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Free Radicals, Antioxidants and Diseases

Edited by Rizwan Ahmad

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Meet the editor

Prof. Rizwan Ahmad is working as a faculty in the College of Medicine, Imam Abdulrahman Bin Faisal University, Saudi Arabia. Dr. Ahmad has established the School of Life and Allied Health Sciences as its founding head at Glocal University, India. He has worked as an associate professor of Biochemistry at Oman Medical College in partnership with WVU, USA, from 2010 to 2013. He has published several research papers in refereed journals. The focus of his research has been on free radical biochemistry and nucleic acid immunology. He has been appointed as a reviewer in many international journals.

Contents

Preface

The idea of this book project comes after the much-acclaimed volume in this series, *Free Radicals and Diseases* published by InTech in 2016 (downloaded more than 11,000 times). Free radicals have been extensively studied in recent years, but with very few exceptions, their roles in the etiologies of specific disorders remain largely undefined. As our understanding of molecular regulation improves, the roles that free radicals have in both preserving and adversely affecting functions will become more clearly defined.

Antioxidants may play a role in the management or prevention of some medical conditions, such as some cancers, macular degeneration, Alzheimer's diseases, and autoimmune disorders. This volume entitled *Free Radicals, Antioxidants, and Diseases* gives an idea of detecting free radicals in vivo by newer techniques and provides insights into the roles played by various antioxidants in combating diseases caused by oxidative stress.

The book covers topics beginning from the basics of free radicals and the role of antioxidants in human health, including mechanisms of their actions in disease prevention such as the role of dimethylformamide in cerebral ischemia, essential oil from *Coleus zeylanicus*, cardioprotective effects of S-nitrosothiols, and physical exercise as prooxidant and antioxidant. Analysis of various antioxidants by EPR is also described. Some of the chapters present reviews on subjects of oxidative DNA damage in chronic renal diseases and aging process. The role of microRNAs as possible regulators of gene expression in aging is also envisaged.

Since this field is rapidly expanding with new discoveries of implications of free radicals in many diseases and roles of various antioxidants being reported every year, we hope that these two books will also provide a reference to free radical research having easy-to-follow approach for the academic use or laboratory manual for students in basic and medical sciences.

I am very much delighted to be involved in this book project. It really gives me the chance to go through the diversified aspects of this astonishing field. I am indebted to Dr. Abdullah Al-Rubaish, President of IAU; Dr. Fahad Al-Muhanna, Vice President; Prof. Ali Al-Sultan, Dean of the College of Medicine; and Prof. Abdulaziz Al-Sebiani, Vice Dean of Academic Affairs for providing me the time and necessary facilities for this accomplishment. Editorial assistance by Ms. Marijana Francetic is gratefully acknowledged.

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Chapter 1

Introductory Chapter: Basics of Free Radicals and Antioxidants

Rizwan Ahmad

Additional information is available at the end of the chapter

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1. Historical Aspect

Do you know the scientist who had discovered the free radical? It was a stunning Professor of Chemistry named Moses Gomberg in 1900 at Michigan State University. In June 25, 2000, after a century the discovery was commemorated by the American Chemical Society as a National Historic Chemical Landmark in a ceremony at the University of Michigan, Michigan [1]. Earlier evidence by a British Chemist Fenton also suggests that hydrogen peroxide which reacts with ferrous sulfate results in violet color which is nothing but oxidation of tartaric acid upon addition of alkali. This is the basis of production of hydroxyl radical, and the reaction is called as Fenton reaction [2].

2. Free Radicals

The most common definition of free radicals is "molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals" [3]. Free radicals are uncharged, very reactive, and short-lived molecules. Human beings contain 10,000–20,000 free radicals which attack each and individual cell of our body. Many of these radicals are beneficial in that they work for immune cells responsible for killing bacterial cells and toning of smooth muscles, which in turn regulate the normal working of blood vessels and internal organs. Uncontrolled generation of free radicals in our body may lead to various ill effects such as autoimmune diseases, heart and neurodegenerative diseases, cancers, etc. [4].

Now, coming to their very existence, one should know the basics. The free radicals are produced during ATP generation through mitochondria. They are generally divided into two

well-known entities: reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS collectively form all the radical and non-radical (oxidants) entities. Radicals are more reactive and less stable than their non-radical counterparts. Non-radical derivatives or oxidants can be easily converted into free radicals by various reactions in living organisms [5].

Oxidative phosphorylation occurring in mitochondria is a nonenzymatic process which results in the production of ROS and RNS. For example, excess generation of hydroxyl radical and peroxynitrite causes damage to cell membranes and lipoproteins, and the process referred to as lipid peroxidation. This results in the formation of mutagenic and cytotoxic compounds such as malondialdehyde (MDA) and other diene derivatives. ROS/RNS affects proteins which may lead to the loss of enzyme activity and structural deformity. These species attack DNA, thus affecting the functional growth and formation of oxidative lesions which can lead to mutagenesis. The body has various enzymes and antioxidants to combat the damage incurred by oxidative stress [6, 7].

3. Antioxidants

Antioxidants are chemicals that bind with free radicals and nullify their effect from causing damage to biological molecules. Endogenous antioxidants are produced by our body which is used to combat various free radicals. However, most of them are obtained from external sources, primarily through diet called as exogenous antioxidants or dietary antioxidants. Major sources of this class of antioxidants are brightly colored vegetables, fruits, and grains. Other very effective sources are berries, green tea, and dark chocolate. Nowadays, many oral supplements are available in the market labeled as dietary antioxidants.

Antioxidants bind with free radical by giving up their own electrons. These results in the termination of oxidative chain reactions, and the free radicals are no longer able to attack the cell. Antioxidant attains free radical state after donating its electron. It can accommodate the change in electrons without becoming reactive, and that's why they are not harmful. There are two lines of antioxidant defense inside the cell. Vitamin E, beta-carotene, and coenzyme Q constitute the first line which is found in the fat-soluble cell membrane. Vitamin E is considered as the most potent chain-breaking antioxidant within the cell membrane. The water-soluble antioxidant is present inside the cell. These are vitamin C, superoxide dismutase (SOD), glutathione peroxidase, and catalase [8].

4. Role in Diseases

Despite the antagonistic role played by various antioxidants, the free radical damage has been implicated in various degenerative conditions such as cardiovascular diseases, neurological disorders, diabetes, ischemia-reperfusion injury, and aging. These diseases may be divided into two groups: (i) the first group includes cancer and diabetes which occurs due to impairing glucose tolerance in which the condition is better known as "mitochondrial oxidative stress" and (ii) the second group is characterized by inflammatory oxidative conditions leading to atherosclerosis

and chronic inflammation. Aging is also a form of the deleterious effect of free radical damage (protein oxidation, lipid peroxidation). Mutation of DNA is a crucial step in carcinogenesis, and elevated levels of DNA lesions are found in many tumors, strongly correlating the oxidative damage in the etiology of cancer. ROS-induced changes are found in various diseases of the heart such as cardiomyopathies, ischemic heart disease, hypertension, atherosclerosis, and heart failure [9].

Free radicals induced cell damage that is found in many autoimmune diseases. Oxidative injury and inflammatory status were proven by raised levels of isoprostanes and prostaglandins in serum and synovial fluid compared to controls [10]. Increased oxidative stress has been proposed as one of the major factors causing hyperglycemia which in turn trigger various diabetic conditions [11]. Oxidative damage advances with age, and this is the reason that it is considered as one of the causative factors in various neurological disorders such as Alzheimer's and Parkinson's diseases [4].

5. Book Overview

In this book, we have tried to incorporate themes and concepts which eventually give the insights into the role of antioxidants in combating various disorders caused by free radical damage. In one of the chapter the author discusses the unique property of essential oil extracted from *Coleus zeylanicus* using the bioautography technique. It reveals the antimicrobial and antioxidant properties of oil from *Coleus zeylanicus* which further increases upon exposure to salinity stress condition.

A novel concept of immune-spin trapping combined with molecular magnetic resonance imaging is also explored in this book. This would give the readers an insight to detect free radicals *in vivo* formed in different tissues. Various therapeutic agents can also be traced to their role in free radical generation and quenching.

The very motive of including a chapter on ethnic aspects of oxidative stress-induced TIDM is that the racial variability was not studied and there is no conclusive evidence about lipid peroxidation and antioxidant defense system in patients of a region. Their comparative study recommends a highly significant approach to this aspect.

Scavenging effect of dimethylformamide (DMF) in cerebral ischemia (CI) is also analyzed. The results and discussion will lead to future research and more clinical trials to establish DMF as an antioxidant in CI.

The role of physical exercise on oxidative stress and vascular diseases is reviewed extensively. Generation of oxidants and simultaneously antioxidants during physical exercise which is in turn responsible for its prevention is summarized.

The recent findings in free radical-induced damage in aging and how antioxidants are employed as an anti-aging agent in the preclinical model are discussed in the book. This would give an idea of their therapeutic potential. Micro-RNAs (miRNAs) are the novel regulators of gene expressions which induce aging process. In the chapter of miRNAs in the regulation of hyperglycemia-induced oxidative stress and cellular senescence, authors explain the critical role of miRNAs in age-related disorders which could open the front of new findings in geriatrics.

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Chapter 2

Detecting *In Vivo* Free Radicals in Various Disease Models

Rheal A. Towner and Nataliya Smith

Additional information is available at the end of the chapter

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Abstract

In vivo free radical imaging in pre-clinical models of disease is now possible. Free radicals have traditionally been characterized by ESR or EPR spin trapping spectroscopy. The disadvantage of the ESR/EPR approach is that spin adducts are short-lived due to biological reductive and/or oxidative processes. Immuno-spin trapping (IST) involves the use of an antibody that recognizes macromolecular DMPO-spin adducts (anti-DMPO antibody), regardless of the oxidative/reductive state of trapped radical adducts. The IST approach has been extended to an *in vivo* application that combines IST with molecular magnetic resonance imaging (mMRI). This combined IST-mMRI approach involves the use of a spin trapping agent, DMPO, to trap free radicals in disease models, and administration of a mMRI probe, an anti-DMPO probe, that combines an antibody against DMPO-radical adducts and a MRI contrast agent, resulting in targeted free radical adduct detection. The combined IST-mMRI approach has been used in several rodent disease models, including diabetes, ALS, gliomas, and septic encephalopathy. The advantage of this approach is that heterogeneous levels of trapped free radicals can be detected directly *in vivo* and *in situ* to pin-point where free radicals are formed in different tissues. The approach can also be used to assess therapeutic agents that are either free radical scavengers or generate free radicals. The focus of this review will be on the different applications that have been studied, advantages and limitations, and future directions.

Keywords: immuno-spin trapping (IST), molecular magnetic resonance imaging (mMRI), targeted free radical imaging, *in vivo*, diabetes, amyotrophic lateral sclerosis (ALS), glioma, septic encephalopathy, mice, rats

1. Introduction

1.1. Free radicals in various diseases

Reactive oxygen and nitrogen species (RONS) lead to structural and functional modifications of cellular proteins and lipids, resulting in cellular dysfunction, such as impaired energy metabolism, altered cell signaling and cell cycle control, impaired cell transport processes and dysfunctional biological activities, immune activation, and inflammation [1]. RONS can be involved in several disease processes as causative agents or result as an effect of the pathogenesis. It is well known that free radicals play a role in the pathogenesis associated with various diseases such as diabetes, septic encephalopathy, neurodegenerative diseases, and cancers, to mention a few.

Nutritional stress, that for instance may result from excessive high-fat and/or carbohydrates, can promote oxidative stress, subsequently forming lipid peroxidation products, protein carbonylation, as well as decreased antioxidant levels [1]. Chronic oxidative stress and inflammation, both associated with obesity, can lead to insulin resistance, dysregulated metabolic pathways, diabetes and cardiovascular diseases, via impaired signaling and metabolism that result in insulin secretion dysfunction, insulin action, and immune responses [1]. In type 1 diabetes mellitus, RONS released from phagocytes may damage adjacent cells, which can lead to excessive inflammation and an autoimmune attack against pancreatic islet β-cells, and contribute to a rapid progression of pathogenesis [1]. Immune system-associated enzymes (such as NADPH oxidase) can trigger the formation of reactive oxygen species (ROS) [1]. Excessive glucose and lipid levels, endocrine factors and numerous pro-inflammatory cytokines are known to activate NADPH oxidase [1]. Pro-inflammatory cytokines can also upregulate nitric oxide synthase 2 (NOS2), producing excessive nitric oxide, which can subsequently lead to the formation of peroxynitrite, and lead to further oxidative stress [1]. In Type 2 diabetes mellitus, excessive RONS production from chronic hyperglycemia increases oxidative stress in tissues that exacerbate the disease, such as pancreatic islets, muscle, adipose and hepatic, as well as influences secondary diabetic complications, including nephropathy, vascular disease and retinopathy, leading to oxidized lipids and proteins [1, 2].

Sepsis-associated encephalopathy pathophysiology is still poorly understood, but a number of mechanisms-of-action (MOA) have been proposed, including mitochondrial and vascular dysfunction, oxidative damage, neurotransmission disturbances, inflammation, and cellular death [3, 4]. Oxidative stress is a central MOA of acute brain damage [3]. Systemic inflammation induces mitochondrial dysfunction, which is involved in both apoptotic and necrotic cell death pathways, and increased glucose uptake by brain tissues, which results in the diversion of glucose to the pentose phosphate pathway that may contribute to oxidative stress by producing excessive superoxide radicals via NADPH oxidase [3, 4]. In addition, microglia activation results in the secretion of nitric oxide, ROS (reactive oxygen species) and matrix metalloproteinases (MMPs) that can all contribute to blood-brain barrier (BBB) and neuronal damage [3]. Regarding brain dysfunction in sepsis, it is thought that RONS, generated during a systemic inflammatory response, triggers lipid peroxidation due to a decreased antioxidant activity [4]. Free radical-induced structural membrane damage also induces neuro-inflammation [4].

The formation of excessive superoxide radicals also depletes ambient nitric oxide in the cerebrovascular bed, forming peroxynitrite, which irreversibly inhibits the mitochondrial electron transport chain, resulting in an increase in mitochondrial release of free radicals, and leads to mitochondrial dysfunction and neuronal bioenergetics failure [4]. Additionally, free radicals trigger apoptosis via altering intracellular calcium homeostasis in brain regions such as the cerebral cortex and hippocampus, further exacerbating local inflammatory responses further [4].

Oxidative stress has been proposed as a contributory factor in the pathogenesis of several neurodegenerative diseases [5]. For instance, in familial ALS (amyotrophic lateral sclerosis) (accounting for 5–10% of ALS cases) there is a mutation in superoxide dismutase 1 (SOD1) which results in dysfunctional superoxide radical clearance, leading to increased oxidative stress [5]. NADPH oxidases have emerged as possible drug targets for the treatment of neurodegeneration, due to their role in generating oxidants and also regulating microglia activation [6].

In cancer cells, RONS accumulation can result in damaging DNA, directly through an increase in cellular mutations and/or increase in oncogenic phenotypes, or indirectly by acting as secondary messengers intracellular signaling cascades [7]. It is thought that impaired cellular repair mechanisms induced by RONS oxidative stress on DNA can lead to cell injury and subsequently to genomic instability, mutagenesis and tumorigenesis [7]. It is also known that ROS can promote cell proliferation activating growth-related signaling pathways [7]. ROS may be involved in the multistep oncogenesis process at various different phases related to tumor initiation and progression, ROS-related mechanisms during tumor promotion, maintenance of the transformed state through extracellular superoxide radical formation by NADPH oxidase 1, and resistance to oxidative stress signals through membrane-associated catalase expression [7].

1.2. Spin trapping and ESR/EPR spectroscopy

For over half a century, free radicals were characterized by electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy coupled with spin trapping. Nitrone spin traps (*N*-oxides of imines), such as PBN (α-phenyl-*tert*-butyl nitrone), DMPO (5,5-dimethyl-pyrroline-*N*-oxide) or 4-POBN (α(4-pyridyl-1-oxide)-N-tert-butyl nitrone), are the most commonly used for biological systems [8–18], and have been administered *in vivo* in various pre-clinical disease models [19–22] for several decades.

The disadvantage of the ESR/EPR approach is that the spin adducts (spin trapping agent — free radical adducts or aminoxyls) are short-lived due to reductive and/or oxidative processes in biological systems [8, 9] (see **Figure 1**).

1.3. Immuno-spin trapping (IST)

Mason *et al*. developed an antibody that recognizes macromolecular DMPO spin adducts, regardless of the oxidative/reductive state of the trapped radical adducts, and called the methodology immuno-spin trapping (IST) [23–28] (see **Figure 2** for an illustrative description), that has been applied in over 80 publications. The anti-DMPO antibody is attached to a fluorescent dye, allowing the *in vitro* or *ex vivo* detection of trapped DMPO-radical adducts,

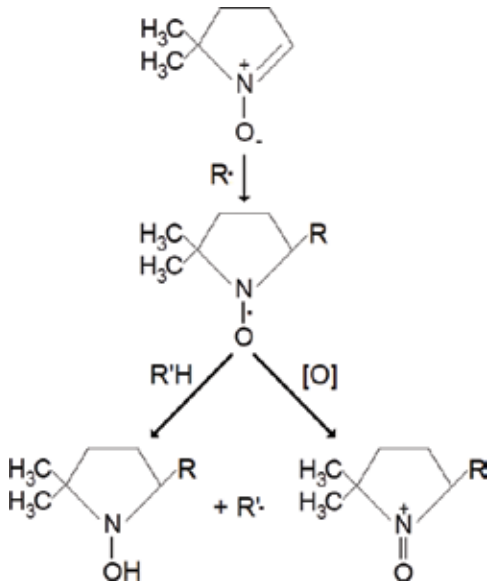

Figure 1. Spin trapping agents (nitrones) can be used to trap free radical compounds (R•) to form a spin adduct (nitroxide or aminoxyl), which are detected by EPR spectroscopy. However, in biological systems spin adducts can be either reduced (R'H) or oxidized ([O]). Reduced or oxidized spin adducts are EPR silent.

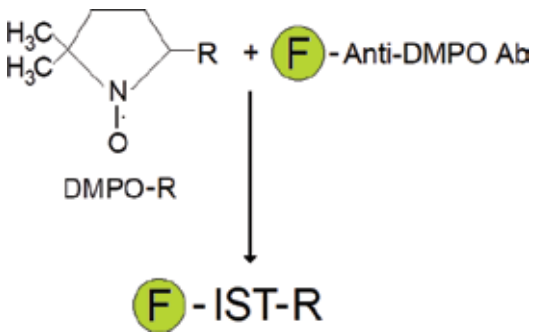

Figure 2. Immuno-spin trapping (IST) involves tagging a fluorescent-labeled-anti-DMPO antibody to DMPO-spin adducts (either as the free radical (aminoxyl), reduced or oxidized products, i.e. regardless of whether they are EPR detectable or not). The "F" designates a fluorescent dye.

either as the free radical (aminoxyl), reduced or oxidized products, with fluorescence microscopy, regardless of whether they are EPR detectable or not.

1.4. Combined IST and molecular MRI (mMRI) detection of *in vivo* and *in situ* free radicals

Towner *et al*. extended the fluorescence *in vitro/ex vivo* approach to an *in vivo* approach that involves the use of immuno-spin trapping (IST) in conjunction with targeted molecular magnetic resonance imaging (mMRI), currently published in six publications [29–34]. This

involves the use of a spin trapping agent, DMPO, which is used to trap free radicals in an oxidative stress-related disease model, and administration of a mMRI probe, called an anti-DMPO probe (see **Figure 3**), that combines an antibody against DMPO-radical adducts and a MRI contrast agent, resulting in targeted free radical adduct mMRI (see **Figure 4** for methodology scheme). The contrast agent used in the Towner approach includes an albumin-Gd-DTPA (gadolinium diethylene tri-amine penta-acetic acid)-biotin construct, where the anti-DMPO antibody is covalently linked to the cysteine residues of albumin, forming an anti-DMPO-adduct antibody-albumin-Gd-DTPA-biotin entity. The Gd-DTPA moiety acts as the MRI signaling component, which will increase MRI signal intensity (SI) in a T_1-weighted morphological MR imaging sequence, and decrease T_1 relaxation in a T_1 map image. Both of these parameters, MRI SI or T_1 relaxation) can be used to assess the presence of the anti-DMPO probe. The biotin moiety can be used for *ex vivo* validation of the presence of the anti-DMPO probe in tissues, by using a streptavidin-fluorescent dye (e.g. Cy3) or streptavidin-HRP (horse radish peroxidase) to tag the biotin in the anti-DMPO probe.

1.5. Other approaches used to detect *in vivo* and *in situ* free radicals in animal models and cells

It is well known that intensity-based fluorescent methods (particularly 2′,7′-dicholorofluorescin [DCFH]) for ROS (includes the non-radical hydrogen peroxide) detection/quantification are sensitive and readily used, however, these agents lack the specificity for ROS or reactive nitrogen species (RNS), and often produce artifacts resulting in false-positive signals [35, 36]. An interesting recent study by Liu et al. used a new fluorescent probe, MPT-Cy2, which can be used to detect endogenous *in vivo* hydroxyl radicals in cells and zebrafish [37]. MPT-Cy2 becomes a fluorescent product, OMPT-Cy2, when it binds hydroxyl radicals [37]. In a similar fashion, Hu et al. reported on a fluorescent probe, HKSOX-1, for the imaging and

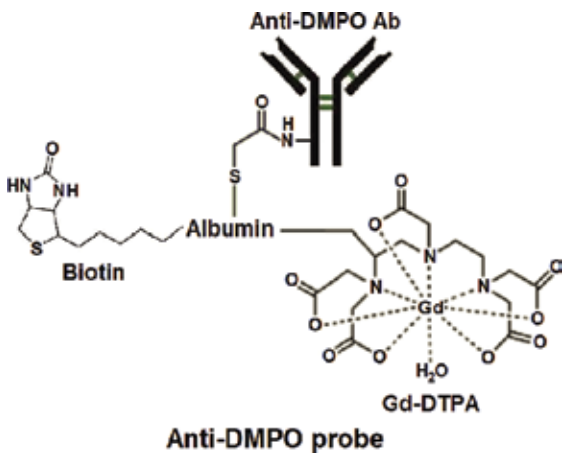

Figure 3. Illustration of the anti-DMPO probe, consisting of an albumin link that binds a MRI contrast agent, Gd-DTPA (for detection by MRI), the anti-DMPO antibody (Ab) (that binds to DMPO-free radical adducts), and biotin (that can be used for ex vivo fluorescence microscopic imaging). Modified from Gomez-Mejiba et al. [28].

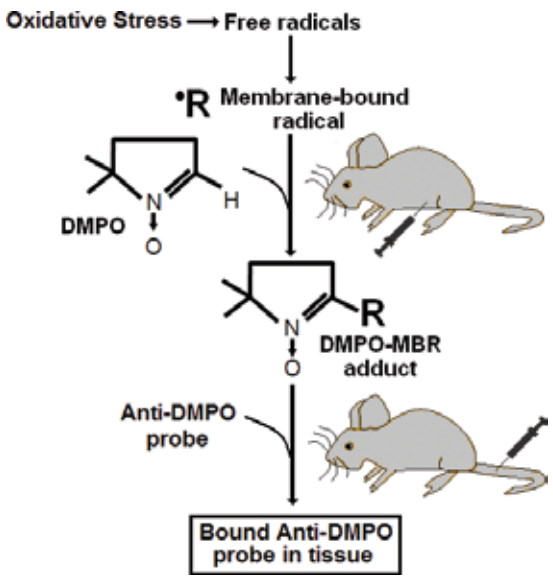

Figure 4. Combined IST and free radical-targeted molecular MRI (mMRI) approach. Initially mice are administered DMPO (i.p.) to trap free radicals resulting from an oxidative stress-associated disease or process. Any cell membrane-bound radicals (e.g. oxidized proteins or lipids) can then be detected with the anti-DMPO probe (administered via a tail-vein catheter). Modified from Towner et al. [29].

detection of endogenous *in vivo* superoxide in cells and zebrafish embryos [38]. A fluorescence probe, o-phenylene diamine-Phe-Phe-OH, has also been developed for the detection of nitric oxide (NO), and used in living cells [39]. Another fluorescence probe, LyNP-NO, was used to detect NO in C6 glioma cells [40]. It was also found that single-walled carbon nanotubes have fluorescent properties, and have been used to detect *in vivo* levels of NO, which quenches the fluorescence signal, in mice [41]. However, for most of the fluorescence probes *in vivo* applications will be limited to a depth-of-penetration detection of the fluorescence signal and may only be applicable to small animal models.

Another group, Li et al. used a near infrared (NIR)-light excited luminescence resonance energy transfer based nanoprobe for *in vivo* detection of hydroxyl radicals [42]. NIR fluorescence probes (Hcy-Mito and Hcy-Biot) were also recently used for the *in situ* detection of superoxide anion and hydrogen polysulfides in living cells and in mouse tumor models [43]. Also, a phosphinate-based NIR fluorescence probe, CyR, was recently also used to detect superoxide radical anion *in vivo* within zebrafish [44]. NIR-fluorescent single-walled carbon nanotubes have also been used to detect *in vivo* NO levels in mice [45]. It should be pointed out, however, that there is ESR spectroscopy evidence for *in vivo* formation of free radicals in the tissues (lungs, heart and liver) of mice exposed to single-walled carbon nanotubes with no oxidative stress [46].

Rayner et al. used a reversible pro-fluorescent probe containing a redox sensitive nitroxide moiety (methyl ester tetraethylrhodamine nitroxide, ME-TRN) for the *in vivo* detection of retinal oxidative status within rat retina following acute ischemia-reperfusion injury [47].

Another interesting approach using nitrone functionalized gold nanoparticles (Au@EMPO, EMPO = 2-(ethoxycarbonyl)-2-methyl-3,4-dihydr-2H-pyrrole 1-oxide)) to trap hydroxyl radicals was demonstrated by Du et al. [48] and may be potentially important for pre-clinical *in vivo* applications in combination with micro-computed tomography (CT).

Recent studies by Berkowitz et al. have used quench-assisted (Quest) $1/T_1$ MRI to measure oxidative stress changes in rodent models [49, 50]. Quest MRI detected pathologic free radical production in MnSOD (manganese superoxide dismutase) knockout mouse retinas with laminar resolution *in vivo*, where in particular dark-adapted RPE-specific MnSOD knockout mice had elevated $1/T_1$ values in the outer retina, compared to relevant controls [49] The Quest MRI technique was also used to report on high levels of free radicals in the hippocampus region in mouse models for neurological diseases such as Alzheimer's disease and Angelman syndrome [50]. However, it should be noted that paramagnetic oxygen [49–51] and hydrogen peroxide [50, 51] can also provide a dominant $1/T_1$ contrast effect, which could complicate the interpretation of the presence of free radicals. In addition, temperature and pH also can influence the rates of proton exchange which will also affect $1/T_1$ contrast [52, 53]. Nonetheless, Quest MRI is an interesting approach that provides information on total free radical burden, somewhat similar to the combined IST and mMRI approach, but without the use of a MRI contrast agent [49, 50].

Endogenous reactive oxygen species (ROS) contrast MRI was also recently used by Tain et al. to detect ROS (measured as a reduction in T_1) in rotenone-treated mouse brains [51]. Another study by Eto et al. used *in vivo* nuclear polarization MRI (DNP-MRI) with nitroxyl radicals (carbamoyl-PROXYL (cell permeable) and carboxy-PROXYL (cell impermeable)) to assess the redox status (measured as an increase in image intensity) in the skeletal muscle of mice that had an acute local inflammation (induced with i.m. injection of bupivacaine) [54]. The signal decay of carbamoyl-PROXYL in bupivacaine-exposed mice was confirmed by *in vivo* L-band EPR spectroscopy [54]. The nitroxyl radical probes, are paramagnetic which broaden the MRI signal, bind free radicals, and thus results in an increase in MRI signal intensity [55]. Another group used DNP-MRI to visualize endogenous free radical intermediates of FMNH (flavin mononucleotide-hydrogen) and FADH (flavin adenine dinucleotide-hydrogen) *in* vitro [56], which could potentially be detected *in vivo* in the future. DNP-MRI, also used for PEDRI (proton electron double-resonance imaging) or OMRI (Overhauser enhanced magnetic resonance imaging), is a relatively new imaging approach for detecting free radical species *in vivo* [57, 58]. Yamamoto et al. recently developed a combined PET (positron emission tomography)/OMRI system to detect radionucleotide and nitroxyl radical probes for small animal imaging [59].

1.6. Overall scope

The focus of this review is on *in vivo* and *in situ* IST-mMRI applications in different experimental oxidative stress-associated animal disease models that have been studied, advantages and limitations of the technique, and future directions in further applications, improvements on the methodology that can be made, and subsequent free radical identification approaches.

2. *In vivo* and *In situ* targeted free radical detection: various models

The combined IST-mMRI approach has been used in several *in vivo* disease models, including multi-tissue assessment in diabetic mice [29] (see **Figure 5**) with further assessment of cardiomyopathy [30] (see **Figure 6**), and in neurological applications, such as rodent models septic encephalopathy [31] (see **Figure 8**), for amyotrophic lateral sclerosis (ALS) [32] (see **Figure 7**), and gliomas [33], to target *in vivo* and *in situ* free radical detection.

2.1. Diabetes

The initial proof-of-concept combined IST and mMRI approach to detect *in vivo* free radicals was assessed in a STZ (streptozotocin)-induced diabetes model in mice. From this study, all major organs excluding the heart, such as the lungs, liver and kidneys, were assessed regarding levels of trapped *in vivo* and *in situ* DMPO-radical adducts [29]. **Figure 5** depicts the data obtained in the liver of diabetic (STZ-induced) and non-diabetic mice (wild-type

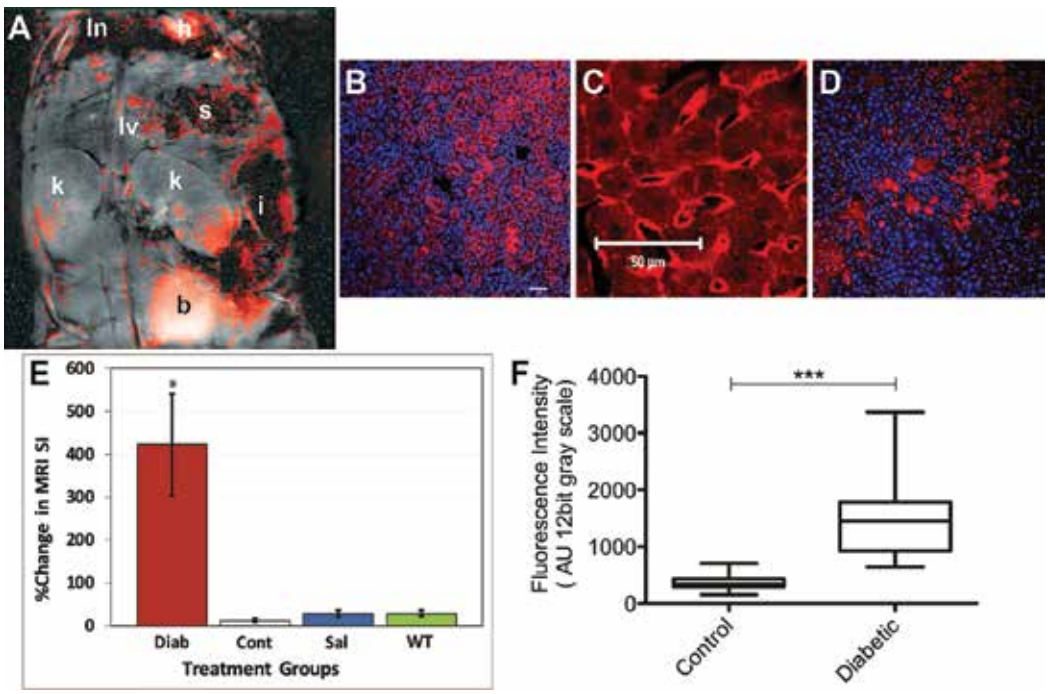

Figure 5. Combined IST and free radical-targeted mMRI in a STZ-induced mouse diabetic model. (A) *In vivo* anti-DMPO probe distribution map (based on MRI signal intensity (SI) change). Anatomical assignments: ln = lung, h = heart, s = stomach, lv = liver, k = kidney, i = intestine, and b = bladder. (B) Stretavidin-Cy3 biotin-tagged *ex vivo* liver image in Diab mice. (C) iNOS immunohistochemistry (IHC) from the liver of a diabetic mouse. (D) Fluorescence intensity of iNOS IHC in non-diabetic (control) and diabetic mouse livers. N = 5 for each. (E) Percent (%) change in MRI SI in diabetic mice administered DMPO + anti-DMPO probe (Diab), DMPO + a non-specific IgG contrast agent (Cont), saline + anti-DMPO probe (Sal), and wild-type non-diabetic mice administered DMPO + anti-DMPO probe. N = 5 for each group. (F) Fluorescent-IST image of the liver of a diabetic mouse. Modified from Towner et al. [29].

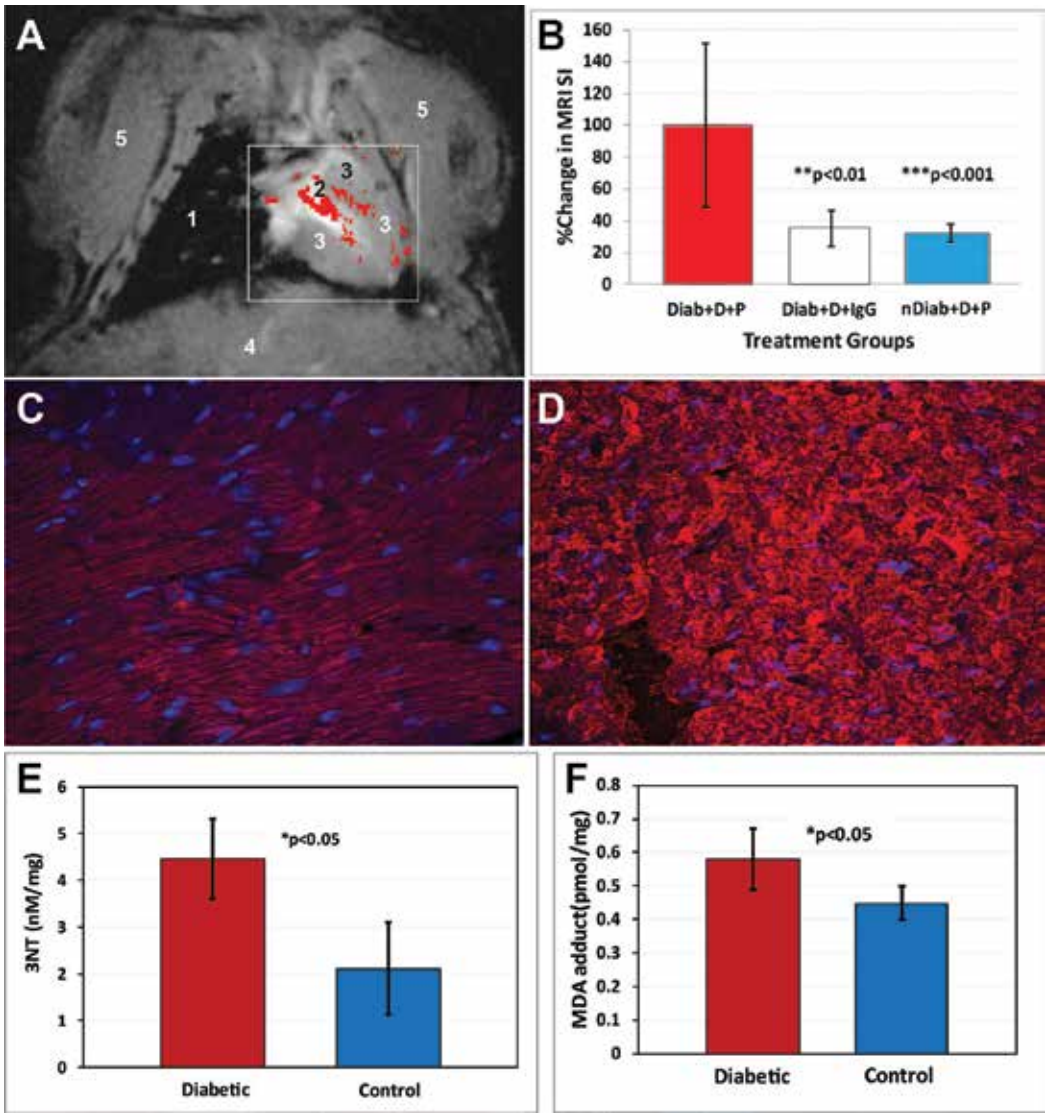

Figure 6. Combined IST and free radical-targeted mMRI in a STZ-induced mouse diabetic heart. (A) MR image of a mouse heart with an *in vivo* anti-DMPO probe cardiac map overlay (based on MRI SI change). Anatomical assignments: 1 = lung, 2 = left ventricle chamber, 3 = cardiac muscle, 4 = liver, and 5 = thoracic muscle. (B) Percent (%) change in MRI SI in diabetic mouse hearts after administered DMPO + anti-DMPO probe (Diab+D + P), DMPO + a non-specific IgG contrast agent (Diab+D + IgG), and non-diabetic mice administered DMPO + anti-DMPO probe (nDiab+D + P). N = 5 for each group. (C) Stretavidin-Cy3 biotin-tagged *ex vivo* cardiac image in Diab mice. (D) Fluorescent-IST image of the heart of a diabetic mouse. (E) 3-Nitrotyrosine (3-NT) adducts and (F) malondialdehyde (MDA) ELISAs from the hearts of diabetic and non-diabetic mice. N = 5 for each. Modified from Towner et al. [30].

mice not administered STZ), and appropriate controls (e.g. diabetic mice given saline rather than DMPO plus the anti-DMPO probe — spin trap control; or a diabetic mouse administered DMPO, but given a non-specific IgG contrast agent [IgG-albumin-Gd-DTPA-biotin] instead

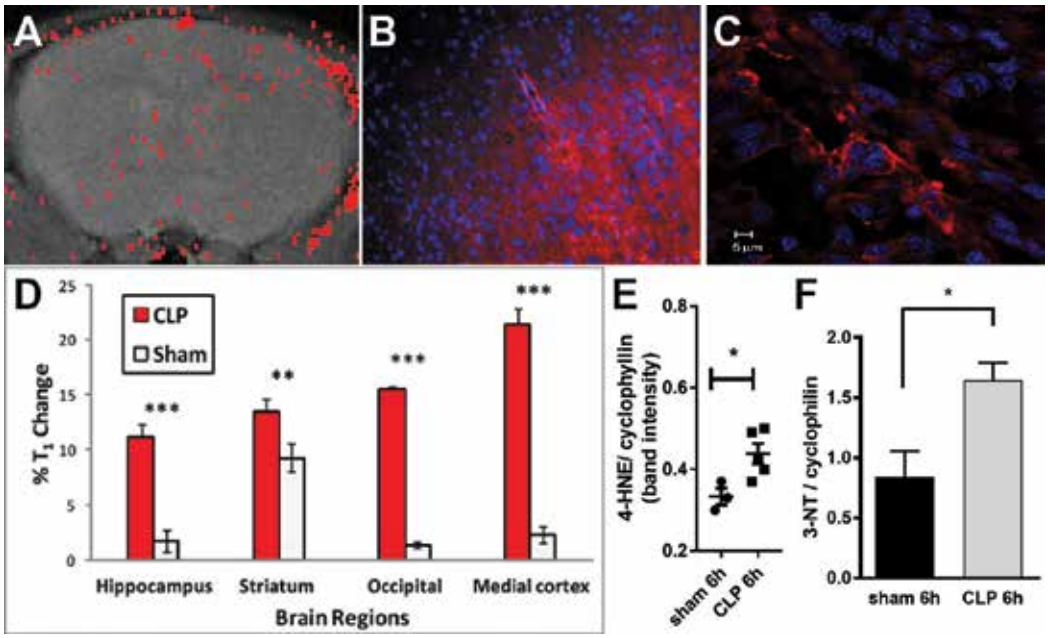

Figure 7. Combined IST and free radical-targeted mMRI in a septic encephalopathy CLP-induced mouse model. (A) *In vivo* anti-DMPO probe brain map of a septic mouse (based on MRI SI change). (B) Stretavidin-Cy3 biotin-tagged *ex vivo* septic brain image. (C) Fluorescent-IST image of the septic brain of a mouse. (D) Percent (%) T1 relaxation change in septic and sham mice administered DMPO + anti-DMPO probe in different brain regions (hippocampus, striatum, occipital lobe, and medial cortex). N = 5 for each group. (E) 4-Hydroxynonenal (4HNE) levels from western blots of brains of septic and non-septic mice. (F) 3-Nitro-tyrosine (3-NT) levels from western blots of brains of septic and non-septic mice. N = 5 for each. Modified from Towner et al. [31].

of the anti-DMPO probe). (**Figure 5**) Diabetic mice that were administered DMPO and the anti-DMPO probe, had significantly higher levels of the anti-DMPO probe (detected by an increase in percent (%) change in MRI SI), than non-diabetic mice or diabetic mouse controls, in their lungs, kidneys and livers (see **Figure 5A** for overall distribution of the anti-DMPO probe in a horizontal image; and **Figure 5E** for quantitative liver data). A post-contrast image minus a pre-contrast image was obtained, and the gray-scale image was false-colored red, and overlaid on top of the morphological image. Non-specific biodistribution of the anti-DMPO was also found in the stomach, intestines, and bladder (**Figure 5A**). From kinetics assessment, it was found that the anti-DMPO probe persisted in certain tissues (e.g. lungs, liver and kidneys) for over 3 hours. Verification of the presence of the anti-DMPO probe in ex vivo tissues was done by using streptavidin-Cy3 which bound to the biotin moiety of the anti-DMPO probe (see **Figure 5B** for example in liver tissue). It was also confirmed that DMPO-radical adducts were formed by using a fluorescent-anti-DMPO antibody (IST approach) in diabetic (see **Figure 5C**) and non-diabetic mouse livers. In addition, inducible nitric oxide synthase (iNOS) levels were assessed in the livers of diabetic and non-diabetic mice (see **Figure 5D** for immunohistochemistry (IHC) detection of iNOS in a diabetic mouse liver; and **Figure 5F** for quantitative levels of iNOS in diabetic and non-diabetic mouse livers) as an additional marker of oxidative stress. This was the first *in vivo* study to demonstrate that diabetic mice had elevated *in situ* free radical levels in organs/tissues such as the lungs, liver and kidneys.

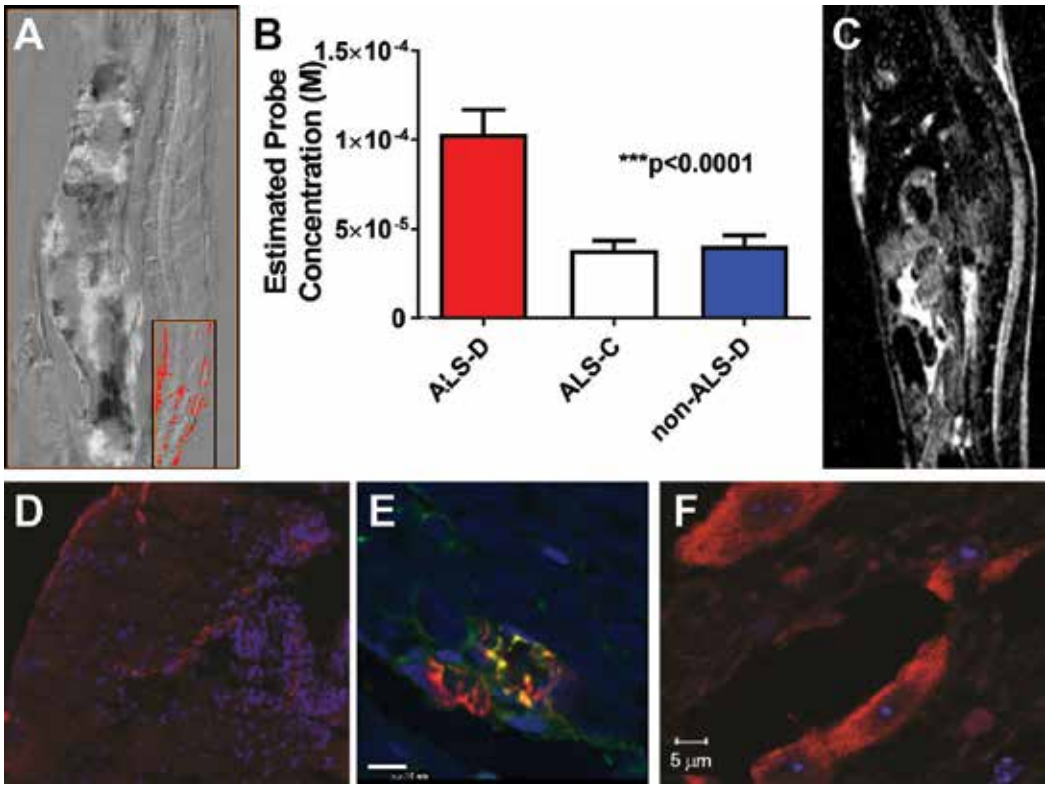

Figure 8. Combined IST and free radical-targeted mMRI in an ALS mouse model. (A) *In vivo* anti-DMPO probe map (based on MRI signal intensity (SI) change). (B) DMPO probe concentration in ALS mice administered DMPO + anti-DMPO probe (ALS-D), DMPO + non-specific IgG contrast agent (ALS-C), and wild-type non-ALS mice administered DMPO + anti-DMPO probe (non-ALS-D). N = 5 for each group. (C) Diffusion-weighted image of an ALS mouse with increased apparent diffusion coefficient (ADC) in lumbar region (outlined). (D) Stretavidin-Cy3 biotin-tagged *ex vivo* spinal cord in ALS-D. (E) Co-localized DMPO probe (red) and neuronal marker (green) fluorescence image in mouse spinal cord. (F) Fluorescent-IST image of the spinal cord of an ALS mouse. Modified from Towner et al. [32].

At a later stage it was found that the cardiac muscle in diabetic mice also retained the anti-DMPO probe [30] (see **Figure 6**). A morphological MR image of a mouse heart is shown in **Figure 6A**. The post-contrast minus pre-contrast image in a diabetic mouse with false coloration is shown in **Figure 6A**, overlaid on top of a horizontal morphological image of the heart.

Significantly higher quantitative levels of the anti-DMPO probe in diabetic (Diab) mice administered DMPO (D) and the anti-DMPO probe (P) were found when compared to diabetic (administered the isotype IgG contrast agent instead of the anti-DMPO probe) and non-diabetic (non-STZ exposed WT mice administered DMPO and the anti-DMPO probe) controls (see **Figure 6B**). Confirmation of the presence of the anti-DMPO probe in cardiac muscle of a diabetic mouse is shown in **Figure 6C**. Verification of the presence of DMPO-radical adducts is shown in **Figure 6D**, where a fluorescent-labeled anti-DMPO antibody was used. In the diabetic cardiomyopathy study, it was also established that diabetic mice had significantly higher levels of 3-nitrotyrosine (3-NT) (oxidized protein marker) (**Figure 6E**) and malondi aldehyde (MDA) adducts (oxidized lipid marker) (**Figure 6F**) in cardiac muscle, when compared

to non-diabetic mice. This was the first *in vivo* study to demonstrate increased *in situ* free radical levels in diabetic cardiomyopathy. The correlation with oxidized lipids and proteins was done, as it is suspected that the combined IST and mMRI approach primarily reports on macromolecular free radicals that are cell-membrane bound. Mason et al. have previously reported on the application of IST to trap oxidized proteins [23–25]. As both an increase in oxidized lipids and proteins were detected in diabetic cardiac muscle via ELISA, it is possible that the combined IST and mMRI method detects both oxidized lipids as well as proteins. Further verification would require a mass spectrometry approach to confirm this assumption.

2.2. Septic encephalopathy

It was then decided to assess the combined IST and mMRI free radical-targeted approach in other disease models, such as septic encephalopathy. Mice with septic encephalopathy (induced by cecal ligation and puncture (CLP)) were also found to have higher levels of trapped DMPO-radical adducts compared to sham animals (abdominal incision without CLP and sutured) [31] (**Figure 7**). **Figure 7A** depicts a MRI SI difference image (false-colored red) overlaid on top of a morphological image of the brain region of a septic mouse. Confirmation of the presence of the anti-DMPO in the cortical brain tissue of a septic mouse is shown in **Figure 7B**, and verification of DMPO-radical adducts in a septic mouse brain is depicted in **Figure 7C**. The distribution of the anti-DMPO probe is dispersed throughout the brain, and was found to be significantly higher in septic mice vs. sham animals in the hippocampus, striatum, occipital lobe and medial cortex regions of the brain (**Figure 7D**), as measured by a % change (overall decrease in T_1 relaxation). Oxidized lipid levels (measured from Western blots for 4-hydroxynonenal (4-HNE) (**Figure 7E**) and oxidized protein levels (measured from Western blots for 3-NT) (**Figure 7F**) were found to be significantly higher in septic mice (CLP) after 6 hours, compared to sham controls. This study also indicates that both oxidized lipids and proteins may play a role in the free radical-associated pathology of ALS. This is the first reported *in vivo* detection of elevated *in situ* free radicals in a mouse model for septic encephalopathy.

2.3. Amyotrophic lateral sclerosis (ALS)

The combined IST and mMRI approach for detecting *in vivo* free radicals was extended to other neurological disorders, such as ALS. High levels of trapped DMPO-radical adducts were also found in a transgenic mouse model for ALS (superoxide dismutase (SOD) mutation) [32] (see **Figure 8**). As SOD1 plays an important role in $O_2^{-\bullet}$ clearance, the loss of SOD1 can lead to increased levels of free radicals [5]. A pre-contrast minus post-contrast difference sagittal image is shown in **Figure 8A** for an ALS mouse, with false coloration depicted in the lumbar region of the spinal cord. From T_1 relaxation values the estimated anti-DMPO probe concentration can be obtained, and it was found that ALS mice administered DMPO and the anti-DMPO probe (ALS-D) had significantly higher levels of the anti-DMPO probe, compared to ALS (administered the IgG contrast agent instead of the anti-DMPO probe) and non-ALS (administered both DMPO and the anti-DMPO probe) controls (see **Figure 8B**). A diffusion-weighted image is shown in **Figure 8C** depicting a high signal intensity in the lumbar region of the spinal cord. *Ex vivo* detection of the anti-DMPO probe in the lumbar region of the spinal cord of an ALS mouse is shown in **Figure 8D**. **Figure 8E** illustrates that a neuronal marker

(NrCAM) (green) co-localizes with the location of the anti-DMPO probe (red) in some regions (yellow). Confirmation of DMPO-radical adducts is depicted in **Figure 8F**. This is the first direct *in vivo* detection of elevated *in situ* free radicals in the lumbar region of ALS-like mice.

2.4. Gliomas

Lastly, the combined IST and mMRI *in vivo* free radical detection method was applied to assess glioma models, both for untreated and anti-cancer agent treated tumors. It was initially demonstrated that free radicals can be detected in a GL261 mouse glioma model [33]. The DMPO-trapped radicals were found to be heterogeneously distributed primarily in the tumor core region, possibly associated with increased cell proliferation [33]. In addition to confirming the presence of the anti-DMPO probe in glioma tumor tissue and detection of anti-DMPO trapped radicals, it was also possible to demonstrate that GL261 gliomas had elevated levels of MDA protein adducts and 3-NT, compared to normal mouse brain tissue [33].

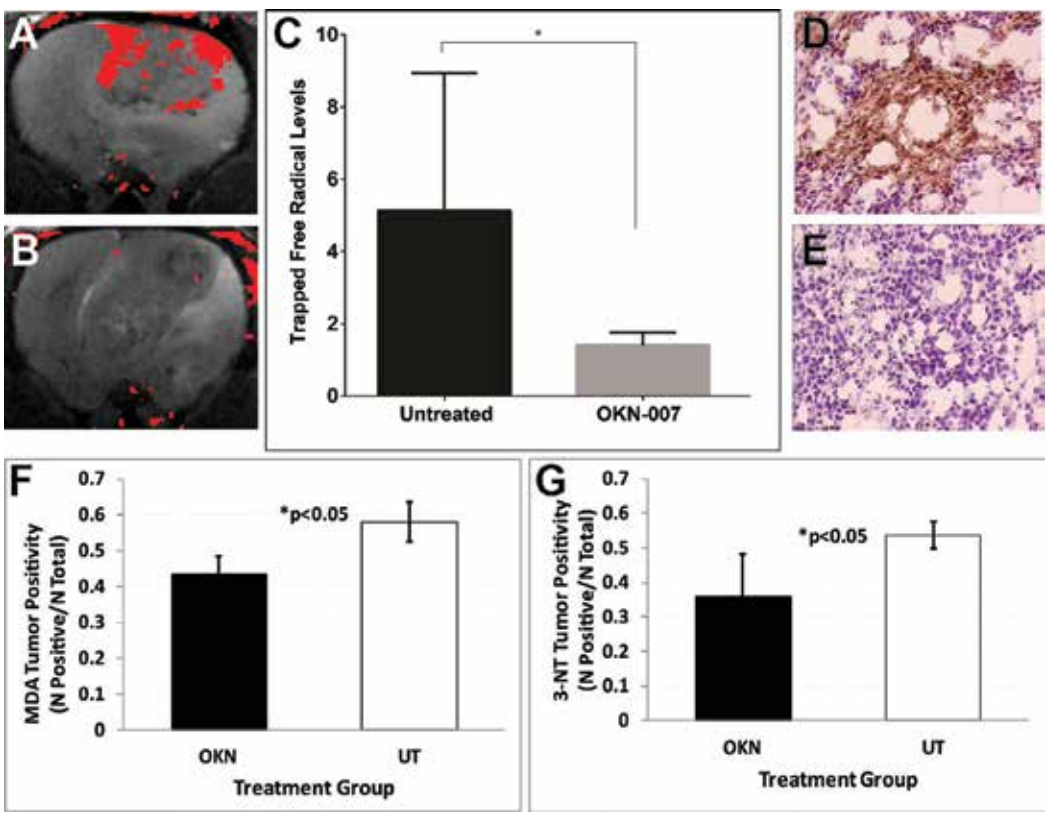

Figure 9. Combined IST and free radical-targeted mMRI in a F98 rat glioma model. *In vivo* anti-DMPO probe brain maps of (A) untreated (UT) and (B) OKN-007-treated rat F98 tumors (based on MRI SI change). (C) Trapped free radical levels (% change in MRI SI) in UT and OKN-007-treated F98 gliomas administered DMPO + anti-DMPO probe. N = 5 for each group. Stretavidin-HRP biotin-tagged ex vivo F98 gliomas that were (D) UT or (E) OKN-007-treated. (F) Malondialdehyde (MDA) levels from IHC of F98 tumors of OKN-007-treated or UT rats. (G) 3-Nitrotyrosine (3-NT) levels from IHC of F98 tumors of OKN-007-treated or UT rats. N = 5 for each. Modified from Coutinho de Souza et al. [34].

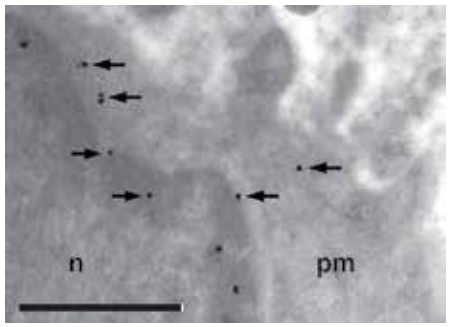

Figure 10. Immuno-electron microscopy detection of the anti-DMPO probe in the plasma membrane/ cytoplasm and cell nuclei in F98 rat gliomas. The biotin moiety of the anti-DMPO probe was targeted with gold-anti-biotin. Gold-anti-biotin colloids were detected within the plasma membrane/cytoplasm (black arrows) and cell nuclei membranes of F98 tumor cells administered the anti-DMPO probe. Scale bar = 1 μm. Magnification = 20,000×. n = nucleus; c = cytoplasm; pm = plasma membrane. Modified from Coutinho de Souza et al. [34].

The combined IST and mMRI approach in glioma models can also be used to assess possible therapeutic agents that are either free radical scavengers or generate free radicals. For example, this approach was used to assess the free radical scavenging ability of an anti-cancer agent, OKN-007, in a rat glioma model [34] (see **Figure 9**). Representative difference images (false-colored red) of an untreated F98 glioma and an OKN-007-treated F98 glioma, overlaid over appropriate morphological images, are shown in **Figure 9A** and **B**, respectively. Quantitative levels of trapped free radical levels (measured from % changes in MRI signal intensities) for untreated and OKN-007-treated F98 gliomas is shown in **Figure 9C**. Significantly lower levels of MDA (**Figure 9F**) and 3-NT (**Figure 9G**) were found for F98 gliomas treated with OKN-007 compared to untreated (UT) tumors. IHC levels for MDA and 3-NT were quantitated in several OKN-007-treated and UT F98 tumor-bearing rats. These results indicate that OKN-007 acts as a free radical scavenger when used as an anti-cancer agent. This is the first time *in vivo* detection of *in situ* free radicals had been reported for an anti-cancer agent with free radical scavenging capability. It was previously demonstrated that OKN-007 can significantly increase animal survival and significantly decrease tumor volumes, when compared to UT animals. The combined IST and mMRI approach can be taken to assess any therapeutic agents that would either increase or decrease free radical levels in different disease models.

Immuno-electron microscopy (IEM) with gold-anti-biotin, targeting the biotin moiety of the anti-DMPO probe, was also used to confirm the *ex vivo* presence of the anti-DMPO probe in the plasma membrane of rat glioma cells following *in vivo* administration [34] (see **Figure 10**). The IEM data is confirmation that the combined IST and mMRI approach is detecting macromolecular membrane-bound (both plasma membrane and possibly nuclear membrane) free radicals.

3. Concluding statements

The novelty of the IST-mMRI approach is that heterogeneous levels of trapped free radicals can be detected directly *in vivo* and *in situ* with high image resolution, and can be used to

pin-point where high levels of free radicals are formed in different heterogeneous regions of specific tissues. It should be noted that MRI has no depth-of-penetration limitation. The current anti-DMPO-albumin-Gd-DTPA-biotin construct allows rapid vascular delivery, and binding to macromolecular DMPO-trapped free radicals in the plasma membrane, as well as *ex vivo* confirmation with microscopy.

This review has discussed all of the current studies that have utilized combined IST and mMRI to detect targeted trapped macromolecular DMPO-radical adducts *in vivo* and *in situ* within various animal models where oxidative stress plays a major role. It was established for all oxidative stress-associated disease models studied thus far, that levels of free radicals were found to be significantly increased in all cases for animals treated with DMPO and the anti-DMPO probe, when compared to controls, including disease controls (e.g. wild-type rodents or shams), non-DMPO controls (i.e. administered saline instead of DMPO), and/or mMRI probe controls (i.e. a non-specific IgG was covalently bound to the albumin of the MRI contrast agent construct instead of the anti-DMPO antibody). An example of assessing an anti-cancer agent with free radical scavenging activity was also presented. The biotin moiety of the anti-DMPO probe also allowed *ex vivo* validation of the presence of trapped DMPO macromolecular adducts in various tissues. IST was also used in all cases to confirm the presence of trapped free radicals with fluorescence or optical (e.g. HRP) microscopy. Finally, IEM was used to confirm the presence of the anti-DMPO probe in plasma and nuclear membranes.

Some of the disadvantages with the methodology include limited access to pre-clinical MRI systems, availability of the anti-DMPO antibody, and further identifying the radical source that is being trapped. For non-neurological studies, this approach can be easily utilized in numerous pathological/toxicological models. However, for neurological studies, the approach will be limited to whether there is BBB permeability, in order to allow the anti-DMPO probe, and possibly DMPO, to access the target tissue.

The IST-mMRI approach can certainly be further applied to study free radicals associated longitudinally in oxidative stress-related disease processes, as well as assess the effect of therapeutic agents that alter free radical levels. Mass spectrometry may need to be used to not only further assess whether the anti-DMPO probe detected in heterogeneous tissue regions are essentially oxidized proteins or oxidized lipids, or a combination of both, before the type of protein or lipid is identified. The current size of the probe may prohibit use in neurological studies with an intact blood-brain barrier (BBB). The development of a smaller nanoparticle-based anti-DMPO probe, which may allow access through an intact BBB, is currently being considered.

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Chapter 3

Oxidative Stress and Vascular Diseases: Effect of Physical Exercise

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Abstract

Investigations have shown that worldwide the vascular diseases are considered independent risk factors for an increased mortality. Despite these diseases being related to factors such as sedentary lifestyle, poor diet and stress, the oxidative stress been the which most strongly explained the genesis of these vascular diseases. In this sense, the body of evidence involving an analysis and understanding of the factors and preventive variables of these diseases available in the literature is necessary. Considering this, we aimed to available information about the role of the oxidants and antioxidants enzymes in the prevention or causes of vascular diseases, and how the physical exercise can prevent the development of these vascular diseases. It was observed that endothelin-1 is an important oxidant involved in the atherosclerotic process, while the hydrogen sulfide and glutathione peroxidase have an antioxidant role. About the physical exercise, there is greater production of oxidants, however, as a mechanism of compensation, there is also greater production and release of antioxidants such as nitric oxide and glutathiones after some sessions when compared with the baseline. We conclude that the stress oxidative is involved in the vascular disease and the physical exercise could be used like prevention.

Keywords: vascular disease, oxidant enzyme, antioxidant enzyme, oxidative stress, physical exercise

1. Introduction

Vascular diseases have been reported worldwide as an independent risk factor for premature mortality [1]. It is now understood that the point in common of all vascular, cardiovascular or chronic non-cardiovascular degenerative diseases is the imbalance between oxidation and reduction profile caused by free radicals culminating in a situation denominated oxidative stress [1, 2]. This reactive oxygen species are generated from NADPH oxidases (NOx), responsible for the bioavailability of nitric oxide (NO) in vascular pathologies, through of a direct inactivation of NO, together with a reduction in NO synthesis and in oxidation of your receptor, denominated guanylyl cyclase soluble [3]. In this sense, the development of studies about the antioxidant action in vascular function are important, considering that alterations in this functions, characterized for one increase in vasoconstrictor responses, decrease in vasodilatory capacity and one increase in reactive oxygen species production, and the reduction of the activity of antioxidants enzymes are associated with the cardiovascular risk factor, as arterial hypertension and atherosclerosis [4–9].

Therefore, researches have analyzed effective therapeutic strategies in the treatment of pathologies that affect the vascular musculature. Among them, drug therapy is still the most indicated, due to its antithrombotic, vasodilatory and hypocholesterolemic pharmacological efficacy, such as statins, capable of improving the endothelial functions, due to yours antioxidants, anti-inflammatories and anti-atherosclerotic properties [10, 11]. Although the drugs administered are effective, their clinical utility is limited due to the development of tolerance and resistance. For that reason, other therapeutic strategies, with fewer collateral effects, have been indicated together with the drug treatment, since they are also able to reduce the deleterious vascular effects, such as nutraceutical biology with a diet rich in fruits, vegetables or red wine, for example [12, 13]. In the present study, the use of polyphenols in the treatment of high levels of polyphenols was studied. Then, it seems plausible to affirm that a better understanding of the relationship between oxidants and antioxidants functions in the prevention or treatment of vascular diseases should have a body of evidence amply constructed to collaborate in the prevention and treatment of diseases.

Physical exercise, in this context, has been presented as one of the elements of a healthy lifestyle capable of modulating oxidative stress, by promoting the increase of endothelial nitric oxide synthase (eNOS) activity, of the early oncogenesis protein tyrosine kinase (c-Src) [73] and the bioavailability of NO and antioxidant enzymes, leading to significant vascular protection [12, 13] .

Although to date the investigations have demonstrated the endogenous antioxidant effect on the prevention of vascular disease, obtained with or without the contribution of short- or long-term physical exercise, other mechanisms are need to be better understood, especially with regard to exercise. It is not yet possible to assert the understanding that in addition to modulation in the antioxidant action, there is also an action on inflammatory factors, reducing the inflammation so present in vascular diseases, if there is modification of the membrane proteins of some vascular cells, or if this regulation also occurs at the downstream level. The collection of the literature regarding these gaps will be best seen in Chapters 2 and 3 of this book.

Considering this, we aimed to avail information about the role of the oxidants and antioxidants enzymes in the prevention or causes of vascular diseases, and how the physical exercise can prevent the development of these same diseases.

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2. What is the role of oxidative stress in vascular diseases?

At first, it is necessary to understand that free radicals are molecules that contain one or more unpaired electrons, are generated independently, and are considered highly reactive due to their ability to accept electrons from other molecules until a terminal reaction occurs [2]. Excessive production of these radicals can trigger cumulative cellular damage in proteins, lipids, deoxyribonucleic acid (DNA) and other components, resulting in several pathological processes [1].

Thus, in vitro studies have demonstrated that these molecules are important intracellular signaling factors that contribute to vascular remodeling, modulating vascular contraction/dilatation, migration, apoptosis and protein turnover of the extracellular matrix [14]. Thus, increased reactive oxygen species (ROS) formation is identified in vascular diseases such as hypertension, atherosclerosis and stroke, and is associated with a reduction in levels of nitric oxide (NO) and other vasodilators, endothelial tissue damage, protein oxidation, DNA damage and increased proinflammatory responses [14].

There are two types of reactive species, one of which is called the reactive oxygen species, a general term that refers not only to radicals derived from superoxide (O_2) metabolism, but also includes non-radical O_2-reactive derivative (e.g., hydrogen—H_2O_2) [15]. Similarly, the other class is known as nitrogen reactive species (RNS), it refers to nitrogen radicals reactive with other molecules in which the reactive center is nitrogen [15]. The most common ROS and RNS are shown in **Table 1** in an order of the ones that are the most to the least reactive in the cell [15].

A variety of enzymatic and non-enzymatic processes can generate the reactive species in mammalian cells [1]. The primary sources are: mitochondrial respiratory chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, dissociated nitric

Oxigen-reactive species	
Singlet oxygen	$^1O_2$
Superoxide anion	$O_2^{-\bullet}$
Hydrogen peroxide	H_2O_2
Hydroxyl radical	$HO^\bullet$
Perhydroxyl radical	$HO_2^\bullet$
Alcoxila radical	$RO^\bullet$
Peroxyl radical	$ROO^\bullet$
Hydroperoxyl radical	$ROOH^\bullet$
Hypochlorous acid	HCLO
Ozone	O_3
Nitrogen reactive species	
Nitric oxide	NO
Nitric oxide	NO_2
Peroxynitrite	ONO_2^-

Table 1. Reactive oxygen and nitrogen species.

oxide synthase (NOS), lipoxygenase and myeloperoxidase (MPO), the first four of which are responsible for aggravating the vascular diseases [1].

The mitochondrial respiratory chain is the main pathway for radical generation in biological systems involving the transport of mitochondrial electrons, where oxygen is used for the production of ATP. Under physiological conditions, most of the oxygen consumed by cells is reduced to water in the mitochondria by serial oxy-reduction reactions through the action of the cytochrome oxidase complex. The reduction of oxygen to water takes place inside it in a way that leaves no intermediates. In fact, it is necessary to receive the oxygen atom to form two molecules of H_2O and this is done when it receives four electrons (H^+) and, upon receiving them, the oxygen goes through intermediate stages: superoxide, hydrogen peroxide, radical hydroxyl and finally water. All this happens inside the cytochrome oxidase complex and it does not let these intermediates leak. However, if there is an accumulation of electron flow in the chain, this increases the probability that some electron will leak out of the chain. From 1 to 5% of the passage of oxygen along the respiratory chain may give rise to $O_2^{\bullet -}$, which results in other non-radical species (H_2O_2) and radicals ($HO^{\bullet}$). This may result from the reduction of an ubiquinone (coenzyme Q) electron, generating ubisemiquinone, which then binds its unpaired electron to O_2 to form $O_2^{\bullet -}$. However, there may be other free radical generation sites in the electron transport chain [2]. Mitochondria also generate NO, which can react with $O_2^{\bullet -}$ to form peroxynitrite ($ONOO^-$), a very potent oxidant [16].

Nicotinamides adenine dinucleotide phosphate oxidases (NADPH) are a family of enzymes with multiple complex subunits that generate $O_2^{\bullet -}$ by reducing one of electron oxygen using NADPH as the source of electrons [17]. They comprise a cytochrome b558 that crosses the plasma membrane, is composed of a large catalytic subunit, gp91^{phox} (nox2), and a small subunit, p22^{phox} (the term "phox" is derived from "phagocytic oxidase") together with cytosolic regulatory subunits p47^{phox}, p67^{phox}, p40^{phox} and the small GTPase Rac [14, 18]. Activation of NADPH oxidase is initiated by phosphorylation (in serine) of the p47^{phox} cytoplasmic subunit, triggering its migration to the membrane, where, along with Rac, it associates with cytochrome b558, initiating the catalytic activity of the enzyme. The identification of subunits homologous to gp91^{phox} resulted in the formation of the Nox family (of "Nonphagocytic NADPH Oxidase") (Nox1, Nox2 [formally known as gp91^{phox}], Nox3, Nox4, Nox5, Duox1 and Duox2 [Dual oxidase]). The main components of the complex enzymatic are nox1, nox2 and nox4 being the major catalytic subunits in vascular endothelial cells, smooth muscle cells, fibroblasts and cardiomyocytes. In the large arteries, p22^{phox}, p47^{phox} and Rac subunits are found. While in cells of small arteries of resistance, gp91^{phox} (nox2), p22^{phox}, p47^{phox} and e p67^{phox}, were identified as the main responsible for the formation of intracellular ROS [19]. In cell stimulation, p47^{phox} becomes phosphorylated and the cytosolic subunits form a complex, which then migrates to the membrane, where it associates with cytochrome b558 to leave the active oxidase, which transfers electrons from the O_2 substrate, leading to formation of de $O_2^{\bullet -}$ [18]. In vascular cells, nox4 is abundantly expressed and plays an important role in the production of de $O_2^{\bullet -}$ and has been associated with vascular pathophysiology [17].

There are three isoforms of nitric oxide synthase (NOS) decoupling enzymes that are termed: neuronal nitric oxide synthase (nNOS) expressed in most neural tissues, endothelial nitric oxide synthase (eNOS) expressed in cardiovascular tissues and inducible iNOS), induced by pro-inflammatory mediators [20]. NO synthesizing enzymes catalyze the conversion of

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L-arginine to L-citrulline and NO. The production of NO via eNOS involves the transfer of electrons from the NADPH cofactor to adenine and flavin dinucleotide and the mononucleotide of adenine and flavin to heme [20]. All can generate $O_2^{\bullet-}$ under substrate (arginine) or cofactor (tetrahydrobiopterin—BH4) conditions. The BH4 enzyme is highly susceptible to oxidative degradation, and the initial oxidative loss of BH4 in response to increased EROS production by NADPH oxidases amplifies oxidative stress through the resulting loss in NO production and an increase in the generation of $O_2^{\bullet-}$ dependent of us. Most of the evidence linking NOS to EROS production belongs to the eNOS isoform [20].

The coupling of the electron flow through the eNOS to L-arginine is dependent on adequate levels of cofactors and under specific circumstances, eNOS can become "decoupled" and reduces the oxygen molecule rather than transfer electrons to L-arginine, generating $O_2^{\bullet-}$. Thus, the impact of eNOS on vasculature may depend on adequate levels of cofactors to support endothelial function. In fact, studies have shown the decoupling of eNOS from the arteries of individuals with diabetes [21] or atherosclerosis [22].

Xanthine and xanthine dehydrogenase are forms of the same enzyme, known as xanthine oxidoreductase. This enzyme is widely expressed in the capillary endothelium and catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid, however, only the oxidase form generates $O_2^{\bullet-}$ and H_2O_2. The enzyme typically exists in the form of dehydrogenase, but under conditions of stress or, for example, in hypoxia induced by a process of atherosclerosis, the oxidase isoform predominates. Therefore, xanthine oxidase has been implicated as a source of EROS after reperfusion of ischemic tissue in various organs [2], and its expression is upregulated by NADPH oxidase [23].

The development of vascular disease originates from an initial injury to the vessel wall by biological or mechanical factors. Both produced in response to injury can stimulate ROS production in macrophages, endothelial cells, smooth muscle cells and adventitial layer. These then impair vessel tone through endothelial dysfunction, which is characterized by inflammatory response, pro-constrictive response, increased migration of smooth muscle cells, proliferation and apoptosis (**Figure 1**), contributing to diseases such as stroke, atherosclerosis and hypertension [5–8, 24].

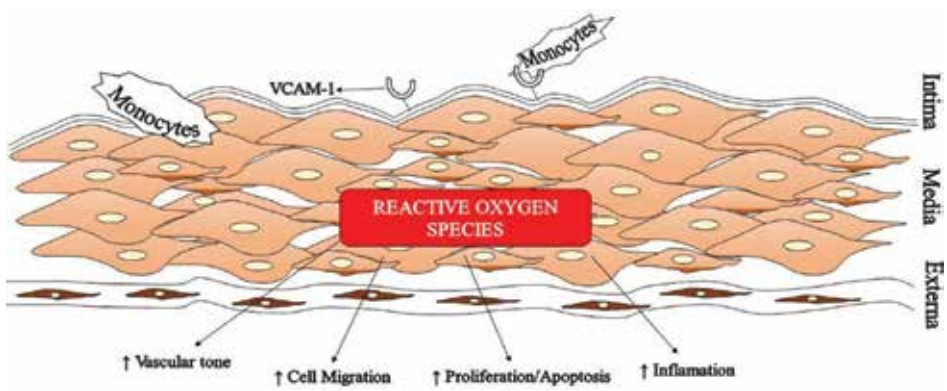

Figure 1. Role of reactive oxygen species in response to an injury.

During vascular injury, when oxidative stress is greatest, there is vessel remodeling, where agonists such as angiotensin II, platelet growth factor, cytokines/chemokines, IL-6 and IL-8, thrombin and endothelin stimulate increased activity of NADPH oxidase and its $p22^{phox}$ domain, increased proliferation, migration and adhesion activity of vascular smooth muscle cells and reduced NO bioavailability [4, 14, 20].

In this sense, it has been observed in diseases, such as hypertension, that in the aorta and mesenteric arteries there is an increase in vascular activation of NADPH oxidase, xanthine oxidase and decoupling of eNOS resulting in an increase in $O_2^{\bullet-}$ generation, whereas levels of glutathione antioxidant and activity of the endotoxin superoxide dismutase (SOD) are reduced [25]. An increase in the local and systemic vascular inflammatory process (C-reactive protein) is also observed [26]. Activation of the renin-angiotensin system stimulates NADPH oxidase activation and production of $O_2^{\bullet-}$ [27]. Vecchione et al. [28] in 2009 have shown that the reduction in endothelium-dependent vasodilation in rat arteries is associated with vascular increase in superoxide production and increased NADPH oxidase activity. However, transgenic mice with overexpression of thioredoxin 2, peroxidase that helps conversion of hydrogen peroxide into water, are resistant to hypertension induced by angiotensin II, oxidative stress and endothelial dysfunction [29]. In this sense, the present chapter aims to present the main vascular diseases, the role of the redox balance and physical exercise, in its prevention.

3. Prevention of vascular diseases

3.1. What is the role of antioxidants?

A vascular inflammation, risk of vascular disease development and oxidative stress have been widely discussed in the literature [30]. In general, oxidative stress and the inflammatory process are closely related to vascular diseases such as atherosclerosis, peripheral obstructive arterial disease, stroke, coronary artery disease and abdominal aneurysm [31].

Vascular diseases are chronic, progressive and multifactorial inflammation in which, at present, the immunological disorder, more precisely inflammatory, is perceived as a factor that plays an important role in the onset and maintenance of diseases [32, 33]. Already, oxidative stress is defined as the state of unbalance without qualifying favoring of oxidants at the expense of antioxidants that culminate in damaging effects on cells and membranes [34]. Oxidants may be referred to as reactive oxygen species, and free radicals such as superoxide ($O_2^{\bullet-}$), peroxynitrite ($ONOO^{\bullet-}$) and hydroxyl ($OH^{\bullet}$), in addition to non-radicals such as hydrogen peroxide (H_2O_2).

Antioxidant defenses consistently protect tissues and body fluids from injury caused by free radicals produced by normal metabolism, disease response or from external sources [15]. For this, they are strategically arranged throughout the cytoplasm, within several organelles, extracellular space and vascular [15].

The first defense mechanism against free radicals is to prevent their formation, mainly by inhibiting chain reactions with iron and copper. A second mechanism is through the interception of free radicals, preventing the attack on the lipids of the cell membrane causing lipid peroxidation, protein amino acids, the double bond of polyunsaturated fatty acids and

DNA bases [18]. Antioxidants obtained from the diet are extremely important in interception. Another mechanism is the repair of the lesions by removing damages of the DNA molecule and the reconstitution of damaged cell membranes [2].

Antioxidant defense mechanisms are grouped into enzymatic systems (are the first to act) and non-enzymatic [17]. Important antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and glutathione-dependent enzymes, such as glutathione peroxidase (GSH-Px), glutathione S-transferase (GST), glutathione reductase (GSH) and glutathione synthetase [17].

There are three types of SOD: SOD-1, found in the cytosol and in the nucleus, dependent on zinc and copper; SOD-2, present in the inner mitochondria, dependent on manganese and SOD-3, found in the extracellular medium. All require a transitional redox active metal in the active site to perform the catalytic decomposition of the superoxide anion.

Physiologically, our body adapts daily so that the concentration of antioxidant/antioxidant substances is balanced. However, in pathological conditions, such as in the development and maintenance of vascular diseases, antioxidant defenses are unable to maintain the oxidoreduction balance, causing higher levels of active reactive oxygen species which have the need of interaction with other cells, substances or membranes [34–37].

In a vicious cycle and difficult to break, oxidative stress can generate an inflammatory process and vice versa, so that there is the progression to an inflamed environment being it internal or external to the cell, resulting in an increase in the concentration of adhesion molecules vascular wall, endothelial dysfunction and onset of atherosclerosis, progressing to stroke, peripheral obstructive arterial disease, diabetic foot, coronary artery disease or abdominal aneurysm [34–37].

One of the oxidizing mechanisms that may explain the onset of vascular diseases such as atherosclerosis and peripheral arterial obstructive disease [38, 39] refers to the exacerbated concentrations of endothelin-1 (ET-1). ET-1 is a peptide with pro-inflammatory and pro-oxidant properties commonly secreted when there is damage to the endothelium as a signaling medium for tissue repair mechanisms. ET-1 directly causes increased NADPH activity and consequent increase in the concentration of reactive oxygen species. This is only the beginning of a reaction cascade that leads to an increase in the activity of adhesion molecules in the vascular cell (VCAM-1), with a consequent increase in macrophage and monocyte infiltration, calcium influx and vasoconstriction. Already indirectly, ET-1 participates in the generational process of atherosclerosis since it decreases the vasodilatory property of the arteries considering that there is redistribution of the eNOS to the mitochondria, thus decreasing the NO bioavailability [38]. In **Figure 2**, we observe the above-mentioned effects of ET-1.

For the antioxidant processes of prevention or deceleration of vascular diseases, proteins, enzymes or transmitting gases are involved [39–43]. In view of this, hydrogen sulfide (H_2S), paraoxonase and glutathione peroxidase (GSH-PX) are reported in the literature as the main antioxidant sources capable of preventing or treating vascular diseases, especially atherosclerosis and obstructive arterial disease peripheral [39–43].

H_2S is currently considered the third gas transmitter after NO and carbon monoxide (CO) [40, 41]. It is known that due to its interaction with ion channels [44], second messengers [45–47], post-translational modification [44, 48, 49] and antioxidant defense [50], this compound plays an important role in the prevention of vascular diseases.

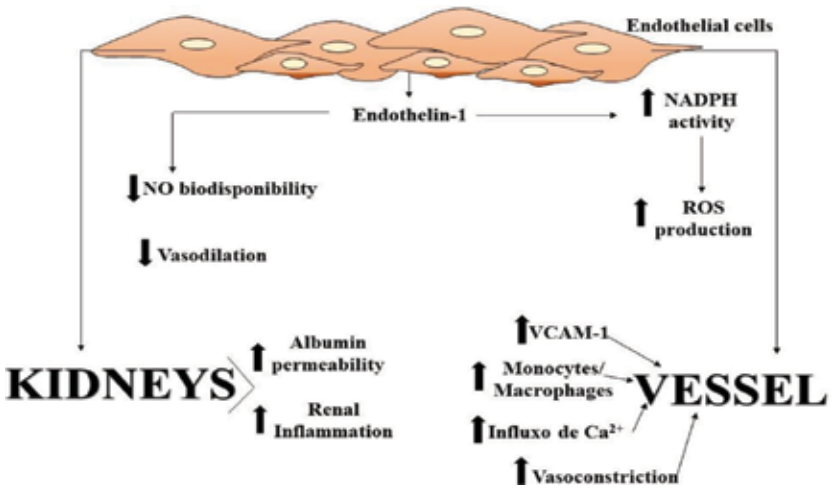

Figure 2. Principal effects of ET-1 on the vascular system and surrounding tissues. VCAM-1, vascular cell adhesion molecule 1.

Vasoprotection promoted by H_2S involves a cascade of effects that culminate in the prevention or deceleration of the atherosclerotic process once it has been initiated. Thus, the effects cascade is composed of: (1) inhibition of atherogenesis by modifying low density lipoprotein (LDL) molecules [51]; (2) inhibition of monocyte aggregation in endothelium [1, 52]; (3) inhibition of proliferation and migration of vascular smooth muscle cells in the atherosclerotic process once it is established in the vascular wall [53, 54]; (4) inhibition of the formation of spongy cells [55]; (5) angiogenesis [56]; (6) improvement of vasorelaxative mechanisms [57]; (7) reduction of vascular wall stiffening or calcification [58] and (8) prevention of platelet aggregation and thrombogenesis [59, 60]. Some of these effects can be visualized in **Figure 3**.

H_2S demonstrates its antioxidant and, consequently, vasoprotective action when it reduces pexonitrite ($ONOO^-$) molecules to nitrous acid ($HSNO_2$) and hydroxyl radical (OH^-) in a chemical representation, proposed by Filipovic et al. [61], as described below:

$$H_2S + ONOO^- \rightarrow HSNO_2 + OH^- \quad (1)$$

Carballal et al. [62] in 2011 proposed that the antioxidant action of H_2S is minimal, or of no physiological significance. However, Filipovic et al. [61] demonstrated that, unlike the initial hypothesis, H_2S has a potent antioxidant and vasoprotective effect and is similar to glutathione.

It is also emphasized that all cells are susceptible to the action of reactive oxygen species, however, the lipid matrix of cell membranes is one of the most affected sites of these active species causing lipid peroxidation [63]. In this sense, although H_2S is able to reduce peroxynitrite molecules, its main and vasoprotective antioxidant action is to reduce lipid hydroperoxides by limiting the pathobiological potential for the development of vascular diseases through lipid peroxidation. The chemical representation can be seen below:

$$LOOH + H_2S + O_2 \rightarrow LOH + SO_2 + H_2O \quad (2)$$

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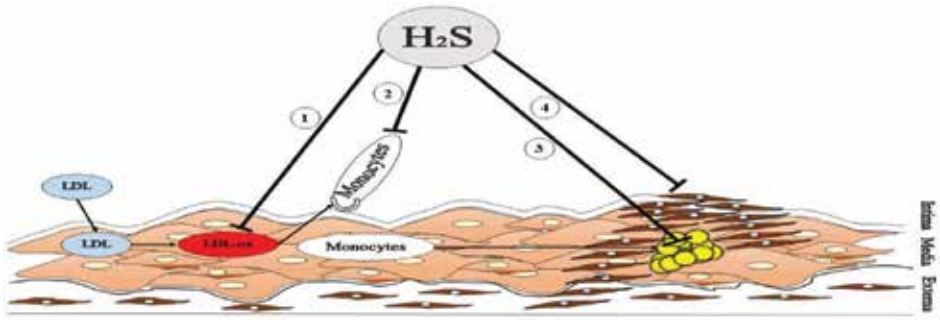

Figure 3. Main effects of hydrogen sulfide (H2S) in vascular system. 1—inhibition oxidation of low density lipoprotein; 2—diminution of monocyte aggregation in tunica intima; 3—inhibition of formation of spongy cells; 4—inhibition of proliferation and migration of vascular smooth muscular cells. The order of these effects was adopted for didactic purposes. LDL, low density lipoprotein; LDL-ox, oxidized low density lipoprotein.

Another proposed antioxidant and vasoprotective mechanism involves the action of paraoxonases, universally accepted proteins as capable of protecting cells from oxidative stress [4]. The paraoxonase family includes paraoxonase-1, 2 and 3 (PON-1, PON-2 and PON-3), with PON-1 and PON-3 being found in plasma and directly associated with high density lipoprotein fractions (HDL) promoting action against the formation of reactive oxygen species, oxidation of low density lipoprotein (LDL) and macrophages leading to blockage or reduction of atherosclerotic lesions [4]. As for PON-2, it is a cell-associated complex, it is not found free in plasma, but in some tissues, especially in the kidneys, in which its antioxidant and anti-inflammatory effects are more evident [42].

In recent years, it has been observed that glutathione peroxidase (GSH-PX), an endogenous antioxidant enzyme, attenuates the development of atherosclerosis in a similar action to H_2S, that is, reducing hydroperoxides [64]. In fact, when blocked, GSH-Px elevates oxidative stress in macrophages and increases ox-LDL activity. In addition, some elements when in non-physiological concentrations decrease GSH-PX activity, such as homocysteine [64]. Porter et al. [65] and Blann et al. [66] demonstrated that volunteers affected by atherosclerotic disease showed a reduction of approximately 29% of the peroxidase activity compared to healthy volunteers, demonstrating that the performance of this enzyme may be more related to prevention than to the repair process after vascular disease.

3.2. Effects of physical activity and physical exercise on oxidative stress: molecular mechanisms and antioxidant effect of physical exercise

The antioxidant defense system has the function of inhibiting and/or reducing the damage caused by the action of free radicals. For this, the mechanisms of action may be the impediment in formation of free radicals or non-radical species (prevention systems), preventing the action of these molecules (sweep systems) or favoring the repair and reconstitution of damaged biological structures [67].

In response to the increased oxygen consumption that occurs in intense physical exercise, the reactive oxygen species (ROS) are generated by activating at least three main mechanisms: mitochondrial, cytoplasmic and favored production by iron and copper ions [68]. At the same time,

physical exercise may also promote adaptation able to reduces the oxidative damage caused by the action of such agents. One such mechanism is the increased expression of the enzyme nitric oxide synthase (eNOS), through phosphorylation of residues by the proto-oncogenesis protein tyrosine kinase (c-SRC) and the activation of eNOS induced by other ROS in response by shear stress; while other mechanisms are triggered by oxidative stress, such as concomitant production of the enzyme superoxide dismutase (SOD), increased glutathione dismutase (GPx) activity, increased NO production induced by adenosine and the NO signaling pathway dependent on cyclic guanosine monophosphate (cGMP) (**Figure 4**). In addition, there is reduced mRNA expression and activity of pro-oxidant enzymes such as NADPH oxidase, angiotensin II receptor type I and increased expression of angiotensin II receptor type II in mammalian arteries [69].

Exercise-induced cardioprotection is probably a multifaceted phenomenon, with potential effector tissues including the myocardium, endothelial cells, inflammatory cells and coronary smooth muscle (CSM) [70]. Several mechanisms explain the positive effects of physical training regarding vascular adaptations [71].

The improvement of endothelial function by physical exercise is dependent on factors such as frequency and magnitude of physical exercise, which can cause shear stress and autoregulation of eNOS expression in endothelial cells [72]. Physical exercise results in increased heart rate and, consequently, increased blood flow and shear stress, which increases the activity of the early oncogenesis protein tyrosine kinase (c-Src) and increased eNOS production.

In the same sense, apparently the adaptations to physical exercise also occur in those vessels where there is no change in perfusion/blood flow during exercise [73]. It is important to highlight that, during exercise, the signal triggering endothelial adaptations in blood vessels perfusing tissues outside actively contracting muscle may not only be increased mean shear stress but also the alteration in shear profiles [74] that result from hemodynamic changes (e.g., heart rate and pressure) during exercise. It is suggested that alterations in the frequency of cyclic shear, and

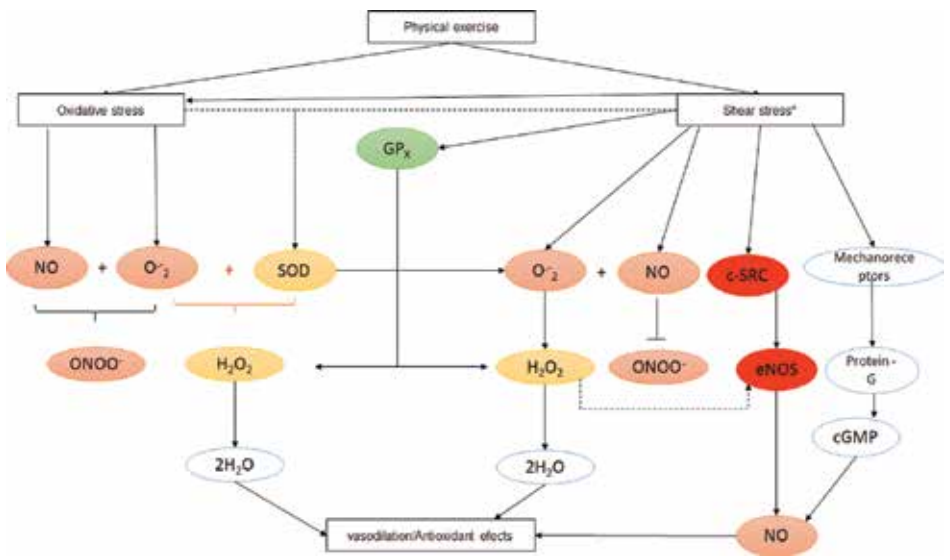

Figure 4. Possible pathways of antioxidant effect occurring as a result of physical exercise.

hence the profile of the shear waveform, may activate highly different signaling pathways than do increases in average shear stress. More research is warranted to isolate the influence of shear patterns from other exercise-related signals to fully evaluate the hypothesis that exercise-induced acute changes in shear waveforms modulate endothelial health systemically with training [75].

Several considerations should be taken into account when viewing the hypothesis that shear stress is an exercise-induced signal for endothelial adaptations in nonworking tissues. Shear stress is directly related to blood flow and viscosity but inversely related to arterial diameter [76]. Given vascular tone (and hence diameter) is constantly regulated by central and local factors (e.g., shear stress), changes in blood flow through a given vessel do not always correspond with alterations in shear stress. In this regard, the extent to which enhanced blood flow or viscosity results in increased shear stress may be dependent on the caliber of the vessel and/or its ability to dilate in response to shear. Contrary to conduit arteries, given the remarkable capacity of arterioles to dilate and constrict, it is unclear to what degree changes in blood flow in the microvasculature translate into alterations in shear [75].

Moreover, shear stress may not be the only hemodynamic exercise-induced signal for systemic endothelial adaptations. Endothelial cells are also exposed to stress from distention of arteries caused by relaxation of smooth muscle in the wall or by increased transmural pressure across the arterial wall. Since endothelial cells are exposed to cyclic distention within each cardiac cycle and during exercise the frequency and magnitude of this distention is augmented, cyclic strain should be considered as a potential exercise-induced signal. In this regard, Awolesi et al. [77] have shown that cyclic strain increases transcription of eNOS in cultured endothelial cells. Similarly, it is demonstrated that distention of isolated arteries is a stimulus for increased expression of the eNOS gene [78].

However, it is important to note that cyclic strain has also been associated with increased production of ROS and expression of adhesion molecules including vascular cell adhesion protein 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), E-selectin and monocyte chemoattractant protein-1 (MCP-1) [79]. Although chronic exposure of endothelial cells increasing cyclic strain (as occurs with hypertension) may produce negative adaptations, based on the classical physiological concept of hormesis, it is plausible that recurring periods of exercise-induced cyclic strain and consequent oxidative stress may increase the tolerance of endothelial cells to withstand subsequent doses and hence stimulate a long-term protective effect [75].

In addition to evidence in the literature that exercise-induced adaptations of the endothelium result from increases in shear stress and/or cyclic strain [80], there is also growing evidence suggesting that changes in chemical signaling (i.e., hormones, cytokines and adipokines) may contribute to systemic benefits of chronic exercise on endothelial cells. Hemodynamic forces may interact with anti-atherogenic mediators such as insulin, adiponectin and IL-6 and with inflammatory cytokines (pro-atherogenic mediators) in the determination of endothelial cell phenotype/function. It appears that substances such as signal remodeling and altered phenotype of endothelial and smooth muscle cells are also released in response to increased shear stress [71].

Regarding the positive adaptations on coronary smooth muscle (CSM) by physical training, some other points need discussion. The beneficial adaptations of physical training can occur both at the sarcoplasmic level (rianodine-sensitive Ca^{2+} channels—RyR-) and sarcolemma (voltage-dependent Ca^{2+} channels and K^{+} channels) [70].

A study by Newcomer et al. [78] demonstrated a postdepolarization, time-dependent decline in the caffeine-releasable SR Ca^{2+} store in cells from exercise-trained animals, but not sedentary control subjects. This phenomenon (termed SR Ca^{2+} unloading) was further determined to result from a slow release of SR Ca^{2+} via RyR. Of noteworth is that the Ca^{2+} released through SR Ca^{2+} unloading seems to be extruded from the cell, not resequestered by the SR or other organelles, and was demonstrated to occur with no increase in bulk Ca_m (myoplasmic free Ca^{2+} concentration) [81]. It was concluded that the net effect of this training-induced SR Ca^{2+} unloading would be a lower SR Ca^{2+} content and an increased subsarcolemmal Ca^{2+} gradient, with no effect on bulk Ca_m. In this way, it was proposed that a lower SR Ca^{2+} content caused by SR Ca^{2+} unloading may contribute to attenuated contractile responses to vasoactive agonists in the exercise trained state because of both diminished SR Ca^{2+} release and increased SR buffering of influx Ca^{2+}.

L-type Ca^{2+} channels are associated with endothelin response, so they may be associated with changes due to physical exercise. It is known that endurance training increases L-type Ca^{2+} channel current density approximately twofold in all three arterial sizes, with no effect on voltage-dependent activation or inactivation characteristics [81]. Additionally, a significant correlation between treadmill endurance time and peak L type Ca^{2+} current density was demonstrated in all three arterial sizes, supporting a direct association between endurance capacity and coronary smooth muscle L-type Ca^{2+} current density. The increase in L-type Ca^{2+} current density could result from an increase in the number of L-type Ca^{2+} channels in the sarcolemmal membrane and/or increased activity of existing channels. Future studies will be necessary to determine the basis for this training-induced adaptation.

Another channels that plays major role in control of smooth muscle tone are K^+ channels, by determining Ca_m, through regulation of membrane potential (V_m) and voltage-gated Ca^{2+} channel activity. Activation of K^+ channels produces membrane hyperpolarization, acting as a negative feedback on voltage-gated Ca^{2+} channel activation to limit contraction or produce vasodilation. Various K^+ channels are expressed in vascular smooth muscle, including large-conductance, Ca^{2+}-activated K^+ channels (K_{Ca}), voltage-dependent K^+ channels (K_v), inward rectifier K^+ channels (K_{ir}) and ATP sensitive K^+ channels (K_{ATP}).

Preliminary research [82] indicates that exercise training increases the relative contribution of both K_{Ca} and K_v channels to regulate basal tone of coronary arteries. Thus, K^+ channels play a greater role in regulating basal coronary tone in the exercise-trained state. Interestingly, it is concluded that stretch is a requisite factor for expression of this training-induced adaptation because K_{Ca} and K_v, currents, determined by voltage-clamp in enzymatically isolated smooth muscle cells, exhibited no difference in current density in cells from exercise trained and sedentary groups.

An intense physical exercise session can generate large amounts of reactive oxygen species, which increases oxidative stress and superoxide production ($O_2^{\bullet -}$) [83]. The superoxide radical is highly reactive, however, it crosses with difficulty the plasma membrane, being converted quickly to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD), whose concentration also increases with physical exercise [84]. Hydrogen peroxide (H_2O_2) is catalyzed by the enzyme glutathione oxidase (GPX), which reduces it to two molecules of water, reducing the oxidant damage of the superoxide radical.

The antioxidant mechanisms induced by shear stress are not yet completely clarified by the literature, so other mechanisms are studied [83]. The mechanism of NO signaling dependent on cyclic

guanosine monophosphate (cGMP), refers to endothelial cells that present mechanoreceptors. These receptors directly activate G-proteins, enzymes and other protein kinases that generate a second messenger, such as cGMP, which leads to vasodilation. Another mechanism increased NO production stimulated by adenosine. Studies have shown that erythrocyte membranes tend to release ATP in response to shear stress and that during the strenuous physical exercise in the cincunflex artery, there is NO production stimulated by adenosine [84].

Despite the exhaustive studies, there are controversies regarding the antioxidant effect of physical exercise related to issues such as eNOS measurement after a training period, eNOS status in the animal and human baseline, and the existence of polymorphisms in the gene promoter of eNOS [83]. It has been shown that eNOS activation induced by shear stress does not depend on the increase of intracellular calcium, but on enzyme phosphorylation [85]. This post-transcription modification occurs at serine 1177 and is mediated by the serine/threonine protein kinase Akt (protein kinase B) [86]. This alters the sensitivity of the enzyme to Ca^{2+}, making its activity maximal at subphysiological concentrations of Ca^{2+}. In the presence of Ca^{2+}/calmodulin, the serine 1177-mediated eNOS phosphorylation occurs in the skeletal and cardiac muscle of rats by the activation of AMPK (activated protein kinase), an enzyme activated by vigorous exercise and ischemic stress [87]. Boo et al. [88] suggested that a coordinated interaction between Akt and PKA may be an important mechanism by regulating eNOS activity in response to shear stress. These results are confirmed in humans with coronary artery disease who underwent 4 weeks of supervised physical exercise training lasting 60 min/day. The increase in the levels of phosphorylation of eNOS-mediated enzyme ser-1177 increased fourfold in the left mammary artery region. This was associated with a two-fold increase in eNOS and a significant increase in endothelium-dependent vasodilation in this artery [89]. Together, the current evidence suggests that phosphorylation induced by shear stress caused by physical exercise contributes to the improvement of endothelium-dependent vasodilation.

The acute effects of aerobic and anaerobic physical exercise are related to the increase of vascular oxidative stress and damages to lipid cells, nucleic acids and the glutathione system (GSH). Very intense physical exercise for 4 weeks may induce increased plasmatic activity of the glutathione peroxidase enzyme (GPx) and decrease of antioxidant substances in the resting plasma, in the pre-exercise period, and mainly, in the post-exercise period and accompanied by a reduced glutathione (GSH) and oxidized glutathione (GSSG, GSH/GSSG) and an increase in plasma thiobarbituric acid reactive substances (TBARS) [90]. Thus, acute periods of exhaustive training may decrease the antioxidative capacity of tissues, such as skeletal muscle and vascular cells [83]. On the other hand, adaptations to moderate exercise appear to occur after a few weeks of training; in fact, endurance training has been shown to be able to reduce oxidative stress, such as lipid peroxidation in membrane erythrocytes, when compared to exhaustive exercise in young men trained [91]. The increase in eNOS expression by physical exercise is followed by increased expression of SOD3 [84]. Self-regulation of SOD by physical exercise not only provides efficient detoxification of superoxide but also reduces the generation of peroxynitrite, a strong oxidant with important pathophysiological effects [92]. While manganese protein levels, superoxide dismutase (SOD2) was not altered, levels of the $p67^{phox}$ protein, a subunit of the pro-oxidant enzyme NADPH oxidase, were reduced by physical training [93]. These observations demonstrate that the antioxidant effects of physical exercise can not only be mediated by increased expression of antioxidant enzymes, but also by reduced expression of pro-oxidant enzymes [83].

Author (date)	Population and/or sample	Characteristics of the intervention*	Frequency and Period of intervention	Main outcomes	Conclusions
Miyazaki et al (2001) [91]	Untrained male	High intensity endurance training-cycle ergometer 60 minutes/session 60r.p.m(15W)/minute	Five times a week for 12 weeks	SOD GPx CAT	↗SOD ↗GPx ↔CAT
de Moffarts et al (2006) [100]	Horses TM: treadmill test RT: run on track	Acute: standard treadmill test (running)	-	SOD GPx GSH GSSG GRR PSH	↔ GPx, SOD, GSSG e GRR As diferenças entre TM e RT foram significantes no E15 para UA, AA e PSH
Wycherley et al(2008) [96]	Overweight and obese patieens with type 2 diabetes	Diet alone (D) or diet plus exercise (DE) Walking/jogging exercise 20 to 60minutes/session Intensity: 60% 80% HRmáx	Four to five times a week for 12 weeks	TAS MDA	**D** ↘MDA ↔TAS **DE** ↘MDA ↔TAS
Venojarvi et al (2013) [94]	Middle-aged men	Aerobic Nordic walking (NW) or resistance exercise training (RT) 60 minutes NW (55 -75% FCR) RT (50 -85% 1RM)	Three times a week for 12 weeks	MDA LPO ORAC	**NW** ↔ MDA ↔LPO ↔ORAC **RT** ↔ MDA ↔LPO ↔ORAC
Mallardé et al (2014) [101]	Diabetic rats	Endurance training (a treadmill run of 60 min/day, 25 m/min)	Five days a week	SOD GPx ORAC	↔ SOD e GPx, ORAC
Duggan et al (2016) [97]	Women Obese	Moderate- to-vigorous intensity aerobic exercise. 45 minutes 70–85% (HRmáx). Activities of ≥4 METs Exercice Diet + Exercice	Five days a week for 12 months	FOP F2-isoprostanes Oxidized-LDL	**-Exercice** ↔FOP ↘F2-isoprostanes ↔ Oxidized LDL **-Diet + Exercice** ↗FOP ↘F2-isoprostanes ↘ Oxidized LDL
Seawright et al. (2016) [103]	Male rats	-Increase in intraluminal pressure and shear stress (SS), to mimic two mechanical signals associated with a bout of exercise -90 (P90) or 130 (P130) cmH2O and exposed to no SS (0 dyn/cm2) or high SS (~65 dyn/cm2) for 1 h	-	L-NNA	-Incubation with L-NNA eliminated flow-induced dilation in Old P130 + SS (soleus muscle feed arteries - SFA)
Chunyan LI et al (2016) [98]	Males Obese adolescents	-Aerobic exercise included jogging, table tennis, badminton, swimming, and aerobics and spinning (exercise training and dietary restriction.) - 60–80% (HRmax)	3 times/day and 6 times/week; 35 min/day for 4 weeks.	MDA SOD GPx	↔ MDA ↗ SOD ↗ GPx
Duntas et al (2016) [95]	Hypertensive elderly women	- Strength training -10 rep. of nine exercises	2 to 3 times/week for 6 weeks.	MDA TAC	↘MDA ↗TAC
Alghadir, Gabr, Al-Eisa (2016) [99]	Healthy older adults	-Moderate aerobic exercise - 45–60 min/day - (60 to 70% of Training heart rate-THR)	24 weeks	MDA TAC	↘MDA ↗TAC
Alaca et al (2018) [102]	Diabetic rats	- Swimming exercise -(CE) Continuous exercise: (30 minutes/day) - (BE) Short bouts of exercise (3x10 minutes/day) - (WWE) Weekend warriors (35+40 minutes/day, 2 days/week).	Five days a week for 6 weeks	MDA	**CE, BR and WWE** ↗MDA

Legend: *Intervention duration/modality, Weekly frequency and session duration and Intensity. Superoxide dismutase, SOD; Glutathione peroxidase, GPx; Glutathione reduced, GSH; Glutathione oxidized, GSSG; Glutathione redox ratio, GRR; Protein thiol, PSH; Catalase, CAT; Total antioxidant status, TAS; Malondialdehyde, MDA; Lipid hydroperoxides, LPO; Absorbance capacity, ORAC; Fluorescent Oxidation Products, FOP; Nitric oxide, NO; No-nitro-l-Arginine, L-NNA; Total antioxidant capacity, TAC; ↗, increased; ↘, decreased; ↔, did not have significant changes.

Table 2. Review of articles about effects of physical exercise and oxidative stress in humans and animals.

A review with studies that verified the influence of physical exercise on oxidative stress is presented in **Table 2**. Overall, human studies with interventions ranging from 4 weeks to 12 months, either with strength training [91, 94, 95] or aerobic training of moderate to vigorous intensity [94, 96–99] have demonstrated an improvement in antioxidant capacity by increasing SOD, CAT, GPx, total antioxidant capacity (TAC) and/or decrease of malondialdehyde (MDA). Only one study that demonstrated the acute effect of horse racing did not show significant changes in GPx, SOD, GSSG and glutathione redox ratio (GRR) [100]. In diabetic rats that performed moderate intensity exercise also did not significantly alter SOD, GPx and oxygen radical absorbance capacity (ORAC) [101] or increased MDA [102].

Considering that the acute effects of vigorous physical exercise are related to the increase in oxidative stress, while the chronic effects of training with moderate exercise can favor changes in gene expression and increase of the antioxidant effect, it is possible to speculate that the antioxidant effect of physical exercise is dependent of the occurrence of oxidative stress in an intermittent way [83]. Briefly, physical exercise may, in the medium term (about 3 weeks, for example) increase vascular hydrogen peroxide and, consequently, eNOS expression [83, 87]. It is possible that physical exercise training in the medium term reduces oxidative stress by the measurement of lipid peroxidation in the erythrocyte membrane in response to strenuous exercise in young, untrained males [91]. Furthermore, eNOS activity was shown to be a crucial factor for vascular expression of the antioxidant enzyme SOD3, and 4 weeks of physical training reduced the expression of potentially pro-oxidant proteins, such as NADPH oxidase and type 1 angiotensin II receptor, while the expression of vascular antioxidant proteins such as angiotensin II receptor type 2 is reduced [104]. Additionally, the potentially beneficial effects of exercise and/or regular physical activity as increased eNOS expression is reversible by a sedentary lifestyle as induced by forced physical inactivity [105].

Thus, regular physical exercise becomes beneficial for healthy people and patients with cardiovascular disease. While exercise training may hinder the development of pro-oxidative vascular gene expression associated with endothelial dysfunction in individuals, it corrects and/or improves already established endothelial dysfunction and increased vascular oxidative stress in cardiovascular diseases such as hypertension, diabetes, encephalic stroke, coronary artery disease and heart failure [83, 103, 106].

4. Conclusion

The present chapter demonstrates that the oxidative stress is heavily involved in most of vascular diseases considering your effect for elevated the aggregation of monocytes in endothelium, low density lipoprotein oxidation, proliferation of vascular smooth muscular cell, among others. On the other hand, endogenous enzymes or compounds like glutathione peroxidase and sulfide hydrogen have the antagonistic effects like inhibition of the pathophysiological processes involved in vascular diseases. Therefore, although the physical exercise be able to elevated the concentration of reactive oxygen species after your practice, is be able to promoted the elevation in the production and secretion of antioxidants like superoxide dismutase, resulting in scavenging effects, responsible for the defenses against the development of the atherosclerotic process, present in the most of the vascular diseases can be observed.

Conflict of interest

There is no conflict of interest.

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Chapter 4

EPR Analysis of Antioxidant Compounds

Betül Çalişkan and Ali Cengiz Çalişkan

Additional information is available at the end of the chapter

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Abstract

A free radical is a molecular species having an unpaired electron and it is a highly reactive entity and unstable. A free radical is a molecule with one or more unpaired electrons in its outer shell. Free radicals can be formed by chemical bond breakage from molecules or by redox reactions. When cells use oxygen, the oxidative stress occurs. The oxidative stress causes free radical formation. Free radicals can also be generated from ionizing radiations, ozone, heavy metal poisoning, cigarette smoking, and chronic alcohol intake. These free radicals are highly reactive and oxidize biomolecules leading to tissue injury and cell death. They also cause toxic effects and diseases. Antioxidants neutralize free radicals resulting from oxidative stress. Antioxidants play an important role in the treatment of diseases. The most suitable method for the analysis of free radicals is the electron paramagnetic resonance (EPR) spectroscopy method. The EPR method detects a paramagnetic center with a single electron. It gives information about the interactions with other nuclei around one electron. It provides information on the structure and environment of radicals.

Keywords: free radical, radiation damage center, antioxidant, electron paramagnetic resonance

1. Introduction

Free radicals are an atom or a molecule that bears an unpaired electron and is extremely reactive, capable of engaging in a rapid change reaction that destabilizes other molecules and generates many more free radicals. In plants and animals, these free radicals are deactivated by antioxidants. These antioxidants act as an inhibitor of the process of oxidation, even at

relatively small concentrations, and thus have diverse physiological roles in the body. The body is constantly exposed to the negative and sometimes lethal effects of oxidants during normal physiological processes. The harmful free radicals such as hydroxyl, peroxyl and the superoxide anion are constantly being produced as a result of metabolic reactions in living systems. On a daily basis, up to 5% of inhaled oxygen may be converted to reactive oxygen species (ROS). These ROS have the ability to bind to cellular structures and have been implicated in a number of pathological processes such as aging, inflammation, reoxygenation of ischemic tissues, atherosclerosis, cancer and even Parkinson's disease in men [1]. Two processes, which produce free radicals in vivo, have been identified and named the Fenton reaction and the Haber-Weiss reaction [2]. Antioxidants play an important role in animal health. Conventional antioxidants have been shown to improve animal performance during conditions characterized by increased tissue oxidant levels such as stress, injury and infections [3].

Free radicals can be classified as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS). Even though free radicals are mainly produced through regular metabolic routes, there are also some external factors that promote their production, including smoking, environmental pollutants, radiation and drugs, among others [4]. In healthy organisms, there is a delicate balance between the production and the removal of free radicals, which guarantees that they remain in adequate concentrations. However, when this balance is broken, these reactive species start producing chemical damages to proteins, lipids, DNA, RNA and sugars generating the so-called oxidative stress of body cells [4]. These processes have been associated with several diseases including cardiovascular, liver, neurological and renal disorders, as well as cancer, auto-immune deficiency and degenerative disorders associated with aging, diabetes, obesity, autism, Alzheimer's, Parkinson's and Huntington's diseases [4]. Although humans have developed different endogenous defense mechanisms to protect cells from the excess of free radicals and to avoid the oxidative stress, often these mechanisms are not enough. Therefore, to increase protection from oxidative damage, dietary supplements with antioxidants are recommended as a way to maintain the concentration of free radicals as low as possible [5].

Antioxidants destroy free radicals. Antioxidants neutralize the radical, and thus the radical-antioxidant association maintains itself in a stationary state. For all these situations, antioxidants must be in a structure that will quench the radical. Therefore, unpaired electrons in the structure of antioxidants must be either radical ions or free radicals. So, they must form a paramagnetic center. Paramagnetic structures are analyzed by electron paramagnetic resonance (EPR) spectroscopy. Thus, the EPR analysis of antioxidants is as valuable and important as the investigation of the paramagnetic centers of radicals. There are many studies done for this purpose. Among these studies, especially the works we have done recently are noteworthy.

Potassium hydroquinone monosulfonate (PHM), succinic anhydride and 3-nitroacetophenone compounds are antioxidants. Paramagnetic centers formed by gamma irradiation effect on single crystals of these materials were analyzed by the EPR method [6–8].

http://dx.doi.org/10.5772/intechopen.74294

2. EPR studies of antioxidant compounds

2.1. Gamma-irradiated potassium hydroquinone monosulfonate single crystal

Free radicals or other odd electrons have often been postulated as intermediates in the decomposition of organic compounds by ionizing radiation. It is still important for formulating mechanisms in radiation chemistry to identify the radicals that are formed. If several organic compounds are irradiated and resulting radicals are identified, one may hope that generalizations can be drawn concerning modes of bond rupture.

The quinone structure is widespread in nature: for example, quinones play an integral role in many biological electron-transfer processes, particularly respiration and photosynthesis [9, 10]. Quinones have long been considered for their fungicide, antibacterial and anticancer properties [11]. The quinone derivatives are especially interesting because they have significant properties due to the ready reversibility of the quinone-semiquinone redox system [12].

The single crystal of PHM was irradiated with gamma rays at room temperature and studied by the EPR technique at 125 K. The spectra were dependent on the orientation of H in the planes, which are perpendicular to each other. The paramagnetic centers formed from PHM are shown in **Figure 1** [6].

The experimental spectrum in **Figure 2** has 1:1:1:2:1 intensity ratios and exhibits the superimposition due to the two PHM anion radicals. Because of the radical A, the spectra exhibit 2-lines with intensity ratios 1:1. Owing to the C6 β-proton, the spectra exhibit a doublet (1:1). Because of the radical B, the spectra exhibit 3-lines with intensity ratios 1:2:1. Owing to the C3 and C4 β-protons, the spectra exhibit a triplet (1:2:1). The spectrum in **Figure 3** has 1:2:2:1 intensity ratios and exhibits the superimposition due to the two PHM anion radicals.

The simulation values of the hyperfine coupling constants of the spectra in **Figures 2** and **3** are given in **Table 1**. The EPR parameters belonging to two semiquinone anion radicals observed in PHM are included in **Tables 2** and **3**.

The EPR measurements have shown the existence of two semiquinone anion radicals. The semiquinone anion radicals were stable. The observed semiquinone anion radicals were obtained from the oxidation of PHM single crystal.

The angular dependences of EPR spectra were obtained for different orientations of the static magnetic field with respect to the crystalline axes. For the radical A and radical B, the spectroscopic splitting factor and the hyperfine coupling constants are anisotropic. For the radical A, the average values of the g-factor and the hyperfine coupling constant were obtained as g_A = 2.01477 and $\left(a_{CH(\beta)}\right)_A$ = 0.326 mT, respectively. For the radical B, the average values of the g-factor and the hyperfine coupling constant were obtained as g_B = 2.01054 and $\left(a_{C_2H_2(\beta)}\right)_B$ = 0.568 mT, respectively.

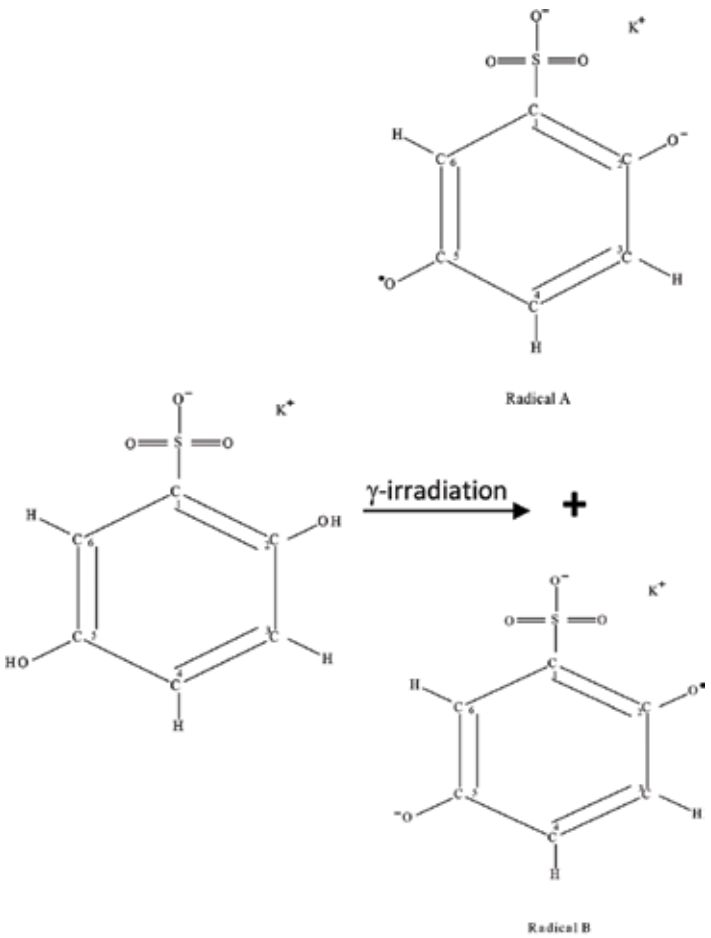

Figure 1. Structure of two semiquinone anion radicals observed in PHM single crystal.

2.2. Gamma-irradiated succinic anhydride single crystal

Succinate:quinone reductase (SQR) of complex II occupies a unique central point in the mitochondrial respiratory system as a major source of electrons driving reactive oxygen species (ROS) production. It is an ideal pharmaceutical target for modulating ROS levels in normal cells to prevent oxidative stress-induced damage or alternatively, increase ROS in cancer cells, inducing cell death [13].

The single crystals of succinic anhydride were irradiated gamma-rays at room temperature and studied by the EPR technique at 125 K [7]. The spectra were dependent on the orientation of H in the planes which are perpendicular to each other The paramagnetic centers formed from succinic anhydride are shown in **Figure 4**.

For only a few angles, the spectra have 1:2:1:1:2:1:2:4:2:2:4:2:1:2:1:1:2:1 intensity ratios. For many angles, the spectra have 1:1:1:1:1:1:1:1:2:2:2:2:2:2:2:2:1:1:1:1:1:1:1:1 intensity ratios. The experimental spectrum in **Figure 5** has 1:2:1:1:2:1:2:4:2:2:4:2:1:2:1:1:2:1 intensity ratios and

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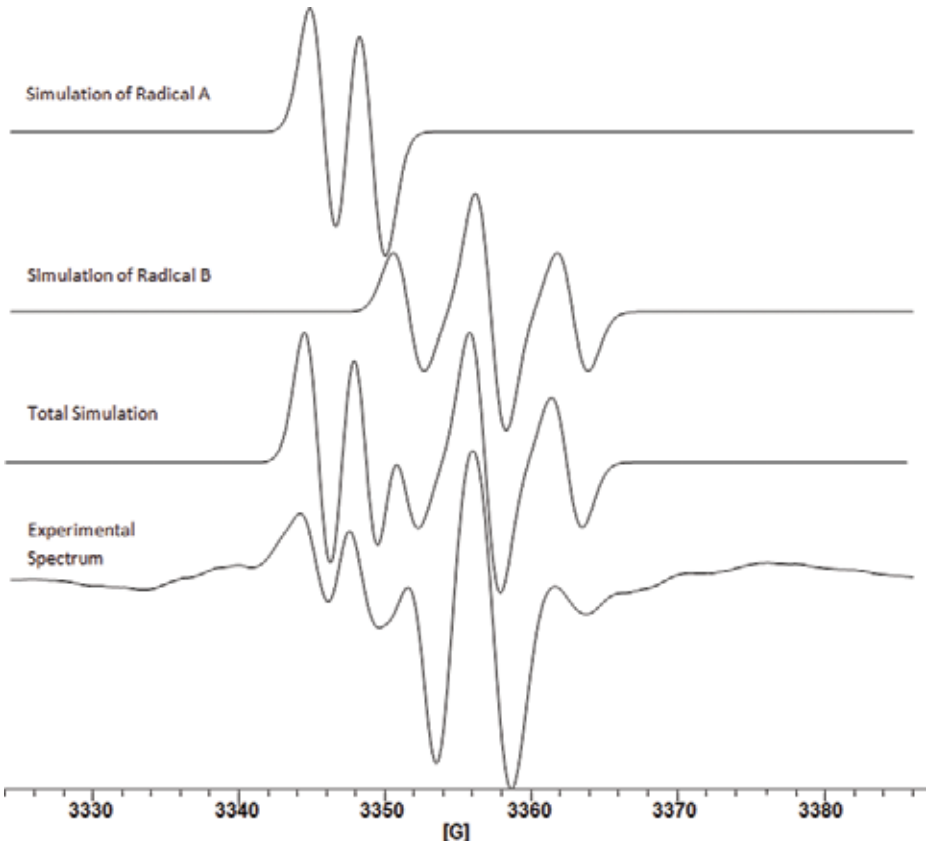

Figure 2. EPR spectrum of gamma-irradiated PHM single crystal at 125 K when the magnetic field is in the ab plane at an angle 0° toward the axis.

exhibits the superimposition due to the two CH_2CH_2 atom groups. Because of the radical A, the spectra exhibit 9-lines with intensity ratios 1:2:1:2:4:2:1:2:1. Owing to the β-protons, the spectra exhibit a triplet (1:2:1). Each of the lines (1:2:1) splits into triplets (1:2:1) due to the two equivalent γ-protons. Because of the radical B, the spectra exhibit 9-lines with intensity ratios 1:2:1:2:4:2:1:2:1. Owing to the β-protons, the spectra exhibit a triplet (1:2:1). Each of the lines (1:2:1) splits into triplets (1:2:1) due to the two equivalent γ-protons. Total 18-lines are observed due to the two anion radicals. The spectrum in **Figure 6** has 1:1:1:1:1:1:1:1:2:2:2:2:2:2:2:2:1:1:1:1:1:1:1:1:1 intensity ratios and exhibits the superimposition due to the two $(CH_2)_\beta CH_{\gamma 1}H_{\gamma 2}$ atom groups. Because of the radical A, the spectra exhibit 12-lines with intensity ratios 1:1:1:1:2:2:2:2:1:1:1:1. Owing to the β-protons, the spectra exhibit a triplet (1:2:1). Each of the lines (1:2:1) splits into doublets (1:1) due to the γ_1-proton. Each of the lines (1:1:2:2:1:1) splits into doublets (1:1) due to the γ_2-proton. The hyperfine structure splittings in the radical A are the same as that of the radical B. The total 24-lines are observed due to the two anion radicals.

We have obtained the computer simulations of the spectra that give the best agreement with experimental values. The simulations of the EPR spectra have been carried out using the Bruker's WINEPR software.

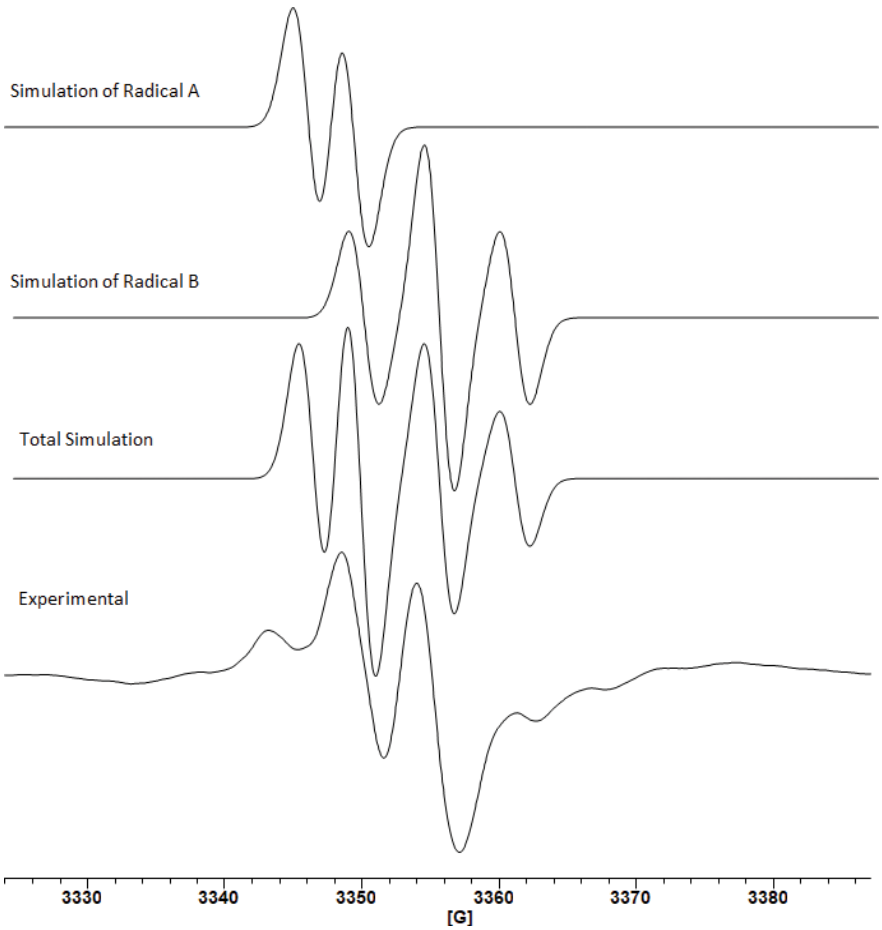

Figure 3. EPR spectrum of gamma-irradiated PHM single crystal at 125 K when the magnetic field is in the ac plane at an angle 70° toward the axis.

	Radical A	**Radical B**
Figure 2	$\left(a_{CH(\beta)}\right)_A$ = 0.328 mT	$\left(a_{CH_{2(\beta)}}\right)_B = \left(a_{H_\beta}\right)_B$ = 0.572 mT
	Center field = 335.5 mT	Center field = 335.5 mT
	ν = 9.38 GHz	ν = 9.408 GHz
	Line width = 0.2 mT	Line width = 0.215 mT
Figure 3	$\left(a_{CH(\beta)}\right)_A$ = 0.327 mT	$\left(a_{CH_{2(\beta)}}\right)_B = \left(a_{H_\beta}\right)_B$ = 0.55 mT
	Center field = 335.5 mT	Center field = 335.5 mT
	ν = 9.38 GHz	ν = 9.401 GHz
	Line width = 0.22 mT	Line width = 0.22 mT

Table 1. EPR parameters of simulated spectra.

	Principal values	Direction cosines		
$\left(A_{CH(\beta)}\right)_A$ (mT)	A_{xx} = 0.347	0.807090	0.452748	−0.378980
	A_{yy} = 0.324	−0.198271	0.812426	0.548318
	A_{zz} = 0.307	0.556143	−0.367402	0.745467
	a_{av} = 0.326			
g_A	g_{xx} = 2.01690	0.745218	0.366680	−0.556952
	g_{yy} = 2.01479	0.353981	0.490298	0.796643
	g_{zz} = 2.01263	0.565109	−0.790667	0.235582
	g_{av} = 2.01477			

Note: The errors are estimated to be ±0.00005 and ±0.005 mT for all the calculated g- and A-values, respectively.

Table 2. The EPR parameters of radical A observed in PHM at 125 K.

	Principal values	Direction cosines		
$\left(A_{C_2H_2(\beta)}\right)_B$	A_{xx} = 0.662	0.618669	0.457813	0.638480
	A_{yy} = 0.56	−0.736026	0.053485	0.674838
	A_{zz} = 0.483	0.274800	−0.887439	0.370051
	a_{av} = 0.568			
g_B	g_{xx} = 2.01119	0.506864	−0.262535	0.821075
	g_{yy} = 2.01056	0.787949	0.527402	−0.317780
	g_{zz} = 2.00986	−0.349608	0.808037	0.474185
	g_{av} = 2.01054			

Note: The errors are estimated to be ±0.00005 and ±0.005 mT for all the calculated g- and A-values, respectively.

Table 3. The EPR parameters of radical B observed in PHM at 125 K.

The simulation values of the hyperfine coupling constants of the spectra in **Figures 5** and **6** are given in **Table 4**. The EPR parameters belonging to two succinic anhydride anion radicals observed in succinic anhydride are included in **Tables 5** and **6**.

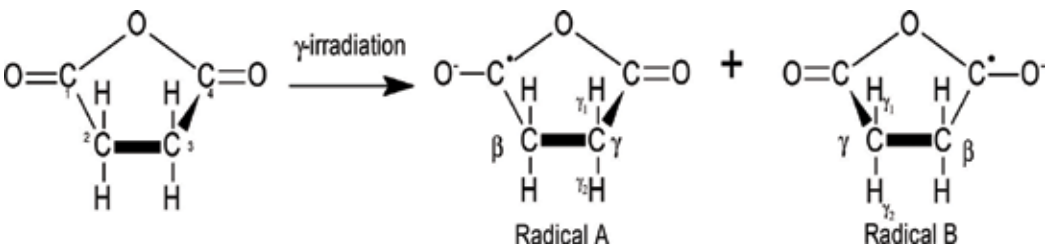

Figure 4. Structure of two succinic anhydride anion radicals observed in succinic anhydride single crystal.

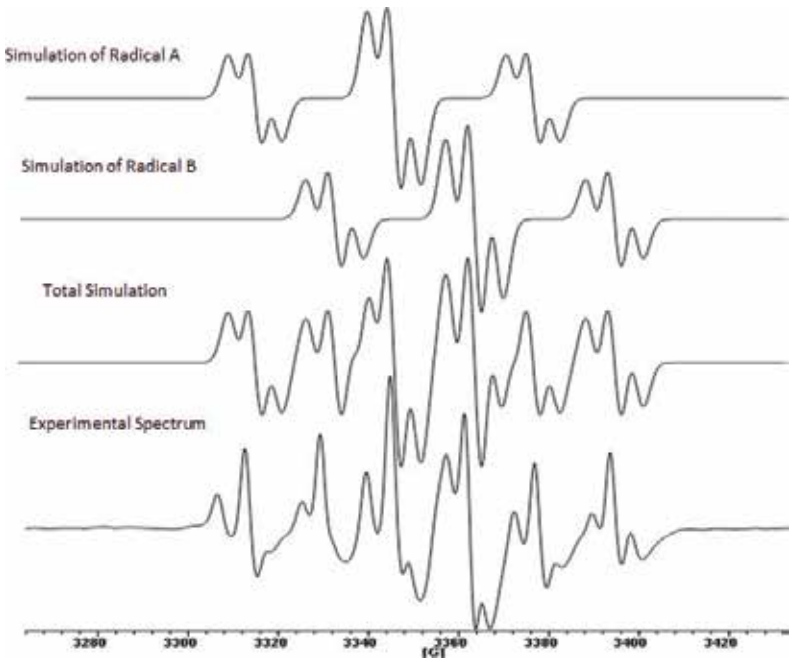

Figure 5. Experimental and simulated EPR spectra of gamma-irradiated succinic anhydride single crystal at 125 K when the magnetic field is in the bc plane at an angle 90° toward the axis.

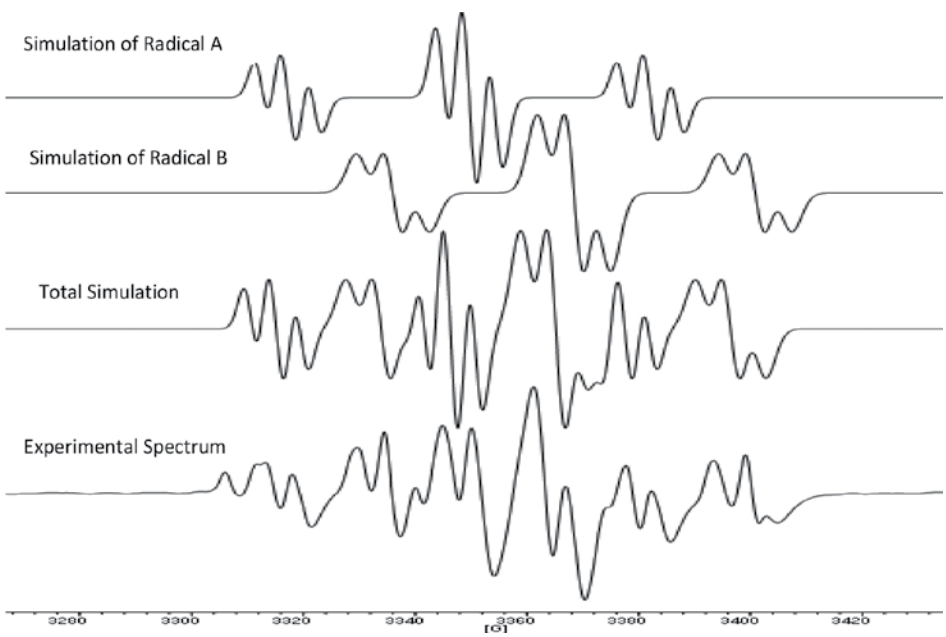

Figure 6. Experimental and simulated EPR spectra of gamma-irradiated succinic anhydride single crystal at 125 K when the magnetic field is in the ac plane at an angle 110° toward the axis.

	Radical A	Radical B
Figure 5	$\left(a_{CH_{2(\beta)}}\right)_A = \left(a_{H_\beta}\right)_A$ = 3.26 mT	$\left(a_{CH_{2(\beta)}}\right)_B = \left(a_{H_\beta}\right)_B$ = 3.281 mT
	$\left(a^{H_{\gamma1}}_{CH_{\gamma1}H_{\gamma2}}\right)_A = \left(a_{H_{\gamma1}}\right)_A$ = 0.47 mT	$\left(a^{H_{\gamma1}}_{CH_{\gamma1}H_{\gamma2}}\right)_B = \left(a_{H_{\gamma1}}\right)_B$ = 0.505 mT
	$\left(a^{H_{\gamma2}}_{CH_{\gamma1}H_{\gamma2}}\right)_A = \left(a_{H_{\gamma2}}\right)_A$ = 0.43 mT	$\left(a^{H_{\gamma2}}_{CH_{\gamma1}H_{\gamma2}}\right)_B = \left(a_{H_{\gamma2}}\right)_B$ = 0.47 mT
	Center field = 335.576 mT	Center field = 335.576 mT
	ν = 9.368 GHz	ν = 9.423 GHz
	Line width = 0.4 mT	Line width = 0.4 mT
Figure 6	$\left(a_{CH_{2(\beta)}}\right)_A = \left(a_{H_\beta}\right)_A$ = 3.245 mT	$\left(a_{CH_{2(\beta)}}\right)_B = \left(a_{H_\beta}\right)_B$ = 3.253 mT
	$\left(a^{H_{\gamma1}}_{CH_{\gamma1}H_{\gamma2}}\right)_A = \left(a_{H_{\gamma1}}\right)_A$ = 0.39 mT	$\left(a^{H_{\gamma1}}_{CH_{\gamma1}H_{\gamma2}}\right)_B = \left(a_{H_{\gamma1}}\right)_B$ = 0.44 mT
	$\left(a^{H_{\gamma2}}_{CH_{\gamma1}H_{\gamma2}}\right)_A = \left(a_{H_{\gamma2}}\right)_A$ = 0.52 mT	$\left(a^{H_{\gamma2}}_{CH_{\gamma1}H_{\gamma2}}\right)_B = \left(a_{H_{\gamma2}}\right)_B$ = 0.5 mT
	Center field = 335.576 mT	Center field = 335.576 mT
	ν = 9.368 GHz	ν = 9.423 GHz
	Line width = 0.3 mT	Line width = 0.42 mT

Table 4. EPR parameters of simulated spectra.

EPR parameters	Principal values	Direction cosines		
$\left(A_{CH_{2(\beta)}}\right)_A = \left(A_{H_\beta}\right)_A$ (mT)	A_{xx} = 3.381	0.194135	0.945231	0.262393
	A_{yy} = 3.285	−0.723568	−0.042637	0.688935
	A_{zz} = 3.227	0.662390	−0.323606	0.675662
	a_{iso} = 3.298			
$\left(A^{H_{\gamma1}}_{CH_{\gamma1}H_{\gamma2}}\right)_A = \left(A_{H_{\gamma1}}\right)_A$ (mT)	A_{xx} = 0.492	0.818223	0.479980	−0.316433
	A_{yy} = 0.44	0.016324	0.530794	0.847344
	A_{zz} = 0. 37	0.574669	−0.698482	0.426473
	a_{iso} = 0.434			
$\left(A^{H_{\gamma2}}_{CH_{\gamma1}H_{\gamma2}}\right)_A = \left(A_{H_{\gamma2}}\right)_A$ (mT)	A_{xx} = 0.62	0.325533	−0.489384	0.809031
	A_{yy} = 0.494	0.775337	0.627894	0.067839
	A_{zz} = 0.414	−0.541185	0.605188	0.583838
	a_{iso} = 0.509			
g_A	g_{xx} = 2.01753	0.998258	0.021593	0.054902
	g_{yy} = 2.01513	−0.054529	0.692912	0.718957
	g_{zz} = 2.01428	−0.022518	−0.720698	0.692883
	g_{iso} = 2.01565			

Table 5. The EPR parameters of radical A observed in succinic anhydride at 125 K.

EPR parameters	Principal values	Direction cosines		
$\left(A_{CH_{2(\beta)}}\right)_B = \left(A_{H_\beta}\right)_B$ (mT)	A_{xx} = 3.36	0.021180	−0.990260	−0.137614
	A_{yy} = 3.283	0.829840	−0.059355	0.554835
	A_{zz} = 3.251	−0.557599	−0.125949	0.820500
	a_{iso} = 3.298			
$\left(A^{H_{\gamma 1}}_{CH_{\gamma 1}H_{\gamma 2}}\right)_B = \left(A_{H_{\gamma 1}}\right)_B$ (mT)	A_{xx} = 0.586	0.066077	0.942855	0.326586
	A_{yy} = 0.485	−0.896800	−0.087383	0.433722
	A_{zz} = 0.379	0.437475	−0.321541	0.839778
	a_{iso} = 0.483			
$\left(A^{H_{\gamma 2}}_{CH_{\gamma 1}H_{\gamma 2}}\right)_B = \left(A_{H_{\gamma 2}}\right)_B$ (mT)	A_{xx} = 0.612	0.759274	−0.102410	0.642663
	A_{yy} = 0.464	−0.632874	0.113810	0.765844
	A_{zz} = 0.288	−0.151571	−0.988210	0.021600
	a_{iso} = 0.455			
g_B	g_{xx} = 2.00660	0.977193	0.210889	−0.024888
	g_{yy} = 2.00410	−0.096452	0.545205	0.832736
	g_{zz} = 2.00300	0.189184	−0.811343	0.553111
	g_{iso} = 2.00457			

Table 6. The EPR parameters of the radical B observed in succinic anhydride at 125 K.

The radical formation mechanism in the present work is the same as that of potassium hydroquinone monosulfonate [6]. The angular dependences of EPR spectra were obtained for different orientations of the static magnetic field with respect to the crystalline axes. For the radical A and radical B, the spectroscopic splitting factor and the hyperfine coupling constants of the H_β, $H_{\gamma 1}$ and $H_{\gamma 2}$ protons are anisotropic. For the radical A, the average values of the g-factor and the hyperfine coupling constants were obtained as g_A = 2.01565, $\left(a_{H_\beta}\right)_A$ = 3.298 mT, $\left(a_{H_{\gamma 1}}\right)_A$ = 0.434 mT, $\left(a_{H_{\gamma 2}}\right)_A$ = 0.509 mT, respectively. For the radical B, the average values of the g-factor and the hyperfine coupling constants were obtained as g_B = 2.00457 and $\left(a_{H_\beta}\right)_B$ = 3.298 mT, $\left(a_{H_{\gamma 1}}\right)_B$ = 0.483 mT, $\left(a_{H_{\gamma 2}}\right)_B$ = 0.455 mT, respectively.

2.3. Gamma-irradiated 3-nitroacetophenone single crystal

Nitroaromatic compounds have widespread actual or potential use in medicine and cancer therapy [14]. Nitroaromatic compounds have been extensively studied and reviewed mainly because of their interesting biological activities as well as their extended use in the chemical industry [15].

The radical in 3NAP identified as 3-nitroacetophenone anion radical is shown in **Figure 7**. The spectra were found to be temperature dependent. When we examined the EPR spectra at a

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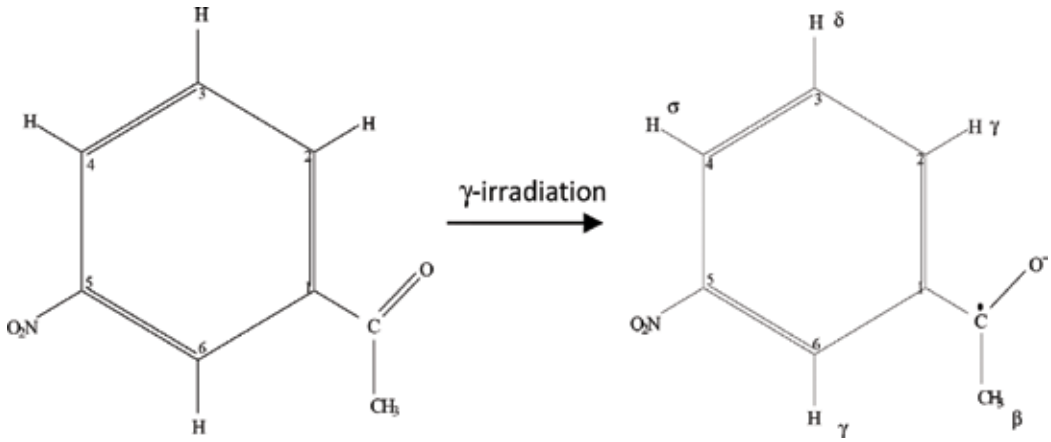

Figure 7. Structure of the 3NAP anion radical observed in 3NAP.

temperature range of 120–360 K, we decided that the radiation damage center in the sample was due to the break of the pi bond in the carbon-oxygen double bond. It was seen that the unpaired electron interacted with all the protons in the molecular structure [8].

In the EPR spectra, especially the impact of the methyl protons was felt. The hyperfine coupling constants of the methyl protons were measured at 300 K. The angular variations of EPR parameters of the 3NAP only at 300 K could be investigated. The spectra were clear only in a few angles, at 120 K. Therefore, only the simulations of these spectra were made, at 120 K. In addition, the simulation was also made for a single angle, at 300 K.

The EPR spectra of irradiated 3-nitroacetophenone single crystals consist of four hyperfine structure lines with a 1:3:3:1 intensity ratio, which is caused by the interaction of the unpaired electron with the three equivalent protons of the methyl group, at most orientations of the crystal in the magnetic field, at 300 K. The H(2) and the H(6) protons are also considered approximately equivalent.

In **Figures 8–11**, the experimental spectra of the gamma-irradiated 3NAP single crystals and their simulation spectra were compared.

The simulation values of the hyperfine coupling constants of the spectra in **Figures 8–11** are given in **Table 7**. The EPR parameters of the 3-nitroacetophenone anion radical are given in **Table 8**.

In the EPR spectra, especially the impact of the methyl protons was felt. Only the hyperfine coupling constants of the methyl protons could be experimentally measured at 300 K. The EPR spectra of irradiated 3-nitroacetophenone single crystals consist of four hyperfine structure lines with a 1:3:3:1 intensity ratio, which is caused by interaction of the unpaired electron with the three equivalent protons of the methyl group, at most orientations of the crystal in the magnetic field, at 300 K. The H(2) and the H(6) protons are also considered approximately equivalent. The hyperfine coupling for the CH_3 group is anisotropic and their average values being a_{CH_3} = 1.402 mT.

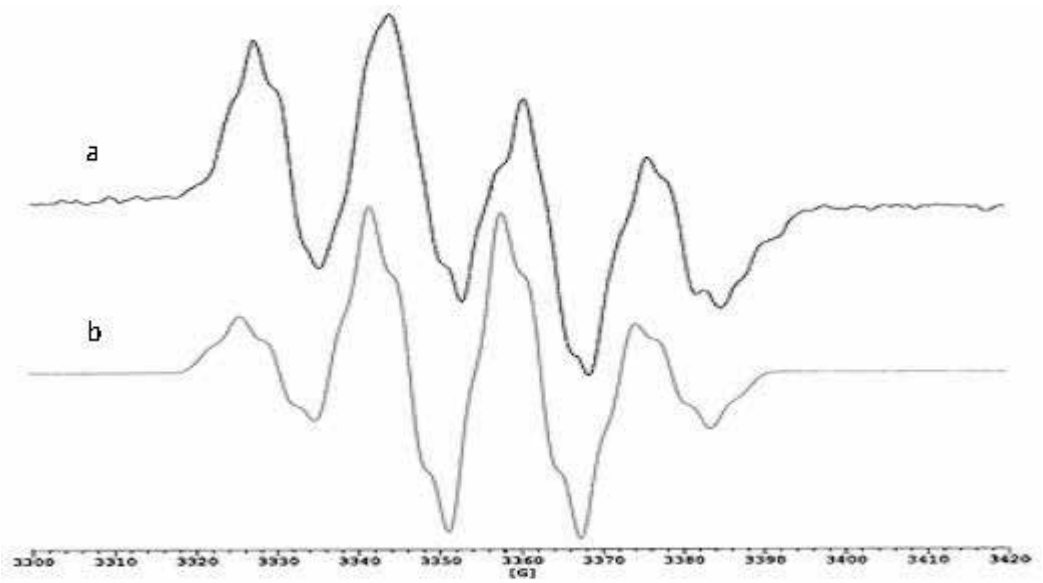

Figure 8. EPR spectra of ^{60}Co-γ irradiated 3NAP single crystal at 300 K when (a) the magnetic field is in the a*b-plane and is away 20° from the a*-axis and (b) simulation of the spectrum; line width is 0.34 mT.

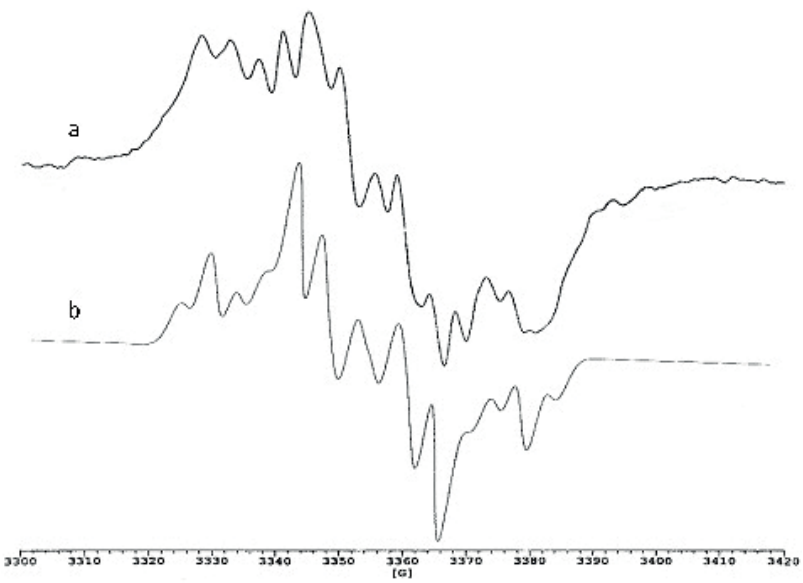

Figure 9. EPR spectra of ^{60}Co-γ irradiated 3NAP single crystal at 120 K when (a) the magnetic field is in the a*c-plane and is away 140° from the a*-axis and (b) simulation of the spectrum; line width is 0.38 mT.

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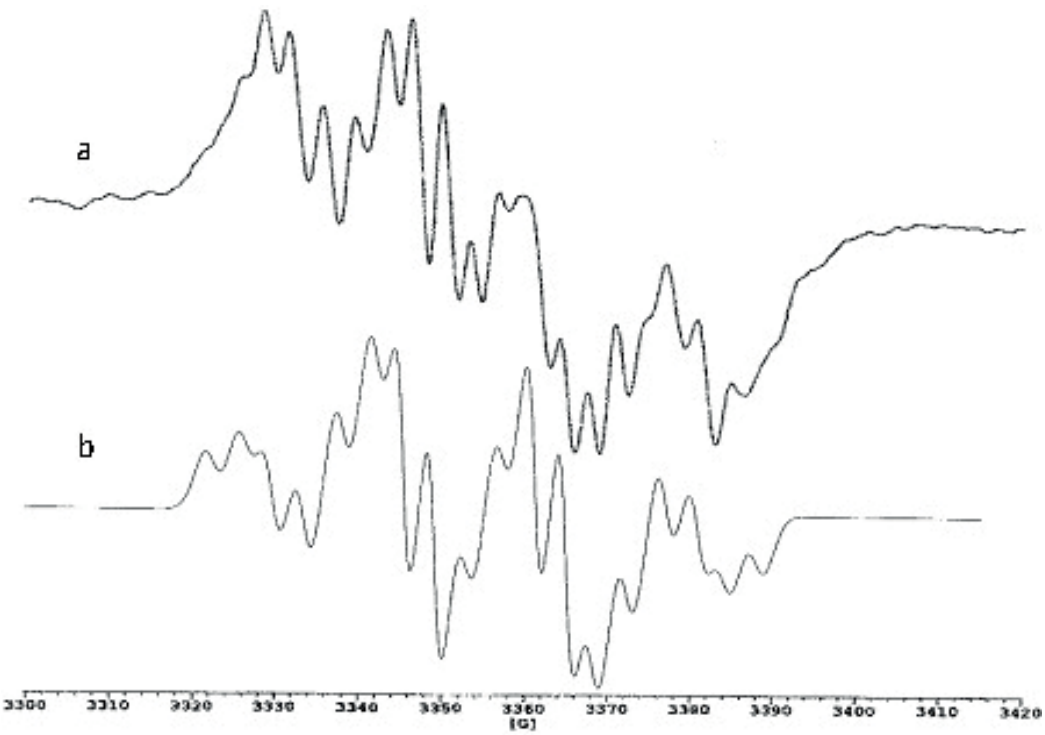

Figure 10. EPR spectra of ^{60}Co-γ irradiated 3NAP single crystal at 120 K when (a) the magnetic field is in the a*c-plane and is away 150° from the a*-axis and (b) simulation of the spectrum; line width is 0.32 mT.

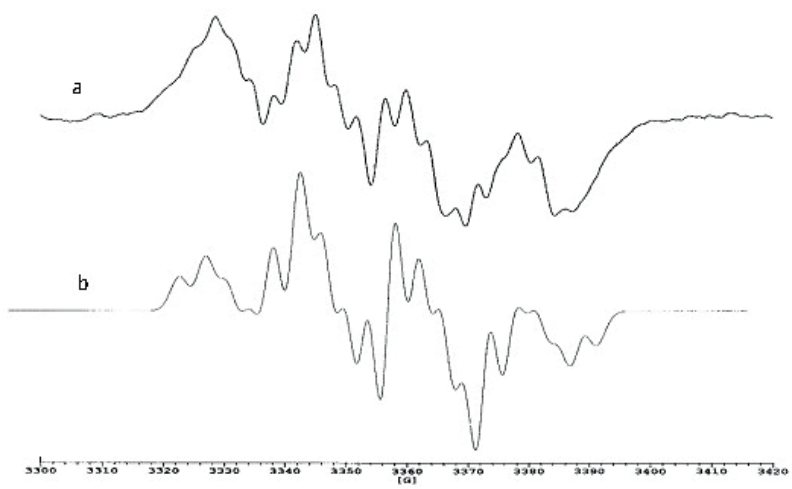

Figure 11. EPR spectra of ^{60}Co-γ irradiated 3NAP single crystal at 120 K when (a) the magnetic field is in the a*c-plane and is away 165° from the a*-axis and (b) simulation of the spectrum; line width is 0.34 mT.

	Figure 8	Figure 9	Figure 10	Figure 11
$a_{CH_3(\beta)}$ mT	1.55	1.36	1.68	1.585
$a_{H(2)} \cong a_{H(6)} = a_{C_2H_2(\gamma')}$ mT	0.38	0.43	0.45	0.46
$a_{H(3)} = a_{H(\delta)}$ mT	0.35	0.68	0.71	0.68
$a_{H(4)} = a_{H(\sigma)}$ mT	0.25	0.39	0.265	0.29

Table 7. Coupling constants values of simulated spectra.

	Principal values	Direction cosines		
$a_{CH_3(\beta)}$ (mT)	A_{xx} = 1.743	0.673208	−0.717129	−0.180325
	A_{yy} = 1.447	0.572490	0.659816	−0.486721
	A_{zz} = 1.015	0.468023	0.224430	0.854743
	a_{iso} = 1.402			
	ρ = 0.52			
g	g_{xx} = 2.006443	0.732137	0.415100	−0.540063
	g_{yy} = 2.006110	0.299886	0.515457	0.802728
	g_{zz} = 2.004067	0.611591	−0.749664	0.252902
	g_{iso} = 2.005540			

Table 8. The EPR parameters of the 3-nitroacetophenone anion radical observed in 3-nitroacetophenone at 300 K.

The simulations of the EPR spectra were carried out using the Win-EPR software.

3. Conclusion

There are many factors that trigger the formation of free radicals. One of these is the radiation effect. The various compounds that undergo radiation effects have the radiation damage centers as a result of breaking bonds in their structures. The radiation damage center is a paramagnetic center, and the detection and motion of these centers are investigated in detail by EPR spectroscopy.

Antioxidants also show paramagnetic structure under radiation effect. EPR analysis of antioxidants is crucial to remove the effect of free radicals that cause many diseases. Potassium hydroquinone monosulfonate (PHM), succinic anhydride and 3-nitroacetophenone compounds show antioxidant properties. When EPR analyses of these materials were made, it was seen that their radical mechanisms were similar to each other. The anion radicals were observed in all three compounds. When the resonance structure and the formation mechanisms are carefully examined, it is seen that the oxygen atom takes an electron and forms a stable anion structure.

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Chapter 5

Oxidative Stress in Type 1 Diabetes Mellitus: Ethnic Aspects

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Additional information is available at the end of the chapter

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Abstract

Numerous researches show that data on an ethnic origin can give additional information for the personified approach in treatment of different diseases. The aim of this study was to evaluate the level of some lipid peroxidation components and antioxidant defense system in Mongoloid and Caucasian patients with Type 1 diabetes mellitus. Conjugated dienes, ketodienes and conjugated trienes, thiobarbituric acid reactants, total antioxidant activity, α-tocopherol, retinol, superoxide dismutase activity, reduced and oxidized glutathione, and oxidative stress coefficient levels were evaluated in 65 patients with type 1 diabetes (38 Mongoloids and 27 Caucasians) and in 82 healthy people (42 Mongoloids and 40 Caucasians). Spectrophotometric and fluorometric methods were used. The intensity of LPO in Mongoloid patients was lower than in Caucasians: the level of primary and intermediate products was by lower 1.53 and 1.83 times, while total antioxidant activity was elevated by 1.44 times, and decreased α-tocopherol level by 1.32 times, which was also supported by oxidative stress coefficient (1.35 in Mongoloids and 2.32 in Caucasians). Activity of the POL-AOD system in Mongoloids is low, which is probably due to the increase of antioxidant defense system work. These results are consistent with clinical characteristics of type 1 diabetes mellitus with infrequent development of complications in Mongoloids living in Eastern Siberia.

Keywords: type 1 diabetes mellitus, oxidative stress, antioxidant defense, ethnos

1. Introduction

Type 1 diabetes mellitus (T1DM) is common in the world and is considered as one of the severe human diseases. This disease is the cause of heart disease, blindness, stroke, kidney

failure, foot ulcers, and so on. A number of studies have shown that complications rate of T1DM depends on different factors including geographic residence and human ethnicity [1–4]. The dominance of T1DM is there in the countries of Scandinavia—Finland and Sweden (63 cases per 100,000 population) [5], while the lowest prevalence is observed in the East and South-East Asia where representatives of the Mongoloid race live [6]. In Russian Federation, there are more than 250,000 patients with T1DM [7]. Some studies revealed that low morbidity of T1DM among the aboriginal people in Arctic and Siberian regions resulted from the presence of the protective allele's genes for this disorder [6]. The prevalence of this form of diabetes among indigenous people in the Buryatia Republic is 24.18 per 100,000, which is below the average level in Russia (224.5 per 100,000) [8, 9].

Oxidative stress is an imbalance between increase reactive oxygen species and an antioxidant ability to detoxify the reactive components. Oxidative stress is thought to be involved in the development of different diseases [10–12].

Many experimental [13] and clinical studies [14] suggest that free-radical processes are activated during different stages and in different types of diabetes mellitus, even in its subclinical forms [15, 16]. In patients with diabetes mellitus, oxidative stress (OS) caused by elevated production of reactive species of oxygen and decrease of antioxidant defense system (AOD) level, leads to activation of lipid peroxidation (LPO) and oxidative lipoprotein modification with increasing atherogenicity [17]. Hyperglycemia can induce damage of β-cells functions with development of OS and decrease of thioredoxine level [18]. Marra et al. suggest that T1DM patients with a short duration of disease and good metabolic control show an early imbalance in their antioxidant capacity and augmented levels of lipid hydroperoxides and conjugated dienes (CDs) [19]. Diabetic women show, independently from other factors, a decreased antioxidant capacity and an increased rate of lipoperoxidation compared with diabetic men [19].

At the same time, the link between certain metabolic characteristics in patients with T1DM and their race remains poorly studied. The **aim** of this study was to evaluate the level of some LPO components and AOD system in Mongoloid and Caucasian patients with T1DM.

2. An evaluation of the level of some lipid peroxidation components and antioxidant defense system in mongoloid and Caucasian patients with type 1 diabetes mellitus

Biochemical parameters in 147 persons (healthy and with T1DM) both Mongoloids (ethnic group is Buryats) and Caucasians (ethnic group is Russians) living in the modern city Ulan-Ude (East-Siberia) were assessed. The diagnosis of T1DM was confirmed in all patients based on clinical and laboratory investigations, severe comorbidities and severe diabetic complications were excluded. Main group's characteristics are presented in **Table 1**. There were no

statistically significant differences in sex, age, duration of disease, Hb $A_{1C,}$ body mass index (BMI), arterial pressure in T1DM groups. There were no statistically significant differences in diets and physical activity between the patients of both ethnic groups with T1DM.

Blood samples were taken after 12 h of fasting during night, then were centrifuged for 5 min at 4°C, and erythrocytes were washed three times with NaCI 0.9% (wt/vol). Aliquots of EDTA plasma and washed erythrocytes were used immediately or kept frozen at −40°C but not more than 1 month. Blood plasma and hemolysate were used as the materials for analysis. The blood was taken from the cubital vein in accordance with accepted requirements. The intensity of LPO was evaluated by the level of diene conjugates (DC), ketodienes (KD), and conjugated trienes (CT). The concentration of DCs detected on absorbance of plasma heptanes extracts at 232 nm (μmol/liter), KD and CT at 278 nm (arb. units) [20]. Thiobarbituric acid reactants (TBARs) levels were identified by fluorometry methods and were considered in μmol/liter [21]. An evaluation of AOD activity was carried out on total radical-trapping antioxidant parameter (TRAP), which was measured by specific methods on 2,2′-azinobis-(3-ethylbensothazoline-6-sulphonate) radical action formation (absorbance at 734 nm) in conditions of exogenous H_2O_2 presence [22].

Also contents of other components of AOD system were determined: α-tocopherol and retinol [23], superoxide dismutase (SOD) [24], and reduced and oxidized glutathione (GSH and the GSSG) [25]. Measurements were carried out on the Shimadzu RF-1501 and Shimadzu RF-1650 spectrofluorometer. For more informative description of the LPO–AOD, coefficient of oxidative stress (COS) was calculated as the ratio of LPO–AOD system values in T1DM patient to the mean control group values. At COS > 1, oxidative stress was stated [26]. In our study,

Clinical	**Mongoloids**		**Caucasians**	
Data	**T1DM**	**Control group**	**T1DM**	**Control group**
n	38	42	27	40
Sex (M / F)	15/23	22/20	15/12	20/20
Age (years)	34.4 ± 11.7	31.4 ± 8.0	32.7 ± 11.9	27.8 ± 7.7
Duration of disease (years)	12.1 ± 3.5	–	12.9 ± 4.0	–
Hb A_{1C} (%)	9.29 ± 3.06	–	8.74 ± 2.24	–
Body mass index (kg/m^2)	23.7 ± 2.1	20.3 ± 1.3	24.6 ± 3.1	21.4 ± 3.4
Total cholesterol (mmol/l)	4.65 ± 1.2	4.28 ± 1.29	5.66 ± 1.22	4.24 ± 1.25
Triglycerides level (mmol/l)	1.01 ± 0.58	0.67 ± 0.21	1.53 ± 0.66	0.54 ± 0.19
Systolic pressure (mm Hg)	115 ± 11	116 ± 12	117 ± 10	113 ± 11
Diastolic pressure (mm Hg)	73 ± 12	71 ± 12	74 ± 9	76 ± 12

Table 1. General characteristics of patients with T1DM and control subjects.

all patients and control groups signed informed consent according to the World Medical Association Declaration of Helsinki (1964, 2000). For statistic analysis of the data, Statistica 6.1 software (StatSoft Inc.) was used. To determine normal distribution of the quantitative data, graphic visual method and Kolmogorov-Smirnov Test with Lilliefors and Shapiro-Wilk corrections were used. The variances equality was verified by Fisher's Test. Descriptive statistics was applied for quantitative data description: mean ± error of the mean. The differences between parameters of groups by parametric Student's Test for independent samples and non-parametric Mann–Whitney Test were analyzed. The critical significance level was considered 5% ($p < 0.05$).

3. Results

The oxidative stress is considered an imbalance in the redox-state with isolated or combined variations in the pro- or antioxidant components concentration. Our study has shown higher concentration of DC (by 1.35 times; $p < 0.01$) in Mongoloid patients as well as higher levels of DC (by 2.4 times; $p < 0.001$) and KD and CT (by 2.71 times; $p < 0.05$) in Caucasian patients in comparison to the corresponding control groups (**Table 2**).

No statistically significant differences between investigated groups in the TBA-reactive products level of lipid peroxidation processes were identified. In conditions of increased generation of LPO products are observed changes permeability of cell membranes for a lot of ions, nonelectrolytes, and macromolecules [27]. This processes in loss of barrier function of cell membranes that is the pathogenesis of different diseases, including development of vascular disorders in T1DM due to lipid peroxidation activation during prolonged hyperglycemia [9, 28]. The observed increase in LPO activity cannot provide sufficient information about the redox-status in patients with T1DM, because it indicates one aspect of the study, LPO excluding of AOD activity. The study of the AOD integral parameter level, TRAP in patients with T1DM, an increase (by 1.54 times; $p < 0.001$) in this indicator in Mongoloid patients in comparison with the control group was shown (**Table 3**).

In Caucasian patients with T1DM, statistically significant differences from the control group included reduced GSH values (by 1.16 times; $p < 0.05$) and increased GSSG level (1.26-times,

Parameters	Mongoloids		Caucasians	
	Control group	T1DM	Control group	T1DM
Diene conjugates, μmol/liter	0.57 ± 0.03	0.77 ± 0.04*	0.51 ± 0.07	1.2 ± 0.11*,+
Ketodienes and conjugated trienes, arb. units	0.31 ± 0.05	0.35 ± 0.04	0.24 ± 0.06	0.65 ± 0.13*,+
TBA-reactive products, μmol/liter	1.57 ± 0.10	1.81 ± 0.11	1.93 ± 0.10	2.05 ± 0.12

Here and in **Table 3**: $p < 0.05$ in comparison with *corresponding control, +Caucasian T1DM patients.

Table 2. Level of LPO products in DM1 mongoloid and Caucasian patients (M ± m).

Parameters	Mongoloids		Caucasians	
	Control group	T1DM	Control group	T1DM
TRAP, arb. Units	14.35 ± 0.72	22.17 ± 1.08*	17.68 ± 1.36	15.42 ± 0.96+
α-tocopherol, μmol/liter	6.85 ± 0.38	6.23 ± 0.32	6.72 ± 0.3	8.21 ± 0.78*,+
retinol, μmol/liter	2.7 ± 0.18	2.29 ± 0.15	2.38 ± 0.13	2.33 ± 0.17
SOD, arb. Units	1.41 ± 0.04	1.26 ± 0.03	1.43 ± 0.44	1.57 ± 0.24
GSH, mmol/liter	2.79 ± 0.16	2.79 ± 0.11	2.90 ± 0.14	2.51 ± 0.13*
GSSG, mmol/liter	2.03 ± 0.15	2.15 ± 0.09	1.72 ± 0.09	2.16 ± 0.10*

Table 3. AOD values in T1DM in mongoloid and Caucasian patients (M ± m).

$p < 0.001$) (**Table 2**). Also in this group in comparison with control, statistically significant differences in activity of SOD, α-tocopherol, and retinol levels were not noted. Initiation of processes of lipid peroxidation at primary and intermediate stages in the absence of natural AOD activity enhancement can lead to function impairment of different components of hemostasis and increased aggregation of blood cell, which will increase blood viscosity, induce thickening of the vascular wall basal membrane, slower blood flow in small and medium vessels, deterioration of microcirculation [24]. The given changes in parameters of lipid peroxidation can attest about the presence of risk factors for microangiopathy development in Caucasian patients with T1DM. Comparison of LPO values in Caucasian and Mongoloid patients showed decreased DC (by 1.56 times; $p < 0.001$), KD and CT (by 1.86 times; $p < 0.05$) values, increased TRAP (by 1.44 times; $p < 0.001$), and decreased α-tocopherol levels (1.22-times, $p < 0.01$) in Mongoloid patients with T1DM in compare with the same values in Caucasian patients with T1DM (**Tables 1** and **2**). Coefficient of oxidative stress (COS) in Mongoloid patients was 1.35, in Caucasian patients was 2.32 ($p < 0.05$). It is believed that COS value>1 indicates activation of oxidative stress. The higher is COS, the more insensitive are processes lipid peroxidation processes and less effective is the antioxidant defense system in the examined patients with different diseases. So, our results indicate increased LPO processes in groups of patients with diabetes mellitus and intensity of LPO processes depends on ethnicity [29]. Noted changes in LPO-AOD system in Mongoloid patients with T1DM were less insensitive than in Caucasian patients, that allows to make a recommendation on highly individualized approaches to the complex therapy.

4. Conclusions

We suppose that ethnic factor plays one of the most important roles in the course of various diseases, including T1DM. It is quite possible that the low incidence in T1DM in Mongoloids is based on less LPO-AOD metabolic imbalance. Further studies of ethnically associated metabolic features can give more opportunities for developing specific approaches for diagnostics, prophylactic and treatment of T1DM in patients of different ethnic groups.

Conflict of interest

There is no conflict of interest.

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Chapter 6

Dimethylformamide Reduces Cerebral Ischaemia in Diabetic Rats Hours after Its Occurrence; A New Horizon

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Additional information is available at the end of the chapter

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Abstract

The antioxidant properties of dimethylformamide (DMF) depend on its interference with the hydroxyl-radical-transduction pathway. Diabetes is a risk factor of cerebral ischaemia (CI), and both entities are associated with oxidative stress (OS). We evaluated DMF's effects on CI in non-diabetic rats (NDRs) and in diabetic rats (DRs). One hour after CI, the animals were divided into two treatment groups (300 μl subcutaneous): either DMF or isotonic saline solution. Treatment effects were analysed in NDRs or DRs without CI. Eight hours after CI, a neurophysiologic score (NS) was determined; CI and OS biomarkers were measured in the ischaemic cerebral hemisphere. Infarct/oedema volumes were measured on dyed brain slices. DMF reduced infarct volume in NDRs and DRs but only improved the NS in DRs. Basal concentrations of all the biomarkers were similar in the NDRs and DRs. Metalloproteinase 9 (MMP9) did not change with DMF. Malondialdehyde (MDA) increased with CI, and DMF only reduced it in DRs. RAGE, nitrite/nitrate and nitrotyrosine increased with CI only in DRs (all prevented by DMF). We conclude that DMF's benefits on CI were greater in the DRs due to a higher susceptibility of diabetic animals to the OS produced by CI. The results open a new horizon in CI treatment since DMF has not been investigated before.

Keywords: cerebral ischaemia, diabetes, oxidative stress, dimethylformamide, antioxidant

1. Introduction

Stroke is one of the leading causes of death and disability worldwide [1]. To date, thrombolysis is the only approved therapy for acute stroke, but the window of time for treatment is short (3–4 h after a stroke). There is an associated risk of haemorrhagic transformation with thrombolysis, which increases the morbidity and mortality of ischaemic stroke [2], and the efficacy of this treatment is small. There is an obvious need for pursuing new treatment strategies to address these deficiencies. Being diabetic is an important risk of suffering a stroke [3]. Oxidative stress (OS) has been associated with both diabetes [4] and stroke [5, 6]. Some antioxidants have shown beneficial effects in animal models of diabetes [7]. However, exogenous antioxidant effects on humans are difficult to evaluate. Indeed, the long-term ingestion of antioxidants included in one's diet seems to provide beneficial health effects [8, 9], whereas there is still some controversy with respect to the usefulness of long-term antioxidant supplements [10].

Most of the positive effects of antioxidants on stroke models are the result of prophylactic strategies (treatment administered before stroke) [11]. The applicability of such approaches in humans yields dubious results because, although there are risk factors that could identify stroke-susceptible patients, it is virtually impossible to predict who would actually suffer a stroke.

N-N-dimethylformamide (DMF) is an amphipathic solvent significantly miscible with water and most organic solvents. It has been shown that DMF significantly protected against neuronal damage produced by dopamine or hydrogen peroxide. Even though the results were attributed to an increase in antioxidant enzymes [12], it seems that DMF interferes with the signal transduction pathway originated by the hydroxyl radical, which includes changes in permeability and ion exchange in the different compartments of mitochondria [13]. Moreover, DMF seems to have anti-ageing effects associated with protein homeostasis, which are independent of its antioxidant properties [14].

Signal transduction mediated by free radicals (including the hydroxyl radical) acts as a double-edge sword. It could be harmful (*i.e.* oxidative damage to biomolecules—proteins, lipids, RNA/DNA-) or beneficial (leading to the synthesis of antioxidant enzymes through nuclear factor kappa B activation) [10]. Free radicals generated during cerebral ischaemia (a first wave within minutes followed by a second wave shortly thereafter) participate in the cascade of events generating neuronal damage, which includes changes in mitochondria function [6]. We postulate that signal transduction mediated by the hydroxyl radical (one of those free radicals liberated during cerebral ischaemia) is one of the harmful events in cerebral ischaemia, and DMF would reduce such ischaemic damage. Therefore, the goal of this study was to precisely investigate the effects of a single dose of DMF, administered 1 h after induced cerebral ischaemia in rats.

2. Material and methods

The Institutional Ethical Committee approved the present work.

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2.1. Diabetes model

Type 1 diabetes mellitus was induced in 4-week old male Wistar rats (79.7 ± 1.8 g), through the intraperitoneal (ip) administration of streptozotocin (STZ, 75 mg/kg, Sigma St. Louis, USA). Diabetes was confirmed 3 days later by the onset of hyperglycaemia (>200 mg/dL). A control group (same characteristics, non-diabetic) was also maintained during the experiment. All the animals were fed Purina chow and water *ad libitum* and kept in light-darkness cycles of 12×12 h. The evolution of diabetes was evaluated by: (1) weighing the rats weekly and comparing the weights of the diabetic and the control animals and (2) measuring glycaemia and glucosylated haemoglobin at the end of the experiment.

2.2. Cerebral ischaemia model

Six weeks after diabetes induction, cerebral ischaemia was produced in diabetic as well as the control animals by slightly modifying the model reported by De Cristóbal et al. [15]. In short, the animals were anaesthetised through intramuscular administration of an anaesthesia cocktail containing acepromazine (5 mg/kg), ketamine (5 mg/kg) and butorphanol (13 mg/kg). The right carotid and the middle cerebral arteries were permanently ligated. One hour later the animals were randomly divided into two treatment groups (eight control and eight diabetic animals per group).

2.3. Experimental groups

1. Isotonic saline solution (ISS, 300 μl) administered subcutaneously (sc)
2. DMF (300 μl, equivalent to 0.9 g/kg, Sigma, St. Louis, USA) also administered sc

2.4. Neurophysiological test

Eight hours after cerebral ischaemia was induced, the neurological signs were scored as follows (sum of all): consciousness (0–3, where 3 was the response only after stimulation), decreased grip of the left forelimb when the tail was pulled (0–3, 3 was complete gripping), failure to grab a bar with the left forelimb (0–3, 0 was strongly grabbing with both forelimbs, 3 was no grabbing at all with the left forelimb), and walking in a circle to the left side (0–3, 3 was walking spontaneously in circles). If the animal did not respond to any stimulus, the score was 12 points.

2.5. Infarct and oedema evaluation

The animals were anaesthetised (pentobarbital, 75 mg/kg, ip). A blood sample (at least 5 ml) was taken from the heart and anticoagulated with ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, USA). The blood glucose and glucosylated haemoglobin were measured. The animals were decapitated after taking the blood samples, and each brain was immediately extracted and sliced (2 mm slices). The slices were incubated in a 1% triphenyltetrazolium chloride (Sigma, St. Louis, USA)/ISS solution, at 37°C for 5 min). Both sides of each slice were photographed against

a millimetre paper background and measured using the ImageJ software (National Institutes of Health, USA). Areas were integrated as volume in mm^3 and infarct volume quantified as a percentage of the right hemisphere volume. Oedema was calculated (in percentage of the left brain hemisphere) by subtracting the left from the right hemisphere volumes.

2.6. Stroke and oxidative stress biomarkers' evaluation

Two groups of animals (diabetic or non-diabetic, n = 12 per group) submitted to cerebral ischaemia (treatments—6 per group—and sacrifice as mentioned earlier) were used to measure biomarkers. Three hundred milligrams of the right hemispheres were homogenised in 1.5 ml of phosphate saline buffer (Sigma, St. Louis, USA; 0.1 M, pH 7.4). The homogenised tissue was centrifuged (13,000 ×*g*, 4°C, 10 min). The supernatant was isolated and kept at −80°C until measurement of the biomarkers was taken (within a month of taking the samples). Ten milligrams of the right hemisphere was homogenised in 0.5 ml of malondialdehyde (MDA) lysis buffer (Abcam, MA, USA) for the measurement of MDA.

Biomarkers evaluated were:

1. Metalloproteinase 9 (MMP 9): It was evaluated through ELISA using a commercial kit (R&D Systems, MN, USA). The concentrations were expressed in ng/ml per mg of protein (proteins were measured using a commercial kit from Cayman Chemical Co, MI, USA).
2. Advanced glycation end-product receptor (RAGE) was measured through ELISA using a commercial kit (Abcam MA, USA). The concentrations were expressed in ng/ml per mg of protein.
3. Nitrite/nitrate (NO_2/NO_3 ratios, an indirect measurement of nitric oxide) were measured by the modified Griess method, using a commercial kit (Cayman Chemical Co, MI, USA). Results were expressed as μM per mg of protein.
4. Nitrotyrosine (NT) was measured through ELISA using a commercial kit (Abcam MA, USA). The concentrations were expressed in ng/ml per mg of protein.
5. MDA was measured using a commercial kit (Abcam MA, USA). The results were expressed in nmol per mg of protein.

To establish reference values, and evaluate the effects of DMF, the biomarkers were measured in the brains of four non-diabetic and four diabetic rats that were not submitted to cerebral ischaemia.

2.7. Statistical analysis

The Kolmogorov-Smirnov test was used to analyse the normality of distribution. The unpaired "t" test was used to compare glycaemia and glucosylated haemoglobin between the non-diabetic and diabetic animals. The two-way ANOVA test (Bonferroni post-hoc) was used to analyse the time-course of weights (6 weeks, diabetic and non-diabetic animals). The one-way

ANOVA test (Tukey post-hoc) was used to compare biomarkers (diabetic, non-diabetic, with or without ischaemia). The neurophysiological score was analysed using the Kruskal-Wallis test (Dunn's multiple comparison post-hoc). Data are shown as the mean ± standard error of the mean (SEM). The $p < 0.05$ was considered significant.

3. Results

3.1. Metabolic evolution

Non-diabetic and diabetic animals had the same weights at the beginning of the experiment (81.6 ± 0.8 g *vs.* 78.8 ± 2.7 g, respectively, $p > 0.05$). The time-course of their weights is shown in **Figure 1**. There was a significant difference beginning the third week after diabetes induction. Glycaemia at the beginning of the experiment (after diabetes induction) was higher in the diabetic animals compared with the non-diabetic animals (456.1 ± 2.3 mg/dL *vs.* 91.1 ± 2.3 mg/dL respectively, $p < 0.0001$). At the end of the experiment both glycaemia and glucosylated haemoglobin were also different: glycaemia 88.7 ± 3.0 mg/dL versus 467.5 ± 12.6 mg/dL (non-diabetic *vs.* diabetic, $p < 0.0001$); glucosylated haemoglobin 4.3 ± 0.1% versus 9.3 ± 0.1% (non-diabetic *vs.* diabetic, $p < 0.0001$).

3.2. Cerebral ischaemia evaluation

3.2.1. Neurophysiological score

The neurophysiological score is shown in **Figure 2**. DMF significantly ($p < 0.05$) improves the neurophysiological scores only in diabetic rats.

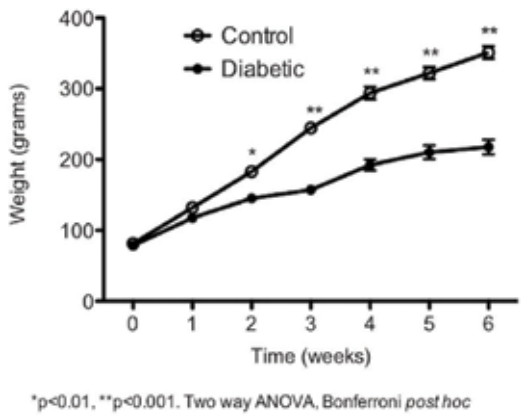

Figure 1. Time-course of the weight. The time-course of the weight was significantly different. Control (non-diabetic rats) gained more weight than diabetic animals during the experiment. The media + SEM are shown. Zero was at the beginning of the experiment, when the rats were 4 weeks old.

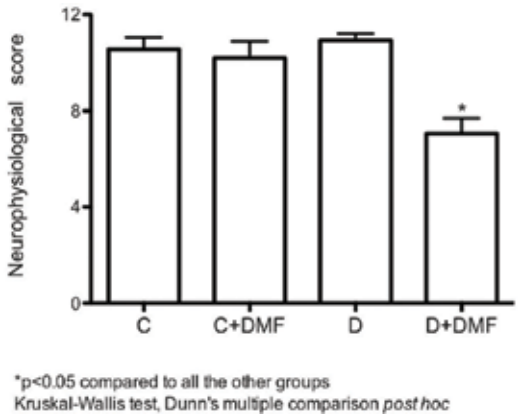

Figure 2. Neurophysiological scores: the neurophysiological score was similar in the control (non-diabetic) rats without (C) or with dimethylformamide treatment (C + DMF), as well as in the diabetic animals without DMF treatment (D). DMF significantly decreased the score in diabetic rats treated with DMF (D + DMF). The media + SEM are shown.

3.2.2. Infarct and oedema volume

DMF significantly decreased the infarct volume in both non-diabetic ($p = 0.02$, **Figure 3**) and diabetic ($p = 0.007$) rats. However, oedema was the same in non-diabetic rats compared to diabetic rats and was not changed by DMF (not shown). **Figure 4** shows an example of the slices, dyed with triphenyltetrazolium.

3.2.3. Cerebral ischaemia and stroke biomarkers

Results are shown in **Figures 5–9**. The results in non-diabetic (Control, C) rats are shown in the left panels whereas results in the diabetic (D) animals are in the right panels. The figures

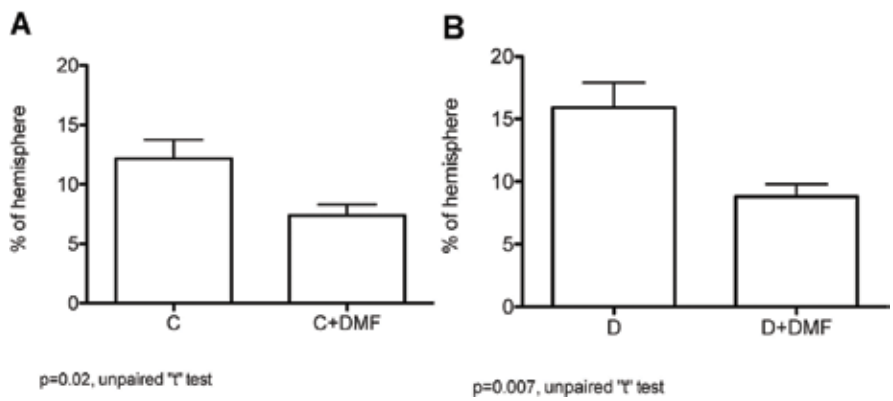

Figure 3. Infarct volume: this figure shows the infarct volume (expressed as percentage of the right hemisphere) in non-diabetic (C, panel A) and diabetic (D, panel B) animals. Dimethylformamide (DMF) significantly reduced infarct volume in non-diabetic as well as in diabetic rats.

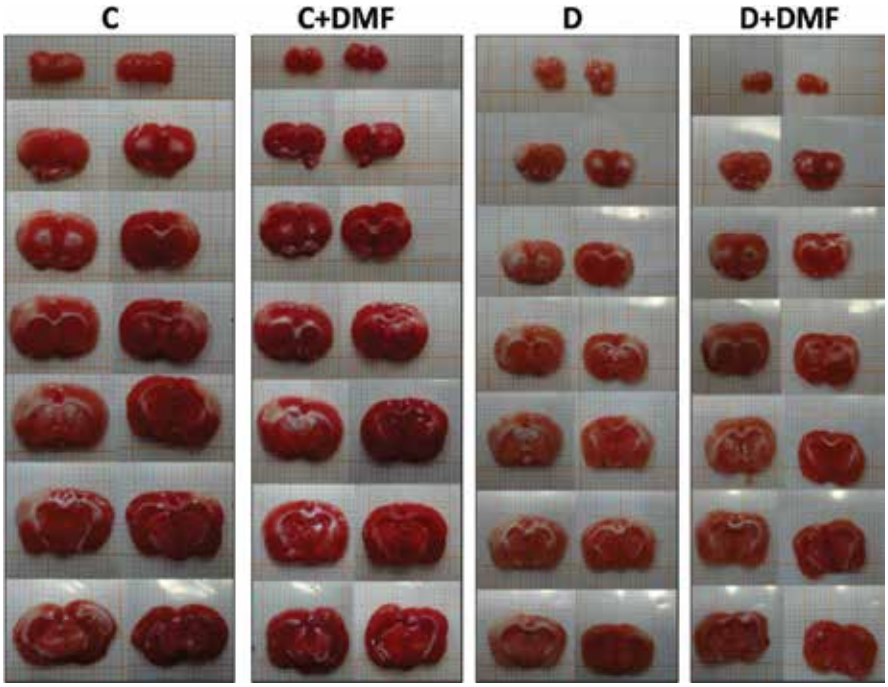

Figure 4. Both sides of the slices of a representative animal per group are shown: C (non-diabetic without treatment), C + DMF (non-diabetic treated with DMF), D (diabetic without treatment), D + DMF (diabetic treated with DMF). The infarct shows no colour (in white), whereas the tissue that is not affected has a red colour. It is noticeable that animals treated with DMF (control and diabetic) have a smaller infarct.

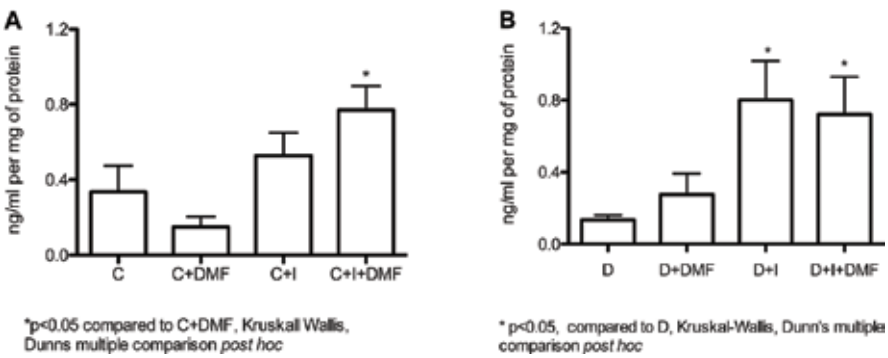

Figure 5. Concentration of metalloproteinase 9 (MMP9) in the brain: The concentration of MMP9 showed significant incremental increases in the brains of diabetic (D, panel B) animals with ischaemia with or without DMF treatment (D + I and D + I + DMF, respectively). DMF did not change MMP9 either in non-diabetic (C + DMF, panel A) or in diabetic (D + DMF, panel B) rats. Non-diabetic (C, panel A) animals with ischaemia and treated with DMF (C + I + DMF) had significantly higher concentrations of MMP9 than non-diabetic animals treated with DMF (C + DMF).

show the basal data (without ischaemia) of animals without (C, D) or with treatment (C + DMF, D + DMF). Animals with ischaemia and without treatment are represented as C + I or D + I. The animals with ischaemia and treatment are shown as C + I + DMF or D + I + DM.

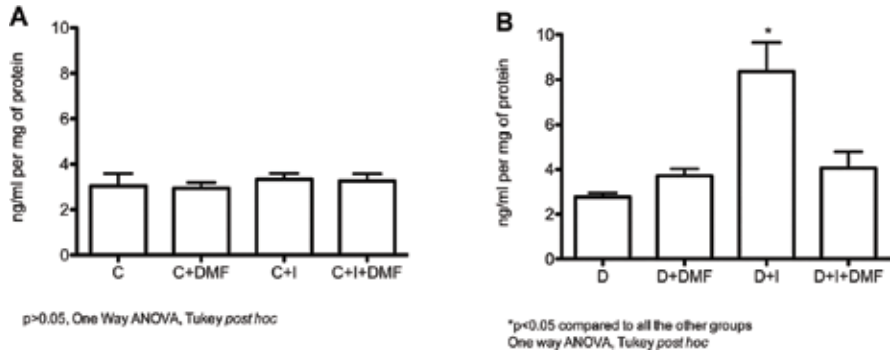

Figure 6. Concentration of the receptor for advanced glycation end products (RAGE) in the brain: RAGE did not change in the brains of non-diabetic (control, C) animals with or without ischaemia (I), either with or without dimethylformamide (C + DMF) treatment (panel A), whereas this biomarker significantly increased in the brains of diabetic animals submitted to ischaemia (D + I, panel B). DMF prevented the effect of ischaemia in diabetic animals (D + I + DMF). The mean + SEM are shown.

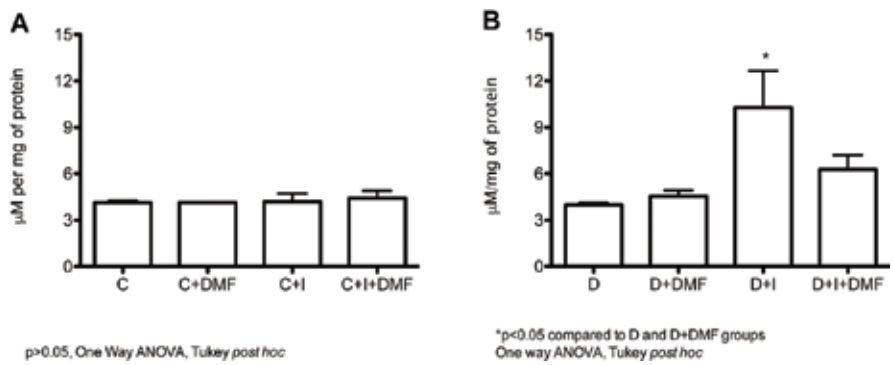

Figure 7. Nitrite and nitrate (NO_2/NO_3) in the brain: NO_2/NO_3 did not change in the brains of non-diabetic (control, C) animals with or without ischaemia (I), neither with nor without dimethylformamide (DMF) treatment (panel A) whereas this biomarker increased significantly in the brains of diabetic (D) rats submitted to ischaemia (panel B). DMF completely prevented the effect of ischaemia in diabetic animals. The mean + SEM are shown.

3.2.3.1. MMP9

Figure 5 shows that MMP9 increased significantly ($p < 0.05$) in the right hemisphere of diabetic rats, and DMF did not prevent such increase. There was a slight (non-significant) increase in the right hemisphere of the non-diabetic rats with ischaemia. Likewise, DMF did not reduce MMP9 in non-diabetic animals with ischaemia. Interestingly, DMF slightly reduced MMP9 in non-diabetic animals without ischaemia; because of that change, non-diabetic animals treated with DMF had different concentrations (significantly higher with than without ischaemia).

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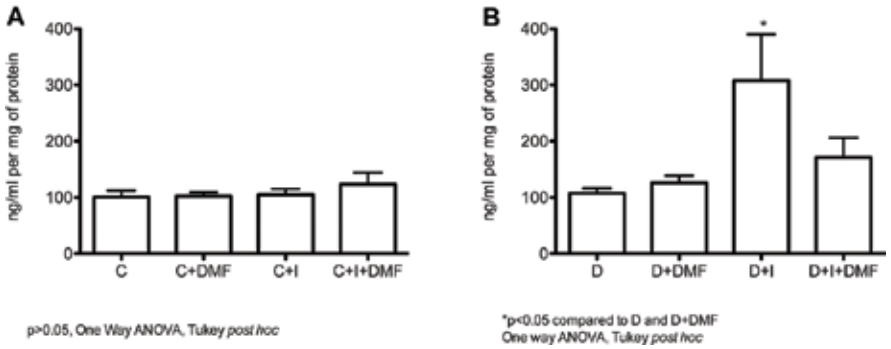

Figure 8. Nitrotyrosine (NT) in the brain: NT did not change in the brains of non-diabetic rats (control, C, panel A), with or without ischaemia (I), neither with nor without dimethylformamide (DMF) treatment, whereas this biomarker increased significantly in the brains of diabetic (D, panel B) rats submitted to ischaemia. DMF prevented the effect of ischaemia in diabetic animals. The mean + SEM are shown.

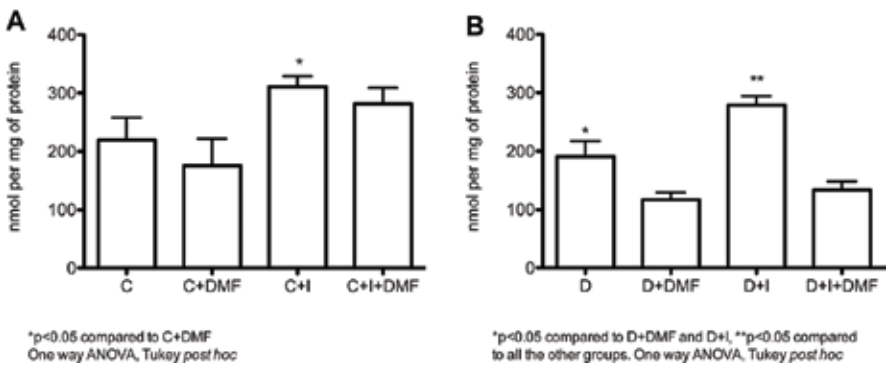

Figure 9. Malondialdehyde (MDA) in the brain: dimethylformamide (DMF) non-significantly reduced MDA in the brains of non-diabetic (control, C, panel A) rats. Ischaemia (I) did not significantly increase MDA in control animals resulting in a significant difference between control rats without ischaemia with DMF treatment (C + DMF) and control rats with ischaemia (C + I). DMF significantly reduced DMF in diabetic (D, panel B) rats without ischaemia (D + DMF). Ischaemia significantly increased MDA in diabetic rats and the change was prevented by DMF. The mean + SEM are shown.

3.2.3.2. RAGE

Figure 6 shows that RAGE did not change in non-diabetic rats, with or without treatment or ischaemia, whereas it significantly increased in diabetic rats with ischaemia ($p < 0.05$), and the effect was prevented by DMF.

3.2.3.3. NO_2/NO_3 and NT

Nitrite/nitrate ratios and nitrotyrosine results are shown in **Figures 7** and **8**. Results were similar to those of RAGE (**Figure 6**). Neither ischaemia nor DMF changes the basal concentrations

of NO_2/NO_3 or NT in the brains of non-diabetic rats, whereas in the diabetic animals nitrates/nitrates as well as NT significantly increased with ischaemia, and the effect was prevented by DMF.

3.2.3.4. MDA

Figure 9 shows the concentrations of MDA in the brains. Basal (no ischaemia, no treatment) concentrations were similar in diabetic and non-diabetic animals. DMF slightly (non-significantly) reduced MDA in non-diabetic rats, whereas it significantly reduced MDA in diabetic animals ($p < 0.05$). Cerebral ischaemia increased MDA in both diabetic and non-diabetic animals but the change was significant only in diabetic rats ($p < 0.05$). DMF suppressed the effect of ischaemia in diabetic rats ($p < 0.05$). Due to the non-significant changes produced by DMF in non-diabetic animals without ischaemia, there was a significant difference ($p < 0.05$) between this group and non-diabetic animals with ischaemia.

4. Discussion

This is the first time that DMF has been used and shown to be beneficial in treating cerebral ischaemia. In the present study DMF significantly reduced infarct volume in both diabetic and non-diabetic animals (similar reductions). It is important to note that infarct volume was evaluated just 8 h after cerebral ischaemia induction; thus, it could be interesting to observe if the similar effect occurs after a longer time (24 or 48 h). It is also important to note that treatment was administered 1 h after the onset of ischaemia, which is different from the experimental strategies that have been used for antioxidants (they usually are administered as a pre-treatment). One hour is a long period and results of the present study open a new horizon in the evaluation of therapeutic strategies that eventually could be used after the therapeutic window of thrombolysis, which is the current treatment (and not very efficient) in cerebral ischaemia.

Even though the reduction of infarct volume was similar in non-diabetic as in diabetic animals, DMF seems to have a better effect in diabetic rats because it significantly improved the neurophysiological score. One of the concerns of using DMF is the possibility of toxic effects. However, the toxic effect noted in workers in the synthetic leather industry is the result of chronic long-term exposure [16, 17]. Moreover, the single dose that we used in the present study (0.9 g/kg) is far from the reported LD_{50} (7.2 g/kg) [18]. Since no apparent toxic effect was noted, it seems that DMF was safe and beneficial. Moreover, the treatment was administered 1 h after ischaemia in non-diabetic as well as diabetic animals, which places the study closer to clinical setting conditions where diabetes is a risk factor for stroke, and it is virtually impossible to predict who would suffer cerebral ischaemia.

The difference of DMF effects between groups seems to be related to special conditions of diabetes pathology. It is known that long-term inflammation and oxidative stress play an important role in the cardiovascular complications of diabetes [8, 19]. Indeed, it was reported that cardiovascular, renal, and neurological complications become apparent at least 2 months after diabetes induction in the rat streptozotocin model and that those complications were attenu-

ated by inhibitors of the renin-angiotensin system, whose effects include the reduction in oxidative stress and probably inflammation [20–22]. In the present study, the diabetic animals had low weights, hyperglycaemia, and high glucosylated haemoglobin. Cerebral ischaemia was produced 5 weeks after the induction of diabetes. This means that diabetic animals were not supposed to have apparent cardiovascular complications of diabetes, although the pathophysiological events were certainly already triggered. Indeed, this could explain why even in basal conditions (brains of animals without ischaemia and without treatment) all the biomarkers had the same concentrations, and notably cerebral ischaemia significantly increased all the biomarkers only in diabetic animals, whereas MDA (biomarkers or OS effects on lipids) was the only biomarker increased in non-diabetic rats with ischaemia.

The expression of MMP9 has been related to various steps of the pathophysiology of cerebral ischaemia, such as inflammation, excitotoxicity, neuronal damage, and blood brain barrier (BBB) disturbance [6, 23]. MMP9 increased clearly in diabetic but not in non-diabetic animals with ischaemia. DMF did not prevent MMP9 incremental increases. The lack of an increment in non-diabetic animals could be due to the short time that passed after ischaemia was induced and the sacrifice (8 h).

Nitrotyrosine (NT) is the fingerprint of peroxynitrite, a nitrogen active species produced by the interaction of superoxide and nitric oxide (NO) [24]. NT is a biomarker of inflammation and OS [24]. NT increases in the brain mitochondria of diabetic animals 21 days after STZ administration [25]. NT also increases in the brains 24 h after ischaemia induction [26]. In the present study, NT was evaluated in homogenised tissue, which could explain why the increment of this biomarker was not observed in the brains of diabetic animals without ischaemia. No increment of NT was observed in the brains of non-diabetic animals, whereas it significantly increased after cerebral ischaemia in diabetic animals. Nitrotyrosine changes in diabetic animals were completely prevented by DMF. Interestingly, nitrite/nitrate (indirect measurement of NO production) and RAGE (a biomarker of neuroinflammation in cerebral ischaemia [27]) changed similarly to NT; they increased after ischaemia only in diabetic animals and such increment was prevented by DMF.

The evaluation of cerebral ischaemia was performed early after arterial occlusion (8 h). It could be the reason for not observing changes in non-diabetic animals (only MDA increment). Diabetic animals were probably more susceptible to inflammation and OS after ischaemia due to the basal conditions produced by hyperglycaemia. Such basal conditions could explain the greater effects of DMF in diabetic animals. It is necessary to study the effects of DMF for longer periods after ischaemia and on other biomarkers. The results of this study open a new horizon in the design of therapeutic strategies for the treatment of stroke.

5. Conclusion

We conclude that DMF has beneficial effects on cerebral ischaemia produced in rats. The protective effects of DMF in diabetic rats could be the result of interference with oxidative and inflammation-triggered pathways, which are exacerbated by diabetes. That could explain the

reason for the better results in diabetic rather than in non-diabetic animals. The mechanisms of the protective effects in non-diabetic rats were not clear. It is necessary to explore other biomarkers and longer periods to elucidate those effects.

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Chapter 7

Cardioprotective Effects of S-Nitrosothiols in Ischemia-Reperfusion: Role for Mitochondria and Calcium Channels

Oscar Arriagada, Adriana V. Treuer and
Daniel R. González

Additional information is available at the end of the chapter

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Abstract

The most important clinical consequence of coronary disease is acute myocardial infarction caused by an occlusion that limits the irrigation to the heart. Although the gold standard treatment is to restore blood flow, this reperfusion causes inherent damage by increasing the size of the infarcted area primarily through the opening of the mitochondrial permeability transition pore (MPTP). The cardioprotective effect of nitric oxide (NO) has been described to operate through S-nitrosylation of several important proteins in the cardiomyocytes such as the calcium channels RyR2 and the L-type Ca^{2+} channel and mitochondrial proteins, including the MPTP. In this sense, an attractive strategy to prevent the ischemia-reperfusion damage is to increase the bioavailability of endogenous S-nitrosothiols. S-nitrosoglutathione reductase (GSNOR) is an enzyme involved in the metabolism of NO through denitrosylation, which would limit the cardioprotective effect of NO. Although inhibition of GSNOR has been studied in different organs, its effects on myocardial reperfusion have not yet been fully elucidated. In this chapter, we review the pathophysiology underlying myocardial reperfusion injury and the opening of the MPTP along with the cardioprotective role of S-nitrosothiols and the potential role for GSNOR.

Keywords: permeability transition pore, heart, GSNOR, nitric oxide, S-nitrosylation, Ca^{2+}

1. Introduction

Coronary heart disease is the leading cause of death worldwide [1]. According to the World Health Organization, in 2008, more than 7 million of deaths worldwide were the result of this

disease (12.8% of all deaths) [2]. The main clinical manifestation of this condition is acute myocardial infarction, which is characterized by a coronary occlusion, most frequently produced by the rupture of an unstable atherosclerotic plaque [3]. Ischemic damage in the myocardium produced by this pathology requires immediate restoration of blood flow to the affected heart area. The injury size of the resulting infarction depends on: (1) the ischemic area at risk, (2) the duration and intermittency of the coronary occlusion and (3) the magnitude of the residual collateral flow and the degree of microvascular dysfunction [4].

The preservation of myocardial functionality after ischemia is critical for the viability of the damaged heart, and although reperfusion is necessary for the survival of the myocardium, paradoxically this reperfusion itself can generate injury or even induce the death of cardiomyocytes. The damage can range from a reversible effect such as "myocardial stunning" to an irreversible one, increasing the size of the infarcted area or increasing microvascular alterations [5]. This phenomenon is known as reperfusion myocardial damage [6, 7].

To combat this condition, most of the current pharmacological strategies of cardioprotection converge to the mitochondria, particularly, to prevent the opening of mitochondrial permeability transition pore (MPTP), which is a large conductance channels that forms during stressing conditions for the heart such as reduction in ATP synthesis, increased Ca^{2+} and increased ROS production [8]. Nitric oxide (NO), a potent signaling molecule with pleiotropic effects in the heart, has long been studied to evaluate its cardioprotective effects. Agents that promote continuous NO releases are among the most commonly used drugs to treat cardiovascular disease [9]. Although its effects on vascular tone and cyclic GMP are well studied [10], S-nitrosylation, a posttranslational modification in which an NO group is added to a cysteine

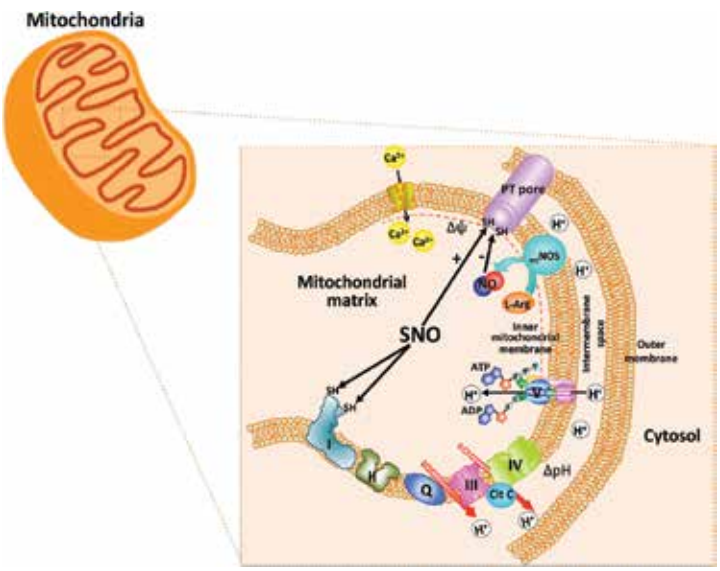

Figure 1. S-nitrosylation in mitochondria. The figure depicts two important targets where S-nitrosothiols (SNO) exert cardioprotective effects within the mitochondria. S-nitrosylation of complex I prevents the burst of reactive oxygen species during reperfusion. CyclophylinD, which interacts with mitochondrial permeability transition pore is also S-nitrosylated, reducing its opening.

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thiol, has showed remarkable cardioprotective effects altering the function of different proteins important for the regulation of cell function and viability.

One of the main cardioprotective effects of S-nitrosylation is the avoidance of the opening of MPTP. The opening of the MPTP triggers a proapoptotic response mediated by the release of cytochrome C and other factors, as well as necrotic cell death. Agents that induce mitochondrial S-nitrosylation of this pore may reduce the infarcted area after ischemia and to maintain the functionality of the heart (**Figure 1**).

2. Reperfusion damage

Myocardial reperfusion injury was first described by Jennings et al. in 1960, using a model of coronary occlusion in dogs [11]. They observed alterations in the sarcolemma and in mitochondria, which accelerated the process of cardiomyocyte necrosis. This reperfusion injury in animal models of acute myocardial infarction can result in up a 50% of infarcted area [1]. During reperfusion, inflammatory mediators are released, such as cytokines, which recruit neutrophils to the affected endothelium, permeabilizing it for the entrance to the injured myocardium of more inflammatory cells. In fact, decreasing this influx of neutrophils confer cardioprotection in animal models [12]. In addition, during the period of ischemia, molecules called "danger signals" are released from the extracellular matrix and damaged cells such as fragments of fibronectin, hyaluronic acid, heat shock proteins and high-mobility group box-1 (HMBG1). HMBG1 is able to interact with toll-like receptors (TLR) such as TLR2, TLR4, TLR9 and the receptor for advanced glycation end products (RAGE). These ligand-receptor interactions lead to the nuclear translocation of NF-kappa B, triggering the transcription of proinflammatory genes [13].

One of the main organelles affected by reperfusion injury is mitochondria, which in pathological conditions may mediate detrimental effects to the cell such as apoptosis or cell necrosis [14].

After ischemia-reperfusion, mitochondria undergo structural as well as functional alterations. One of the most important alterations is the formation of MPTP, a pore of nonselective permeability, stimulated by high concentrations of mitochondrial Ca^{2+}, adenosine and reactive oxygen species (ROS) [15]. The function of this pore is thought to be the regulation of Ca^{2+} concentrations, preventing the overload of this ion at the mitochondrial level [16]. In fact, inhibition of the mitochondrial Ca^{2+} exchanger showed cardioprotective effects in isolated hearts of newborn [17] as well as in adult rabbits [18].

3. Mitochondrial permeability transition pore

In situations such as Ca^{2+} overload, alterations in ATP production, mitochondrial membrane depolarization, or inhibition of respiration, mitochondria may undergo a swelling process that represents a permeabilization of the internal membrane, product of the opening of nonspecific channels known as the mitochondrial permeability pore [19]. This pore, which allows the passage of molecules up to 1.5 kDa, mediates the rupture of the outer mitochondrial membrane

and the release of proapoptotic substances such as endonuclease G and the mitochondrial apoptosis inducing factor (AIF) allowing the exit of elements such as the cytochrome C [20].

What is the function of this mitochondrial pore? The pathological effects of the presence of the pore have been well described and usually associated with long-term openings since short-term openings do not have a major impact on cell viability [21]. On the contrary, it is believed that its transient opening can play a very important role both in the physiological regulation of Ca^{2+} and redox homeostasis in the generation of ROS [22].

3.1. Constituents of the transition pore of mitochondrial permeability

3.1.1. Cyclophilin D

Cyclophilin D (Cyp-D) is an 18 kDa protein encoded by the Ppif gene, synthesized in the cytosol and transported to the mitochondria to form part of the pore. It is inhibited by cyclosporine A, an immunosuppressant, decreasing its sensitivity to elevations in Ca^{2+} concentration [23]. CypD is a member of a family of cyclophilin proteins that have peptidylprolyl isomerase (PPIase) activity, catalyzing the cis-trans isomerization of peptidylprolyl bonds. In studies in knockout mice for the Ppif gene, mitochondria were more resistant to swelling and decreased the ability to open the pore [24]. It was also observed that overexpression of this gene caused an increase in swelling and cell death after ischemia-reperfusion [25]. Canceling the expression of this protein would, in theory, desensitize to the mitochondria for Ca^{2+} and will be protective. Nevertheless, it was described that when Ca^{2+} concentration is high enough, the pore can be opened independent of the presence of CypD [26]. It has been postulated that both CypD and the mitochondrial Ca^{2+} pore opening participate in the regulation of mitochondrial Ca^{2+} homeostasis, since the pore opening directly depends on the Ca^{2+} concentrations reached and its opening would try to reinforce the effect of the Na^{+}/Ca^{2+} through an efflux of Ca^{2+} to prevent the overload of this cation [27].

3.1.2. Adenine nucleotide traslocase

Adenine nucleotide traslocase (ANT) is a member of the long list of mitochondrial carriers. It shows a tripartite conformation of repeated sequences of 100 amino acids, with ANT1 being the isoform present in the heart [28]. Given the importance of the production of ATP by the mitochondria and its use mainly in the cytosol, a mechanism of fast and effective transport between the two compartments is necessary: the exit of ATP to the cytosol and transport of the ADP to the mitochondria. Therefore, ANT1 is one of the most abundant proteins inside the mitochondria and more than 10% of the energy used by the cardiomyocyte is used to maintain this transport [28]. Regarding to the role of ANT in the pore formation, it has been observed that inhibition of binding of ATP and ADP to ANT increases the sensitivity of MPTP in response to variations in $[Ca^{2+}]_i$ [19]. Evidence shows that ANT has the ability to bind CypD and that this binding is important for pore opening, with ANT acting as a sensor of $[Ca^{2+}]_i$. Although this protein has been categorized as a component of the MPTP, its importance in the pore gating is controversial since inhibition of both ANT 1 and 2 in mouse liver could still constitute the mitochondrial pore, although with a higher increase in the $[Ca^{2+}]_i$ [29].

3.1.3. Mitochondrial phosphate carrier

The mitochondrial phosphate carrier is encoded by the gene SLC25A3 and is a primary transport system of inorganic phosphate (Pi) by a proton transporter or an exchanger with hydroxyl ions [16]. It has been described that Pi is a potent modulator for the MPTP opening. In an energized condition, Pi can induce the opening of the pore. This contrasts with other studies postulating Pi as an inhibitor of pore opening [30]. This carrier could be considered as a structural component of the MPTP, but studies are still lacking to confirm its actual function.

3.1.4. Voltage-dependent anion channel

This is a channel present in the external mitochondrial membrane that serves as a pore for different substances in or out of the mitochondria. Like ANT, voltage-dependent anion channel (VDAC) can be considered a structural element in the conformation of the MPTP. Inhibiting VDAC activity, inhibits Bax (a proapoptotic protein) function and the release of cytochrome C, decreasing the apoptotic process [31].

3.1.5. F_1F_0-ATP synthase

More recent investigations have shown that a dimer of the mitochondrial F_1F_0-ATP-synthase is essential to form the core of the mPTP. F_1F_0-ATP-synthase interacts with CyPD and can reversibly undergo a Ca^{2+}-dependent transition to form a channel with the characteristics of the mPTP [20].

3.2. MPTP opening in ischemia/reperfusion damage

3.2.1. Ischemia

During the period of ischemia, the hypoxic condition makes the mitochondria unable to produce ATP through oxidative phosphorylation, leading to a rapid depletion of cellular ATP reserves, with the concomitant increase of ADP and Pi [15]. There is a change in ATP production from oxidation of fatty acids (most common) to glucose metabolism, but through an anaerobic route, a less efficient mechanism, which leads to increased lactate, NADH and H^+ [32] with a decrease in pH. This inhibits phosphofructokinase and activates the H^+/Na^+ symporter. This compensatory mechanism is unable to deliver enough ATP to supply cardiomyocytes, and specially pumps such as Na^+/K^+ ATPase. The accumulation of Na^+ facilitates the accumulation of Ca^{2+} in the cell because the Na^+/Ca^{2+} exchanger works in a reverse mode [15]. Then, oxidative damage alters the sarcoplasmic reticulum, releasing Ca^{2+}, further contributing to the overload of this ion [1]. The small amount of oxygen that remains serves for the oxidation of xanthine by the enzyme xanthine oxidase, mainly in the endothelium but also in the cardiomyocyte, which produces superoxide and other ROS. The duration of the ischemia period is an important factor; in short periods of ischemia (less than 15 min), reperfusion manages to restore homeostatic values with respect to mitochondrial membrane potential, cardiomyocyte contracture and Ca^{2+} concentration. However, in periods longer than 25 min, all these values are altered in a way that cell death is promoted [33].

3.2.2. Reperfusion

In reperfusion, other factors beside an increase in Pi, ROS and Ca^{2+} and the decrease in ATP come into play. Although all of them contribute to the opening of the mitochondrial pore, the diminished pH would be a "protective" factor in the conformation of this pore [34]. In fact, a decrease in pH inhibits the H^+/Na^+ symporter, which would prevent accumulation of Ca^{2+} in the cell [35].

Recovery of oxygen levels re-energizes the mitochondria to restore its role in the production of ATP via fatty acids metabolism. An alteration of the membrane potential causes Ca^{2+} to enter the mitochondria, while the oxygen entering it contributes to the formation of more ROS due to the reduced respiratory chain [19]. Reperfusion favors the recovery of pH in a period close to 3 min, time in which lactate diffuses. During the first few minutes, the damage caused by reperfusion is small; nevertheless, as reperfusion is delayed, the infarcted area grows. Once the pore has been established as a result of the abovementioned alterations, mitochondria may become swollen and the rupture of the outer membrane occurs, which will trigger the release of cytochrome C and other proapoptotic substances leading to cell death [15, 36–38].

4. Mitochondrial ROS release and reperfusion injury

ROS release by mitochondria is one of the major events that trigger ischemia-reperfusion injury. Although this event is thought to be produced by an alteration in the mitochondrial electron transport chain during reperfusion, it is further believed that these reactive species can be produced by an altered metabolic process during ischemia, whose consequences appear in the reperfusion period. For example, in a study by Couchani et al., they observed the accumulation of succinate, a metabolic intermediate of the Krebs cycle, during ischemia, as product of the reversal activity of succinate dehydrogenase. During reperfusion, the accumulated succinate is rapidly re-oxidized by succinate dehydrogenase, driving extensive ROS generation by reverse electron transport at mitochondrial complex I [39].

4.1. Inhibition of mitochondrial pore opening protects the heart against ischemia-reperfusion injury

The importance of MPTP opening in myocardial damage makes it an excellent target for therapies aimed to reduce reperfusion injury. In studies in animal models in which the mitochondrial pore opening was inhibited, a reduction of the infarcted area was observed in more than 50% [1, 40], highlighting the impact of the presence of this pore on the magnitude of myocardial damage. These types of treatments are currently in development and in the search for safe and specific pore opening inhibitors [1, 38, 41].

In this context, different mechanisms have been developed to mitigate cell damage induced by reperfusion, and although improvements have been achieved with regard to the response of the infarcted region, there is no effective therapy yet to completely avoid reperfusion injury [2].

An interesting target to achieve inhibition of the opening of the pore is cyclophilin D. As mentioned earlier, the inhibition of CypD with cyclosporine in isolated hearts has shown

protective effects in ischemia-reperfusion [42]. A decrease in infarcted area was also demonstrated in knockout mice for CypD compared control mice [43].

Interestingly, CypD can be S-nitrosylated on cysteine 203. This S-nitrosylation reduces the activity of the MPTP [23].

There are other constituents of the mitochondrial pore that, being inhibited, contribute to avoid the gating of the pore. Unfortunately pore elements such as ANT and the carrier of mitochondrial phosphate are indispensable for other vital cellular functions, and therefore their inhibition would add the cell an additional risk after ischemia-reperfusion. The opening of the pore can also be avoided indirectly. By decreasing the influx of Ca^{2+} from the extracellular space, the overload of this cation is avoided and thus the pore opening.

5. Cardioprotective function of nitric oxide

Nitric oxide is a molecule that participates in different processes, including neurotransmission, vasodilatation, defense against pathogens and depending on cellular conditions, in apoptosis [44–46]. This molecule is produced by the conversion of L-arginine to L-citrulline, a reaction catalyzed by one of three types of enzyme nitric oxide synthase (NOS) [47]. Neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are constitutively expressed enzymes and inflammatory nitric oxide synthase (iNOS) is inducible. The activity of NO as a cell signaling molecule via cyclic GMP is well known. The cGMP formed by NO has three targets: protein kinase G (PKG), cyclic nucleotide-dependent channels and phosphodiesterase (PDE) [48] achieving well-studied vasoactive effects. However, recent studies suggest that NO can modify proteins posttranslationally, via a nitrosative pathway, known as S-nitrosylation [49]. S-nitrosylation consists in the addition a NO group to a cysteine residue generating nitrosothiols (SNO). Different studies have shown that this nitrosative modification of proteins alters their function, reversibly [49]. In addition, the S-nitrosylated cysteines of proteins are in a chemical equilibrium with S-nitrosoglutathione (GSNO), a low molecular weight nitrosothiol. In this manner, GSNO functions as a reserve of NO that can be exchanged with GSH in a reaction termed transnitrosylation and in this way participates in part of NO bioactivity [50, 51].

5.1. S-nitrosylation and cardioprotection

Although the cardioprotective effects of NO by their dependent cGMP pathway are known, recent research suggests a relationship between increased SNO formation and cardioprotection [52]. In cardiomyocytes, S-nitrosylation occurs in a large number of proteins [53].

In ischemia-reperfusion studies, S-nitrosylation has been associated with an increase in the recovery of developed ventricular pressure and ventricular work [54]. This has been observed in both preconditioning and ischemic postconditioning studies [55], resulting in an increase in S-nitrosylation associated with an improvement in contractile activity. This recovery of functionality has also been appreciated with the use of S-nitrosothiols donors such as Mito-SNO (targeted to mitochondria) or S-nitroso-N-acetylpenicillamine (SNAP) [56]. Using GSNO to generate a preconditioning effect in an ischemia-reperfusion experiment in mice resulted in a greater

recovery of developed ventricular pressure and a smaller infarcted area compared to controls [54]. Furthermore, it was observed that the inhibition of caspase-3 by S-nitrosylation decreases its proapoptotic activity [57]. In a study in mice undergoing cardiac ischemia-reperfusion, it was observed that an increase in S-nitrosylation of the cardiac L-type Ca^{2+} channel reduced Ca^{2+} entry, thereby reducing Ca^{2+} overload, subsequently reducing cardiac damage by reperfusion [58]. On the other hand, in transgenic mice with iNOS overexpression submitted to cardiac ischemia-reperfusion, a smaller infarcted area was observed in addition to a lower Ca^{2+} overload and a smaller opening of the mitochondrial pore [59]. Mito-SNO (5-(2-acetylamino-3-methyl-3-nitrosothiobutyrylamino)-pentyl]-triphenylphosphonium methanesulfonate) was used in a study in mice and it was observed that the application of this donor was able to improve cardiac function and also to decrease the infarcted area compared to controls [60]. This cardioprotective effect was due to the reversible S-nitrosylation of the mitochondrial complex I. This effect would slow mitochondrial activation in the first few minutes of reperfusion, a crucial moment in ROS release, decreasing it and limiting reperfusion injury [61]. Similar effects were found in ischemia-reperfusion in rat hearts, where another S-nitrosothiol donor, S-nitroso-2-mercaptopropionyl glycine (SNO-MPG) was used, resulting in inhibition of the complex I, mainly during the late stage of ischemia and early reperfusion, thereby reducing ROS production by mitochondria, similar to the previous case. In addition, it was observed that in hearts treated with the nitrosylating agent they were more resistant to the opening of the transition pore [62]. In studies of ischemic preconditioning in mouse hearts, a relationship between increased S-nitrosylation and cardioprotection was observed, where an elevation of the nitrosothiols with GSNO treatment, developed positive ventricular pressure and reduced total infarcted area [54].

In mitochondria, complex I is the primary site of ROS production, with the generation of superoxide anion. S-nitrosylation of complex I was shown to inhibit its activity, which reduces ROS production during reperfusion [63]. However, SNAP reversibly inactivated the mitochondrial complex I, resulting in an increase in H_2O_2 production [64]. On the other hand, S- nitrosylation decreased the Ca^{2+} elevation produced during reperfusion through the modification of the L-type Ca^{2+} channel [54], which prevented the opening of the mitochondrial pore.

An interesting question arises as whether nitrosylation occurs equally in all mitochondria. Sun et al. found higher S-nitrosylation in the sub sarcolemmal mitochondria than in the interfibrillar in mice hearts subjected to ischemic preconditioning. Both types of mitochondria differ in protein and lipid composition, in addition to the capacity for protein synthesis and oxygenation [65]. This suggests that subsarcolemmal mitochondria are more susceptible to ischemia-reperfusion injury, with the MPTP more prone to ischemia-reperfusion injury and also being exposed to a higher oxygen gradient during reperfusion [66]. This difference in mitochondrial response has already been observed with the use of other cardioprotective agents such as diazoxide [67].

5.2. S-nitrosoglutathione reductase

The cellular levels of S-nitrosothiols are governed by the enzyme S-nitrosoglutathione reductase (GSNOR), an alcohol dehydrogenase type III enzyme, whose function is to remove NO groups from the cysteine thiols in proteins [68] through denitrosylation, degrading GSNO and

increasing levels of glutathione (GSH), thereby reducing nitrosylation [69]. This enzyme is highly conserved from bacteria to mammals and has the function of modulating the S-nitrosylating function of NO and thus avoiding for example nitrosative stress in the presence of excessive NO.

Although this enzyme does not act directly on SNO-protein substrates, its deficiency causes an increase in the intracellular concentrations of nitrosylated proteins (**Figure 2**). This means that there is an equilibrium based on the transfer of SNO groups between low molecular weight nitrosothiols and protein cysteines. It is also thought that the role of GSNO is not only a transitrosilation (from one thiol to another) but also function as a source of NO independent of the action of NOS.

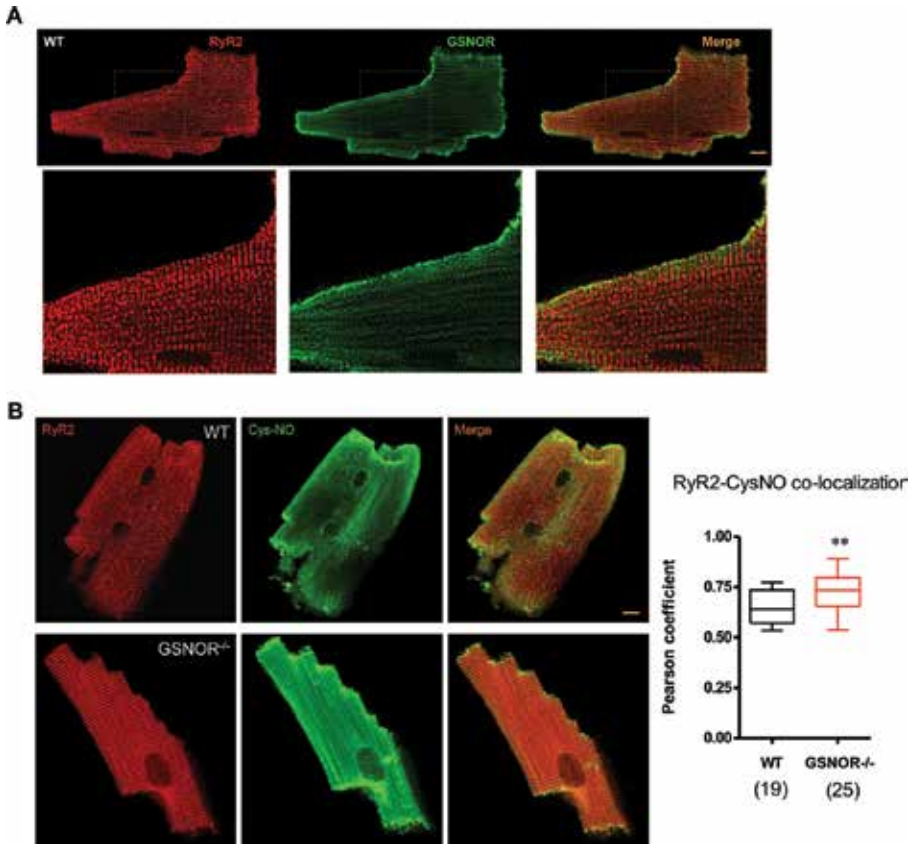

Figure 2. GSNOR in the cardiac cell. Immunocytochemical analysis of S-nitrosoglutathione reductase in cardiac myocytes. (A) The pictures depicts the localization in a mouse cardiomyocytes of GSNOR (green) compared to that of the ryanodine receptor (RyR2, red) using confocal microscopy. The sections demarked by dotted lines are amplified below. Notice that GSNOR partially co-localizes with RyR2, suggesting association in the sarcoplasmic reticulum. (B) Co-localization analysis for RyR2 (red) and S-nitrosylated thiols (Cys-NO, green) using a specific antibody against Cys-NO in wild type (WT) and GSNOR-deficient ($GSNOR^{-/-}$) mice. The bar graph shows the Pearson analysis for co-localization of RyR2 and CysNO. ** indicates $p < 0.001$ using T test. Number of cells analyzed is indicated between parentheses. The scale bar indicates 10 μm.

While most proteins have cysteine residues and NOS is expressed in almost all cells, substrate specificity is an important feature of endogenous S-nitrosylation of proteins. Moreover, in all proteins possessing more than one S-nitrosylation site, the single modification of one cysteine thiol can modify their function under physiological conditions [70].

Apparently, the activity of GSNOR is increased lung endothelial cell of females compared to males [71]. In the heart the results are similar. Females possess a higher activity of GSNOR than males [72], without differences in the activity of this enzyme in sexually immature mice. Although there are no significant differences in the expression of GSNOR for either sex, estrogens stimulate the activity of eNOS, which would lead to increased S-nitrosylation, an important factor that would stimulate GSNOR activity. On the other hand androgens may play a role, since in neutered male mice the activity of the GSNOR seems to be similar to that of the females. This mechanism of GSNOR activation is not known [71].

5.3. The complex role of GSNOR

Given the anti-inflammatory and muscle relaxant properties of NO, inhibition of GSNOR is already being tested for the treatment of associated pathologies, mainly respiratory [73]. For example, in bronchoscopy samples from different subjects, GSNOR activity was increased in certain types of asthma [74], which makes it a good target for the treatment of this pathology. In a mice model of asthma, the GSNOR inhibitor SPL-334 was evaluated, resulting in a reduction of bronchial inflammation, airway hyperreactivity, mucus production and eosinophil accumulation and allergen-specific T [75]. Also, in mice lung smooth muscle, inhibition of GSNOR decreased the contractile response in the methacholine test, in addition to anti-inflammatory effects [76]. On the other hand, GSNOR appears to protect lung immune cells from nitrosative stress, since its deficiency tends to increase lung susceptibility for *Klebsiella pneumoniae* infection [77].

Skeletal muscle tissue has also been evaluated for GSNOR function. With the use of GSNOR knockout mice, the tibialis anterior muscle of $GSNOR^{-/-}$ mice was found to be more resistant to fatigue, with no alterations in mitochondrial function or in capillary density, associated with hypernitrosylation ryanodine receptor (RyR1), which could increase skeletal muscle contractility without altering mitochondrial function [78]. However, another study using $GSNOR^{-/-}$ mice showed the opposite effect, since the silencing of GSNOR revealed a muscular atrophy and loss of muscle mass, associated with increased S-nitrosothiols, along with mitochondrial fragmentation and depolarization [79].

GSNOR also appears to have a role in breast cancer. The study by Cañas et al. indicates that the antiproliferative action exerted by trastuzumab on breast cancer cells overexpressing HER2 is suppressed when GSNOR is inhibited. This indicates that an increase in SNOs would provide a survival advantage for cancer in HER2 + tumors and may constitute a mechanism of resistance to this targeted treatment in breast cancer [80]. On the other hand, other studies have shown that inhibition of GSNOR can be detrimental. Rizza et al. observed that the silencing of GSNOR was shown to protect SH-SY5Y cells from toxins characteristic of Parkinson's disease. However, it was also observed that overexpression of this enzyme is a resistance factor for the treatment of amyotrophic lateral sclerosis [81]. It has also been shown in other investigations

that the absence of GSNOR may be harmful and that this increase in S-nitrosylation that would cause its absence would be pathological. Rizza et al. observed that hepatocytes from GSNOR-deficient mice had mitochondrial alterations, characterized by an increase in the levels and activity of the enzyme succinate dehydrogenase, mediating this alteration in a greater proclivity to the development of hepatocarcinoma [82]. This relationship had already been studied previously by Wei et al. who found that human hepatocarcinoma cells showed less GSNOR activity than noncancerous liver cells [83], inferring that the absence of GSNOR can alter DNA repair and thus induce tumor growth. In lung cancer, studies show that GSNOR is reduced in lung cancer samples relative to normal lung tissue [84]. This emphasizes the relationship between nitrosative stress and the development of neoplasia and how this enzyme can participate in its development.

GSNOR also appears to be important in the immune system. A study in GSNOR-deficient mice shows that the absence of this enzyme would cause a pathological nitrosylation causing apoptosis in thymus cells and a reduction of B and T lymphocytes. All this was mediated by the uncontrolled activity of iNOS [85].

5.4. GSNOR and cardioprotection

Given the large number of benefits delivered by S-nitrosylation in the heart, there is currently a search for therapies that pursue a greater availability of S-nitrosothiols in order to reduce cardiac damage and improve ventricular performance after ischemia. Therefore, NO donor agents, ischemic pre- and postconditioning, and modulation of GSNOR activity have been tested.

In the cardiomyocyte of knockout mice for GSNOR, a decrease in the response to a β-adrenergic agonist and a decrease in cytosolic Ca^{2+} concentration were observed [86], highlighting the role of endogenous S-nitrosothiols in cardiac function. In these GSNOR it was observed S-nitrosylation of β-arrestin and G protein receptor kinase 2, two proteins involved in the regulation of the β-adrenergic receptors pathway [87], besides the nitrosylation of different Ca^{2+} channels, such as the L-type Ca^{2+} channel, ryanodine receptor RyR2 and the pump SERCA [88] (**Figure 3**). Lima et al., using a model of myocardial infarction in GSNOR-deficient mice, observed a reduction in the infarcted area, maintenance of ventricular function, with an increase in vascular density compared to wild type mice. This proangiogenic effect of GSNOR inhibition was explained as a result of increased activity of transcription factor hypoxia inducible factor-1 (HIF-1 α) due to increased S-nitrosylation in normoxic conditions, which resulted in increased binding to the VEGF gene promoter [50].

In a similar study in GSNOR-deficient mice that underwent myocardial infarction, the improved contractile function and increased angiogenesis were confirmed. Importantly, these authors reported that after myocardial infarction, the GSNOR knockout mice hearts showed increased proliferation of endogenous cardiac stem cells and increased mitosis in cardiac myocytes compared to wild type mice [89].

On the other hand, pharmacological inhibition of GSNOR has been shown to improve endothelial function in rats. In a study by Chen et al. it was observed that using the GSNOR

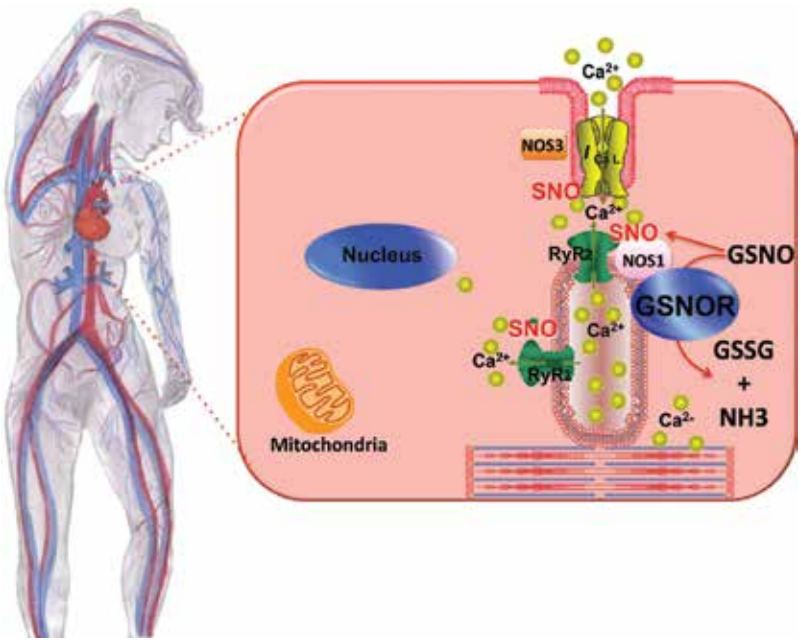

Figure 3. Regulation of intracardiac S-nitrosothiols by GSNOR. Schematic model of the regulation of S-nitrosylation within the cardiac cell by GSNOR. S-nitrosoglutathione reductase is able to metabolize endogenous S-nitrosothiols by controlling the fate of S-nitrosoglutathione (GSNOR). The RyR2 and the L-type (*I* Ca) calcium channels are nitrosylated (SNO) by the activity of the nitric oxide synthases 1 (NOS1) and 3 (NOS3) located in the sarcoplasmic reticulum and plasmalemma, respectively. SNO are in equilibrium with nitrosoglutathione (GSNO). Thereby, by controlling the fate of GSNO, GSNOR regulates the levels of S-nitrosylation of these channels, important for the reperfusion damage. GSSH, oxidized glutathione, NH_3, ammonia.

inhibitor N6338 at a single dose maintained flow-mediated arterial dilation versus inhibition of NOS. In hypertensive rats, application of N6338 was shown to decrease blood pressure and vascular resistance, as well as to restore altered flow-mediated dilatation [90].

Then, the question arises whether inhibition of GSNOR is cardioprotective. Much of the evidence suggests that this would be the case. However, the protective role of GSNOR in preventing pathological S-nitrosylation should be considered, as there are studies that suggest that the enzyme is essential for cardiovascular homeostasis. For example, Sips et al. used mice that overexpressed GSNOR specifically in cardiomyocytes and observed an improvement in myocardial properties post induction of myocardial damage after sepsis, a condition that is associated with nitrosative stress derived from the activity of iNOS [91]. Nevertheless, in conditions where increased bioavailability of S-nitrosothiols is required, such after ischemia, the inhibition of GSNOR appears as an attractive therapeutic strategy. This would increase the S-nitrosylation for example, of calcium-handling proteins, which, in a setting of ischemia-reperfusion would prevent the calcium overload. In addition, increased S-nitrosylation of mitochondrial proteins may prevent the generation of ROS and a reduction in mitochondrial permeability transition pore activity, reducing cell death. Nevertheless, these effects remain to be probed experimentally.

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6. Conclusions

Although reperfusion is essential for the recovery of the heart after ischemia, it can itself initiate myocardial damage and cause death of the cardiomyocyte. A key event in this cellular disorder of this alteration is the opening of the MPTP, which induces mitochondrial dysfunction and ultimately leads to cardiomyocyte death. For this reason, therapies are being developed in order to limit the opening of the pore and reduce the damage by reperfusion. One important mechanisms of cardioprotection is S-nitrosylation, which consists of adding NO groups to cysteine residues of proteins, modifying their function. S-nitrosylation of mitochondrial complex I and MPTP using NO donors and S-nitrosylating agents has shown to induce cardioprotection. It is known that agents or genetic alterations that increase SNO are cardioprotective and in this context the modulation of GSNOR is of relevance, since this protein is a critical regulator of endogenous SNOs. This enzyme participates in the redox balance, regulating the levels of nitrosylation/denitrosylation and its inhibition can increase the bioavailability of S-nitrosothiols, being this a potential cardioprotection element. So far, in the heart, genetic cancelation of GSNOR has indeed shown positive results in the context of damage by infarction. For this reason, more studies are needed in order to assess the impact of inhibition of GSNOR on global cellular functioning. This will indeed help in the development of new knowledge for the treatment and prevention of myocardial diseases.

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Chapter 8

Antimicrobial and Antioxidant Properties of Essential Oil Isolated from *Coleus zeylanicus* under Normal and Salinity Stress Conditions

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Additional information is available at the end of the chapter

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Abstract

Essential oils can be used as antibacterial additives and are generally recognized as safe. *Coleus zeylanicus* is one of the medicinal aromatic plant serves as a source of essential oils. Antimicrobial and antioxidant activities of essential oils obtained from the control and salinity stressed *Coleus zeylanicus* plant was investigated in the present study. Essential oils from the control and salinity stressed *Coleus zeylanicus* plant was extracted using Clevenger apparatus. The composition of essential oils was identified using gas chromatography mass spectrometry, which showed a few compounds expressed differentially. The antibacterial activity of the isolated essential oils was studied by using the agar well diffusion method, showing potent inhibitory activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The antioxidant and antimicrobial constituents of the essential oils were spotted using the bioautography method, revealing that the antioxidant and antimicrobial properties in the essential oils of *Coleus zeylanicus* were increased upon exposure to salinity stress.

Keywords: agar well diffusion method, salinity stress, essential oils, bioautography method, thin layer chromatography

1. Introduction

In the recent years, the increasing resistance and the wide spread of multi-drug resistant microbes are leading to a serious health problem to the human population. The emergence of resistant microbes is due to the indiscriminate use of antibiotics [1]. Hence there is a need

to identify new drugs with effective antimicrobial and antioxidant properties to overcome this problem and to replace the usage of synthetic drugs responsible for the cause of side effects in patients. The resistance to antibiotics can be reduced by the use of resistance inhibitors isolated from plants. Salt stress is a vital abiotic stress factor that affects the growth and productivity of plants. In general, salinity refers to the presence of different salts like sodium chloride, calcium sulfate, magnesium, and bicarbonates in water and soil [2]. The uptake of water and absorption of essential nutrients by plants are restricted due to the presence of soluble salts exerting high osmotic pressure which ultimately affects the growth of plants [3]. Plants have adopted a mechanism to tolerate salt stress by the accumulation of solutes such as glycine, betaine, proline, sugar alcohols, polyols, and soluble sugars and by eliminating the toxic Na^+ ions in the cytoplasm [4]. Plant bioactive compounds are low molecular weight secondary metabolites distributed largely in plants that play a major role in the adaptation of plants to different environmental changes and in overcoming stress constraints. Medicinal plants exhibit pharmacological properties as they are known to possess various bioactive compounds called secondary metabolites like tannins, terpenes, alkaloids, steroids, flavonoids, glycosides, saponins, etc. These compounds play a major role in protecting the plants from various stress factors. Secondary metabolites that act as active components exhibit a wide range of antimicrobial activity [5]. The target sites shown by plant extracts are active against drug resistant microbes than those used by the antibiotics. It was discovered that the non-antibiotic substances such as essential oils have shown good fighting potential against drug resistant microbes. Essential oils change the rate of an enzyme reaction by interfering with the metabolism of microbes; thereby influencing the uptake of nutrients from the medium and affect the synthesis of enzymes or by changing the membrane structures that leads to the death of microbes. Thus, with the discovery of many natural products from plant species due to advancements in science and technology made a remarkable progress in the field of medicine. Now-a-days, many researchers are interested in isolating the biologically active compounds from plant species for developing the novel drugs in order to combat the microbes responsible for dreadful diseases [6].

Essential oils are a heterogeneous group of complex mixture of organic compounds synthesized within plants as secondary metabolites with characteristic flavor and odor. The quantity and quality of oil vary depending on the ecological and growth conditions of plant chosen for extraction. The various other factors also influence the yield of essential oils. The different parts of the plant such as leaves, root, stem, seeds, bark, woods, twigs, buds, fruits, and flowers can be used for the extraction of aromatic oily liquids called essential oils [7]. Essential oils are a mixture of compounds principally terpenoids like (C_{10}) monoterpenes, (C_{15}) sesquiterpenes, and (C_{20}) diterpenes, also contains lactones or acyclic esters, low molecular weight aliphatic hydrocarbons, aldehydes, alcohols, acids and rarely may contain coumarins, nitrogen-, and sulfur-containing compounds and homologs of phenylpropanoids [8]. Essential oils are produced commercially by the method of steam distillation; whereas the other methods of extraction, fermentation, and expression can also be performed to obtain oils [9]. Essential oils possess antibacterial, antifungal, antiviral, anticancer, antioxidant, and insecticidal properties [10]. Essential oils from medicinal plants possess antioxidant activity, which plays an important

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role in neutralizing free radicals benefiting human health. Essential oils also showed a potent inhibitory effect against Gram-positive bacteria, Gram-negative bacteria, filamentous fungi, and yeast. The highest inhibitory activity of *C. zeylanicus* oil against a wide spectrum of bacteria and fungi was reported [11].

Coleus zeylanicus is a perennial aromatic herb belonging to the family of *Lamiaceae*. It has astringent and stomachic properties used in the treatment of fever, common cold, asthma, dysentery, diarrhea, vomiting, burning sensation, small pox, eye diseases, worm diseases, chronic ulcers, dental diseases, and thirst [12]. The juice obtained from the stem and leaves of *C. zeylanicus* are used to treat diarrhea when taken along with honey [13]. It acts as diuretic, diaphoretic, and cholagogue which are useful for chronic and acute congestion of the liver. *C. zeylanicus* is also used to develop potential biodegradable micro-biocides [14]. The medicinal properties of this plant were moderately understood, but not much work was done on the properties of the essential oils isolated from the leaves exposed to salinity stress. In the present study, essential oils were isolated from the control and salinity stressed leaves of *Coleus zeylanicus* and their compositions were determined using gas chromatography mass spectrometry. Antibacterial and antioxidant properties of these essential oils were studied.

2. Material and methods

2.1. Plant material and salt stress treatment

Coleus zeylanicus plants (Herbarium specimen no. 21904) were propagated in the GITAM University botanical garden in 12 inch pots under 720 min natural photoperiod [irradiance (400–700 nm) of 1600–1800 μmol m^{-2} s^{-1}] with day/night temperatures of 30/23°C with an approximate air humidity of 60%. The pots were arranged in rows 1 m apart and the plants were irrigated daily and fertilized weekly with Hoagland solution. Three month old plants with uniform growth were selected for this study. Plants were then separated into two groups. Control plants were watered daily and salt stressed plants were treated with 250 ml of 300 mM NaCl solution twice a day for a period of 1 week. The plant materials were dried separately in shade and powdered, which were used for the extraction of essential oils.

2.2. Extraction of essential oil

The essential oils were extracted using Clevenger apparatus. Distillation is carried out for a period of 4 h by immersing the dried leaf powder directly into a round bottom flask filled with water. The contents were boiled and the vapors were condensed, allowing the essential oils to separate based on their difference in immiscibility and density. After extraction, the organic phase was separated and dried over Na_2SO_4. The percentage of the oils was calculated using the following formula:

Oil (% v/w) = observed volume of oil (ml)/weight of sample (g) × 100.

2.3. Identification of compounds using GC-MS

The identification of the major chemical composition of the isolated essential oil was performed by GC-MS (Sathyabama University, Chennai). The extracted solution containing essential oil was separated using diethyl ether and the injected sample volume was 1.0 μl for control and 0.5 μl for the salt stress sample as it was more concentrated. A Shimadzu GC-MS QP2010, a polyethylene glycol (Carbowax), and model Rtx-Wax (RESTEC) (30 m to 0.25 mm i.d., film thickness 0.25 lm) capillary column were used for the analysis. The temperature was first held at 40°C and then raised to 250°C (10 min, 2°C/min). The carrier gas was helium at a flow rate of 3 ml min^{-1}. The components of the oil were identified based on the comparison of their retention indices and mass spectra with the fragmentation patterns for computer matching with the NIST (National Institute of Standards and Technology) library.

2.4. Antimicrobial studies of the isolated essential oils

2.4.1. Microorganisms used

Three bacterial strains, *Escherichia coli* (MTCC 1652), *Staphylococcus aureus* (MTCC 3160), and *Pseudomonas aeruginosa* (MTCC 1688), were used in the present study obtained from Microbial Type Culture Collection Centre, Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.4.2. Preparation of inoculum

The colonies of test organisms were inoculated into 0.85% normal saline and the turbidity adjusted to 0.5 McFarland using the standard, which is equal to 1.5×10^8 CFU/ml.

2.4.3. Antibacterial activity by the agar well diffusion method

The antibacterial activity of the essential oils isolated from *Coleus zeylanicus* control and salinity stressed plants was analyzed by an agar well diffusion method. The essential oil was tested against the selected bacterial strains *E. coli*, *S. aureus*, and *P. aeruginosa*. Sterile Muller Hinton agar medium was poured into each Petri dish and allowed to solidify. After solidification, culture was spread over the plate by a spread plate technique using sterile cotton swab. Wells of 5 mm size were made in the agar plates with the help of sterile cork borer; the wells were then loaded with 200 μl of sample (essential oil dissolved in solvent), solvent alone as negative control and antibiotic as positive control. All the plates were incubated at 37°C for 24–48 h. After incubation, the plates were observed for the zone of inhibition around the well [15]. The zone of inhibition was calculated by measuring the diameters of the inhibition zone around the well.

2.5. Identification and separation of compounds using TLC

The essential oils obtained by the method of steam distillation are subjected to thin layer chromatography to identify and separate the bioactive compounds present in both the samples of

control and salt stress. Both the control and salt stress samples were applied to the TLC plate separately. The solvent system used in the TLC analysis was toluene:ethyl acetate in the ratio of 93:7. TLC was carried out using TLC silica gel 60 F_{254} aluminum sheets (Merck). After complete elution, the spots were identified and R_f values were calculated for each spot.

2.6. Screening of antioxidant activity

Several TLC techniques have been developed successfully for the analysis of antioxidants both quantitatively and qualitatively. The use of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical reagent for the analysis of antioxidant activity by the TLC method is one among them. TLC bioautography assay for screening of antioxidants possess several advantages that include high throughput, simplicity, and flexibility. In the present study, the antioxidant activity of essential oils is evaluated using the method of TLC bioassay. The components of essential oils were separated on the TLC plate and sprayed with DPPH solution. Antioxidant reduces the radical and produces creamy spots against a purple background.

2.7. Screening of antimicrobial activity

To screen the antibacterial activity of essential oils from control and salt stress, the method of direct bioautography is performed. Initially, the components were separated on the TLC plate and allowed to air dry. The test organisms were allowed to grow on the TLC plate by dipping the plate into the respective medium containing the organism, followed by incubation at 37°C for 24 h. After incubation, the plates were sprayed with 2 mg/ml solution of p-iodonitrotetrazolium violet dye. A clear zone indicated the inhibition of growth of the organism.

2.8. Statistical analysis

Results mentioned are reported as the mean ± standard error (SE) values of five independent experiments, conducted on five different plants in each experiment. SE values were calculated directly from the data according to standard methods. Data analyses were carried out using the SPSS package. Mean values were compared by Duncan's multiple range test and P-values which are less or equal to 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Essential oil analysis

The essential oils obtained by the hydrodistillation of *Coleus zeylanicus* leaf powder under control as well as salinity stress conditions was 1% (v/w) and 0.93% (v/w), respectively, based on the gram dry weight of the leaf powder. Analysis of the essential oil was carried out using GC-MS, which identified a total of 14 compounds in the essential oil isolated from the control *Coleus zeylanicus* plants and 7 compounds from the oil isolated from the *Coleus zeylanicus* leaves exposed to 300 mM salinity stress (**Figures 1** and **2**). Majority of the compounds present

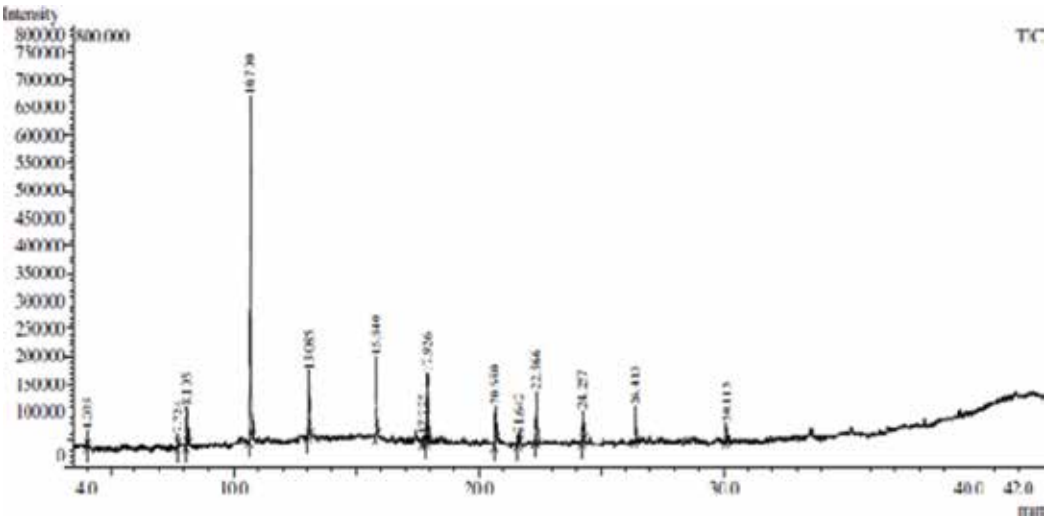

Figure 1. GC-MS result of essential oil obtained from *coleus zeylanicus* control.

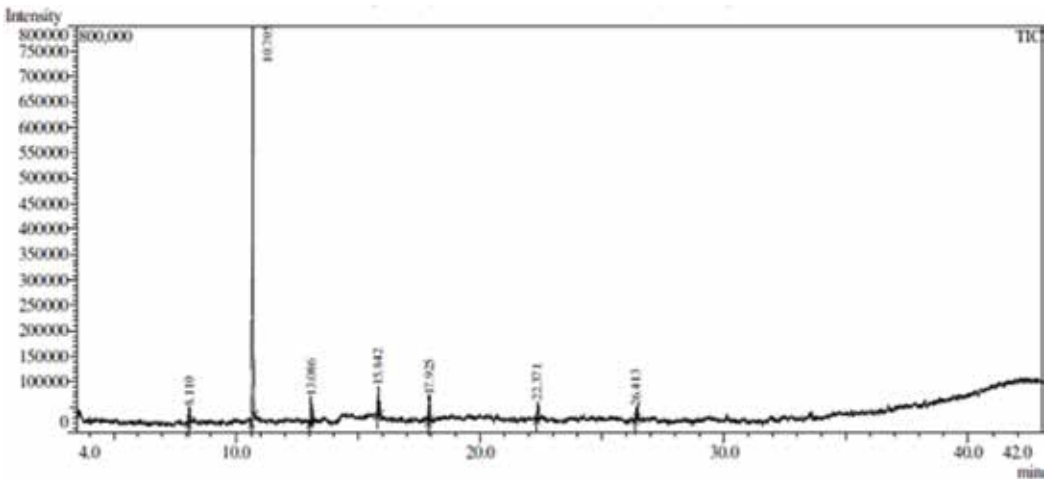

Figure 2. GC-MS result of essential oil obtained from *coleus zeylanicus* salinity stress plant.

in the essential oil of *Coleus zeylanicus* under control conditions was not identified in the essential oil extracted from the leaves exposed to the salinity stress, which made the essential oil composition of the salinity stressed *Coleus zeylanicus* different from that of *Coleus zeylanicus* plant growing under control conditions (**Tables 1** and **2**). Only compounds that were identified to be common in both the essential oils were Z-5-nonadecene and 2,4-di-tert-butylphenol. Thymol and carvone are found to be present in higher percentages of control and salinity stressed essential oils of *Coleus zeylanicus*. These two terpenoids were the major components of the essential oil extracted from *Lippia* species showing antiprotozoan properties [16]. The percentage of Z-5-nonadecene and 1-nonadecene components was decreased in the salinity stress treated essential oil of *Coleus*

zeylanicus. The difference in the composition of essential oils might be due to salinity stress [17]. Long chain fatty acids 1-heptadecene and 1-nonadecene isolated from the root ethanol extracts of *S. longepedunculata* possess antifungal and antibiotic properties [18, 19]. In the present study, the essential oils extracted from the *Coleus zeylanicus* leaves during control and salinity stress had a different chemical composition, which were tested for their antimicrobial and antioxidant properties.

3.2. Antimicrobial activity

The antibacterial activities of essential oils extracted from the control and salinity stressed *Coleus zeylanicus* leaves were tested against *E. coli*, *S. aureus* and *P. aeruginosa*, which have shown moderately high inhibition (**Table 3**). The antimicrobial susceptibility testing was done using the disc diffusion method. Essential oils showed effective inhibitory activity against *E. coli* than that of *S. aureus* and *P. aeruginosa* with zone of inhibition increasing from 12 to 16 mm, respectively. The essential oil of *C. furcata* showed inhibition zones of 14 and 13 mm against the Gram-negative strains of *E. coli* and *P. aeruginosa*; 14 mm against the Gram-positive strain *S. aureus* [20]. The oxygenated monoterpenes exhibit strong antimicrobial activity than hydrocarbon derivatives as they are less water soluble limiting the diffusion through medium. Essential oils containing hydroxyl group exhibits strong antimicrobial activity which might be due to the capability of binding easily to the enzyme at the active site and altering their metabolism. The combination of electrolyzed NaCl and essential oil with 0.5% carvacrol and 0.5% thymol reported to be effective in controlling microbial population and chemical deterioration

Peak No.	R. time	Area	Area (%)	Name of the compound
1	4.035	44,267	1.29	1-Decene
2	7.724	45,372	1.32	endo-Borneol
3	8.105	147,076	4.28	1-Dodecene
4	10.700	1,366,605	39.74	Thymol
5	13.085	261,154	7.59	9-Octadecene
6	15.840	353,293	10.27	2,4-Di-tert-butylphenol
7	17.725	69,757	2.03	(−)-Caryophyllene oxide
8	17.926	296,236	8.61	1-Heptadecene
9	20.680	180,412	5.25	Dibenzo[a,h]cyclotetradecene
10	21.642	48,642	1.41	6-Methyl-5-(1-methylethylidene)
11	22.366	235,008	6.83	Z-5-Nonadecene
12	24.257	143,316	4.17	(−)-Isolongifolol, methyl ether
13	26.413	159,508	4.64	Behenic alcohol
14	30.113	87,997	2.56	1-Heptacosanol

Table 1. Composition of the essential oil extracted from the *Coleus zeylanicus* control plant.

Peak No.	R. Time	Area	Area (%)	Name of the compound
1	8.110	55,347	2.14	3-Tetradecene
2	10.705	2,000,267	77.23	Carvone
3	13.086	129,091	4.98	1-Pentadecene
4	15.842	147,692	5.70	2,4-Di-tert-butylphenol
5	17.925	119,179	4.60	E-14-Hexadecenal
6	22.371	82,567	3.19	Z-5-Nonadecene
7	26.413	55,879	2.16	1-Nonadecene

Table 2. Composition of the essential oil extracted from the salinity stressed *Coleus zeylanicus* plant.

Strain name	*C. zeylanicus* control (mm)	*C. zeylanicus* salt stress (mm)	Positive control (ampicillin) (mm)	Negative control (solvent) (mm)
S. aureus	17 ± 2.87	17 ± 2.82	32 ± 2.65	No zone
E. coli	12 ± 2.10	16 ± 3.12	No zone	No zone
P. aeruginosa	17 ± 2.75	17 ± 1.80	No zone	No zone

Table 3. Antimicrobial activity of both the essential oils by agar well diffusion method.

[21]. Essential oils act indirectly on membranes by secreting toxins which play an important role in controlling the microbial population of *S. aureus* and *B. cereus*. These essential oils can also be used in combination with other antibacterial agents to enhance their activity. Addition of lysozyme enhanced the synergistic activity between carvone and nisin [22]. The antimicrobial activity of essential oils depends on the chemical composition and the amount of single compound present. These compounds occur in the active form in plants or can be activated by specific enzymes when subjected to biotic or abiotic stress. Mechanism of antimicrobial activity of the essential oils includes the damage of the cytoplasmic membrane, degradation of cell wall, decreased ATP synthesis, membrane protein damage, reducing the proton motive force, and increasing the membrane permeability by reducing the membrane potential [23]. In the present study, the antibacterial activity of the essential oil of *C. zeylanicus* under both the conditions of control and salinity stress was effective against the tested strains. It was also observed that the activity was enhanced when the plant is subjected to salinity stress.

Majority of the essential oils has shown effective inhibitory activity against Gram-positive strains [24], might be due to the presence of hydrophilic outer membrane, which prevents the entry of hydrophobic compounds into the target cell membrane, thereby acquiring resistance to the antimicrobial drugs [25]. Another possible reason could be the inhibition of microbial respiration and increased membrane permeability by essential oils resulting in the death of microbes after massive ion leakage [26, 27]. Therapy with traditional herbs is practiced with the plant species containing medicinal properties. Secondary metabolites such as terpenoids, tannins, alkaloids, phenols, and flavonoids rich in plants are found to be responsible for

antimicrobial properties *in vitro*. Plant phytochemicals serve in defense mechanisms against predation by herbivores, insects and microorganisms. Quinones and tannins were responsible for plant pigment, whereas terpenoids responsible for plant odor and flavor. Highly oxygenated phenols were found to be highly toxic to microorganisms. The search for new compounds with antimicrobial property derived from natural plant sources has gained much attention to replace synthetic drugs. The growth of microbes can be controlled with the use of phytochemicals derived from plant source which are more effective and less toxic [28, 29].

3.3. Screening of antioxidant and antimicrobial activity by the TLC bioautography method

Bioautography technique was employed to detect the antimicrobial and antioxidant activity [30]. In our study, the two essential oils spotted on the TLC plate were separated into distinct bands with different R_f values. Essential oil from control and salt stressed *C. zeylanicus* have characterized by the presence of three antimicrobial compounds with the R_f values of 0.34, 0.54 and 0.72. The developed TLC plates were used separately for determining the presence of antimicrobial and antioxidant compounds present in the essential oils of *Coleus zeylanicus*. The antioxidant activity of the compounds present in the essential oil was also determined by developing the TLC plate with 20 μg/ml of DPPH solution after the separation of compounds (**Figure 3**). Similar results were reported with the essential oil of *Eucalyptus lanceolatus* [31]. By using the agar overlay bioautography method, several antimicrobial compounds were identified and isolated from the husk, cotyledons and tubers of *Tylosema esculentum* [32].

Figure 3. The antioxidant activity of the compounds present in the essential oil determined by developing the TLC plate with 20 $\mu g/ml^{-1}$ of DPPH. Lane 1: essential oil isolated from control leaves of *C. zeylanicus*. Lane 2: essential oil isolated from salinity stressed leaves of *C. zeylanicus*.

In this method of TLC bioautography, a developed TLC plate is dipped into the respective broth containing microbes of pure culture and incubated under humid conditions. The microbes grow directly on the TLC plates except in the regions where the bioactive compounds exhibit the antimicrobial property. The zones of inhibition with creamy spots against purple background are visualized after spraying the plates with INT (p-iodonitrotetrazolium violet) dye. The tetrazolium salts are converted into a purple colored compound called formazan by the dehydrogenase activity of living microbes. Once the activity is located on the TLC plate, the samples can be analyzed by GC-MS to identify the presence of known or unknown compounds responsible for the activity [33]. This method is considered to be convenient for obtaining the reliable information on the activity of single compounds as the plant extracts possess numerous bioactive compounds. The analytical determination of compounds present in plant extracts and the characterization of their biological properties are made possible with the optimized antimicrobial assays. Separation of compounds in plant extracts is necessary to avoid study on fractions with no biological activity. Detection of antimicrobial compounds by this method is rapid, uncomplicated and effective in saving money and time [34]. Apart from the search of bioactive compounds, this method is also used to find out best solvent for the extraction of compounds and for the selection of mobile phase to separate compounds. It was reported that the thymol and carvacrol were responsible for the antimicrobial property present in the essential oils of *T. vulgaris* L. using the dot blot test [35].

The TLC bioautography method also used to detect the compounds exhibiting antioxidant activity. The developed TLC plate sprayed with DPPH (2,2-diphenyl-1-picrylhydrazyl radical) solution produces clear creamy yellow spots against a purple background. The DPPH decreases upon the reduction reaction with a radical scavenger leading to the color change which can be observed in a TLC bioassay. The reaction has been depicted in **Figure 3**. The assay depends on the measurement of antioxidants scavenging activity, where the DPPH is characterized as a stable free radical. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants present in the plant extracts to the corresponding hydrazine. Rosmarinic acid, luteolin, chrysoeriol and apigenin were the four different antioxidant compounds isolated from the fruit of *Perilla frutescens var. acuta* by a TLC bioautography method using DPPH as a detection reagent [36].

4. Conclusion

The results of this study showed that the essential oils of *C. zeylanicus* showed good antibacterial activity against the tested pathogenic strains. The essential oils containing compounds with antimicrobial and antioxidant activity were identified by the method of bioautography technique. Chemical profiling by GC-MS showed thymol and carvone as major components in control and salt stressed essential oil. The present study mainly focused on to observe the change in the level of bioactive compounds in the essential oils of *coleus zeylanicus* when the plant is subjected to salinity stress for determining its commercial value. Our study concludes that the antimicrobial and antioxidant activity remained to be effective even under stress conditions.

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Chapter 9

Vascular Calcification and Oxidative DNA Damage as Nontraditional Cardiovascular Risk Factors in Chronic Renal Disease

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Abstract

The number of CKD sufferers that require renal replacement techniques (RRTs) is increasing. The severity of cardiovascular disease (CVD) is disproportionate in these kinds of patients and contributes considerably to mortality in CKD patients. We evaluated the association between oxidative DNA damage, antioxidant activity and vascular calcification (VC) in CKD. An analytical cross-sectional study was performed. Two simple plaques were taken for each patient (pelvis-hip, and hands-wrists). The presence of VC was scored as presence (1) and absence (0). Oxidative stress was determined by activity of catalase, superoxide dismutase (SOD) and oxidative DNA damage by determination of 8-OHdG marker. Eighty-one patients were included. The RRT type was similar for hemodialysis (HD) and peritoneal dialysis (PD). Thirty-eight patients (47%) presented VC ($p < 0.01$); in 61%, the VC was severe (≥3 points). VC prevalence in women was significantly higher, (67%) ($p < 0.001$), and (29%) men. Sixty four percent of the patients submitted to HD presented VC and 27% to PD ($p < 0.001$). The activity of the catalase enzyme was significantly decreased in CKD vs. the healthy control (HC) ($p < 0.0001$). The oxidative DNA damage in CKD was greater vs. HC ($p < 0.0001$). In conclusion, the VC was frequent (47%) in CKD, and decreased catalase activity and greater oxidative DNA damage.

Keywords: end-stage renal disease, vascular calcification, oxidative stress, antioxidants, oxidative DNA damage

1. Introduction

1.1. Chronic kidney disease

Diabetes mellitus (DM) and systemic arterial hypertension are the predominant risk factors for chronic kidney disease (CKD). CKD has become a public health problem. In recent years there has been a progressive increase in the incidence and prevalence of CKD, as well as the number of sufferers who reach the most advanced stage and require renal replacement therapy (RRT); all of which has led to CKD being considered a real epidemic [1]. CKD has the capacity to produce cardiovascular disease (CVD), increasing the risk of hospitalization, morbidity, and mortality [2]. Renal patients are extremely vulnerable to cardiovascular pathology. The mortality is 10–30 times greater in patients with end-stage renal disease (ESRD) compared to the general population. Despite the prevalence of the traditional risk factors being very high, the broadness and severity of cardiovascular complications are clearly disproportionate to the risk profile that these patients have [3]. CVD contributes considerably to the mortality of patients with CKD, including patients with renal transplant (RT) [4]. Therefore, in the past two decades, the interest in nontraditional or emerging risk factors has increased in mineral bone disorders (MBD), inflammation, malnutrition, and oxidative stress. There is evidence that MBD in CKD plays an important role in the increase of cardiovascular morbidity and mortality by favoring the development of vascular calcifications (VC) [5]. MBD increases the risk of CKD-associated CVD with the onset of hyperphosphatemia, VC, and increased levels of fibroblast growth factor 23 [6–8]. When renal function is impaired, it increases the potential for aggravating traditional risk factors (hypertension, dyslipidemia, inflammation, and oxidative stress). The concomitant deterioration of mineral homeostasis and bone metabolism is probably the key factor leading to accelerated CVD [9]. In CKD, MBD alterations occur from the early stages of the disease (stage 2) [10]. MBD alterations favor vascular rigidity by increasing systolic blood pressure, pulse wave velocity, and left ventricular mass in patients with CKD [11]. The structural and functional abnormalities that occur in the vasculature by early CKD cause endothelial dysfunction progressing to VC.

The majority of the vascular pathologies that are present in patients with CKD are secondary to the development of VC. They are characterized as being a multifactorial pathological process with an increase in calcium phosphate deposits in a form of hydroxyapatite. The aforementioned is a result of the imbalance in mineral metabolism that kidney patients have [12]. VC is a common phenomenon in the aging of the general population; however, in CKD, the onset is rapidly accelerated, promoting the development of left ventricular hypertrophy, increasing cardiovascular risk with increased cardiac mortality in patients with CKD [13]. VC can present in the tunica media or in the tunica intima (atherosclerosis or sclerosis of Mönckeberg) of the arterial vessels. VC causes thickening of the intima-media with the formation of atherosclerotic plaques and lesions of the tunica intima, producing local inflammation, dyslipidemia, and accumulation of foam cells [14]. Atherosclerosis is restricted to medium or large arteries and is related to other risk factors such as smoking, obesity, dyslipidemia, hypertension, and aging. Calcification of the tunica media can occur in arteries of any size, is a typical process related to age, and is associated with greater arterial stiffness and reduction of

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the damping function [15]. Although initial events leading to calcification of the tunica media or the tunica intima differ, both calcifications reflect an active and highly regulated process that closely resembles endochondral and/or intramembranous bone formation [16].

The oxidative stress is characterized by imbalance between the productions of reactive oxygen species (ROS) that surpasses the capacities of the antioxidants. CKD has been shown to be a prooxidant disease [17]. Atherosclerosis, CVD, and CKD are associated with oxidative stress, inflammation, and reduced availability of nitric oxide (NO). Oxidative stress and inflammation are considered as nontraditional risk factors [18]. The oxidizing compounds have physiological defense mechanisms in the organism, but when there is an imbalance in the generation of the oxidants, it results in damage to the tissues. Oxidative stress has the ability to induce endothelial dysfunction by promoting the progression of atherosclerosis and reducing the availability of NO [19]. There is growing evidence in the general population that indicates correlation between oxidative stress and VC development [20]. Macrophages, endothelial cells, and smooth muscle cells produce ROS (hydrogen peroxide (H_2O_2) and superoxide anion (O^{-2})) in response to diverse stimuli. The free radicals of (NO) are generated in the vascular endothelium from L-arginine by the NO synthase enzyme with the capacity to produce hydroxyl or peroxyl radicals [21].

The most important cellular targets of ROS are DNA damage characterized by rupture of DNA strands, point mutations, and compromise of telomere integrity [22]. Shortening of telomeres and mitochondrial DNA damage is related to the rapid onset of atherosclerosis [23]. Increased oxidative stress is a prominent factor in the pathogenesis of VC, while some evidence suggests that increased DNA damage affects contractility of the vascular tunica media [24]. The ROS are capable of damaging macromolecules and DNA bases (purine, adenine, guanine, cytosine, and thymine). Other DNA bases may also be oxidized in a similar manner by the hydroxyl radical. The important consequences of oxidative damage to DNA bases are produced by mutations by the AT↔GC transition and by GC↔TA transversion. These mutations, if not repaired, can lead to changes in the gene expression of proteins [25]. Oxidative DNA damage can be measured by the 8-hydroxy-2′-deoxyguanosine (8-OHdG) marker. Antioxidants play an important role in the proper balance of oxidative stress. Monitoring the levels of antioxidants and oxidative DNA damage products in patients with CKD and the interpretation of the relationship between these markers could contribute better management of patients with CKD [26]. For the above, we set out to evaluate the association between oxidative DNA damage, antioxidant activity, and VC in patients with CKD.

2. Materials and methods

An analytical cross-sectional study was performed. Patient population > 16 years of age who were undergoing RRT and meet the selection criteria while being attended to at the Division of Nephrology-Transplants of the Subspecialty Medical Unit at the Specialties Hospital of the National Occidental Medical Centre, Mexican Social Security Institute (*División de Nefrología-Trasplantes de la Unidad Médica de Alta Especialidad (UMAE) del Hospital de Especialidades, CMNO, del Instituto Mexicano del Seguro Social (IMSS)*), in Guadalajara, Jalisco, Mexico. Patient

demographics, biochemical date, and time of RRT were recorded. Results were determined: hemoglobin (Hb), hematocrit (Hto), sodium (Na), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg), chlorine (Cl), alkaline phosphatase, intact parato-hormone (iPTH), creatinine (Cr), urea, albumin, vitamin D3, and specific C-reactive protein (CRP). VC was determined using simple x-ray plaques according to the method of Dr. Adragao [27]. Two simple plaques were taken for each patient (one of the pelvis and hip and one of hands and wrists); the presence or absence of VC was evaluated in the anatomical territories of the iliac-femoral and radial-digital arteries. VC was scored as 1 (presence) and 0 (absence). The highest score to obtain could be 8 and minimum 0. All plaques were checked by the same radiologist blinded to the clinical characteristics of the patients.

Oxidative stress was determined by the concentrations of antioxidants, and catalase and superoxide dismutase (SOD) and the oxidative DNA damage by determination of the 8-OHdG marker (ELISA technique). Because there were no normal parameters for the reagents, 10 mL extra blood from 20 healthy volunteers (healthy control group) (blood donors) with similar age and gender was used to determine the normal concentrations of the reagents.

2.1. Superoxide dismutase

The kit manufacturer's instructions were followed (SOD No. 706002, Cayman Chemical Company®, USA). The detection of O_2^- generated by the xanthine oxidase and hypoxanthine enzymes was through the reaction of tetrazolium salts. The serum samples were diluted in sample buffer 1:5 in sample buffer: 200 μL of the radicals' detector, diluted 1:400, was placed, and 10 μL of the sample was then added. After slow agitation, 20 μL of xanthine oxidase was added to the wells. The microplate was incubated for 20 min at room temperature, and the absorbency was read at a wavelength of 440 nm.

2.2. Catalase-520

The determination of the antioxidant activity of catalase was performed according to the manufacturer's commercial kit (Bioxytech® Catalase-520™, USA). The spectrophotometric assay was performed by adding 30 μL of the diluted standards or samples into the corresponding tubes. Five hundred microliter of the substrate (10 mM H_2O_2) was added to each tube and then incubated for 1 min at room temperature, and 500 μL of the stop reagent was added to each tube. The tubes were capped and mixed by inversion, and 20 μL of the mixture was added. Two milliliter of the HRP chromogenic reagent was added to each tube, mixed inversely, and incubated for 10 min at room temperature. The absorbance was obtained at 520 nm of optical density.

2.3. 8-Hydroxy-2′-deoxyguanosine

Instructions for the ELISA kit were followed (8-hydroxy-2-deoxyguanosine No. ab10124 Abcam®, Cambridge, United Kingdom). The plasma sample, the EIA buffer, the standards, and the 8-OHdG-AChE tracer were added to all the wells except the blank. The monoclonal antibody 8-OHdG was added, and the plate was incubated for 18 h at 4°C and washed with buffer for the recommended times, and 200 μL of Ellman's reagent was added to each well. The optical density was read at 405 nm.

2.4. Statistical analysis

The data were analyzed using SPSS software (Statistical Package for the Social Sciences, v. 20, SPSS Inc., Chicago, IL). The Kolmogorov–Smirnov test was used to determine the distribution of the study variables. The quantitative variables are expressed in mean ± standard deviation/error or median (percentile 25–75%) and Spearman's correlation test. The qualitative variables are expressed in frequencies and percentages and were analyzed with Chi^2 or Fisher's exact test for the intragroup analysis. A value of $p \leq 0.05$ was considered statistically significant, with a confidence interval of 95%.

2.5. Ethical considerations

The study was carried out according to the General Law of Health of Mexico in the Field of Research for Health. The study was classified as category III, that is to say, research with a risk greater than the minimum, for which it was necessary to sign the consent under information. Confidentiality of data and patient outcomes was respected. The project was evaluated and accepted by the Local Committee of Research and Ethics in Health of the UMAE, HE, CMNO, Guadalajara, Jalisco, with folio R-2016-1301-97.

3. Results

We included 81 patients with CKD candidates for TR who had previously received dialysis. The patients were young adults, barely older than 30 years of age. The male–female ratio was 1:1. The RRT type was similar for hemodialysis (HD) and peritoneal dialysis (PD). Seventy-five of patients had at least 1.5 years on dialysis. In general, patients had regular control of azoles, serum electrolytes, lipid profile, and albumin. PCR and iPTH were elevated without clinical evidence of infection. Vitamin D3 levels were found to be decreased (**Table 1**).

3.1. Vascular calcification

Of the 81 patients evaluated, 38 (47%) had VC, and in 61% of them VC was severe (≥3 points). Women had a significantly higher prevalence of VC: 39 women (67%) had VC, while 42 (29%) of male had VC, although age, dialysis time, lipid profile, urea, creatinine, and albumin were similar in patients with and without VC. iPTH, P, and alkaline phosphatase were significantly higher in patients with VC. Vitamin D3 and calcium levels showed no significant difference (**Table 2**). Patients were compared according to the type of RRT to which they were subjected since the onset of CKD. Sixty-four percent of patients submitted to HD presented VC, and 27% of those submitted to PD. The HD patients were older and were predominantly women, had better control of azoles, and had higher levels of vitamin D3.

3.2. The activity of the antioxidant enzyme

Catalase was found to be significantly decreased ($p < 0.0001$) in CKD. The SOD activity was not found to be decreased in CKD ($p = 0.58$). Regarding the type of RRT and oxidative

	n-81
Age (years)	30.6 ± 9.7
Gender (%)	
Female	39 (48)
Male	42 (52)
Time of dialysis (months)	19 (15–30)
Type of dialysis (n (%))	
Hemodialysis	44 (54)
Peritoneal dialysis	37 (46)
Urea (mg/dL)	121 ± 45
Creatinine (mg/dL)	13 ± 4.1
Phosphorus (mg/dL)	3.5 ± 2.2
Calcium (mg/dL)	9.1 ± 1.1
Sodium (mg/dL)	139 ± 4.0
Total cholesterol (mg/dL)	160 ± 39
Triglycerides (mg/dL)	149 ± 86
LDL (mg/dL)	85 ± 35
Albumin (g/dL)	3.5 ± 0.7
Alkaline phosphatase (U/I)	125 (91–240)
iPTH (pg/mL)	557 (173–911)
Vitamin D3 (ng/mL)	21.1 (16.2–27.4)
PCR (mg/mL)	3.5 (3.0–9.6)

Mean ± SD, median (percentile 25–75%).

Table 1. Demographic and biochemical characteristics of the patients.

stress, we evidenced significantly higher levels of the marker of oxidative damage to DNA (8-OHdG) in patients submitted to PD and more consumed SOD activity ($p < 0.0001$). Serum catalase concentrations were similar between HD and DP (**Table 3**). However, when comparing the results between the antioxidants and the enzymes, we found to decrease significantly the activity of the catalase in CKD vs. the healthy controls ($p < 0.0001$). The activity of SOD was similar between CKD and healthy controls. We found greater oxidative damage to DNA in CKD vs. healthy controls ($p < 0.0001$) (**Table 4**).

To determine the association between oxidative stress data with the MBD results, we used the Spearman correlation test. Catalase showed a negative trend with alkaline phosphatase levels. SOD correlated negatively with the marker of oxidative damage to DNA (8-OHdG),

	CV n-38	No CV n-43	*p*
Age (years)	29.9 ± 9.2	31.3 ± 10.2	0.51
Gender Male (n (%))	12 (29)	30 (71)	
Female (n (%))	26 (67)	13 (33)	**0.001**
Time in dialysis (months)	18.5 (14.8–30)	22 (13–34)	0.76
BMI (Kg/m^2)	24.2 ± 3.7	23.5 ± 3.0	0.50
Urea (mg/dL)	124 ± 47	120 ± 45	0.73
Creatinine (mg/dL)	12.8 ± 4.2	13.2 ± 4.2	0.70
Albumin (g/dL)	3.7 ± 0.6	3.8 ± 0.7	0.79
PCR (mg/mL)	3.8 (3.0–9.8)	3.0 (3.0–13.9)	0.66
Total cholesterol (mg/dL)	160 ± 43	159 ± 35	0.95
Triglyceride (mg/dL)	160 ± 100	139 ± 69	0.29
LDL (mg/dL)	86 ± 36	85 ± 33	0.94
MBD in CKD			
Vitamin D3 (ng/mL)	21.0 (16.2–26.8)	21.4 (16.2–29.2)	0.91
iPTH (pg/mL)	675 (237–980)	428 (114–650)	**0.04**
Phosphorus (P) (mg/dL)	4.2 ± 2.3	2.7 ± 1.7	**0.001**
Calcium (mg/dL)	9.0 ± 1.0	9.2 ± 1.1	0.53
Alkaline phosphatase (UI/L)	141 (106–317)	100 (77–152)	**0.01**

BMI, body mass index; LDL, low-density lipoproteins; ALP, alkaline phosphatase; iPTH, intact parato-hormone (data shown as average ± SD or median (percentile 25–75%)).

Table 2. Demographic and biochemical data with and without CV.

and VC score showed a positive correlation with alkaline phosphatase and P. iPTH correlated positively marginally. 8-OHdG was negatively correlated with VC, alkaline phosphatase, and P (**Table 5**). The strength of association between VC and oxidative stress was performed through uni- and multivariate logistic regression analysis. Being female and being ≥30 years of age confer greater risk for VC (**Table 6**).

Demographic, biochemical, and gender-specific differences in MBD in CKD are shown in age (**Table 7**). Women had significantly higher VC frequency (67%) and were predominantly found in HD, although they had significantly lower levels of creatinine and higher concentrations of P compared to men. Age was similar in patients with and without VC. Significantly, RRT (HD) predominated in patients aged ≥30 years with less time in dialysis.

	HD	DP	*p*
	n-44	**n-37**	
Vascular calcification (n (%))	28 (64)	10 (27)	**0.001**
Age (years)	30.0 (25.0–37.0)	26.0 (23.3–30.8)	**0.05**
Gender (male) (n (%))	12 (27%)	30 (82%)	**<0.0001**
BMI (Kg/m^2)	22.3 (21.4–24.3)	23.6 (21.6–26.6)	0.29
Time in dialysis (months)	17.0 (6.8–23.5)	21.5 (15.3–33.0)	0.30
Urea (mg/dL)	108 (78–120)	120(102–157)	**0.03**
Creatinine (mg/dL)	11.0 (9.0–12.1)	13.9 (10.1–16.8)	**0.005**
Albumin (g/dL)	3.9 (3.4–4.2)	3.6 (3.0–4.1)	0.18
Total cholesterol (mg/dL)	149 (128–174)	160 (142–198)	0.07
Triglycerides (mg/dL)	130 (98–179)	134 (82–190)	0.84
LDL (mg/dL)	72 (57–87)	92 (73–120)	**0.002**
MBD and CKD			
iPTH (pg/mL)	593 (173–911)	509 (126–918)	0.66
Antioxidants			
Catalase (U/mg)	*13.5 (8.5–19.0)*	*18.7 (9.5–25.7)*	*0.19*
SOD (UI/mL)	7.9 (4.3–13.3)	3.3 (2.3–4.6)	**<0.0001**
Oxidative DNA damage			
8-OHdG (ng/mL)	4.4 (3.1–6.4)	9.4 (7.3–10.8)	**<0.0001**

MBD, metabolic body disease; iPTH, intact parato-hormone; SOD, superoxide dismutase; 8-OHdG, 8-hydroxi-2′-deoxiguanosina; mean ± SD or median (percentile 25–75%).

Table 3. Type of dialysis with and without vascular calcification.

	CKD	Healthy control	*p*
Antioxidants			
Catalase (U/mg)	17.7 ± 9.0	94.17 ± 1.58	***<0.0001***
SOD (UI/mL)	8.2 ± 7.7	10.2 ± 1.9	0.58
Oxidative DNA damage			
8-OHdG (ng/mL)	9.4 ± 11.1	4.7 ± 1.0	***<0.0001***

SOD, superoxide dismutase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; mean ± SD or median (percentile 25–75%).

Table 4. Oxidative stress in CKD vs. healthy controls.

	r	p
Catalase (U/mg)	−0.29	0.08
Alkaline phosphatase (mg/dL)		
SOD (UI/mL)	−0.40	**0.002**
8-OHdG (ng/mL)	−0.27	**0.03**
Score of VC	0.24	0.06
iPTH (pg/mL)	0.30	**0.02**
Alkaline phosphatase (mg/dL)	0.50	**<0.0001**
Phosphorus (P) (mg/dL)		
8-OHdG (ng/mL)	−0.52	**<0.0001**
Score of VC	−0.37	**0.003**
Alkaline phosphatase (mg/dL)	−0.55	**<0.001**
Phosphorus (P) (mg/dL)		

SOD, superoxide dismutase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; iPTH, intact parato-hormone.

Table 5. Correlation between MBD and CKD.

	OR	IC 95%	*p*
SOD (UI/mL)	1.08	0.99–1.20	0.09
Age (≥30 years)	1.1	1.02–1.20	**0.02**
Gender (female)	20.8	2.5–173.70	**0.00**

SOD, superoxide dismutase. Oxidative DNA damage markers: 8-OHdG, PCR, vitamin D3, iPTH, age, gender, phosphorus, LDL, albumin, alkaline phosphatase.

Table 6. Predictors of VC ($Chi^2 = 13.38$; $p = 0.004$).

	Male (n-42)	Female (n-39)	*p*
VC (n (%))	12 (29%)	26 (67%)	**0.001**
Age (years)	26.0 (23.5–31.5)	30.0 (25.0–38.0)	0.13
RRT (HD/DP (%))	12/30 (29/71)	32/7 (82/18)	**<0.0001**
Time in dialysis (months)	21.5 (15.3–30.0)	15.0 (4.5–67.5)	0.37
BMI (Kg/m^2)	22.9 (21.9–25.2)	23.6 (20.7–26.9)	0.96
Urea (mg/dL)	114 (96–144)	105 (79–165)	0.54
Creatinine (mg/dL)	13.4 (11.3–16.7)	9.7 (7.3–10.9)	**0.001**
Total cholesterol (mg/dL)	149 (129–184)	155 (131–183)	0.9
Triglycerides (mg/dL)	134 (81–194)	130 (98–179)	0.76

	Male (n-42)	Female (n-39)	*p*
LDL (mg/dL)	79 (62–112)	81 (68–88)	0.43
PCR (mg/mL)	3.1 (3.0–17.3)	3.9 (3.0–8.9)	0.94
Vitamin D3 (ng/mL)	21.2 (16.2–31.1)	21.0 (15.9–26.4)	0.51
Calcium (mg/dL)	9.2 (8.5–9.9)	8.9 (8.4–9.4)	0.33
Phosphorus (P) (mg/dL)	2.1 (1.6–2.5)	5.3 (3.2–6.5)	**<0.0001**
iPTH (pg/mL)	369 (137–615)	650 (203–950)	0.09
Alkaline phosphatase (U/I)	106 (78–138)	150 (95–329)	**0.02**
Demographic, biochemicals, and MBD in CKD by age data			
	<30 years (n-48)	**≥30 years (n-33)**	*p*
VC (n (%))	24 (50)	14 (42)	0.45
RRT (HD/DP (%))	21/27 (45/55)	23/10 (70/30)	**0.03**
Time in dialysis (months)	24 (17–32)	12 (2–15)	**0.02**
BMI (Kg/m^2)	23.2 (21.5–25.2)	22.6 (21.6–28.0)	0.44
Urea (mg/dL)	108 (93–144)	123 (94–144)	0.46
Creatinine (mg/dL)	13.4 (10.8–15.8)	12.0 (9.2–15.7)	0.15
Total cholesterol (mg/dL)	153 (129–187)	152 (133–182)	0.86
Triglycerides (mg/dL)	131 (98–187)	127 (88–176)	0.45
LDL (mg/dL)	81 (68–98)	74 (60–102)	0.52
PCR (mg/mL)	4.3 (3.0–13.6)	3.0 (3.0–7.2)	0.12
Vitamin D3 (ng/mL)	20.5 (15.7–28.0)	21.7 (16.4–27.2)	0.73
Calcium (mg/dL)	9.0 (8.4–9.8)	9.2 (8.6–9.8)	0.37
Phosphorus (P) (mg/dL)	2.3 (1.8–4.1)	3.7 (2.1–5.9)	0.06
iPTH (pg/mL)	557 (222–770)	526 (125–955)	0.64
Alkaline phosphatase (U/I)	117 (89–179)	145 (94–320)	*0.33*

VC, vascular calcification; RRT, renal replacement therapy; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCR, C-reactive protein; iPTH, intact parato-hormone.

Table 7. Demographic, biochemicals, MBD, and CKD data.

4. Discussion

VC appears as an independent cardiovascular risk factor of the state of hypercholesterolemia and atherosclerosis that present the patient [28]. In the general population, this previously described the protective effect of estrogens against VC [29]. The estrogen-related receptors (ERR) are closely related to estrogen receptors, sharing high homology in the DNA-binding domain, although they do not bind to estrogen [30]. The ERR subfamily consists of three members, ERRα,

ERRβ, and ERRγ (NR3B1–NR3B3), which bind to the classical estrogen response elements and to the extended middle site sequences (ERNA TNAAGGTCA; ERR) as monomers or dimers [31]. ERRα is strongly expressed throughout the differentiation of osteoblasts and regulates the expression of osteopontin through a noncanonical ERRα response element [32]. ERRγ is expressed in osteoblast progenitors and negatively regulates osteoblast differentiation induced by BMP2 and bone formation [33]. ERRγ plays a key role in VC through the positive regulation of the BMP2 signaling pathway, suggesting that inhibition of ERRγ could be a potential therapeutic strategy for VC prevention [34]. Previously, the influence of hormonal *status* in patients with ESRD prevalent in HD was evidenced by the association between follicle-stimulating hormone and MBD in CKD, particularly with bone mineral density. This association could also reflect alterations in the appearance of VC [35]. The majority of patients who suffer VC tend to be of advanced ages and have multiple metabolic, renal, and inflammatory complications. Although in recent years knowledge of the pathophysiology of arteriosclerosis and its close relationship to the mechanisms of oxidative stress has increased considerably, there is still little known on the influence these inflammatory processes have on the development of VC, but it is thought that there is a close link between them [36].

The secondary hyperparathyroidism, hyperphosphatemia, hypercalcemia, and other inherent factors of ESRD, like the state of uremia, inflammation, and the oxidative stress, all of them play a relevant role in the pathophysiology of these vascular alterations. Patients with ESRD present with a chronic inflammatory state that is associated with VC, increased morbidity, and cardiovascular mortality. As well as promoting simultaneous changes in mineral metabolism, the inflammation favors vascular damage that in the long term could lead to the development of VC [37].

Over time, VC has been considered a passive or degenerative illness where minerals spontaneously deposited in the vascular tissue. However, a large number of studies in recent years have contributed to the understanding of the underlying mechanisms of VC and have demonstrated that they form part of a regulated process that carries with it the phenotypical transformation of the vascular smooth muscle cells in osteogenic cells, in response to diverse calcifying stimuli. Thus, it is thought that VC in renal patients is not only due to disturbances in mineral metabolism but that there are other risk factors involved like oxidative stress [38]. The inflammatory mediators must be considered because they are often increased with capacity to activate the renin-angiotensin system in CKD, which probably contributes to increase ROS production and accelerates atherosclerosis. Therefore, promoters of VC increase and inhibitors are reduced, which favors metastatic VC in ESRD. Accelerated atherosclerosis will lead to a higher prevalence of coronary artery disease, heart failure, stroke, and peripheral arterial disease. Consequently, subjects with CKD are exposed to increased morbidity and mortality as a result of CVD [39].

In our study, the predictors of VC were significantly the woman being aged ≥30 years. Patients in HD had a higher age and a higher percentage of VC compared to patients undergoing PD, which to some extent was expected due to the higher degree of atherosclerosis and secondary vascular damage that occurs in patients with HD. On the other hand, MBD variables in CKD were evaluated in patients with and without VC; iPTH and alkaline phosphatase levels were elevated in patients with VC (no significant difference) as expected.

Vitamin D 3 levels were slightly decreased in patients with VC. This behavior reflects changes of secondary hyperparathyroidism in CKD. The VC is a complex phenomenon, with many causal associations yet to be fully elucidated, despite the fact that current therapeutic strategies still fail to improve the impact on cardiovascular morbidity and mortality in patients with CKD [40].

Alkaline phosphatase and P were significantly increased in patients with HD. These results agree with previous studies reporting a lower prevalence of VC in patients submitted to PD, which could be a reflection of factors such as lower age, lower anterior vascular damage, and better residual renal function [35]. The prooxidant status of the ESRD was evaluated; our results were consistent with what was previously reported by other authors [41]. The marker levels of oxidative DNA damage (8-OHdG) were significantly increased in patients with ESRD compared to healthy controls. The antioxidant catalase was found to be significantly decreased in patients with ESRD. We cannot explain why the levels of SOD enzyme activity showed no significant difference, since like catalase reflects the activity of the antioxidant system. This could be a result of the magnitude of the defense system; that is to say that large amounts of SOD are required and therefore small changes are not enough showing mathematical differences. We also do not know if the members of the antioxidant defense system operate jointly or independently in CKD, which could explain such results. However, more specific studies are needed to elucidate such findings. Theoretically, there is a link between oxidative stress and VC in ESRD, since both conditions converge in the uremic environment; however, so far there is insufficient evidence in the available literature in human beings about this association. Among their main experimental theories in the study, the possible role of ROS as promoters of VC in patients with ESRD is postulated [42].

With the obtained information, we cannot explain these results, and we have some questions: The levels of the 8-OHdG marker appear to be a compensatory consequence of the SOD activity in the presence of VC. These findings could be due to the limitation of the type of study performed, because no causal association can be determined. However, it would be logical to think that in those patients with VC that showed better antioxidant activity (SOD); its effect with 8-OHdG levels is reflected, although there are no cellular and/or tissue evaluations to prove it. In addition, we cannot know if those patients with VC and 8-OHdG levels previously had them before the presence of VC. In addition, we wonder if there is another associated determinant in patients with VC, which influences such findings. Regarding this, one of the most studied theories is the presence of repair systems of oxidative damage to DNA. Of these systems, it is known that eukaryotic cells can activate mechanisms to repair damage, modify transcriptional activity, and stop the cell cycle [43]. These mechanisms may act independently but frequently act together. If the level of damage exceeds the mechanisms intended to repair DNA, the cell can activate mechanisms of cell death by apoptosis. Among the major DNA repair enzymes are endonucleases, exonucleases, ligases, and DNA glycosylase. In mammals, an important mechanism of DNA repair is the base cleavage repair system which operates as follows: a DNA glycosylase removes the damaged base, generating an apurinic/apirimidic site or abasic site. Subsequently, the sugar/phosphate residue remaining in the abasic site is eliminated by the activity of the APE1 enzyme. Through the activity of DNA polymerase B and ligase III, the correct nucleotide is incorporated and the 3 ‘to 5’ ends of the DNA strand is

repaired [44]. If the abasic sites are not repaired, they determine breaks in the DNA strands, and the induction of cellular apoptosis occurs [45].

When performing the correlation tests between oxidative stress and MBD results in patients with CKD, we found that SOD were correlated negatively with the 8-0HdG marker and the VC score and the oxidative stress correlated positively with alkaline phosphatase and phosphorus. The 8-OHdG correlated negatively with VC, alkaline phosphatase, and P. Based on the findings, the importance of the association between oxidative stress and VC in patients with ESRD, and the recent studies evidencing the persistence of alterations in oxidative status even after TR, it is necessary to evaluate the usefulness of antioxidant therapeutic strategies in this population of patients [46]. The results of this study, being the first in our country to describe the association between oxidative stress behavior and VC, contribute to the future evaluation of the oxidative status of the ESRD for prognostic and therapeutic purposes.

5. Conclusions

The VC frequency was 47%. Compared with healthy subjects, patients with ESRD had decreased catalase activity and increased marker levels of oxidative DNA damage (8-OHdG). The correlations of the oxidative stress markers were significant weak; in the case of SOD, it had negative significance with the VC score and the 8-OHdG score. There was a positive correlation with alkaline phosphatase and P. The 8-OHdG marker was negatively correlated with VC, alkaline phosphatase, and P. The significant predictors of VC were being female aging ≥30 years.

Conflict of interest

No conflict of interest.

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The current book entitled *Free Radicals, Antioxidants, and Diseases* gives an idea of detecting free radicals in vivo by newer techniques and provides insights into the roles played by various antioxidants in combating diseases caused by oxidative stress. The chapters included in this volume showcase new investigation in this field by the research groups around the world.

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