Oxidative Stress in Veterinary Medicine

Guest Editors: Cristina Castillo Rodríguez, Fernando Wittwer Menge, and José Joaquin Cerón

Oxidative Stress in Veterinary Medicine

Oxidative Stress in Veterinary Medicine

Guest Editors: Cristina Castillo Rodríguez,
Fernando Wittwer Menge, and José Joaquin Cerón

Editorial Board

Contents

Editorial

Oxidative Stress in Veterinary Medicine

Cristina Castillo Rodríguez,[1] Fernando Wittwer Menge,[2] and José Joaquin Cerón[3]

[1] *Departamento de Patología Animal, Facultad de Veterinaria de Lugo, Universidad de Santiago de Compostela, 27002 Lugo, Spain*
[2] *Instituto de Ciencias Clínicas Veterinarias, Universidad Austral de Chile, Casilla 567, Valdivia, Chile*
[3] *Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, Espinardo, 30100 Murcia, Spain*

Correspondence should be addressed to Cristina Castillo Rodríguez, cristina.castillo@usc.es

Oxidative stress can be regarded as an imbalance between prooxidant/free radical production and opposing antioxidant defenses. There is growing evidence that oxidative stress (OS) significantly impairs organic function and plays a major role in the aetiology and pathogenesis of several metabolic diseases in veterinary medicine. In many of these cases, it is unclear if oxidants trigger the disease or if they are produced as a secondary consequence of the disease and from general tissue damage.

In this special issue on oxidative stress in veterinary medicine, we have invited a few papers that address novel approaches about this matter, taking into account not only the pathogenic mechanisms of diseases but also new specific laboratorial tools helping in the OS measurement.

The first paper on this special issue addresses the advantages of measuring hepatic oxidative status in liver biopsy, helping in diagnosis of hepatic dysfunction and reflecting the degree of deterioration in the liver tissues. Thus, liver biopsy aids in recommending antioxidant's therapy in patients that had a hepatic disease with derangement in hepatic antioxidant constituents.

On the other hand, an increasing body of evidence suggests that OS is involved in the pathogenesis of a wide range of cardiovascular diseases. Nevertheless, it is still a matter of debate whether this increased OS has a primary causative role in cardiovascular disease pathogenesis or rather is a vascular sequel of disease progression. The establishment of the specific role of OS in cardiovascular diseases will help to choose the antioxidant therapy that will prove beneficial in combating these problems. The second paper performs a wide revision regarding the pathogenesis of OS and cardiac diseases in dog, and how supplementation can play a protective role, avoiding cell disorganisation and cellular damages. The authors describe there the effect of proper antioxidant supplementation (coenzyme-Q10, polyphenols, or omega-3 fatty acids) increasing the concentration of antioxidants in heart cells and making them less sensitive to free radicals.

Finally, a number of vitamins and trace minerals are involved in the antioxidant defense system and a deficiency of any of these nutrients may depress immunity. Some vitamins (such as E or C) are important antioxidants that have been shown to play an important role in immunoresponsiveness and health. A number of trace minerals are required for functioning of enzymes involved in the antioxidant defense system, and certain trace minerals may also affect immune cells via mechanisms distinct from antioxidant properties. Two reports analyze the protective effects of Zn or vitamin C in different species (chickens and mice) in different diseases (parasitic infections and haematological disturbances).

Finally, OS has been implicated in the pathogenic mechanism of some heavy metals (such as lead or cadmium), causing many disease conditions and toxicities in animals. Several ameliorative measures to counteract the oxidative damage to the body system aftermath or during exposure to these toxicants have been assessed with the use of antioxidants. The last report focuses on this aspect.

This is a novel field of research and it is expected an increased number of studies in the future.

Cristina Castillo Rodríguez
Fernando Wittwer Menge
José Joaquin Cerón

Review Article

The Role of Liver Biopsy in Detection of Hepatic Oxidative Stress

Mahmoud Rushdi Abd Ellah

*Clinical Laboratory Diagnosis, Department of Animal Medicine, Faculty of Veterinary Medicine, Assiut University,
Assiut 71526, Egypt*

Correspondence should be addressed to Mahmoud Rushdi Abd Ellah, mahmoudrushdi@hotmail.com

The goal of the current paper is to explore the role of liver biopsy as a tool in detection of hepatic oxidative stress, with brief notes on different types of free radicals, antioxidants, hepatic and blood oxidative stress, and lipid peroxidation. Hepatic oxidative stress was investigated for many years in human and animals, but most of the studies performed in animals were concerned with studying oxidative status in the liver tissues after slaughtering or euthanasia. However, in human medicine, a large number of studies were implemented to investigate the status of antioxidants in liver biopsy specimens. Similar studies are required in animals, as the changes in hepatic antioxidants and formation of lipid peroxide give a good idea about the condition of the liver. On the other hand, hepatic disease may present without significant effect on blood oxidative status, and, consequently, the best way to detect the status of hepatic oxidants and antioxidants is through measuring in liver biopsy. Measuring antioxidants status directly in the liver tissues gives an accurate estimation about the condition of the liver, permits the diagnosis of hepatic dysfunction, and helps to determine the degree of deterioration in the hepatic cells.

1. Introduction

Free radicals are highly reactive substances produced continuously during metabolic processes. They participate mainly in physiological events such as the immune response, metabolism of unsaturated fatty acids, and inflammatory reaction. The balance between free radicals and antioxidants is disrupted in many diseases. This disruption may be attributed to a number of factors such as the inability of the cells to produce sufficient amounts of antioxidants, the nutritional deficiency of minerals or vitamins, and the excess production of reactive oxygen species [1]. Free radical excess results in impairment of DNA, enzymes, and membranes and induces changes in the activity of the immune system and in the structure of basic biopolymers which, in turn, may be related to mutagenesis and aging processes [2].

The involvement of oxidative stress in the pathogenesis of hepatic dysfunction in human [2–15] and animals [1, 16–25] has been investigated for many years. Some of the liver diseases were associated with an increase [21, 24, 26] or decrease [17, 22, 27–29] in antioxidant's contents. Usually hepatic antioxidants increase at the beginning of hepatic

disease and decrease in severe hepatic injury. The advantages of measuring hepatic oxidative status in liver biopsy are that it helps in diagnosis of hepatic dysfunction, reflects the degree of deterioration in the liver tissues, and helps to determine the severity of hepatic injury, and, also, it aids in recommending antioxidant's therapy in patients that had a hepatic disease with derangement in hepatic antioxidant constituents. The main purpose of the current paper is to explore the value of liver biopsy as a tool for detection of hepatic oxidative stress. A focus was done on different types of free radicals, antioxidants, lipid peroxidation, and hepatic and blood oxidative status in hepatic dysfunction.

2. Free Radicals

2.1. Types of Free Radicals. Free radicals can be defined as molecules containing a single unpaired electron in atomic or molecular orbits. These molecules have an important role in the pathogenesis of tissue damage in various disorders [30], such as hepatic dysfunction, mastitis, kidney damage, inflammation, immune injury, and carcinogenesis

[1]. The most important free radicals include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^\bullet OH$), and hypochlorous acid (HOCL) [31]. HOCL is produced by the reaction of hydrogen peroxide (H_2O_2) with chloride ions and plays an important role in the leukocyte respiratory burst, which is involved in the host defense system [32]. Nitric oxide ($NO^\bullet$) acts as a free radical and as a biological mediator in biochemical reactions. Physiologically it is synthesized from L-arginine by NO synthase employing cofactor NADPH. In the host, $NO^\bullet$ arises in some pathological situations, such as sepsis, stroke, myocardial depression, and inflammatory responses [33].

Superoxide anion induces important reducing reactions in biological materials via Fenton-like reactions, which are catalyzed by redox cycling metal ions, including iron, copper, chromium, and vanadium [34]. These metal ions have the ability to accept and donate single electrons, making them important catalysts of free radical reactions; the most widely distributed and most commonly studied transition metal ions are the cations iron and copper [31]. Superoxide anion reduces Fe^{3+} in metalloproteins such as ferritin. The reduction of protein bound iron is an important reaction in biological material, because if there is sufficient H_2O_2 available, a reaction between the resultant Fe^{2+} and H_2O_2 occurs and gives rise to the highly reactive $^\bullet OH$ [32], and H_2O_2 traverses biological membranes and intracellularly targets phospholipids, carbohydrates, metalloproteins, and DNA and causes damage via Fenton's reaction [35].

2.2. Sources of Free Radicals. Free radicals may be released in the liver as a subsequence to hepatic detoxification of drugs, chemicals, and toxic materials [36, 37]. The formation of oxygen free radicals may be physiological as in phagocytosis (superoxide and H_2O_2 are used by phagocytic cells to kill bacteria), a side effect of metabolic pathways, or may occur in pathological conditions due to toxic agents as in the case of ischemia, inflammation, and disease, or due to decreased antioxidant defenses [38].

Mitochondria are considered a major source for the production of $O_2^{\bullet-}$ and H_2O_2; about 2-3% of consumed oxygen is constantly converted into reactive oxygen/reactive nitrogen species (ROS/RNS) in the mitochondria; hepatocytes contain many mitochondria and therefore generate excess ROS/RNS [31].

In many liver diseases, including the wide range of neonatal hepatitis, the tissue inflammatory infiltrates are likely to be responsible for the formation of $O_2^{\bullet-}$, H_2O_2, $^\bullet OH$, HOCL, and the highly cytotoxic monochloramine [39, 40]. In turn, the superoxide anion attracts further neutrophils to the inflammatory site by a chemotactic activity, causing an increase in tissue injury [41]. In addition, activated macrophages, Kupffer cells, and vascular endothelium can generate nitric oxide, which may react with superoxide generating peroxynitrite. The latter is responsible for the inhibition of mitochondrial respiration and DNA synthesis [42].

Liver damage due to iron (hemochromatosis) and copper overload is believed, at least partially, to derive from the catalytic activity of these metals in the Fenton reaction leading to the generation of ROS and increased lipid peroxidation with consequent abnormal mitochondrial function [43–45].

3. Antioxidants and Free Radicals

The cells contain a variety of antioxidant mechanisms that play a central role in the protection against reactive oxygen species [46, 47]. The antioxidant system consists of antioxidant enzymes (superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px)), glutathione, ancillary enzymes (glutathione reductase (GR), glutathione S-transferase, and glucose 6-phosphate dehydrogenase (G6PD)), metal-binding proteins (transferrin, ceruloplasmin, and albumin), vitamins (alpha-tocopherol, ascorbate, and beta-carotene), flavonoids, and urate [48].

Pathological free radical reactions do not necessarily cause cell and tissue damage, as antioxidants of cells and tissues are able to prevent free radical injury [36]. On the intracellular level, ROS formation and metabolism can be summarized as shown in Figure 1.

4. Hepatic Oxidative Stress and Lipid Peroxidation

Oxidative stress results when reactive forms of oxygen are produced faster than they can be safely neutralized by antioxidant mechanisms [49] and/or from a decrease in antioxidant defense, which may lead to damage of biological macromolecules and disruption of normal metabolism and physiology [50]. This condition can contribute and/or lead to the onset of health disorders [38] and play a damaging role in a number of liver disorders, for example, in anoxic and reoxygenation injury during transplantation, activated phagocytes and xanthine oxidase formed during ischemia, catalyzing the formation of superoxide during reperfusion [39, 51–53].

Lipid peroxidation is implicated in the pathogenesis of several hepatic disorders in human [2, 26] and animals [18, 22]. Hepatic failure in cattle was associated with decreased antioxidant mechanisms inside the cells, which led to the increase in the reactive oxygen species, especially H_2O_2. The decrease in hepatic GSH-Px activity in severe fatty degeneration, for example, results in the increase of H_2O_2 [22], which can initiate free radical formation through Fenton's reaction. In addition, the decrease in hepatic vitamin E level, which is an important chain-breaking antioxidant, results in lipid peroxidation and failure to regenerate the ascorbic acid [17, 18]. Increased hepatic oxidative stress was also reported in cows suffering from glycogen degeneration [22], sawdust liver, and liver abscesses [19, 21]. The authors contended that the antioxidant defense was high in the case of sawdust liver, glycogen degeneration, and liver abscess, which indicated that the body can combat the increased free radical stress.
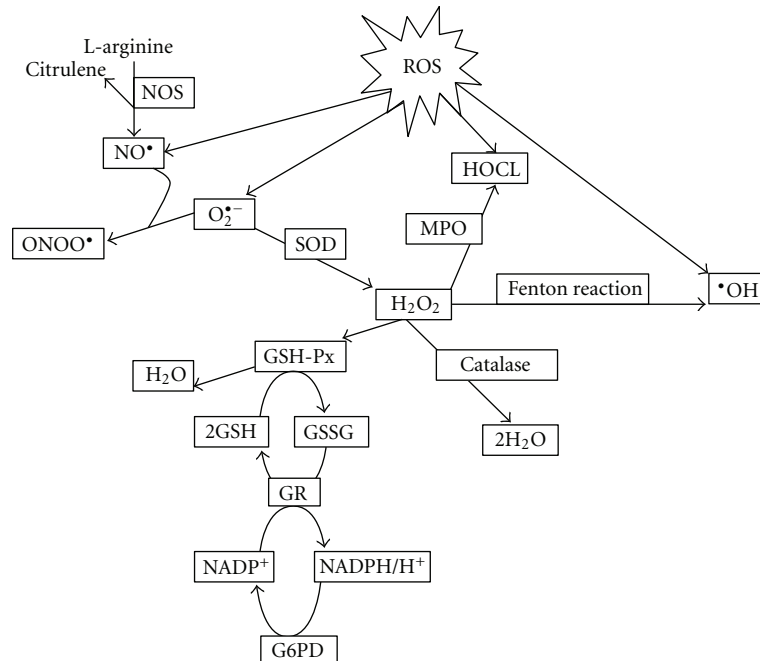
FIGURE 1: Shown are different types of reactive oxygen species (ROS). Abbreviations: GSH-Px: Glutathione peroxidase; HOCl: Hypochlorous acid; H_2O_2: Hydrogen peroxide; MPO: Myeloperoxidase; $NO^\bullet$: Nitric oxide; NOS: NO synthase; $O_2^{\bullet-}$: Superoxide anion; $^\bullet OH$: Hydroxyl radical; $ONOO^\bullet$: Peroxynitrite anion; SOD: Superoxide dismutase; NADPH: Nicotinamide adenine diphosphate; GSH: Reduced glutathione; GR: Glutathione reductase; G6PD: Glucose-6-phosphate dehydrogenase.

Liver abscesses in fattening steers occur mainly due to intensive feeding of highly concentrated rations. Consumption of a carbohydrate-rich diet stimulates G6PD expression in endothelial and parenchymal cells [16, 19]. Since G6PD supports reactive oxygen metabolism, the response may represent an antioxidant pathway in the hepatic cell populations that targets sinusoid born reactive oxygen species during infections [19, 21].

Underfeeding in cattle was reported to induce changes in the antioxidant systems in liver manifested by lowering hepatic G6PD and SOD activities. This results in depletion of antioxidant defense mechanisms and renders the hepatocytes more susceptible to the lethal effects of endogenous or exogenous peroxides, and it indicates that the generation of lipid peroxides in cattle in poor nutritional condition exceeds the antioxidant capacity of the liver cells, generating a situation of oxidative stress and peroxidation [20].

The leading mechanism of free radical toxicity is the peroxidation of membrane phospholipids, which is initiated by the formation of lipid peroxide or hydroperoxides, and peroxy radicals are formed in the presence of oxygen to start a chain reaction (propagation) [2, 54, 55]. Various pathogenic effects occur as the result of degradation of membrane lipids [31]. Chiefly, the hydroxyl radical and to a lesser extent the superoxide anion leads to peroxidation of membrane lipids thereby causing production of malondialdehyde (MDA) and 4-hydroxyalkenals (4HNE). These substances directly induce hepatocytes damage with generation of proinflammatory cytokines, activation of spindle cells, and fibrogenesis

[56, 57] and may bind to various molecules, impairing their functions [5] and therefore lead to membrane damage, protein damage, enzyme dysfunction, and DNA or RNA damage [58]. It is well known that persistent oxidant stress causes mutative effects on cell DNA and increases fibroblastic activity, leading to cirrhosis and carcinoma. Many studies have shown that oxidative stress takes part in the pathogenesis of cholestasis by way of cytokines [8, 11–13] and lipid peroxidation [6].

The role of lipid peroxidation in liver fibrosis was assessed. Lipid peroxidation products in the form of MDA adduct were detected in areas of active fibrogenesis. It has been shown that lipid peroxidation products can stimulate fibrogenesis by inducing collagen gene expression, and detection and prevention of lipid peroxidation could be of major interest in preventing fibrosis and cirrhosis in this disease [9].

Increased lipid peroxidation may be caused by inflammation related to viral infection and decreased antioxidant levels. The lipid peroxides formed may be chemotactic for the neutrophils causing increased inflammation, which further drives oxidant-mediated injury in the liver [59]. Previous studies have demonstrated an increase in MDA levels and decrease of the antioxidant capacity in acute and chronic hepatitis [3, 4, 55]. Mitochondrial lipid peroxidation takes place at varying levels in liver disorders independent of etiology [44, 60]. Increased lipid, protein, and nucleic acid peroxidation in the blood and liver biopsy specimens from patients with chronic hepatitis has been demonstrated [7, 15, 26].

TABLE 1: Methods for preparation of liver biopsy implemented in different studies.

Tissue preparation	Buffer used	Homogenization	Oxidative stress marker	Reference
Liver biopsy samples were washed twice in cold 0.9% salt solution	Tris-HCL (50 mM) pH 7.5	The liver biopsy was homogenized in 20 volumes of cold buffer, and then the supernatant was harvested after centrifugation at 5000 g for 30 min at 4°C.	SOD, CAT and GSH	[24, 25]
	Chilled potassium chloride (1.17%)	Liver biopsy was homogenized in chilled buffer. The homogenates were centrifuged at 800 g for 5 min at 4°C to separate the nuclear debris. The obtained supernatant was recentrifuged at 10,500 g for 20 min at 4°C to get the postmitochondrial supernatant.	SOD, CAT and MDA	[68]
	Ice-cold PBS buffer (20 mM), pH 7.3 with 10 ml of 5 mM butylated hydroxyl toluene	The tissue was homogenized in 290 ml ice-cold buffer. Following this, the suspension was centrifuged and supernatant was fractioned for analysis.	LPO and AOP	[69]
	Tris-HCl (50 mM), pH 7.5, 5 mM EDTA, 1 nM dithiothreitol	The tissue was homogenized in 5 ml/g cold buffer. The homogenate was centrifuged at 10,000 g for 15 minutes at 4°C. The supernatant was removed for assay.	GSH-Px	[70]
	Potassium phosphate (0.05 M) and 0.1 mM EDTA, pH 7.8	The tissue was homogenized in 200 μL buffer and centrifuged at 15,000 g for 30 minutes at 4°C. The supernatant was used for analysis.	SOD	[70]

5. Oxidative Stress and Hepatic Dysfunction: Role of Liver Biopsy

5.1. Blood and Hepatic Oxidative Stress.
Antioxidant status of blood does not reflect hepatic oxidative stress only, but their levels change in response to diseases in other organs. Studying the effect of hepatic dysfunction on blood oxidative status in cows revealed that hepatic glycogen degeneration, fatty degeneration, or liver abscesses had no effect on erythrocytic oxidative status, as indicated by the insignificant changes in erythrocytes GSH-Px and G6PD activities [21, 22]. Many studies had been performed on humans to determine the effect of hepatic dysfunction on erythrocytic oxidative status; some of these studies had reported no significant changes in erythrocytes GSH-Px activity in patients suffered from liver cirrhosis and alcoholic liver disease [61–63]. Other studies had demonstrated that a red cell GSH-Px activity significantly decreased in patients with chronic liver disease [64–66]. In addition, lower activities of erythrocytes GSH-Px and SOD activities have been reported in patients with acute hepatitis B [67]. The cause of such contradictory results may be related to the degree of hepatic dysfunction or the presence or absence of selenium deficiency. Significant decreases in plasma selenium level and erythrocytes GSH-Px had been reported in patients with chronic liver disease [64].

Increased oxidative stress had been reported in the liver of cattle with naturally occurring fatty liver [18, 22], with liver abscessation [21], and in animals on restricted feed intake [20], without significant changes in blood oxidative status; this means that hepatic disease may present without effect on blood oxidative status and also that detection of hepatic oxidative stress is best done through measuring oxidative stress markers in the hepatic tissues by means of liver biopsy.

5.2. Preparation of Liver Biopsy for Antioxidants Measurements.
The principles for preparation of liver biopsy are that liver biopsy must be prepared directly after collection, otherwise stored at −80°C, liver biopsy must be washed twice in a cold saline or cold buffer before homogenization, blot dry, and then homogenized in a cold buffer at certain pH. After centrifugation, the supernatant is harvested and used to measure hepatic antioxidant enzyme activities, which can be performed using commercial test kits (Table 1).

5.3. Liver Biopsy and Oxidative Stress.
Oxygen free radicals might play a role in the pathogenesis of tissue damage in many pathological conditions and have been implicated in a variety of liver diseases. It, therefore, may participate in the pathogenesis of toxic liver diseases and other hepatic alterations [10]. Oxidative stress is a major pathogenetic event occurring in several liver disorders ranging from metabolic to proliferated ones and is a main cause of liver damage in ischemia/reperfusion during liver transplantation [14].

The involvement of oxidative stress in the pathogenesis of liver injury has been investigated for many years [2–4]. Some of these studies were conducted using liver biopsy in human [70, 71] and animals [24, 25]. But most of the studies in animals measured hepatic oxidative stress after slaughtering or euthanasia. Examples include measuring hepatic G6PD activity in chemically induced hepatocellular carcinoma in rat liver [72] and in liver of rat with macronodular cirrhosis induced by long-term thioacetamide administration [73]. In cattle, hepatic GSH-Px activity [22]

and vitamin E [17, 18] were measured in cows suffering from severe fatty degeneration. In addition, hepatic GSH-Px and G6PD activities were determined in cows suffering from glycogen degeneration [22], sawdust liver, and liver abscesses [16, 19, 21]. Furthermore, hepatic G6PD and SOD activities were measured in cows with restricted feed intake [20].

Recently, liver biopsy was applied as a tool for detecting hepatic oxidative stress in cattle from the viewpoint of the status of hepatic antioxidant enzymes after injection of a potent hepatotoxic (DL-ethionine), data published in [24, 25]; the supernatant of liver homogenate was used to measure hepatic SOD, catalase [25], total glutathione level and glutathione reductase activity [24].

Many studies were performed to establish the importance of liver biopsy from the viewpoint of oxidative stress in a variety of liver disorders in human. Examples in human include the following oxidative stress-related parameters were investigated in liver biopsy from NAFLD patients and used to assay activities of CAT and GSH-Px [28]. Oxidative stress status in children with glycogen storage disease [74] and with cholestatic chronic liver disease [70] was investigated by measuring GSH-Px, SOD, and CAT activities in liver biopsy samples. Activities of SOD, CAT, and GSH-Px were measured in liver biopsy specimens from patients with various liver diseases, including chronic persistent hepatitis, chronic active hepatitis, nonalcoholic cirrhosis, alcoholic cirrhosis, and acute hepatitis [71].

Increased hepatic oxidative stress had also been detected in liver biopsy from patients with cirrhosis and hepatocellular carcinoma, shown by the decrease of GSH-Px activity, hepatic and blood glutathione (GSH) levels, along with an increase in the oxidized glutathione/glutathione ratio in cirrhotic [26, 27] and liver cancer tissues [29], which reflects a decrease in both the synthesize capacity of liver and the antioxidant defense.

It is clear from the above review of the literature that liver biopsy can be used for measuring oxidative status of the liver tissues and that significant changes were detected in different hepatic dysfunctions. Antioxidant activities in liver biopsy can be used to diagnose liver disease and as a prognostic factor for the liver disease under investigation.

6. Conclusion

Most of the studies done in animals were concerned with studying the hepatic oxidative stress after slaughtering or euthanasia. Studying the hepatic oxidative status in liver biopsy is lacking in animals. In human medicine, a large number of studies were implemented to achieve this goal. Hepatic disease may present without significant effect on blood oxidative status. Consequently, the best way is to measure hepatic oxidants and antioxidants in liver biopsy, which reflects the actual status of the liver.

Abbreviations

4HNE: 4-hydroxyalkenals
AOP: Antioxidant potential
CAT: Catalase
EDTA: Ethylenediaminetetraacetic acid
G6PD: Glucose-6-phosphate dehydrogenase
GSH-Px: Glutathione peroxidase
GR: Glutathione reductase
H_2O_2: Hydrogen peroxide
$^{\bullet}OH$: Hydroxyl radical
HOCl: Hypochlorous acid
MDA: Malondialdehyde
MPO: Myeloperoxidase
NADPH: Nicotinamide adenine diphosphate
$NO^{\bullet}$: Nitric oxide
NOS: NO synthase
NAFLD: Nonalcoholic fatty liver disease
$ONOO^{\bullet}$: Peroxynitrite anion
ROS/RNS: Reactive oxygen/reactive nitrogen species
GSH: Reduced glutathione
$O_2^{\bullet-}$: Superoxide anion
SOD: Superoxide dismutase
LPO: Total lipid peroxide.

References

[1] M. R. Abd Ellah, "Involvement of free radicals in animal diseases," *Comparative Clinical Pathology*, vol. 19, no. 6, pp. 615–619, 2010.

[2] G. Poli, "Liver damage due to free radicals," *British Medical Bulletin*, vol. 49, no. 3, pp. 604–620, 1993.

[3] M. Comporti, "Lipid peroxidation and cellular damage in toxic liver injury," *Laboratory Investigation*, vol. 53, no. 6, pp. 599–623, 1985.

[4] G. Poli, E. Albano, and M. U. Dianzani, "The role of lipid peroxidation in liver damage," *Chemistry and Physics of Lipids*, vol. 45, no. 2–4, pp. 117–142, 1987.

[5] M. Zern, M. Czaja, and F. Weiner, "The use of molecular hybridization techniques as tools to evaluate hepatic fibrogenesis," in *Connective Tissue in Health and Disease*, M. Rojkind, Ed., pp. 99–122, Boca Raton, Fla, USA, CRC Press, 1990.

[6] L. Y. Tsai, K. T. Lee, S. M. Tsai, S. C. Lee, and H. S. Yu, "Changes of lipid peroxide levels in blood and liver tissue of patients with obstructive jaundice," *Clinica Chimica Acta*, vol. 215, no. 1, pp. 41–50, 1993.

[7] N. De Maria, A. Colantoni, S. Fagiuoli et al., "Association between reactive oxygen species and disease activity in chronic hepatitis C," *Free Radical Biology and Medicine*, vol. 21, no. 3, pp. 291–295, 1996.

[8] J. A. Gonzalez-Correa, J. P. De La Cruz, E. Martin-Aurioles, M. A. Lopez-Egea, P. Ortiz, and F. Sanchez De La Cuesta, "Effects of S-adenosyl-L-methionine on hepatic and renal oxidative stress in an experimental model of acute biliary obstruction in rats," *Hepatology*, vol. 26, no. 1, pp. 121–127, 1997.

[9] V. Paradis, P. Mathurin, M. Kollinger et al., "In situ detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features," *Journal of Clinical Pathology*, vol. 50, no. 5, pp. 401–406, 1997.

[10] J. Fehér, G. Lengyel, and A. Blázovics, "Oxidative stress in the liver and biliary tract diseases," *Scandinavian Journal of Gastroenterology*, vol. 228, pp. 38–46, 1998.

[11] J. L. Wallace and M. J. S. Miller, "Nitric oxide in mucosal defense: a little goes a long way," *Gastroenterology*, vol. 119, no. 2, pp. 512–520, 2000.

[12] C. Spiral, M. H. Nathanson, R. Fiorotto et al., "Proinflammatory cytokines inhibit secretion in rat bile duct epithelium," *Gastroenterology*, vol. 121, no. 1, pp. 156–169, 2001.

[13] G. Alpini, J. M. McGill, and N. F. LaRusso, "The pathobiology of biliary epithelia," *Hepatology*, vol. 35, no. 5, pp. 1256–1268, 2002.

[14] L. Cesaratto, C. Vascotto, S. Calligaris, and G. Tell, "The importance of redox state in liver damage," *Annals of Hepatology*, vol. 3, no. 3, pp. 86–92, 2004.

[15] E. Jabłonowska, H. Tchórzewski, P. Lewkowicz, and J. Kuydowicz, "Reactive oxygen intermediates and serum antioxidative system in patients with chronic C hepatitis treated with IFN-α and thymus factor X," *Archivum Immunologiae et Therapiae Experimentalis*, vol. 53, no. 6, pp. 529–533, 2005.

[16] A. A. Khan, D. Lovejoy, A. K. Sharma, R. M. Sharma, M. G. Prior, and L. E. Lillie, "Effects of high dietary sulphur on enzyme activities, selenium concentrations and body weights of cattle," *Canadian Journal of Veterinary Research*, vol. 51, no. 2, pp. 174–180, 1987.

[17] P. Mudron, J. Rehage, H. P. Sallmann, M. Mertens, H. Scholz, and G. Kovac, "Plasma and liver alpha-tocopherol in dairy cows with left abomasal displacement and fatty liver," *Zentralblatt für Veterinärmedizin. Reihe A*, vol. 44, no. 2, pp. 91–97, 1997.

[18] P. Mudron, J. Rehage, K. Qualmann, H. P. Sallmann, and H. Scholz, "A study of lipid peroxidation and vitamin E in dairy cows with hepatic insufficiency," *Zentralblatt für Veterinärmedizin. Reihe A*, vol. 46, no. 4, pp. 219–224, 1999.

[19] Z. Spolarics, "A carbohydrate-rich diet stimulates glucose-6-phosphate dehydrogenase expression in hepatic sinusoidal endothelial cells," *Journal of Nutrition*, vol. 129, no. 1, pp. 103–108, 1999.

[20] A. Sansinanea, S. Cerone, G. Virkel, S. Streitenberger, M. Garcia, and N. Auza, "Nutritional condition affects the hepatic antioxidant systems in steers," *Veterinary Research Communications*, vol. 24, no. 8, pp. 517–525, 2000.

[21] M. R. Abd Ellah, K. Nishimori, M. Goryo, K. Okada, and J. Yasuda, "Glucose 6-phosphate dehydrogenase and glutathione peroxidase activities in hepatic abscesses of cattle," *Veterinary Biochemistry*, vol. 39, no. 2, pp. 25–30, 2002.

[22] M. R. Abd Ellah, K. Nishimori, M. Goryo, K. Okada, and J. Yasuda, "Glutathione peroxidase and glucose6-phosphate dehydrogenase activities in bovine blood and liver," *Journal of Veterinary Medical Science*, vol. 66, no. 10, pp. 1219–1221, 2004.

[23] M. R. Abd Ellah, M. Goryo, K. Okada, and J. Yasuda, "Glutathione peroxidase and glucose6-phosphate dehydrogenase activities in bovine blood and liver," *Journal of Veterinary Medical Science*, vol. 66, no. 10, pp. 1219–1221, 2004.

[24] M. R. Abd Ellah, K. Okada, M. Goryo, S. Kobayashi, A. Oishi, and J. Yasuda, "Total glutathione and glutathione reductase in bovine erythrocytes and liver biopsy," *Journal of Veterinary Medical Science*, vol. 70, no. 8, pp. 861–864, 2008.

[25] M. R. Abd Ellah, K. Okada, M. Goryo, A. Oishi, and J. Yasuda, "Superoxide dismutase activity as a measure of hepatic oxidative stress in cattle following ethionine administration," *Veterinary Journal*, vol. 182, no. 2, pp. 336–341, 2009.

[26] F. Farinati, R. Cardin, N. De Maria et al., "Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis," *Journal of Hepatology*, vol. 22, no. 4, pp. 449–456, 1995.

[27] G. Barbaro, G. D. Lorenzo, M. Ribersani et al., "Serum ferritin and hepatic glutathione concentrations in chronic hepatitis C patients related to the hepatitis C virus genotype," *Journal of Hepatology*, vol. 30, no. 5, pp. 774–782, 1999.

[28] L. A. Videla, R. Rodrigo, M. Orellana et al., "Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients," *Clinical Science*, vol. 106, no. 3, pp. 261–268, 2004.

[29] H. Czeczot, D. Ścibior, M. Skrzycki, and M. Podsiad, "Glutathione and GSH-dependent enzymes in patients with liver cirrhosis and hepatocellular carcinoma," *Acta Biochimica Polonica*, vol. 53, no. 1, pp. 237–241, 2006.

[30] B. Dalgiç, N. Sönmez, G. Biberoğlu, A. Hasanoğlu, and D. Erbaş, "Evaluation of oxidant stress in Wilson's disease and non-Wilsonian chronic liver disease in childhood," *Turkish Journal of Gastroenterology*, vol. 16, no. 1, pp. 7–11, 2005.

[31] S. J. Stohs, "The role of free radicals in toxicity and disease," *Journal of Basic and Clinical Physiology and Pharmacology*, vol. 6, no. 3-4, pp. 205–228, 1995.

[32] J. Lunec, "Free radicals: their involvement in disease processes," *Annals of Clinical Biochemistry*, vol. 27, no. 3, pp. 173–182, 1990.

[33] D. S. Bredt and S. H. Snyder, "Nitric oxide: a physiological messenger molecule," *Annual Review of Biochemistry*, vol. 63, pp. 175–195, 1994.

[34] S. J. Stohs and D. Bagchi, "Oxidative mechanisms in the toxicity of metal ions," *Free Radical Biology and Medicine*, vol. 18, no. 2, pp. 321–336, 1995.

[35] A. Samuni, M. Chevion, and G. Czapski, "Unusual copper-induced sensitization of the biological damage due to superoxide radicals," *Journal of Biological Chemistry*, vol. 256, no. 24, pp. 12632–12635, 1981.

[36] J. Feher, A. Vereckei, and G. Lengyel, "Role of free-radical reactions in liver diseases," *Acta Physiologica Hungarica*, vol. 80, no. 1–4, pp. 351–361, 1992.

[37] T. Ogino and S. Okada, "Oxidative damage of bovine serum albumin and other enzyme proteins by iron-chelate complexes," *Biochimica et Biophysica Acta*, vol. 1245, no. 3, pp. 359–365, 1995.

[38] J. K. Miller, E. Brzezinska-Slebodzinska, and F. C. Madsen, "Oxidative stress, antioxidants, and animal function," *Journal of Dairy Science*, vol. 76, no. 9, pp. 2812–2823, 1993.

[39] P. A. Southorn and G. Powis, "Free radicals in medicine. II. Involvement in human disease," *Mayo Clinic Proceedings*, vol. 63, no. 4, pp. 390–408, 1988.

[40] J. M. McCord, "Oxygen-derived free radicals," *New Horizons*, vol. 1, no. 1, pp. 70–76, 1993.

[41] W. F. Petrone, D. K. English, K. Wong, and J. M. McCord, "Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 2, pp. 1159–1163, 1980.

[42] S. Moncada and A. Higgs, "The L-arginine-nitric oxide pathway," *New England Journal of Medicine*, vol. 329, no. 27, pp. 2002–2012, 1993.

[43] R. J. Sokol, M. W. Devereaux, K. O'Brien, R. A. Khandwala, and J. P. Loehr, "Abnormal hepatic mitochondrial respiration and cytochrome C oxidase activity in rats with long-term copper overload," *Gastroenterology*, vol. 105, no. 1, pp. 178–187, 1993.

[44] R. J. Sokol, D. Twedt, J. M. McKim et al., "Oxidant injury to hepatic mitochondria in patients with Wilson's disease and Bedlington terriers with copper toxicosis," *Gastroenterology*, vol. 107, no. 6, pp. 1788–1798, 1994.

[45] B. R. Bacon, R. O'Neill, and R. S. Britton, "Hepatic mitochondrial energy production in rats with chronic iron overload," *Gastroenterology*, vol. 105, no. 4, pp. 1134–1140, 1993.

[46] A. Par and T. Javor, "Alternatives in hepatoprotection: cytoprotection-influences on mono-oxidase system—free radical scavengers. (A review)," *Acta Physiologica Hungarica*, vol. 64, no. 3-4, pp. 409–423, 1984.

[47] B. Halliwell, "Reactive oxygen species in living systems: source, biochemistry and role in human disease," *American Journal of Medicine*, vol. 91, p. 1422, 1991.

[48] B. Halliwell, "Free radicals, antioxidants, and human disease: curiosity, cause, or consequence?" *Lancet*, vol. 344, no. 8924, pp. 721–724, 1994.

[49] H. Sies, *Oxidative Stress: Oxidants and Antioxidants*, Academic Press, London, UK, 1991.

[50] M. Trevisan, R. Browne, M. Ram et al., "Correlates of markers of oxidative status in the general population," *American Journal of Epidemiology*, vol. 154, no. 4, pp. 348–356, 2001.

[51] R. J. Nauta, E. Tsimoyiannis, M. Uribe, D. B. Walsh, D. Miller, and A. Butterfield, "Oxygen-derived free radicals in hepatic ischemia and reperfusion injury in the rat," *Surgery Gynecology and Obstetrics*, vol. 171, no. 2, pp. 120–125, 1990.

[52] C. A. Brass, J. Narciso, and J. L. Gollan, "Enhanced activity of the free radical producing enzyme xanthine oxidase in hypoxic rat liver," *Journal of Clinical Investigation*, vol. 87, no. 2, pp. 424–431, 1991.

[53] B. G. Rosser and G. J. Gores, "Liver cell necrosis: cellular mechanisms and clinical implications," *Gastroenterology*, vol. 108, no. 1, pp. 252–275, 1995.

[54] M. J.P. Arthur, I. S. Bentley, and A. R. Tanner, "Oxygen-derived free radicals promote hepatic injury in the rat," *Gastroenterology*, vol. 89, no. 5, pp. 1114–1122, 1985.

[55] G. Bianchi, G. Marchesini, A. Fabbri, M. Ronchi, R. Chianese, and G. Grossi, "Lipoperoxide plasma levels in patients with liver cirrhosis," *Hepatogastroenterology*, vol. 44, no. 15, pp. 784–788, 1997.

[56] D. Pessayre, A. Berson, B. Fromenty, and A. Mansouri, "Mitochondria in steatohepatitis," *Seminars in Liver Disease*, vol. 21, no. 1, pp. 57–69, 2001.

[57] Z. M. Younossi, A. M. Diehl, and J. P. Ong, "Nonalcoholic fatty liver disease: an agenda for clinical research," *Hepatology*, vol. 35, no. 4, pp. 746–752, 2002.

[58] P. Vajdovich, "Measurements of oxidative stress," *Veterinary Clinical Pathology*, vol. 30, p. 158, 2001.

[59] J. Deutsch, "G6PD assay," in *Methods in Enzymatic Analysis*, H. U. Bergmeyer, Ed., vol. 3, p. 190, Academic Press, New York, NY, USA, 1983.

[60] A. Mansouri, I. Gaou, B. Fromenty et al., "Premature oxidative aging of hepatic mitochondrial DNA in Wilson's disease," *Gastroenterology*, vol. 113, no. 2, pp. 599–605, 1997.

[61] I. Akkuş, F. Gültekin, M. Aköz et al., "Effect of moderate alcohol intake on lipid peroxidation in plasma, erythrocyte and leukocyte and on some antioxidant enzymes," *Clinica Chimica Acta*, vol. 266, no. 2, pp. 141–147, 1997.

[62] U. Johansson, F. Johnsson, and B. Joelsson, "Selenium status in patients with liver cirrhosis and alcoholism," *British Journal of Nutrition*, vol. 55, no. 2, pp. 227–233, 1986.

[63] A. R. Tanner, I. Bantock, and L. Hinks, "Depressed selenium and vitamin E levels in an alcoholic population. Possible relationship to hepatic injury through increased lipid peroxidation," *Digestive Diseases and Sciences*, vol. 31, no. 12, pp. 1307–1312, 1986.

[64] J. Czuczejko, B. A. Zachara, E. Staubach-Topczewska, W. Halota, and J. Kedziora, "Selenium, glutathione and glutathione peroxidases in blood of patients with chronic liver diseases," *Acta Biochimica Polonica*, vol. 50, no. 4, pp. 1147–1154, 2003.

[65] M. H. Yasa, M. Kacmaz, H. S. Ozturk, and I. Durak, "Antioxidant status of erythrocytes from patients with cirrhosis," *Hepatogastroenterology*, vol. 46, no. 28, pp. 2460–2463, 1999.

[66] A. M. Chrobot, A. Szaflarska-Szczepanik, and G. Drewa, "Antioxidant defense in children with chronic viral hepatitis B and C," *Medical Science Monitor*, vol. 6, no. 4, pp. 713–718, 2000.

[67] S. G. Pak and E. V. Nikitin, "Status of the processes of free radical oxidation and antioxidation system in patients with a severe course of hepatitis B," *Klinicheskaya Meditsina*, vol. 69, no. 9, pp. 54–57, 1991.

[68] S. Noori, N. Arendt, M. Qureshi, and T. Mahboob, "Reduction of carbon tetrachloride-induced rat liver injury by coffee and green tea," *Pakistan Journal of Nutrition*, vol. 8, no. 4, pp. 452–458, 2009.

[69] J. Madill, B. M. Arendt, E. Aghdassi et al., "Hepatic lipid peroxidation and antioxidant micronutrients in HCV liver transplant patients with and without disease recurrence," *Transplantation Proceedings*, vol. 41, no. 9, pp. 3800–3805, 2009.

[70] N. A. Ismail, S. H. Okasha, A. Dhawan, A. O. Abdel-Rahman, O. G. Shaker, and N. A. Sadik, "Antioxidant enzyme activities in hepatic tissue from children with chronic cholestatic liver disease," *Saudi Journal of Gastroenterology*, vol. 16, no. 2, pp. 90–94, 2010.

[71] H. Togashi, H. Shinzawa, H. Wakabayashi et al., "Activities of free oxygen radical scavenger enzymes in human liver," *Journal of Hepatology*, vol. 11, no. 2, pp. 200–205, 1990.

[72] J. S. S. G. De Jong, W. M. Frederiks, and C. J. F. Van Noorden, "Oxygen insensitivity of the histochemical assay of glucose-6-phosphate dehydrogenase activity for the detection of (pre)neoplasm in rat liver," *Journal of Histochemistry and Cytochemistry*, vol. 49, no. 5, pp. 565–571, 2001.

[73] N. Sanz, C. Díez-Fernández, A. M. Valverde, M. Lorenzo, M. Benito, and M. Cascales, "Malic enzyme and glucose 6-phosphate dehydrogenase gene expression increases in rat liver cirrhogenesis," *British Journal of Cancer*, vol. 75, no. 4, pp. 487–492, 1997.

[74] N. A. Ismail, S. H. Okasha, A. Dhawan, A. M. O. Abdel Rahman, O. G. Shaker, and N. A. H. Sadik, "Glutathione peroxidase, superoxide dismutase and catalase activities in hepatic tissue from children with glycogen storage disease," *Archives of Medical Science*, vol. 5, no. 1, pp. 86–90, 2009.

Review Article

Oxidative Stress in Dog with Heart Failure: The Role of Dietary Fatty Acids and Antioxidants

Emmanuelle Sagols[1] and Nathalie Priymenko[2]

[1] *ENVT, 2 allée des Cèdres 66330 Cabestany, France*
[2] *ENVT, INRA, UMR 1089, Université de Toulouse, 31076 Toulouse, France*

Correspondence should be addressed to Emmanuelle Sagols, emma.sagols@yahoo.fr

In dogs with heart failure, cell oxygenation and cellular metabolism do not work properly, leading to the production of a large amount of free radicals. In the organism, these free radicals are responsible of major cellular damages: this is oxidative stress. However, a suitable food intake plays an important role in limiting this phenomenon: on the one hand, the presence of essential fatty acids in the composition of membranes decreases sensitivity of cells to free radicals and constitutes a first protection against the oxidative stress; on the other hand, coenzyme Q10, vitamin E, and polyphenols are antioxidant molecules which can help cells to neutralize these free radicals.

1. Introduction

Life expectancy of domestic carnivores has been increasing a lot these last years, probably due to veterinary medicine progresses, leading to an increase of the old-age diseases. Nowadays, heart failure is one of the main causes of death in domestic carnivores: approximately 10% of dogs have heart troubles, which can evolve to a cardiac insufficiency and, in the end, to the animal death [1]. Although treatments can slow down this evolution, a well-adapted nutrition constitutes a major asset to improve the well-being and life expectancy of these animals.

Besides the energetic protein or salt requirements commonly expressed by the literature, a suitable food intake of fatty acids and antioxidants can play an important role in maintaining cardiac cells homeostasis and especially in managing oxidative stress.

2. Oxidative Stress and Heart Failure

In the organism, free radicals ($O_2°$, $OH°$, $H_2O_2...$) are produced permanently via cellular metabolism. In physiological conditions, antioxidant enzymes (super oxide dismutase, glutathione peroxidase...) regulate this phenomenon.

However, when a large amount of free radicals is produced, these enzymes are overloaded, and free radicals induce major cellular damages: this is oxidative stress.

2.1. Free-Radical Production during Heart Failure. In dogs with heart failure, oxygenation cells do not occur properly [2], which leads to cellular metabolism dysfunction: the main free-radical sources in heart failure are mitochondria, where an uncoupling of the respiratory chain leads to the increase in free-radical production [3], Xanthine oxidases and NADPH oxidases [4]. This cellular metabolism dysfunction occurs at two levels. In a chronic way, the heart work of a dog with heart failure is insufficient to provide the normal oxygenation of all cells, which implies a dioxygen cellular chronic deficit and thus a regular production of free radicals. In an acute way, during the phenomenon of ischemia reperfusion, the cell ischemia induces a massive production of free radicals that are released in the organism when reperfusion occurs.

2.2. Cellular Consequences of Oxidative Stress in Heart Cells. Free radicals are molecules very unstable and toxic for cells. The fatty acids of cell membranes are the first molecules damaged by free radicals: indeed, free radicals induce

a membrane lipid peroxidation and alter the stability and the permeability of these membranes. They also induce DNA damages and protein denaturation with oxidation and fragmentation of polypeptidic chains [5]. These cellular lesions are so important that they can lead to the apoptosis of cells [6]. As well as these effects on cellular components, free radicals also act as intracellular messenger leading to cardiac hypertrophy and myocardium remodelling, worsening cardiac insufficiency [4].

So, cardiac insufficiency is at the origin of a noxious state of cellular oxidative stress, which increases the cardiac cell dysfunctions and thus amplifies the phenomenon of oxidative stress.

3. Role of Omega 3 Essential Fatty Acids in the Management of Oxidative Stress

Fatty acids are carboxylic acids with long carbon chain with an even number of carbon atoms. They can be saturated or not according to the presence or absence of double bounds between the carbon atoms. Three fatty acid groups could be distinguished with the saturated fatty acids (not any double bound), the monounsaturated fatty acids (one double bound), or the polyunsaturated fatty acids (several double bounds). Some fatty acids are called "essentials" as mammals do not synthesize them, and they must then be present in the food to satisfy the organism needs. This is the case of long omega 3 and omega 6 fatty acids that possess their first double bound between the 3rd and the 4th carbon and between the 6th and the 7th carbon of the carbon chain, respectively.

3.1. Role of Essential Fatty Acids in the Management of Oxidative Stress. The unsaturated fatty acids of cell membranes are the first damaged by the free radicals produced during cardiac insufficiency, this results in cell disorganization and cellular dysfunction. The presence of essential fatty acids in the composition of cell membranes decreases their sensitivity to free radicals and constitutes a first protection against the oxidative stress [7].

Essential fatty acids also play a role in scavenging free radicals produced within cells, which participate to protection of cell constituents. Indeed, in rats, a 7% daily supplementation in omega 3 essential fatty acids increases the cellular concentration of super oxide dismutase in heart, making it more available to neutralize the free radicals produced by cellular metabolism [8]. Another study led in rats shows as well that a 60 mg/kg α-lipoïc injection decreases significantly the amount of free radicals produced during an oxidative stress phenomenon and is associated with an increase of the level of gluthatione peroxidase in cells [9]. However, all essential fatty acids are not equivalent: the omega 6 fatty acids, which are commonly known to be inflammation activators, do not induce the same benefits for the cardiac cells [1].

3.2. Source of Essential Fatty Acids. Omega 3 fatty acids can be found mainly in fat fishes, soya, colza, or linen oils (Table 1) [10]. Although the optimal dose of omega 3

essential fatty acids still remains to be determined, it would seem that the ratio omega 6 : omega 3 is very important. Indeed, if the quantity of fatty acids omega 3 brought is too important, there is a risk of lipid peroxidation. Then, the most adapted ratio seems to be 5 : 1 ratio [11].

Concerning cardiac insufficiency, essential fatty acids in cell membranes play a protective role against free radicals produced by cardiac cells also against free radicals released by other cells, especially when ischemia reperfusion phenomena occurs. However, antioxidants are also playing an important role to neutralize the free radicals upstream before cell membranes infringement.

4. Role of Antioxidants in the Management of Oxidative Stress

4.1. Coenzyme Q10

4.1.1. Roles of Coenzyme Q10 in the Management of Oxidative Stress. It is now well known that heart function is improved by a supplementation of coenzyme Q10 [12]. Indeed, the coenzyme Q10, which is a part of the respiratory chains of mitochondria, plays a role in the cell energy production. However, oxidative stress results from a dioxygen misuse by cells. So, we understand why the coenzyme Q10, which enters in the composition of respiratory chains of mitochondria, improves the cell energy production [2].

Nevertheless, the heart functioning improvement is also due to antioxidant properties of coenzyme Q10, particularly if an ischemia phenomenon occurs in the myocardium. The coenzyme Q10, with its antioxidant properties [13], helps to fight against the free radicals released later during reperfusion, thanks to two mechanisms: directly, by scavenging free radicals produced [14], and indirectly, by regenerating the active form of other antioxidant molecules [15]. Moreover, the coenzyme Q10 also intervenes in the energy production resumption after an ischemia phenomenon, improving the myocytes functioning [16].

4.1.2. Sources of Coenzyme Q10. Coenzyme Q10 can be synthesized from phenylalanine, acetyl Coenzyme A, tyrosine and by seven vitamins (B_2, B_3, B_5, B_6, B_9, B_{12}, and C). However, food can also supply coenzyme Q10: meat is particularly rich in coenzyme Q10 (Table 2) [17, 18]. An intake from 30 to 90 mg by oral route, twice a day, is the dose most frequently recommended [1].

4.2. Vitamine E and Selenium

4.2.1. Antioxidant Properties of Vitamin E and Selenium. All the eight vitamin E isomers are fat-soluble vitamins with antioxidant properties, but the most active form is α-tocopherol. By its antioxidant properties, vitamin E plays a cellular barrier role for the oxidant molecules produced by oxidative stress. In a study led in rats, a vitamin E supplementation allows an increase of its incorporation in the cell membranes and a decrease of the quantity of proteins oxidized in cells [19]. When the vitamin E is exceeded,

glutathione peroxidase relieves vitamin E to neutralize the oxidant molecules. The main cofactor of this enzyme is selenium, and it is easily understandable which impact could have a deficit in selenium on its activity. On the other hand, an increase of the selenium intake seems to have no effect on the activity of glutathione peroxidase [20].

4.2.2. Sources of Vitamin E. Vitamin E is a molecule synthesized by plants. The minimum food contribution recommended by AAFCO is 50 UI/kg DM. Nevertheless, its intestinal absorption is widely influenced by the ration composition: the presence of polyunsaturated fatty acids oxidant agents implies an increase of vitamin E need; on the contrary, a ration containing monounsaturated fatty acids or selenium leads to a decrease of vitamin E necessary. So, there is no vitamin E recommended intake to obtain an antioxidant effect. However, in dog food, a maximum of 1000 UI/kg was fixed by the AAFCO. Indeed, vitamin E is a little toxic, and we can imagine an interesting antioxidant effect with such concentrations [21].

4.3. Polyphenols

4.3.1. Role of Polyphenols in the Management of Oxidative Stress. Polyphenols have recently attracted many scientists' attention. Indeed, for a long time, these molecules were considered as uninteresting for the organism, concerning nutritional level or health, but the progress of the analysis techniques allowed to understand better the role of these organic compounds. These molecules are synthesized by plants to respond to an environmental stress. They compose a wide family of chemical compounds, which contains more than 8000 different organic molecules. All the polyphenols are characterised by one or more benzene nuclei, where are fixed one or more alcohol groups (–OH) [22].

Polyphenols have antioxidant properties which allow them to catch the free radicals produced during oxidative stress [23]. In a study led in rat, a contribution of 35 mL of a 90% pure polyphenols extract, once a day during 14 days, allows an increase of the quantity of polyphenols in the cardiomyocyte membranes and decreases significantly the noxious effects of the ischemia-reperfusion phenomena: improvement of the myocardium contractibility, decrease of cellular edema, and limit the dysfunctions in heart cells [24, 25]. In another study in rat, 50 mg/kg of quercetin neutralize the free radicals produced during ischemia reperfusion, enabling a decrease of cellular damages due to oxidative stress and an increase in the concentrations of glutathione peroxidase and glutathione reductase, which confirms the antioxidant properties of polyphenols [26].

4.3.2. Sources of Polyphenols. The available data concerning the polyphenol place in the food and their beneficial effects on health are at least vague. In food, polyphenols are mainly found in vegetables (Table 3). However, the determination of the food polyphenols composition is difficult because of the important variety of molecules. There is a big difference between the quantity of polyphenols contained

Table 1: Main omega 3 fatty acid sources in food [10].

Source	Omega 3 fatty acids (g/100 g)
Salmon	3,2
Linen oil	20,3
Colza oil	9
Soya oil	5

Table 2: Coenzyme Q10 content in food [17, 18].

Food		Coenzyme Q10 (μg/100 g wet weight) [17]	Coenzyme Q10 (μg/100 g wet weight) [18]
Meat	Beef	3100	3030–4010
	Chicken	1700	1710–2500
Fish	Herring	430–2700	180–1300
Vegetables	Broccoli	660	701
	Potato	52	105
Milk		—	31
Egg		150	73

Table 3: Polyphenol food availability in food [22].

Food	Polyphenols (mg/kg)
Tea	100–800
Black grape	300–7500
Potato	100–190
Soya	200–900
Soya flour	800–1800
Corn flour	310
Wheat flour	70–90
Apple	50–600
Beans	350–550
Tomato	2–15

in the food and the quantity effectively absorbed and used by the body: cooking, peeling fruits and vegetables, and preservation are so many factors which decrease the effective contribution of polyphenols in the body [22]. Moreover, the digestibility of polyphenols depends on their structure: the highly polymerized polyphenols cross with difficulty the intestinal wall. The small polymers are more easily absorbed and have a better systemic action, which implies a better nutritional value [22–27].

There is no study on the effect of polyphenols in domestic carnivores, but it is likely that the quantity of polyphenols ingested by dogs and cats via industrial or domestic food is very low. On the other hand, in a study led in rat, 50 mg/kg of weight of quercetin allow to neutralize the free radicals produced during ischemia reperfusion and so enables an antioxidant effect [26].

5. Conclusion

Although food has been recognized for several years to be an asset for the management of heart failure in animals,

the place of antioxidants and essential fatty acids remains very limited. However, a suitable supplementation can play a major role against oxidative stress occurring during heart failure, avoiding cell disorganisation and cellular damages: on the one hand, thanks to a proper supplementation of antioxidants like Coenzyme Q10, vitamins, and polyphenols that neutralize free radicals produced by oxidative stress, and on the other hand, through omega 3 fatty acids that take part in the composition of cell membranes increasing the concentration of antioxidants in heart cells and making them less sensitive to free radicals. Although the studies led until now seem very promising, they were mainly led *in vitro* and/or in rats, and unfortunately, few studies have been led in dog and even less in the cardiac insufficient dog. Other studies should be led in domestic carnivores to confirm these first data.

In practice, dietary feed for cardiac insufficient animals are only supplemented with essential fatty acids with an omega 6/omega 3 report which can vary from 2 to 10. Regarding the antioxidant molecule supplementations, they are often absent or below the contributions needed to have benefit for the heart. This is probably due to the difficulty to preserve a sufficient amount of antioxidants during the manufacturing process. In these conditions, the establishment of a home-made ration could be the best means to have a proper antioxidant supplementation although more complicated for the owner.

References

[1] L. M. Freeman and J. E. Rush, "Maladies cardiovasculaires : influence de l'alimentation," in *Encyclopédie de la Nutrition Clinique Canine. Royal Canin*, P. Pibot, V. Biourge, and D. Elliot, Eds., pp. 316–347, 2006.

[2] F. L. Rosenfeldt, S. Pepe, A. Linnane et al., "Coenzyme Q protects the aging heart against stress: studies in rats, human tissues, and patients," *Annals of the New York Academy of Sciences*, vol. 959, pp. 355–359, 2002.

[3] T. Ide, H. Tsutsui, S. Kinugawa et al., "Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium," *Circulation Research*, vol. 85, no. 4, pp. 357–363, 1999.

[4] J. A. Byrne, D. J. Grieve, A. C. Cave, and A. M. Shah, "Oxidative stress and heart failure," *Archives des Maladies du Coeur et des Vaisseaux*, vol. 96, no. 3, pp. 214–221, 2003.

[5] S. Orrenius, "Mechanisms of oxidative cell damage," in *Free Radicals: From Basic Science to Medicine*, G. Poli, E. Albano, and M. U. Dianzani, Eds., pp. 47–64, MCBU, Bern, Switzerland, 1993.

[6] J. M. Hare, "Oxidative stress and apoptosis in heart failure progression," *Circulation Research*, vol. 89, no. 3, pp. 198–200, 2001.

[7] L. M. Freeman, J. E. Rush, J. J. Kehayias et al., "Nutritional alterations and the effect of fish oil supplementation in dogs with heart failure," *Journal of Veterinary Internal Medicine*, vol. 12, no. 6, pp. 440–448, 1998.

[8] R. Luostarinen, R. Wallin, and T. Saldeen, "Dietary (n-3) fatty acids increase superoxide dismutase activity and decrease thromboxane production in the rat heart," *Nutrition Research*, vol. 17, no. 1, pp. 163–175, 1997.

[9] A. Goraca, A. Piechota, and H. Huk-Kolega, "Effect of alpha-lipoic acid on LPS-induced oxidative stress in the heart," *Journal of Physiology and Pharmacology*, vol. 60, no. 1, pp. 61–68, 2009.

[10] M. Feinberg, J. C. Favier, and J. Ireland-Ripert, "Répertoire général des aliments, Table de compositon des corps gras (tome 1)," in *Technique et Documentation*, INRA, Ciquel-Regal, 1987.

[11] R. C. Wander, J. A. Hall, J. L. Gradin, S. H. Du, and D. E. Jewell, "The ratio of dietary (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation and vitamin E status in aged dogs," *Journal of Nutrition*, vol. 127, no. 6, pp. 1198–1205, 1997.

[12] P. H. Langsjoen and A. M. Langsjoen, "Overview of the use of coenzyme Q10 in cardiovascular disease," *BioFactors*, vol. 9, no. 2-4, pp. 273–284, 1999.

[13] K. A. Weant and K. M. Smith, "The role of coenzyme Q10 in heart failure," *Annals of Pharmacotherapy*, vol. 39, no. 9, pp. 1522–1526, 2005.

[14] J. S. Joo, "Coenzyme Q10 and cardiovascular health: to take or not to take, that is the question," *Nutrition Bytes*, vol. 10, p. 4, 2005.

[15] P. J. Quinn, J. P. Fabisiak, and V. E. Kagan, "Expansion of antioxidant function of vitamin E by coenzyme Q," *BioFactors*, vol. 9, no. 2–4, pp. 149–154, 1999.

[16] O. Hano, S. L. Thompson-Gorman, J. L. Zweier, and E. G. Lakatta, "Coenzyme Q enhances cardiac functional and metabolic recovery and reduces Ca^{2+} overload during postis-chemic reperfusion," *American Journal of Physiology*, vol. 266, no. 6, pp. H2174–H2181, 1994.

[17] C. Weber, A. Bysted, and G. Hølmer, "Coenzyme Q10 in the diet-daily intake and relative bioavaibility," *Molecular Aspects of Medicine*, vol. 18, pp. S251–S254, 1997.

[18] H. Kubo, K. Fuj II, T. Kawabe, S. Matsumoto, H. Kishida, and K. Hosoe, "Food content of ubiquinol-10 and ubiquinone-10 in the Japanese diet," *Journal of Food Composition and Analysis*, vol. 21, no. 3, pp. 199–210, 2008.

[19] J. M. Berthiaume, P. J. Oliveira, M. W. Fariss, and K. B. Wallace, "Dietary vitamin E decreases doxorubicin-induced oxidative stress without preventing mitochondrial dysfunc-tion," *Cardiovascular Toxicology*, vol. 5, no. 3, pp. 257–267, 2005.

[20] G. Flores-Mateo, A. Navas-Acien, R. Pastor-Barriuso, and E. Guallar, "Selenium and coronary heart disease: a meta-analysis," *American Journal of Clinical Nutrition*, vol. 84, no. 4, pp. 762–773, 2006.

[21] K. L. Gross, K. J. Wedekind, and C. S. Cowell, "Nutriments," in *Nutrition Clinique des Animaux de Compagnie*, M. S. Hand, C. D. Thatcher, R. L. Remillard, and P. Roudebush, Eds., pp. 87–109, Mark Morris Institute, Turin, Italy, 4th edition, 2000.

[22] C. Manach, A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez, "Polyphenols: food sources and bioavailability," *American Journal of Clinical Nutrition*, vol. 79, no. 5, pp. 727–747, 2004.

[23] P. Stanely Mainzen Prince and M. Karthick, "Preventive effect of rutin on lipids, lipoproteins and ATPase in normal and isoproterenol-induced myocardial infarction in rats," *Journal of Biochemical and Molecular Toxicology*, vol. 21, pp. 1–6, 2007.

[24] S. Mirwa, K. Yamazaki, S.-H. Hyon, and M. Komeda, "A novel method of "preparative" myocardial protection using green tea polyphenols in oral uptake," *Interactive CardioVascular and Thoracic Surgery*, vol. 3, pp. 612–615, 2004.

[25] J. Nečas, L. Bartošíková, T. Florian et al., "Protective effects of the flavonoids osajin and pomiferin on heart ischemia—

reperfusion," *Ceska a Slovenska Farmacie*, vol. 55, no. 4, pp. 168–174, 2006.

[26] M. Ikizler, N. Erkasap, S. Dernek, T. Kural, and Z. Kaygisiz, "Dietary polyphenol quercetin protects rat hearts during reperfusion: enhanced antioxidant capacity with chronic treatment," *Anadolu Kardiyoloji Dergisi*, vol. 7, no. 4, pp. 404–410, 2007.

[27] A. Scalbert and G. Williamson, "Dietary intake and bioavailability of polyphenols," *Journal of Nutrition*, vol. 130, no. 8, pp. 2073S–2085S, 2000.

Research Article

Zinc Supplementation against *Eimeria acervulina*-Induced Oxidative Damage in Broiler Chickens

Nedyalka V. Georgieva,[1] Margarita Gabrashanska,[2] Ventsislav Koinarski,[3] and Zvezdelina Yaneva[1]

[1] *Department of Pharmacology, Animal Physiology and Physiological Chemistry, Faculty of Veterinary Medicine, Trakia University, Student's Campus, 6000 Stara Zagora, Bulgaria*

[2] *Institute of Experimental Pathology and Parasitology, Bulgarian Academy of Sciences, Acad G.Bonchev Street Bl. 25, 1113 Sofia, Bulgaria*

[3] *Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Student's Campus, 6000 Stara Zagora, Bulgaria*

Correspondence should be addressed to Nedyalka V. Georgieva, nvgeorgieva@vmf.uni-sz.bg

This study was undertaken to determine the dietary supplements of Zn containing diet on the antioxidant status in chickens experimentally infected with *Eimeria acervulina*. The antioxidant status was monitored via determination of MDA concentrations and erythrocyte SOD and CAT activities, as well as vitamin E, vitamin C, Cu, and Zn in liver, muscle, and serum. The results showed increased MDA ($P < .05$), CAT ($P < .001$), and decreased SOD ($P < .001$) in the infected birds. Significant changes in Cu and Zn concentrations and dramatically reduction of vitamin C and E concentrations in the infected chickens were found. The observed deviations in the studied enzymes and nonenzymatic parameters evidence the occurrence of oxidative stress following the infection and impaired antioxidant status of chickens, infected with *Eimeria acervulina*. Our results proved the ameliorating role of $CuZn(OH)_3Cl$ (0.170 g per kg food) against *Eimeria acervulina*-induced oxidative damage in infected chickens.

1. Introduction

It is widely accepted that the balance between the reactive oxygen species (ROS) in cells, tissues, and physiological fluids determines their red/ox status. Under usual conditions, the production of ROS and their elimination are in a dynamic equilibrium. This balance could be disturbed when the generation of ROS becomes higher than the protection capacity of systemic antioxidant defense. The impaired equilibrium in favour of oxidants is named oxidative stress and it is involved in the pathogenesis of numerous diseases, including parasitic infections [1–4]. Prime targets of ROS are the polyunsaturated fatty acids (PUFA) in the membrane lipids. This attack causes lipid peroxidation. Further, the decomposition of peroxidized lipids yields a wide variety of end-products, including malondialdehyde (MDA) that is widely used in practice as an indicator of free radical

damages [5–7]. Antioxidant system comprising vitamins A, C, and E, and metal enzymes CuZn-superoxide dismutase (SOD) and catalase (CAT) have a cellular protective action against oxidative stress. Reduction of vitamin E, C, and A levels was established during eimeriosis [8–11]. Dietary trace elements/antioxidants can help maintain appropriate antioxidant balance in a lot of infections [12, 13]. Zinc has been shown to play a significant role as an antioxidant [14]. Burke and Fenton [15] have established that Zn deficiency causes increased lipid peroxidation in liver. The reduction of the trace elements/antioxidants such as Zn leads to a decrease in activity of antioxidant enzyme [16]. The mechanism of Zn action as an antioxidant manifests into acute and chronic effects [17]. Chronic effects involve exposure of an organism to Zn on a long-term basis, resulting in induction of some other substance that is the ultimate antioxidant, such as the metallothioneins. Acute effects involve two mechanisms:

(1) protection of protein sulphydryl groups or (2) reduction of $^\bullet OH$ formation from H_2O_2 due to Zn antagonism to redoxactive transition metals, such as iron and copper [18]. Administration of pharmacological doses of Zn *in vivo* has shown to have a protective effect against general and liver-specific prooxidants. Hence Zn gained an increasing attention to be applied in diseases accompanied with ROS generation [14, 15, 19, 20].

In our previous studies, we observed an impaired blood antioxidant status in broiler chickens infected with *Ascaridia galli* [21], *Eimeria tenella* [22], *Eimeria acervulina* [23], and beneficial effect of $2Gly \cdot ZnCl_2 \cdot 2H_2O$ compound upon blood antioxidant status in broiler chickens experimentally infected with *Eimeria acervulina* [24].

Therefore, the activity of our investigation was oriented toward finding new sources of trace elements/antioxidants with regards to antioxidant requirements of the infected host and their possible use in the control of the parasitoses.

The aim of the present study was to determine the effect of Zn-Cu hydroxichloride-mixed crystals, $(Cu_{0.78}/Zn_{0.22})_2(OH)_3Cl$, on antioxidant status in broiler chickens experimentally infected with *Eimeria acervulina*. For this purpose, we investigated blood MDA concentrations and erythrocyte SOD and CAT activities and vitamins E and C, copper and zinc in liver, muscles and serum from all experimental groups of chickens at the end of the experiment.

2. Materials and Methods

2.1. Compounds Tested. Zn-Cu hydroxichloride-mixed crystals, $(Cu_{0.78}/Zn_{0.22})_2(OH)_3Cl$, were synthesized by method of continuous coprecipitation under standard conditions with pH = 7 [25]. Diluted solutions of zinc and copper chloride and sodium hydroxide were used. Crystals were highly soluble in mineral acids but not in water.

2.2. Animal Studies and Treatment Schedules. The study was performed on 60 clinically healthy 20-day-old broiler chickens, Cobb 500 hybrids, weighing 288.0–411.0 g. Up to the age of 11 days, they were housed in cages on slat floors under conditions excluding an additional *Eimeria* infection and received a standard diet without antibiotics or coccidiostatics. At the age of 12 days, three groups of 20 birds each were formed. The first experimental group was healthy untreated and uninfected (negative controls). The second and the third experimental groups were infected three times with 3×10^5 sporulated *Eimeria acervulina* oocysts, at 2-day intervals (at 12th, 14th, 16th day), using an ingluvial tube [26]. The third experimental group was treated with double basic salt $CuZn(OH)_3Cl$- 0.170 g per kg food. It was given starting lasting 10 days (2 days before infection and 8 after infection).

The experiment was approved by the Committee on Animal Experimentation at Trakia University, Stara Zagora, Bulgaria and was performed according to the recommendations of Directive 86/609/EC of November 24, 1986.

2.2.1. Infectious Material. *Eimeria acervulina* oocysts were obtained from naturally infected chickens, passed through 2-week-old broiler chickens, and stored in 2.5% potassium bichromate solution in refrigerator (4°C).

2.3. Analyses of MDA, SOD, and CAT in Blood. Blood for biochemical analyses (2 mL) for MDA, SOD, and CAT assays was sampled from *v. subcutanea ulnaris* or *v. brachialis* at postinfection day 8 (of every chicken from the experimental groups). Ethylenediaminetetraacetic acid (EDTA) was used as anticoagulant.

Peripheral Blood Processing. Collected blood was centrifuged at 3000 g for 15 min and plasma was separated. Then, the plasma was deproteinized with 25% trichloroacetic acid by continuous mixing for 5 min and centrifugation at 2000 g for 15 min. The deproteinized plasma was used for lipid peroxidation products determination.

2.3.1. Determination of Products of Lipid Peroxidation. The total amount of lipid peroxidation products in plasma was assayed using the thiobarbituric acid (TBA) method, measuring spectrophotometrically malondialdehyde (MDA) reactive products at 532 nm [27].

Erythrocyte Processing. The erythrocyte pellet was washed three times with saline and lysed. The hemoglobin was separated by precipitation with ethanol/chloroform mixture. The mixture was continuously shaken for 5 min and centrifuged at 2500 g for 20 min. The obtained supernatants were used for determination of enzyme activity.

2.3.2. Determination of Superoxide Dismutase (SOD) Activities. CuZn-SOD activity was determined as described by Sun et al. [28] with minor modifications. Briefly, the xanthine/xanthine oxidase system was used to generate the superoxide anion-radical $(O_2^{\bullet -})_x$. This anion reduces nitroblue tetrazolium (NBT) to formazan, which is monitored at 560 nm. SOD in the sample sremoves the $O_2^{\bullet -}$ and inhibits the reduction. The level of this reduction is used as a measure of SOD activity. One unit of enzymatic activity is defined as the amount of enzyme causing 50% inhibition of the reduction of NBT to formazan observed. Results were expressed as units per g haemoglobin (U/gHb).

2.3.3. Determination of Catalase (CAT) Activities. CAT activity was assessed in the erythrocyte lysats by the method described by Beers and Sizer [29]. Briefly, hydrogen peroxide (30 Mm) was used as a substrate, and the decrease in H_2O_2 concentration at 22°C in phosphate buffer (50 mM, pH 7.0) was followed spectroscopically at 240 nm for 1 min. Results are presented as units per g haemoglobin (U/gHb). One unit of CAT activity is defined as the amount of enzyme that degrades 1 μmol H_2O_2 per minute.

2.4. Determination of the Levels of Cu, Zn, and Vitamins E and C. The levels of Cu and Zn and these of vitamins E and C were detected in the livers, serum, and breast musculature at the end of the experiment (8 days after the infection).
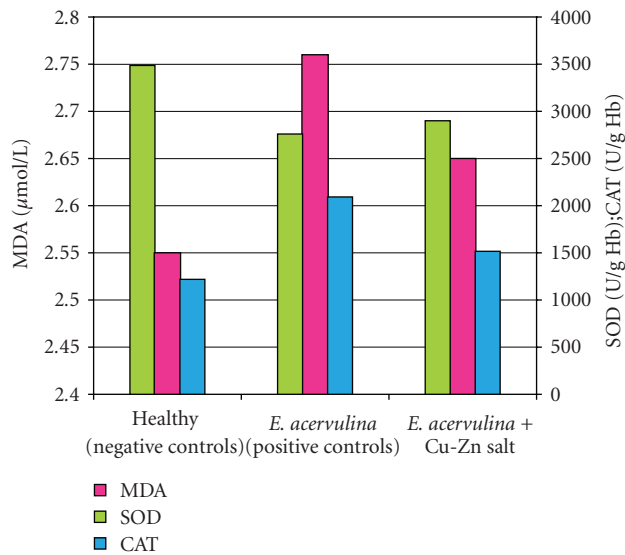
FIGURE 1: Blood MDA concentrations and erythrocyte SOD and CAT activities in chickens.

The content of Cu and Zn was determined using an atomic absorption spectrophotometry, Varian Spectr. AA 220, Madrid [30]. Vitamin E concentration was detected fluorometrically [31] and vitamin C concentration spectrophotometrically [32].

2.5. Haemoglobin Concentrations. Haemoglobin concentrations of lysates were determined spectrophotometrically at 546 nm by the cyanmethemoglobin method of Mahoney et al. [33].

2.6. Statistical Analysis. The results are reported as means ± SD for the experimental groups of chickens. Statistical analysis was performed with Student's t-test and multiple regression analysis. $P < .05$ was considered statistically significant.

3. Results

The blood MDA concentrations and the activities of antioxidant enzymes SOD and CAT in studied birds are presented in Figure 1.

The data showed a statistically significant increase of MDA concentrations—a marker of radical-induced damage, in chickens infected with *E. acervulina* versus the healthy birds (2.76 μmol/L versus 2.55 μmol/L, $P < .05$, Figure 1). The results of lipid peroxidation products measured by the formation of MDA in plasma in groups of chickens infected with *E. acervulina* and treated with double basic salt were found to be not significantly different in comparison to the healthy controls (2.65 μmol/L versus 2.55 μmol/L, $P > .05$, Figure 1) and were significantly reduced, compared to MDA of positive control group (2.65 μmol/L versus 2.76 μmol/L, $P < .05$, Figure 1). SOD activities were significantly lower in infected chickens than in negative controls (2759.4 U/gHb
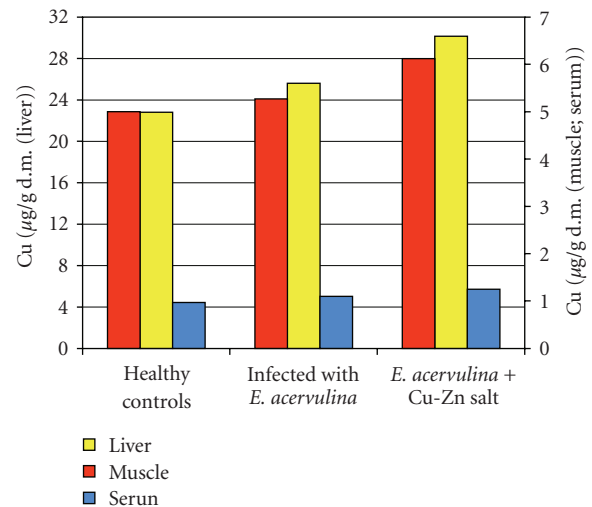
FIGURE 2: Cu level in broiler chickens.
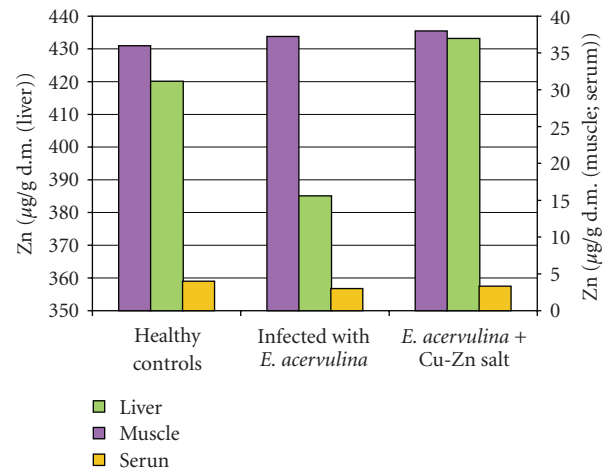
FIGURE 3: Zn level in broiler chickens.

versus 3486.5 U/gHb, $P < .001$, Figure 1). The erythrocyte SOD activity in chickens infected with *E. acervulina* and treated with double basic salt was found to be increased, as compared to the infected chickens (2900 U/gHb versus 2759 U/gHb, $P < .001$, Figure 1) and lower to the healthy birds (3486 U/gHb, Figure 1). A significant increase of CAT activity was observed in infected, compared to healthy birds (2092.0 U/gHb versus 1218.4 U/gHb, $P < .001$, Figure 1). The supplementation of the basic salt restored the levels of CAT in lysate of infected with *E. acervulina* and treated with $CuZn(OH)_3Cl$ broiler chickens (1515 U/gHb versus 1218 U/gHb, $P > .05$, Figure 1).

The results showed that *E. acervulina* infection increased the liver Cu level (Figure 2).

The levels of Cu and Zn in the musculature were slightly reduced as well as in the serum (Figures 2 and 3) and reduced the liver Zn level (Figure 3).

The contents of vitamin C (10.05 mg% versus 19.45 mg%, $P < .05$, Figure 4) and vitamin E (2.40 mg% versus 4.20 mg%, $P < .001$, Figure 5) were decreased in the liver

Table 1: SD, %, values for the parameters MDA, SOD, CAT, Cu, Zn, and Vitamins E and C.

Groups	MDA	SOD	CAT	Cu			Zn			Vitamin E			Vitamin C		
				liver	muscle	serum	liver	muscle	serum	liver	muscle	serum	liver	muscle	serum
Healthy controls	0.07	63.60	117.60	7.100	0.800	0.350	55.600	7.600	0.990	0.330	0.250	0.300	3.330	0.055	0.099
Infected with *E. acervulina*	0.04[1]	106.20[3]	115.20[3]	8.140	0.990	0.330	90.450	4.100	0.990	0.750	0.140	0.040	3.300	0.042	0.092
E. acervulina + Cu-Zn salt	0.05*	211.00***	152.00***	5.000	1.450	0.100	81.000	1.200	1.100	1.120	0.210	0.090	2.900	0.080	0.073

[1] $P < .05$; [2] $P < .01$; [3] $P < .001$ versus healthy (negative) controls
*$P < .05$; **$P < .01$; ***$P < .001$ versus infected (positive) controls.

Figure 4: Content of vitamin C in broiler chickens.

Figure 5: Content of vitamin E in broiler chickens.

but they did not change in the musculature in the infected chickens, as compared to the healthy broiler chickens ($P > .05$, Figure 5 and $P > .01$, Figure 4).

There was a significant decrease in the concentration of vitamin E (0.80 mg% versus 1.10 mg%, Figure 5, $P < .01$, Figure 5) and no significant change in the vitamin C ($P > .05$, Figure 4) levels in the serum in the infected chickens, compared to the healthy controls.
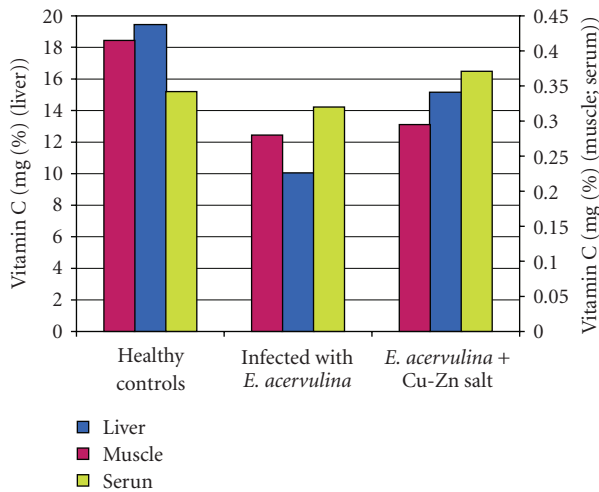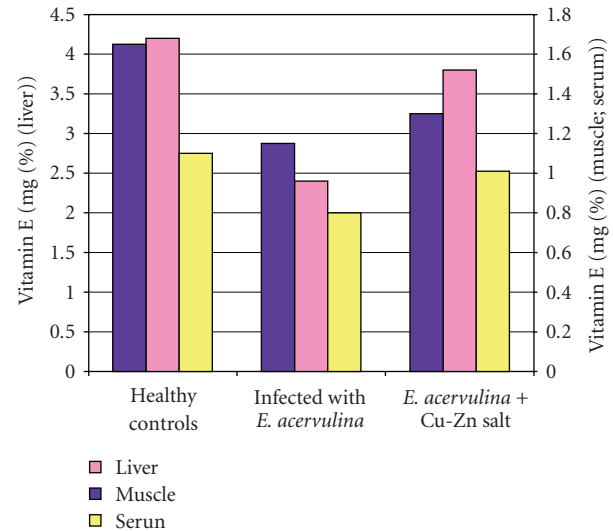
The supplementation of the basic salt restored the levels of both vitamins to the controls, increased significantly the Cu level under the control ($P < .05$, Figure 2), and restored the Zn level ($P > .05$, Figure 3) in the infected and treated chicks. The vitamin C ($P < .05$, Figure 4) and vitamin E ($P < .05$, Figure 5) levels as well as those of Cu ($P < .05$, Figure 2) and Zn ($P < .05$, Figure 3) in serum from the infected and uninfected chickens were significantly increased after the treatment.

SD, %, values for the parameters Cu, Zn, Vitamin E, and Vitamin C are present in Table 1.

4. Discussion

The production of ROS as by-products of metabolism that have the potential to damage or destroy cellular structures is

in a dynamic equilibrium under normal conditions in living organisms. It has been demonstrated that concentrations of ROS are increased in many parasitoses [1, 22, 23, 34–36]. The observed deviations in the studied enzymes and nonenzymatic parameters evidence the occurrence of oxidative stress following the infection and impaired the ecological oxidative balance (EOB) between antioxidants and pro-oxidants of chickens, infected with *E. acervulina*. In a state of impaired EOB and oxidative stress, biological systems are not protected against the oxidative radical challenge that could result in toxic damage or death of the aerobic organisms [37]. The deviations in the antioxidant status of Eimeria acervulina-infected birds, compared to healthy controls, allowed us to extend the studies upon the mechanism of avian eimeriosis, and how oxidative stress in broiler chickens could be reduced using a substances with proved antioxidant properties. Superoxide dismutase is involved in the antioxidant defense system in a first attempt (or approach) to control and eliminate the toxic ROS [38]. According to Amstad et al. [39], the decrease in the activities of antioxidant enzymes could have a negative impact on cellular resistance against the oxidant-induced damage of cell genome and cell killing. On the other hand Speranza, et al. [40] and Popova and Popov [41] reported that the antioxidant enzyme CAT was important for adaptation of

cells to oxidative stress and preserved cells via degradation of the reactive hydrogen peroxide. In the present study, the increases of plasma MDA concentrations and the marked reduction of the blood SOD activity in *E. acervulina* infected birds evidenced the occurrence of an oxidative stress due to infection and the impairment of antioxidant/pro-oxidant equilibrium in favour of pro-oxidants. The number of facts evidencing the existence of a changed expression of the principal antioxidant enzymes in various diseases is increasing, but the reports are rather conflicting [12, 42–44]. The concomitant increase of CAT activity (Figure 1) would be compensatory mechanism in infected birds against *E. acervulina*-induced oxidative damage. Similar increased CAT activity was found in birds infected with *E. tenella* [22]. The application of Cu-Zn basic salt restored the CAT enzyme antioxidant defense system in chickens infected with *E. acervulina*, but SOD activities were significantly different compared to negative controls. Probably Cu-Zn basic salt produced ROS and this finding was compromised by reduction of the SOD activity in chickens of this group compared to healthy birds ($P < .05$, Figure 1). The impaired enzyme antioxidant system may favour accumulation of ROS, which probably induced *E. acervulina* infection, too. Free radicals including ROS are known to be toxic to some parasites [45]. A more logical interpretation of increased both CAT activity and ROS production, after the salt application, is difficult to be done now. It is envisaged that ROS may also be useful in combating other kinds of skin infections and Cu-Zn basic salt to minimize the possible negative effects of *E. acervulina*. This would agree with our observation of decreased levels of MDA—marker of oxidative stress, in plasma of chickens treated with $CuZn(OH)_3Cl$.

Vitamin E is one of the antioxidants widely used in poultry diets and has been proposed as a major antioxidant in plasma membranes of all cells and subcellular organs, functioning as a chain-breaker and free radical scavenger. Poultry cannot synthesize vitamin E and its concentration is reduced under stress conditions. Vitamin C and E concentrations were dramatically reduced in infected chickens (liver-$P < .05$, serum-$P < .01$, and muscle-$P < .05$, Figure 4 and liver-$P < .001$, serum-$P < .01$ and muscle-$P < .05$, resp. Figure 5). Higher vitamin E reduction and that of vitamin C are comparable with that established in chickens infected with *Eimeria* sp. [24]. Antioxidant imbalance, increase CAT and decrease SOD, vitamins C and E levels in blood of chickens due to the eimeriosis were based on the liver hypovitaminoses C and E and reduced Zn level (Figures 3, 4, 5). The antioxidant imbalance, comprising decreased levels only of vitamins/antioxidants (vitamin E, C, retinal, and carotene), has been found in parasitize goats (34). Significant changes in activity/concentrations of antioxidant parameters in acute phase of fascioliasis in rats were observed by Kolodziejczyk et al. [46]. The authors established decreased activity of liver enzymes SOD, GSH-Px and GSSG-R, as well as a reduction of vitamin C, E, A and glutathatione levels. Vitamin E plays the most important role in the antioxidant system because it is an excellent biological chain-breaking antioxidant that protects cells and tissue from lipoperoxidative damage induced by free radicals.

Vitamin C enhances antioxidant activity of vitamin E by reducing the tocopheroxyl radicals back to their active form of vitamin E or by sparing available vitamin E [9, 13]. Regarding antioxidant property there is a synergistic effect of vitamins C and E on the antioxidant defense system in infected with *E. acervulina* chickens. Vitamins A, C, and E, Zn, and Cu act as a coordinated and balanced system to protect tissues from damage by reactive oxygen species and each relies on the action of the others [13]. There was a little information about the effect of trace elements supplementation on the antioxidant status in parasitoses. Recently, a positive effect of Zn-Cu mixed basic salt on the antioxidant imbalance in chicks infected with *Ascaridia galli* [21] and in rabbits infected with *Fasciola hepatica* [47] was established. The authors investigated the levels of vitamins C and E, the levels of Zn and Cu, as well as SOD-activity in liver of hosts (chickens and rabbits). Developed hypovitaminoses C and E and reduced Zn and Cu levels in infected chickens were restored by Zn-Cu salt supplementation. The differences in the rates of depletion of Zn, as well as vitamins C and E, depended on the parasite and host species, the parasite localization, their life cycle, the biological role, and the possible store of the elements in the host organism. The Cu level in infected with *Eimeria acervulina* and treated chickens was higher, than that in the healthy controls ($P < .001$ for liver, $P < .01$ for muscle and $P < .05$ for serum, Figure 2), but without any toxic signs. Additional studies are required to establish the optimum Cu : Zn ratio for mixed Zn-Cu crystals for an application in *eimeriosis* without any copper accumulation.

5. Conclusion

The observed deviations in the studied enzymes and nonenzymatic parameters evidence the occurrence of oxidative stress following the infection and impaired the EOB between antioxidants and pro-oxidants of chickens, infected with *Eimeria acervulina*. Our results proved the ameliorating role of $CuZn(OH)_3Cl$ (0.170 g per kg food) against *Eimeria acervulina*-induced oxidative damage in infected chickens.

References

[1] P. C. Allen, "Production of free radical species during Eimeria maxima infection in chichens," *Poultry Science*, vol. 76, no. 6, pp. 814–821, 1997.

[2] R. G. Allen and M. Tresini, "Oxidative stress and gene regulation," *Free Radical Biology and Medicine*, vol. 28, no. 3, pp. 463–499, 2000.

[3] D. Costantini and A. P. Møller, "Does immune response cause oxidative stress in birds? A meta-analysis," *Comparative Biochemistry and Physiology A*, vol. 153, no. 3, pp. 339–344, 2009.

[4] A. K. Bansal and G. S. Bilaspuri, "Impacts of oxidative stress and antioxidants on semen functions," *Veterinary Medicine International*, vol. 2011, Article ID 686137, 7 pages, 2011.

[5] D. B. Marks, A. D. Marks, and C. M. Smith, "Oxygen-metabolism and oxygen toxycity," in *Basic Medical Biochemistry. A Clinical Approach*, J. Velker, Ed., pp. 327–340, Williams & Wilkins, Baltimore, Md, USA, 1996.

[6] P. M. Abuja and R. Albertini, "Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins," *Clinica Chimica Acta*, vol. 306, no. 1-2, pp. 1–17, 2001.

[7] W. Dröge, "Free radicals in the physiological control of cell function," *Physiological Reviews*, vol. 82, no. 1, pp. 47–95, 2002.

[8] C. Lauridsen, C. Jensen, K. Jakobsen et al., "The influence of vitamin C on the antioxidative status of chickens in vivo, at slaughter and on the oxidative stability of broiler meat products," *Acta Agriculturae Scandinavica A*, vol. 47, no. 3, pp. 187–196, 1997.

[9] P. C. Allen and R. H. Fetterer, "Interaction of dietary vitamin E with Eimeria maxima infections in chickens," *Poultry Science*, vol. 81, no. 1, pp. 41–48, 2002.

[10] R. A. Dalloul, H. S. Lillehoj, T. A. Shellem, and J. A. Doerr, "Effect of vitamin A deficiency on host intestinal immune response to Eimeria acervulina in broiler chickens," *Poultry Science*, vol. 81, no. 10, pp. 1509–1515, 2002.

[11] R. A. Dalloul, H. S. Lillehoj, T. A. Shellem, and J. A. Doerr, "Intestinal immunomodulation by vitamin A deficiency and lactobacillus-based probiotic in Eimeria acervulina infected chickens broiler," *Avian Diseases*, vol. 47, no. 4, pp. 1313–1320, 2003.

[12] J. K. Miller, E. Brzezinska-Slebodzinska, and F. C. Madsen, "Oxidative stress, antioxidants, and animal function," *Journal of Dairy Science*, vol. 76, no. 9, pp. 2812–2823, 1993.

[13] P. Evans and B. Halliwell, "Micronutrients: oxidant/antioxidant status," *British Journal of Nutrition*, vol. 85, no. 2, pp. S67–S74, 2001.

[14] T. M. Bray and W. J. Bettger, "The physiological role of zinc as an antioxidant," *Free Radical Biology and Medicine*, vol. 8, no. 3, pp. 281–291, 1990.

[15] J. P. Burke and M. R. Fenton, "Effect of a zinc-deficient diet on lipid peroxidation in liver and tumor subcellular membranes," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 179, no. 2, pp. 187–191, 1985.

[16] R. Bou, F. Guardiola, A. C. Barroeta, and R. Codony, "Effect of dietary fat sources and zinc and selenium supplements on the composition and consumer acceptability of chicken meat," *Poultry Science*, vol. 84, no. 7, pp. 1129–1140, 2005.

[17] S. R. Powell, "The antioxidant properties of zinc," *Journal of Nutrition*, vol. 130, no. 5, pp. 1447–1454, 2000.

[18] M. Gabrashanska, S. E. Teodorova, and M. Anisimova, "Oxidative-antioxidant status of Fasciola hepatica-infected rats supplemented with zinc. A mathematical model for zinc bioaccumulation and host growth," *Parasitology Research*, vol. 104, no. 1, pp. 69–78, 2008.

[19] M. P. Richards and P. C. Augustine, "Serum and liver zinc, copper, and iron in chicks infected with *Eimeria acervulina* or *Eimeria tenella*," *Biological Trace Element Research*, vol. 17, pp. 207–219, 1988.

[20] N. Georgieva, K. Stoyanchev, N. Bozakova, and I. Jotova, "Combined effects of muscular dystrophy, ecological stress and selenium on blood antioxidant status in broiler chickens," *Biological Trace Element Research*. In press.

[21] M. Gabrashanska, M. Galvez Morros, N. Tsocheva-Gaytandzieva, S. Pollet, S. Ermidou-Pollet, and M. Mitov, "Antioxidant status in Ascaridia galli infected chicks treated with zinc-copper double basic salts," in *Proceedings of the 4th International Symposium on Trace Elements: New Perspectives*, pp. 845–854, Athens, Greece, October 2003.

[22] N. V. Georgieva, V. Koinarski, and V. Gadjeva, "Antioxidant status during the course of *Eimeria tenella* infection in broiler chickens," *Veterinary Journal*, vol. 172, no. 3, pp. 488–492, 2006.

[23] V. Koinarski, N. Georgieva, V. Gadjeva, and P. Petkov, "Antioxidant status of broiler chickens, infected with *Eimeria acervulina*," *Revue de Medecine Veterinaire*, vol. 156, no. 10, pp. 498–502, 2005.

[24] V. Koinarski, M. Gabrashanska, N. Georgiva, and P. Petkov, "Anitioxidant parameters in *Eimeria acervulina* infected chicks after treatment with a new zinc compound," *Bulletin of the Veterinary Institute in Pulawy*, vol. 50, no. 1, pp. 55–61, 2006.

[25] L. Markov, *Synthesis and thermal decomposition of Me(II)-Co(II) hydroxonitrate mixed crystals with layer structure type (Me=Mg, Ni, Cu, Zn)*, Ph.D. thesis, Bulgarian Academy of Sciences, Sofia, Bulgaria, 1987.

[26] G. M. Willis and D. H. Baker, "*Eimeria acervulina* infection in the chicken: a model system for estimating nutrient requirements during coccidiosis," *Poultry science*, vol. 60, no. 8, pp. 1884–1891, 1981.

[27] Z. A. Placer, L. L. Cushman, and B. C. Johnson, "Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems," *Analytical Biochemistry*, vol. 16, no. 2, pp. 359–364, 1966.

[28] Y. Sun, L. W. Oberley, and Y. Li, "A simple method for clinical assay of superoxide dismutase," *Clinical Chemistry*, vol. 34, no. 3, pp. 497–500, 1988.

[29] R. F. Beers and I. W. Sizer, "A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase," *The Journal of biological chemistry*, vol. 195, no. 1, pp. 133–140, 1952.

[30] Anonnymous, *Analytical methods for atomic absorption spectrophotometry*, Perkin-Elmer, Norwalk. Conn, USA, 1982.

[31] J. Bieri and T. Tolliver, "On the occurance of alpha-tocopherol in rat tissues," *Lipids*, vol. 16, pp. 777–779, 1981.

[32] S. T. Omaye, J. D. Turnbul, and H. E. Saveberlich, "Ascorbic acid analysis. II. Determination after derivatisation with 2,2-dinitrophenylhydrazine. Selected methods for determination of ascorbic acid in animal cells,tissues and fluids," *Method in Enzymology, London*, vol. 62, p. 7, 1979.

[33] J. J. Mahoney, H. J. Vreman, D. K. Stevenson, and A. L. Van Vessel, "Measurements of carboxyhaemoglobin by five spectrophotometers (cooximeters) in comparison with reference methods," *Clinical Chemistry*, vol. 39, pp. 1693–1700, 1993.

[34] S. Dede, Y. Değer, S. Değer, T. Kahraman, M. Alkan, and M. Cemek, "Oxidation products of nitric oxide and the concentration of antioxidant vitamins in parasitise goats," *Acta Veterinaria Brno*, vol. 71, no. 3, pp. 341–345, 2002.

[35] H. S. Lillehoj and G. Li, "Nitric oxide production by macrophages stimulated with coccidia sporozoites, lipopolysaccharide, or interferon-γ, and its dynamic changes in SC and TK strains of chickens infected with Eimeria tenella," *Avian Diseases*, vol. 48, no. 2, pp. 244–253, 2004.

[36] H. Karamouz, H. Shahriar, and R. Doust, "Response of male broiler to different levels of food industries residual oil on serum lipoproteins, lipid peroxidation and total antioxidant status," *American-Eurasian Journal of Agricultural & Environmental Sciences*, vol. 6, no. 2, pp. 252–256, 2009.

[37] N. V. Georgieva, "Oxidative stress as a factor of disrupted ecological oxidative balance in biological systems—a review," *Bulgarian Journal of Veterinary Medicine*, vol. 8, no. 1, pp. 1–11, 2005.

[38] J. M. McCord, "The superoxide free radical: its biochemistry and pathophysiology," *Surgery*, vol. 94, no. 3, pp. 412–414, 1983.

[39] P. Amstad, R. Moret, and P. Cerutti, "Glutathione peroxidase compensates for the hypersensitivity of Cu,Zn- superoxide dismutase overproducers to oxidant stress," *Journal of Biological Chemistry*, vol. 269, no. 3, pp. 1606–1609, 1994.

[40] M. J. Speranza, A. C. Bagley, and R. E. Lynch, "Cells enriched for catalase are sensitized to the toxicities of bleomycin, adriamycin, and paraquat," *Journal of Biological Chemistry*, vol. 268, no. 25, pp. 19039–19043, 1993.

[41] M. P. Popova and C. S. Popov, "Effect of heavy metal salts on the activity of rat liver and kidney catalase and lysosomal hydrolases," *Journal of Veterinary Medicine Series A*, vol. 45, no. 6-7, pp. 343–351, 1998.

[42] J. Challey, "The effect of caecal coccidiosis infections and experimental hemorhage upon adrenal ascorbic acid levels in the chickens," *The Journal of Parasitology*, vol. 46, pp. 727–731, 1960.

[43] A. M. Baker, L. W. Oberley, and M. B. Cohen, "Expression of antioxidant enzymes in human prostatic adenocarcinoma," *Prostate*, vol. 32, no. 4, pp. 229–233, 1997.

[44] J. M. Matés, C. Pérez-Gómez, and I. N. De Castro, "Antioxidant enzymes and human diseases," *Clinical Biochemistry*, vol. 32, no. 8, pp. 595–603, 1999.

[45] G. M. Rosen, S. Pou, C. L. Ramos, M. S. Cohen, and B. E. Britigan, "Free radicals and phagocytic cells," *FASEB Journal*, vol. 9, no. 2, pp. 200–209, 1995.

[46] L. Kolodziejczyk, E. Siemieniuk, E. Skrzydlewska, and A. Machoy-Mokrzynska, "Antioxidant abilities of rat liver in acute fascioliasis," in *Proceedings of the 9th European Multicolloquium of Parasitology*, p. 538, Valencia, Spain, July 2004.

[47] N. Tsocheva-Gaytandzhieva, M. Gabrashanska, M. Galvez-Morros, B. Soto de Zaldivar, S. Pollet, and S. Ermidou-Pollet, "Liver antioxidant status under acute fascioliasis and $(Zn_xCu_{1-x})2Cl$ supplementation in rabbits," in *Proceedings of the 22nd Macro and Trace Elements Workshop*, vol. 1, pp. 619–624, Jena, Germany, 2004.

Research Article

Hemotoxicity Induced by Chronic Chlorpyrifos Exposure in Wistar Rats: Mitigating Effect of Vitamin C

Suleiman F. Ambali,[1] Joseph O. Ayo,[1] King A. N. Esievo,[2] and Samuel A. Ojo[3]

[1] *Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria 800007, Nigeria*
[2] *Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria 800007, Nigeria*
[3] *Department of Veterinary Anatomy, Ahmadu Bello University, Zaria 800007, Nigeria*

Correspondence should be addressed to Suleiman F. Ambali, fambali2001@yahoo.com

The study evaluated the ameliorative effect of vitamin C on chronic chlorpyrifos-induced hematological alterations in Wistar rats. Twenty adult male rats divided into 4 groups of 5 animals each were exposed to the following regimens: group I (S/oil) was administered soya oil (2 mL/kg b.w.), while group II (VC) was given vitamin C (100 mg/kg b.w.); group III was dosed with CPF (10.6 mg/kg b.w.); group IV was pretreated with vitamin C (100 mg/kg) and then exposed to CPF (10.6 mg/kg b.w.), 30 minutes later. The regimens were administered by oral gavage once daily for a period of 17 weeks. Blood samples collected at the end of the study revealed reduction in the levels of pack cell volume, hemoglobin, red blood cells, leukocytes (attributed to neutropenia, lymphopenia, and monocytopenia), and platelets in the CPF group, which were ameliorated in the vitamin C- pretreated group. The elevated values of malonaldehyde, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and neutrophil/lymphocyte ratio in the CPF group were restored in those pretreated with vitamin C. The study has shown that chronic CPF-induced adversity on hematological parameters of Wistar rats was mitigated by pretreatment with vitamin C.

1. Introduction

Organophosphate (OP) insecticides are used in the agricultural and domestic pest control [1], accounting for 50% of the global insecticidal use [2]. Their use is, however, accompanied by widespread toxicity in nontarget organisms, including man. Chlorpyrifos (CPF) is one of the most widely used OP insecticides until 2000 when the United States Environmental Protection Agency restricted some of its domestic uses due to its toxicity. Despite this, CPF remains one of the most widely used OP insecticides. Anemia and alteration in other hematological parameters have been recorded following repeated CPF exposure [3, 4]. Although the mechanism of acute CPF toxicity involves acetylcholinesterase (AChE) inhibition, other mechanisms unrelated to AChE inhibition, including the induction of oxidative stress, have been implicated [4–8]. As a lipophilic molecule, CPF easily passes through the cells into the cytoplasm [9]. Once inside the cell, CPF induces damage to the cellular molecules [10].

Oxidative damage primarily occurs through production of reactive oxygen species (ROS) which causes damage to macromolecules such as lipids, proteins, and DNA. Under normal circumstances, the body copes with oxidative assault through the repair of the damage or the invocation of the indigenous antioxidant enzymatic and nonenzymatic systems to reduce the pro-oxidation states. However, in situation of increased and accelerated oxidative challenge by CPF as previously reported [4–8], the natural antioxidant mechanisms are overwhelmed thereby resulting in damage. Therefore, supplementation with exogenous source of antioxidant is likely to reduce the oxidative burden, hence tissue damage. Vitamin C is one of the most widely available and affordable nonenzymatic antioxidant molecules that have been used to mitigate oxidative damage. It is an important water-soluble antioxidant in biological fluids [11, 12]. It readily scavenges physiological ROS such as superoxide, hydroxyl, and aqueous peroxyl radicals, as well as nonradical species such as singlet oxygen and ozone, as well as reactive

nitrogen species (RNS) such as peroxynitrite, nitrosating species (N_2O_3/N_2O_4), nitroxide radicals, and nitrogen dioxide [13, 14]. The reduction in CPF-induced toxicity following vitamin C supplementation has been reported previously [6, 7]. The aim of the present study is therefore to evaluate the mitigating effect of vitamin C on hematological changes induced by chronic CPF exposure in Wistar rats.

2. Materials and Methods

2.1. Animals and Housing. Twenty young adult male Wistar rats weighing 95–110 g were obtained from the Laboratory Animal Unit of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. They were housed in metal cages and fed on standard rat chow, and water was provided *ad libitum*. The animals were allowed to acclimatize for at least one week. The housing and management of the animals and the experimental protocols were conducted as stipulated in the Guide for Care and Use of Laboratory Animals [15].

2.2. Chemicals. Commercial grade CPF (Termicot, 20% EC, Sabero Organics, Gujarat, India) was dissolved in soya oil (Grand Cereal, Jos, Nigeria), while each tablet of vitamin C, Med Vit C (100 mg/tablet; Dol-Med Laboratories Limited, Lagos, Nigeria), was dissolved in 1mL of distilled water to obtain 100 mg/mL suspension, just prior to its daily administration.

2.3. Experimental Protocol. The rats were weighed using digital weighing balance and then assigned randomly into 4 groups of 5 rats in each group. Rats in group I served as the control group (S/oil) and were given only soya oil (2 mL/kg b.w.), while those in group II (VC) were dosed with vitamin C (100 mg/kg b.w.). Rats in group III (CPF) were administered with CPF only (10.6 mg/kg b.w. ~1/8th LD_{50} of 85 mg/kg) [16], while those in group IV (VC+CPF) were pretreated with vitamin C (100 mg/kg) and then dosed with CPF (10.6 mg/kg b.w), 30 min later. The different regimens were administered once daily by oral gavage for a period of 17 weeks. At the end of the study period, the rats were sacrificed by severing the jugular vein after light ether anesthesia.

2.4. Hematological Evaluation. Two milliliters of blood collected into heparinized sample bottles were analyzed for hematological parameters such as pack cell volume (PCV), hemoglobin (Hb), total red blood cells (RBCs), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cell (WBC), and total platelets count using an automatic hematological assay analyzer, Advia 60 Hematology system (Bayer Diagnostics Europe Ltd, Ireland). Blood smears were also stained with Giemsa for absolute differential WBC count [17], while the neutrophil-lymphocyte ratio was calculated.

2.5. Evaluation of Erythrocytes Malonaldehyde Concentration. The erythrocyte malonaldehyde (MDA) concentration, as a marker of lipid peroxidation, was determined by the double heating method of Draper and Hadley [18], as we described previously [4, 6]. The principle of the method was spectrophotometric measurement of the colour produced during the reaction of thiobarbituric acid (TBA) with MDA. One milliliter of heparinized blood samples obtained from each animal was centrifuged at 600 g and the plasma discarded. Erythrocyte packets were prepared by washing erythrocytes three times in cold isotonic saline (0.9% w/v). The washed erythrocytes were used to analyze for MDA concentrations. Briefly, 2.5 mL of 100 g/L trichloroacetic acid was added to 0.5 mL of erythrocytes in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at $1000 \times$ g for 10 min, and 2 mL of the supernatant was added to 1 mL of 6.7 g/L TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water, and its absorbance measured using a UV spectrophotometer (Jenway, 6405 model, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex, $1.56 \times 10^5\ cm^{-1}\ M^{-1}$, and expressed in nanomoles per gram of hemoglobin. The hemoglobin concentration was determined using the method of Dacie and Lewis [19].

2.6. Statistical Analysis. Values obtained as mean ± SEM were subjected to one-way analysis of variance (ANOVA) followed by Tukey test using GraphPad Prism version 4.0 for windows from GraphPad Software, San Diego, California, USA). Values of $P < .05$ were considered significant.

3. Results

3.1. Effects of Treatments on Pack Cell Volume. The PCV recorded for rats in the CPF group was significantly lower compared to either the S/oil ($P < .05$) or the VC ($P < .01$) group. There was no significant change in the PCV of rats in the VC+CPF group compared to any of the other groups (Table 1).

3.2. Effect of Treatments on Hemoglobin Concentration. The Hb concentration was significantly lower in the CPF group compared to either the S/oil ($P < .05$) or the VC ($P < .01$) group. There was no significant difference ($P > .05$) in the Hb of VC+CPF group compared to either the S/oil, VC, or CPF group (Table 1).

3.3. Effect of Treatments on Total Red Blood Cell Concentration. A significantly lower RBC concentration was recorded in the CPF group compared to either the S/oil ($P < .01$), VC ($P < .01$), or VC+CPF ($P < .05$) group. The RBC concentration in VC+CPF group was significantly lower ($P < .05$) compared to those recorded in the VC group, but was marginally higher than in the CPF group (Table 1).

3.4. Effect of Treatments on Red Blood Cell Indices. The effect of treatments on MCV, MCH, and MCHC is shown in Table 1. The MCV and MCH in the CPF group were

Table 1: Effect of chronic exposure to soya oil (S/oil), vitamin C (VC), and/or chlorpyrifos (CPF) on pack cell volume (PCV), red blood cell (RBC) and hemoglobin (Hb) concentrations, and erythrocyte indices in Wistar rats.

Parameters	S/oil	VC	CPF	VC+CPF
PCV (%)	42 ± 1.6	44 ± 1.3	35 ± 1.2[ab]	40 ± 0.95
Hb (g/dL)	14 ± 0.53	15 ± 0.64	12 ± 0.37[ab]	13 ± 0.31
RBC count ($\times 10^{12}$/L)	5.8 ± 0.49	6.8 ± 0.3	3.7 ± 0.15[abc]	5.1 ± 0.07[d]
MCV (fL/cell)	79 ± 11	63 ± 6.0	9.3 ± 3.1[b]	7.9 ± 3.0
MCH (pg/cell)	25 ± 2.7	24 ± 2.2	33 ± 2.1[b]	26 ± 0.84
MCHC (g/dL)	33 ± 1.5	33 ± 2.2	34 ± 2.0	33 ± 0.8

[a]$P < .01$ versus soya oil group; [b]$P < .01$ versus vitamin C group; [c]$P < .05$ versus vitamin C+chlorpyrifos group; [d]$P < .01$ versus vitamin C group. Values are mean ± SEM of 5 animals per group. NB-MCV: mean cell volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

significantly elevated ($P < .05$) compared to those recorded in the VC group. There was no significant change ($P > .05$) in MCHC in between the groups. Anisocytosis was also observed in the CPF group compared to normocytosis in the other groups.

3.5. Effect of Treatments on Total and Absolute Differential White Blood Cell Counts.

There was a significant decrease ($P < .01$) in the WBC counts of CPF group compared to either S/oil or VC group. The WBC concentration in the VC+CPF group was significantly lower ($P < .05$) compared to those recorded in the VC group (Table 2).

The neutrophil count in the CPF group was significantly lower compared to the S/oil ($P < .01$), VC ($P < .01$) and VC+CPF ($P < .05$) groups, respectively. The neutrophil count in the VC+CPF group was significantly lower ($P < .01$) compared to either the S/oil or VC group. The lymphocyte count in the CPF group was significantly lower ($P < .01$) compared to either the S/oil, VC, or VC+CPF group. The lymphocyte count of VC+CPF group was significantly lower compared to either S/oil ($P < .05$) or VC ($P < .01$) group. The monocyte count in the CPF group was significantly lower in the CPF group compared to either S/oil ($P < .05$) or VC ($P < .01$) group. The monocyte count in the VC+CPF group was significantly lower ($P < .05$) compared to the VC group (Table 2).

3.6. Effect of Treatments on Neutrophil/Lymphocyte Ratio.

The neutrophil/lymphocyte ratio in the CPF group was significantly higher compared to either the S/oil ($P < .05$), VC ($P < .05$), or VC+CPF ($P < .01$) group. The neutrophil/lymphocyte ratio of VC+CPF group was not significantly different ($P > .05$) from those obtained in either the S/oil or VC group (Table 2).

3.7. Effect of Treatments on Platelet Count.

The platelet count in the CPF group was significantly lower compared to either the S/oil ($P < .01$), VC ($P < .01$), or VC+CPF ($P < .05$)
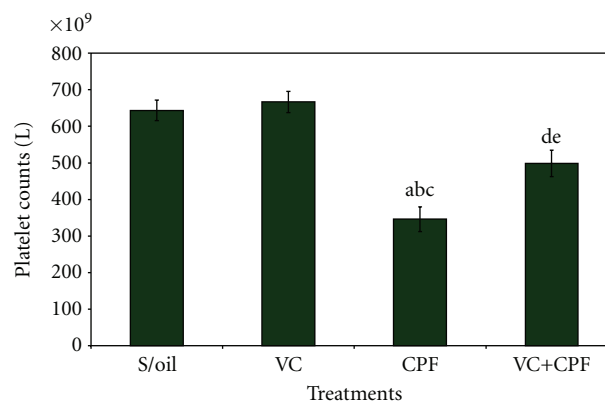
Figure 1: Effect of chronic exposure to soya oil (S/oil), vitamin C (VC), and/or chlorpyrifos (CPF) on platelet counts in Wistar rats. [a]$P < .01$ versus soya oil group; [b]$P < .01$ versus vitamin C group; [c]$P < .05$ versus vitamin C+chlorpyrifos group; [d]$P < .05$ versus soya oil group; [e]$P < .05$ versus vitamin C group. Values are mean ± SEM of 5 animals per group.
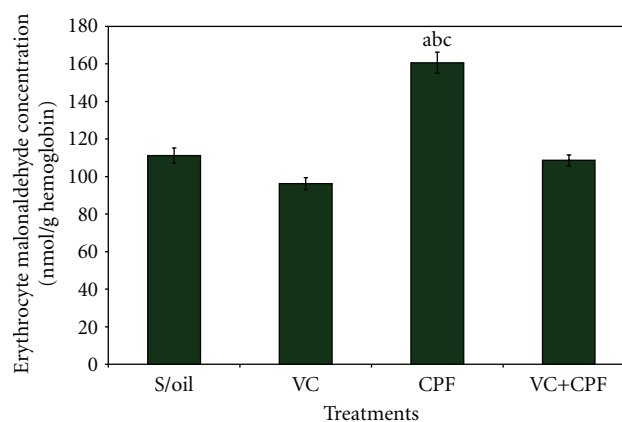
Figure 2: Effect of chronic exposure to soya oil (S/oil), vitamin C (VC), and/or chlorpyrifos (CPF) on erythrocytes malonaldehyde concentration. [abc]$P < .01$ versus soya oil, vitamin C, and vitamin C+chlorpyrifos groups, respectively.

group. The platelet count recorded in the VC+CPF group was significantly lower ($P < .05$) relative to either the S/oil or VC group (Figure 1).

3.8. Effect of Treatments on Erythrocyte Malonaldehyde Concentration.

The erythrocyte MDA concentration in the CPF group was significantly higher ($P < .01$) compared to those obtained in the soya oil, VC, and VC+CPF groups, respectively. The MDA concentration in VC+CPF group was not significantly different from those recorded in either the VC or the S/oil group (Figure 2).

4. Discussion

The low hematological parameters of PCV, Hb, and RBC show that chronic CPF administration causes anemia. This

TABLE 2: Effect of chronic exposure to soya oil (S/oil), vitamin C (VC), and/or chlorpyrifos (CPF) on absolute total and differential leukocyte count in Wistar rats.

Parameters ($\times 10^9$/L)	S/oil	VC	CPF	VC+CPF
Total leukocyte count ($\times 10^9$/L)	9.9 ± 0.81[a]	9.9 ± 0.89	5.4 ± 0.37[ab]	8.4 ± 0.5[i]
Neutrophils count ($\times 10^9$/L)	4.4 ± 0.12	4.9 ± 0.19	2.7 ± 0.15[abc]	3.4 ± 0.16[de]
Lymphocytes count ($\times 10^9$/L)	5.3 ± 0.18	5.9 ± 0.16	3.0 ± 0.13[abf]	4.5 ± 0.19[eg]
Monocytes count ($\times 10^9$/L)	0.07 ± 0.01	0.09 ± 0.02	0.0 ± 0,0[ch]	0.03 ± 0.01[i]
Band cells count ($\times 10^9$/L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.01 ± 0.003
Neutrophil : Lymphocyte ratio	0.84 ± 0.05	0.82 ± 0.09	1.0 ± 0.0[abc]	0.76 ± 0.04

[a]$P < .01$ versus soya oil group; [b]$P < .01$ versus vitamin C group; [c]$P < .05$ versus vitamin C+chlorpyrifos group; [d]$P < .01$ versus Soya oil group; [e]$P < .01$ versus Vitamin C group; [f]$P < .01$ versus vitamin C+chlorpyrifos group; [g]$P < .05$ versus Soya oil group; [h]$P < .05$ versus soya oil group; [i]$P < .05$ versus vitamin C group. Values are mean ± SEM of 5 animals per group.

agreed with earlier findings [3, 4, 20, 21]. Goel et al. [3] attributed the anemia to the ability of CPF to reduce serum iron concentration, thereby compromising the synthesis of Hb. The anemia may also be related to interference with Hb synthesis and shortening of RBC lifespan [22]. We have earlier shown that chronic CPF exposure causes increased erythrocyte fragility, partly due to increased lipoperoxidation of the erythrocyte membranes [4, 7, 8]. The increased lipoperoxidation in the CPF group, reflected by significant MDA concentration, may have caused increased vulnerability of the RBC to destruction, but may directly destroy the erythrocytes thereby leading to anemia. MDA is a major oxidation product of peroxidized polyunsaturated fatty acids (PUFAs), and increased MDA content is an important indicator of lipid peroxidation [23].

The RBC is susceptible to lipoperoxidative changes because of its direct association with molecular oxygen, high content of metal ions catalyzing oxidative reactions, and availability of high amount of PUFAs, which are susceptible to lipid peroxidation. Inability to repair membrane damage and regenerate due to lack of nucleus and poor antioxidant enzymes composition of the plasma medium in which they are bathed [24, 25] are some of the other factors responsible for the increased vulnerability of RBC to lipoperoxidation. Therefore, CPF-induced oxidative damage to the erythrocyte membrane may have contributed to the anemia recorded in the CPF group. This is because the process of lipid peroxidation impairs the functions and homeostasis of the erythrocyte membranes through decrease in hydrophobic characteristics of bilayer membrane, and altering the affinity and interaction of proteins and lipids [26]. ROS can equally affect the proteins resulting in modification of enzymes activity, and damage to the membrane transport proteins may produce disturbed cellular ionic homeostasis, leading to alterations in intracellular calcium and potassium that triggers a series of changes in the cell [27]. ROS can directly affect the conformation and/or activities of all sulfhydryl-containing molecules, by oxidation of their thiol moiety [28, 29]. The combined effect of these ROS-triggered cellular changes may eventually lead to cellular dysfunction and ultimate destruction.

Anisocytosis observed in the CPF group in the present study had also been recorded in earlier studies [3, 4].

The increased MCV may reflect the presence of immature RBCs in the peripheral blood, perhaps arising from the body compensatory mechanism to cater for the CPF-induced deficit in RBC concentration. The increased presence of immature RBCs may be similarly responsible for the anisocytosis observed in the CPF group. The significant increase in MCH in the CPF group shows that the amount of Hb in this group is high, while the apparently normal MCHC indicates normal Hb concentration. Therefore, the OP insecticide can be said to induce macrocytic anemia.

The lack of significant increase in PCV and concentrations of RBCs and Hb recorded in group pretreated with vitamin C when compared to the S/oil or VC group was an indication of the attenuation of CPF-evoked anemia by the antioxidant vitamin. In its reduced form, vitamin C has been shown to improve the absorption of iron from the gut [30, 31], thereby increasing its serum concentration of iron essential for heme synthesis. This is by facilitating the reduction of ferric iron to the ferrous form [32]. Besides, vitamin C has also been shown to be beneficial in the management of anemia [33]. Furthermore, the amelioration of the anemia in the group pretreated with vitamin C may be due to reduction in lipoperoxidative damage to the erythrocyte membrane as demonstrated by its low MDA concentration in the present study. Similarly, the low erythrocyte fragility observed in our earlier study following vitamin C supplementation of rats chronically exposed to CPF [7] may have contributed to the mitigation of anemia in the present study.

The present study also revealed leucopenia apparently due to lymphopenia, neutropenia, and monocytopenia in the CPF group. Previous studies have attributed CPF-induced leucopenia to neutropenia [6] and lymphopenia [3, 4]. Ambali et al. [4] reported neutrophilia following CPF exposure, in contrary to neutropenia recorded in the present study. Levine et al. [34] attributed monocytopenia recorded in workers exposed to OP to inhibition of a monocyte esterase, [alpha]-naphthyl butyrate esterase. Many pesticides have been shown to induce immunotoxicity either via the induction of apoptosis or necrosis [35, 36]. CPF exposure has been shown to induce immunotoxicity via the induction of apoptosis partly mediated through the activation of caspase 3 [37]. Chronic CPF exposure has been associated with

abnormality of the immune system including depression of T-lymphocytes [38]. Immunotoxicity in OPs has been associated with either inhibition of serine hydrolases or esterases in components of the immune system, through oxidative damage to immune organs, or by modulation of signal transduction pathways controlling immune functions [39]. Free radical-induced oxidative damage that has been widely implicated in the molecular mechanism of CPF cytotoxicity is an initiator of apoptosis [35, 40], which may have been involved in the depletion of the components of the WBC in the group exposed to the OP in the present study.

Vitamin C pretreatment was able to mitigate the CPF-induced immunotoxicity by restoring the concentration of leukocytes and its components. The ability of vitamin C to restore subchronic CPF-induced leucopenia has been demonstrated in our earlier study [6]. Vitamin C has been shown to enhance immune response via numerous mechanisms, including lymphocytes proliferation [41, 42]. Besides, the antioxidant function of the vitamin has been shown to inhibit apoptosis [43, 44].

The increase in the neutrophil/lymphocyte ratio (NLR) in the CPF group recorded in the present study has been reported previously in our laboratory following subchronic CPF exposure [7]. NLR provides an indication of inflammatory status in patients [45] and has been used as a prognostic factor in predicting clinical outcomes of a disease process and in the situation of increased stress or inflammation [45–47]. NLR correlates well with the magnitude of total leukocyte response and may provide a parameter that is more sensitive than the total leukocyte count in a disease process [48]. The elevated NLR in the CPF group in this study is a demonstration of ongoing stress and inflammatory process in rats from this group, predicating bad clinical outcomes. The NLR in the group pretreated with vitamin C was not significantly different from those observed in the group administered either soya oil or vitamin C only, indicating amelioration of CPF-induced stress and inflammatory process in the group, partly due to protection from oxidative damage by the antioxidant vitamin.

The significant decrease in platelet count in the CPF group shows that chronic exposure to the insecticide caused thrombocytopenia. This finding contradicted what we reported earlier [4] that recorded thrombocytosis following subchronic CPF exposure. The reason for the discrepancy is not clear but may be related to the duration of exposure. Thrombocytopenia may be related to CPF-induced oxidative damage to the platelet membranes. A direct relationship between oxidative stress and thrombocytopenia has been demonstrated in patients infected with malaria parasites [49]. The significant improvement in the level of thrombocytes in group pretreated with the vitamin further underscored the role of oxidative stress in CPF-induced thrombocytopenia.

In conclusion, the present study has shown that vitamin C pretreatment ameliorated the chronic CPF-induced hemotoxicity in Wistar rats. This may be partly due to free radical scavenging properties of the antioxidant vitamin, which attenuated CPF-evoked lipoperoxidation to the blood cellular constituents. However, the other nonantioxidant role of vitamin C may have also complemented this antioxidant mechanism of cytoprotection. Therefore, the results of this study give an indication that vitamin C supplementation may mitigate hemotoxicity in individuals who are at risk of prolonged CPF exposure.

References

[1] D. Donaldson, T. Kiely, and A. Grube, "Pesticides industry sales and usage 1998 and 1999 market estimates," U.S. Environmental Protection Agency, Washington, DC, USA, 2002, http://www.epa.gov/opp00001/pestsales/99pestsales/market_estimates1999.pdf.

[2] J. E. Casida and G. B. Quistad, "Organophosphate toxicology: safety aspects of nonacetylcholinesterase secondary targets," *Chemical Research in Toxicology*, vol. 17, no. 8, pp. 983–998, 2004.

[3] A. Goel, V. Dani, and D. K. Dhawan, "Role of zinc in mitigating the toxic effects of chlorpyrifos on hematological alterations and electron microscopic observations in rat blood," *BioMetals*, vol. 19, no. 5, pp. 483–492, 2006.

[4] S. F. Ambali, A. T. Abubakar, M. Shittu, L. S. Yaqub, S. B. Anafi, and A. Abdullahi, "Chlorpyrifos-induced alteration of hematological parameters in Wistar rats: ameliorative effect of zinc," *Research Journal of Environmental Toxicology*, vol. 4, no. 2, pp. 55–66, 2010.

[5] F. Gultekin, N. Delibas, S. Yasar, and I. Kilinc, "*In vivo* changes in antioxidant systems and protective role of melatonin and a combination of vitamin C and vitamin E on oxidative damage in erythrocytes induced by chlorpyrifos-ethyl in rats," *Archives of Toxicology*, vol. 75, no. 2, pp. 88–96, 2001.

[6] S. Ambali, D. Akanbi, N. Igbokwe, M. Shittu, M. Kawu, and J. Ayo, "Evaluation of subchronic chlorpyrifos poisoning on hematological and serum biochemical changes in mice and protective effect of vitamin C," *Journal of Toxicological Sciences*, vol. 32, no. 2, pp. 111–120, 2007.

[7] S. F. Ambali, J. O. Ayo, S. A. Ojo, and K. A. N. Esievo, "Ameliorative effect of vitamin C on chlorpyrifos-induced increased erythrocyte fragility in Wistar rats," *Human and Experimental Toxicology*, vol. 30, no. 1, pp. 19–24, 2010.

[8] S. F. Ambali, A. T. Abubakar, M. Shittu, L. S. Yaqub, P. I. Kobo, and A. Giwa, "Zinc ameliorates chlorpyrifos-induced increased erythrocyte fragility and lipoperoxidative changes in Wistar rats," *New York Science Journal*, vol. 3, pp. 117–122, 2010.

[9] F. G. Uzun, F. Demir, S. Kalender, H. Bas, and Y. Kalender, "Protective effect of catechin and quercetin on chlorpyrifos-induced lung toxicity in male rats," *Food and Chemical Toxicology*, vol. 48, no. 6, pp. 1714–1720, 2010.

[10] S. Ncibi, M. Ben Othman, A. Akacha, M. N. Krifi, and L. Zourgui, "*Opuntia ficus indica* extract protects against chlorpyrifos-induced damage on mice liver," *Food and Chemical Toxicology*, vol. 46, no. 2, pp. 797–802, 2008.

[11] B. Frei, L. England, and B. N. Ames, "Ascorbate is an outstanding antioxidant in human blood plasma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 16, pp. 6377–6381, 1989.

[12] B. Frei, R. Stocker, L. England, and B. N. Ames, "Ascorbate: the most effective antioxidant in human blood plasma," *Advances in Experimental Medicine and Biology*, vol. 264, pp. 155–163, 1990.

[13] B. Halliwell, "Mechanisms involved in the generation of free radicals," *Pathologie Biologie*, vol. 44, no. 1, pp. 6–13, 1996.

[14] A. Carr and B. Frei, "Does vitamin C act as a pro-oxidant under physiological conditions?" *FASEB Journal*, vol. 13, no. 9, pp. 1007–1024, 1999.

[15] NRC, *Guide for the Care and Use of Laboratory Animals*, National Research Council, Academic Press, Washington, DC, USA, 1996.

[16] S. F. Ambali, *Ameliorative effect of vitamins C and E on neurotoxicological, hematological and biochemical changes induced by chronic chlorpyrifos in Wistar rats*, Ph.D. Dissertation, Ahmadu Bello University, Zaria, Nigeria, 2009.

[17] J. V. Gulye, J. Z. Camicas, and A. M. Diouf, "Ticks and blood parasites in Senegal (Sahlian zone)," *Revue d Elevage et de Medecine Veterinaire des Pays Tropicaux*, vol. 40, pp. 119–125, 1988.

[18] H. H. Draper and M. Hadley, "Malondialdehyde determination as index of lipid peroxidation," *Methods in Enzymology*, vol. 186, pp. 421–431, 1990.

[19] J. V. Dacie and S. M. Lewis, *Practical Haematology*, Churchill Livingstone, London, UK, 7th edition, 1991.

[20] T. Barna-Lloyd, J. R. Szabo, and N. L. Davis, "Chlorpyrifos-methyl (Reldan R) rat subchronic dietary toxicity and recovery study," Unpublished Report TXT K-046193-026, Dow Chemical, Tex, USA, submitted to WHO by Dow Elanco, Ind, USA, 1990.

[21] T. Barna-Lloyd, J. R. Szabo, and N. L. Davis, "Chlorpyrifos-methyl (Reldan R) rat chronic dietary toxicity/oncogenicity study," Unpublished Report TXT K-046193-031, Dow Chemical, Tex, USA, submitted to WHO by Dow Elanco, Ind, USA, 1991.

[22] D. E. Ray, *Pollution and Health*, Wiley Eatern Ltd., New Delhi, India, 1992.

[23] I. Celik and H. Suzek, "Effects of subacute exposure of dichlorvos at sublethal dosages on erythrocyte and tissue antioxidant defense systems and lipid peroxidation in rats," *Ecotoxicology and Environmental Safety*, vol. 72, no. 3, pp. 905–908, 2009.

[24] S. L. Marklund, E. Holme, and L. Hellner, "Superoxide dismutase in extracellular fluids," *Clinica Chimica Acta*, vol. 126, no. 1, pp. 41–51, 1982.

[25] Ö. Etlik and A. Tomur, "The oxidant effects of hyperbaric oxygenation and air pollution in erythrocyte membranes (hyperbaric oxygenation in air pollution)," *European Journal of General Medicine*, vol. 3, no. 1, pp. 21–28, 2006.

[26] R. Dargel, "Lipid peroxidation—a common pathogenetic mechanism?" *Experimental and Toxicologic Pathology*, vol. 44, no. 4, pp. 169–181, 1992.

[27] L. D. Kerr, J. I. Inoue, and I. M. Verma, "Signal transduction: the nuclear target," *Current Opinion in Cell Biology*, vol. 4, no. 3, pp. 496–501, 1992.

[28] K. A. Webster, H. Prentice, and N. H. Bishopric, "Oxidation of zinc finger transcription factors: physiological consequences," *Antioxidants and Redox Signaling*, vol. 3, no. 4, pp. 535–548, 2001.

[29] D. E. Wilcox, A. D. Schenk, B. M. Feldman, and Y. Xu, "Oxidation of zinc-binding cysteine residues in transcription factor proteins," *Antioxidants and Redox Signaling*, vol. 3, no. 4, pp. 549–564, 2001.

[30] G. M. Wardlaw, *Perspectives in Nutrition*, McGraw-Hill, New York, NY, USA, 4th edition, 1999.

[31] K. Iqbal, A. Khan, and M. A. K. Khattak, "Biological significance of ascorbic acid (Vitamin C) in human health—a review," *Pakistan Journal of Nutrition*, vol. 3, no. 5, pp. 5–13, 2004.

[32] M. H. Sayers, S. R. Lynch, P. Jacobs et al., "The effects of ascorbic acid supplementation on the absorption of iron in maize, wheat and soya," *British Journal of Haematology*, vol. 24, no. 2, pp. 209–218, 1973.

[33] K. Gastaldello, A. Vereerstraeten, T. Nzame-Nze, J. L. Vanherweghem, and C. Tielemans, "Resistance to erythropoietin in iron-overloaded haemodialysis patients can be overcome by ascorbic acid administration," *Nephrology Dialysis Transplantation*, vol. 10, supplement 6, pp. S44–S47, 1995.

[34] M. S. Levine, N. L. Fox, and B. Thompson, "Inhibition of esterase activity and an undercounting of circulating monocytes in a population of production workers," *Journal of Occupational Medicine*, vol. 28, no. 3, pp. 207–211, 1986.

[35] G. B. Corcoran, L. Fix, D. P. Jones et al., "Apoptosis: molecular control point in toxicity," *Toxicology and Applied Pharmacology*, vol. 128, no. 2, pp. 169–181, 1994.

[36] C. L. Rabideau, *Pesticide mixtures induce immunotoxicity: potentiation of apoptosis and oxidative stress*, M.S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, Va, USA, 2001.

[37] A. Nakadai, Q. Li, and T. Kawada, "Chlorpyrifos induces apoptosis in human monocyte cell line U937," *Toxicology*, vol. 224, no. 3, pp. 202–209, 2006.

[38] J. D. Thrasher, R. Madison, and A. Broughton, "Immunologic abnormalities in humans exposed to chlorpyrifos: preliminary observations," *Archives of Environmental Health*, vol. 48, no. 2, pp. 89–93, 1993.

[39] T. Galloway and R. Handy, "Immunotoxicity of organophosphorous pesticides," *Ecotoxicology*, vol. 12, no. 1–4, pp. 345–363, 2003.

[40] D. J. McConkey, M. B. Jondal, and S. G. Orrenius, "Chemical-induced apoptosis in the immune system," in *Immunotoxicology and Immunopharmacology*, J. H. Dean, M. I. Luster, A. E. Munson, and I. Kimber, Eds., pp. 473–485, Raven, NewYork, NY, USA, 2nd edition, 1994.

[41] H. Hemilä, "Vitamin C, respiratory infections and the immune system," *Trends in Immunology*, vol. 24, no. 11, pp. 579–580, 2003.

[42] E. S. Wintergerst, S. Maggini, and D. H. Hornig, "Immune-enhancing role of Vitamin C and zinc and effect on clinical conditions," *Annals of Nutrition and Metabolism*, vol. 50, no. 2, pp. 85–94, 2006.

[43] I. Stoian, A. Oros, and E. Moldoveanu, "Apoptosis and free radicals," *Biochemical and Molecular Medicine*, vol. 59, no. 2, pp. 93–97, 1996.

[44] J. A. Knight, "Review: free radicals, antioxidants, and the immune system," *Annals of Clinical and Laboratory Science*, vol. 30, no. 2, pp. 145–158, 2000.

[45] K. J. Halazun, A. Aldoori, H. Z. Malik et al., "Elevated preoperative neutrophil to lymphocyte ratio predicts survival following hepatic resection for colorectal liver metastases," *European Journal of Surgical Oncology*, vol. 34, no. 1, pp. 55–60, 2008.

[46] S. R. Walsh, E. J. Cook, F. Goulder, T. A. Justin, and N. J. Keeling, "Neutrophil-lymphocyte ratio as a prognostic factor in colorectal cancer," *Journal of Surgical Oncology*, vol. 91, no. 3, pp. 181–184, 2005.

[47] A. Papa, M. Emdin, C. Passino, C. Michelassi, D. Battaglia, and F. Cocci, "Predictive value of elevated neutrophil-lymphocyte ratio on cardiac mortality in patients with stable coronary artery disease," *Clinica Chimica Acta*, vol. 395, no. 1-2, pp. 27–31, 2008.

[48] D. A. Goodman, C. B. Goodman, and J. S. Monk, "Use of the neutrophil:lymphocyte ratio in the diagnosis of appendicitis," *American Surgeon*, vol. 61, no. 3, pp. 257–259, 1995.

[49] C. F. Araujo, M. V. G. Lacerda, D. S. P. Abdalla, and E. S. Lima, "The role of platelet and plasma markers of antioxidant status and oxidative stress in thrombocytopenia among patients with vivax malaria," *Memorias do Instituto Oswaldo Cruz*, vol. 103, no. 6, pp. 517–521, 2008.

Review Article

Oxidative Stress in Lead and Cadmium Toxicity and Its Amelioration

R. C. Patra,[1, 2] Amiya K. Rautray,[1] and D. Swarup[2, 3]

[1] *Department of Medicine, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar 751003, India*
[2] *Division of Medicine, Indian Veterinary Research Institute, Izatnagar 243122, India*
[3] *Central Institute for Research on Goats, Makhdoom 281122, UP, India*

Correspondence should be addressed to R. C. Patra, rcpatra@gmail.com

Oxidative stress has been implicated to play a role, at least in part, in pathogenesis of many disease conditions and toxicities in animals. Overproduction of reactive oxygen species and free radicals beyond the cells intrinsic capacity to neutralize following xenobiotics exposure leads to a state of oxidative stress and resultant damages of lipids, protein, and DNA. Lead and cadmium are the common environmental heavy metal pollutants and have widespread distribution. Both natural and anthropogenic sources including mining, smelting, and other industrial processes are responsible for human and animal exposure. These pollutants, many a times, are copollutants leading to concurrent exposure to living beings and resultant synergistic deleterious health effects. Several mechanisms have been explained for the damaging effects on the body system. Of late, oxidative stress has been implicated in the pathogenesis of the lead- and cadmium-induced pathotoxicity. Several ameliorative measures to counteract the oxidative damage to the body system aftermath or during exposure to these toxicants have been assessed with the use of antioxidants. The present review focuses on mechanism of lead- and cadmium-induced oxidate damages and the ameliorative measures to counteract the oxidative damage and pathotoxicity with the use of supplemented antioxidants for their beneficial effects.

1. Introduction

The diverse deleterious health effect upon exposure to toxic heavy metals in the environment is a matter of serious concern and a global issue. Much emphasis has been given to elucidate the mechanism of toxicity due to common environmental toxicants and to develop a safer chemotherapeutic approach to mitigate the toxic effects. Lead and cadmium are the two most abundant toxic metals in the environment. The coexposure to these two toxic metals has synergistic cytotoxicity that may, at times, turn to antagonistic effects, because exposure to higher mixture concentrations may enhance cellular defense mechanisms [1, 2], including induction of metallothioneins synthesis upon exposure to cadmium. The concurrent higher levels of lead and cadmium have been recorded in several field situations. The common sources of lead and cadmium are diverse in nature including natural and anthropogenic processes such as combustion of coal and mineral oil, smelters, mining and alloy processing units, paint industries, and so forth. [2–5]. The quantity of lead used in the present decade far exceeds the total amount consumed in all previous eras [2]. The anthropogenic activities and vehicular emissions contribute to the entry of toxic metals to humans and other animals food chains [6].

Cadmium is an important environmental pollutant present in soil, water, air and food. Anthropogenic sources add 3–10 times more cadmium to the atmosphere than natural sources [7]. Major occupational exposure occurs from nonferrous smelters during production and processing of cadmium, its alloys, and compounds, and the exposure is increasingly common during recycling of electronic waste.

Lead and cadmium do not have any detectable beneficial biological roles. On the contrary, their detrimental effects on physiological, biochemical, and behavioral dysfunctions have been documented in animals and humans by several

investigators [8, 9]. The higher levels affect the central and peripheral nervous systems [10], haemopoietic system [11], cardiovascular system [12], kidneys [13], liver [14], and reproductive systems [15, 16]. Cadmium is more toxic than lead and causes renal and hepatic damage in exposed animals [13, 14].

Of late, lead- and cadmium-induced tissue damages have been attributed, at least in part, to toxicant-induced oxidative stress [17, 18]. Cadmium stimulates the formation of metallothioneins and reactive oxygen species (ROS), thus causing oxidative damage to erythrocytes and various tissues resulting in loss of membrane functions [19]. Long-term exposure to Cd increases lipid peroxidation and causes inhibition of SOD activity indicating oxidative damage in liver, kidney and testes [20]. The various toxic effects induced by Cd in biological systems have been linked to increased lipid peroxidation, an as early and sensitive consequence of Cd exposure. The increase in lipid peroxidation due to Cd toxicity have been attributed to alterations in the antioxidant defense system which includes enzymes such as glutathione peroxidase (GPx), glutathione-S-transferase, superoxide dismutase (SOD), and catalase (CAT), and nonenzymatic molecule like glutathione, which normally protect against free radical toxicity.

2. Lead-Induced Oxidative Stress

The mechanism of lead-induced oxidative stress involves an imbalance between generation and removal of ROS (reactive oxygen species) in tissues and cellular components causing damage to membranes, DNA and proteins. The presence of double bonds in the fatty acid on cell membrane weakens the C–H bonds on the carbon atom adjacent to the double bonds and makes H removal easier. Therefore, fatty acids containing zero to two double bonds are more resistant to oxidative stress than polyunsaturated fatty acids with more than two double bonds [21]. The above fact was substantiated after incubation of linoleic, linolenic, and arachidonic acid with lead in which the concentration of a final product of oxidative stress, malondialdehyde (MDA) was increased with the number of double bonds of fatty acid [22].

The intrinsic mechanism underlying lead-induced oxidative damage to membranes is associated with changes in its fatty acid composition [23]. The fatty acid chain length and unsaturation are the determinant for membrane susceptibility to peroxidation, and lead induced arachidonic acid elongation might be responsible for the enhanced lipid peroxidation of the membrane [24]. Thus, lead affects membrane-related processes such as the activity of membrane enzymes, endo- and exocytosis, transport of solutes across the bilayer, and signal transduction processes by causing lateral phase separation [25].

Lead accumulation in tissues causes oxidative DNA damages including strand break, although the evidence of lead-induced oxidative damage to DNA is less conclusive [18]. The δ-aminolevulinic acid dehydrase (ALAD) is highly sensitive to the toxic effects of lead [26]. The accumulation of δ-aminolevulinic acid (ALA) upon exposure to lead

induces generation of ROS [27, 28] and resultant oxidative stress [29]. The final oxidation product of ALA, 4,5-dioxovaleric acid is an effective alkylating agent of the quinine moieties within both nucleoside and isolated DNA [30]. Increased level of 8-oxo-7, 8-dihydro-2-deoxyguanosine and 5-hydroxyl-2-deoxycytidine following chronic treatment with ALA in rats has been attributed for ALA-induced DNA damage [31]. There are recent data suggesting lead-induced alteration in gene expression [32] and it appears to interact with zinc-binding sites on an important DNA-associated protein, human protamine [33].

The effect on the antioxidant defense systems of cells is the second mechanism for lead-induced oxidative stress. Lead and other metals such as Hg and Cd have a high affinity for sulfhydryl (SH) groups. Mercaptides are formed with the SH group of cysteine, that are less stable complexes [34]. Lead is shown to alter antioxidant activities by inhibiting functional SH groups in several enzymes such as ALAD, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glucose-6-phosphate dehydrogenase (G6PD) [35–38]. G6PD contains many SH group and supplies cells with most of the extramitochondrial NADPH through the oxidation of glucose-6-phosphate to 6-phosphogluconate. G6PD is inhibited by lead [39]. However, there are more complex effects of lead on G6PD, as evidenced by *in vivo* studies. G6PD activity increases in RBCs of lead-treated rats [40] and lead-exposed workers [41]. The most important regulation of the pentose phosphate pathway is the NADP-/NADPH ratio, which is known to change in favor of the oxidized form under oxidative stress conditions. Therefore, lead exposure results in an increase or decrease in G6PD activity depending on the concentration and duration of lead exposure, and magnitude of oxidative stress inside the cell [42].

GPx, CAT, and SOD are potential targets for lead toxicity because these antioxidant enzymes depend on various essential trace elements for proper molecular structure and activity [43]. Since lead-associated reduction in selenium uptake may increase the susceptibility of the cell to oxidative stress, an antagonistic effect between selenium and lead was found to affect GPx activity that requires selenium as a cofactor [44]. On the other hand, administration of selenium before lead exposure produces significant prophylactic action against lead-induced oxidative stress by means of increasing SOD, glutathione reductase (GR) activity, and glutathione (GSH) content in male rats [45]. In summary, impaired antioxidant defenses can be a result of the inhibitory effects of lead on various enzymes, which in turn causes the cells to be more susceptible to oxidative insult.

3. Cadmium and Oxidative Stress

Cadmium is a well-recognized environmental pollutant with numerous adverse health effects. It principally affects lung, liver, kidney, and testes following acute intoxication, and nephrotoxicity, immunotoxicity, osteotoxicity and tumors on prolong exposures. Reactive oxygen species (ROS) are often implicated in Cd-induced deleterious health effects. There are direct evidence of the generation of free radicals

in animals following acute Cd overload, and indirect evidence of involvement of ROS in chronic Cd toxicity and carcinogenesis. Cd-generated superoxide anion, hydrogen peroxide, and hydroxyl radicals *in vivo* have been detected by the electron spin resonance spectra, which are often accompanied by activation of redox sensitive transcription factors (e.g., NF-κB, AP-1 and Nrf2) and alteration of ROS-related gene expression. It is generally agreed upon that oxidative stress plays important roles in acute Cd poisoning.

However, direct evidence for oxidative stress is often obscure following long-term and environmentally-relevant low levels of Cd exposure. Alterations in ROS-related gene expression during chronic exposures are also less significant compared to acute Cd poisoning. This is probably due to induced adaptation mechanisms such as overexpression of metallothionein and glutathione following chronic Cd exposures, which in turn diminish Cd-induced oxidative stress. In chronic Cd-transformed cells, less ROS signals are detected with fluorescence probes. Acquired apoptotic tolerance renders damaged cells to proliferate with inherent oxidative DNA lesions, potentially leading to tumorogenesis. Thus, ROS are generated following acute Cd overload that play an important roles in tissue damage. Adaptation to chronic Cd exposure reduces ROS production, but acquired Cd tolerance with aberrant gene expression plays important roles in chronic Cd toxicity and carcinogenesis.

The basic mechanisms involved in cadmium carcinogenesis are gene regulation of proto-oncogenes [46], oxidative stress [47–51], disruption of cadherins, inhibition of DNA repair and interference with apoptosis [52]. Cadmium is a potent cell poison, and known to cause oxidative stress by increasing lipid peroxidation and/or by changing intracellular glutathione levels. It affects the ubiquitin/ATP-dependent proteolytic pathway. However, the cellular mechanisms involved in cadmium toxicity are still not well understood, especially in neuronal cells. The treatment of neuronal cells culture with different concentrations of the metal ion to examine the relationship between cadmium-induced oxidative stress and the ubiquitin/ATP-dependent pathway revealed decreased glutathione levels, and marked increases in protein-mixed disulfides (Pr-SSGs) [53]. The increases in intracellular levels of Pr-SSGs were concurrent with increases in the levels of ubiquitinated proteins (Ub proteins) when the HT4 cells were subjected to lower (25 μm or less) concentrations of cadmium. However, higher concentrations of cadmium (50 μm) led to increases in Pr-SSGs but inhibited ubiquitination, probably reflecting inhibition of ubiquitinating enzymes.

The cadmium-induced changes in Pr-SSGs and Ub proteins are not affected when more than 85% of intracellular glutathione is removed from the cells by the glutathione synthetase inhibitor l-buthionine-(S, R)-sulfoximine. However, the reducing agent dithiothreitol, that prevents build-up of Pr-SSGs in the cell also blocks the accumulation of Ub proteins induced by cadmium. In addition, dithiothreitol blocks the effects of higher (50 μm) concentrations of cadmium on cytotoxicity and on glutathione, Pr-SSGs, and Ub proteins. Together, these results strongly suggest that changes in the levels of intracellular Pr-SSGs and ubiquitin-protein

conjugates in neuronal cells are the responses closely associated with the disruption of intracellular sulfhydryl homeostasis caused by cadmium-mediated oxidative stress.

The testis is the important target organ of Cd toxicity. Many studies indicate that Cd induces testicular damage in many species of animals including mice, hamsters, rabbits, guinea pigs and dogs [54, 55]. Cadmium has profound effect on sex organ weight, a primary indicator of possible alteration in androgen status [56, 57]. Several mechanisms of Cd-induced testicular toxicity have been proposed. Lafuente et al. [58] reported increased Cd accumulation in the hypothalamus, pituitary, and testis and decreased plasma levels of follicle stimulating hormone in rats, suggesting a possible effect of Cd on the hypothalamic-pituitary-testicular axis.

Several studies also suggest participation of reactive oxygen species (ROS) in Cd-induced testicular damage [59]. Both acute and chronic Cd exposure is associated with elevated lipid peroxidation in the lung, brain, kidney, liver, erythrocytes, and testis [60–64]. The reactive oxygen species (ROS) play both beneficial and harmful roles in living organisms [65]. ROS can be generated by both exogenous and endogenous sources. Cadmium is one of the exogenous sources shown to indirectly produce ROS in various cell lines [66–68]. The production and accumulation of ROS inhibit the electron transfer chain in mitochondria [69]. In general, the accumulated ROS consists of various amounts of hydrogen peroxide, hydroxyl ions, singlet oxygen, superoxide anions, lipid hydroperoxides, phospholipid hydroperoxides, and so forth. Excessive production of ROS disturbs the balance between the ROS and antioxidant agents (enzymes and antioxidant substances) in the cells. Hydrogen peroxide is the common substrate for catalase and GPx enzymes in the cells. While catalase decomposes H_2O_2 into water and oxygen, GPx oxidizes GSH to GSSG by utilizing H_2O_2. Glutathione reductase (GR) is another enzyme required for the antioxidant defense mechanism in cells. It reduces GSSG into GSH. Both GPx and GR work in tandem in the cells in order to maintain the GSH/GSSG ratio at a steady state level. When the cells are under oxidative stress, catalase, GR and GPx respond by altering their activities.

4. Amelioration of Lead- and Cadmium-Induced Oxidative Stress

Abetment of lead and cadmium toxicity with rebalancing the impaired prooxidant/antioxidant ratio through supplementation of antioxidant nutrients are still not completely clear. However, evidences suggest significant protective effects of antioxidant nutrients such as vitamin-C, carotenoids, selenium, vitamin-E, and so forth.

Vitamin C is a major antioxidant that scavenges the aqueous ROS by very rapid electron transfer that inhibits lipid peroxidation [21]. Administration of vitamin C significantly inhibits the lipid peroxidation levels of liver and brain, and increased the CAT levels of kidney in lead-exposed rats [17]. Lead-induced ROS production as examined by rat sperm chemiluminescence generation reduced by 40% with supplementation of 500 mg vitamin C/l drinking water

[70]. Vitamin C supplementation in lead-exposed animals significantly reduces blood, liver, and renal lead levels, and associated biochemical changes indicating a significant protective action of vitamin C against toxic effects of lead on heme synthesis and drug metabolism [71]. The combination of vitamin C and thiamine have been proved effective in reducing lead levels in blood, liver, and kidney along with reduction in lead-induced inhibition in the activity of blood d-ALAD and blood zinc protoporphyrin [72].

There has been considerable debate concerning the relationship between vitamin C nutritional status and lead-induced toxic effects. Early reports suggest vitamin C as a possible chelator of lead, with similar potency to that of EDTA [8]. Vitamin and/mineral supplementation in African American women was found to reduce blood lead level (BLL) from 5.1 to 1.1 mg/dl, which was negatively correlated with serum levels of vitamin E and C [73]. Ascorbic acid increases urinary elimination of lead and reduces the hepatic and renal lead burden in rats [74].

A cross-sectional study analyzed 4213 young and 15365 adult Americans with mean BLL of 2.5–3.5 mg/dl, respectively. The BLL had inverse relationship with serum vitamin C [75]. Vitamin C supplementation resulted in small reductions in lead retention in 85 human volunteers who consumed a lead-containing drink [76]. However, workers occupationally exposed to lead, and with BLL ranged from 28.9 to 76.4 mg/dl, supplementation of vitamin C and zinc did not significantly reduce BLL [77]. The vitamin C supplementation did not alter the blood and sperm lead levels in lead-treated rats with BLL of 36 mg/dl [70]. A recent report stated that rats treated with ascorbic acid did not reduce lead burden in the liver, kidney, brain, and blood [17]. Although it is biologically plausible that vitamin C may affect lead absorption and excretion, the effect is more obvious in low-exposed subjects with higher vitamin C supplementation. In humans and animals exposed to high levels of lead, the reduction of BLL by vitamin C is less significant.

Vitamin E is the generic term used to describe at least eight natural-occurring compounds that possess the biological activity of α-tocopherol. The group is comprised of α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol. RRR-α-tocopherol has the highest biological activity [78], the other tocopherols and tocotrienols are less biologically active but they are at least as abundant as α-tocopherol in certain foods [79]. Vitamin E is nature's major lipid soluble chain-breaking antioxidant that is known to protect biological membranes and lipoproteins from oxidative stress [80]. The main biological function of vitamin E is its direct influence on cellular responses to oxidative stress through modulation of signal transduction pathways [81]. One of the protective roles of vitamin E on lead-induced damage is prevention of lipid peroxidation and inhibition of SOD and CAT activities in liver [82]. In lead-exposed rats, supplementation of vitamin E and/or C reduced sperm ROS generation, prevented loss of sperm motility and oocyte penetration capacity [70]. The interaction between vitamin E and other antioxidants might have a more efficient protective action against lead toxicity. Vitamin E and C jointly protect

lipid structures against peroxidation [83]. Although vitamin E is located in membranes and vitamin C in aqueous phases, vitamin C is able to recycle oxidized vitamin E [84]. Vitamin C repairs the tocopherol radical, thus recovering the chain-breaking antioxidant capacity of vitamin E [83]. Vitamin E alone or in combination with conventional chelator, CaNa$_2$EDTA has been reported to decrease the lead-induced lipid peroxide levels in liver and brain in rats [17].

Carotenoids play a significant role in reduction of lead-induced stress in all species. The reaction of carotenoids with radicals is partly due to its roles in photosynthesis, thus electron transfer from β-carotene to P680, with the β-carotene being oxidized to its radical cation CAR [85]. Dietary β-carotene mediates prevention of lipid peroxidation, and reduces the incidence of many diseases including cancer, atherosclerosis, age-related macular degeneration, and multiple sclerosis [86, 87]. However, the generally accepted beneficial roles of carotenoids as antioxidants have been seriously challenged by the results from clinical trials that suggest deleterious effects of administered β-carotene in heavy smokers [88]. There have been considerable recent investigations in the interaction of β-carotene and other antioxidants [89].

The antioxidant effects of *Spirulina fusiformis*, bluegreen algae rich in β-carotene and SOD, against lead toxicity have been examined in the testes of Swiss mice. The antioxidant nutrients scavenged the free radicals after lead administration and ROS generation in mice testes [90]. Supplementation with multiple antioxidants including vitamin C, vitamin E, β-carotene, selenium, and zinc resulted in significant increase of SOD and GPx in 36 workers exposed to lead [91]. The interaction of carotenoids and carotenoid radicals with other antioxidants is of importance with respect to anti- and, possibly, pro-oxidative reactions of carotenoids. The nature of the reaction between the tocopherol (TOH) and various carotenoids shows a marked variation depending on the specific tocopherol homologue, of which a-TOH is the most active. β-carotene radical interacts with vitamin C in the aqueous phase, and carotenoid radical are efficiently reconverted to the parent carotenoid by vitamin C [89, 92].

Interactions between zinc and lead have been investigated at absorptive and enzymatic sites [93]. Zinc and lead compete for similar binding sites on the metallothionein-like transport protein in the gastrointestinal tract [94]. The competition between zinc and lead might decrease the absorption of lead, thus reducing lead toxicity. Dietary supplementation with zinc and in combination with ascorbic acid [95] and thiamine [96] reduces lead toxicity in humans. Zinc has an important role in spermatogenesis in the male reproductive system, and the most probable site of action is the primary spermatocyte. Zinc supplementation competes for and effectively reduces the availability of binding sites for lead uptake. In another study, zinc was administrated to lead-exposed rats along with chelating agents CaNa$_2$EDTA, succimer, and D-penicillamine. Zinc enhanced the efficacy of lead chelation by reducing the blood, hepatic and renal lead level, and overturning the inhibited activity on blood ALAD [97]. A recent study has shown prevention of δ-ALAD inhibition and increased cellular SOD in the testis of

lead-exposed rats following zinc supplementation [98]. The protective effects of zinc against testicular damage caused by lead might be due to competition between lead and zinc. There is still no strong and direct evidence to conclude that the beneficial effects of zinc are mediated by antioxidation.

Zinc is a trace element essential for living organisms. More than 300 enzymes require Zn for their activity. It plays an important role in the DNA replication, transcription, and protein synthesis, influencing cell division and differentiation [99]. Zn has a relationship with many enzymes in the body and can prevent cell damage through activation of the antioxidant system [100–102]. It is an essential component of the oxidant defense system and functions at many levels [103]. Zn deficiency increases lipid peroxidation in various rat tissues, whereas Zn supplementation corrects the impairment [102]. Cotreatment with Cd and Zn prevents damage to the testes from Cd exposure [104]. This suggests Cd interference in Zn-related metabolic functions. The competitive mechanism of interaction and Zn-induced metallothionein induction are the plausible mechanism behind protective effects of Zn against Cd toxicity. This is substantiated by the findings that Cd treatment decreases the testicular Zn concentration and elevates the levels of hepatic and renal metallothioneins [105]. Zn pretreatment can prevent of cadmium-induced testicular tumors which may be attributed to the ability of Zn to reduce the cytotoxicity of Cd in interstitial cells by enhancing efflux of Cd and decreasing accumulation of Cd in the nuclei of this target cell population in the rat testis [106]. So, Cd altered testicular function mediated through induction of oxidative stress could be reversed by administration of Zn.

Selenium is a cofactor of GPx, a cyto-antioxidant enzyme. Selenium enhances the availability of GSH, which is one of the most abundant intrinsic antioxidants that helps in preventing lipid peroxidation and resultant cell damage. Lead exposure decreases the activity of GPx due to the interaction of lead with the essential selenocysteine moiety of the enzyme [107]. Protection against liver and kidney damage by selenium is attributed to enhanced antioxidant capacity of cells, as evidenced by increased SOD and GR activities and elevated GSH content following selenium supplementation [45]. The combination of selenium and other antioxidants has been shown to reduce oxidative stress in animals. DNA damage in the liver and spleen induced by fumonisin B1 was protected by the mixture of antioxidants coenzyme Q10, L-carnitine, vitamin E and selenium in rats [108]. Combined administration of antioxidants containing selenium, vitamin C, vitamin E, β-carotene, and N-acetyl cysteine has been reported to prevent both the diabetes- and galactosemia-induced elevation of oxidative stress in rats [109]. Despite the above findings, the beneficial role of selenium alone on lead-induced oxidative stress is still unclear in human studies.

5. Conclusion

Generation of highly reactive oxygen species aftermath of lead and cadmium exposure may result in systematic mobilization and depletion of the cell intrinsic antioxidant defenses. Formation of reactive oxygen intermediates

beyond the scavenging capacity of these antioxidant defense mechanisms results in accumulation of harmful free radicals and likelihood of oxidative damage to critical biomolecules, such as enzymes, proteins, DNA, and membrane lipids. Several mechanisms have been proposed to mediate the oxidative stress caused by lead and cadmium, including disrupted pro-oxidant/antioxidant balance. Although many investigators have shown lead-induced oxidative damage, and some antioxidants were found to reduce lead toxicity, the mechanisms of dietary supplementation of antioxidants remain to be further clarified in lead-exposed humans or animals.

Evidences suggest that in presence of varying concentrations of cadmium, the mitochondrial enzymes are more effective in reducing various ROS than their cytoplasmic counterparts. This observation reveals that most oxido-reduction reactions take place in the mitochondria, leading to the formation of several ROS. As less ROS are produced in the cytoplasm, the activities of antioxidant enzymes in the cytoplasm are not as high as the mitochondrial enzymes with cadmium treatments. Thus, more oxidative stress is observed in the mitochondria than in the cytoplasm. Each antioxidant enzyme shows its own pattern of activation or inhibition upon exposure of cells to different concentrations of lead and cadmium.

References

[1] D. S. Bae, C. Gennings, W. H. Carter, R. S. H. Yang, and J. A. Campain, "Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes," *Toxicological Sciences*, vol. 63, no. 1, pp. 132–142, 2001.

[2] C. Phillips, Z. Gyori, and B. Kovács, "The effect of adding cadmium and lead alone or in combination to the diet of pigs on their growth, carcase composition and reproduction," *Journal of the Science of Food and Agriculture*, vol. 83, no. 13, pp. 1357–1365, 2003.

[3] R. C. Patra, D. Swarup, R. Naresh, P. Kumar, P. Shekhar, and R. Ranjan, "Cadmium level in blood and milk from animals reared around different polluting sources in India," *Bulletin of Environmental Contamination and Toxicology*, vol. 74, no. 6, pp. 1092–1097, 2005.

[4] D. Swarup, R. C. Patra, R. Naresh, P. Kumar, and P. Shekhar, "Blood lead levels in lactating cows reared around polluted localities; transfer of lead into milk," *Science of the Total Environment*, vol. 347, no. 1–3, pp. 106–110, 2005.

[5] R. C. Patra, D. Swarup, R. Naresh et al., "Tail hair as an indicator of environmental exposure of cows to lead and cadmium in different industrial areas," *Ecotoxicology and Environmental Safety*, vol. 66, no. 1, pp. 127–131, 2007.

[6] I. A. Okada, A. M. Sakuma, F. D. Maid, S. Dovidemskas, and O. Zenebon, "Evaluation of lead and cadmium in milk due to environmental contamination in Paraiba valley region of South Estern Brazil," *Raissade-Saude-Publica*, vol. 31, pp. 140–143, 1997.

[7] R. J. Irwin, M. Van Mouwerik, L. Stevend, M. D. Seese, and W. Basham, "Environmental contaminants encyclopedia," National Park Service, Water Resources Division, Fort Collins, Colorado. Distributed within the federal government as electronic document, February 2003.

[8] R. A. Goyer and M. G. Cherian, "Ascorbic acid and EDTA treatment of lead toxicity in rats," *Life Sciences*, vol. 24, no. 5, pp. 433–438, 1979.

[9] H. A. Ruff, M. E. Markowitz, P. E. Bijur, and J. F. Rosen, "Relationships among blood lead levels, iron deficiency, and cognitive development in two-year-old children," *Environmental Health Perspectives*, vol. 104, no. 2, pp. 180–185, 1996.

[10] J. Dressier, K. A. Kim, T. Chakraborti, and G. Goldstein, "Molecular mechanisms of lead neurotoxicity," *Neurochemical Research*, vol. 24, no. 4, pp. 595–600, 1999.

[11] P. E. De Silva, "Determination of lead in plasma and studies on its relationship to lead in erythrocytes," *Brazilian Journal of Indigenous Medicine*, vol. 38, pp. 209–217, 1981.

[12] F. Khalil-Manesh, H. C. Gonick, E. W. J. Weiler, B. Prins, M. A. Weber, and R. E. Purdy, "Lead-induced hypertension: possible role of endothelial factors," *American Journal of Hypertension*, vol. 6, no. 9, pp. 723–729, 1993.

[13] D. J. Humphreys, "Effects of exposure to excessive quantities of lead on animals," *British Veterinary Journal*, vol. 147, no. 1, pp. 18–30, 1991.

[14] R. P. Sharma and J. C. Street, "Public health aspects of toxic heavy metals in animal feeds," *Journal of the American Veterinary Medical Association*, vol. 177, no. 2, pp. 149–153, 1980.

[15] W. N. Rom, "Effects of lead on reproduction," in *Proceedings of the Workshop on Methodology for Assessing Reproductive Hazards in the Workplace*, P. F. Infante and M. S. Legator, Eds., pp. 33–42, Washington, DC, USA, 1980.

[16] I. Lancranjan, H. I. Popescu, O. GAvănescu, I. Klepsch, and M. Serbănescu, "Reproductive ability of workmen occupationally exposed to lead," *Archives of Environmental Health*, vol. 30, no. 8, pp. 396–401, 1975.

[17] R. C. Patra, D. Swarup, and S. K. Dwivedi, "Antioxidant effects of α tocopherol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats," *Toxicology*, vol. 162, no. 2, pp. 81–88, 2001.

[18] H. Fu, X. B. Ye, J. L. Zhu et al., "Oxidative stress in lead exposed workers," in *IARC Gargnano Conference*, p. 2.3, 1999.

[19] S. Sarkar, P. Yadav, and D. Bhatnagar, "Lipid peroxidative damage on cadmium exposure and alterations in antioxidant system in rat erythrocytes: a study with relation to time," *BioMetals*, vol. 11, no. 2, pp. 153–157, 1998.

[20] R. C. Patra, D. Swarup, and S. K. Senapat, "Effects of cadmium on lipid peroxides and superoxide dismutasein hepatic, renal and testicular tissue of rats," *Veterinary and Human Toxicology*, vol. 41, no. 2, pp. 65–67, 1999.

[21] B. Halliwell and J. M. C. Gutteridge, "Protection against oxidants in biologyogical systems: the superoxide theory of oxygen toxicity," in *Free Radical in Biology and Medicine*, B. Halliwell and J. M. C. Gutteridge, Eds., pp. 86–123, Clarendon Press, Oxford, UK, 1989.

[22] S. J. Yiin and T. H. Lin, "Lead-catalyzed peroxidation of essential unsaturated fatty acid," *Biological Trace Element Research*, vol. 50, no. 2, pp. 167–172, 1995.

[23] S. O. Knowles and W. E. Donaldson, "Dietary modification of lead toxicity: effects on fatty acid and eicosanoid metabolism in chicks," *Comparative Biochemistry and Physiology C*, vol. 95, no. 1, pp. 99–104, 1990.

[24] L. J. Lawton and W. E. Donaldson, "Lead-induced tissue fatty acid alterations and lipid peroxidation," *Biological Trace Element Research*, vol. 28, no. 2, pp. 83–97, 1991.

[25] V. N. Adonaylo and P. I. Oteiza, "Pb promotes lipid oxidation and alterations in membrane physical properties," *Toxicology*, vol. 132, no. 1, pp. 19–32, 1999.

[26] J. P. Farant and D. C. Wigfield, "Biomonitoring lead exposure with δ-aminolevulinate dehydratase (ALA-D) activity ratios," *International Archives of Occupational and Environmental Health*, vol. 51, no. 1, pp. 15–24, 1982.

[27] M. Hermes-Lima, V. G. R. Valle, A. E. Vercesi, and E. J. H. Bechara, "Damage to rat liver mitochondria promoted by δ-aminolevulinic acid-generated reactive oxygen species: connections with acute intermittent porphyria and lead-poisoning," *Biochimica et Biophysica Acta*, vol. 1056, no. 1, pp. 57–63, 1991.

[28] M. Hermes-Lima, "How do Ca^{2+} and 5-aminolevulinic acid-derived oxyradicals promote injury to isolated mitochondria?" *Free Radical Biology and Medicine*, vol. 19, no. 3, pp. 381–390, 1995.

[29] E. J. H. Bechara, "Oxidative stress in acute intermittent porphyria and lead poisoning may be triggered by 5-aminolevulinic acid," *Brazilian Journal of Medical and Biological Research*, vol. 29, no. 7, pp. 841–851, 1996.

[30] T. Douki, J. Onuki, M. H. G. Medeiros, E. J. H. Bechara, J. Cadet, and P. D. Mascio, "DNA alkylation by 4,5-dioxovaleric acid, the final oxidation product of 5-aminolevulinic acid," *Chemical Research in Toxicology*, vol. 11, no. 2, pp. 150–157, 1998.

[31] T. Douki, J. Onuki, M. H. G. Medeiros, E. J. H. Bechara, J. Cadet, and P. Di Mascio, "Hydroxyl radicals are involved in the oxidation of isolated and cellular DNA bases by 5-aminolevulinic acid," *FEBS Letters*, vol. 428, no. 1-2, pp. 93–96, 1998.

[32] T. G. Rossman, "Cloning genes whose levels of expression are altered by metals: implications for human health research," *American Journal of Industrial Medicine*, vol. 38, no. 3, pp. 335–339, 2000.

[33] B. Quintanilla-Vega, D. Hoover, W. Bal, E. K. Silbergeld, M. P. Waalkes, and L. D. Anderson, "Lead effects on protamine-DNA binding," *American Journal of Industrial Medicine*, vol. 38, no. 3, pp. 324–329, 2000.

[34] B. L. Vallee and D. D. Ulmer, "Biochemical effects of mercury, cadmium, and lead," *Annual Review of Biochemistry*, vol. 41, no. 10, pp. 91–128, 1972.

[35] J. M. Hsu, "Lead toxicity as related to glutathione metabolism," *Journal of Nutrition*, vol. 111, no. 1, pp. 26–33, 1981.

[36] Y. Ito, Y. Niiya, and H. Kurita, "Serum lipid peroxide level and blood superoxide dismutase activity in workers with occupational exposure to lead," *International Archives of Occupational and Environmental Health*, vol. 56, no. 2, pp. 119–127, 1985.

[37] C. McGowan and W. E. Donaldson, "Changes in organ non-protein sulfhydryl and glutathione concentrations during acute and chronic administration of inorganic lead to chicks," *Biological Trace Element Research*, vol. 10, no. 1, pp. 37–46, 1986.

[38] M. Chiba, A. Shinohara, K. Matsushita, H. Watanabe, and Y. Ihaba, "Indices of lead-exposure in blood and urine of lead-exposed workers and concentrations of major and trace elements and activities of SOD, GSH-Px and catalase in their blood," *Tohoku Journal of Experimental Medicine*, vol. 178, no. 1, pp. 49–62, 1996.

[39] N. A. Lachant, A. Tomoda, and K. R. Tanaka, "Inhibition of the pentose phosphate shunt by lead: a potential mechanism for hemolysis in lead poisoning," *Blood*, vol. 63, no. 3, pp. 518–524, 1984.

[40] H. Gurer, H. Ozgunes, R. Neal, D. R. Spitz, and N. Ercal, "Antioxidant effects of N-acetylcysteine and succimer in red

blood cells from lead-exposed rats," *Toxicology*, vol. 128, no. 3, pp. 181–189, 1998.

[41] P. Cocco, S. Salis, M. Anni, M. E. Cocco, C. Flore, and A. Ibba, "Effects of short term occupational exposure to lead on erythrocyte glucose-6-phosphate dehydrogenase activity and serum cholesterol," *Journal of Applied Toxicology*, vol. 15, no. 5, pp. 375–378, 1995.

[42] H. Gurer and N. Ercal, "Can antioxidants be beneficial in the treatment of lead poisoning?" *Free Radical Biology and Medicine*, vol. 29, no. 10, pp. 927–945, 2000.

[43] B. B. Gelman, I. A. Michaelson, and J. S. Bus, "The effect of lead on oxidative hemolysis and erythrocyte defense mechanisms in the rat," *Toxicology and Applied Pharmacology*, vol. 45, no. 1, pp. 119–129, 1978.

[44] G. N. Schrauzer, "Effects of selenium antagonists on cancer susceptibility: new aspects of chronic heavy metal toxicity," *Journal of UOEH*, vol. 9, pp. 208–215, 1987.

[45] A. I. Othman and M. A. El Missiry, "Role of selenium against lead toxicity in male rats," *Journal of Biochemical and Molecular Toxicology*, vol. 12, no. 6, pp. 345–349, 1998.

[46] D. Hanahan and R. A. Weinberg, "The hallmarks of cancer," *Cell*, vol. 100, no. 1, pp. 57–70, 2000.

[47] F. Thévenod and J. M. Friedmann, "Cadmium-mediated oxidative stress in kidney proximal tubule cells induces degradation of Na$^+$/K$^+$-ATPase through proteasomal and endo- /lysosomal proteolytic pathways," *FASEB Journal*, vol. 13, no. 13, pp. 1751–1761, 1999.

[48] A. Piqueras, E. Olmos, J. R. Martínez-Solano, and E. Hellín, "Cd-induced oxidative burst in tobacco BY2 cells: time course, subcellular location and antioxidant response," *Free Radical Research*, vol. 31, pp. S33–S38, 1999.

[49] S. J. Stohs, D. Bagchi, E. Hassoun, and M. Bagchi, "Oxidative mechanisms in the toxicity of chromium and cadmium ions," *Journal of Environmental Pathology, Toxicology and Oncology*, vol. 20, no. 2, pp. 77–88, 2001.

[50] W. Wätjen and D. Beyersmann, "Cadmium-induced apoptosis in C6 glioma cells: influence of oxidative stress," *BioMetals*, vol. 17, no. 1, pp. 65–78, 2004.

[51] C. O. Ikediobi, V. L. Badisa, L. T. Ayuk-Takem, L. M. Latinwo, and J. West, "Response of antioxidant enzymes and redox metabolites to cadmium-induced oxidative stress in CRL-1439 normal rat liver cells," *International Journal of Molecular Medicine*, vol. 14, no. 1, pp. 87–92, 2004.

[52] C. M. Shih, W. C. Ko, J. S. Wu et al., "Mediating of caspase-independent apoptosis by cadmium through the mitochondria-ROS pathway in MRC-5 fibroblasts," *Journal of Cellular Biochemistry*, vol. 91, no. 2, pp. 384–397, 2004.

[53] M. E. Figueiredo-Pereira, S. Yakushin, and G. Cohen, "Disruption of the intracellular sulfhydryl homeostasis by cadmium- induced oxidative stress leads to protein thiolation and ubiquitination in neuronal cells," *Journal of Biological Chemistry*, vol. 273, no. 21, pp. 12703–12709, 1998.

[54] K. W. Hew, W. A. Ericson, and M. J. Welsh, "A single low cadmium dose causes failure of spermiation in the rat," *Toxicology and Applied Pharmacology*, vol. 121, no. 1, pp. 15–21, 1993.

[55] G. Xu and X. Z. Jiang, "Male reproductive toxicity of cadmium," *Chinese Journal of Public Health*, vol. 15, pp. 17–18, 1996.

[56] N. M. Biswas, R. Sengupta, G. R. Chatopadhyay, A. Choudhury, and M. Sarkar, "Effect of ethanol on cadmium-induced testicular toxicity in male rats," *Reproductive Toxicology*, vol. 15, pp. 699–704, 2001.

[57] J. W. Laskey and P. V. Phelps, "Effect of cadmium and other metal cations on in vitro Leydig cell testosterone production," *Toxicology and Applied Pharmacology*, vol. 108, no. 2, pp. 296–306, 1991.

[58] A. Lafuente, N. Márquez, M. Pérez-Lorenzo, D. Pazo, and A. I. Esquifino, "Pubertal and postpubertal cadmium exposure differentially affects the hypothalamic-pituitary-testicular axis function in the rat," *Food and Chemical Toxicology*, vol. 38, no. 10, pp. 913–923, 2000.

[59] P. I. Oteiza, V. N. Adonaylo, and C. L. Keen, "Cadmium-induced testes oxidative damage in rats can be influenced by dietary zinc intake," *Toxicology*, vol. 137, no. 1, pp. 13–22, 1999.

[60] J. Klimczak, J. M. Wisniewska-Knypl, and J. Kolakowski, "Stimulation of lipid peroxidation and heme oxygenase activity with inhibition of cytochrome P-450 monooxygenase in the liver of rats repeatedly exposed to cadmium," *Toxicology*, vol. 32, no. 3, pp. 267–276, 1984.

[61] N. Sugawara and C. Sugawara, "Selenium protection against testicular lipid peroxidation from cadmium," *Journal of Applied Biochemistry*, vol. 6, no. 4, pp. 199–204, 1984.

[62] D. Manca, A. C. Ricard, B. Trottier, and G. Chevalier, "Studies on lipid peroxidation in rat tissues following administration of low and moderate doses of cadmium chloride," *Toxicology*, vol. 67, no. 3, pp. 303–323, 1991.

[63] T. Koizumi and Z. G. Li, "Role of oxidative stress in single-dose, cadmium-induced testicular cancer," *Journal of Toxicology and Environmental Health*, vol. 37, no. 1, pp. 25–36, 1992.

[64] D. Bagchi, P. J. Vuchetich, M. Bagchi et al., "Induction of oxidative stress by chronic administration of sodium dichromate [chromium VI] and cadmium chloride [cadmium II] to rats," *Free Radical Biology and Medicine*, vol. 22, no. 3, pp. 471–478, 1997.

[65] M. Valko, M. Izakovic, M. Mazur, C. J. Rhodes, and J. Telser, "Role of oxygen radicals in DNA damage and cancer incidence," *Molecular and Cellular Biochemistry*, vol. 266, no. 1-2, pp. 37–56, 2004.

[66] D. J. Price and J. G. Joshi, "Ferritin. Binding of beryllium and other divalent metal ions," *Journal of Biological Chemistry*, vol. 258, no. 18, pp. 10873–10880, 1983.

[67] A. Szuster-Ciesielska, A. Stachura, M. Słotwińska et al., "The inhibitory effect of zinc on cadmium-induced cell apoptosis and reactive oxygen species (ROS) production in cell cultures," *Toxicology*, vol. 145, no. 2-3, pp. 159–171, 2000.

[68] M. Watanabe, K. Henmi, K. Ogawa, and T. Suzuki, "Cadmium-dependent generation of reactive oxygen species and mitochondrial DNA breaks in photosynthetic and non-photosynthetic strains of Euglena gracilis," *Comparative Biochemistry and Physiology*, vol. 134, no. 2, pp. 227–234, 2003.

[69] Y. Wang, J. Fang, S. S. Leonard, and K. M. K. Rao, "Cadmium inhibits the electron transfer chain and induces reactive oxygen species," *Free Radical Biology and Medicine*, vol. 36, no. 11, pp. 1434–1443, 2004.

[70] P. C. Hsu, C. C. Hsu, M. Y. Liu, L. Y. Chen, and Y. L. Guo, "Lead-induced changes in spermatozoa function and metabolism," *Journal of Toxicology and Environmental Health A*, vol. 55, no. 1, pp. 45–64, 1998.

[71] A. G. Vij, N. K. Satija, and S. J. S. Flora, "Lead induced disorders in hematopoietic and drug metabolizing enzyme system and their protection by ascorbic acid supplementation," *Biomedical and Environmental Sciences*, vol. 11, no. 1, pp. 7–14, 1998.

[72] S. J. S. Flora and S. K. Tandon, "Preventive and therapeutic effects of thiamine, ascorbic acid and their combination in lead intoxication," *Acta Pharmacologica et Toxicologica*, vol. 58, no. 5, pp. 374–378, 1986.

[73] W. L. West, E. M. Knight, C. H. Edwards et al., "Maternal low level lead and pregnancy outcomes," *Journal of Nutrition*, vol. 124, no. 6, pp. 981–986, 1994.

[74] M. Dhawan, D. N. Kachru, and S. K. Tandon, "Influence of thiamine and ascorbic acid supplementation on the antidotal efficacy of thiol chelators in experimental lead intoxication," *Archives of Toxicology*, vol. 62, no. 4, pp. 301–304, 1988.

[75] J. A. Simon and E. S. Hudes, "Relationship of ascorbic acid to blood lead levels," *Journal of the American Medical Association*, vol. 281, no. 24, pp. 2289–2293, 1999.

[76] E. B. Dawson and W. A. Harris, "Effect of ascorbic acid supplementation on blood lead levels," *Journal of the American College of Nutrition*, vol. 16, p. 480, 1997.

[77] R. Lauwerys, H. Roels, and J. P. Buchet, "The influence of orally-administered vitamin C or zinc on the absorption of and the biological response to lead," *Journal of Occupational Medicine*, vol. 25, no. 9, pp. 668–678, 1983.

[78] H. Weiser and M. Vecchi, "Stereoisomers of α-tocopheryl acetate. II. Biopotencies of all eight stereoisomers, individually or in mixtures, as determined by rat resorption-gestation tests," *International Journal for Vitamin and Nutrition Research*, vol. 52, no. 3, pp. 351–370, 1982.

[79] A. J. Sheppard, J. A. T. Pennington, and J. L. Weihrauch, "Analysis and distribution of vitamin E in vegetable oils and foods," in *Vitamin E in Health and Disease*, L. Packer and J. Fuchs, Eds., pp. 9–13, Marcel-Dekker, New York, NY, USA, 1993.

[80] L. Packer, "Protective role of vitamin E in biological systems," *American Journal of Clinical Nutrition*, vol. 53, no. 4, pp. 1050S–1055S, 1991.

[81] A. Azzi, D. Boscoboinik, and C. Hensey, "The protein kinase C family," *European Journal of Biochemistry*, vol. 208, no. 3, pp. 547–557, 1992.

[82] S. S. Chaurasia and A. Kar, "Protective effects of vitamin E against lead-induced deterioration of membrane associated type-I iodothyronine 5'-monodeiodinase (5'D-I) activity in male mice," *Toxicology*, vol. 124, no. 3, pp. 203–209, 1997.

[83] G. R. Buettner, "The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate," *Archives of Biochemistry and Biophysics*, vol. 300, no. 2, pp. 535–543, 1993.

[84] B. Frei, "Ascorbic acid protects lipids in human plasma and low-density lipoprotein against oxidative damage," *American Journal of Clinical Nutrition*, vol. 54, no. 6, pp. 1113–1118, 1991.

[85] A. Telfer, S. M. Bishop, D. Phillips, and J. Barber, "Isolated photosynthetic reaction center of photosystem II as a sensitizer for the formation of singlet oxygen. Detection and quantum yield determination using a chemical trapping technique," *Journal of Biological Chemistry*, vol. 269, no. 18, pp. 13244–13253, 1994.

[86] J. A. Mares-Perlman, W. E. Brady, R. Klein et al., "Serum antioxidants and age-related macular degeneration in a population-based case-control study," *Archives of Ophthalmology*, vol. 113, no. 12, pp. 1518–1523, 1995.

[87] E. Giovannucci and S. K. Clinton, "Tomatoes, lycopene, and prostate cancer," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 218, no. 2, pp. 129–139, 1998.

[88] G. S. Omenn, G. E. Goodman, M. D. Thornquist et al., "Effects of a combination of beta carotene and vitamin A on

lung cancer and cardiovascular disease," *New England Journal of Medicine*, vol. 334, no. 18, pp. 1150–1155, 1996.

[89] A. Mortensen, L. H. Skibsted, and T. G. Truscott, "The interaction of dietary carotenoids with radical species," *Archives of Biochemistry and Biophysics*, vol. 385, no. 1, pp. 13–19, 2001.

[90] D. Shastri, M. Kumar, and A. Kumar, "Modulation of lead toxicity by Spirulina fusiformis," *Phytotherapy Research*, vol. 13, no. 3, pp. 258–260, 1999.

[91] V. Machartova, J. Racek, J. Kohout, V. Senft, and L. Trefil, "Effect of antioxidant therapy on indicators of free radical activity in workers at risk of lead exposure," *Vnitrni Lekarstvi*, vol. 46, pp. 444–446, 2000.

[92] A. Mortensen and L. H. Skibsted, "Relative stability of carotenoid radical cations and homologue tocopheroxyl radicals. A real time kinetic study of antioxidant hierarchy," *FEBS Letters*, vol. 417, no. 3, pp. 261–266, 1997.

[93] S. J. S. Flora, V. K. Jain, J. R. Behari, and S. K. Tandon, "Protective role of trace metals in lead intoxication," *Toxicomanies*, vol. 13, no. 1-2, pp. 51–56, 1982.

[94] J. H. Kagi and B. L. Vallee, "Metallothionein: a cadmium and zinc-containign protein from equine renal cortex. II. Physico-chemical properties," *The Journal of biological chemistry*, vol. 236, pp. 2435–2442, 1961.

[95] R. Papaioannou, A. Sohler, and C. C. Pfeiffer, "Reduction of blood lead levels in battery workers by zinc and vitamin C," *Journal of Orthomolecular Psychiatry*, vol. 7, no. 2, pp. 94–106, 1978.

[96] S. J. S. Flora, S. Singh, and S. K. Tandon, "Thiamine and zinc in prevention or therapy of lead intoxication," *Journal of International Medical Research*, vol. 17, no. 1, pp. 68–75, 1989.

[97] S. J. S. Flora and S. K. Tandon, "Beneficial effects of zinc supplementation during chelation treatment of lead intoxication in rats," *Toxicology*, vol. 64, no. 2, pp. 129–139, 1990.

[98] N. Batra, B. Nehru, and M. P. Bansal, "The effect of zinc supplementation on the effects of lead on the rat testis," *Reproductive Toxicology*, vol. 12, no. 5, pp. 535–540, 1998.

[99] C. J. Frederickson, "Neurobiology of zinc and zinc-containing neurons," *International review of neurobiology*, vol. 31, pp. 145–238, 1989.

[100] S. R. Powell, "The antioxidant properties of zinc," *Journal of Nutrition*, vol. 130, no. 5, pp. 1447s–1454s, 2000.

[101] A. Ozturk, A. K. Baltaci, R. Mogulkoc et al., "Effects of zinc deficiency and supplementation on malondialdehyde and glutathione levels in blood and tissues of rats performing swimming exercise," *Biological Trace Element Research*, vol. 94, no. 2, pp. 157–166, 2003.

[102] G. Ozdemir and F. Inanc, "Zinc may protect remote ocular injury caused by intestinal ischemia reperfusion in rats," *Tohoku Journal of Experimental Medicine*, vol. 206, no. 3, pp. 247–251, 2005.

[103] M. Sato and I. Bremner, "Oxygen free radicals and metallothionein," *Free Radical Biology and Medicine*, vol. 14, no. 3, pp. 325–337, 1993.

[104] A. A. Shaheen and A. A. El-Fattah, "Effect of dietary zinc on lipid peroxidation, glutathione, protein levels and superoxide dismutase activity in rat tissues," *The International Journal of Biochemistry & Cell Biology*, vol. 27, pp. 89–95, 1995.

[105] E. Bonda, T. Włostowski, and A. Krasowska, "Testicular toxicity induced by dietary cadmium is associated with decreased testicular zinc and increased hepatic and renal

metallothionein and zinc in the bank vole (Clethrionomys glareolus)," *BioMetals*, vol. 17, no. 6, pp. 615–624, 2004.

[106] T. Koizumi and M. P. Waalkes, "Effects of zinc on the distribution and toxicity of cadmium in isolated interstitial cells of the rat testis," *Toxicology*, vol. 56, no. 2, pp. 137–146, 1989.

[107] A. Valenzuela, J.-M. Lefauconnier, J. Chaudiere, and J.-M. Bourre, "Effects of lead acetate of cerebral glutathione peroxidase and catalase in the suckling rat," *NeuroToxicology*, vol. 10, no. 1, pp. 63–70, 1989.

[108] F. Atroshi, A. Rizzo, I. Biese et al., "Fumonisin B1-induced DNA damage in rat liver and spleen: effects of pretreatment with coenzyme Q10, L-carnitine, α-tocopherol and selenium," *Pharmacological Research*, vol. 40, no. 6, pp. 459–467, 1999.

[109] R. A. Kowluru, R. L. Engerman, and T. S. Kern, "Diabetes-induced metabolic abnormalities in myocardium: effect of antioxidant therapy," *Free Radical Research*, vol. 32, no. 1, pp. 67–74, 2000.

Review Article

Antioxidants and the Integrity of Ocular Tissues

Marcela P. Cabrera[1] and Ricardo H. Chihuailaf[2]

[1] *Escuela de Graduados, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, P.O. Box 567, 5110566 Valdivia, Chile*
[2] *Instituto de Ciencias Clínicas Veterinarias, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, P.O. Box 567, 5110566 Valdivia, Chile*

Correspondence should be addressed to Ricardo H. Chihuailaf, rchihuailaf@uach.cl

Oxygen-derived free radicals are normally generated in many pathways. These radicals can interact with various cellular components and induce cell injury. When free radicals exceed the antioxidant capacity, cell injury causes diverse pathologic changes in the organs. The imbalance between the generation of free radicals and antioxidant defence is known as oxidative stress. The eye can suffer the effect of oxidative damage due to the etiopathogenesis of some pathological changes related to oxidative stress. This paper reviews the role of oxidative stress in the onset and progression of damage in different eye structures, the involvement of the antioxidant network in protecting and maintaining the homeostasis of this organ, and the potential assessment methodologies used in research and in some cases in clinical practice.

1. Introduction

The eye is an organ that captures light stimuli of the environment and transforms these light signals into nerve impulses that travel through the optic nerve to be processed in images by the brain. Today, there has been an increase in visits for eye diseases in both human medicine and veterinary medicine. The main causes of this increase can be attributed to xenobiotics derived of environmental pollution [1], increasing of the ultraviolet radiation intensity [2], and feeding based on fats and carbohydrates associated with physical inactivity and increasing of degenerative diseases such as diabetes and cardiovascular problems [3, 4].

All the above factors can generate harmful chemicals to ocular tissues and are called oxidants agents or free radicals (FRs). To protect against external agents, the eye has several nonspecific defence mechanisms, such as eyelids, tear film, cornea, and lens. When harmful agents have overcome these barriers, other specific mechanisms operate based on molecules called antioxidants [5].

This paper reviews the role of oxidative stress in the onset and progression of damage in different eye structures, the involvement of the antioxidant network in protecting and maintaining the homeostasis of this organ, and the assessment methodologies used in research and in some cases in clinical practice.

1.1. Free Radicals. An FRs represents any chemical species that has one or more unpaired electrons rotating in its external atomic orbits [6]. Several authors have classified FRs according to the functional group in their molecule. The most frequent one is an oxygen FRs, in which oxygen is the functional centre. This species is called reactive oxygen species (ROS) and is the most relevant in eye damage. ROS is a generic name that includes FRs and those chemical species that act like oxidants but that are not FRs. The first group includes superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet-}$), peroxyl radical ($LOO^{\bullet}$), and alkoxyl ($LO^{\bullet}$) [3, 7].

Nonradical species behave as oxidants or are easily converted into FRs. Within this group are hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), singlet oxygen ($^1O_2$), and ozone (O_3) [8].

ROS are generally formed from normal metabolic reactions and exogenous factors can increase them [3, 9]. The first ones are mainly those formed in the mitochondria during ATP synthesis, in the peroxisomes during β-oxidation of fatty acids and D-amino acids, as consequence of activation of P-450 enzyme system or by macrophages

and neutrophils as part of the immune response [10–12]. The exogenous factors can include an excessive intake of some transition metals such as iron (Fe) and copper (Cu), ultraviolet radiation, drugs, and pollution [12, 13].

1.2. Oxidative Stress. This alteration is produced when there is an imbalance in FRs generation and the antioxidant defence mechanism. Normal metabolic processes generate large amounts of ROS. However, when FRs generation exceeds the capacity of adaptation and cellular defence, a condition known as oxidative stress is produced. This does not define if the alteration is due to an increase in FRs or to a decrease in a homeostatic response in tissues. Given that oxidative injury can be related to a deficiency of protective substances and several protective substances are nutrients, a close relationship between oxidative stress and nutritional state can be established. Oxidative stress affects the cell integrity, because biomolecules such as DNA, proteins, and lipids are damaged consequence of this process. Almost every biological macromolecules can be oxidized by ROS; however, lipids and proteins are the most labile biomolecules presents in the eyeball [3, 9].

1.2.1. Lipid Peroxidation. The action of ROS on lipids is known as lipoperoxidation or lipid peroxidation (LPO), and the mechanism, measurement, and interpretation have been widely reviewed. The LPO is particularly destructive, for it develops as a self-perpetrating chain reaction. This process is initiated when ROS removes a hydrogen atom from the methylene group of a polyunsaturated fatty acid (PUFA) and form a lipid FRs. Quickly, it adds oxygen molecule and becomes a fatty acid peroxyl FRs and oxidized to other PUFA initiating new reactions. This mechanism is facilitated by the presence of transition metal ions (Cu and Fe) and the double bonds contained in the chain of PUFA [7, 8]. The end products of LPO are degraded and originate new cytotoxic compounds such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). The consequences of oxidative damage to PUFA are more evident when they are part of cellular or subcellular membranes, because it alters its cohesion, fluidity, permeability, and metabolic function [6, 9, 14].

1.2.2. Protein Oxidation. While proteins, peptides, and amino acids are also targets for ROS, its modification is less harmful than lipids, because the progress of reaction is slow. However, the ocular tissue has a high percentage of proteins, and then, any alteration on the proteins is very important [15, 16]. It has been observed that the presence of significant amounts of aromatic and sulfur amino acids in a protein structure makes it more vulnerable to the FRs [6]. This condition is observed in the lens, whose protein composition contains high proportions of tryptophan, tyrosine, phenylalanine, histidine, methionine, and cysteine amino acids that can be modified by ROS, producing adducts and aggregation and altered enzyme function. Peptide bonds are also susceptible to be attacked by FRs. These bonds can be modified after the oxidation of

proline residues. In addition, end products could amplify the initial damage [13].

2. Antioxidant Protection Mechanisms Associated to the Eye

The ocular tissue has a protection system against oxidative damage that can be classified into enzymatic and nonenzymatic antioxidants [17].

2.1. Enzymatic Antioxidants. Enzymatic antioxidants catalyze electron transference from a substrate toward ROS. Later, the substrates or reducing agents used in these reactions are regenerated to be used again, and they achieve this by using the NADPH produced in different metabolic pathways [6]. The main antioxidant enzymes protecting the eye against ROS are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Each of these enzymes catalyzes the reduction of a particular type of ROS [18].

2.1.1. Superoxide Dismutase (EC 1.15.1.1, SOD). Superoxide dismutase catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2. This enzyme is a metalloprotein and has three isoforms, which have different cellular locations and employ different cofactors. The isoforms Cu-SOD and Zn-SOD are located in the cytosol and extracellular fluid, whereas the isoform Mn-SOD is located in the mitochondrial matrix [9, 19]. All isoforms have been identified in the cornea (epithelium and endothelium) [18, 20–22], lens epithelium [23], aqueous humor [24], iris, ciliary body [25], and retina (inner segment layer of photoreceptor cells and pigment epithelium) [26, 27].

2.1.2. Glutathione Peroxidase (EC 1.11.1.9, GPx). This selenoprotein can reduce H_2O_2 and organic hydroperoxides into water and alcohol, respectively, using reduced glutathione (GSH) as electron donor. Four GPx isoforms have been described, and they are all found in different locations: cellular GPx, extracellular or plasmatic GPx, phospholipid hydroperoxide GPx, and gastrointestinal GPx. All GPx are an important defence against ROS-mediated damage to lipid membranes and other molecules susceptible to oxidation [6]. GPx has been detected in cornea (epithelium and endothelium) [18, 28], lens epithelium, aqueous humor [24], ciliary body, choroid, and retina (inner segment layer of photoreceptors and retinal pigment epithelium) [21, 27, 29].

2.1.3. Catalase (EC 1.11.1.6, CAT). This hemoprotein contains four heme groups. The enzyme is present in peroxisomes mitochondria and cytoplasm and catalyzes the conversion of H_2O_2 into H_2O and O_2. This function is shared with GPx, but CAT has higher affinity when H_2O_2 is found in high concentrations [14]. CAT has been detected in the cornea (epithelium and endothelium) [18, 28], lens epithelium, aqueous humor [24], ciliary body, iris [25], and retina [26, 27] of rabbits and rats [30].

2.2. Nonenzymatic Antioxidants.

2.2. Nonenzymatic Antioxidants. These antioxidants constitute a heterogeneous group, and they act by donating an electron to an FRs in order to stabilize it and create chemical species that are less noxious to cell integrity [6]. The main nonenzymatic antioxidants present in the globe are ascorbic acid, vitamin E, vitamin A, and GSH [5].

2.2.1. Ascorbic Acid. It is an antioxidant that is soluble at physiological pH in most tissues as ascorbate anion. Its antioxidant role is to reduce $O_2^{\bullet-}$, $OH^{\bullet-}$ and lipid hydroperoxide into more stable forms [14]. Another function of ascorbate is related with the recycling of α-tocopheryl radical to α-tocopherol. However, this process transforms ascorbate anion into dehydroascorbate anion radical, which can be reduced by dehydroascorbate reductase and GSH returning it to native state. Also, ascorbate can act as a pro-oxidant in the presence of excessive concentrations of ions Fe^{+3} and Cu^{+2} [6, 7, 14]. Ascorbate was detected in cornea [31], aqueous humor [32, 33], lens [34], vitreous humor [35], and retina [29].

2.2.2. Vitamin E. The term vitamin E is a generic name for a family of eight compounds, four tocopherols, and four tocotrienols, of which the α-tocopherol is the most active antioxidant and the primary defence liposoluble antioxidant in membranes. The α-tocopherol converts $O_2^{\bullet-}$, $OH^{\bullet-}$ and $LOO^{\bullet}$ into less reactive molecules. The phenolic hydroxyl on the chroman ring is responsible for the antioxidant function [9]. In parallel, α-tocopherol can stop the chain reaction of ROS during the attack on cell membranes [36]. To stabilize ROS, the α-tocopherol is converted into the α-tocopheryl radical, whose shape is stable and does not react with biomolecules. α-tocopheryl radical can be regenerated to its original through reactions mediated by vitamin C, GSH, and lipoic acid [6]. The antioxidant ability of α-tocopherol depends on the concentrations of those compounds, which keep α-tocopherol in its reduced state in instances of oxidative stress [36]. It is possible that an overproduction of ROS can cause a significant drop of active vitamin E tissue concentration. The α-tocopherol has been detected in lens [37], aqueous humor, and retina [29].

2.2.3. Vitamin A. This generic term includes those compounds from animals that show vitamin A biological activity. The major precursor of vitamin A is β-carotene, which is the most efficient neutralizer of $^1O_2$. The antioxidant properties of vitamin A derive from its chemical structure, which consists of long chains of conjugated double bonds, this allows to convert $O_2^{\bullet-}$ y $LOO^{\bullet}$ into less reactive substances [14]. Carotenoids are effective antioxidants but differ in their concentrations in ocular tissues. Other carotenoids, with the exception of lutein/zeaxanthin, are found only in trace quantities in the ocular tissues except in the ciliary body, where aqueous humor is produced. In contrast, lutein and zeaxanthin are found in high concentrations in some ocular tissues, such as the macula, retina, and lens [37, 38].

2.2.4. Glutathione (GSH). Its reduced form corresponds to a tripeptide (gamma glutamyl-cysteinyl-glycine) with a sulfhydryl group (-SH) in the active site. GSH transfers electrons to oxidized specie such as hydroxyl radicals and carbonyls, becoming in turn an oxidized product (GSSG) [9, 14]. During this reaction, GSH donates a pair of H so that two GSH molecules are oxidized to produce GSSG. Also, GSH acts as cosubstrate of GPx in the removal of H_2O_2 and organic peroxides, and it can reduce tocopheryl FRs and dehydroascorbate returning them to original form [6, 36].

GSH is important for the maintenance of lenticular proteins in a reduced state [30]. This has been found in lens [39, 40], cornea, and retina [41], being next to ascorbic acid one of the main mechanism of defence against photo-oxidation [15].

3. Oxidative Stress and Globe

The eye is affected by oxidative stress due to its physical and metabolic characteristics. The eye is a metabolically active organ, consuming large amount of ATP. In addition, the transparency of the cornea, aqueous humor, lens, vitreous and retina allows a constant photochemical ROS generation [17, 32]. All ocular tissues and fluids are susceptible to damage by oxidative stress; however, those described below affect severely the function of the eye due to the physical changes they undergo [18].

3.1. Cornea. The main ROS generation occurs in the cornea due to high exposure to ultraviolet radiation [18]. The cornea is the main barrier which stops ultraviolet radiation (UVA-UVB). It absorbs 92% of UV-B and 60% of UV-A, and the highest degree of absorption occurs in the surface layers [42, 43].

UV radiation produces changes in the cornea, such as blocking epithelial cells proliferation and reduced epithelial thickness [44], decrease in corneal antioxidant such CAT, GPx, and SOD [18], decreased Na^+/K^+ ATPase in the corneal epithelium and endothelium, causing significant increase in corneal hydration and changes in the transparency of the cornea and release of proinflammatory cytokines [2, 20, 45, 46]. UV radiation also causes alteration of the physiological properties of glycosaminoglycans in the stroma, becoming more susceptible to degradation by tissue enzymes from stimulated phagocytic cells [18, 47].

It has been determined that the primary antioxidant defence is ascorbic acid (highly concentrated in the corneal centre, right in the pupil area) and SOD activity. CAT and GPx enzymes have a secondary role [33]. However, when an episode of oxidative stress is triggered, the enzymatic activity begins to reduce, first CAT activity, then GPx activity, and finally SOD activity begin to reduce, thereby increasing the amount of H_2O_2 further damaging the cornea [18, 20].

3.2. Aqueous Humor. ROS generation in the aqueous humor is due primarily to UV radiation [18] and inflammatory processes that occur in adjacent structures [48]. Aqueous humor contains ascorbic acid, proteins, and some amino acids (tyrosine, phenylalanine, cysteine, and tryptophan), involved in UV-B absorption by allowing only a small fraction of these radiation reach the posterior segment of globe [18]. The UV radiation absorption in the aqueous

humor causes an increase in H_2O_2 concentration, which, in turn, decreases the metabolism of GSH. It has been determined that ascorbic acid plays a fundamental role as UV filters in mammals, having a greater concentration in the aqueous humor from diurnal animals than nocturnal animals [17]. Also, ascorbic acid concentration in aqueous humor is higher than blood plasma [32, 48, 49].

Another source of formation of large amounts of ROS was observed after surgery, paracentesis, or uveitis due to an increase in the amount of proteins and cells in aqueous humor [32, 48]. There is evidence that after lenticular surgery, the total antioxidant capacity of aqueous humor decreased up to 40%, mainly due to the decreased concentration of ascorbic acid [32, 48]. This fact was also observed in human patients with idiopathic acute anterior uveitis [50].

The increase in the H_2O_2 concentration in the aqueous humor may cause damage to corneal endothelium, lens, and ciliary body, especially trabecular network. *In vitro* studies have shown that a decrease in the facility of aqueous humor outflow occurs when an increased of H_2O_2 concentration is present, and this may be grounds for diseases such as glaucoma [51].

3.3. Lens. The lens is most affected by oxidative damage, because it is an avascular structure and has a constant and spare production of lenticular proteins. It is highly exposed to UV radiation and shows a marked reduction of antioxidants levels in the lenticular nucleus. In addition, changes on the aqueous humor composition can affect the (inflammation of adjacent structures and metabolic disorders such as diabetes) [5, 51].

Lenticular metabolism is related to energy production for protein synthesis and maintenance of osmotic balance. The pentose phosphate pathway, via glucose-6-phosphate dehydrogenase (G-6-PD) activity, provides reducing equivalents (NADPH) for keeping the lens in a reduced state [40].

The mechanism proposed to explain the lenticular opacity is the oxidation of the crystalline (α, β, and γ-crystalline), mainly lens proteins [51, 52]. The photo-oxidation of thiol groups on lens crystallins produce disulfide adducts and molecules that lead to protein aggregation and hence the cataract development [5]. Besides protein aggregation, there are also alterations in the Na^+/K^+ ATPase [51]. It has been determined that damage at the protein level by oxidative processes is increases with age in human lens and is significantly greater in those with cataracts than normally transparent crystalline [16]. An increase of cystine levels (disulfide groups) and a decrease in cysteine concentration (sulfhydryl groups) during the cataract genesis have been demonstrated. Also an increase in disulfide-sulfhydryl ratio from soluble and insoluble proteins of human cataractous lenses is higher when the lenticular opacification increases [53].

Ascorbic acid and GSH are major defence mechanisms against photo-oxidation in the lens and aqueous humor [15]. The concentration of GSH in the lenticular epithelium is as high as that in the liver and its concentration decreases by exposure to UV radiation [54] and cataracts cases [40].

No differences were found between erythrocyte antioxidant enzymes from animals with and without cataracts, but MDA plasma levels are increased and ascorbic acid concentrations decreased from animals with cataracts [49]. Others antioxidants present in lens, like lutein-zeaxanthin, retinoid, and tocopherol contents, do not show differences in concentration between normal and cataractous lenses [37], also, one study shows that concentration of α-tocopherol was significantly higher in cataract lenses than clear lenses [55].

3.4. Retina. The retina is the neurosensorial tissue of the eye, and it is extremely rich in polyunsaturated lipids. This characteristic makes it particularly sensitive to ROS [56]. The main generation of ROS in the retina is due to the presence of cells with high oxygen consumption rate [57], exposure to UV radiation, and the presence of diseases that directly affect the vascular irrigation such as glaucoma [27].

In the retina, the light is focused directly on cells group located in a richly oxygenated place. The presence of a variety of pigments (melanin, lipofuscin, and lutein) provides an optimum condition for photosensitizing reactions, generating ROS. The outer segment photoreceptor membrane is rich in polyunsaturated lipids and is where most damage occurs [51].

The final common pathway of a group of diseases associated to decreased sensitivity and function of retinal ganglion cells, cells death, enlargement of the optic nerve head, visual field reduction, and blindness is glaucoma. Oxidative stress may contribute to the etiology and progression of glaucoma [58], the ischemia, and reperfusion process affects the retina increasing the nitric oxide production and other FRs in vitreous and aqueous humor [59]. The LPO is triggered, and it is considered the cause of injury and death of retinal ganglion cells and subsequent optic nerve damage [60]. Antioxidant protection exists in the retina, and this is mainly due to C and E vitamin, carotenoids, GPx, SOD and CAT enzymes, and GSH compound [29, 51, 61].

4. Evaluation of Oxidative Stress

The measurement of biomarkers of oxidative stress varies depending on the structure to be evaluated in the globe. Oxidant and antioxidant agents vary in the different ocular tissues and fluids. It is possible evaluate the protection and/or existing damage [32].

4.1. Protection Assessment. The antioxidant protection can be estimated by measurements of antioxidant compound concentrations (proteins, peptides, or vitamins), antioxidant enzyme activities, or the antioxidant capacity as a whole in each tissue or fluid of globe [32, 68].

The presence of SOD, GPx, and CAT in the globe can be evaluated *in vitro* using biochemical and immunohistochemical methods [18, 29, 30]. Also, it is possible to determine the SOD activity *in situ* [18]. Ascorbic acid, carotenoids, and tocopherols can be determined by high-pressure liquid chromatography (HPLC) [29, 32, 37, 48]. The activity of fat-soluble antioxidants can be estimated by quantifying the resistance to oxidation of low density lipoprotein [6].

TABLE 1: Incidence of eye disease compared with antioxidant intake and/or plasma levels of antioxidants molecules in humans.

Authors	Objective	Antioxidants	Conclusion
Berendschot et al. (2002) [62]	376 subjects of 18 to 75 years. To investigate whether serum levels of antioxidants influence the lens optical density.	Lutein, zeaxanthin, Vit. C, and α-tocopherol.	High serum levels of lutein and zeaxanthin may retard aging of the lens.
Delcourt et al. (1999) [63]	2584 subjects, >60 years. To determine plasma levels of antioxidant enzymes related with cataract and age-related macular degeneration.	Enzymes: GPx and SOD.	High levels of plasma GPx were associated with age-related macular degeneration and cataract prevalence. High levels of plasma SOD were associated with high cataract prevalence.
Delcourt et al. (2003) [64]	1947 subjects, >60 years. To determine the association between antioxidant enzymes activity and incidence of cataract.	Enzymes: SOD and GPx.	High levels of plasma GPx and SOD were associated with high cataract incidence.
Gale et al. (2001) [65]	372 subjects of 66 to 75 years. To determine plasma levels of some vitamins and carotenoids related with cataract risk incidence.	Vit. C, Vit. E, α and β-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin.	High levels of α-carotene, β-carotene, lycopene and lutein were associated with low risk of cataract.
Jacques et al. (2001) [66]	478 women of 53 to 73 years. To assess the relation between usual nutrient intake, plasma vitamins concentration and subsequently diagnosed age-related nuclear lens opacities.	Vit. C, Vit. E, riboflavin, β-carotene, lutein, and zeaxanthin.	High Vit. C intake is associated with low risk of cataract incidence. High Vit. C and Vit. E plasma concentrations are associated with low lenticular opacity.
Lyle et al. (1999) [67]	400 subjects of 50 to 86 years, 7 years followup. To assess the relation of serum carotenoids and tocopherols levels to the incidence of cataract.	Carotenoids, α-tocopherol, and γ-tocopherol.	High serum levels of tocopherols are associated with low risk of cataract.

Vit. C: vitamin C; Vit. E: vitamin E; GPx: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase.

TABLE 2: Animal research comparing antioxidants and/or plasma levels of antioxidants molecules with degenerative changes in dogs.

Authors	Objective	Antioxidants	Conclusion
Barros et al. (1999) [49]	To determine the erythrocytic enzymatic antioxidants, plasma Vit. C and MDA in normal and cataractous English Cocker Spaniel dogs.	Enzymes: SOD, CAT, GPx, and G6PD. Vit. C.	Decrease levels of plasma Vit. C are related with cataract.
Barros et al. (2003) [32]	To determined the antioxidant status of the aqueous humor after extracapsular lens extraction.	Total antioxidant status and Vit C.	Lens surgical procedures reduced total antioxidant status and Vit. C levels.
Barros et al. (2004) [69]	To determined levels of enzymatic and nonenzymatic antioxidants in blood and aqueous humor of cataractous and noncataractous poodles.	Enzymes: SOD, CAT, GPx, and G6PD. Vit. C.	Activity of SOD, G6PD, and CAT was significantly higher in noncataractous poodles than in cataractous poodles. There was no difference in mean plasma Vit. C concentration between cataractous and noncataractous dogs.
De Biaggi et al. (2006) [48]	To determined the antioxidant status of the aqueous humor after phacoemulsification.	Total antioxidant status and Vit. C.	Lens surgical procedures reduced total antioxidant status and Vit. C levels.

Vit. C: vitamin C; GPx: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde; G6PD: glucose-6-phosphate dehydrogenase.

To evaluate the total antioxidant capacity is accepted to determine the capacity of the tissue or fluid to inhibit a specific free radical [32, 48, 68] or determine the GSH/GSSG ratio and ascorbic acid/dehydroascorbate ratio [6].

4.2. Damage Assessment. To assess the damage, we can use the identification and quantification of proteins, lipids, and other substances from damaged cell. The biomarkers frequently used are carbonyl and sulfhydryl levels [16]. In the course of an oxidative process, carbonyl groups are formed into the amino acid chain mainly in the lysine, arginine, proline, and histidine residues. These carbonyl can be detected by spectrophotometric methods, high-pressure liquid chromatography (HPLC), or enzyme-linked

immunosorbent assay (ELISA) technique. To assess the integrity of lipids, the measurement of membrane lipid peroxidation is a key indicator [70]. The most common method for assessing LPO, due to its simplicity and low cost, is the measurement of MDA as thiobarbituric acid reactive substance (TBARS) [6, 70].

5. Conclusions

As mentioned, the eye is exposed to oxidizing conditions that lead to an alteration of ocular tissue, impaired vision, and, in turn, the animal's relationship with the surrounding environment. The importance of assessing oxidative stress in the globe lies in identifying the magnitude and extent of damage that occurs in this condition. It is necessary to establish treatment regimens to prevent and repair damage by ROS. This last point is being addressed by some research groups inducing or determining the incidence of ocular diseases and comparing the intake of antioxidants and/or blood levels of these (Table 1). It is established that it is important to have an optimal level of antioxidants to maintain ocular tissues safe, but it is not clear if consumption of those antioxidant could help to achieve this gold. Veterinary literature in this topic seems to be limited (Table 2).

Models to study oxidative stress have yielded promising results in relation with the use of antioxidants in diet. However, the results cannot be completely confirmed because of the inability to measure accurately the intake of antioxidants in humans, and also it is unlikely to extrapolate results from studies made in laboratory animals. There are other drawbacks to recommend antioxidant intake due to the fact that it is possible that an excessive consumption can lead to other diseases such as formation of bladder stones by eating high concentrations of vitamin C. Negligent administration of supplements without having more knowledge of dosages and good employment conditions may actually speed up rather than suppress oxidative damage.

It would be desirable to know the normal antioxidant status of the eye in each animal species and have better or less invasive ways to evaluate it, such as measure blood antioxidant profile and determine how it relates to the antioxidant profile or damage of a specific ocular tissue. It is also an important goal find different ways to strengthening antioxidant defences and stop oxidative damage. There is an open door to start new research in this area with many questions to resolve.

References

[1] R. Saxena, S. Srivastava, D. Trivedi, E. Anand, S. Joshi, and S. K. Gupta, "Impact of environmental pollution on the eye," *Acta Ophthalmologica Scandinavica*, vol. 81, no. 5, pp. 491–494, 2003.

[2] K. M. Newkirk, H. L. Chandler, A. E. Parent et al., "Ultraviolet radiation-induced corneal degeneration in 129 mice," *Toxicologic Pathology*, vol. 35, no. 6, pp. 817–824, 2007.

[3] J. K. Willcox, S. L. Ash, and G. L. Catignani, "Antioxidants and prevention of chronic disease," *Critical Reviews in Food Science and Nutrition*, vol. 44, no. 4, pp. 275–295, 2004.

[4] P. P. Lee, Z. W. Feldman, J. Ostermann, D. S. Brown, and F. A. Sloan, "Longitudinal prevalence of major eye diseases," *Archives of Ophthalmology*, vol. 121, no. 9, pp. 1303–1310, 2003.

[5] D. L. Williams, "Oxidation, antioxidants and cataract formation: a literature review," *Veterinary Ophthalmology*, vol. 9, no. 5, pp. 292–298, 2006.

[6] R. Chihuailaf, P. Contreras, and F. Wittwer, "Patogénesis del estrés oxidativo: consecuencias y evaluación en salud animal," *Veterinaria México*, vol. 33, pp. 265–283, 2002.

[7] B. Halliwell, J. M. C. Gutteridge, and C. E. Cross, "Free radicals, antioxidants, and human disease: where are we now?" *Journal of Laboratory and Clinical Medicine*, vol. 119, no. 6, pp. 598–620, 1992.

[8] B. Halliwell and S. Chirico, "Lipid peroxidation: its mechanism, measurement, and significance," *American Journal of Clinical Nutrition*, vol. 57, supplement 5, pp. 715S–724S, 1993.

[9] D. Benítez, "Vitaminas y oxidorreductasas antioxidantes: defensa ante el estrés oxidativo," *Revista Cubana de Investigaciones Biomedicas*, vol. 25, no. 2, pp. 1–8, 2006.

[10] N. Punchard and F. Kelly, *Free Radicals-A Practical Approach*, Oxford University Press, New York, NY, USA, 1997.

[11] C. M. Deaton and D. J. Marlin, "Exercise-associated oxidative stress," *Clinical Techniques in Equine Practice*, vol. 2, no. 3, pp. 278–291, 2003.

[12] S. Galecio, *Lipoperoxidación y daño muscular producto del ejercicio en equinos criollo Chileno y su relación con la actividad sanguínea de glutatión peroxidasa*, Memoria Magíster en Ciencias. thesis, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile, 2007.

[13] M. Martínez-Cayuela, "Toxicidad de xenobióticos mediada por radicales libres de oxígeno," *Ars Pharmaceutica*, vol. 39, no. 1, pp. 5–18, 1998.

[14] B. Yu, "Cellular defenses against damage from reactive oxygen species," *Physiological Reviews*, vol. 74, no. 1, pp. 139–162, 1994.

[15] A. Taylor, P. F. Jacques, and E. M. Epstein, "Relations among aging, antioxidant status, and cataract," *American Journal of Clinical Nutrition*, vol. 62, supplement 6, pp. 1439s–1447s, 1995.

[16] F. Boscia, I. Grattagliano, G. Vendemiale, T. Micelli-Ferrari, and E. Altomare, "Protein oxidation and lens opacity in humans," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 9, pp. 2461–2465, 2000.

[17] S. D. Varma, "Scientific basis for medical therapy of cataracts by antioxidants," *American Journal of Clinical Nutrition*, vol. 53, supplement 1, pp. 335s–345s, 1991.

[18] J. Čejková, S. Štípek, J. Crkovská et al., "UV rays, the prooxidant/antioxidant imbalance in the cornea and oxidative eye damage," *Physiological Research*, vol. 53, no. 1, pp. 1–10, 2004.

[19] L. L. Ji, "Oxidative stress during exercise: implication of antioxidant nutrients," *Free Radical Biology and Medicine*, vol. 18, no. 6, pp. 1079–1086, 1995.

[20] J. Čejková, S. Štípek, J. Crkovská, and T. Ardan, "Changes of superoxide dismutase, catalase and glutathione peroxidase in the corneal epithelium after UVB rays. Histochemical and biochemical study," *Histology and Histopathology*, vol. 15, no. 4, pp. 1043–1050, 2000.

[21] A. Bilgihan, K. Bilgihan, Ö. Yis, C. Sezer, G. Akyol, and B. Hasanreisoglu, "Effects of topical vitamin E on corneal superoxide dismutase, glutathione peroxidase activities and polymorphonuclear leucocyte infiltration after photorefractive keratectomy," *Acta Ophthalmologica Scandinavica*, vol. 81, no. 2, pp. 177–180, 2003.

[22] A. Behndig, K. Karlsson, B. O. Johansson, T. Brännström, and S. L. Marklund, "Superoxide dismutase isoenzymes in the normal and diseased human cornea," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 10, pp. 2293–2296, 2001.

[23] B. Ozmen, D. Ozmen, E. Erkin, I. Güner, S. Habif, and O. Bayindir, "Lens superoxide dismutase and catalase activities in diabetic cataract," *Clinical Biochemistry*, vol. 35, no. 1, pp. 69–72, 2002.

[24] A. Satici, M. Guzey, B. Gurler, H. Vural, and T. Gurkan, "Malondialdehyde and antioxidant enzyme levels in the aqueous humor of rabbits in endotoxin-induced uveitis," *European Journal of Ophthalmology*, vol. 13, no. 9-10, pp. 779–783, 2003.

[25] A. C. Phylactos and W. G. Unger, "Biochemical changes induced by intravitreally-injected doxorubicin in the iris-ciliary body and lens of the rabbit eye," *Documenta Ophthalmologica*, vol. 95, no. 2, pp. 145–155, 1998.

[26] R. N. Frank, R. H. Amin, and J. E. Puklin, "Antioxidant enzymes in the macular retinal pigment epithelium of eyes with neovascular age-related macular degeneration," *American Journal of Ophthalmology*, vol. 127, no. 6, pp. 694–709, 1999.

[27] C. D. Agardh, C. Gustavsson, P. Hagert, M. Nilsson, and E. Agardh, "Expression of antioxidant enzymes in rat retinal ischemia followed by reperfusion," *Metabolism: Clinical and Experimental*, vol. 55, no. 7, pp. 892–898, 2006.

[28] J. Čejková, M. Vejražka, J. Pláteník, and S. Štípek, "Age-related changes in superoxide dismutase, glutathione peroxidase, catalase and xanthine oxidoreductase/xanthine oxidase activities in the rabbit cornea," *Experimental Gerontology*, vol. 39, no. 10, pp. 1537–1543, 2004.

[29] J. S. Penn, L. A. Thum, and M. I. Naash, "Oxygen-induced retinopathy in the rat: vitamins C and E as potential therapies," *Investigative Ophthalmology and Visual Science*, vol. 33, no. 6, pp. 1836–1845, 1992.

[30] S. Zhang, F. Y. Chai, H. Yan, Y. Guo, and J. J. Harding, "Effects of N-acetylcysteine and glutathione ethyl ester drops on streptozotocin-induced diabetic cataract in rats," *Molecular Vision*, vol. 14, pp. 862–870, 2008.

[31] R. F. Brubaker, W. M. Bourne, L. A. Bachman, and J. W. McLaren, "Ascorbic acid content of human corneal epithelium," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 7, pp. 1681–1683, 2000.

[32] P. S. M. Barros, C. F. Padovani, V. V. Silva, L. Queiroz, and S. B. M. Barros, "Antioxidant status of dog aqueous humor after extracapsular lens extraction," *Brazilian Journal of Medical and Biological Research*, vol. 36, no. 11, pp. 1491–1494, 2003.

[33] A. Ringvold, E. Anderssen, and I. Kjonniksen, "Distribution of ascorbate in the anterior bovine eye," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 1, pp. 20–23, 2000.

[34] Y. Ohta, T. Niwa, and T. Yamasaki, "Short-term ascorbic acid deficiency does not impair antioxidant status in lens of guinea pigs," *Journal of Nutritional Science and Vitaminology*, vol. 50, no. 2, pp. 149–153, 2004.

[35] K. Koide, X. M. Zhang, K. Ohishi, Y. Usami, Y. Hotta, and T. Hiramitsu, "Ascorbic acid concentration in rabbit vitreous measured by microdialysis with HPLC-electrochemical detection before and after vitreous surgery," *Experimental Eye Research*, vol. 82, no. 5, pp. 868–873, 2006.

[36] S. K. Powers and S. L. Lennon, "Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle," *Proceedings of the Nutrition Society*, vol. 58, no. 4, pp. 1025–1033, 1999.

[37] K. J. Yeum, A. Taylor, G. Tang, and R. M. Russell, "Measurement of carotenoids, retinoids, and tocopherols in human lenses," *Investigative Ophthalmology and Visual Science*, vol. 36, no. 13, pp. 2756–2761, 1995.

[38] P. S. Bernstein, F. Khachik, L. S. Carvalho, G. J. Muir, D. Y. Zhao, and N. B. Katz, "Identification and quantitation of carotenoids and their metabolites in the tissues of the human eye," *Experimental Eye Research*, vol. 72, no. 3, pp. 215–223, 2001.

[39] H. Sasaki, F. J. Giblin, B. S. Winkler, B. Chakrapani, V. Leverenz, and S. Chu-Chen, "A protective role for glutathione-dependent reduction of dehydroascorbic acid in lens epithelium," *Investigative Ophthalmology and Visual Science*, vol. 36, no. 9, pp. 1804–1817, 1995.

[40] H. Sasaki, F. J. Giblin, B. S. Winkler, B. Chakrapani, V. Leverenz, and S. Chu-Chen, "A protective role for glutathione-dependent reduction of dehydroascorbic acid in lens epithelium," *Investigative Ophthalmology and Visual Science*, vol. 36, no. 9, pp. 1804–1817, 1995.

[41] R. Kannan, D. Tang, J. B. Mackic, B. V. Zlokovic, and J. C. Fernandez-Checa, "A simple technique to determine glutathione (GSH) levels and synthesis in ocular tissues as GSH-bimane adduct: application to normal and galactosemic guinea-pigs," *Experimental Eye Research*, vol. 56, no. 1, pp. 45–50, 1993.

[42] S. Zigman, "Ocular light damage," *Photochemistry and Photobiology*, vol. 57, no. 6, pp. 1060–1068, 1993.

[43] L. Kolozsvari, A. Nogradi, B. Hopp, and Z. Bor, "UV absorbance of the human cornea in the 240- to 400-nm range," *Investigative Ophthalmology and Visual Science*, vol. 43, pp. 2165–2168, 2002.

[44] E. Haaskjold, W. M. Olsen, R. Bjerknes, and K. Kravik, "Early cell kinetic effects of a single dose of narrow-banded ultraviolet B irradiation on the rat corneal epithelium," *Photochemistry and Photobiology*, vol. 57, no. 4, pp. 663–666, 1993.

[45] S. Löfgren and P. G. Söderberg, "Lens lactate dehydrogenase inactivation after UV-B irradiation: an in vivo measure of UVR-B penetration," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 8, pp. 1833–1836, 2001.

[46] J. Čejková and Z. Lojda, "Histochemical study on xanthine oxidase activity in the normal rabbit cornea and lens and after repeated irradiation of the eye with UVB rays," *Acta Histochemica*, vol. 98, no. 1, pp. 47–52, 1996.

[47] R. Carubelli, R. E. Nordquist, and J. J. Rowsey, "Role of active oxygen species in corneal ulceration. Effect of hydrogen peroxide generated in situ," *Cornea*, vol. 9, no. 2, pp. 161–169, 1990.

[48] C. P. De Biaggi, P. S. M. Barros, V. V. Silva, D. E. Brooks, and S. B. M. Barros, "Ascorbic acid levels of aqueous humor of dogs after experimental phacoemulsification," *Veterinary Ophthalmology*, vol. 9, no. 5, pp. 299–302, 2006.

[49] P. S. M. Barros, A. C. Angelotti, F. Nobre, A. Morales, D. T. Fantoni, and S. B. M. Barros, "Antioxidant profile of cataractous English Cocker Spaniels," *Veterinary Ophthalmology*, vol. 2, no. 2, pp. 83–86, 1999.

[50] M. L. Cheng, T. Z. Liu, F. J. Lu, and D. T. Y. Chiu, "Simultaneous detection of vitamin C and uric acid by capillary electrophoresis in plasma of diabetes and in aqueous humor in acute anterior uveitis," *Clinical Biochemistry*, vol. 32, no. 6, pp. 473–476, 1999.

[51] U. Andley, "Photodamage to the eye: yearly review," *Photochemistry and Photobiology*, vol. 46, pp. 1057–1066, 1987.

[52] R. J. W. Truscott, "Age-related nuclear cataract—oxidation is the key," *Experimental Eye Research*, vol. 80, no. 5, pp. 709–725, 2005.

[53] O. P. Kulshrestha, S. N. Kulshrestha, K. P. Khuteta, and Y. Shukla, "Study of cysteine, cystine and methionine in normal and cataractous human lenses," *Indian Journal of Ophthalmology*, vol. 31, no. 3, pp. 267–269, 1983.

[54] V. C. Mody Jr., M. Kakar, A. Elfving, P. G. Söderberg, and S. Löfgren, "Ultraviolet radiation-B-induced cataract in albino rats: maximum tolerable dose and ascorbate consumption," *Acta Ophthalmologica Scandinavica*, vol. 84, no. 3, pp. 390–395, 2006.

[55] K. Krepler and R. Schmid, "Alpha-tocopherol in plasma, red blood cells and lenses with and without cataract," *American Journal of Ophthalmology*, vol. 139, no. 2, pp. 266–270, 2005.

[56] M. Miranda, M. Muriach, J. Roma et al., "Oxidative stress in a model of experimental diabetic retinopathy: the utility of peroxinytrite scavengers," *Archivos de la Sociedad Espanola de Oftalmologia*, vol. 81, no. 1, pp. 27–32, 2006.

[57] L. Wang, M. Kondo, and A. Bill, "Glucose metabolism in cat outer retina: effects of light and hyperoxia," *Investigative Ophthalmology and Visual Science*, vol. 38, no. 1, pp. 48–55, 1997.

[58] J. H. Kang, L. R. Pasquale, W. Willett et al., "Antioxidant intake and primary open-angle glaucoma: a prospective study," *American Journal of Epidemiology*, vol. 158, no. 4, pp. 337–346, 2003.

[59] M. E. Källberg, D. E. Brooks, K. N. Gelatt, G. A. Garcia-Sanchez, N. J. Szabo, and G. N. Lambrou, "Endothelin-1, nitric oxide, and glutamate in the normal and glaucomatous dog eye," *Veterinary Ophthalmology*, vol. 10, no. 1, pp. 46–52, 2007.

[60] L. A. Levin, J. A. Clark, and L. K. Johns, "Effect of lipid peroxidation inhibition on retinal ganglion cell death," *Investigative Ophthalmology and Visual Science*, vol. 37, no. 13, pp. 2744–2749, 1996.

[61] Y. Nakajima, Y. Inokuchi, M. Shimazawa, K. Otsubo, T. Ishibashi, and H. Hara, "Astaxanthin, a dietary carotenoid, protects retinal cells against oxidative stress in-vitro and in mice in-vivo," *Journal of Pharmacy and Pharmacology*, vol. 60, no. 10, pp. 1365–1374, 2008.

[62] T. T. J. M. Berendschot, W. M. R. Broekmans, I. A. A. Klöpping-Ketelaars, A. F. M. Kardinaal, G. Poppel, and D. Norren, "Lens aging in relation to nutritional determinants and possible risk factors for age-related cataract," *Archives of Ophthalmology*, vol. 120, no. 12, pp. 1732–1737, 2002.

[63] C. Delcourt, J. P. Cristol, C. L. Léger, B. Descomps, and L. Papoz, "Associations of antioxidant enzymes with cataract and age-related macular degeneration: the POLA study," *Ophthalmology*, vol. 106, no. 2, pp. 215–222, 1999.

[64] C. Delcourt, I. Carrière, M. Delage, B. Descomps, J. P. Cristol, and L. Papoz, "Associations of cataract with antioxidant enzymes and other risk factors: the French age-related eye diseases (POLA) prospective study," *Ophthalmology*, vol. 110, no. 12, pp. 2318–2326, 2003.

[65] C. R. Gale, N. F. Hall, D. I. W. Phillips, and C. N. Martyn, "Plasma antioxidant vitamins and carotenoids and age-related cataract," *Ophthalmology*, vol. 108, no. 11, pp. 1992–1998, 2001.

[66] P. F. Jacques, L. T. Chylack Jr., S. E. Hankinson et al., "Long-term nutrient intake and early age-related nuclear lens opacities," *Archives of Ophthalmology*, vol. 119, no. 7, pp. 1009–1019, 2001.

[67] B. J. Lyle, J. A. Mares-Perlman, B. E. K. Klein et al., "Serum carotenoids and tocopherols and incidence of age-related nuclear cataract," *American Journal of Clinical Nutrition*, vol. 69, no. 2, pp. 272–277, 1999.

[68] A. Nemec, M. Drobnič-Košorok, M. Skitek, Z. Pavlica, S. Galac, and J. Butinar, "Total antioxidant capacity (TAC) values and their correlation with individual antioxidants in serum of healthy beagles," *Acta Veterinaria Brno*, vol. 69, no. 4, pp. 297–303, 2000.

[69] P. S. M. Barros, A. M. V. Safatle, L. Queiroz, V. V. Silva, and S. B. M. Barros, "Blood and aqueous humour antioxidants in cataractous poodles," *Canadian Journal of Ophthalmology*, vol. 39, no. 1, pp. 19–24, 2004.

[70] H. Esterbauer and K. H. Cheeseman, "Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal," *Methods in Enzymology*, vol. 186, pp. 407–421, 1990.