified.

6. Conclusion

Several prerequisites are necessary for model development in classical QSAR: the compounds to be studied should be closely related congeners, thereby increasing the probability of having the same mechanism of action; the biological activity data to be used in modelling should be accurate and measured under uniform conditions; and the activity parameter must be intrinsically additive [21]. Thus, in certain restricted way, it may be indicated that QSAR relies on read-across approach, since it evaluates the substance of interest based on according substances that have been evaluated and for which a structure-action relationship has been identified.

Conflict of interest

There is no conflict of interest.

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Chapter 5

Genotoxicity by Electromagnetic Fields

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Additional information is available at the end of the chapter

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Abstract

Modern life implies a constant exposure of living organisms to many sources of radiation, especially electromagnetic fields (EMFs) generated by our technological devices. The question of whether or not EMFs in the non-ionizing extremely low frequency (ELF) range can induce genotoxic effects is currently a subject of interest. People of industrialized societies are commonly exposed to EMFs and waves in a very broad range of frequencies, including power lines, telecommunications, and domestic and industrial equipment. In this review, we present controversial evidence from our research group and others of genotoxicity induced by ELF-EMFs, since scientific community consider EMF devices produce marginal amounts of energy, which does not justify any DNA alterations, together with conflicting laboratory results and few epidemiological studies. However, in 2002 the International Agency for Research on Cancer (IARC) categorized ELF-EMFs as being potential carcinogenic and genotoxic agents to humans. The aim of the present chapter is to discuss the role of ELM-EMFs on human genotoxicity.

Keywords: genotoxicity, human DNA, non-ionizing radiation, electromagnetic fields, low-frequency radiation, extremely low frequency radiation

1. Introduction

Human exposure to electromagnetic fields (EMFs) and waves is a common feature of modern life. We have learned to understand the physical characteristics of these energy forms, and have applied them in many ways to embellish our ways of life and our standards of living. Furthermore, individuals have become dependent on them for health, safety, information, comfort, and conveyance. In fact, it has been determined that living in a major metropolitan region will increase at least three-fold exposure to environmental EMFs, than that of people living in suburban of rural areas, although the level of exposure depends on the proximity and time of exposure to a radiation source.

EMFs and waves are reported to produce direct and indirect effects on genes and chromosomes of living beings, which depend on many physical, chemical, and biological factors. They may be visible or not soon after exposure. However, there may be subtle changes only detectable upon careful laboratory study, or be apparent after a long period of time.

Our understanding of the interaction of EMFs with living systems is progressing in a wide range of areas. Nowadays, the increasing amount of research related to the evaluation of EMFs genotoxic effects, lead to consider the potential risk associated with EMFs exposure. In the last 4 decades, research on the genotoxic and cytotoxic effects and health implications of EMFs, not only has expanded, but also has become a subject of a public concern and private debate worldwide.

It is known that the interaction of EMFs and waves with biological systems is frequency-dependent. High frequencies possess more energy and different interaction mechanisms than the low ones. The focus of this chapter is on the recent developments and our experience on living systems interaction of very-low and extremely-low EMF frequencies.

2. Electromagnetic fields and waves

Although gamma and X-rays, ultraviolet rays, visible light, infrared radiation, microwaves, radiofrequency, and slowly varying electric and magnetic fields are typical of the electromagnetic spectrum, they differ in their interaction with physical materials and living organisms. This difference mainly resides in their specific wavelength, since they all travel at the speed of light. The energy of these waves propagates in bundles of photons, and the energy in a photon is inversely proportional to the wavelength. Therefore, the shorter the wavelength, the higher the energy per photon. Clearly, the photon energies vary over a wide range of values [1].

Gamma rays and X-rays possess high amounts of energy and are capable of ionization, that is, they produce ions by ejection of orbital electrons from the atoms of the material thorough which they travel [2]. Their biological effects, therefore, result largely from the produced ionization. On the other hand, in the non-ionizing region of the spectrum, ultraviolet radiation is important for a number of biological processes and has also been shown to have deleterious effects on many biological activities [3]; one common effect of ultraviolet radiation is sunburn. Ultraviolet is known to kill several microorganisms and is reported to have carcinogenic effects as well. It transmits its energy to atoms or molecules almost entirely by excitation, that

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is, it promotes electrons to higher orbits. Consequently, some of the effects produced by ultraviolet radiation may resemble the changes resulting from ionizing radiation. In fact, ultraviolet radiation is considered the limit value between ionizing and non-ionizing radiation.

In regard to non-ionizing radiation, photosynthesis, plant growth, and vision depend on visible light. These wavelengths are no capable of ionization nor excitation, but they produce photochemical or photobiological reactions. Infrared radiation from the sun is the major source of the Earth's heat, and it is also emitted by all living beings. There is little evidence showing that photons in the infrared region are capable of initiating photochemical reactions in biological materials [4]. Although thermochemical reactions may follow photochemical reactions, changes in vibrational modes are responsible for absorption in the infrared region. The absorbed energy increases the kinetic energy of the system, which in turn, is dissipated in the form of heat. Thus, the primary response of biological systems to infrared exposure is thermal. It is customary to use frequency instead of wavelength to denote electromagnetic energy at the microwave range and below. The microwave region extends from 300 mHz to 300 GHz, radiofrequency from 30 kHz to 300 MHz, VLF (very low frequency) from 3 to 30 kHz, and ELF (extremely low frequency) ranges from 3 Hz to 3 kHz, which includes the power system frequency of 50–60 Hz. Microwave and radiofrequency fields primarily increase the kinetic energy of the exposed systems, upon absorption by the biological materials [5]. In this case, the increased energy is due to changes of the vibrational or rotational energy state that dissipates into heat.

At VLF and ELF frequencies, EMFs have wavelengths that are much larger than typical dimensions of life bodies. The electric and magnetic fields become quasi-static and can be considered separately. Because the living organisms contain almost no magnetic material, lower frequency magnetic fields can penetrate without attenuation. Furthermore, electric fields are induced within the life bodies by these time-varying magnetic fields and cause the so-called "Eddy currents" to flow inside the body. These currents represent the primary biological effect attributed to such ELF-EMFs.

An important characteristic of the effect of ionizing and non-ionizing (photochemical reactions produced by absorbed light) energies on humans is that they are cumulative. However, at present there is no scientific evidence indicating any cumulative effect due to exposure to electromagnetic energy in the microwave, radiofrequency, and the lower frequency region. Available information suggests that the observed effects diminish as the intensity is reduced to a low level and repeated exposures do not seem to deviate from this conclusion [5]. This is probably because at low levels, the organism has an opportunity for recovery to an injury (if any) from exposure; however, it is uncertain if prolonged exposure to low level ELF fields has seriously harmful biological effects, such as genotoxic or carcinogenic is still open.

Penetration of ELF-EMFs to tissues increases with frequencies. For instance, at 50–60 Hz, the internal electric field (IEF) of a person standing under a powerline is about 10^{-6} times weaker than that with the external field strength. The marginal IEF under such conditions become one of the reasons why research on ELF health effects has focused on magnetic fields. In contrast, low-frequency magnetic fields are not attenuated by biological tissues, thus the internal and external magnetic field intensities are identical. EMF energy absorption by humans is the highest between 30 and 300 mHz, which is the resonance range for the whole body. Above this, EMFs penetrate the human tissues as electromagnetic waves, rather than

separate electric and magnetic fields. Furthermore, at the microwave range, electromagnetic waves penetration depth decreases with increasing frequency [6].

In order to understand the genotoxic and cytotoxic effects attributed to ELF-EMFs, it is necessary to consider the electromagnetic induction. Magnetic fields induce electrical currents in conductors. Cells and tissues are affected by these currents (as mentioned before, the so-called "Eddy Currents"), with the increase of frequencies the magnitude of the induced current increases. Strong magnetic fields lead to changes in orientation, rotation and movement, and deformation and fusion or destruction of cells. Quantum processes are not possible, because the quantum energy of the fields is not big enough to break molecular bonds, as an ionizing radiation does [7].

3. Carcinogenesis

Electromagnetic fields at very- and extremely-low frequency regions, were classified as "possibly carcinogenic" by the International Agency for Research on Cancer [8], based on pooled analyses of epidemiological research that reported an association between exposure to low-level magnetic fields and several types of cancer.

As genetic damage is very often a signal for cancer, some publications have reported associations between EMF exposure and DNA damage [9–11], but other studies showed conflicting results [12–14]. In this regard, several epidemiological studies support a weak to moderate association between exposure to magnetic fields in residential or occupational environments and the incidence of cancer [15–17], particularly acute leukemia and brain cancer. An association between EMFs exposure and cancer has been suggested, but it has not been evidenced, however, a number of well-designed residential and occupational studies are underway. A solid case for causality will depend on various factors including consistent associations of magnetic fields and cancer, improved exposure characterization, dose-response data, and full evaluation of potential confounding factors. The very complex phenomenon of carcinogenesis suggests that ELF-EMFs can alter cell growth in many ways involving hormone secretion. Melatonin, the principal pineal hormone, exerts a suppressive action on other endocrine glands. Reduced circulating concentrations of melatonin can result in increased prolactin release by the pituitary and increased estrogen and testosterone release by the gonads. ELF-EMFs have been reported to suppress melatonin production by the pineal gland [18–20]. On the basis of these findings, it may be postulated that magnetic fields may increase the risk of certain hormone-dependent cancers, i.e. breast and prostatic carcinomas.

At present, the available information suggests that ELF-EMFs may cause cancer. Much research (epidemiology, animal bioassays, mechanistic studies, and basic biology), however, remains to be done in order to assess the full carcinogenesis potential of ELF-EMFs and to evaluate, in quantitative terms, the level of risk of ELF-EMFs intensities in the order of magnitude to which humans are currently being exposed. ELF-EMFs then represent one of the priority issues on environmental and occupational carcinogenesis. Our knowledge of the carcinogenicity of ELF-EMFs leads to a wider concern about possible similar effects of non-ionizing electromagnetic radiation other than ELF-EMFs. In future characterizations of possible cancer risk, considerable reliance will probably be given to laboratory investigations which will, on demand, include genotoxicity research.

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4. The genotoxic potential of magnetic fields

Biological effects of magnetic fields have been widely discussed during recent years. The question has been raised as to whether exposure to such fields causes genetic damage. Many researchers agree that life bodies could be genetically affected by exposure to magnetic fields [21–25]. Nevertheless, the issue of ELM-EMFs genotoxic potential is controversial, mainly related to the fact that many scientists believe ELF-EMF devices emit low energy and are therefore too weak to have any effect on cells. Because of the low energy levels in molecular interactions, it is physically highly improbable that ELF-EMFs cause direct genetic damage. However, it has been theorized that these fields may enhance such damage from other sources, e.g. endogenous radicals [26]. Furthermore, the inconclusive nature of laboratory experiments turns this concern more conflicting. Regarding the issue that weak fields may have too low energy to cause genotoxic effect or DNA damage, it has been proposed that because low frequency electromagnetic radiation does not transmit enough energy to alter chemical bonds, ELF-EMFs do not directly damage DNA [13, 27, 28]. However, several hypotheses of the indirect effect of EMFs on DNA structure, have been suggested. For this, secondary currents and, hence, a movement of electrons in DNA might be induced [29, 30]. This may, in turn, produce guanine radicals, which, upon reaction with water, induce oxidative DNA damage [31, 32]. Recently, Focke et al. [33] reported that exposure of human primary fibroblasts to a 50 Hz EMF at 1.0 mT caused a slight, but significant increase of DNA fragmentation, as tested by the Comet assay. They also showed that EMF-induced responses in this assay were dependent on cell proliferation, suggesting that processes of DNA replication, rather than the DNA itself may be affected.

Three important reviews published in the 90s [6, 34, 35] and recently by Maes and Verschaeve [26], concluded that ELF-EMFs do not directly cause genotoxic effects. Only a small minority of the reported studies indicate potential of these fields to cause genetic changes in biological systems. A few studies have addressed the possibility that ELF-EMFs could enhance the action of known genotoxic chemicals or ionizing radiation. There is some evidence that ELF-EMFs might enhance the genotoxic potential of gamma radiation [36], X-rays [37]; or mutagenic chemicals [38].

A critical review by Vijayalaxmi and Obe [39] concluded that 22% of previous studies of ELF-EMF-induced genotoxicity indicated a genotoxic effect, whereas 46% did not and 32% of the studies were inconclusive. Recently, Dominici et al. [40] reported a significant high micronuclei frequency in human blood cells of welders exposed to ELF-EMF, in a dose-dependent manner. Yaguchi et al. [41, 42] also showed that exposure to 5, 50, and 400 mT ELF-EMF can induce sister chromatid exchanges and chromosomal aberrations in murine m5S cells. Similarly, Lai and Singh [43] observed genotoxic effects of these fields, finding that exposure of rats for 2 h to a 60 Hz magnetic field (0.1, 0.25, and 0.5 mT) increased DNA strand breaks in brain cells in a dose-dependent fashion, indicating a clastogenic effect.

In relation to ELF-EMF long exposures, Rageh et al. [44] observed a significant increase in rat bone marrow micronuclei continuously exposed for 30 days to 50 Hz and 0.5 mT magnetic fields, suggesting an association between ELF-EMF exposure time and DNA damage. In

contrast, Abramsson-Zetterberg and Grawé [45], reported that an 18-day exposure to 50 Hz and 14 μT magnetic fields did not significantly alter micronucleated red cells frequency in fetal and adult mice.

Despite the large number of published works in recent years, there is no conclusive evidence supporting causality of exposure to ELF-EMFs and genotoxicity.

5. Our experience regarding genotoxicity induced by ELF-EMFs

In view of these conflicting results, several years ago we developed some studies aimed to evaluate the genotoxic and cytotoxic potential of ELF-EMFs. In a report by Heredia-Rojas et al. [46], lack of genotoxic effect of 60 Hz magnetic fields on sister-chromatid exchange (SCE) frequency of cultured human peripheral blood lymphocytes, but altered cell proliferation, as measured by proliferation (PI) and mitotic (MI) indexes, were observed; exposed lymphocytes showed higher PI and MI than controls. It was also shown no synergistic effect of magnetic fields and mitomycin-C (a well-known genotoxic agent) on SCE frequency. However, PI and MI in cultures treated with mitomycin-C and exposed to magnetic fields were higher than those in cultures treated with mitomycin-C alone, indicating that proliferating activity increase may increase the overall risk of mitomycin-C induced genomic damage [46].

The issue of potential genotoxic and cytotoxic effects of magnetic fields in the ELF region has developed almost completely from cytological studies in somatic cells. However, meiotic cells offer a good model to establish a relationship between magnetic field exposure and cytotoxicity. Based on this, we evaluated the effect of *in vivo* exposure of mice to a 60 Hz sinusoidal magnetic field at 2.0 mT on male germ cells. No statistically significant differences on meiotic chromosome aberrations in spermatocytes and sperm morphology were observed between magnetic field-exposed and control animals. Furthermore, when animals were co-exposed to magnetic fields and mitomycin-C, an antagonistic effect in terms of meiotic chromosome aberrations and sperm morphology abnormalities were observed. Treated animals showed, in spite of mitomycin-C genotoxicity, lower percentages of meiotic chromosome aberrations and sperm morphology abnormalities, when compared with animals treated with mitomycin-C alone [47].

5.1. Effects of ELF-EMFs on immune function

It has been proposed that the effects of ELF-EMFs depend on the biological-functional state of the cells, in particular on the degree of cellular activation for cells of the immune system, and this is correlated with genetic damage. For that reason, we decided to perform bioassays trying to demonstrate the ELF-EMFs effects on immune parameters. We have previously reported absence of proliferation of murine thymic lymphocytes, production of nitric oxide and phagocytosis of *Candida albicans* by peritoneal murine macrophages effects after 60 Hz and 1.0 mT treatment [48]. In contrast, 72 h exposure to 60 Hz and 2.0 mT oscillating magnetic fields significantly increased number of apoptotic-like cells and cellular immune response in *Trichoplusia ni* (Lepidoptera:Noctuidae) larvae [49]. Furthermore, conflicting results on the effect of ELF-EMFs on the immune system have been reported [50, 51].

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5.2. Effects of ELF-EMFs on gene expression

It has been reported that 50–60 Hz magnetic fields with flux densities ranging from microTesla to milliTesla, induce changes in gene expression, and this in turn, can increase the overall risk for genotoxicity, which is considered in the search of gene-environment interactions. Based on this, we have evaluated the effect of 60 Hz sinusoidal magnetic fields at 8.0 and 80.0 μT on expression of the luciferase gene, contained in an own gene construct labeled as electromagnetic field-plasmid (pEMF), which was transfected into HeLa and BMK16 cell lines, later exposed to magnetic fields; this vector included the hsp70 promotor containing the 3 nCTCTn sequences, previously described for the induction of hsp70 expression by magnetic fields, as well as the reporter of the luciferase gene [52]. For this bioassay, a positive control of thermal shock treated cells was included. Interestingly, we observed an increased luciferase expression after exposure to magnetic fields and thermal shock, compared with controls. Furthermore, a synergistic effect between two factors on luciferase gene expression, was observed [52]. In another study, Heredia-Rojas et al. [53], demonstrated that a magnetic field with characteristics aforementioned, increased luciferase gene expression and activity in INER-37 cells. However, this treatment had no effect on RMA E7 cells [53]. Recently, Rodríguez-de la Fuente et al. [54], showed significant luciferase expression increase in mice exposed to ELF-EMFs (80 μT and 60 Hz frequency) for 2 h a day for 7 days, with prior pEMF vector electro-transferred to BALB/c mice quadriceps muscles, as compared with controls. Our work of magnetic field effects on gene expression was summarized in a recent book chapter [55].

Taking together, the resulting research data, along with other reports by others, suggest that ELF-EMFs are involved in DNA damage.

5.3. ELF-EMFs in pre-clinical studies

There is an increasing interest in the use of magnetic fields in medicine. The notion that magnetic fields can be used for therapeutic purposes has existed long before they were understood or were controllable. Particularly, pulsed electromagnetic fields (PEMFs) technology was based on 2 decades of studies related to the electromechanical properties of bone and other connective tissues. Effectiveness of such fields to treat a number of health conditions has been recently demonstrated.

We have evaluated the genotoxic effect induced in PEMF-exposed rats, using a patented medical device (US patent 6,235,251 B1). The cytological endpoints included acridine orange fluorescent-staining micronucleus test and male germ cells analysis and the observed results showed that the applied magnetic fields generated by the therapeutic device did not have any detectable genotoxic effect in exposed rats, compared with the unexposed controls [56].

In addition, we have reported a clastogenic effect of 60 Hz magnetic fields on mice bone marrow, as assessed by *in vivo* micronucleus (MN) test [57]. As mentioned before, it is accepted that ELF-EMFs do not cause breaks in DNA because they are unable to transfer energy to cells in sufficient amounts to damage DNA directly and thus, in the past they were considered to be non-genotoxic. Nevertheless, we observed a higher MN frequency in mice exposed to 60 Hz magnetic fields at 1.5 and 2.0 mT, compared with controls. Recommendations by Vijayalaxmi

and Obe [39] due to the conflicting nature of laboratory experiments and variability, we decided to develop three independent experiments at three different times and in three different laboratories located in our Department of Exact Sciences and Human Development in Biological Sciences School at Autonomous University of Nuevo Leon, Mexico, the Department of Pathology in Medicine School at this University, and the Institute of Biomedicine at the Mexican Institute of Social Security, Mexico. A high MN frequency in exposed animals was observed in all bioassays. For acute treatments, an exposure time of 72 h was chosen because it is generally accepted that the period of differentiation from stem cells to mature erythrocytes in mice is about 72 h [58]. In addition, it was reported the same antagonistic effect between ELF-EMF exposure and mitomycin-C, as previously observed in cultured human lymphocytes and mice germ cells mentioned above [46, 47].

6. Concluding remarks

It is clear that the current knowledge of bioeffects of weak magnetic fields is limited and inconclusive to establish a causality with genotoxic effects. However, evidence is beginning to accumulate both from epidemiological studies and laboratory work that might be enlightening to define genotoxic risks involved with exposure to ELF-EMFs. Further studies are needed to clarify this and consequently the interaction mechanisms involved.

The absence of independent replication has been a consistent feature of experimental studies searching for biological effects of weak ELF-EMFs. It remains to be determined whether the present reports on DNA damage will be substantiated, demonstrating a potential relevance to a chain of events leading to genotoxicity, which is considered the gold standard to define if an environmental factor is a carcinogen, but the currently available data for extremely-low frequency time-varying magnetic fields remain conflicting. As an environmental stimulus, the effect of ELF-EMFs on cellular DNA may be subtle. Therefore, a more sensitive method and systematic research strategy are warranted to evaluate genotoxicity. Meanwhile, we believe it would be a good practice to adopt the discreet avoidance strategy. Environmental EMFs are generated, in part, by the transmission and distribution of 60 Hz electric power using overhead lines and by those of electricity in residential buildings, and in the workplace. Other sources, in particular inductive devices such as electric motors, also generate localized, relatively intense magnetic fields. Regarding the workplace or home environment, this could include the choosing of low emission appliances when new equipment is considered, and switching off the apparatus when not in use.

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Conflict of interest

The authors claim they have no conflicts of interest to declare.

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Chapter 6

Genotoxic Risk in Human Populations Exposed to Pesticides

Delia Aiassa

Additional information is available at the end of the chapter

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Abstract

The importance of early detection of genetic damage is that it allows taking the necessary measures to reduce or suppress the exposure to the deleterious agent when it is still reversible, thus decreasing the risk of developing diseases. For this reason, genotoxicity tests should be considered as indispensable tools in the implementation of a complete medical surveillance in people potentially exposed to various environmental pollutants and especially those who live in the same place with people who have already developed some type of neoplasia at early ages in order to prevent the occurrence of tumors of environmental origin and work-related. On the other hand, the application of these tests is useful to detect possible long-term effects of substances that are introduced to the market without knowing exactly their capacity to affect human and environmental health.

Keywords: genotoxicity, pesticides, Argentina

1. Introduction

1.1. Pesticides

Pesticides are a heterogeneous group of chemical compounds used in the production of food and considered one of the major sources of contamination by synthetic substances generated as a result of agricultural activity. For more than a decade, many of them have been classified as potential carcinogens [1, 2].

The Food and Agriculture Organization of the United Nations (FAO [3]) defines a pesticide as any substance or mixture of substances intended for preventing, destroying or controlling any plague, including vectors of human or animal diseases, unwanted species of plants and

animals that cause harm or interfere in any other way in the production, processing, storage, transportation or commercialization (marketing) of food, agricultural products, wood and its derivates [4].

The benefits obtained by the use of pesticides are certainly numerous, however, the dissemination of large amounts of these compounds to the environment, has led to problems affecting both the environment and human health [5]. Particularly, in agricultural activities, agrochemicals are widely used products, and its use without the necessary protection can lead to genetic alterations and the possible development of some types of neoplasia [6, 7].

Exposure to these substances results in acute poisoning. Poisoning is the body's reaction to a toxic agent, and it is described as acute poisoning when the symptoms occur after a recent exposure to the chemical. In this kind of intoxication, the diagnosis is relatively easy, fast and with an established treatment.

Chronic health effects have been associated to pesticide exposure, including neurological disorders, reproductive or developmental problems and cancer. Epidemiological studies on farmers, pesticide manufacturers, pesticide sprayers and on accidentally exposed industrial workers or residents have shown that exposure to pesticides may increase the risk of site-specific cancers. Also, increased risks have been detected for leukemia, Ewing's bone sarcomas, kidney cancer, soft tissue sarcoma, non-Hodgkin's lymphoma, and testicular, colorectal, endocrine glands and brain cancers in children exposed to pesticides in their home or whose parents were occupationally exposed to pesticides [8].

1.2. DNA damage

Experimental data reveal that the chemical substances used in food production contain many components that affect the genetic material of organisms—they are genotoxic agents—([9–16]) and they may be responsible for the high incidence of different types of cancer (both in children and adults), reproductive problems or malformations in the offspring of populations occupationally and/or environmentally exposed to these compounds.

It has been observed that the offspring of agricultural workers have a higher risk of congenital anomalies. However, congenital anomalies in the mid-1990s represented around 20% of deaths during the first year of life in some countries, and in other countries, they represented almost 40% of deaths [17].

A genotoxic agent is described as a physical, chemical or biological agent that can interact with the genetic material (DNA) of organisms causing alterations, damage or ruptures.

This term includes agents that interact both directly and indirectly with the DNA causing ruptures and, also, those that interfere with enzymatic processes of repair, genesis or polymerization of proteins involved in chromosome segregation. Consequently, they may change the structure of a specific genome. Genotoxic agents can bind directly to DNA or act indirectly by affecting the enzymes involved in the physiological modifications of DNA during replication or transcription. These alterations could lead to impaired embryonic development or be the initial steps in the development of cancer. Genotoxic agents are not necessarily carcinogenic, but most carcinogens are genotoxic.

Genomic damage is probably the most important and fundamental cause of neurodegenerative disorders, reproductive effects and developmental problems [8]. It is also well established that genomic damage is produced by exposure to environmental contaminants (e.g., metals, pesticides), medical procedures (e.g., radiation and chemicals), micronutrient deficiency (e.g., folate), lifestyle factors (e.g., alcohol, smoking, drugs and stress), and genetic factors such as inherited defects in DNA metabolism and/or repair (Holland et al [18–20]).

Therefore, in recent years, there has been an increase in the number of studies that seek to understand and evaluate, using biomarkers, the possible consequences that exposure to pesticides has on the environment and mainly on human beings [21–23].

1.3. Genotoxicity biomarkers

Biomarkers are biological parameters that provide information about normal or pathological states of an individual or a population, and they are used for monitoring different aspects of a disease such as: treatment, prevention, diagnosis and progression of the disease, responses to the therapy, experimental toxicological evaluation of drugs or pesticides, environmental and epidemiological risk measurement, as well as evaluation of therapeutic intervention, among others [24].

In this sense, the use of genotoxicity biomarkers—chromosomal aberrations (CA), micronuclei (MN), sister chromatid exchanges (SCEs) and comets (CO)—has been relevant to analyze the potential risk of a substance, as they reveal the damage to the DNA, the molecule that transmits genetic information through generations. Therefore, they are considered suitable biomarkers to evaluate the risk of a potentially harmful substance and, in addition, their carcinogenic risk [66].

The chromosomal aberration test detects numerical (aneugenic effect) or structural (clastogenic effect) alterations at the chromosomal level. The importance of this test lies in experimental and epidemiological evidence suggesting that structural aberrations are involved in the carcinogenesis process, and, therefore, a high frequency of chromosomal aberrations is associated with an increased risk of developing cancer in the future ([26–30]).

Micronucleus test detects breaks at the chromosomal level and alterations of the mitotic apparatus, allowing the identification of compounds with aneugenic and clastogenic effects. The simplicity of the test is an advantage and the number of cells scored (1000 cells) gives statistical significance to the study.

The prospective analysis of a database of 6700 subjects from 20 laboratories representing 10 different countries have confirmed that a high frequency of micronuclei is predictive of an increased risk of cancer (Bonassi et al. [31–33]).

Sister chromatid exchanges (SCEs) are another cytogenetic assay to evaluate alterations at the chromosomal level. The exchange between sister chromatids occurs precisely by the reciprocal exchange of DNA between two sister chromatids in a duplicated chromosome. The frequency of exchange in eukaryotic cells is increased by the exposure to genotoxic agents that induce DNA damage by interfering with its replication, but it is not increased by those agents that only induce breaks in the DNA strands [28]. However, the formation mechanisms of these alterations are not completely elucidated, and, therefore, their biological significance is still uncertain [28].

The main molecular studies are (1) molecular cytogenetics to detect inversions, translocations, or to identify the chromosomal origin of micronuclei and (2) comet assay in lymphocytes.

The comet assay is an electrophoresis technique in agarose microgels considered to be highly sensitive to detect DNA damage in single cells. It detects DNA single- and double-strand breaks, labile alkali sites, and DNA-DNA or DNA-protein cross linking associated with repair sites by incomplete excision. When the nucleus is subjected to electrophoresis, the DNA fragments migrate in a pattern that resembles a comet, hence the name of this assay [34].

The studies that define the mechanisms of action and/or the cytotoxic and genotoxic effects can be performed at different levels of complexity. In vitro assays are very useful to detect the genotoxic effects of various agents in human cellular systems. Although these models do not include the toxicokinetics of substances (absorption, distribution, metabolization and excretion), it is possible to evaluate their potential effects using a wide range of biomarkers [25].

In vivo genotoxicity, studies provide a physiological framework to the activity of different agents with genotoxic potential. This allows to evaluate, under controlled conditions, a systemic response to the agent in question and to discern the effects according to the route of entry of the agent to the organism. These studies bring the results one step closer to real human exposure.

Finally, epidemiological studies use different genotoxicity biomarkers for the study of populations exposed to toxic agents. At the international level, there are numerous studies evaluating the effect of pesticides on the genetic material of exposed populations; however, in Argentina, these are still scarce [21, 35, 36].

1.4. Populations human exposed to pesticides

Studies conducted in populations exposed to pesticides, mostly in European applicators, show positive association between exposure to a complex mixture of agrochemicals and the presence of CA, SCEs, MN and/or CO [21, 37–39].

Argentine populations are exposed to complex mixtures of pesticides. In the province of Córdoba, the most commonly used mixtures contain glyphosate, cypermethrin, chlorpyrifos, and others as active ingredients ([40, 41]). Evaluating the genotoxic potential of components of the mixtures is the initial step to study its behavior to check possible antagonistic or synergistic effects that could modify the effect.

There are few reports regarding the genotoxic potential of glyphosate, cypermethrin and chlorpyrifos. Glyphosate herbicide has been studied in our research group by [12], Bosch et al. [16] and Barbosa et al. [42]. On the other hand, Kocaman and Topaktaş [43] reported on the effects of a commercial formulation of cypermethrin on peripheral blood lymphocytes, this is the only genotoxic and cytotoxic study in the available literature. Rahman et al. [44] and Vindas et al. [45] analyzed the genotoxic effects of chlorpyrifos on human cells performing the comet assay.

Therefore, there is a clear need to assemble a set of tests that cover different complexity levels so that we can have a more accurate approximation of the genotoxic potential of an agent on human population.

In this sense, it is important to highlight that a large part of the toxicity of many chemical substances is explained by their capacity to generate oxidative processes that can damage various cellular structures, including DNA; oxidative damage is, therefore, an important cause of genotoxicity [46]. One of the most commonly used techniques to evaluate the capacity of a substance to generate oxidative damage is through the quantification of thiobarbituric acid reactive substances (TBARS). A large number of reports in the literature show the oxidative effects of pesticides used in food production ([14, 47, 48]).

Given the impact of the problem raised, it is necessary to approach it not only from the biological sciences aspect (toxicological genetics), as discussed here, but to support it from the social sciences' perspective (legislation and environmental education).

The evidence of genetic risk as a result of exposure due to the intensive use of pesticides indicates the need to review the law enforcement, in order to develop educational programs aimed to control the use of these substances and/or implement prevention and protection measures.

Argentine legislation on pesticides is, in some cases, incomplete, permissive and/or obsolete [49, 50]. On the other hand, there is no participation of the Ministry of Health in the approval of pesticides registration for agricultural use. To the gaps or defects in legislation and the lack of control, is added the deficiency of measures that contemplate the effects of pesticides and their mixtures.

In the light of the foregoing, it is necessary to increase the scientific evidence regarding the toxicity and genotoxicity effects of chemical substances applied in our country. This will allow extending the legislation, adapting it to the real problems and, if necessary, modifying the permitted levels of pesticides and its mixtures in the environment.

In many countries, measuring the frequency of genetic damage in human groups exposed to environmental agents has been, for decades, a priority in public health studies, and the increased rates of chromosomal aberrations (CA) is commonly interpreted as evidence of genotoxic exposure and early biological effect on DNA.

It has long been known that there is a strong link between DNA alterations and cancer or chronic degenerative diseases. The carcinogenic process is initiated and promoted by alterations/mutations in areas where oncogenes, tumor suppressor genes and DNA repair systems are located [51, 52].

1.5. Genotoxic effects in children

Regarding the age groups and the DNA effects caused by these chemical substances, we must differentiate between adults and children. Children may be more sensitive to toxic agents compared to adults and the genetic damage occurring at an early age may represent adverse effects on adolescent or adult health (Landrigan et al. [53]; Roberts and Karr [54]). However, the information about the genotoxic effects in children is scarce, although in recent years, the number of studies has increased [35, 55, 56].

Children are a high-risk group concerning the effects of air pollution on health [53, 54, 57–59]. Some studies suggest that early childhood exposure to pollutants can lead to the development of chronic diseases in adulthood. The earlier the exposure, the higher the risk of developing a chronic disease, cancer included [60].

Among the adverse effects in children exposed to various environmental hazards, genetic damage receives special attention after it has been shown that an increased frequency of DNA damage in childhood is predictive of the development of cancer in healthy adults [61]. Children are still in an active development phase, and in this condition, their response to environmental risks may differ from that of the adults. The effects of this environment could manifest themselves many years, even decades, after exposure.

The clinical symptoms of acute pesticide poisoning are rarely pathognomonic; they can simulate an acute respiratory disease, conjunctivitis, gastrointestinal disease, cutaneous manifestations, among others.

In this sense, it agrees with Salameh et al. [62], Salam et al. [63], Alarcón et al. [64] and other authors who indicate that pesticide poisoning are commonly under-diagnosed.

Several studies show that an increase in the risk of developing cancer has been observed at high rates of both chromosomal aberrations and micronuclei [31, 32, 65, 66].

The presence of MN represents alterations that are the result of cell exposure to genotoxic contaminants.

The MNi originate from chromosome fragments or whole chromosomes that are lag behind during cell division and left outside the daughter nuclei. MNi can be assessed in different tissues such as blood and epithelial tissue, and they can be easily visualized through the optical microscope. In particular, nasal and buccal exfoliated epithelial cells have been used as biological control in people exposed to airborne contaminants since they are similar to epithelial cells of the respiratory tract and are easier to collect [18–20, 67–69].

The oral mucosal epithelial cells are the first barrier for substances introduced into the body by inhalation or ingestion; therefore, it is a suitable tissue to detect the genotoxic effects induced by airborne contaminants. Studies have shown a strong correlation between the MN frequency in buccal epithelial cells and blood cells, also related to the subsequent risk of developing cancer. Collecting the samples from this tissue is especially recommended for pediatric population due to the ease of the procedure ([70–72]).

The results from international and national publications are mostly consistent with the conclusion that environmental contaminants lead to increasing the MN frequency in children [35, 49, 55, 56, 73, 74]).

Gómez Arroyo et al. [36] evaluated the potential genotoxic risk in two groups: one group of 125 children (52 female and 73 male) from the state of Sinaloa (Mexico) whose houses are close to areas of intense agricultural activity which are sprayed with mixtures of pesticides; and a control group of 125 children (57 female and 68 male) living in the city of Los Mochis, Sinaloa; in both groups, micronuclei (MN) test in oral mucosal cells was used as a biomarker. The results showed a significant increase in the frequency of MN. Other nuclear abnormalities

associated with cytotoxicity or genotoxicity were detected; in all cases, the differences were significant when compared to the control group.

Benitez-Leite et al. [75] analyzed oral mucosa samples of 48 children from Paraguay potentially exposed to pesticides and 46 children not exposed, in order to determine genetic damage through the frequency of micronuclei (MNi). These authors found that the mean frequency of micronuclei was higher in the group potentially exposed to pesticides. This research provides evidence of genetic damage in a population of children potentially exposed to pesticides sprayed in the environment.

Our research group GeMA, performed monitoring studies in children from different locations in the Province of Córdoba through the micronuclei test (cytoma approach) in oral mucosa samples. All the locations are surrounded by fields which are sprayed with chemical agents and where there is a perception of damage caused by the use of agrochemicals. We studied 19 children between 5 and 12 years old, from the towns of Oncativo and Marcos Juárez (Province of Córdoba) which are surrounded by fields cultivated with soy and corn and with regular applications of pesticides [35]. Significant differences were found in the frequency of cells with nuclear abnormalities -buds- and the frequency of MN among the exposed groups and between them and the control groups. This work concludes that genotoxic monitoring constitutes the basis to a proper medical surveillance in populations at risk due to occupational or environmental exposure to chemical substances, such as pesticides.

Another study from Argentina [55] compares the MNi frequencies in oral exfoliated epithelial cells of environmentally exposed (by inhalation) children from urban areas with children that live in urban regions far from the area sprayed. The studied population consisted in 50 children from the town of Marcos Juárez (Córdoba), living at different exposure distances to the spraying site, and 25 children from Río Cuarto city (Córdoba), considered not exposed to such products. The MNi frequency was significantly different between the exposed children (500 m or less) when compared to the group of children not exposed. Forty percent of exposed individuals suffer some kind of persistent condition, which could be associated with chronic exposure to pesticides. The results indicated genetic damage in the group of children exposed to genotoxic substances when compared to the other group, and highlight the relevance of the MN assay in buccal mucosa for genetic biomonitoring and public health surveillance. The performed test detects a level of damage that is still reversible.

The limited number of published articles may indicate that carrying out adequately designed studies in children populations is difficult, more research in this segment of the human population is necessary.

Overall, the evidence of genetic damage in children due to early environmental exposure is strong, and every effort should be made to prevent them and pregnant women from such exposures and protect their health.

The most studied adult populations are those occupationally exposed to pesticides.

Several studies have been carried out in the agricultural sector, since it is considered the group with the highest risk of exposure to these compounds, with the purpose of evaluating the genotoxic risk they imply, especially for agricultural workers [76].

Aiassa et al. [21] and Gómez-Arroyo et al. [77] conducted a review work from studies performed on groups of people exposed to pesticides in Latin America.

In this paper from Aiassa et al. [21], we reviewed the main concepts in the field, the usefulness of genotoxicity assays and we compiled studies of genetic monitoring performed in the last 20 years in people occupationally exposed to pesticides. We think that genotoxicity tests, that include chromosomal aberrations, micronuclei test, sister chromatid exchanges and comet assays, should be considered essential tools for a complete medical monitoring in people exposed to potential environmental pollutants, particularly for those living in the same place as others who have already developed some type of malignancy. This screening is particularly important at early stages to prevent the occurrence of tumors, especially from environmental origins.

This work reviews 100 reports from different parts of the world, including 21 investigations from South America in the following countries: Argentina (6), Bolivia (2), Chile (3), Brazil (7), Colombia (2), Ecuador (1); 14 from North America: Mexico (7), United States (6), British Columbia (1); 4 from Central America: Costa Rica (3), Cuba (1); 37 from Europe: Hungary (4), Czech Republic (1), Russia (2), Spain (8), Greece (5), Italy (8), Denmark (1), Portugal (1), former Yugoslavia (1), Finland (1), Croatia (3), Poland (1), European Countries (1); 2 from Oceania: Australia (2); 2 from Africa: Egypt (2); and 10 from Asia: Syria (1), Turkey (3), Pakistan (1), Taiwan (2), Israel (1), India (2).

According to the analysis of these publications, 90% of the workers exposed to spraying are in contact with mixtures of pesticides, therefore, it is difficult to establish a correlation between a single pesticide and the damage observed. This leads to the issue of evaluating the risk of pesticides mixtures. The combined action of mixtures may result in noninteraction or interaction. If the toxicological capacity of each component of the mixture is different, the interaction between them may result either in an enhancement, when the combined effect is greater than the additive effect; or in an antagonistic effect, when the combined effect is less than the additive effect. The studies regarding the problem of pesticide mixtures are rare.

So far in Argentina, eight studies were carried out using CA, MN, SCEs and CO as biomarkers [35].

Dulout et al.'s study [78] is the first reported study from Argentina and one of the first carried out in Latin America, which was performed in floriculturists using CA and SCEs tests, obtaining negative results for CA and positive for SCEs. In 1987, the same author [79], in another population of floriculturists, analyzed CA, obtaining negative results and subsequently [80], in a new study reported positive results for SCEs. These results are followed by studies on rural workers (pesticide applicators) performed by Mañas et al. [60] that evaluate CA with positive results, Simoniello et al. [81] analyzed CO with positive results, Peralta et al. [82], who study CA, MN (in blood samples) and CO in both occupationally and environmentally exposed people, reported positive results for all biomarkers. Gentile et al. [41] also analyzed MN in blood samples with positive results. In all cases, the workers were exposed to several pesticides mixtures, making difficult to attribute the damage to a single compound and also impeding the comparison between different investigations due to the large number and variety of products applied. In none of these works was reported any exposure to other sources (confounding factors) that could interfere with the expressed results.

Gentile et al. [41] concluded that the exposure to pesticides in the study group of rural workers could induce levels of genetic damage detectable in peripheral blood lymphocytes by micronucleus assay. Age is a factor that increases both the frequency of binucleated cells with micronuclei and the total amount of micronuclei. Another factor, such as the years of exposure, does not affect these variables. Notwithstanding the above, all the potentially confounding factors must be considered when performing a cytogenetic evaluation.

As shown, in Argentina, problems derived from the use of pesticides have little attention in the health system. This situation is related to an underreporting of intoxications [83]. A high percentage of the Argentine population is engaged in agriculture and lives in rural areas where large quantities of substances are used to control plagues. It is also known that a high proportion of the population is actually and potentially exposed to these pesticides not only because they participate directly in work activities, but also because of the sprays that involuntarily reach urban areas, increasing the possibilities of harmful effects on their health.

According to Gómez Arroyo et al. [36], several studies about the genotoxic effects of pesticides have been carried out in diverse countries of Latin America from 1985 to 2013, using the four biomarkers; 41 of these studies were analyzed: 6 corresponding to Argentina, 2 to Bolivia, 10 to Brazil, 4 to Colombia, 5 to Costa Rica, 1 to Cuba, 2 to Chile, 3 to Ecuador, and 8 to Mexico. In most of the cases, workers from different countries of Latin America were in contact with products that are included in the list of highly dangerous pesticides, and it is remarkable that such individuals were mostly agricultural workers who were exposed to mixtures of pesticides. Results obtained in the studies performed in human populations demonstrate that CA, MN, SCE and CO are suitable tests to evaluate the risk associated with exposure to pesticides, showing a high percentage of positive results. Moreover, the studies carried out using CA and MN biomarkers have been correlated as predictors of cancer risk.

The genetic, molecular and biochemical methodologies that currently exist facilitate us to detect changes or alterations that act as a warning signal, allowing us to implement appropriate measures to minimize the risk on health [52].

One of the problems regarding adults population monitoring is confounding factors such as the habit of smoking, the consumption of alcohol and the occupational risk, that interfere in the analysis of the obtained results. These confounding effects are minimized and even absent during childhood. However, monitoring of children populations may consider potentially genotoxic factors including indoor tobacco smoke, regional ozone level, airborne nanoparticles, food contaminants such as pesticide residues and compounds generated by cooking (Holland et al. [84]), natural sources of ionizing and non-ionizing radiation, environmental pollutants, fuel and hydrocarbon emissions, which can vary significantly between rural or urban environmental settings [85].

2. Conclusions

Early detection of genetic damage is crucial to implement the necessary measures to reduce or suppress the exposure to deleterious agent when the damage is still reversible, thus reduce the

risk to suffer diseases. Therefore, genotoxicity tests should be considered as essential tools for a complete medical surveillance of people potentially exposed to environmental pollutants and, especially for those who inhabit places where other people have already developed any type of neoplasia at young ages, in order to prevent the occurrence of tumors of environmental or occupational origin. On the other hand, the application of these tests is useful to detect possible long-term effects of substances that are introduced to the market without knowing their capacity to affect human and environmental health.

The large number of studies performed in both occupationally exposed populations and environmentally exposed children provides important information to create a list of recommendations in order to avoid the genetic damage due to the exposure to pesticides and other contaminants.

It is recommended against the situations mentioned below:

- Disrupt the exposure to the potential risk in the workplace or in the proximity of residential areas until further studies are performed, and protection measures can be implemented to preserve the health of people (especially children) and the environment where they live.
- Control the sources of pollution, with the main objective of decreasing, removing or, ideally, eliminating the exposure.

The removal of sources of pollution away from residential areas is a matter of wide discussion, since it is difficult to control the drift of pesticides due to the environmental conditions in some provinces of Argentina such as Córdoba. Despite the toxicological classification of pesticides, the damage they cause to the genetic material of populations exposed to these chemicals should be taken into account.

The available literature shows an increased damage to the genetic material of children from Paraguay exposed to pesticides and living 50 m from the source of contamination [75].

The study carried out in Argentina with children from Marcos Juárez (Córdoba) did not found statistically significant differences in the micronuclei frequencies between the group that lives less than 500 m from the contamination source and the group that lives 500–1500 m from it. The results of Marcos Juárez study showed that the pesticides could spread by air and reach the entire town. Therefore, the vulnerable population of children is subject to an extremely high concentration and continuous exposure of pesticides, given that they live surrounded by crops fields. Up to 1095 m from the sprayed site, no significant differences were found in the MNi frequencies between the children of both groups. This information should be considered when establishing environmental safeguards in locations that are surrounded by crops fields and regularly sprayed [55].

Agricultural industry is one of the main economic activities in many regions of our country; however, despite the benefits it provides, it is responsible of environmental issues, risk to human health and damaging other organisms, so the result is a negative overall balance.

Reducing the environmental pollutants that affect human health, such as metals, pesticides, organic solvents, food additives, some natural products, and especially their derivative effects, would produce a remarkable improvement in health conditions of exposed populations.

- Establishing a monitoring protocol that tracks genotoxicity biomarkers to determine whether the biological indicators of cell damage are persistent through time, at least for 1 year and with two sampling in the absence of contaminants.

Genotoxicological monitoring in humans is a useful tool for estimating the genetic risk of exposure to a compound or complex mixtures of chemicals and constituting an early warning system for genetic diseases, reproductive problems and cancer. It also allows developing adequate control measures that can be implemented to protect human populations and the environment.

- Conduct studies of contaminants in urine, blood and environmental matrices such as air, water, sediments and soil.
- Educate the community with information campaigns about human and environmental health to promote a culture of care, foresight and prevention in the area.

It is essential to focus our attention on the children population. The WHO Task Force for the Protection of Children's Environmental Health has stated: "Children are not small adults," the premise behind this principle is that children have an exceptional vulnerability to the acute and chronic effects of environmental hazards and that they are disproportionately susceptible compared to adults [60, 86]. It has been recognized that children are a group, within the population, that has particular characteristics of exposure and special vulnerability to environmental toxins, and it is required a strategy for risk assessment that considers their particular features [53, 54].

The health of a society can be judged by the health of its children. This implies the early identification of preventable risks and the immediate translation of this knowledge into effective protection policies.

The evidence of the effects of environmental exposure at an early age is so substantial that every effort should be made to avoid such exposures in children and pregnant women as well as to protect their present and future health (Holland et al. 2011).

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This book is designed to provide an overview of the different genotoxicants and their effects on living organisms, including humans. The contributions made by the specialists in this field of research are gratefully acknowledged. We hope that the information presented in this book will meet the expectations and needs of all those interested in the different aspects of the genotoxicity field. The publication of this book is of great importance to those scientists, pharmacologists, physicians and veterinarians, as well as engineers, teachers, graduate students and administrators of environmental programmes, who make use of these investigations to understand both the basic and applied genotoxic aspects of known and new xenobiotics, and to guide them in their future investigations.

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