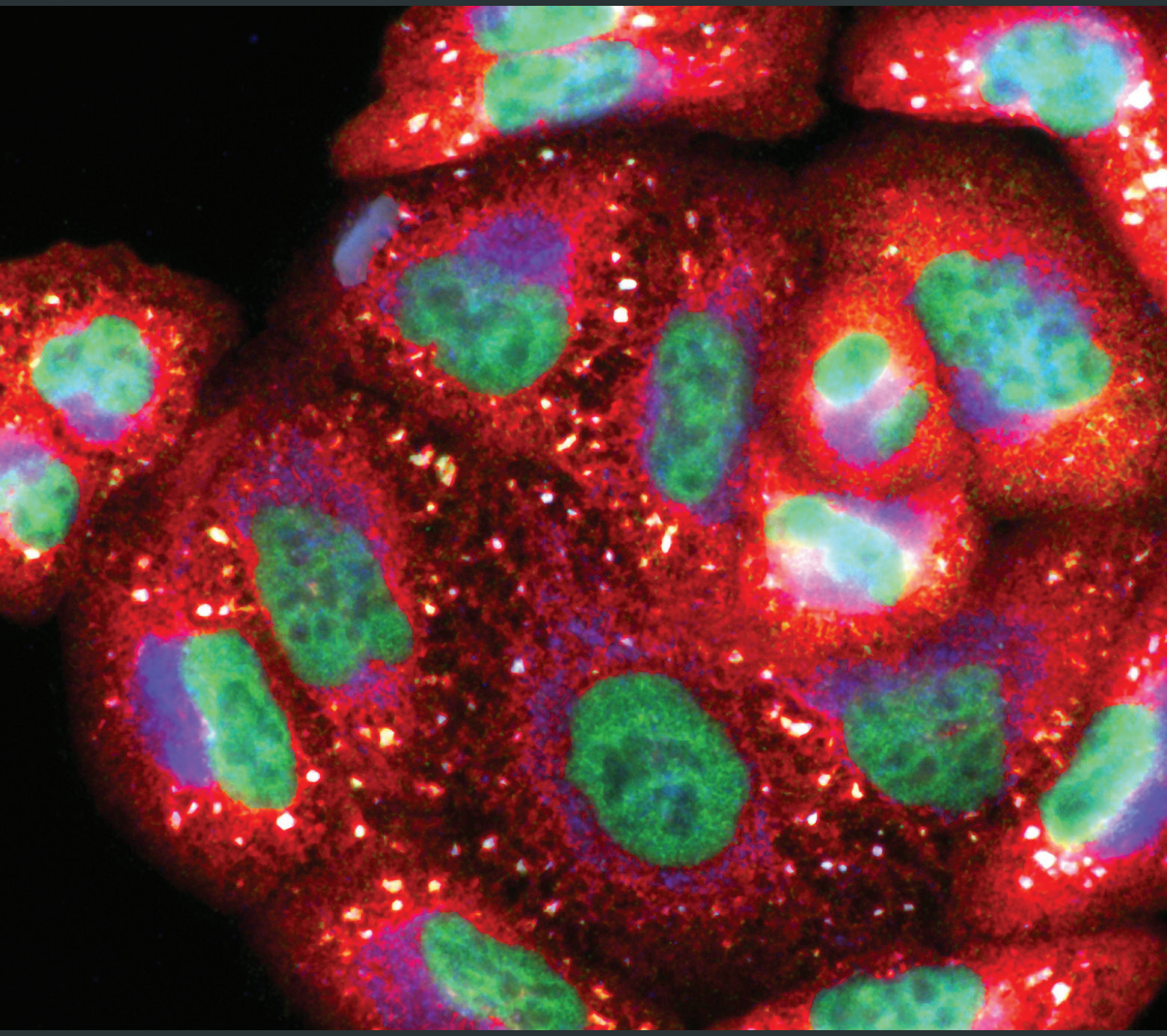
Oxidative Medicine and Cellular Longevity

Lipid Peroxidation Products in Human Health and Disease 2014

Guest Editors: Kota V. Ramana, Sanjay Srivastava, and Sharad S. Singhal



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Editorial Board

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Editorial

Lipid Peroxidation Products in Human Health and Disease 2014

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Lipid peroxidation is a complex chain reaction process due to the oxygen-free radicals mediated attack of cell membrane lipids such as polyunsaturated fatty acids (PUFA) resulting in cell damage and dysfunction. The end products of lipid peroxidation yield a variety of highly reactive electrophilic aldehydes, which can act as endogenous danger signals that alter important cell signaling pathways responsible for disease pathogenesis. Recent studies identified potential role of lipid peroxidation products as markers of oxidative stress and biomarkers of human diseases. Indeed, a number of preclinical and clinical studies suggest the involvement of lipid peroxidation products in numerous pathological conditions such as inflammation, atherosclerosis, diabetes, ageing, neurodegenerative diseases, and cancer. The wealth of knowledge we are gathering from the past decade or so will significantly help us to better understand the mechanisms by which lipid peroxidation products trigger pathological aspects and will help to identify novel potential targets for future therapeutic strategies.

The 2014 special issue of lipid peroxidation products in human health and disease compiles 16 excellent manuscripts, including clinical studies, research articles and reviews, which provides comprehensive evidence demonstrating the significance of lipid peroxidation products in various pathological conditions.

The 3 review articles of this issue discuss how lipid peroxidation products are involved in cell signaling which leads to various pathological conditions. An excellent review article by A. Ayala et al. described in depth how lipid peroxidation-derived aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are formed from PUFA and their metabolism. Further, they described how MDA and HNE are involved in cell signaling that causes either cell survival or cell death. At the end, they also discussed various *in vivo* mammalian model systems used for investigating lipid peroxidation. The review article by E. Miller et al. described the significance of isoprostanes and neuroprostanes as biomarkers of oxidative stress in various neurodegenerative diseases. Specifically, authors have nicely discussed the relationship between F2-isoprostanes and F4-neuroprostanes as biomarkers of lipid peroxidation in the pathogenesis of human neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, Huntington's disease, and Creutzfeldt-Jakob disease. Joshi and Pratico in their review article discussed how lipid peroxidation is involved in the pathophysiology of psychiatric diseases. Specifically, the authors have nicely described recent clinical data supporting the involvement of lipid peroxidation aldehydes in schizophrenia, bipolar, and other major depressive disorders. The review articles in this special issue provide widespread information on the formation of lipid peroxidation-derived aldehydes and their involvement in the neurological and psychiatric diseases.

The research article by M. E. Soto et al. investigates the role of oxidative stress in various aortopathies. In this study, aorta fragments from patients with systemic arterial hypertension, Marfan's syndrome, Turner's syndrome and Takayasu's arteritis were evaluated for oxidative stress enzymes and structural and functional proteins. This study indicates that the activities of glutathione peroxidase and

glutathione-S-transferase were decreased and the rate of lipid peroxidation was increased in all types aortopathies. However, activities of other oxidative and functional proteins showed variations depending upon the type of aortopathy. A cross-sectional study by F. Moreto et al. examines the relationship between plasma lipid peroxidation product MDA and metabolic syndrome in 148 free-living human subjects. Authors found interesting data that subjects with higher plasma MDA showed higher prevalence of metabolic syndrome accompanied by higher waist circumference, higher values of glucose, triglycerides, insulin resistance, and higher dietary sugar-intake. These studies indicate that MDA is a major determinant of glucolipotoxic state in subjects with metabolic syndrome.

Studies by P. Stiuso et al. report oxidant/antioxidant status and lipidomic profile in the serum of NASH patients at the basal conditions and after one-year treatment with the silibinin-based food integrator Realsil, they found that chronic treatment with Realsil significantly changed the basal severity of the disease as determined by NAS scores and most importantly decreased the serum lipid peroxidation. These data indicate that lipidomic status in the serum of patients with NASH could be a useful prognostic marker for the antioxidant therapies. In another study by S. Dziegielewska-Gesiak et al. investigated the role of lipid peroxidation products, plasma total antioxidant status and Cu-, Zn-SOD as biomarkers of oxidative stress in elderly prediabetic subjects. Based on the data obtained from 52 elderly persons, this study identified SOD-1 and TAS as initial stage biomarkers and thiobarbituric acid-reacting substances (TBARS) as later stage biomarkers of oxidative stress in the elderly prediabetics.

Another study by P. Sutkowy et al. examined the influence of exercise combined with whole-body cryotherapy on lipid peroxidation products formation in healthy kayakers. This study reports data on various oxidative stress and antioxidative markers such as MDA, conjugated dienes, protein carbonyls, total antioxidant capacity, vitamin E, cortisol, and testosterone in 16 kayakers of the Polish National Team. The findings indicate that combining exercise during longer training cycles with whole-body cryotherapy could be advantageous in kayakers. A multicentric research study reported by L. M. Gomez-Olivan et al. examined the impact of involuntary exposure of antineoplastic drugs in nurses in Mexican hospitals. When compared to occupationally exposed nurses with control subjects who are not occupationally exposed to antineoplastic drugs, the occupationally exposed nurses show significantly increased levels of oxidative stress markers, including lipid peroxidation levels, protein carbonyl content, super oxide dismutase, catalase, and glutathione peroxidase. This study again addresses the significance of lipid peroxidation products as biomarkers of oxidative stress in antineoplastic exposed subjects.

M. Saenz-de-Viteri et al. created an interesting model to investigate phototoxicity in the rabbits exposed to light. By using this model, they found that retinas from rabbits exposed to light showed higher levels of lipid peroxidation than unexposed controls. They demonstrate that light damage causes an increase in the retinal oxidative stress immediately after light exposure that decreases with time of exposure, although, some morphological and apoptotic events still appear days after light exposure. Buttari et al. investigated the effect of polyphenolic compound resveratrol to regulate the 7-oxo-cholesterol-triggered proinflammatory signaling in M1 and M2 human macrophage subsets. These studies demonstrate that in the M1 subset, resveratrol prevents the 7-oxo-choesterol-induced downregulation of CD16 and the upregulation of MMP-2 extracts whereas in M2 subset, resveratrol prevents the upregulation of CD14, MMP-2, MMP-9, and downregulation of endocytosis. These studies also indicate that regulation of NF-kB activation is the main signaling mechanism by which 7-oxo-choesterol and resveratrol mediate inflammatory effects.

S. A. Ganie et al. in their article reported the antioxidant and cytotoxic activities of *Arnebia benthamii*, an endangered medicinal plant of Kashmir Valley. By using rat liver microsomes and human cancer cell lines, authors have shown the antioxidant potential of this plant extract. Specifically, they have shown that extract of Arnebia inhibits Fe2+/ascorbic acid-induced lipid peroxidation in rat liver microsomes and also shows cytotoxic effects towards various cancer cell lines. The effect of antihypertensive drug Enalapril on oxidative stress markers and antioxidant enzymes in the kidneys of spontaneously hypertensive rats was reported by G. Chandran et al. in their article. Particularly, they demonstrate that Enalapril treatment significantly enhanced total antioxidant status and superoxide dismutase and decreased the TBARS levels in the kidneys of hypertensive rats. These studies indicate that Enalapril, besides its antihypertensive effect, also decreases the oxidative stress and lipid peroxidation in the hypertensive rat kidneys. In another research article by S. Ojha et al. reported the effect of *Withania coagulans* fruit extract on oxidative stress and inflammatory response in the kidneys of streptozotocin-induced diabetic rats. They have shown that treatment of diabetic rats with *Withania* plant extract significantly decreased hyperglycemia, glutathione levels, inflammatory cytokines such as IL-1b, IL-6, and TNF-α, and subsequent renal injury. These studies indicate potential antioxidative and anti-inflammatory actions of this fruit extract in prevention of diabetic complications.

S. Perrone et al. in their clinical study investigated the hypothesis that neonatal supplementation of lutein in the first hours of life reduces neonatal oxidative stress in the immediate postpartum period. This hypothesis was tested in a randomized, double-blinded clinical trial conducted among 150 newborns by investigating the levels of total hydroperoxides, advanced oxidation protein products, and biological antioxidant potential in the blood samples collected from the cord. Their findings indicate that neonatal supplementation of lutein in the first hours of life increases biological antioxidant potential and reduce total hydroperoxides when compared to babies without lutein supplementation. In another interesting randomized cross-over clinical study, L. D. Renzo et al. evaluated the outcome of consumption of McDonald's meal and a Mediterranean meal without and with red wine in healthy population. Red wine decreased the ox-LDL and increased the expression of antioxidant enzymes in people taking McDonald's or Mediterranean meals. These studies

indicate the positive effect of red wine intake combined with widely consumed meal types on oxidative and inflammatory gene expressions.

As an end note, it is obvious from the recently published studies and current special issue papers that lipid peroxidation products play a major role in human health and disease. Lipid peroxidation products are now recognized as biomarkers of oxidative stress and endogenous danger mediators of multiple cell signaling pathways. Although, over the past several years, substantial research has shown that lipid peroxidation has a crucial pathophysiological role in the development of various human diseases, the exact nature of the significance of lipid peroxidation on cellular homeostasis that maintain cell survival, differentiation, and death leading to pathological consequences and their responses to antioxidant therapies requires further detailed investigations. Newly developed technologies such as lipid finger printing/lipidomics and metabolomics are important tools that will help to define how the lipid peroxidation products adapt and provide a buffer against increased oxidative stress in various pathological conditions. Therefore, the molecules that interrupt or neutralize the effects of lipid peroxidation products-mediated signaling pathways could be the next important targets for future drug discovery studies.

Acknowledgments

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Research Article

Withania coagulans Fruit Extract Reduces Oxidative Stress and Inflammation in Kidneys of Streptozotocin-Induced Diabetic Rats

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The present study was carried out to investigate the changes in oxidative and inflammatory status in streptozotocin-induced diabetic rat's kidneys and serum following treatment with *Withania coagulans*, a popular herb of ethnomedicinal significance. The key markers of oxidative stress and inflammation such as inflammatory cytokines (IL-1β, IL-6, and TNF-α) and immunoregulatory cytokines (IL-4 and IFN-γ) were increased in kidneys along with significant hyperglycemia. However, treatment of four-month diabetic rats with *Withania coagulans* (10 mg/kg) for 3 weeks significantly attenuated hyperglycemia and reduced the levels of proinflammatory cytokines in kidneys. In addition, *Withania coagulans* treatment restored the glutathione levels and inhibited lipid peroxidation along with marked reduction in kidney hypertrophy. The present study demonstrates that *Withania coagulans* corrects hyperglycemia and maintained antioxidant status and reduced the proinflammatory markers in kidneys, which may subsequently reduce the development and progression of renal injury in diabetes. The results of the present study are encouraging for its potential use to delay the onset and progression of diabetic renal complications. However, the translation of therapeutic efficacy in humans requires further studies.

1. Introduction

Diabetes, a rising epidemic throughout the world, has no signs of abatement and remains one of the most challenging health problems. People with diabetes suffer from the detrimental vascular which accounts for high morbidity and mortality [1]. Among several vascular complications, chronic renal failure and end stage renal diseases appear first and often associated with metabolic and hemodynamic alternations. The development and progression of diabetes and associated vascular complications are largely precipitated by chronic hyperglycemia-induced oxidative stress [2]. In addition to oxidative stress, immune-mediated low grade chronic inflammatory mechanisms have been demonstrated to play a significant role in pathogenesis of renal injury in long term diabetes [3]. Convincing number of studies demonstrates that oxidative stress and immune inflammatory processes intimately linked together causing renal damage through multiple mechanisms [3–6].

The management of diabetic renal complications is based on the approaches to delay the development and progression by keeping strict control of blood pressure or plasma glucose [7]. However, controlling the blood pressure and plasma glucose levels to prevent the renal complications is far from satisfactory [7]. This imperfection points to the need for newer therapeutic agents that have potential

to target these intimately linked cascade; oxidative stress-inflammatory cytokine signaling and delay the progression and development of renal complications in diabetes [7]. Therefore, in search of newer therapeutic agents, medicinal plants considered as a major source of drug discovery from natural origin have been extensively explored [1].

Subsequently, many plant-derived natural products have the potential to be effective in diabetic renal complications by attenuating oxidative stress and proinflammatory and immunoregulatory cytokines [8–10]. The challenge is to identify the most promising compounds and evaluate their protective mechanism. The fruits of *Withania coagulans* belonging tofamily Solanaceae have received considerable attention for their benefits in chronic degenerative diseases including diabetes. The plant, *Withania coagulans*, commonly known as Indian Rennet, vegetable rennet (English), Panir dodi (Hindi), and Ning gu shui qie (Chinese), has been reported to possess a variety of ethnomedicinal uses [11]. The extract has shown potential activities, namely, anticancer [12], wound healing [13], immunomodulating [14], antihyperglycemic [15], and hypolipidemic [16] activities.

Despite several reports of its benefits in diabetes [11, 15, 17–19] and considering its potential to target the complex interplay of oxidative stress and inflammatory and immunoregulatory cytokines in diabetic renal complication it is worthwhile to study the effect of *Withania coagulans* in kidneys of streptozotocin- (STZ-) induced diabetes. In order to understand the mechanism, the present study examined the effect of *Withania coagulans* on antioxidant defense, lipid peroxidation, and immunoregulatory and proinflammatory cytokines.

2. Material and Methods

2.1. Chemicals. STZ, sodium citrate, citric acid, bovine serum albumin, 5-sulfosalicylic acid (SSA), naphthalene diamine dihydrochloride, sulphanilamide, phosphoric acid, HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sucrose, 1,4-dithiothreitol (DTT), CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, sodium chloride, protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), tween 20, sodium nitrate, 3,3,5,5′-Tetramethylbenzidine (TMB), glutathione (GSH) assay kit, and all other required chemicals, if not specified, were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). All chemicals used in the present study were of analytical grade. Malondialdehyde (MDA) assay kit was purchased from Northwest Life Science Specialties (WA, USA). Cytokines duo set ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). EnzChek myeloperoxidase (MPO) activity assay kit was purchased from Life Technologies (NY, USA).

2.2. Animals and Diet. Male Wistar rats (230 to 250 g) bred in the animal research facility of College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, UAE, were used. The animals were housed under standard laboratory conditions (22 ± 2°C and 65 ± 5% humidity) and maintained on a 12-hour light/dark cycle. The animals had free access to food and water and were fed commercially available standard rat diet. A maximum of four rats were housed per cage and acclimatized to the laboratory conditions prior to the commencement of the experiment. The experimental protocols were approved by the Institutional Animal Ethics Committee of College of Medicine and Health Sciences (IAEC CMHS), United Arab Emirates University, Al Ain, UAE, and conducted according to the criteria outlined in the guide for the care and use of laboratory animals by the National Academy of Sciences.

2.3. Preparation of the Withania coagulans Aqueous Fruit Extract. A standard protocol was followed for the extraction of *Withania coagulans*. The fruits of *Withania coagulans* (0.28 g/100 mL) were soaked in distilled water overnight followed by a mechanical dispersion using a sterile cotton wood (Hardwood Products Company, Guilford, CT, USA) and filtration through cheese cloth. The dose of 10 mg/kg was selected based on a dose response pilot study in our laboratory. A total of five doses (0, 10, 125, 625, and 1250 mg/kg) were screened to find out the optimal dose following a dose response curve in a postprandial glucose test based dose response study. Five groups of six STZ diabetic rats each were fasted overnight and used in the experiment. Group I served as diabetic control and received vehicle (distilled water only). Rats of groups II, III, IV, and V received doses of 10, 125, 625, and 1250 mg/kg, respectively, of aqueous fruit extract suspended in distilled water. The level of baseline blood glucose was measured at 0 hr, followed by an oral administration of either distilled water (diabetic control group) or *Withania coagulans* extract. The rats were allowed to have free access to food and water. The blood samples were collected from tail vein at 1, 2, 3, and 4 hrs after giving the extract using an ACCU-CHEK performa glucometer. Among the doses studied, the dose of 10 mg/kg was found most potent in exhibiting the antihyperglycemic activity (results not shown). For further experiments, the dose of 10 mg/kg was chosen and a detailed study was performed.

2.4. Induction of Experimental Diabetes in Rats. A single dose of 60 mg/kg STZ was dissolved in freshly prepared citrate buffer (pH 4.5; 0.1 M) and injected intraperitoneally to induce diabetes. The age matched control rats received an equal amount of citrate buffer and were used along with the diabetes control group. Diabetes was confirmed by using Accucheck performa glucometer (Roche Diagnostics, NSW, Australia), after 48 hours of STZ injection. The rats having plasma glucose levels of >350 mg/dL were considered as diabetics and were used in the present study. The rats injected with STZ provide a relatively inexpensive and easily accessible rodent model that is not extremely obese and simulates the natural history and metabolic characteristics of patients with diabetes mellitus [20].

2.5. Experimental Design. The rats were divided into three experimental groups, each consisting of six rats. Group 1 served as nondiabetic controls group. The group 2 and 3

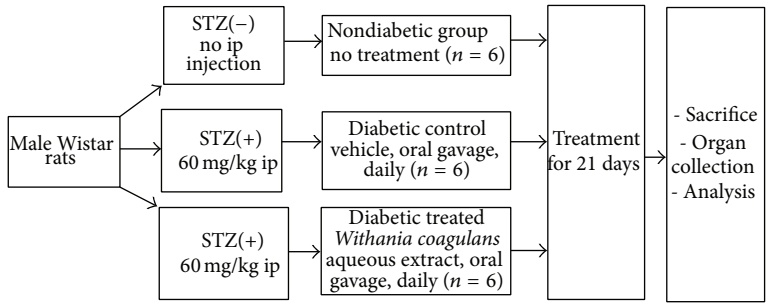

Figure 1: Schematic diagram of the experimental groups and treatment protocol.

rats were four-month diabetic at the start of the experiment. Group 2 served as STZ-induced diabetic group; group 3 served as diabetic group treated orally with *Withania coagulans* (10 mg/kg/day b.w. for 3 weeks). The schematic representation of the experimental groups and treatment procedure are presented in Figure 1. During the experimental period, the body weight and blood glucose were determined at regular intervals. The blood glucose level was measured before treatment and after the 3-week treatment over a period of 4 h. At the end of the experimental period, rats were euthanized and the kidneys were removed and processed for the estimation of reduced glutathione (GSH), malondialdehyde (MDA), nitric oxide (NO), and cytokines (IL-1β, IL-4, IL-6, TNF-α, and IFN-γ) using the specific kits.

2.6. Preparation of Kidney Tissue Homogenate. The kidneys were removed, weighed, washed in ice-cold PBS, and minced into 2–5 mm fragments followed by homogenization using a polytron homogenizer (IKA Laboratory, Germany), with 5 volumes of ice-cold buffer containing 100 mM HEPES, pH 7.5, 10% sucrose, 10 mM DTT, 0.1% CHAPS, 150 mM NaCl, protease inhibitors tablet, and 1 mM PMSF. The samples were centrifuged at 10000 ×g for 10 min and the obtained supernatant was removed and stored at −80°C until the assessment of MPO activity and cytokines using ELISA kits.

2.7. Determination of Oxidative Stress Markers. The levels of GSH and MDA were determined using commercially available kits in serum and kidney. The level of NO was measured only in kidney tissues.

2.8. Estimation of Reduced Glutathione (GSH). The GSH content in serum and kidney homogenate was estimated following manufacturer protocol of the assay kit. Briefly, the measurement of GSH uses a kinetic assay in which catalytic amounts (nmoles) of GSH cause a continuous reduction of 5,5-dithiobis (2-nitrobenzoic acid) to nitrobenzoic acid (TNB), and the glutathione disulfide (GSSG) formed was recycled by glutathione reductase and NADPH. The yellow color product, 5-thio-2-TNB, was measured spectrophotometrically at 412 within 5 min of 5,5-dithiobis(2-nitrobenzoic acid) addition, against a blank with no homogenate. GSH concentration was expressed as μM of GSH per milligram of tissue or per 0.01 mL of serum.

2.9. Estimation of Malondialdehyde (MDA). The lipid peroxidation product, MDA, in the kidney homogenate from each group was measured using the MDA assay kit. Briefly, the assay is based on the reaction of MDA with thiobarbituric acid (TBA) to form a MDA-TBA adduct that absorbs strongly at 532 nm. Briefly, the deproteinated tissue sample was added to 1 M phosphoric acid and butylated hydroxytoluene in ethanol and then the mixture was heated at 60°C for 60 min. The suspension was cooled to room temperature and centrifuged at 10000 ×g for 2-3 min and the pink colored supernatant was taken for spectroscopic measurements at 532 nm for the assay of MDA. The concentration of MDA was expressed as μM per 10 milligram of tissue or per 0.1 mL serum.

2.10. Assay of Myeloperoxidase (MPO) Activity. The chlorination assay for MPO activity in serum and kidney homogenate (ng/mg tissue wet weight) was performed in a microtiter plate using the EnzChek MPO activity assay kit. Briefly, 50 μL of 2 × 3′-(*p*-aminophenyl) fluorescein working solution was added to 50 μL of sample. The reaction mixture was then incubated in the dark at 37°C for 20 min. The fluorescence intensity of each sample was recorded at 485 nm excitation and 530 nm emission on a Perkin Elmer luminescence spectrofluorometer.

2.11. Estimation of Nitric Oxide (NO). Accumulation of nitric oxide was used to determine the production of NO according to the Griess reagent (0.2% naphthylene diamine dihydrochloride and 2% sulphanilamide in 5% phosphoric acid) method. Briefly, 100 μL of sample was mixed with an equal volume of Griess reagent and incubated at room temperature for 10–15 min. The absorbance at 492 nm was measured in an automated microplate reader (Tecan Group Limited, Männedorf, Switzerland). The nitrite concentration was quantitated using $NaNO_2$ as standard and was expressed as micromolar concentrations of NO per mg tissue.

2.12. Determination of Proinflammatory Cytokines in Kidney. Enzyme immunoassay of IL-1β, IL-4, IL-6, TNF-α, and IFN-γ in kidney homogenate was performed by using commercial sandwich R&D duoset ELISA kit (Minneapolis, USA). Briefly, the wells of a 96-well microtiter plate were coated with respective primary antibody in phosphate buffer saline

Table 1: Effect of *Withania coagulans* on weight changes of body and kidney to body weight ratio. Twenty-one-day treatment with *Withania coagulans* extract caused a significant improvement in the body weight and kidney to body weight ratio compared to diabetic controls.

Groups	Body weight (gms)		Kidney weight: body weight
	Before treatment	During treatment	
Nondiabetic controls	368.166 ± 17.20	419.33 ± 22***	0.0029 ± 0.00012
Diabetic controls	266.4 ± 5.61	259.2 ± 5.39***	0.00469 ± 0.00017***
W. coagulans treated	269.57 ± 7.09	292 ± 12.49$^{**,\#}$	0.00408 ± 0.000084$^{***,\#}$

Results are means ± SEM; $n = 6$ rats; $^{**}P < 0.01$, $^{***}P < 0.001$ from nondiabetic controls; $^{\#}P$ 0.05 from diabetic controls.

(PBS), (100 μL/well), overnight at room temperature, washed with phosphate-buffered saline containing 0.05% Tween-20 (PBST), and then blocked with 1% bovine serum albumin in PBS for one hour. After washing, plates were incubated with serum, kidney homogenates, and respective standards for 2 hours. After washing with PBST, a detection antibody was added for 2 hours and 100 μL of HRP was added for half an hour, after the washing. The TMB-ELISA substrate was added and the color intensity read at 450 nm with a microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Cytokines levels were expressed as pg per milligram of tissue wet weight and per mL of serum.

2.13. Statistical Analysis. Data was analyzed statistically using SPSS 19.0 software. The means of the data are presented with the standard error mean (SEM). The results were analyzed using one-way ANOVA to determine the significance of the mean between the groups. Values of $P < 0.05$ were considered significant.

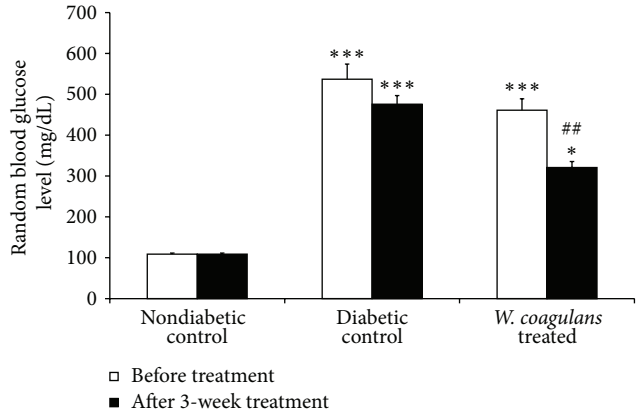

Figure 2: Effect of *Withania coagulans* on blood glucose level. The diabetic treated rats showed significant decrease in blood glucose levels compared to diabetic controls. Results are means ± SEM; $n = 6$ rats; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ from nondiabetic controls; $^{\#\#}P < 0.01$, from diabetic controls.

3. Results

3.1. Effect of Withania coagulans on Body Weight and Kidney to Body Weight Ratio. Table 1 shows the changes in body weight and the ratio of kidney/body weight in different experimental groups. There was a significant ($P < 0.001$) decrease in the body weight of rats administered STZ in comparison with rats of nondiabetic control group. Diabetic rats treated with *Withania coagulans* show a significant ($P < 0.05$) improvement in body weight when compared to diabetic control rats. Ratio of kidney/body weight is an index of renal hypertrophy and a significant ($P < 0.001$) increase in kidney/body weight indicates renal injury in STZ administered rats. However, treatment with *Withania coagulans* to the diabetic rats has significantly ($P < 0.05$) reduced renal hypertrophy as evidenced by reduction of kidney/body weight when compared to the diabetic control.

3.2. Effect of Withania coagulans on Blood Glucose, BUN, and Creatinine. The changes in the level of blood glucose and serum insulin in the rats of different experimental groups are represented in Figure 2. A significant ($P < 0.001$) and persistent rise in plasma glucose level was observed in STZ administered rats as compared with nondiabetic control group. However, a significant ($P < 0.001$) reduction was observed in the plasma glucose level of diabetic rats treated with *Withania coagulans* when compared to diabetic controls The BUN and creatinine levels were not different between the different groups (results not shown).

3.3. Effect of Withania coagulans on Glutathione. Animals administered STZ showed a significant ($P < 0.05$) decrease in the serum GSH level when compared to the nondiabetic control group (Figure 3(a)). However, no significant change in kidney GSH level was observed in diabetic rats when compared to the nondiabetic control group. Treatment with *Withania coagulans* extract significantly ($P < 0.05$) induced the level of GSH, both in serum and in kidney of diabetic rats when compared to diabetic control group (Figure 3(a)).

3.4. Effect of Withania coagulans Lipid Peroxidation. The rats administered STZ showed a significant increase in the MDA levels of serum ($P < 0.05$) and kidney ($P < 0.001$) as compared to the nondiabetic control group (Figure 3(b)). However, treatment with *Withania coagulans* has not reduced the level of MDA in serum and showed a slight nonsignificant decrease in the kidney compared to diabetic control group (Figure 3(b)).

3.5. Effect of Withania coagulans on MPO Activity. A modest but insignificant increase in MPO levels in kidney of the diabetic control group was observed when compared to nondiabetic control group (Figure 3(c)). However, treatment with

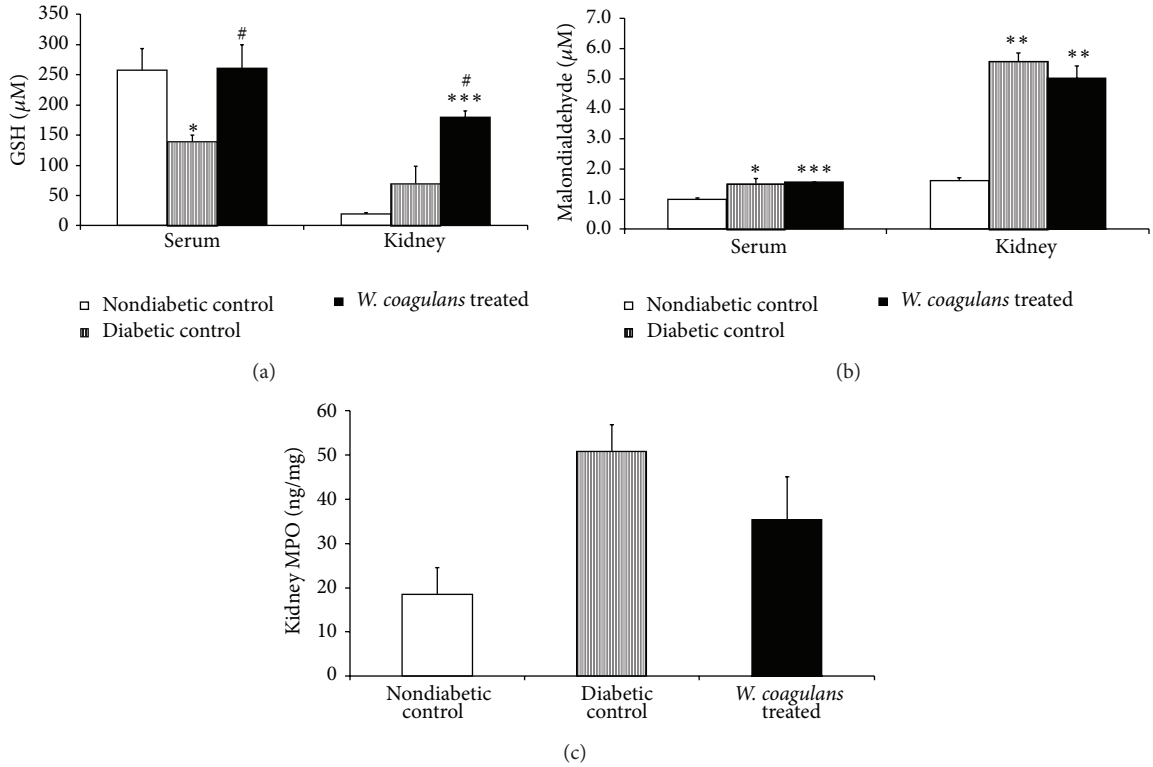

Figure 3: Effect of *Withania coagulans* on serum and kidney levels of (a) GSH, (b) MDA, and (c) MPO. Results are means ± SEM; $n = 6$ rats; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ from nondiabetic controls; $^{\#}P$ 0.05 from diabetic controls.

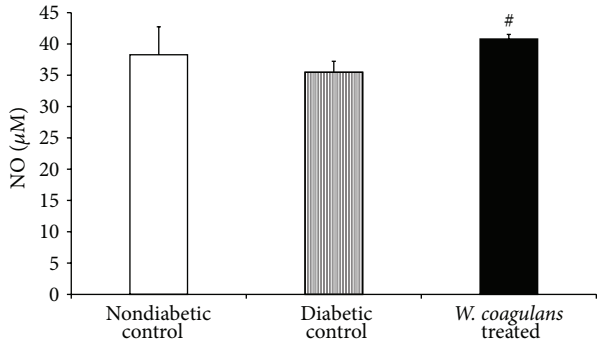

Figure 4: Effect of *Withania coagulans* on levels of NO in kidney. Results are means ± SEM; $n = 6$ rats; $^{\#}P$ 0.05 from diabetic controls.

Withania coagulans was found todecrease MPO levels in kidney as compared to the diabetic control group (Figure 3(c)). The decrease in MPO levels was not significant in any group.

3.6. Effect of Withania coagulans on Nitric Oxide. A modest nonsignificant decrease in NO levels in kidney of the diabetic control group was observed when compared to nondiabetic control group (Figure 4). However, treatment with *Withania coagulans* has significantly ($P < 0.05$) increased the NO levels in kidney as compared to the diabetic control (Figure 4).

3.7. Effect of Withania coagulans on Proinflammatory Cytokines. Figures 5(a)–5(c) represent the levels of kidney proinflammatory cytokines such as IL-1β, IL-6, and TNF-α of different experimental groups: nondiabetic control, diabetic control, and *Withania coagulans* treated. There was a significant increase in the level of IL-1β ($P < 0.001$), IL-6 ($P < 0.001$), and TNF-α ($P < 0.05$) in kidneys of STZ-induced diabetic rats when compared to nondiabetic control group. A significant decline in the kidney levels of IL-1β ($P < 0.05$), IL-6 ($P < 0.05$), and TNF-α ($P < 0.01$) was observed on treatment with *Withania coagulans* when compared to diabetic control.

3.8. Effect of Withania coagulans on Immunoregulatory Cytokines. The levels of IL-4 and IFN-γ in kidneys of different experimental groups are presented in Figure 6. Though the change in IFN-γ levels was not altered significantly, a significant ($P < 0.05$) increase in the IL-4 level was observed in STZ-induced diabetic rats when compared to the nondiabetic control group. However, treatment with *Withania coagulans* extract has significantly reduced the levels of IL-4 ($P < 0.05$) and IFN-γ ($P < 0.01$) in kidneys as compared to diabetic rats.

4. Discussion

In the present study, STZ-injected rats show significant rise in plasma glucose level along with decrease in serum insulin and body weight and increase in kidney weight in comparison with nondiabetic control rats, indicating the development of diabetes as characterized by chronic and persistently elevated plasma glucose level. Decreased body weight in STZ-induced diabetic rats is believed to be due

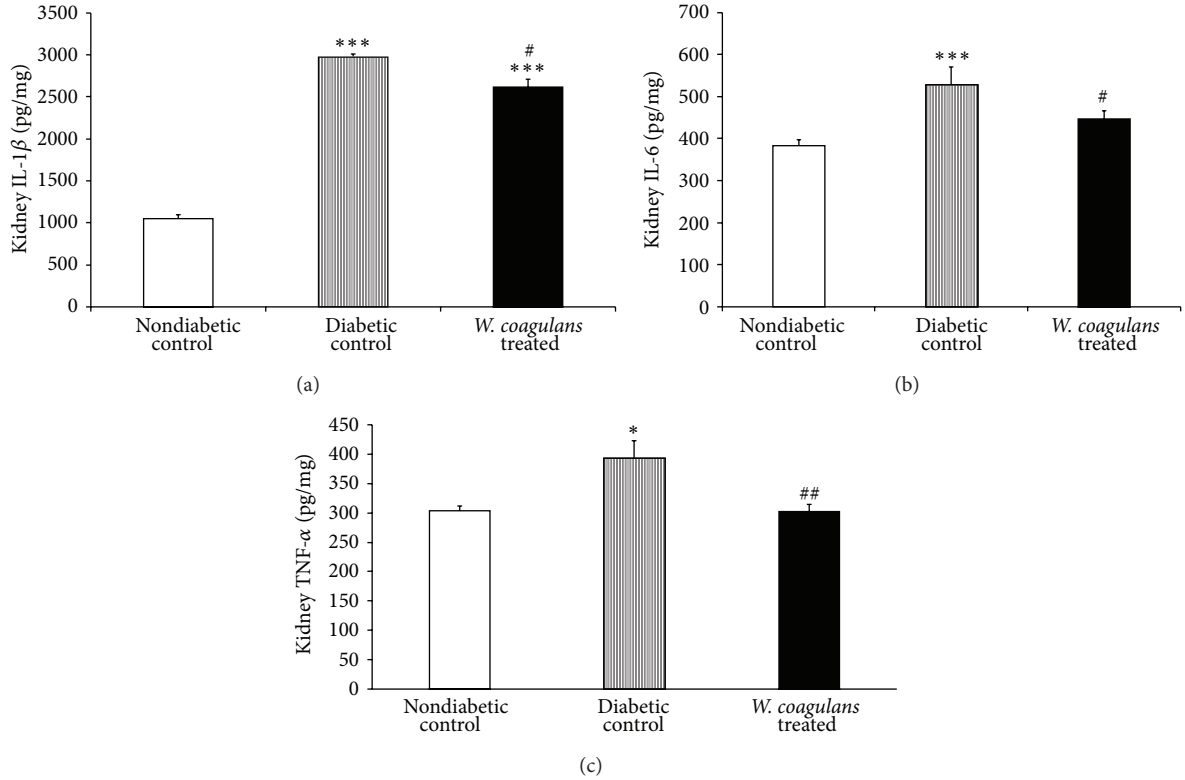

Figure 5: Effect of *Withania coagulans* on kidney levels of (a) IL-1β, (b) IL-6, and (c) TNF-α. Diabetic controls showed significantly elevated kidney IL-1β (a), IL-6 (b), and TNF-α (c) cytokines levels, compared to nondiabetic controls. *Withania coagulans* treatment significantly decreased the IL-1β (a), IL-6 (b), and TNF-α (c) compared to diabetic controls. Results are means ± SEM; $n = 6$ rats; $^{*}P < 0.05$, $^{***}P < 0.001$ from nondiabetic controls; $^{\#}P$ 0.05, $^{\#\#}P < 0.01$ from diabetic controls.

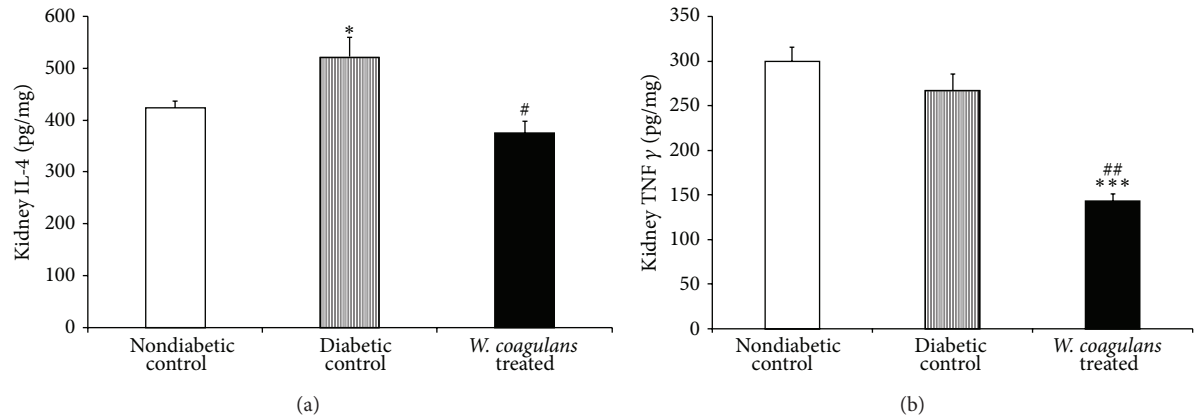

Figure 6: Effect of *Withania coagulans* on kidney levels of (a) IL-4 and (b) IFN-γ. *Withania coagulans* treatment significantly decreased the kidney IL-4 and IFN-γ compared to diabetic controls. Results are means ± SEM; $n = 6$ rats; $^{*}P < 0.05$, $^{***}P < 0.001$ from nondiabetic controls; $^{\#}P$ 0.05, $^{\#\#}P < 0.01$ from diabetic controls.

to dehydration, breakdown, and catabolism of fats and proteins. Increased catabolic reactions after STZ administration leads to muscle wasting and decreased body weight. STZ induces diabetes by selectively destroying insulin producing pancreatic endocrine cells and damages kidney similar to early stage diabetic nephropathy [20, 21]. This is in agreement with various other observations that STZ-induced animals exhibit diabetic renal complications [8, 9, 22]. However, treatment with *Withania coagulans* restored body weight, kidney weight, and reduced hyperglycemia, as well as enhancing survival and general body growth of diabetic rats. Ratio of kidney/body weight is an index of renal hypertrophy and a significant increase in kidney/body weight indicates renal injury in STZ administered rats. However, treatment with

Withania coagulans to the diabetic rats has markedly reduced renal hypertrophy as evidenced by reduction of kidney/body weight when compared to the diabetic control. These results demonstrate that the extract of *Withania coagulans* exhibits antihyperglycemic effects through modulation of insulin and related enzyme activities in consonance with other studies demonstrated antihyperglycemic as well as protective effect in other organs apart from kidneys [15–17].

Pathogenic mechanisms underlying the progressive renal diseases in diabetics are known to be multifactorial including oxidative stress, inflammation, and immune-dysfunction [5, 6]. Oxidative stress ultimately triggers inflammation and modulates immunologic cascade in progression of renal damage from genesis to progression [2, 3]. Hyperglycemia-induced oxidative stress and inflammation unleash a cascade of events that affect cellular proteins, gene expression, and cell surface receptor expression, ultimately resulting in progressive pathologic changes in diabetic kidneys [4]. To counteract oxidative stress, the first line of defense against reactive oxygen species (ROS) is GSH, an intracellular nonprotein thiols compound, which also participate in second line of defense as a substrate or cofactor for GSH-dependent enzymes to detoxify ROS generated toxic byproducts and prevent propagation of free radicals [23]. In the present study, decreased levels of GSH in serum of STZ-injected rats might be explained by depletion or consumption of GSH in removing the hyperglycemia generated peroxides. Following treatment with *Withania coagulans*,the improvement in GSH level demonstrates its antioxidant activity in agreement with other studies where *Withania coagulans* was shown to ameliorate oxidative stress [15–17]. Although no significant change in renal GSH levels was observed in the STZ administered rats, a significant rise in kidney GSH levels was obtained following treatment with *Withania coagulans* indicating increased production of GSH.

Furthermore, ROS, by impairing antioxidant defense, renders the kidneys more susceptible to lipid peroxidation. ROS induced lipid peroxidation is a marker of cellular oxidative damage and is an important pathogenic event in renal injury [24]. In our study, increased level of lipid peroxidation product, MDA, clearly indicates oxidative stress in diabetic kidneys. Following treatment with *Withania coagulans*,the inhibition of lipid peroxidation as evidenced by decreased albeit not significant MDA levels in kidney demonstrates the antioxidant effect of *Withania coagulans* in agreement with previous studies which showed its antilipid peroxidation activity [15–17]. In addition to reduction of hyperglycemia, the ability of *Withania coagulans* to prevent GSH depletion and lipid peroxidation seems to be advantageous to mitigate the oxidative stress and may delay the development and progression of renal complications in diabetes.

In addition, change in MPO activity has been demonstrated to play role in degenerative and immunologic changes of the kidney [25]. In this study, we did not observe a significant change in MPO activity. Changes in renal NO levels have been linked to the pathogenesis of diabetes and associated complications [26]. The complex oxidative milieu in diabetes triggers several pathophysiologic mechanisms that simultaneously stimulate or suppress NO production at a given stage of the disease. Many studies demonstrated that decrease in renal NO levels are partly results of enhanced oxidative stress and partly of decreased NOS expression [27]. However, treatment of diabetic rats with *Withania coagulans* significantly increased NO levels in the kidneys. This effect is supported by the reduction of oxidative stress and could be ascribed to the induction of NOS following a counterbalance of NOS activity under the oxidative burst in accordance with previous other studies [21, 24].

Recent studies have shown that long-term, innate immune system activation resulting in chronic low grade inflammation is associated with the risk of developing renal complications, implying that immunologic and inflammatory mechanisms play a significant role in disease development and progression [4–6]. Studies suggest that proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and IFN-γ (Th1) and IL-4 (Th2) act as pleiotropic polypeptides that are independently associated and exert an important diversity of actions in diabetic kidneys from development to progression [2, 6]. Both infiltrating immune cells (mainly monocytes and macrophages) and renal resident cells (endothelial, mesangial, dendritic, and epithelial) produce proinflammatory cytokines such as IL-1β, IL-6, and TNF-α [28]. The release of these cytokines may lead to renal injury through several mechanisms [6]. Being chemotactic in nature, the produced chemokines recruit more inflammatory cells and activate fibroblasts and matrix production, therefore, inducing the development of diabetic renal complications [2, 6]. Further, IFN-γ secreted by activated T cells and NK cells in conjunction with proinflammatory cytokines activates macrophages and stimulates chemokine production which result in pathological lesions of diabetic renal diseases. Increased IL-1β in kidney is known to increase the subsequent expression of chemotactic factors and adhesion whereas increased IL-6 levels are known to alter endothelial permeability, induce proliferation, and increase fibronectin expression [6, 9]. In the present study, a significant increase in cytokine levels, IL-1β, IL-4, IL-6, and TNF-α,, in kidneys of rats injected STZ are in agreement with previous studies [21]. Following treatment with *Withania coagulans*, significant reduction in the level of these cytokines is clearly suggestive of its anti-inflammatory effect in diabetic kidney. Thus, the attenuation of proinflammatory cytokines and lipid peroxidation along with diminution of hyperglycemia and improved antioxidants by *Withania coagulans* treatment is clearly suggestive of its beneficial effects in diabetic kidney.

Recent evidences in alternative medicine have encouraged that whole herb formulation is an effective therapeutic modality in chronic diseases including diabetes due to their multitudes of synergistic bioactivities and nutritional properties [29]. The current concept has revealed a new class of agents, known as adaptogens which increase resistance of the organism to aversive stimuli threatening to perturb internal homeostasis. The adaptogens have the potential to reverse stress induced immunity deregulation and organ dysfunction by sparing the antioxidants and modulating the immune system [29]. The immunoregulatory cytokines play an essential role in downmodulating adaptive and innate immune responses leading to chronic inflammation [4].

Several studies have demonstrated the adaptogenic activity of *Withania species* by inducing immune-surveillance [14]. In the present study, the decreased levels of immunoregulatory cytokines, IL-4 and IFN-γ are strongly suggestive of the immunomodulatory and associated adaptogenic potential of *Withania coagulans* in consonance with therapeutic benefits of adaptogenic medicines in chronic diseases [30]. *Withania* described in Indian Ayurvedic medicine as Rasayana drugs is believed to produce its positive health impact through immune-enhancing, longevity promotion, and molecular nutritive effect [29].

Based on the present study findings and supportive data from ethnomedicinal, clinical, and preclinical studies [11, 15, 17–19], *Withania coagulans* holds promise for its potential in delaying the progression of renal complications in diabetes. Being a natural agent and due to its time tested use since ancient time is supportive of its relative safety. This is encouraging for *Withania coagulans* to be used in prevention and treatment of preventing renal complications in diabetes. Coupled with multiple pharmacological effects such as antihypertensive, hypolipidemic, hypoglycemic, immunosuppressive, antioxidant, anti-inflammatory, and adaptogenic activity, *Withania coagulans* might be a good therapeutic agent against renal complications of diabetes which involves multifactorial aetiopathogenesis.

To conclude, the results of our study demonstrate that treatment with *Withania coagulans* reduces the occurrence of oxidative stress and inflammation and improves hyperglycemia owing to its synergistic and polypharmacological properties. Further studies are encouraged for the translational application in humans.

Conflict of Interests

There are no patents, products in development, or marketed products to declare. This study was supported by grants from College of Medicine & Health Sciences, UAE University, UAE. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

Authors' Contribution

Authors who contributed significantly, read, and approved the paper are Shreesh Ojha, Juma Alkaabi, Naheed Amir, Azimullah Sheikh, Ahmad Agil, Mohamed Abdelmonem Fahim, and Abdu Adem. Authors who conceived and designed the experiments are Shreesh Ojha, Juma Alkaabi, Ahmad Agil, Mohamed Abdelmonem Fahim, and Abdu Adem. Authors who performed the experiments are Naheed Amir, and Azimullah Sheikh. Authors who analyzed the data are Shreesh Ojha, Naheed Amir, Juma Alkaabi, Azimullah Sheikh and Abdu Adem. Authors who contributed reagents/materials/analysis tools are Juma Alkaabi, Abdu Adem and Ahmad Agil. Authors who wrote the paper are Shreesh Ojha, Juma Alkaabi, Naheed Amir, and Abdu Adem. Shreesh Ojha and Juma Alkaabi contributed equally.

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Research Article

Effect of the Antihypertensive Drug Enalapril on Oxidative Stress Markers and Antioxidant Enzymes in Kidney of Spontaneously Hypertensive Rat

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Oxidative stress has been suggested to play a role in hypertension and hypertension induced organ damage. This study examined the effect of enalapril, an antihypertensive drug, on oxidative stress markers and antioxidant enzymes in kidney of spontaneously hypertensive rat (SHR) and Nω -nitro-L-arginine methyl ester (L-NAME) administered SHR. Male rats were divided into four groups (SHR, SHR+enalapril, SHR+L-NAME, and SHR+enalapril+L-NAME). Enalapril (30 mg kg^{-1} day^{-1}) was administered from week 4 to week 28 and L-NAME (25 mg kg^{-1} day^{-1}) was administered from week 16 to week 28 in drinking water. Systolic blood pressure (SBP) was measured during the experimental period. At the end of experimental periods, rats were sacrificed; urine, blood, and kidneys were collected for the assessment of creatinine clearance, total protein, total antioxidant status (TAS), thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), and catalase (CAT), as well as histopathological examination. Enalapril treatment significantly enhanced the renal TAS level ($P < 0.001$) and SOD activity ($P < 0.001$), reduced the TBARS levels ($P < 0.001$), and also prevented the renal dysfunction and histopathological changes. The results indicate that, besides its hypotensive and renoprotective effects, enalapril treatment also diminishes oxidative stress in the kidneys of both the SHR and SHR+L-NAME groups.

1. Introduction

Hypertension is a global chronic health condition in which systemic arterial pressure is persistently elevated. It is of great public concern as prolonged, uncontrolled hypertension leads to cardiovascular diseases and organ damage including the kidneys, resulting in nephropathy, chronic renal disease, and ultimately renal failure [1]. This makes it the leading behavioural and physiological risk factor for attributable deaths, accounting for 13% of global deaths [2].

The pathogenesis of essential hypertension is multifactorial and highly complex as various factors modulate the blood pressure in the body [3]. In this respect, free radical mediated oxidative damage has been proposed as an important predisposing pathogenic mechanism in the development and progression of hypertension and its complications including organ damage [4, 5]. Free radicals and their metabolites, reactive oxygen species (ROS), are constantly formed in the body by several mechanisms. These substances, being reactive, can cause oxidative damage to biological molecules. The body possesses antioxidant systems that are very important to protect cellular components from free radical induced oxidative damage. These consist of nonenzymatic and enzymatic systems including SOD and CAT [6]. Under physiological conditions, ROS produced in the course of metabolism are contained by the body's antioxidant defence mechanism. When these defence mechanisms are inadequate, either due to increased ROS production or diminished antioxidant

levels, oxidative stress occurs [7]. Oxidative stress which leads to damage of biological molecules, such as lipids, proteins, carbohydrates, and DNA, can inflict tissue injury and dysfunction [8]. Several reports have documented that hypertension is associated with increased free radical production as well as reduction of antioxidant capacity [9, 10]. High levels of lipid peroxidation biomarkers [11, 12] as well as hydrogen peroxide [13] in patients with essential hypertension suggest the probable involvement of free radicals in this disease and its long term complications.

As hypertension contributes to organ damage, antihypertensive drug treatment aims to reduce blood pressure and hypertension induced organ damage including the kidneys. In this respect, studies have shown that certain antihypertensive drugs, in particular those that target the renin-angiotensin system, are able to blunt the progression of renal disease in hypertension [14–17]. Some studies have suggested that the therapeutic benefit of antihypertensive drugs including renoprotection could be in part due to their antioxidant properties whereby there is inhibition of free radical production. These studies involving both human and animal models, including the SHR, have demonstrated that certain groups of antihypertensive drugs, such as the angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARB), and calcium channel blockers (CCB), lower blood pressure and cause changes in the oxidative status [18–22].

Even though antihypertensive drug treatments have been shown to reduce blood pressure and certain oxidative stress parameters, the studies concerned were not comprehensive as no in-depth study on the effect of these antihypertensive drug treatments on the antioxidant mechanisms involved as kidney damage progresses has been carried out. As such, the biochemical mechanisms by which these antihypertensive drugs might inhibit oxidative stress, especially in the kidneys, are not well known. Further studies are needed to clarify whether these antihypertensive drugs function by affecting the antioxidant defence mechanisms in the kidneys or just primarily correct the altered mechanical forces that cause structural changes in the kidney.

The SHR is a suitable model for the study of essential hypertension as the natural progression of hypertension and organ damage including the kidneys is remarkably similar to man. As in humans, kidney damage and progressive decline in glomerular filtration rate (GFR) occur at a much later stage in the SHR. Time-course studies until this stage of renal damage require maintaining SHR until an advanced age which would take a very long time and is costly. This is overcome by the usage of the L-NAME administered SHR model which produces renal damage similar to those seen in human hypertensive nephropathy [23]. This model has been used for studies on hypertensive nephropathy [24–26].

Overall the effect of ACEi in lowering blood pressure on oxidative stress parameters and related protective mechanisms in the kidney has not been well studied neither in humans nor in SHR. As such, this study was undertaken to see the effect of enalapril, a widely used ACEi class antihypertensive drug, on the control of hypertension and the role of oxidative stress and antioxidant defence mechanisms in hypertension, as the subsequent renal damage progresses in SHR and L-NAME administered SHR.

2. Methods

2.1. Animals. Male SHR and Wistar-Kyoto (WKY) rats aged just below 4 weeks, obtained from the Animal Research and Service Centre (ARASC), Health Campus, Universiti Sains Malaysia, Kelantan, Malaysia, were used for the study.

2.2. Experimental Protocols. The experimental protocols used in this study were approved by the Animal Ethics and Welfare Committee of Universiti Sains Malaysia, Kelantan, Malaysia.

SHR were divided into 4 different groups of six rats each:

(1) SHR (untreated): SHR,

(2) SHR treated with enalapril (age: 4 weeks–28 weeks): SHR+E,

(3) SHR administered L-NAME (age: 16 weeks–28 weeks): SHR+LN,

(4) SHR treated with enalapril (age: 4 weeks–28 weeks) and L-NAME (age: 16 weeks–28 weeks): SHR+E+LN.

Control normotensive WKY rats were similarly divided into 4 groups ($n = 6$/group) and treated in the same manner as SHR groups. Each rat was housed in individual cage in standard controlled environment: room temperature of 25–27°C under 12-hour-light and 12-hour-dark cycle (lights on 0700–1900 hours). The animals were fed with standard commercial rat food and water *ad libitum*.

2.3. Enalapril and L-NAME Administration. After acclimatization of the rats in the cages, the average daily water intake of rats was determined. Both enalapril (Ranbaxy, Malaysia) and L-NAME (Sigma Chemicals, USA) were given to rats through their daily drinking water in the following doses: enalapril 30 mg kg^{-1} day^{-1}, L-NAME 25 mg kg^{-1} day^{-1}. Both dosage formulations were prepared freshly each day by dissolving the compounds in slightly less volume of daily water consumption to ensure their complete dosage intake. The daily water consumption was monitored to ensure the dosage was adhered to. Extra drinking water was provided after the required dosage had been taken. Concentration of both compounds in water was adjusted accordingly to match the age-related increase in body weight of the rats.

2.4. Physical Parameter Measurements. Body weight of rats was measured every week using a top pan balance by placing the rat in a small weighed cage. SBP was measured every two weeks in conscious rats during the experimental period by the noninvasive (indirect) blood pressure (NIBP) tail plethysmography method, using an automated cuff inflator-pulse detection system (Model 6R22931, IITC Life Science, USA). An average of three readings was taken for each measurement.

2.5. Specimen Collection and Processing. One to two days before 4 weeks, 16 weeks, and 28 weeks of age, the rats

were placed in metabolic cages for collection of 24-hour urine. Collected urine was stored at −80°C until analysis. Rats were weighed and sacrificed at the end of 28 weeks. Blood samples were collected in plain tubes, allowed to clot, centrifuged to obtain serum, and then stored at −80°C until analysis. Kidneys were rapidly removed, washed in saline, decapsulated, blot-dried, and weighed. One kidney was cut transversely and one half was used for histopathology examination. Other kidney tissues were used for kidney homogenate preparation.

2.6. Histopathology Examination. Routine histopathology procedures were followed whereby kidney sections were fixed with 10% neutral buffered formaldehyde for 2 days, dehydrated, and then embedded in paraffin. Paraffin sections were made at 3 μm and stained with haematoxylin/eosin (HE) for microscopic study to assess any glomerular, tubular, and vascular changes.

2.7. Preparation of Kidney Homogenates. A weighed amount of kidney tissue was homogenized to make 10% homogenates (w/v) in ice cold (0–4°C) 0.05 M sodium phosphate buffer pH 7.4, using an ice-chilled glass homogenizing vessel in a homogenizer fitted with Teflon pestle (Glass-Col, USA) at 900 rpm. The homogenates were centrifuged in a refrigerated centrifuge at 1,000 ×g at 4°C for 10 minutes to remove nuclei and debris [27]. The supernatant obtained was used for biochemical assays. TBARS assay was carried out on the day of sacrifice. Homogenates were kept frozen at −80°C until analysis for the other assays.

2.8. Biochemical Assays

2.8.1. Total Protein. Protein concentration of urine and kidney homogenates was determined using the Micro TP kit (Wako Pure Chemicals, Japan) according to the method of Watanabe et al. [28]. This method is a pyrogallol dye-binding spectrophotometric assay with bovine serum albumin (BSA) as the standard. To 1 mL of the Micro TP reagent 0.01 mL of sample or BSA standard was added and mixed. The reaction mixtures were left at room temperature for 15 minutes before absorbance was read at wavelength of 600 nm using a spectrophotometer (Ultrospec 1100 Pro, UK). Protein concentration in mg/day (for urine) and mg/L (for kidney homogenates) was calculated using the BSA standard.

2.8.2. Creatinine. Serum and urine creatinine were determined by the kinetic alkaline picrate method using a commercial reagent kit (Randox Laboratories, Crumlin, UK). Creatinine clearance was calculated from these data.

2.9. Oxidative Stress Markers

2.9.1. TAS. TAS was assessed according to the method of Koracevic et al. [29]. It is based on the principle that a standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton-type reaction, leading to the formation of hydroxyl radicals. These reactive oxygen species degrade benzoate, resulting in the release of TBARS. Antioxidants from the added sample of kidney homogenate cause suppression of the production of TBARS that was proportional to their concentration. This reaction is measured spectrophotometrically at 532 nm and the inhibition of colour development is defined as the TAS. The assay was performed as follows.

10 μL of kidney homogenate was pipetted in a test tube containing 0.49 mL of 100 mM sodium phosphate buffer. This was followed by the addition of 0.5 mL of 10 mM sodium benzoate solution, 0.2 mL of Fe-EDTA mixture, and 0.2 mL of 10 mM H_2O_2 solution. Negative control (with phosphate buffer instead of the kidney homogenate) containing similar reagents as in sample test tubes was also prepared. The test tubes were vortexed and incubated at 37°C for 60 minutes. This was followed by the addition of 1 mL of 20% acetic acid and 0.8% (w/v) thiobarbituric acid (TBA). The reaction tubes were incubated at 100°C for 10 minutes. After cooling to room temperature, the absorbance of the mixture was measured spectrophotometrically at 532 nm against distilled water. TAS in the kidney homogenates was calculated using uric acid as standard. TAS was expressed as μmol uric acid equivalent per mg protein.

2.9.2. TBARS. Lipid peroxidation was determined as TBARS according to the method of Chatterjee et al. [30]. MDA, an end product of fatty acid peroxidation, reacts with TBA to form a coloured complex which has maximum absorbance at 532 nm. 1,1,3,3-Tetraethoxypropane (TEP), a form of MDA, was used as standard in this assay. Briefly, 1.5 mL of 20% glacial acetic acid (pH 3.5), 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 0.8% (w/v) thiobarbituric acid (TBA), 0.7 mL of distilled water, and 0.1 mL of kidney homogenate or MDA standard were pipetted into test tubes. The test tubes were vortexed (Stuart, UK) and then kept in a boiling water bath (Memmert, Germany) at 95°C for 60 minutes with a marble on top of each test tube. After cooling, the test tubes were centrifuged at 3000 ×g for 10 minutes. One mL of each supernatant was transferred to cuvette and absorbance was read at 532 nm on a spectrophotometer (Ultrospec 1100 Pro, UK). The concentration of each sample was determined from a standard curve based on its absorbance. TBARS levels were represented as μmol MDA equivalent per mg protein.

2.10. Antioxidant Enzymes

2.10.1. SOD. SOD activity was assayed according to the method of Dogan et al. [31]. The oxidation of epinephrine is followed in terms of the production of adrenochrome which exhibits an absorption maximum at 480 nm. SOD removes $O_2^{\cdot-}$ from reaction mixtures by catalyzing its dismutation to O_2 and H_2O_2 thereby inhibiting autoxidation of epinephrine. Measurement of autoxidation of epinephrine was determined by pipetting 2 mL of 0.08 M sodium bicarbonate buffer solution (pH 10.2) into a cuvette, followed by 0.5 mL of 0.75 mM ethylenediaminetetraacetic acid (EDTA) solution. The reaction was started by adding 0.5 mL of 4.37 mM

epinephrine into the cuvette and changes in absorbance were measured at wavelength of 480 nm at 30-second intervals for 6 minutes after adding epinephrine. Measurement of SOD in kidney homogenate was performed by pipetting 1.95 mL of 0.08 M sodium bicarbonate buffer solution (pH 10.2), 0.5 mL of 0.75 mM EDTA solution, and 0.05 mL of kidney homogenate into a cuvette. Changes in absorbance were read at wavelength 480 nm every 30 seconds over a period of 6 minutes after adding 0.5 mL of 4.37 mM epinephrine solution, using a spectrophotometer. SOD activity was expressed as unit per mg protein. One unit (U) of SOD was defined as the amount of enzyme that inhibits the rate of autoxidation of epinephrine by 50%.

2.10.2. CAT. CAT activity was assayed according to the method of Goth [32]. The method is based on the enzyme-catalyzed decomposition of hydrogen peroxide and assay of the remaining hydrogen peroxide. Hydrogen peroxide and molybdate ions formed a yellowish complex which has maximum absorbance at 405 nm. The assay requires 4 reaction tubes: Blank 1, Blank 2, Blank 3, and the sample. For Blank 1, Blank 2, and Blank 3, the reagents were added in sequence. Blank 1 contains 0.5 mL substrate (65 mM hydrogen peroxide in 60 mM sodium-potassium phosphate buffer, pH 7.4), 0.5 mL 32.4 mM ammonium molybdate solution, and 0.1 mL kidney homogenate; Blank 2 contains 0.5 mL substrate, 0.5 mL ammonium molybdate solution, and 0.1 mL sodium-potassium phosphate buffer; Blank 3 contains 0.6 mL sodium-potassium phosphate buffer and 0.5 mL ammonium molybdate. For sample tubes, 0.1 mL kidney homogenate was incubated in 0.5 mL substrate at 37°C for 60 seconds. The enzymatic reaction was stopped with 0.5 mL ammonium molybdate solution and the yellow complex of molybdate and hydrogen peroxide was measured at wavelength of 405 nm against Blank 3. CAT activity was expressed as unit per mg protein. One unit of CAT was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol of hydrogen peroxide per minute.

2.11. Statistical Analysis. Data were analyzed by one-way ANOVA with post hoc Tukey test using Statistical Package for the Social Science (SPSS) software version 20. Significant level was set ($P < 0.05$). Data are expressed as mean and standard error mean (mean ± SEM) for six animals in each group.

3. Results

3.1. Kidney Weight and Kidney to Body Weight Ratio. There was no significant difference in the absolute kidney weight of SHR and SHR+E at 16 weeks. However at 28 weeks the absolute kidney weight of SHR, SHR+LN, and SHR+E+LN was significantly increased ($P < 0.01$, a**) when compared to SHR+E. There was no significant difference in absolute kidney weight among SHR, SHR+LN, and SHR+E+LN at 28 weeks (Figure 1). The kidney to body weight ratio for SHR+E was significantly reduced at both 16 weeks and 28 weeks when compared to SHR, SHR+LN, and SHR+E+LN ($P < 0.01$, b**). Kidney to body weight ratio was also significantly reduced in SHR+E+LN at 28 weeks when compared to untreated SHR+LN ($P < 0.01$, c**) (Figure 2).

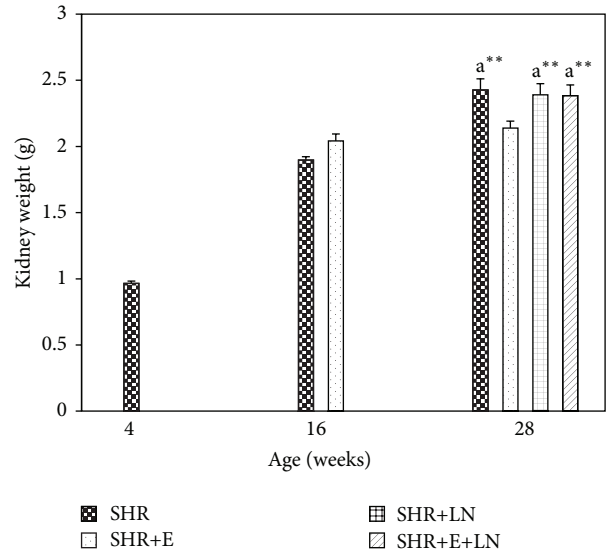

Figure 1: Kidney weight of enalapril treated and untreated SHR and SHR administered L-NAME. a$^{**}P < 0.01$ SHR+E compared to SHR, SHR+LN, and SHR+E+LN.

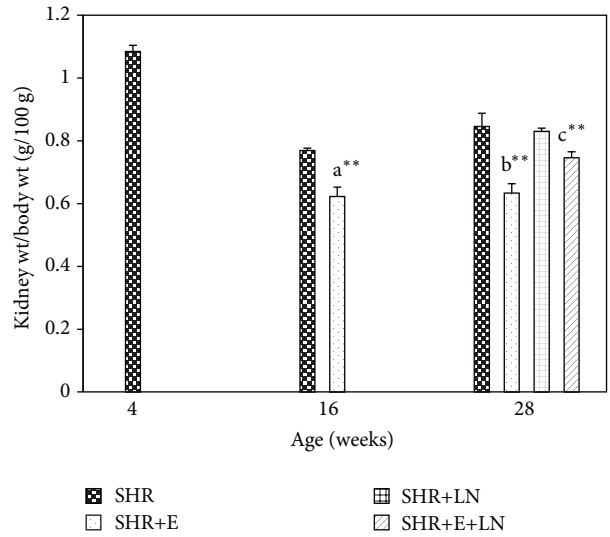

Figure 2: Kidney to body weight ratio of enalapril treated and untreated SHR and SHR administered L-NAME. a$^{**}P < 0.01$ SHR+E compared to SHR (16 weeks), b$^{**}P < 0.01$ SHR+E compared to SHR (28 weeks), and c$^{**}P < 0.01$ SHR+E+LN compared to SHR+LN.

3.2. SBP. The SBP of enalapril treated and untreated SHR and SHR+LN are presented in Figure 3. SBP of SHR treated with enalapril (SHR+E) were significantly lower from the age of 8 weeks until that of 28 weeks when compared to untreated SHR ($P < 0.001$, a***). L-NAME was administered to rats at the age of 16 weeks onwards. After administration of L-NAME, SHR+LN showed significant increase in SBP

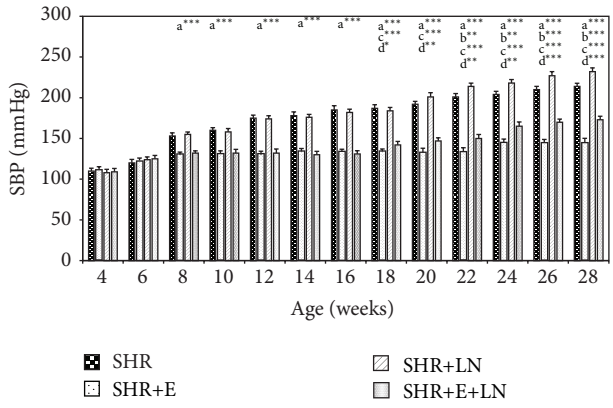

Figure 3: SBP of enalapril treated and untreated SHR and SHR administered L-NAME. $a^{***}P < 0.001$ SHR compared to SHR+E, $b^{**}P < 0.01$, $b^{***}P < 0.001$ SHR+LN compared to SHR, $c^{***}P < 0.001$ SHR+E+LN compared to SHR+LN, $d^{*}P < 0.05$, $d^{**}P < 0.01$, and $d^{***}P < 0.001$ SHR+E+LN compared to SHR+E. (Note that from week 4 to week 16 the data for the groups SHR+LN and SHR+E+LN are approximately similar to that of SHR and SHR+E rats, respectively, that have not been treated with L-NAME yet.)

from week 22 until week 28 compared to SHR ($P < 0.01$ b^{**}; $P < 0.001$, b^{***}). The SHR+LN group treated with enalapril (SHR+E+LN) showed significant decrease compared to untreated SHR+LN ($P < 0.001$, c^{***}) from week 18 until week 28. However the SBP levels of this SHR+E+LN group were still above normal at weeks 22, 24, 26, and 28. When compared to the SHR+E group, the SBP of the SHR+E+LN group showed significant increase from week 18 onwards until week 28 ($P < 0.05$, d^{*}, $P < 0.01$, d^{**}, and $P < 0.001$, d^{***}).

3.3. Histopathological Examination. Histopathological examination showed no pathological glomerular, tubular, or blood vessel changes in SHR at 4 and 16 weeks. However at 28 weeks, SHR showed some presence of minimal blood vessel medial hypertrophy. SHR+LN at 28 weeks showed significant pathological changes in the glomerulus, tubules, and blood vessels: glomerulosclerosis, shrunken or collapsed glomeruli, increased mesangial cells, presence of inflammatory cells, tubular atrophy and dilatation with casts, and blood vessel hypertrophy. These pathological changes were prevented by enalapril treatment (Figure 4).

3.4. Biochemical Parameters

3.4.1. Urinary Protein. Figure 5 shows the urinary protein levels in enalapril treated and untreated SHR and SHR+LN. Urinary protein was significantly increased in the untreated SHR group at 28 weeks when compared to the SHR+E group ($P < 0.01$, a^{**}). The greatly increased proteinuria in the SHR+LN group was significantly reduced when treated with enalapril (SHR+E+LN group: $P < 0.001$, b^{***}).

3.4.2. Creatinine Clearance. Figure 6 shows the creatinine clearance levels in enalapril treated and untreated SHR and SHR+LN. Creatinine clearance was significantly reduced in the untreated SHR+LN group when compared with the SHR+E+LN group ($P < 0.001$, b^{***}).

3.4.3. TAS. Figure 7 represents the kidney TAS levels in enalapril treated and untreated SHR and SHR+LN. There was no significant difference in TAS levels between SHR and SHR+E at 16 and 28 weeks. However the SHR+LN group showed significantly reduced TAS levels at 28 weeks when compared with the other groups ($P < 0.001$, a^{***}).

3.4.4. TBARS. The kidney TBARS levels of enalapril treated and untreated SHR and SHR+LN are shown in Figure 8. There was no significant difference in TBARS levels between SHR and SHR+E at 16 weeks. However at 28 weeks, SHR showed significant increase in TBARS when compared to SHR+E ($P < 0.001$, a^{***}). SHR+LN had the highest TBARS levels at 28 weeks, showing significant increase when compared to all the other groups ($P < 0.001$, b^{***}). Results also showed that enalapril treatment successfully prevented the increase in TBARS levels in both SHR and SHR+LN rat groups at 28 weeks.

3.4.5. SOD. There was no significant difference in kidney SOD activity between SHR and SHR+E at 16 weeks. At 28 weeks, SHR showed significant decrease in SOD when compared to SHR+E ($P < 0.05$, a^{*}). SHR+LN had the lowest SOD levels at 28 weeks, showing significant decrease when compared to all the other groups ($P < 0.001$, b^{***}). Results also showed that enalapril treatment successfully enhanced SOD levels in both SHR+E and SHR+E+LN rat groups at 28 weeks (Figure 9).

3.4.6. CAT. Kidney CAT activity (Figure 10) was significantly increased in SHR at 16 weeks ($P < 0.001$, a^{***}) and 28 weeks ($P < 0.001$, b^{***}) when compared to SHR+E group. CAT activity was the lowest in the SHR+LN group at 28 weeks and significantly reduced when compared to the other groups ($P < 0.001$, c^{***}).

3.5. SBP, Biochemical, Oxidative Stress Parameters, and Antioxidant Enzyme Levels in Enalapril Treated and Untreated WKY and WKY+L-NAME. WKY rats showed normal SBP throughout the study period for the WKY and WKY+E groups. L-NAME administration in WKY significantly increased the SBP from 20 weeks onwards ($P < 0.001$) when compared to untreated WKY. Enalapril treatment significantly reduced the SBP in WKY+LN when compared to untreated WKY+LN; however the level was still slightly above normal (data not shown).

Table 1 shows the urinary protein, creatinine clearance, and kidney oxidative stress parameters and antioxidant enzymes SOD and CAT of treated and untreated normotensive WKY and WKY+LN rats. At 28 weeks, WKY+LN had significantly reduced TAS levels when compared to the other groups ($a^{***}P < 0.001$). Similarly, SOD was significantly

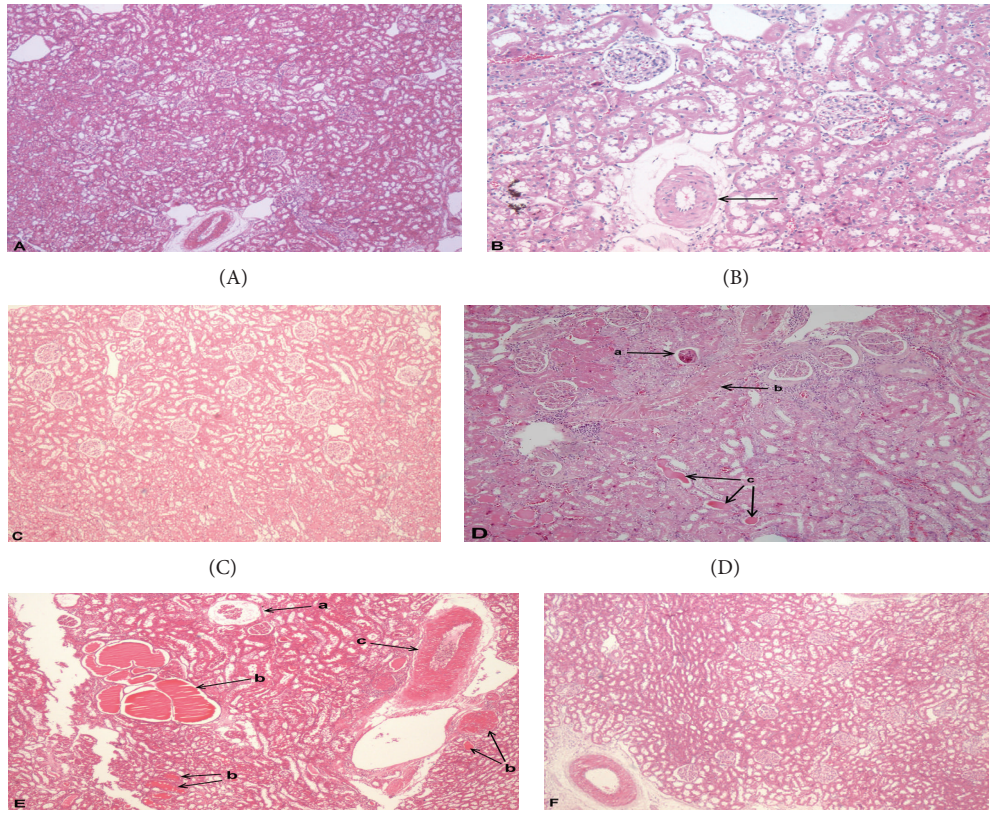

Figure 4: Kidney section HE stains of enalapril treated and untreated SHR and SHR administered L-NAME. (A) SHR at 16 weeks showing no abnormal changes (40x); (B) SHR at 28 weeks showing presence of mild blood vessel changes, medial hypertrophy (100x); (C) SHR+E at 28 weeks showing no abnormal changes (40x); (D) SHR+LN at 28 weeks showing collapsed glomerulus (a), blood vessel hypertrophy (b), and tubular casts (c)(40x); (E) SHR+LN at 28 weeks showing collapsed glomerulus (a), casts (b), and blood vessel hypertrophy (c) (100x); (F) SHR+E+LN at 28 weeks showing no abnormal changes.

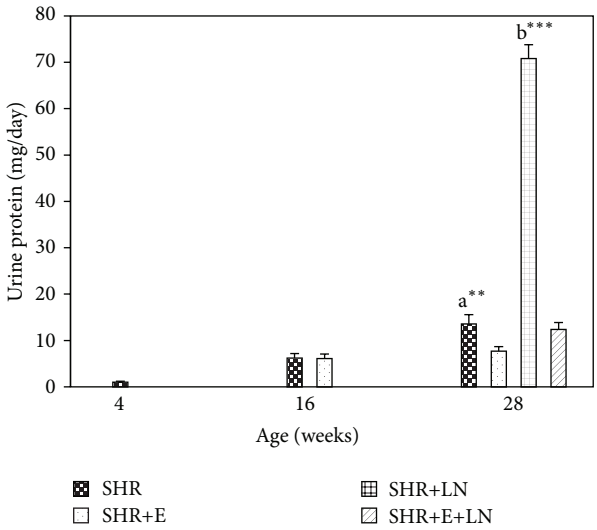

Figure 5: Urine protein levels in enalapril treated and untreated SHR and SHR administered L-NAME. a$^{**}P < 0.01$ SHR compared to SHR+E; b$^{***}P < 0.001$ SHR+LN compared to SHR+E+LN.

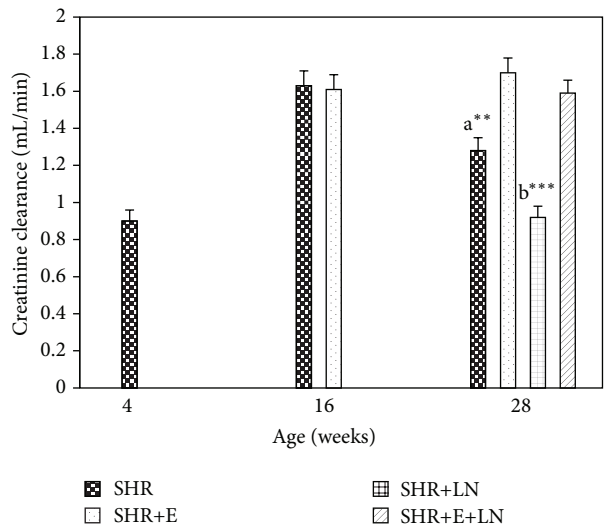

Figure 6: Creatinine clearance levels in enalapril treated and untreated SHR and SHR+LN. a$^{**}P < 0.01$ SHR compared to SHR+E; b$^{***}P < 0.001$ SHR+LN compared to SHR+E+LN.

Table 1: Effect of enalapril treatment on urinary protein, creatinine clearance, kidney TAS, TBARS, SOD, and CAT levels in treated and untreated WKY and WKY administered L-NAME rats.

Parameters	Age	Groups			
		WKY	WKY+E	WKY+LN	WKY+E+LN
TAS (umol/mg protein)	4 weeks	2.52 ± 0.03	—	—	—
	16 weeks	3.21 ± 0.06	3.34 ± 0.06	—	—
	28 weeks	3.47 ± 0.04	3.48 ± 0.05	2.67 ± 0.12^{a***}	3.68 ± 0.06
TBARS (umol/mg protein)	4 weeks	1.87 ± 0.05	—	—	—
	16 weeks	1.96 ± 0.11	1.94 ± 0.16	—	—
	28 weeks	2.28 ± 0.11	2.23 ± 0.13	3.05 ± 0.12^{a***}	2.15 ± 0.06
SOD (U/mg protein)	4 weeks	82.50 ± 5.8	—	—	—
	16 weeks	78.50 ± 3.94	82.33 ± 2.53	—	—
	28 weeks	79.50 ± 3.10	78.83 ± 2.20	61.33 ± 1.17^{a**}	74.83 ± 0.95
CAT (U/mg protein)	4 weeks	857 ± 21	—	—	—
	16 weeks	781 ± 14	706 ± 7	—	—
	28 weeks	768 ± 12	698 ± 15	725 ± 22	699 ± 16
Urinary protein (mg/day)	4 weeks	0.98 ± 0.22	—	—	—
	16 weeks	5.16 ± 0.41	6.08 ± 0.42	—	—
	28 weeks	5.28 ± 0.34	5.95 ± 0.43	9.19 ± 0.39^{a**}	5.80 ± 0.30
Creatinine clearance (mL/min)	4 weeks	0.59 ± 0.05	—	—	—
	16 weeks	1.69± 0.06	1.67 ± 0.07	—	—
	28 weeks	1.79± 0.08	1.77 ± 0.08	1.47 ± 0.03^{a**}	1.76 ± 0.07

Values are expressed as mean ± SEM ($n = 6$ per group).
WKY: WKY with no treatment, WKY+E : WKY+enalapril, WKY+LN : WKY+L-NAME, and WKY+E+LN : WKY+enalapril+L-NAME.
$^{a**}P < 0.01$ and $^{a***}P < 0.001$ WKY+LN compared to WKY, WKY+E, and WKY+E+LN.

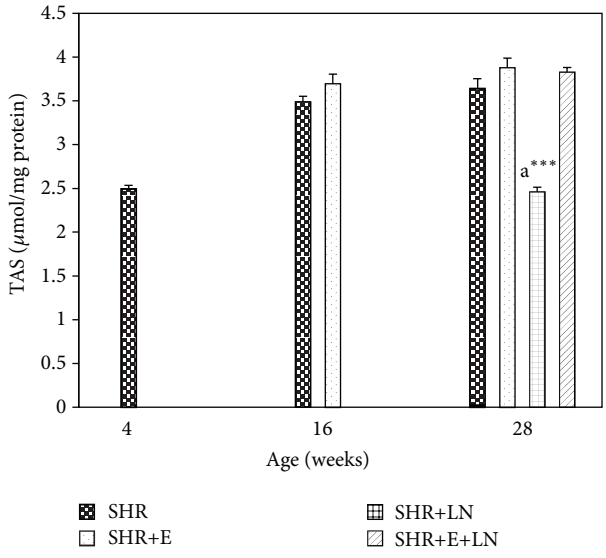

Figure 7: Kidney TAS levels in enalapril treated and untreated SHR and SHR administered L-NAME. $a^{***}P < 0.001$ SHR+LN compared to SHR, SHR+E, and SHR+E+LN.

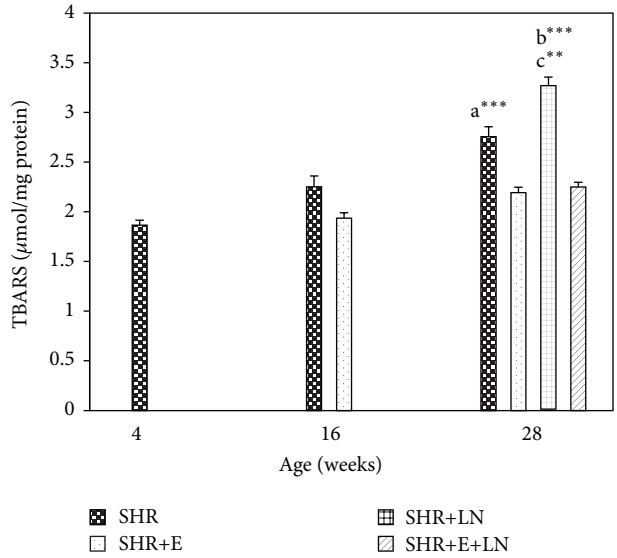

Figure 8: Kidney TBARS levels in enalapril treated and untreated SHR and SHR administered L-NAME. $a^{***}P < 0.001$ SHR compared to SHR+E, $b^{***}P < 0.001$ SHR+LN compared to SHR+E and SHR+E+LN, and $c^{**}P < 0.01$ SHR+LN compared to SHR.

reduced ($a^{**}P < 0.01$) and TBARS significantly increased ($a^{***}P < 0.001$) in the WKY+LN group at 28 weeks when compared to the other groups. No significant difference was seen in CAT activity between the different groups at 16 and 28 weeks. At 28 weeks, urinary protein was significantly increased ($a^{**}P < 0.01$) and creatinine clearance significantly decreased ($a^{**}P < 0.01$) in the WKY+LN group when compared to the other groups.

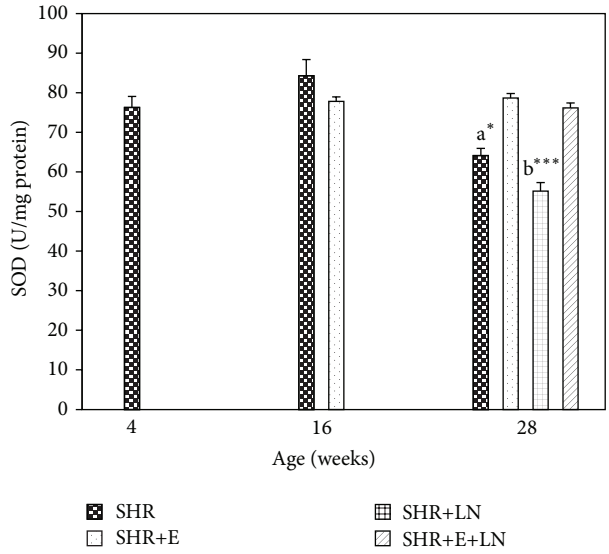

Figure 9: Kidney SOD activity in enalapril treated and untreated SHR and SHR administered L-NAME. a$^{*}P < 0.05$ SHR compared to SHR+E; b$^{***}P < 0.001$ SHR+LN compared to SHR, SHR+E, and SHR+E+LN.

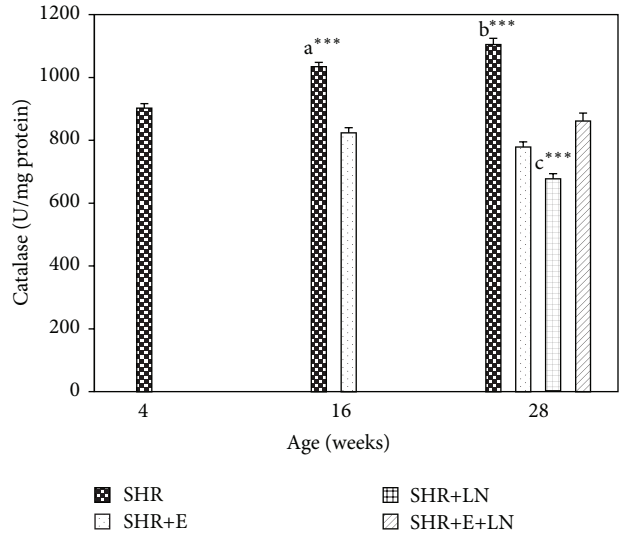

Figure 10: Kidney CAT activity in enalapril treated and untreated SHR and SHR administered L-NAME (16 weeks: a$^{***}P < 0.001$ SHR compared to SHR+E; 28 weeks: b$^{***}P < 0.001$ SHR compared to SHR+E and c$^{***}P < 0.001$ SHR+LN compared to SHR, SHR+E, and SHR+E+LN).

4. Discussion

This study utilized the SHR to look into the relationship between oxidative stress, kidney damage, and blood pressure lowering effect of enalapril in hypertension in a time-course manner, as this model has been shown to be excellent for the study of hypertension [33]. The SHR+L-NAME model was incorporated into the study so as to hasten the kidney damage and thereby shorten the study period. Zhou and Frohlich [23] showed the suitability of this model whereby they started the L-NAME inhibition on 17-week-old SHR, producing clear nephropathy in 3 weeks. Similarly, in our study, L-NAME inhibition was commenced at around the same age, that is, at 16 weeks, and continued for 12 weeks until 28 weeks so as to ensure that significant and extensive nephropathy occurs, which was confirmed by our histopathology, proteinuria, and creatinine clearance results. L-NAME inhibition was not started at an earlier age as enhancing hypertension rapidly at a younger age might affect the survival rate of the rats. Overall the study time points of 4 weeks, 16 weeks, and 28 weeks were selected so as to observe the changes from prehypertension to established hypertension and finally hypertensive kidney damage periods. The 16- and 28-week study time points were also selected as our previous research showed that this age period had greater increase in blood pressure and antioxidant changes [34]. Enalapril, a widely used ACEi class of antihypertensive drugs, was used for this study as it has been said to have renoprotective properties but the exact mechanism for this is not known [35]. The enalapril and L-NAME doses used in this study are similar to what has been used by other researchers [36, 37].

Results obtained showed that SBP of untreated SHR was already elevated to hypertensive levels at 8 weeks of age. This is in agreement with other researchers who noted hypertension in SHR at around this age [38, 39]. This elevated blood pressure increased progressively with a sharp increase occurring between the ages of 16 and 28 weeks. Chronic inhibition of nitric oxide synthase with L-NAME to SHR, initiated at 16 weeks, caused significant increase in SBP from 20 weeks onwards when compared to untreated SHR. SBP exceeded more than 200 mmHg after 4 weeks of administration and reached more than 220 mmHg at the end of the experimental period at 28 weeks. This confirmed the effect of L-NAME on SBP as obtained by other researchers [24–26]. Enalapril administration to SHR succeeded in lowering the SBP within normal limits. However, for the SHR+LN group, enalapril administration did not effectively reduce the SBP to normal whereby the values were about 155 mmHg at 24 to 28 weeks (Figure 3). The blood pressure of enalapril treated and untreated WKY rats was normal and relatively unchanged throughout the study. However the WKY+LN group showed elevated SBP, almost similar to the SHR+LN group (data not shown). Here, again, enalapril administration did not effectively reduce the SBP to normal. The reason for both these situations could be because the enalapril dose used was insufficient to overcome the inhibition effects caused by L-NAME.

SHR showed significantly lower body weight than age-matched WKY from 10 weeks onwards (data not shown). This could be due to various factors including metabolic changes associated with hypertension, stress, and poorer appetite. The absolute kidney weight of untreated SHR, SHR+LN, and SHR+E+LN at 28 weeks was significantly higher than SHR+E. The kidney to body weight ratio was also increased in a similar pattern (Figures 1 and 2). This is probably due to hypertrophy of various structures in the kidney brought about by hypertension which causes the kidney weight to increase as well as the lower body weight of these groups

which results in a reduced kidney to body weight ratio. Similar findings were reported by researchers experimenting on different animal models of hypertension [40, 41].

The histopathology results of this study confirmed the effect of L-NAME in producing kidney damage as clear pathological changes were seen in the glomerulus, tubules, and blood vessels at 28 weeks (Figures 4(D)-4(E)). Besides this, urine protein was markedly increased and the creatinine clearance was greatly reduced. Enalapril treatment managed to prevent this damage, confirming its renoprotective effect through blood pressure lowering as mentioned by other researchers [35].

Oxidative stress has been implicated in the pathogenesis and progression of hypertension with some studies suggesting it is the cause while others suggest it is a consequence of hypertension [4, 5, 42, 43]. Results from this study showed that TAS levels are significantly reduced at 28 weeks in SHR+LN rats when compared to the other groups. Also, TBARS levels are significantly raised in SHR and SHR+LN rats during the same time period. These findings indicate the presence of oxidative stress in the kidneys of these groups. The SBP of these rat groups during this time period was also very high, exceeding 200 mmHg, indicating a strong relationship with oxidative stress. Enalapril treatment, besides reducing the SBP, also managed to prevent this oxidative stress by reducing the TBARS levels as well as enhancing the TAS levels (Figures 5 and 6). SOD levels at 28 weeks were significantly reduced in the SHR and SHR+LN rats. These levels were restored to earlier 4-week and 16-week levels when enalapril was administered (Figure 7). CAT activity in the SHR group was significantly raised at 16 weeks and 28 weeks when compared to the other groups. It is possible that this overexpressed CAT activity during this time might be a compensatory mechanism to protect the kidney from the deleterious effects of free radicals involved in causing oxidative stress. The CAT activity in the SHR+LN group was significantly reduced at 28 weeks when compared to the other groups. This could be due to some unknown mechanism in the oxidative stress process that has affected its activity. Enalapril treatment managed to normalise the CAT activity to earlier levels (Figure 8). All these findings clearly indicate that enalapril has antioxidative properties. This study also supports the view that enalapril has renoprotective properties which might be conferred through the reduction or elimination of oxidative stress in the kidney as has been shown for other antioxidants [44].

5. Conclusion

This study showed that enalapril, in addition to blood pressure lowering properties, also has beneficial effect in reducing oxidative stress in the kidneys.

Conflict of Interests

No conflict of interests, financial or otherwise, is declared by the authors.

Acknowledgments

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Research Article

Analysis of Oxidative Stress Enzymes and Structural and Functional Proteins on Human Aortic Tissue from Different Aortopathies

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The role of oxidative stress in different aortopathies is evaluated. Thirty-two tissue samples from 18 men and 14 women were divided into: 4 control (C) subjects, 11 patients with systemic arterial hypertension (SAH), 4 with variants of Marfan's syndrome (MV), 9 with Marfan's syndrome (M), 2 with Turner's syndrome, and 2 with Takayasu's arteritis (TA). Aorta fragments were homogenized. Lipoperoxidation (LPO), copper-zinc and manganese superoxide dismutase (Mn and Cu-Zn-SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), endothelial nitric oxide synthase (eNOS), nitrates and nitrites (NO_3^-/NO_2^-), and type IV collagen, and laminin were evaluated. There was an increase in Mn- and Cu-Zn-SOD activity in SAH, MV, M, and Turner's syndrome. There was also an increase in CAT activity in M and Turner' syndrome. GPx and GST activity decreased and LPO increased in all groups. eNOS was decreased in SAH, MV, and M and NO_3^-/NO_2^- were increased in SAH and TA. Type IV collagen was decreased in Turner's syndrome and TA. Laminin γ-1 was decreased in MV and increased in M. In conclusion, similarities and differences in oxidative stress in the different aortopathies studied including pathologies with aneurysms were found with alterations in SOD, CAT, GPx, GST, and eNOS activity that modify subendothelial basement membrane proteins.

1. Introduction

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of the biological system. It requires rapid detoxification of intermediate reactants or the repair of the damage and it alters the essential processes possibly becoming the origin of tissue damage in the organism [1–3].

Aortic pathologies, characterized by the loss of contractile function and endothelium-mediated relaxation, are related to oxidative stress and vascular dysfunction [4–6]. Besides, the mechanical properties of the vessel transform [7], leading to pseudoaneurysm or aneurysm formation, to obstruction or destruction of the vessel [8, 9]. Even though the origin of aortic damage might be multifactorial, molecular oxygen could play a critical role in vascular tonicity, cardiac contractility,

and other parameters. Molecular oxygen participates in ROS genesis and/or can induce irreversible damage and even death. However, it can also have beneficial effects, participating in cellular signaling processes [10]. Oxidative stress has been involved in cardiovascular diseases [2, 11, 12], such as arrhythmias, coronary arterial disease, left ventricular hypertrophy, aortic dilatation, aortic dissection, and congestive heart failure. ROS and reactive nitrogen species (RNS) are produced in these diseases through different pathways such as mitochondrial xanthine oxidase and NADPH oxidase (Nox). Endothelial nitric oxide synthase (eNOS) also plays a relevant role in them [13–24].

Structural and functional damage is present in the aortic walls in different human diseases such as hypertension (SAH), atherosclerosis, Takayasu's arteritis (TA), Turner's syndrome, and Marfan's syndrome (M) and its variants (MV) [25–28]. In them, aortic damage, stenosis, occlusion, aneurysms or pseudoaneurysms [29–31], and endothelial dysfunction [32–34] have been described. These findings have been described independently for each disease having a specific genetic background, appearing at different ages and in subjects of different genders and exposed to different environments such as infections or trauma. However, they all require similar surgical treatment [35].

The role of oxidative stress in aortic damage progression in some pathologies has already been described [7, 36]; however, in others, human tissues are scarce [37, 38]. Therefore, a study of oxidative stress and antioxidants in the aorta from patients with aortic damage is justified.

The aim of this paper is to evaluate the role of oxidative stress in human aortas from patients with different pathologies where aortic damage is present. Tissue was obtained by elective surgery or emergency. We also evaluate subendothelial basement membrane proteins.

2. Materials and Methods

2.1. Patients. 18 men and 14 women treated at our institution and who met a surgical criteria for either elective or emergency surgery by Bentall and Bono's method [39] were included in a consecutive way as soon as aortic root dilatation (>50 mm) was demonstrated by computed tomography angiography.

The samples collected were taken from patients with M, classified by the Ghent's criterion in 1996 [40], and TA diagnosed by the standards of the American College of Rheumatology (ACR) [41] and patients with hypertension (SAH) and Turner's syndrome [42]. Once patients fulfilled the inclusion criteria they went through a thorough clinical examination to determine the extent of their cardiac pathology. The results were analyzed and presented in a clinic and pathological conference where cardiovascular specialists assessed the risks and benefits of a surgical intervention. The subjects were submitted to a preoperative protocol that included coagulation tests, X-rays, electrocardiogram, anesthesia evaluation, and individualized medical intervention. Cases were dealt with caution, to avoid including patient undertaking treatments with antioxidants, allopurinol, or probable inhibitors of pathways involved in ROS production. Aspirin, warfarin, clopidogrel, and other antiplatelet or anticoagulant medications were suspended. Control tissues were obtained from patients who underwent surgery for aortic stenosis, who had no syndromic pathology diagnosed, and in whom there was no suspicion of inflammatory diseases such as TA or atherosclerosis nor presence of degenerative disorders such as diabetes mellitus, arterial hypertension, thyroid, or autoimmune diseases. The surgery performed implied substitution of aortic valves and there was a need to perform plasty or resection of aortic tissue surrounding the valvular area. Control subjects were evaluated previously to surgery by an expert cardiologist and rheumatologist to verify that none of the above mentioned diseases was present and routine laboratory tests were made to determine acute phase reactants, triglycerides, and HDL cholesterol. Additionally to image studies by echocardiography, computerized tomography or magnetic resonance studies were done to discard aortic damage additional to valvular damage. None of the control subjects was taking anti-inflammatory drugs or statins. The research protocol was approved by the Research and Ethics Committee of our institution (Institutional protocol number: 09654). Informed consent of patients and controls was obtained for the anesthetic procedure and surgery and to obtain a tissue sample, according to the Declaration of Helsinki [43]. Once the surgery was performed, the tissue was placed in liquid nitrogen and was kept at −70°C until use.

2.2. Thoracic Aorta Homogenization. A sample from thoracic aorta was taken for homogenization in liquid nitrogen, it was mixed with a sucrose solution in the presence of protease inhibitors (1 mM PMSF, 2 μM pepstatin A, 2 μM leupeptin, and 0.1% aprotinin), and the preparation was kept on ice. The thoracic aorta homogenate was centrifuged at 900 × g for 10 min at 4°C. The supernatant was separated and kept at −70°C until required. Protein concentration in the thoracic aorta homogenate was determined by the method of Lowry et al. [44].

2.3. Immunofluorescence. 3 mm of aortic sections was quickly frozen in Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA). Sections were fixed with acetone and were blocked with PBS/azide 0.02%/BSA 1% for 30 min.

Subsequently, these sections were left for 2 hours at room temperature with a rabbit polyclonal antibody against type IV collagen (1 : 20), fibronectin, laminin-γ-1, iNOS, and eNOS (1 : 50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Primary antibodies were detected with goat, anti-rabbit, FITC (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), at room temperature for 60 min. Negative controls were prepared by substituting the primary antibody with an irrelevant antibody. Immunofluorescence was examined using a fluorescence microscope (Nikon Eclipse TE2000-U, Digital Nikon sight chamber D5-U3). Images were processed with SigmaScan Pro program, Image Analysis version 5.0.0, 1987–1999 SPSS Inc.

Table 1: Demographic characteristics of the patients.

Characteristics	Control $n = 4$	SAH $n = 11$	Marfan's variants $n = 4$	Classic Marfan $n = 9$	Turner's syndrome $n = 2$	Takayasu's arteritis $n = 2$
Median age (range)	59 (42–62)	59 (34–72)	46 (28–55)	30 (17–56)	33 (31–34)	33 (26–40)
Women *n* (%)	1 (25)	4 (37)	1 (25)	4 (44)	2 (100)	2 (100)
Men *n* (%)	3 (75)	7 (64)	3 (75)	5 (56)	0	0
Median BMI (range)	22 (18–24)	31 (30–32)	24 (23–32)	22 (17–30)	28 (23–32)	19 (16–23)
Median LVEF (range)	44 (30–65)	45 (25–65)	60 (56–75)	50 (40–65)	59 (50–67)	65 (65-65)
Median aortic diameter (range)	55 (55-55)	52 (26–65)	66 (62–70)	75 (55–120)	81 (67–96)	50 (50-50)
SAH *n* (%)	1 (50)	11 (100)	1 (25)	2 (22)	2 (100)	1 (50)
Tobacco smoking *n* (%)	1 (50)	4 (37)	2 (100)	2 (22)	0	1 (50)

SAH: systemic arterial hypertension, BMI: body mass index, and LVEF: left ventricular ejection fraction.

2.4. Lipoperoxidation (LPO). LPO, a marker of damage by free radicals, was measured by a standard method [45]. One mg of protein from the thoracic aorta homogenate was used.

2.5. Superoxide Dismutase (SOD) Activity. SOD enzyme activity was determined in the thoracic aorta homogenate by nondenaturing gel electrophoresis and nitroblue tetrazolium staining as described by Flohe and Otting [46]. 60 μg of the thoracic aorta homogenate was used. SOD activity was 5600 units/mg, calculated following the technique described by Pérez et al. [45]. Riboflavin and TEMED in the presence of UV light and oxygen produce ROS; nitroblue tetrazolium and SOD compete with them. Where SOD is present, the gel remains transparent, whereas reduced nitroblue tetrazolium turns it into purple-blue.

2.6. Catalase (CAT) Activity. 60 μg of thoracic aorta homogenate was analyzed by native-gel electrophoresis with 8% polyacrylamide [47]. Protein CAT activity was 25000 units/mg, calculated following the technique described by Pérez et al. [45]. The gels of CAT and SOD were analyzed by densitometry by the image analyzer with SigmaScan Pro program, Image Analysis version 5.0.0, 1987–1999 SPSS Inc.

2.7. Assay of Glutathione S-Transferase (GST). The specific activity of cytosolic GST was determined spectrophotometrically at 340 nm by the method of Beutler [48]. 100 μg from aorta homogenate was used per sample. The specific activity of GST is expressed in micromoles of CDNB-GSH conjugate formed/min/mg protein.

2.8. Assay of Glutathione Peroxidase (GPx). The enzyme GPx activity was measured spectrophotometrically at 340 nm by the method of Flohé and Günzler [49]. 100 μg from aorta homogenate was used per sample.

2.9. Nitrates and Nitrites. Nitrates and nitrites were measured spectrophotometrically at 540 nm by the method described by Pérez et al. [45]. 100 mg of protein from aorta homogenate was used per sample.

2.10. Immunoblotting. 100 μg from aorta homogenate was used per sample. The immunoblotting was done according to the method described by Pérez et al. [45]. A dilution of 1/500 of the rabbit primary IgG polyclonal antibodies against eNOS, iNOS, laminin-γ-1, and COL4A2 (NOS3 antibody rabbit IgG [C-20]: sc-654, NOS2 antibody rabbit IgG [C-19]: sc-649, laminin-γ-1 antibody rabbit IgG [H-190]: sc-5584, and COL4A2 antibody rabbit IgG [N-14]: sc-70244; Santa Cruz Biotechnology, Inc.) was used.

2.11. Statistical Analysis. A descriptive analysis was performed. Univariate test includes age, gender, pathology types, and variables, and dichotomic and nominal variables were described as relative frequencies (percentages) and compared by chi-squared distribution or Fisher's exact test, as convenient. For bivariate analysis, continuous quantitative variables of normal distribution were compared by *t* student, and nonparametric ones by Mann-Whitney *U* test.

3. Results

3.1. General Characteristics. Out of the 32 patients, 14 were women, and 18 were men. The demographical characteristics of the patients are shown in Table 1, and the clinical data are shown in Table 2.

Measurements were made individually and they were grouped afterwards to be analyzed.

3.2. Lipoperoxidation. Aortic tissues from patients with SAH, MV, M, Turner's syndrome, and TA showed significantly increased LPO ($P \leq 0.05$), when compared to C subjects (Figure 1).

3.3. Activity of Antioxidant Enzymes. In SAH, MV, M, and Turner's syndrome, aortas showed an increase in Mn-SOD activity and Cu-Zn-SOD ($P < 0.05$) in comparison to C subjects (Figures 2(a) and 2(b)). CAT activity increased in M and Turner's syndrome patients ($P = 0.05$) in comparison to C subjects (Figure 3). Figure 4(a) shows the GPx activity in SAH, MV, M, Turner's syndrome, and TA patients which was significantly increased ($P < 0.05$) when compared to C

Table 2: Clinical finding, surgery type, and report diameters aortic.

Sex	Age	Diagnosis	Clinical findings	Aortic diameter mm
H	68	SAH	SVAo and aortic reduction plastic	26
M	55	SAH	Aortic dissection and Ao. I (BB)	55
H	63	SAH	Hypertensive cardimyopathy and abdominal aneurysm (BB)	55
H	59	SAH	Aortic arc substitution and stent subclavian artery (BB)	69
M	53	SAH	Aortic root aneurysm (BB)	50
H	46	SAH	B and B aortic arc substitution and revascularization of brachiocephalic trunk	55
M	59	SAH	Ascending aorta dissection (BB)	60
M	55	SAH	Aortic valve substitution + plastia aorta + Revascularization Coronary: Internal thoracic artery-DA, Venous Hemoduct	65
H	34	SAH	Abdominal aneurysm (BB)	100
H	72	SAH	B and B aortic arc substitution and revascularization of brachiocephalic trunk	70
H	63	SAH	Ascending aortic aneurysm (David)	51
H	31	Marfan's syndrome	Ascending Ao. A, coarctation. Bivalve aorta (BB)	75
H	17	Marfan's syndrome	Aortic dissection and aortic insufficiency (BB)	87
H	56	Marfan's syndrome	Aortic dissection and aortic insufficiency (BB)	94
H	17	Marfan's syndrome	Aortic dissection and Ao. A (BB)	68
H	42	Marfan's syndrome	Aortic dissection and Ao. A (BB)	120
M	38	Marfan's syndrome	Aortic dissection and Ao. A (BB)	55
M	21	Marfan's syndrome	Aortic ascending aneurysm (BB)	67
H	23	Marfan's syndrome	Bicuspid aortic valve and ascending aorta A (BB)	96
H	23	Marfan's syndrome	Aortic root dilation and aneurysm (BB)	50
M	28	Marfan's variant	Ascending aorta aneurysm (BB)	88
H	46	Marfan's variant	Ascending aorta aneurysm (BB)	70
H	46	Marfan's variant	Thoracic aneurysm and ascending aorta (BB)	55
H	55	Marfan's variant	Ascending aortic and aortic root aneurysm (BB)	57
M	31	Turner's syndrome	Ascending aorta aneurysm and infradiaphragmatic aorta (By)	67
M	34	Turner's syndrome	Acute aortic syndrome and dissection (BB)	100
M	26	Takayasu's arteritis	Acute aortic syndrome and dissection of aneurysm (BB) SVAo	54
M	40	Takayasu's arteritis	Autopsia complications of IRCT	52
H	62	Control	Aortic stenosis	50
M	60	Control	Aortic stenosis	54
H	42	Control	Aortic stenosis	52
H	60	Control	Aortic stenosis	60

F: female, M: man, Ao. A: aortic aneurysm, SAH: systemic arterial hypertension, Ao. I: aortic insufficiency, BB: surgery Bental and Bono, David: David surgical procedure, Descending artery: DA, SVAo: aortic valve substitution.

subjects. Figure 4(b) shows the GST activity in SAH, MV, M, Turner's syndrome, and TA. It was significantly increased in these groups ($P < 0.05$) when compared to C subjects.

3.4. Endothelial Nitric Oxide Synthase and NO_3^-/NO_2^-. In patients with SAH, MV, and M, eNOS expression was elevated ($P = 0.01$) in comparison to C subjects (Figure 5(a)). NO_3^-/NO_2^- significantly increased in SAH and TA ($P = 0.01$) in comparison to C subjects (Figure 5(b)).

3.5. Variations in Structural and Functional Proteins. Figure 6(a) shows the localization of type IV collagen in aortas from patients. A decrease in type IV collagen was observed in Turner's syndrome and TA ($P = 0.01$) when compared to C subjects. Fibronectin did not show changes in any group (results not shown). Figure 6(b) shows that laminin-γ-1 in aorta of MV was decreased while in M patients it was increased ($P = 0.03$) when compared to C subjects.

3.6. Inducible Nitric Oxide Synthase. In patients with SAH, MV, M, Turner syndrome, and TA iNOS expression was elevated ($P < 0.05$) in comparison to C subjects (Figure 7).

3.7. Immunoblotting. Figure 8 shows eNOS, iNOS, type IV collagen, and laminin-γ-1 expression in the homogenized

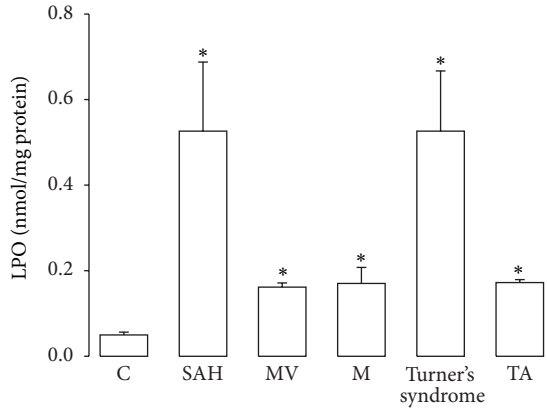

Figure 1: Comparison of LPO results in control subjects and in patients with the different pathologies studied. $^{*}P \leq 0.05$; C versus SAH, MV, M, Turner's syndrome, and TA. Abbreviations: SAH: systemic arterial hypertension; MV: variants of Marfan's syndrome; M: Marfan's syndrome; Turner's syndrome; and TA: Takayasu's arteritis.

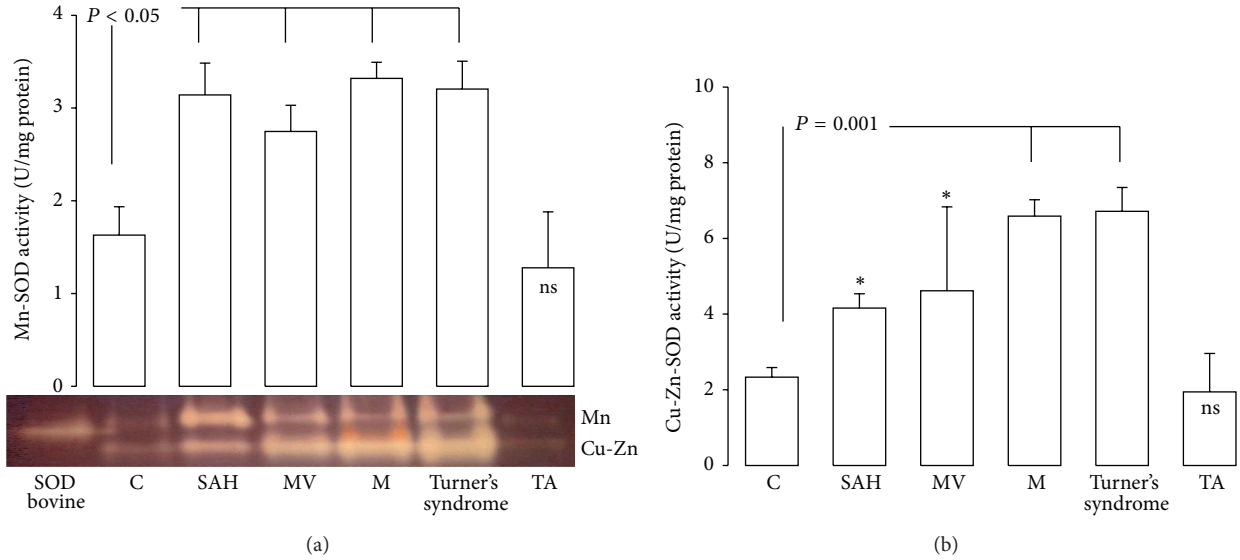

Figure 2: Mn-SOD and Cu-Zn-SOD activity in control subjects and in patients with the different pathologies studied. $^{*}P \leq 0.05$; C versus MV and SAH.

aorta from the patients with aortopathies. The eNOS, iNOS, type IV collagen, and laminin-γ-1 expression showed the same trend as the one found by immunofluorescence.

4. Discussion

The mechanisms through which oxidative stress and inflammatory processes might produce vascular abnormalities are unknown. In this paper we studied variations in the activity of antioxidant enzymes and in structural and functional proteins in several illnesses in which there is vascular dysfunction and altered mechanical properties that lead to pseudoaneurysms/aneurysms or obstruction. The disease renders it necessary for the patients to undergo surgery. The exact source of ROS and RNS in the pathophysiological pathways of these pathologies has not been clearly described.

ROS and LPO lead to DNA oxidative damage and to high levels of 8-iso- prostaglandin $F_{2\alpha}$ (8- iso-$PGF_{2\alpha}$) in patients having essential hypertension [50]. Serum of patients with TA shows a similar tendency [51]. The role of ROS in the onset and progression of aortic damage has been described in animal models of different illnesses [52] and ROS are important mediators in the signaling pathways of inflammation and atherogenesis [53, 54]. An imbalance between the prooxidative agents and antioxidants leads to changes in the redox state. Mouse models for M have vasomotor dysfunction in the thoracic aorta associated with oxidative stress, which correlates with an increase in eNOS and a diminished production of Mn- and Cu-Zn-SOD [4, 55]. Our results show an increase in Mn-SOD and Cu-Zn-SOD activity in SAH, MV, M, and Turner's syndrome. The increased expression and activity found suggest that, due

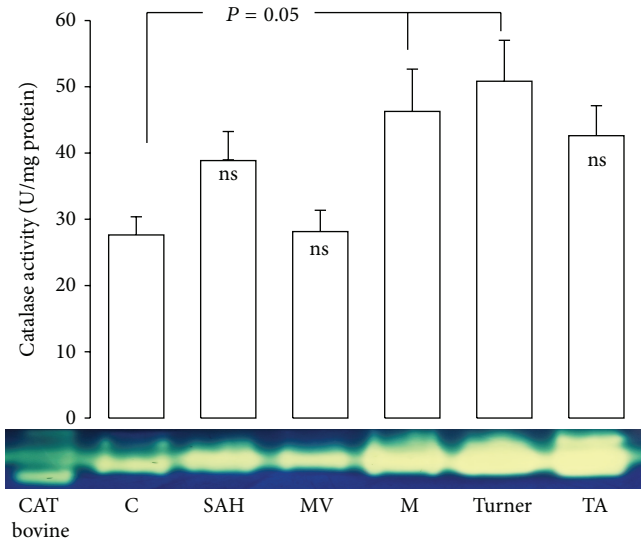

Figure 3: Catalase activity in control subjects and in patients with the different pathologies studied.

to the increase in oxidative stress, the patient's antioxidant system is overexpressed in an attempt to counteract the imbalance. The vascular protection by the expression and activity of the isoforms of the SOD in the vascular wall has been evaluated in mouse models, finding either SOD deficiency or overproduction [56]. An increase in vascular permeability or reperfusion injury after ischemia is related to SOD deficiency, and the overexpression of the Cu-Zn-SOD protects against reperfusion injury [57]. Also, cytosolic and extracellular SOD expression alterations might impact on vascular and other tissue structures, because they inhibit vascular and myocardial hypertrophy [58].

The beneficial function of SOD as antioxidant in diverse illnesses [59] both in animal models and in patients with or without an active inflammatory disease has been described [60]. The mechanisms inducing inflammation may act through different stimulus and receptors among which the type 1 angiotensin receptor seems to play an important role [61]. Our results showed an increase in Mn- and Cu-Zn-SOD enzyme activity in SAH, MV, M, and Turner's syndrome without significant changes in TA when compared to C subjects. These results suggest that SOD isoforms increase their activity to protect against oxidative stress.

SOD isoforms are important within the vascular wall in normal conditions and in diseased states in humans. Cu-Zn-SOD expression is relatively high in all cell types, including blood cells, and it accounts for 50% to 80% of the total activity, being, therefore, the predominant isoform. Mn-SOD is responsible for 2% of the activity and the remaining 12% of the activity may be due to extracellular SOD (SOD-EC). The functional importance of Cu-Zn-SOD is further evidenced when there is deficiency of SOD-EC [62]. In mice with Cu-Zn-SOD deficiency there is an increase in vascular permeability and ischemia related to hypertrophy of cerebral arterioles [63]. In our study, the Cu-Zn-SOD deficiency found in hypertensive patients with aortic dilatation, Turner's syndrome, and TA could be related to vascular alterations and to the clinical condition of these patients. In genetically modified mice with Cu-Zn-SOD overexpression there is a protective effect against vascular dysfunction [64].

Under normal conditions, Mn-SOD is the first line of defense against oxidative stress. Its localization, induction mechanisms, vascular expression, and activity are known [65] and might be altered under physiological and pathophysiological conditions, particularly under overregulation of oxidative stress [66]. Mn-SOD expression is altered at certain stages of disease that are associated with vascular oxidative stress.

Proinflammatory cytokines and LPS-mediated inflammation in vascular tissue cause an increase in superoxide (O_2^-) production and in Mn-SOD expression [67]. In atherosclerosis, vascular expression of Cu-Zn-SOD and Mn-SOD mRNA increases at the onset of the disease and diminishes over time [68]. In chronic hypertensive models, the expression of vascular Mn-SOD is also increased [69]. There are many conditions that elevate peroxynitrite concentrations which, in turn, inactivate Mn-SOD. These include inflammation, diabetes, hypertensive atherosclerosis, subarachnoid hemorrhage, and age. However, in recent studies, Mn-SOD was found to protect from mitochondrial vascular damage and atherosclerosis development [70].

In this study, all of the aortopathy groups had an increase in Mn- and Cu-Zn-SOD, which correlated with increased LPO with the exception of TA. Mn-SOD overexpression reduces superoxide levels [71] and improves endothelial function in some models, thereby preventing endothelial injury [72]. Moreover, we consider that, in these patients, the compensatory mechanisms were diminished and oxidative stress was increased. In other vascular illnesses, these compensatory response mechanisms vary and could be associated with other factors such as evolution of the pathology, etiopathogenic mechanisms, and host response capacity [73]. TA is associated with tuberculosis infection which has only been proven in isolated cases [74–77]. In patients with confirmed tuberculosis, an important decrease in Mn-SOD has been found, which improves with treatment [78]. Recent studies proposed Mn-SOD as an important antioxidant modulator after vascular injury. These results suggest that it might be employed as a promising therapeutic strategy for vascular injury prevention and in proliferative diseases where there is stenosis [79].

CAT is another important antioxidant enzyme, which is found in the liver, kidney, and aorta. CAT uses two H_2O_2 molecules to break them into O_2^-; one acts as a reducing agent and the other as an oxidant. Overexpression of CAT prevents the stimulation of ROS [80]. Our results showed that CAT increased in the patients with M and Turner's syndrome. These results suggest that overexpression of CAT may be due to overproduction of H_2O_2 in the aorta from these patients. The increase in CAT activity in our study may be attributed, in part, to continuous exposure to hydroperoxides. Although different studies have shown that in hypertension CAT activity is low [81], in our study CAT activity showed no significant change in SAH, MV, and TA when compared to C subjects.

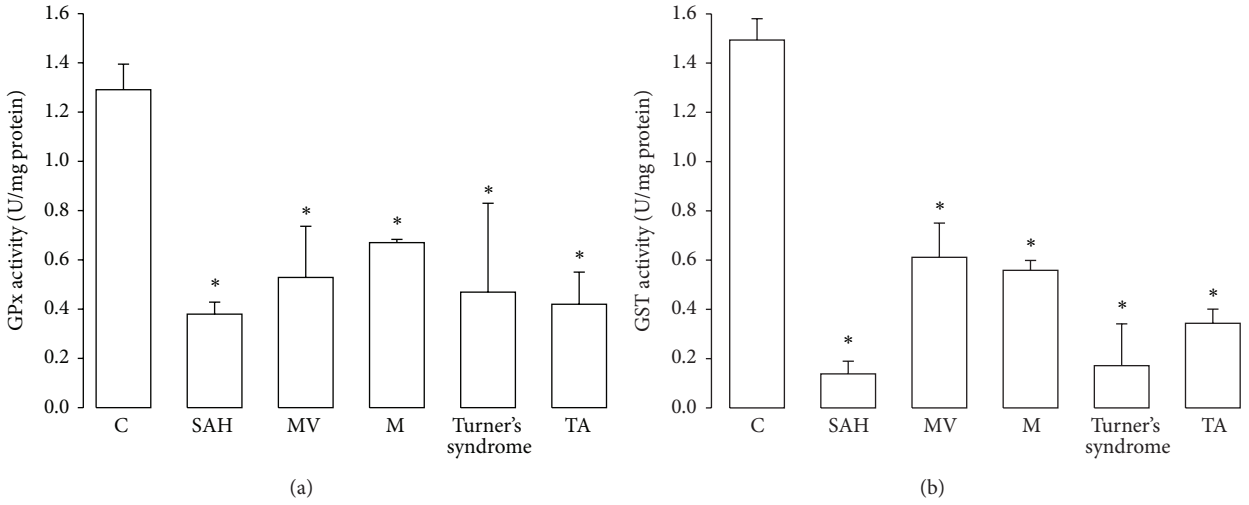

Figure 4: Glutathione peroxidase and glutathione S-transferase activity in controls and in the pathologic conditions studied. $^{*}P = 0.05$, C versus SAH, MV, M, Turner's syndrome, and TA. Abbreviations: SAH: systemic arterial hypertension, MV: variants of Marfan's syndrome, M: Marfan's syndrome; Turner's syndrome; and TA; Takayasu's arteritis.

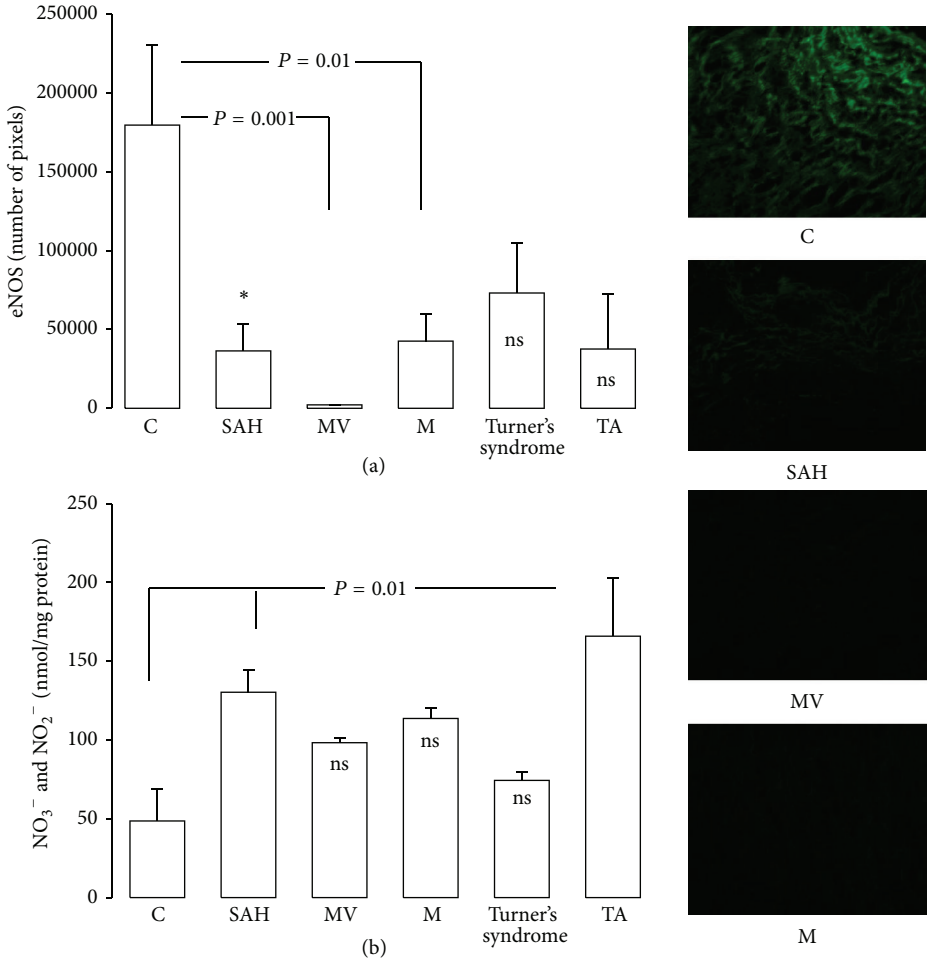

Figure 5: Comparison of the eNOS activity and NO_3^-/NO_2^- in control subjects and in patients with the different pathologies studied $^{*}P = 0.05$, C versus SAH. Pictures show the eNOS immunofluorescence that were significantly different.

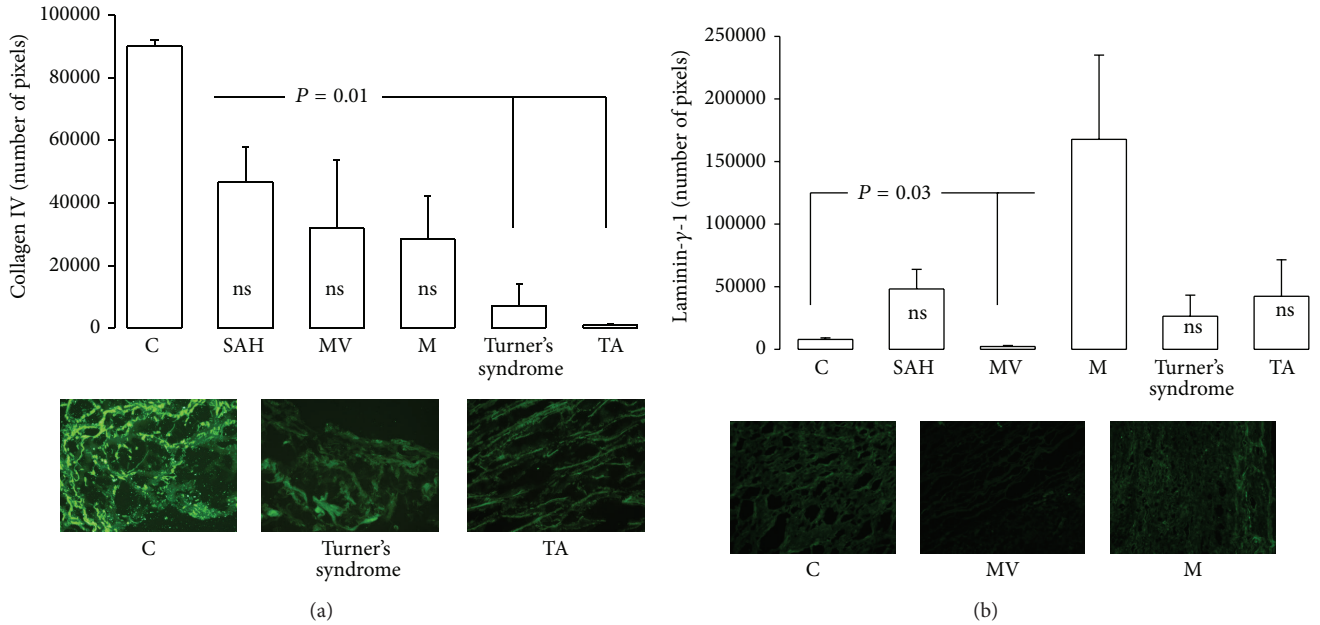

Figure 6: Immunofluorescence results for collagen and laminin-γ-1 in control subjects and in patients with the different pathologies studied. Pictures show type IV collagen and laminin-γ-1 immunofluorescence with statistically significant differences.

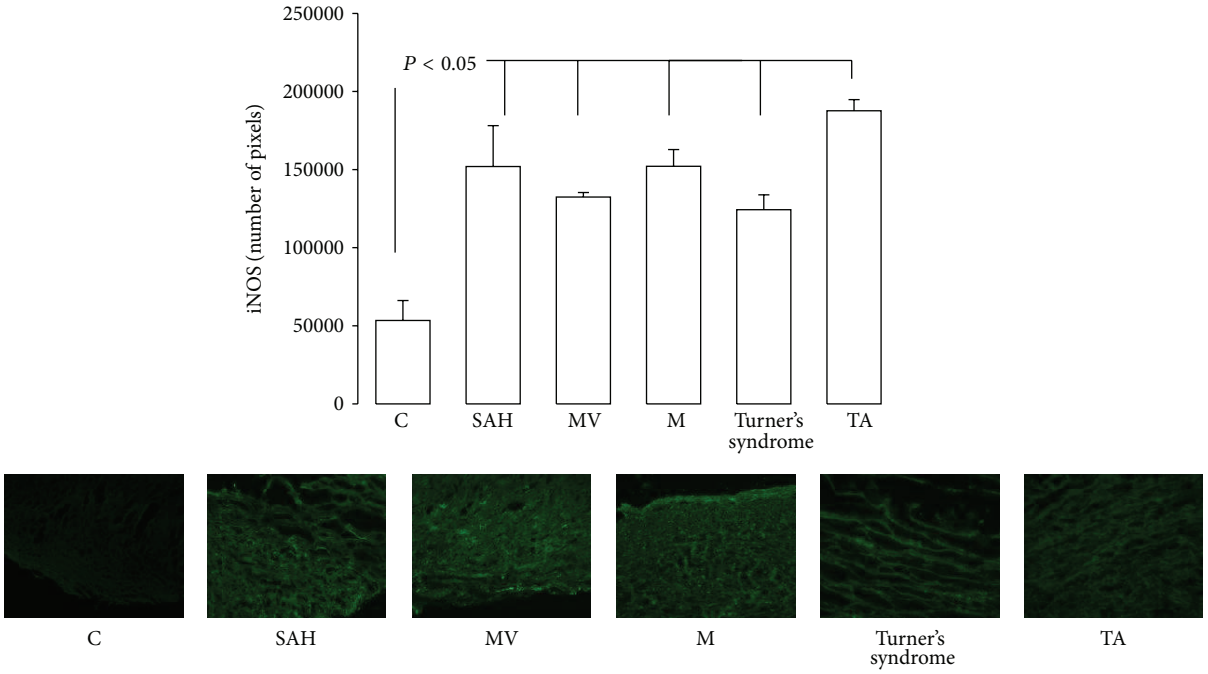

Figure 7: Immunofluorescence results and comparison of the iNOS expression in control subjects and in patients with the different pathologies studied. Pictures show the iNOS immunofluorescence with significant differences.

The enzyme GST conjugates GSH to electrophilic xenobiotics, chemicals, and toxic compounds like malondialdehyde which is an end product of the LPO process in phospholipids, leading to an increase in the rigidity of the cellular membrane, forming a thioether bond [82]. Patients with hypertension usually show a decrease in GST activity [83]. Our results show a decrease in GST activity in SAH, MV, M, Turner's syndrome, and TA in comparison to C subjects. A similar tendency was found in GPx activity. GPx detoxifies low levels of hydrogen peroxide with the help of GSH, causing its

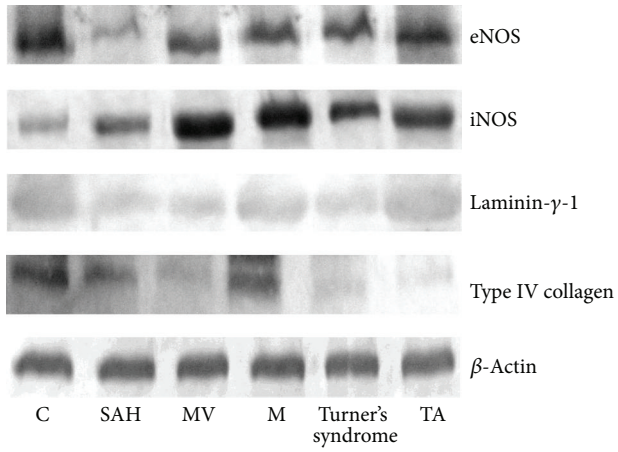

Figure 8: eNOS, iNOS, type IV collagen, and laminin-γ-1 expression in homogenized aorta. Abbreviations: SAH: systemic arterial hypertension, MV: variants of Marfan's syndrome, M: Marfan's syndrome, Turner's syndrome, and TA: Takayasu's arteritis.

oxidation [84]. Hypertension is associated with decreased activity of many antioxidant enzymes including GST and GPx.

GPx can also be inactivated in conditions of oxidative stress; O_2^- can inhibit the function of this enzyme [83]. These results suggest that the decrease in GST and GPx activity can be due to oxidative stress in these patients. In this study we did not analyze glutathione concentration, but the decrease in GPx and GST activity may be due in part to a decrease of glutathione and to the accumulation of ROS. When hypertension advances into stages II and III, even the defense of GPx might deteriorate because of the increased production of ROS [85].

LPO is increased in animal models, in serum from patients with atherosclerosis [86], and in TA. Our results show that LPO is increased in SAH, MV, M, Turner's syndrome, and TA, which is consistent with previous reports in the literature [87]. In essential hypertension and M an increase in the LPO levels in comparison to healthy subjects has been described [88, 89]. 8-iso-$PGF_{2\alpha}$, a marker of stress oxidative, also increases in TA [37]. Our results on MDA levels suggest that the pathologies studied show a different grade of oxidative stress with respect to C subject, modifying the activity of the antioxidant enzyme system.

The endothelium constitutively releases a number of vasoactive mediators including NO that regulate smooth muscle contractility and thus vascular smooth muscle tone and mechanical properties. Endothelial dysfunction and downregulated NO would contribute to the stiffness, the reduced distensibility, and the aortic complications that have been described in MV and M in mice models. Alteration of fibrillin-1 impairs the integrity of elastic fibers within the endothelial layer and endothelial permeability is impaired in M [90]. The reduction of NO production also decreases cGMP levels which act as downstream second messengers of NO signaling. In addition, MV and M are associated with elevated plasma levels of homocysteine, which attenuates endothelial function and limits NO bioavailability, production, and reduction. NO alterations increase the susceptibility to aortic complications [91]. Our results showed that SAH, MV, and M showed a decrease in eNOS expression. These results suggest that eNOS metabolism is decreased and that its participation is reduced in these pathologies. An explanation for the decline of eNOS is that the highly oxidative environment decreases its activity. However, our results show an increase of the NO_3^- and NO_2^- ratio, which are metabolites of NOS. The increase in NO_3^- and NO_2^- ratio may be due to inducible nitric oxide synthase (iNOS); this enzyme could be a mediator in some stages of the disease [92]. The immunofluorescence shows an increase of iNOS expression in all of the pathologies studied in comparison to control subjects. These results suggest that iNOS produces NO mainly during inflammatory processes and iNOS contributes significantly to the tissue NO_3^-/NO_2^- ratio. It may participate in protein matrix degradation and play a causal role in aneurysm formation [93]. iNOS is widely expressed in diverse cell types that are under transcriptional regulation by inflammatory mediators and has been implicated in the pathogenesis of many disorders including atherosclerosis, stroke, arthritis, and aneurisms [94].

In the pathologies studied, the presence of aneurisms contributes to endothelial dysfunction and the increase in the NO_3^- and NO_2^- ratio can favor the increase of peroxynitrites. In addition, altered NO bioavailability contributes to the modified vasomotion in hypertension [95] where an increased production of ROS is associated with an elevated production of peroxynitrite in coronary blood vessels. Endothelial dysfunction also promotes an increase in the generation of O_2^- leading to an enhanced NO inactivation against peroxynitrites [93]. Additionally, peroxynitrites impair NO production through oxidation of BH_4, a NOS cofactor [96]. Furthermore elevated peroxynitrites are associated with elevations in the myogenic tone, vasoconstriction, and deterioration of the endothelium-mediated relaxation [64].

Our results show significant changes in some proteins of the extracellular matrix such as laminin-γ-1 and type IV collagen. A decrease in these proteins could explain, at least in part, the deterioration of vascular mechanical function in these patients. Oxidative stress could deteriorate the endothelium and favor the synthesis of subendothelial proteins. In animal models, changes in the signaling pathways promote alterations such as proliferation, migration, and remodeling of the extracellular matrix in vascular smooth muscle cells having as a consequence an increase in vascular wall thickness, inflammation, and susceptibility to develop atherosclerosis. Some animal models have been proposed to explain the structural changes in vascular pathology [52]. However, it is not clear if these mechanisms operate in a similar way in human tissues [97].

Finally, this study has, as an important limitation, the use of aortas from patients with rare conditions. This renders impractical the monitoring of each patient prospectively for a long time. The retrospective study only allows the evaluation of some aspects but does not give the opportunity to correct

some biases. Prospective studies should be undertaken in a systematic way to evaluate several aspects such as the role of the oxidative stress, antioxidant therapy, and participation of factors in reducing aortic dilatation.

The relevance of this study lies in the presence of aneurysms and cardiovascular damage as an outcome in all of the conditions studied. It stimulates research interest in intervention maneuvers and preventive aspects. The study generates hypothesis from the genetic, environmental, and therapeutical points of view. The major limitation of this study is the small size of the aortic sample in diseases like MV, M, Turner's syndrome, and TA that occur with an incidence of 2-3 per 10000 individuals, being autosomal dominant disorders of the connective tissue caused by mutations. Furthermore, the causal associations between cellular and mechanical processes in the formation of aortic aneurysms have not been completely defined, so that a specific therapy has not been proposed. However, some studies have shown that simvastatin decreases free radicals, NF-κB, and improves the antioxidant condition [98]. In this study, patients with M had an increase in CAT that could be associated with laminin, but this could be due to the fact that they were receiving simvastatin. In some cases with TA, hypertension, and myocardial ischemic injury, LPO was increased and antioxidant enzymes decreased, suggesting an increase in the production of ROS. In this group none of the patients received simvastatin; this observation sets up a possible future therapeutic hypothesis in these cohorts.

5. Conclusions

These preliminary findings show similarities and differences in the role of oxidative stress in the pathologies studied. It is necessary to implement appropriate studies and methodological strategies to assess oxidative stress in each condition, as each pathogenesis can influence the cellular redox state. Prooxidant damage mechanisms seem to be specific and a common pathway for injury and aortic deterioration. Compensatory mechanisms, in chronic stages of aortic damage, are inversely related, since in the presence of LPO there is a low antioxidant activity. However, in early stages, prooxidant and antioxidant agents seem to develop in parallel, as a response to the imbalance. In these pathologies, whose ultimate damage is the aorta, therapeutic maneuvers acting upon antioxidants should be started since diagnosis, independent of the cause of aortic damage. The cohort design, retrospective and prospective, should be appropriate for each group.

Abbreviations

CAT: Catalase
SOD: Superoxide dismutase
GPx: Glutathione peroxidase
GST: Glutathione S-transferase
ROS: Reactive oxygen species
eNOS: Endothelial nitric oxide synthese
iNOS: Inducible nitric oxide synthese
LPO: Lipoperoxidation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Higher Plasma Malondialdehyde Concentrations Are Determined by Metabolic Syndrome-Related Glucolipotoxicity

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This study aimed to elucidate the determinants of higher plasma malondialdehyde (MDA) in free-living adults. In a cross-sectional study we evaluated 148 free-living subjects (54 ± 11 years, 78% women) at high risk for or with metabolic syndrome (MetS). They were assessed by anthropometry and body composition, dietary intake, and clinical and laboratorial analysis. The analysis of plasma MDA was performed by HPLC, and concentration values were used to provide four groups according to percentile distribution. Subjects with higher plasma MDA showed higher prevalence of MetS and higher values of waist circumference (WC), glucose, triglycerides (TG), γ-glutamyltransferase (γ-GT), and higher energy intake. Multiadjusted logistic regression analysis identified as determinants of higher plasma MDA the altered values of WC and γ-GT followed by hypertriglyceridemia, hyperglycemia, insulin resistance, higher dietary sugar-intake, and presence of MetS. In conclusion, the glucolipotoxic state predisposed by the presence of MetS seems to be the major determinant of higher plasma MDA concentrations.

1. Introduction

Lipid peroxidation (LPO) is a phenomenon where instable molecules are responsible for oxidizing lipids, proteins, and nucleic acids, resulting in cell malfunctions with generalized responses [1]. LPO has been characterized as a natural process of lipid degradation. In cell membranes, LPO begins when electrons from lipids are kidnapped by unstable free radicals promoting a chain reaction with successive oxidations that results in lipid instability and formation of by-products such as malondialdehyde (MDA) [2]. MDA is formed by enzymatic and/or free-radical peroxidation of PUFAs like arachidonic acid and docosahexaenoic acid by cleavage of its double bounds and releasing bis-aldehyde malonaldehyde [3]. The presence of factors accelerating free-radical production and loss or failure in neutralizing damaging processes (antioxidants) characterizes oxidative stress.

Several factors are associated with oxidative imbalance in the human organism. Among them, behavioral (e.g., smoking, nutrition, and exercise) and pathological (e.g., metabolic syndrome, type 2 diabetes, and dyslipidemia) factors can be pointed out. Epidemiological evidences have shown associations between dietary sugar-intake and increased risk for developing metabolic syndrome [4], type 2 diabetes [5], obesity [5, 6], and body adiposity [7], and the pathophysiology of these complications includes ectopic fat deposition with glucotoxic and lipotoxic actions [8]. To our knowledge there are few studies evaluating direct effects on LPO accessed by plasma MDA concentrations in humans. Plasma MDA can be easily assessed in large-scale people groups. Thus, knowledge of the interaction between behavioral and pathological processes in the initiation of lipoperoxidative activity can generate important tools for the prevention of pathological processes derived from lifestyle. So, this study aimed to elucidate the determinants of the higher plasma

MDA concentrations in free-living adults at high risk for or with MetS.

2. Methods

2.1. Study Design and Subjects. The subjects were beginners at the Botucatu Longitudinal Study (BLS) on Healthy Lifestyle Promotion Program called "Move for Health" as primary care for chronic noncommunicable diseases. This program is conducted by multidisciplinary staff from Center for Nutritional and Exercise Metabolism (CeMENutri) at UNESP Medical School (Botucatu, SP, Brazil). In this cross-sectional study with convenience sample, 541 adults were admitted to the program and 278 subjects were eligible for the study. The inclusion criteria were 35 years old or older and at high risk for (or presenting) MetS, without history of complications from cardiovascular, hepatic, renal, inflammatory, and autoimmune diseases or cerebral stroke. We excluded subjects who did not complete all assessments and those using vitamin supplements, inflammatory drugs, and chronic alcoholics. One hundred and thirty subjects did not achieve inclusion criteria or were excluded, so 148 subjects were included in this study. Written informed consent was obtained from all subjects and this study was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human subjects were approved by the Ethics Committee of the Botucatu School of Medicine (Of 557/2011).

2.2. Assessments. During medical evaluation subjects were screened for chronic diseases and submitted to assessments of physical activity readiness (PAR-Q). The presences of diseases or clinical history that would preclude the subjects' participation in the study according to inclusion and exclusion criteria were also taken. Also, at this time smoking status was self-reported by the subjects and the measures of systolic and diastolic blood pressures were made using the auscultatory method. Cardiorespiratory fitness was assessed estimating maximal oxygen consumption (VO_{2max}) by an equation proposed by the American College of Sports Medicine. This equation considers the total time of maximal treadmill test using the Balke protocol [9].

Body weight and height were obtained for subsequent Body Mass Index (BMI) calculation. Waist circumference (WC) was measured using a millimeter metal tape according to WHO recommendations. Body fat percentages and lean mass were estimated by equations considering electric resistance and reactance of the body provided by a bioelectric impedance device (Biodynamics, model 450, USA). Muscle mass in kilograms was estimated using the equation proposed by Janssen et al. [10], and these values were used to calculate the Muscle Mass Index in kg/m^2.

Subjects were submitted to nutritional history through 24-hour recall. Dietary data obtained in household measures were converted to grams and milliliters to enable chemical analysis of food consumption. Subsequently, data were processed in a nutritional analysis program (NutWin, Support Program for Nutrition, version 1.5, UNIFESP, 2002). To assess the dietary quality we used the adapted Healthy Eating Index (HEI), compiled from the American HEI [11]. This index assesses the quality of the diet by assigning points according to the individual food intake [12].

Blood samples were obtained after overnight fasting by vacuum venipuncture. Laboratory analysis of lipid parameters (total and HDL-cholesterol and triglycerides), glucose, uric acid, and γ-glutamyltransferase (γ-GT) was performed within 4 hours after blood collection by dry chemistry method (Vitros 5600, Ortho Clinical Diagnostics, Johnson & Johnson Company, Raritan, NJ, USA). The LDL-cholesterol concentrations were estimated using the formula proposed by Friedewald. Serum concentrations of insulin were quantified by a chemiluminescent method (Immulite 2000, Siemens Healthcare Diagnostics, Marburg, Germany) and used for subsequent calculation of the Insulin Resistance Index HOMA-IR. Serum C-reactive protein (CRP) concentrations were measured by a high-sensitivity immunonephelometric assay (Siemens Healthcare Diagnostics, Marburg, Germany). Plasma MDA concentrations were performed by high performance liquid chromatography with fluorometric detection (HPLC, system LC10A, Shimadzu, Japan) as previously described [13].

The criteria used for MetS diagnosis were described by the American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement [14].

2.3. Statistical Analysis. The statistical analysis was conducted with the statistical analysis software (SAS version 9.1.3, SAS Institute, USA), and the α of significance was set at 5% ($P < 0.05$). Initially, the normality of the data using the Kolmogorov-Smirnov test was tested. Data are presented as mean ± standard deviation (parametric variables) or median and interquartile range (nonparametric variables). The percentile values p25 (0.593 μmol/L), p50 (0.857 μmol/L), and p75 (1.058 μmol/L) of plasma MDA were used to obtain four groups: very low MDA (those with plasma MDA < 0.593 μmol/L), low MDA (those with plasma MDA between 0.593 and 0.856 μmol/L), increased MDA (plasma MDA between 0.857 and 1.057 μmol/L), and higher MDA (plasma MDA > 1.057 μmol/L). The comparison among groups was performed by one-way ANOVA for parametric variables and Kruskal-Wallis ANOVA for nonparametric variables, both with Dunn's post hoc test when significant. The predictors for elevating plasma MDA in the presence of alterations were performed by multiadjusted logistic regression models (odds ratio (OR) with 95% confidence interval (CI)). For this analysis, all parameters were categorized and alterations were set as reference values according to age and gender. Adjustment models were also performed including age, gender, smoking status, medicine use, energy intake, and BMI.

3. Results

The sample was predominantly characterized by females (78%), aged 35 to 65 years old (70%), nonsmokers (76%), and overweight or obese (80%). The prevalence of MetS in this sample was 34%. Some subjects showed dyslipidemia

Table 1: The assessed biomarkers according to groups of plasma malondialdehyde (MDA).

	MDA groups			
	Very low (<0.593 μmol/L) $n = 37$	Low (0.593–0.856 μmol/L) $n = 37$	Increased (0.857–1.057 μmol/L) $n = 37$	Higher (>1.058 μmol/L) $n = 37$
Anthropometry and body composition				
Body Mass Index (kg/m^2)	29.6 ± 5.9	30.2 ± 5.6	30.9 ± 6.4	30.8 ± 4.6
Muscle Mass Index (kg/m^2)	8.1 ± 1.4	8.5 ± 1.5	8.5 ± 1.7	8.4 ± 1.4
Waist circumference (cm)	95.6 ± 13.8	94.0 ± 15.3	96.8 ± 14.6	102.5 ± 13.2*
Body fat (%)	32.1 (27.2–44.7)	32.3 (29.0–42.9)	32.3 (30.0–45.0)	37.0 (30.6–45.0)
Blood pressures and fitness				
Systolic BP (mmHg)	129 ± 21	127 ± 18	126 ± 14	126 ± 17
Diastolic BP (mmHg)	79 ± 10	79 ± 10	78 ± 11	80 ± 10
VO_{2max} (mL/kg/min)	30.3 ± 7.8	29.1 ± 6.1	29.0 ± 5.8	26.6 ± 5.2
Dietary intake and quality				
Variety (items)	11.8 ± 3.8	13.0 ± 4.2	13.9 ± 3.7	13.3 ± 3.8
Energy intake (kcal)	1197 (892–1801)	1113 (974–1654)	1190 (982–1715)	1575 (1184–1955)*
Carbohydrates (%)	52.5 ± 10.4	51.5 ± 9.0	54.2 ± 9.3	55.0 ± 11.2
Sugar (servings)	0.5 (0.0–1.8)	0.5 (0.0–2.0)	0.7 (0.0–2.0)	1.0 (0.2–2.5)
Proteins (%)	17.9 ± 6.5	16.4 ± 5.6	17.4 ± 5.0	18.7 ± 5.1
Total fat (%)	29.6 ± 9.1	30.7 ± 14.5	28.3 ± 9.1	30.0 ± 9.5
Saturated fat (%)	8.3 ± 3.4	8.6 ± 5.9	7.7 ± 3.0	7.9 ± 3.8
Monounsaturated fat (%)	9.8 ± 4.1	8.8 ± 4.4	7.9 ± 3.0	10.4 ± 12.9
Polyunsaturated fat (%)	7.9 ± 4.0	7.2 ± 2.9	7.0 ± 2.9	7.6 ± 3.3
Fibers (g)	13.7 (9.0–17.3)	15.0 (10.0–19.2)	13.3 (9.1–20.2)	15.0 (9.4–20.8)
HEI (points)	83.0 ± 13.6	79.6 ± 14.8	78.7 ± 16.0	77.5 ± 12.0
Blood markers				
Glucose (mg/dL)	98.9 ± 42.4	99.5 ± 29.3	97.1 ± 21.2	107.5 ± 31.6*
HOMA-IR	1.53 (0.91–4.08)	1.47 (1.08–4.01)	1.98 (1.35–3.19)	2.78 (1.33–4.50)
Total cholesterol (mg/dL)	198.9 ± 33.5	200.2 ± 39.3	186.0 ± 42.3	199.3 ± 37.2
HDL-cholesterol (mg/dL)	48.7 ± 11.3	47.6 ± 13.6	48.4 ± 11.5	46.8 ± 11.5
LDL-cholesterol (mg/dL)	122.8 ± 30.9	124.4 ± 36.0	107.7 ± 37.0	118.8 ± 30.9
Triglycerides (mg/dL)	127.0 (108.3–160.8)	133.5 (102.0–180.0)	134.0 (88.0–179.0)	152.0 (106.5–221.5)*
Uric acid (mg/dL)	4.7 ± 1.6	4.9 ± 1.9	4.9 ± 1.8	5.0 ± 1.4
γ-GT (U/L)	23.0 (16.3–38.5)	27.0 (15.8–49.3)	21.0 (17.0–33.0)	33.5 (22.5–46.0)*
C-reactive protein (mg/L)	2.6 (1.7–7.9)	3.0 (1.7–6.0)	3.3 (1.6–6.3)	3.5 (2.0–6.7)

*Different from other groups ($P < 0.05$).

(39%), hypertension (31%), and hyperglycemia (25%) and were under drug therapy (46%), which was considered when diagnosing MetS.

The higher plasma MDA group differed from other groups by showing higher values of WC, fasting blood glucose, TG, γ-GT, and energy intake. Total body fat, BMI, blood pressures, cardiorespiratory fitness, and cholesterol fractions were similar among groups (Table 1). Also, higher plasma MDA group was discriminated by showing higher and significant prevalence of MetS (50%) compared to other groups (29% in very low, 29% in low, and 38% in increased groups). Moreover, plasma MDA concentrations were significantly ($P < 0.01$) different between the presence (0.947 ± 0.339 μmol/L) and absence (0.803 ± 0.283 μmol/L) of MetS. Also, those with MetS showed higher dietary sugar-intake (0.83 [0.0–2.47] versus 0.5 [0.0–1.5], $P < 0.01$), higher WC measures (104.0 [93.5–110.9] versus 93.0 [85.0–104.0], $P < 0.001$), higher HOMA-IR (3.07 [1.63–5.77] versus 1.34 [0.89–2.32], $P < 0.001$) and higher TG (199.0 [148.0–248.5] versus 116.0 [88.3–139.8], $P < 0.001$), blood glucose (98.5 [90.0–125.0] versus 88.0 [82.0–94.8], $P < 0.001$), and γ-GT (33.0 [21.0–46.0] versus 23.0 [16.0–40.0], $P < 0.01$) concentrations than those without MetS.

The multiadjusted logistic regression analysis showed that MetS presence was identified as an independent predictor for higher plasma MDA concentrations (OR 2.07, CI 1.04 to 4.51). Likewise, alterations in MetS components such as WC (OR 2.94, CI 1.01 to 10.0), glucose (OR 2.46, CI 1.16 to 5.92), and TG (OR 2.20, CI 1.01 to 4.85) were also identified as predictors for higher plasma MDA (Figure 1(a)). BMI, muscle

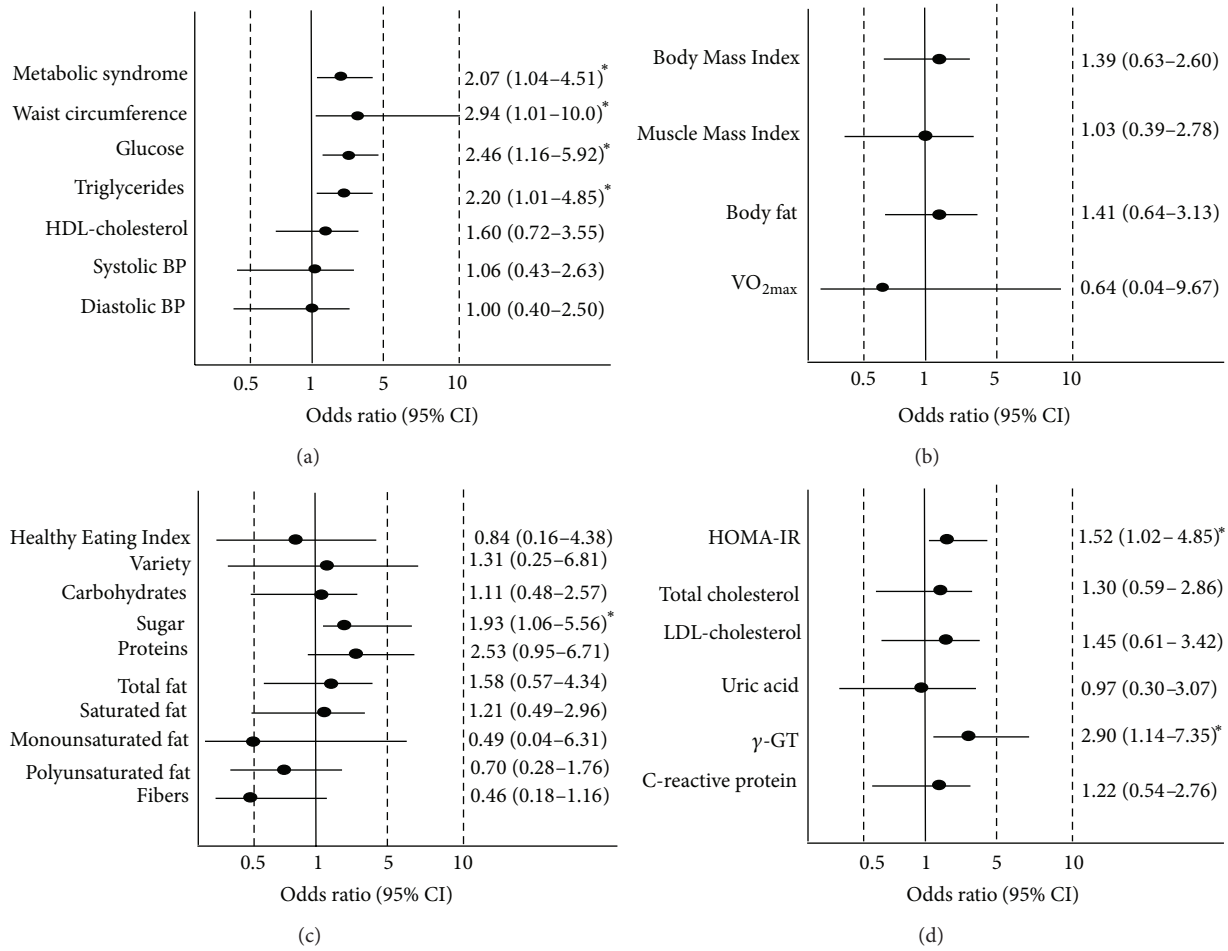

FIGURE 1: Logistic regression analysis identifying the main predictors for higher plasma MDA concentrations; (a) metabolic syndrome and its components; (b) anthropometry, body composition, and fitness; (c) dietary intake; (d) blood markers. Analyses were adjusted for age, gender, smoking status, medicine use, BMI, and energy intake. Black circles are odds ratio and traces are confidence interval. $^{*}P < 0.05$.

mass, and body fat showed no association with higher plasma MDA (Figure 1(b)); however, the higher values of HOMA-IR (OR 1.52, CI 1.02 to 4.85), γ-GT (OR 2.90, CI 1.14 to 7.35) (Figure 1(c)), and dietary sugar-intake (OR 1.93, CI 1.06 to 5.65) (Figure 1(d)) were also identified as predictors for higher plasma MDA concentrations.

4. Discussion

This study elucidated the major determinants of the higher plasma MDA concentrations in free-living adults at high risk for or with MetS. Altered values of WC and γ-GT were strongly associated with higher plasma MDA concentrations. Altered concentrations of TG and glucose, higher sugar/energy intake, insulin resistance, and the presence of MetS were also associated with higher plasma MDA concentrations. From the used plasma markers, blood glucose (and HOMA-IR), LDL-cholesterol, and TG are referred to as risk factors for lipoperoxidative activity with higher CRP (systemic inflammatory marker) and γ-GT (steatohepatitis marker) concentrations as its probably causes. On the other hand, higher plasma concentrations of uric acid are indicative of enhanced extracellular hydrosoluble antioxidant response whereas HDL-cholesterol presents both antioxidant and anti-inflammatory actions. From this point of view, these markers can be markedly influenced by lifestyle conditions like sedentary and inadequate nutrition.

Oxidative stress and chronic low-grade inflammation are common comorbidities of MetS. Age and gender showed no differences among plasma MDA groups whereas MetS prevalence was greater in subjects with higher plasma MDA concentrations. Increasing adiposity is determinant to the development of MetS with proinflammatory effects [15]. Hypertrophic adipocytes secrete cytokines (IL-6, TNF-α) and monocyte chemoattractants (MCP-1) and are characterized by macrophage infiltration generating global proinflammatory profile [16]. Additionally, macrophage activation leads to NADPH oxidase overexpression and activation, implicated in ROS production [17]. These ROS can oxidize the cell membrane lipids breaking their molecules with consequent increase in their plasma by-products. This proinflammatory

state would be in conjunction with the occurrence of oxidative stress [18]; however, no associations between C-reactive protein concentrations and plasma MDA among groups were observed.

This study showed an independent association between higher dietary sugar-intake and higher plasma MDA, suggesting that sugar-intake is directly involved in the generation of oxidative stress. High sugar-intake induces hyperglycemic peaks with subsequent hyperinsulinemia [19]. We observed that hyperglycemia and HOMA-IR were associated with higher plasma MDA concentrations even after adjusting for smoking and obesity. Hyperglycemia-induced oxidative stress is characterized by the presence of advanced glycation end-products (AGEs) [20]. AGEs can oxidize lipids in cell membranes leading them to instability and consequent degradation to LPO by-products [21]. Besides MDA is considered a limited marker to assess overall oxidative stress; the analysis of plasma MDA performed by HPLC with fluorometric detection is very sensitive and widely used in scientific research assessing LPO [22]. Therefore, exposure to hyperglycemia and insulin resistance may be decisive for the development of LPO.

In the present study, subjects with higher dietary sugar-intake in our sample were characterized by increased intake of sweetened beverages including soft drinks (like soda) or industrialized fruit juices and candies. In Brazil, the predominant sugar-sweetening of these products is sucrose. An elegant meta-analysis showed that higher consumption of sweetened beverages is closely related to higher risk for developing MetS and type 2 diabetes [4]. Elevated sugar/energy intake is a predisposing condition to MetS development due to increasing adiposity [23] and the link between high sugar/energy intake and metabolic abnormalities seems to be the ectopic fat deposition [24]. Although cardiorespiratory fitness was not associated with plasma MDA concentrations, combating sedentary lifestyle with physical activity and nutritional adequacy can prevent fat deposition and MetS development, with consequent impact over plasma markers of oxidative stress [25].

The hypertriglyceridemic waist phenotype is considered a more simple way to diagnosing metabolic complications and is closely related to the development of insulin resistance and liver steatosis [26]. We observed an independent association of the higher plasma MDA concentrations with the higher TG concentrations, and, more strongly, with elevated WC and γ-GT concentrations. Visceral ectopic fat deposition coexists with hypertriglyceridemia promoting intracellular lipotoxicity, especially in hepatocytes and muscle cells [8]. In hepatocytes, increased fatty acids supply does not essentially result in activation of β-oxidation [27]. Hepatocyte accumulation of esterified fatty acids constitutes a stressful stimulus that result in mitochondrial dysfunction with increased ROS production [28], and therefore becoming a crucial situation for liver injury that can be identified by serum γ-GT alterations. Elevations in γ-GT concentrations is also used to identify chronic alcohol consumption and is related to inflammatory markers [29], blood glucose [30], and metabolic syndrome [31]. In addition, elevations of γ-GT concentrations could be an indirect marker of antioxidant response to increased ROS production once γ-GT acts on the glutathione metabolism by regulating the oxidized glutathione clearance [32]. This would be possible explication for the strong associations between higher plasma MDA and higher γ-GT. However, the precise mechanism to explain this association remains unknown. Different phenotypes of MetS combining hyperadiposity, hyperglycemia and insulin resistance, dyslipidemia, and hypertension results in multifactorial responses such as the high risk for hepatic steatosis, type 2 diabetes, and cardiovascular diseases. Our results showed that hyperadiposity and dyslipidemia are main determinants of higher plasma MDA concentrations, but hyperglycemia and insulin resistance can also contribute to higher MDA concentrations, supporting the hypothesis that MetS-related glucolipotoxicity sets the raising of MDA concentrations in this population. Overall, the lipoperoxidation and MDA formation might be consequence of dysfunctional glycated proteins, AGEs, and glycooxidative stress glycol-oxidative stress (hyperglycemic glycotoxicity) and consequence of lipotoxicity when lipid is forced into organ cells (e.g., liver, skeletal, and heart muscle and pancreas) significantly impairing functions. MetS is a model of metabolism homeostasis breakdown presenting glucolipotoxicity along with abnormalities in blood lipids, glucose, blood pressure, coagulation, and inflammation [33].

Some limitations must be explicit. Antioxidants such as uric acid and plasma HDL-C in the current study did not offer protection against LPO, suggesting that antioxidant protection is important at the time and place of lipoxidation occurrence. So a limitation was the lack of measurement of some fat-soluble vitamins involved in the protection of cell membranes. The practice of physical activity (which is also related to combating inactivity) is known as an important inducer of antioxidant capacity [34]. However, our study subjects were classified as sedentary, the reason that cardiorespiratory fitness values were not used in the adjustment models. Finally, the association found between higher MDA concentrations and the presence of MetS is arithmetical without any causal understanding once this is a cross-sectional approach. Intervention studies focusing on MDA-formation inhibitors must be considered for further investigation in MetS.

5. Conclusion

Elevated central adiposity (WC) and γ-GT concentrations were the main determinants of the higher plasma MDA concentrations. Hyperglycemia, insulin resistance, hypertriglyceridemia, and higher sugar-intake were also associated with higher plasma MDA concentrations. These markers are directly related to the development of the glucolipotoxic states predisposed by the presence of MetS and seem to be the major determinant of plasma MDA concentration in this pathologic condition. Lifestyle modifications are indicated to these subjects in order to reduce MetS and its comorbidities developments; however, the benefits on higher plasma MDA concentrations are still unknown and it is possible that they will follow modulations on glucolipotoxic states.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Serum Oxidative Stress Markers and Lipidomic Profile to Detect NASH Patients Responsive to an Antioxidant Treatment: A Pilot Study

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Liver steatosis can evolve to steatohepatitis (NASH) through a series of biochemical steps related to oxidative stress in hepatocytes. Antioxidants, such as silybin, have been proposed as a treatment of patients with nonalcoholic fatty liver disease (NAFLD) and NASH. In this study, we evaluated, in patients with histologically documented NASH, the oxidant/antioxidant status and lipid "fingerprint" in the serum of NASH patients, both in basal conditions and after 12 months of treatment with silybin-based food integrator Realsil (RA). The oxidant/antioxidant status analysis showed the presence of a group of patients with higher basal severity of disease (NAS scores 4.67 ± 2.5) and a second group corresponding to borderline NASH (NAS scores $= 3.8 \pm 1.5$). The chronic treatment with RA changed the NAS score in both groups that reached the statistical significance only in group 2, in which there was also a significant decrease of serum lipid peroxidation. The lipidomic profile showed a lipid composition similar to that of healthy subjects with a restoration of the values of free cholesterol, lysoPC, SM, and PC only in group 2 of patients after treatment with RA. *Conclusion.* These data suggest that lipidomic and/or oxidative status of serum from patients with NASH could be useful as prognostic markers of response to an antioxidant treatment.

1. Introduction

It is well known that nonalcoholic fatty liver disease (NAFLD) is manifested by a "metabolic" chronic liver damage due to an impaired "traffic" of lipids among adipose tissue, muscle, gut, and liver [1].

The occurrence of NAFLD is associated with numerous changes in the lipid composition of the liver [2] and the shift of these patients towards definitive steatohepatitis (NASH) is associated with changes in plasma lipidomic profile [3]. The clinical importance of NASH is related to its capacity to evolve in liver cirrhosis and cancer [4]. The principal risk factor for the development of NASH is insulin resistance [5–7] that increases lipolysis and releases free fatty acids (FFA) causing liver injury [8–10] by excessive liver lipid accumulation. Oversupply of free fatty acids induces an increase in mitochondrial H_2O_2 production that, in turn, oxidizes mitochondrial membranes and regulates activity of uncoupling protein 2 and carnitine palmitoyl transferase 1 [11]. Mitochondria play a key role in hepatocyte metabolism,

being the site of β-oxidation and oxidative phosphorylation. Using a metabolomic approach, it has recently been shown that NASH is also characterized by decreased phosphatidylcholine (PC) and altered n3 and n6 polyunsaturated fatty acid (PUFA) metabolism [2, 3, 12]. Importantly, the levels of arachidonic acid (20:4 n6), the precursor of many biologically active eicosanoids, appear to be depleted [3]. It is not known if these changes can cause a variation in the circulating lipidome and if NASH can be, consequently, associated with a distinct lipidomic signature. Presently, there is no proven treatment for NASH and the introduction of drugs directly able to reduce oxidative stress, in association with lowering lipid accumulation, could be important in the control of these disorders. Silybin is a natural flavonoid and the main component of silymarin. Its derivative Realsil (RA) is a compound in which silybin is conjugated with phosphatidylcholine (PC) and vitamin E to enhance its intestinal absorption and its consequent bioavailability together with antioxidant and antifibrotic activity [13]. Silybin has a marked antioxidant activity both *in vitro* and *in vivo,* thus regulating glucose homeostasis in hepatocytes [13–15]. We have recently reported that a chronic treatment (for 12 months) with a dietary supplement of RA given orally twice a day significantly improves both liver damage plasma marker levels (AST, ALT, and γGT) and liver histology in about 50% of patients with NAFLD and NASH [16]. In this study, we also observed that, despite the fact that no significant changes were observed in the global population for both dietetic regimen and body composition, in patients treated with RA, about 15% had a reduction of BMI values and 35% a reduction of blood glucose and HOMA test (marker of insulin resistance).

In this retrospective study, we addressed the effects of the chronic treatment with RA on both oxidative stress plasma markers and lipidomic profile in patients with NASH. Moreover, we have also evaluated the *in vitro* effects induced by sera from NASH patients on lipid accumulation in hepatoblastoma HepG2 cells.

2. Subjects and Methods

The study was performed after approval by the Ethic Committee according to Helsinki Declaration. The trial was registered with the European Clinical Trials Database (EudraCT, reference 2005-000860-24). We selected for our purpose frozen serum at −80° of 30 patients with histological documented NASH according to literature data [17] and treated for 12 consecutive months with Realsil (IBI-Lorenzini, Italy, RA) (active components: silybin 94 mg, phosphatidyl choline 194 mg, and vitamin E acetate 50% (α-tocopherol 30 mg) 89.28 mg) orally twice daily. Baseline clinical characteristics of the study population are summarized in Table 1. The histological diagnosis was established using H&E and Masson trichrome stains of formalin-fixed paraffin-embedded liver and graded in a blinded fashion according to the NAFLD scoring system proposed by the National Institute of Diabetes and Digestive and Kidney Diseases NASH Clinical Research Network. A NAFLD activity score (NAS) ≥5 corresponded to a diagnosis of "definitive NASH", a score of 3-4 corresponded to "borderline NASH", and a score of <3 corresponded to "simple steatosis" [16, 18].

Table 1: Main findings of patients with NASH and controls.

	NASH patients	Control subjects
Number	30	10
Age (yr)	40.8 ± 10.3	40 ± 12
Gender (M/F)	15/15	7/3
BMI (kg/m^2)	29.9 ± 4.6	25.1 ± 2
Obesity	6/30	0
Diabetes mellitus	4/30	0
Hypercholesterolemia	4/30	0
Hypertriglyceridemia	3/30	0

2.1. Extraction of Serum Lipid and MALDI-TOF MS Analysis. Phospholipids were extracted in chloroform-methanol according to Bligh and Dye [19]. Methanol-chloroform (2 : 1 v/v; 800 μL) was added to the serum (200 μL). Phase separation is induced by adding 200 μL of water. The mixture was centrifuged at 1000 g for 10 min. The upper phase was discarded and the lower chloroform phase was evaporated to dryness under a stream of nitrogen. The lipids were dissolved in 100 μL of chloroform. A 2 μL aliquot was used for MALDI-TOF MS determination. MALDI-TOF MS experiments were carried out by loading lipid mixtures (1 mL from a solution 0.02 mg/mL in H_2O/0.1% v/v TFA) on the stainless steel target together with 1 μL of matrix 2,5-dihydroxybenzoic acid (10 mg in 1 mL MetOH/0.1% v/v TFA). Spectra were acquired on a PerSeptive Biosystems (Framingham, MA, USA) Voyager DE-PRO mass spectrometer, equipped with a N_2 laser (337 nm, 3 ns pulse width) operating either in linear or in reflector positive ion mode, using the delayed extraction technology. In the analysis of lipids, laser power was maintained at the lowest possible values in order to prevent insource fragmentation. To check repeatability, spectra were acquired in triplicate at least.

2.2. Thiobarbituric Acid-Reactive Species (TBARS) Levels. Samples were incubated with 0.5 mL of 20% acetic acid, pH 3.5, and 0.5 mL of 0.78% aqueous solution of thiobarbituric acid. After heating at 95°C for 45 minutes, the samples were centrifuged at 4000 r.p.m. for 5 minutes. In the supernatant fractions TBARS were quantified by spectrophotometry at 532 nm [20]. Results were expressed as TBARS μM/μg of serum protein. Each data point is the average of triplicate measurements, with each individual experiment performed in duplicate.

2.3. Nitrite Levels. NO is rapidly converted into the stable end products nitrite and nitrate. Nitrite was measured by the Griess reaction as reported in literature [21]. Briefly, 10 μL of serum was mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 2.5% H_3PO_4, and 0.05% naphthylethylene diamine in H_2O) and incubated for 10 min at room temperature. Absorbance was assayed at 550 nm and

compared with a standard curve obtained using sodium nitrite.

2.4. Catalase Activity. Catalase (CAT) activity was measured using Catalase Assay Kit (Cayman Chemical Ann Arbor, MI) according to the manufacturer's protocol. Each data point was performed in triplicate, and the results were reported as mean absorption ± standard deviation.

2.5. Superoxide Dismutase (SOD) Activity. Activity of superoxide dismutase (SOD) was measured with a superoxide dismutase assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol [22, 23]. Each data point was performed in triplicate, and the results were reported as mean absorption ± standard deviation.

2.6. Treatment of the HepG2 Cells with Sera from NASH Patients. The histological definition of steatosis is the visible accumulation of lipid droplets in more than 5% of hepatocytes. To determine if the serum of patients NASH may induce steatosis, HepG2 cells were cultured for 72 hours with pools sera of groups 1 and 2 of patients. We used both sera from T0 and T12 times. Oil Red O (ORO) methods were utilized for detecting intracellular lipids.

2.7. Statistical Analysis. Values are expressed as the mean ± SE. The significance of the difference between the control and each experimental test condition was analysed by unpaired Student's t-test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of Serum Oxidative Stress Markers and Metabolic Parameters in NASH Patients in Basal Conditions and after the Chronic Treatment with RA. Although we do not find any significant difference between NASH patients and controls as mean values, due to both high interindividual variability and sample size, the individual analysis of oxidative stress markers (TBARS and NO) and antioxidant enzyme activities (SOD and CAT) showed the presence of two distinct groups of patients. In the first group (group 1) of NASH patients (11/30), we found very low levels of TBARS if compared to those of healthy controls. In this group, the treatment with RA significantly (about 5-fold, $P < 0.0001$) increased mean serum levels of both TBARS and NO that overcame the mean values recorded in healthy subjects ($P < 0.0001$). The second group (group 2, 19/30) of NASH patients presented very high mean basal (T0) values of TBARS if compared to those of healthy subjects; in these patients the treatment with RA significantly decreased the TBARS mean values (2-fold, $P < 0.0001$), while NO mean values were almost unaffected by the pharmacological treatment (Table 2). In Table 2, we also reported both superoxide dismutase (SOD) and catalase (CAT) activity in the two previously defined groups before and after 12 months of RA treatment. In group 1 a significant decrease ($P = 0.01$) of mean values of CAT activity was found if compared to those of normal subjects, while in group 2 a significant increase of mean values of SOD activity ($P = 0.01$) was recorded. A separate system of scoring the features of NAFLD, called NAFLD activity score (NAS), was developed as a tool to measure changes in NAFLD during the therapeutic trials [18]. Interestingly, group 1 presented higher NAS scores (4.67±2.5) when compared to that of group 2 (3.8 ± 1.5) indicating a higher basal severity of the disease. The chronic treatment with RA changed the NAS score in both groups (Table 3); in group 1 we observed a nonsignificant decrease (NAS = 3.6 ± 1.0, $P = 0.057$) while was significantly decreased in group 2 (NAS = 2.5 ± 0.51, $P = 0.0058$). NASH has been reported to be a component of the so-called "metabolic syndrome," that is, a cluster of closely associated abnormalities related to the insulin-resistant phenotype [24]. In group 1 the mean BMI, insulin, and HOMA values did not significantly change after the treatment ($P > 0.05$), while mean glucose blood concentration significantly decreased ($P = 0.05$). On the other hand, in group 2 mean BMI ($P = 0.005$), insulin, and HOMA values significantly decreased ($P = 0.001$), while the concentration of glucose did not change after the pharmacological treatment.

3.2. Lipidomic Profile of Controls and NASH Patients before and after the Chronic Treatment with RA. The sera of each group of subjects enrolled in the study (healthy subjects and groups 1 and 2 of patients) were separately pooled for the analysis of lipidomic profile by positive ion MALDI-TOF MS. In Figure 1, the positive ion MALDI-TOF mass spectra of the organic lipid extracts from sera of healthy subjects (CTR), group 1, and group 2 of NASH patients at T0 and after the chronic administration of RA (T12) are shown. The level of lysophosphatidylcholine (lysoPC) within palmitic acid (m/z = 496,36) was not significantly different among the three studied groups. However, the lipid species as free cholesterol, sphingomyelins (SM), and PCs in NASH patients at T0 (group 1 and group 2) were basally decreased if compared to those of healthy subjects. Only the NASH patients of group 2 after 12 months of RA treatment (T12) showed a lipid profile similar to that of healthy subjects with a restoration of the values of free cholesterol, lysoPC, SM, and PC. In Table 4 the identification and quantification of major plasma circulating lipids in healthy subjects, group 1, and group 2 at T0 and T12 are reported. The peaks with m/z between 520 and 524 were identified as LysoPC within linoleic (18:2), oleic (18:1), and stearic acid (18:0). In the healthy subjects, the three classes of lipids were uniformly represented, while both groups of NASH patients showed a major percentage of lysoPC within stearic acid (18:0) if compared to LysoPC 18:1 and 18:2, even after the treatment with RA. The ratio between PC percentage of healthy subject and that of NASH patients at T12 was reported in Figure 2. Only in Group 2 the ratio was about 1 after the chronic administration of RA. These findings may be due to an increase of both Δ 9 stearoyl-coA desaturase (SCD) and elongase activity. Importantly, in the NASH patients the levels of PC with arachidonic acid (Table 4), the precursors of many biologically active eicosanoids, were very low, but they

Table 2: Serum TBARS, NO levels, and SOD and Catalase enzyme activities in two groups of NASH patients basal conditions (T0) and after 12 months of treatment with RA (T12).

Parameters	Group1		Group2		
	T0	T12	T0	T12	CTR
TBARS (μM/μg prot.)	0.0044 ± 0.0003	0.021 ± 0.001	0.074 ± 0.006	0.047 ± 0.004	0.01 ± 0.002
NO (nmol/μg prot.)	0.011 ± 0.003	0.025 ± 0.003	0.135 ± 0.03	0.13 ± 0.027	0.0002 ± 0.00001
SOD activity (U/ng prot.)	0.121 ± 0.03	0.103 ± 0.02	0.159 ± 0.02	0.32 ± 0.056	0.15 ± 0.06
CAT activity (nmol/ng prot.)	1.3 ± 0.3	0.92 ± 0.08	1.5 ± 0.38	1.33 ± 0.185	1.5 ± 0.2

TBARS: thiobarbituric acid-reacting substances; NO: nitric oxide; SOD: superoxide dismutase; CAT: catalase.

Table 3: Metabolic data in two groups of NASH patients basal conditions (T0) and after 12 months of treatment with RA (T12).

Parameters	Group 1 T0	Group 1 T12	% variation	P	Group 2 T0	Group 2 T12	% variation	P
BMI	30 ± 1.86	30 ± 1.8	0	ns	28 ± 0.50	26 ± 0.50	9	0.005
Glucose	116 ± 10	105 ± 8.2	−10	0.05	99 ± 2.15	99 ± 2	0	ns
Insulin	18 ± 2.26	17 ± 3.77	−8	ns	23 ± 4.34	14 ± 1.9	−40	0.001
HOMA	5 ± 1	4.5 ± 1.0	−11	ns	5.97 ± 0.6	3.43 ± 0.5	−42	0.001
AST	40 ± 19	27 ± 7	−33	0.05	72 ± 31	41 ± 14	−42	0.01
ALT	40 ± 17	35 ± 10	−14	ns	72 ± 39	50 ± 9	−31	0.05
GGT	67 ± 31	43 ± 12	−35	0.05	101 ± 81	84 ± 75	−16	ns
Steatosis score	1.86 ± 0.90	1.8 ± 0.7	−0.01	ns	1.8 ± 0.8	1.2 ± 0.6	−33	0.01
NAS score	4.67 ± 1.5	3.6 ± 1.15	−29	ns	3.8 ± 1.5	2.5 ± 0.51	−70	0.001
Portal infiltration	1.33 ± 0.8	1.0 ± 0.2	−25	ns	1.2 ± 0.7	0.5 ± 0.3	−58	0.001
Fibrosis	1.35 ± 0.8	0.67 ± 0.5	−50	0.01	1.2 ± 0.7	0.5 ± 0.3	−60	0.001

increased only in group 2 at T12. RA treatment induced in both groups an increase of PC 18:0/20:3, with a partial restoration of their levels to those of healthy subjects.

3.3. In Vitro Effects of Serum from NASH Patients on Lipid Accumulation in HepG2 Cells. The histological definition of steatosis is the visible accumulation of lipid droplets in more than 5% of hepatocytes. To determine if the serum of NASH patients may induce steatosis and if RA can be involved in lipid cell accumulation, HepG2 cells were cultured for 72 hours with pooled sera from group 1 or 2 at T0 and T12 or from healthy subjects. Oil Red O (ORO) method was used for the detection of intracellular lipids (Figure 3). ORO staining microscopy revealed lipid droplets accumulation in the cytoplasm of HepG2 cells after treatment with sera (groups 1 and 2) at T0 and a decrease of intracellular lipid only in the cells incubated with group 2 serum at T12 (Figures 3(a) and 3(b)). No changes were recorded in the cells exposed to the sera from healthy subjects (data not shown). In order to quantitatively assess lipid accumulation in HepG2 cells, we performed ORO colorimetric assay [25]. In Figure 3 panel (c), the quantitative ORO colorimetric assay on HepG2 cells after 72 h of incubation with NASH sera from groups 1 and 2 at T0 and T12 is shown. The effects of the sera of both groups 1 and 2 at T0 determined an about 2.5-fold increase of the lipid droplets if compared to those of untreated HepG2 cells ($P < 0.001$). The sera of group 2 NASH patients after treatment with RA (T12) induced an about 40% significant decrease of lipids accumulation if compared to that of HepG2 treated with the sera from T0 ($P < 0.001$).

4. Discussion

The diagnosis of NASH is defined by the presence of specific histological abnormalities determined at liver biopsy. Therefore, in all studies and trials on NAFLD, liver histology is the gold standard for the evaluation of response to treatments [17]. Serum markers of lipid peroxidation are generally used to evaluate the "oxidative stress" status *in vivo* in patients with NASH. The data of the present study suggest that, despite apparently similar clinical, biochemical, and histological characteristics that were found in all patients, two distinct groups of patients can be detected according to the modification of parameters of oxidative stress and lipid profiling. These two groups of patients have also a different sensitivity to the treatment with RA. Group 1 was characterized by lower lipid peroxidation as evaluated by TBARS assay, not due to increased SOD and CAT activity, while group 2 showed higher values of TBARS again with normal activity values of the scavenger enzymes. Insulin resistance (IR) was a common feature of both groups. Moreover, group 1 presented higher basal histological score (4.67 ± 0.5) corresponding to a greater severity of disease, while group 2 had a NAS score of 3.8 ± 0.6 corresponding to borderline NASH [17]. The excessive liver lipid accumulation in the pathogenesis of NASH can result from one or a combination of the following metabolic alterations: (i) decreased β-oxidation of fatty acids; (ii) increased fatty acid synthesis due to upregulation of lipogenic pathway; (iii) increased delivery of fatty acids from adipose and other organs due to lipolysis associated with peripheral insulin resistance (IR) and inhibition of VLDL-triglyceride [16].

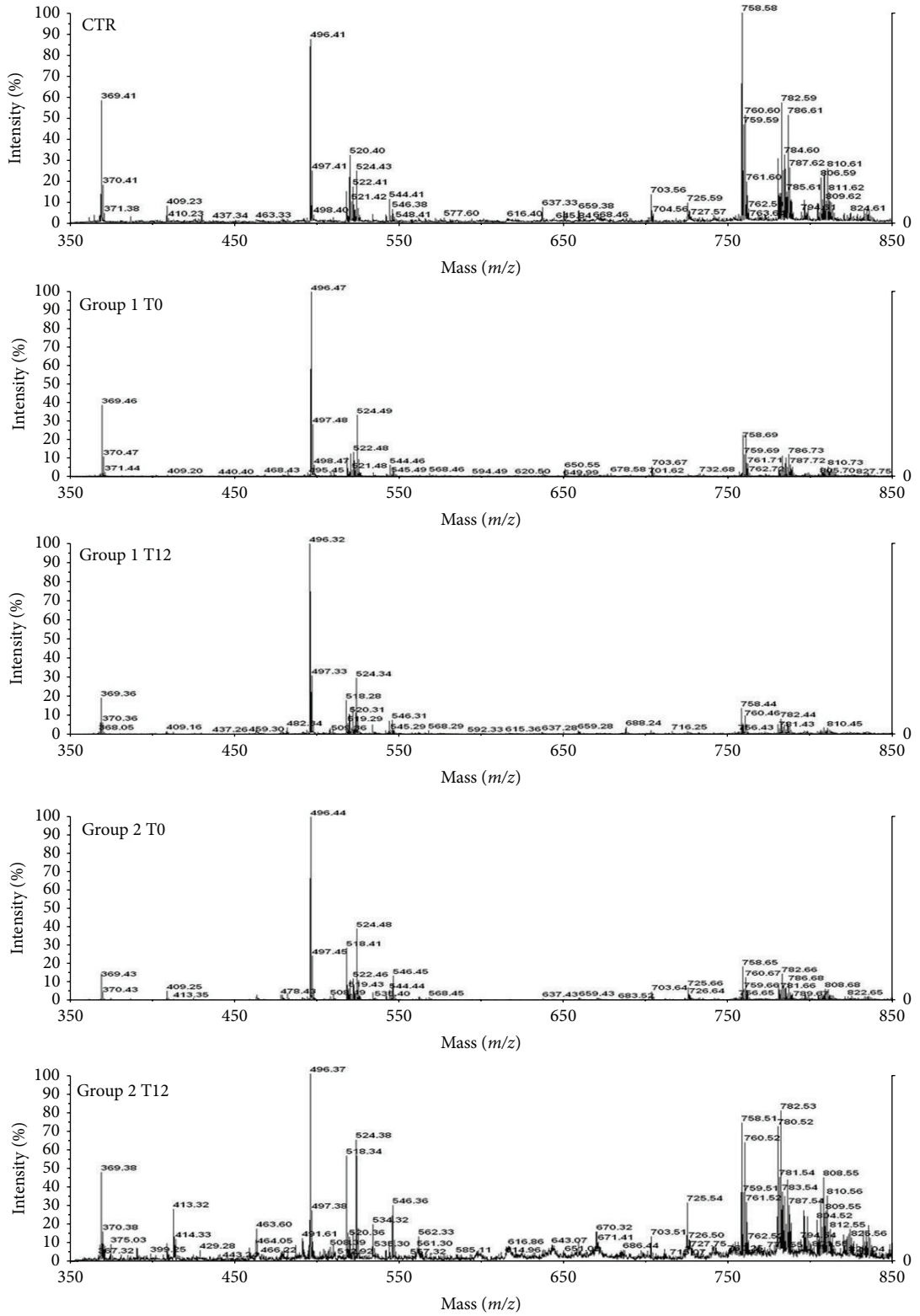

Figure 1: Positive ion MALDI-TOF MS mass spectra of choline phospholipid molecular species in lipid extracts from healthy individuals (CTR), group 1 and group 2 NASH patients at T0 and after 12 months of RA administration. Aliquots of chloroform extracts were analyzed directly by MALDI-TOF MS as described in Section 2.1 Selected peaks are indicated by their m/z values. For detailed peak assignments see Table 3.

Table 4: Assignments of the *m/z* ratios detected in the positive ion MALDI-TOF mass spectra of the organic extracts of serum patient NASH before (T0) and after 12 months of chronic administration of RA (T12).

m/z (MH^+)	Identity	CTR	Group 1		Group 2	
			T_0	T_{12}	T_0	T_{12}
496,36	lyso PC 16:0	100	100	100	100	100
369,37	CL ($-H_2O$) (H^+)	68 ± 5	35 ± 3	16 ± 1	15 ± 7	41 ± 5
520,4	lyso PC 18:2	37 ± 3	12 ± 2	2 ± 1	11 ± 3	9 ± 4
522,41	lyso PC 18:1	30 ± 3	18 ± 5	17 ± 5	17 ± 4	15 ± 6
524,37	lyso PC 18:0	31 ± 4	32 ± 5	30 ± 3	30 ± 4	59 ± 6
703,5	SM 16:0	20 ± 5	5 ± 6	1 ± 1	8 ± 5	18 ± 2
758,65	PC 16:0/18:2	104 ± 4	21 ± 5	8 ± 2	19 ± 4	64 ± 10
760,51	PC 16:0/18:1 (H^+)	42 ± 5	24 ± 4	13 ± 5	8 ± 2	27 ± 4
784,66	PC 18:1/18:2	43 ± 5	13 ± 3	5 ± 2	9 ± 4	34 ± 4
786,53[#]	PC 18:0/18:2 (H^+)/ PC 18:1/18:1 (H^+)	40 ± 6	9 ± 1	4 ± 1.5	6 ± 2	18 ± 3
804,52	PC 18:2/18:2 (Na^+)	9 ± 1	2 ± 0.5	1 ± 0.75	2 ± 1	10 ± 0.5
808,55[#]	PC 18:0/18:2 (Na^+)/PC 18:1/20:4 (H^+)	19 ± 2	3 ± 0.5	9 ± 1	5 ± 1	19 ± 2
810,55	PC 18:0/20:4	25 ± 3	5 ± 1	3 ± 1	5 ± 2	18 ± 3
812,62	PC 18:0/20:3	12 ± 1	5 ± 0.5	22 ± 5	3 ± 1	16 ± 4

Quantitative determination in % made only on the basis of the value of H^+ or Na^+.
[#]Identification is not unique (there are two possible identities).
CL = free cholesterol; lyso-PC = lysophosphatidylcholine; PC = phosphatidylcholine; SM = sphingomyelin.

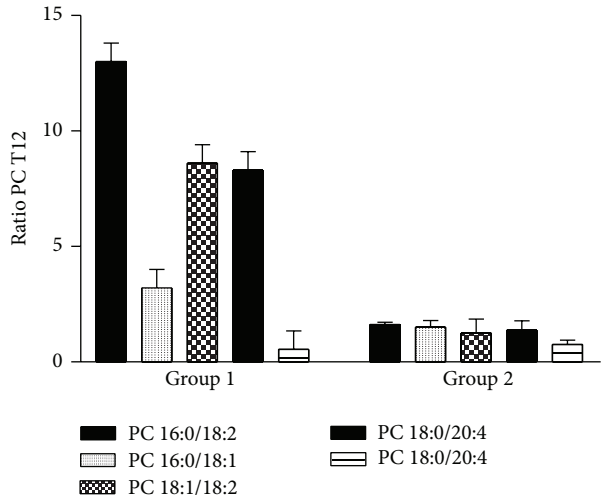

Figure 2: PC ratio of healthy subject and NASH patients after chronic administration of Realsil.

Lower TBARS values in the serum of group 1 may be due to a greater hepatic intracellular accumulation of circulating FFA, mobilized by IR, which are not metabolized, as demonstrated by the significant 3-fold increase of ORO values of cells treated with group 1 T0 sera. In group 2 higher TBARS values could be correlated to the hepatocytes accumulation of circulating FFA, mobilized by IR, that are partially metabolized by β-oxidation with production of toxic aldehydes and their subsequent release into the circulation. Interestingly, low levels of serum TBARS were correlated to higher NAS score, while higher TBARS levels corresponding to NAS score were correlated to a milder disease. The chronic treatment with RA induced changes of serum oxidative status, metabolic parameters, and NAS score in both groups. In group 1, we observed an increase in TBARS values, presenting values higher than the control ones, a decrease of fasting glucose, a variation of NAS score that corresponded to borderline NASH (see Table 3), and a decrease of about 30% of ORO values compared to the T0 sera-treated cells. Instead, group 2 showed a significant decrease in TBARS value, BMI, insulin levels, HOMA test, and ORO values that resulted slightly higher than the control-treated cells, after RA treatment. These results demonstrate that 12 months of chronic administration of RA significantly improves group 2 disease as shown by NAS score variation from 3.6, that corresponded to "borderline NASH", to 2.5 ± 0.51 corresponding to "simple steatosis."

In the present study, we have also evaluated the effects of the treatment on serum "lipidomics" by MALDI-TOF mass spectrometry. More specifically, phospholipids are important components of all mammalian cells and have a variety of biological functions: (i) they form lipid bilayers that provide structural integrity necessary for protein function; (ii) they function as an energy reservoir (e.g., triglycerides); and (iii) they serve as precursors for various second messengers. In this light, lipid and phospholipid metabolism have an important role in the determination of NASH and the study of the modifications in the sera could reflect the lipidic metabolism in the liver [2, 3]. In fact, the study of the circulating "lipidome" does not provide direct information about changes in the liver but it is a tool to determine the effect of chronic treatment on whole-body lipid metabolism. We have found that lipid species as free cholesterol, SM, and PC in NASH patients at T0 (group 1 and group 2) were decreased compared to those of healthy subjects. In group 2 NASH patients, the chronic treatment with RA restored

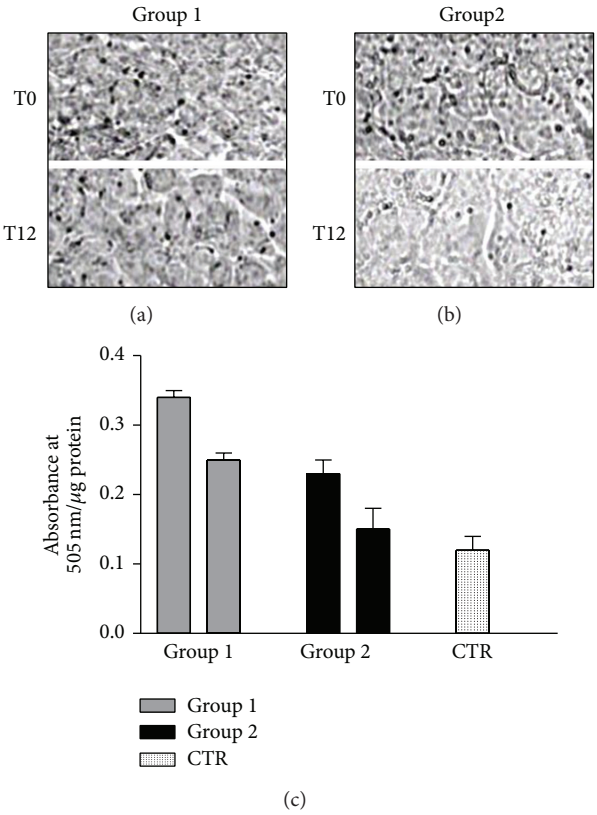

Figure 3: Serum NASH-induced steatosis in HepG2 cells determined by ORO staining ((a) and (b)) and ORO colorimetric assay (c). (a) The HepG2 cells were treated for 72 h with serum of group 1 at T0 and T12. (b) The HepG2 cells were treated with serum of group 2 at T0 and T12. (c) Oil red O colorimetric assay was determined on HepG2 cells after 72 h of incubation with NASH sera of groups 1 and 2 at T0 and T12.

the levels of cholesterol and phospholipids to normal values. It is noteworthy that SMs are synthesized in the lumen of the Golgi apparatus [26] and move to the outer leaflet of the plasma membrane by vesicular membrane transport [27]. Moreover, SMs have high affinity for cholesterol and form a complex with cholesterol in the outer leaflet of the plasma membrane. RA treatment induces, only in group 2, the release of free cholesterol and SMs into the serum. It can be hypothesized that this release can be due to the increase of production of Lyso-PC 18:0 that is an amphipathic molecule and possesses "detergent-like" properties likely promoting the cholesterol-SM efflux [28]. These efflux-promotive effects of lyso-PC were confirmed by the fact that group 2 T12 sera-treated cells showed lower ORO staining than the basal cells (HepG2 treated with group 2 T0 sera). These results suggest that lyso-PC may inhibit the lipid accumulation in liver and the development of NASH disease or enhance its regression by stimulating cholesterol-SM efflux.

In conclusion, this is the first study, at least to our knowledge, that suggests that 12 months of treatment with RA can be useful in order to ameliorate the metabolic asset of patients affected by mild NASH. Finally, our findings suggest that the treatment of these patients with RA induces specific changes of lipidomic profile likely due to a different metabolic response of the patients that should be stratified also for other metabolic alterations (age, sex, AST, ALT, GGT levels, etc.). The understanding of the metabolic alterations at the basis of NASH could be useful in the future to have powerful predictive serum markers that can drive the clinicians in the treatment of this disease.

Abbreviations

ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
γGT: γ-glutamyltransferase
BMI: Body mass index
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
HCC: Hepatocellular carcinoma
FFA: Free fatty acids
LPC: Lysophosphatidylcholine
SM: Sphingomyelin
PC: Phosphatidylcholine
ROS: Reactive oxygen species
NO: Nitric oxide
HSP27: Heat shock protein 27
TBA: Thiobarbituric acid
NAS: NAFLD activity score
PUFA: Polyunsaturated fatty acid.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Oxidative Stress and Histological Changes in a Model of Retinal Phototoxicity in Rabbits

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Photochemical damage occurs after an exposure to high energy radiation within the visible spectrum of light, causing morphological changes in the retina and the formation of superoxide anion. In this study we created a model of phototoxicity in rabbits. Animals were exposed to a light source for 120 minutes and were sacrificed immediately or one week after exposure. Outer nuclear layer and neurosensory retina thickness measurements and photoreceptor counting were performed. Caspase-1 and caspase-3 were assessed by immunohistochemistry. Dihydroethidium was used to evaluate in situ generation of superoxide and thiobarbituric acid reactive substances were measured in retinal homogenates as indicators of lipid peroxidation. The total antioxidant capacity and oxidative ratio were also determined. Retinas from rabbits exposed to light showed higher levels of lipid peroxidation than the unexposed animals and a decrease in outer nuclear layer and neurosensory retina thickness. Our study demonstrates that light damage produces an increase in retinal oxidative stress immediately after light exposure that decreases one week after exposure. However, some morphological alterations appear days after light exposure including apoptotic phenomena. This model may be useful in the future to study the protective effect of antioxidant substances or new intraocular lenses with yellow filters.

1. Introduction

Photochemical damage occurs after an exposure to high energy radiation with a wavelength within the visible spectrum of light. It has been demonstrated that the blue portion of the visible spectrum of light is the most harmful, producing disturbances of the outer blood retinal barrier in the retinal pigmented epithelium (RPE) [1]. At the retinal level, exposure to light causes an increase in phagocytosis of photoreceptor outer segments (POS) [2] and induces the formation of superoxide anion by the RPE [3].

Tissues with a high proportion of membrane lipids and a high tissue oxygen concentration are most sensitive to damage by oxidative stress [4]. The retina has a high oxygen tension (70 mmHg) which makes it very vulnerable to oxidative stress [5]. Moreover, the retina and more specifically the POS possess very high levels of polyunsaturated fatty acids which further increases the sensitivity to oxidative damage and lipid peroxidation of cell membranes [6, 7] as well as phenomena of cell death (apoptosis or necrosis) [8].

Evidence suggests that excessive light exposure plays an important role in the development and progression of age related macular degeneration (AMD) [9–13]. Lipofuscin, a target molecule for phototoxic damage, accumulates in the retina with age, making elderly people more susceptible to light damage [9]. On the contrary, the human lens accumulates yellow chromophores with aging that reduce the transmission of blue and UV light to the retina [9, 10]. However, removal of the lens by cataract surgery, a common

procedure in the elderly, restores the amount of visible radiation that is incident upon the retina [13].

Various experimental studies have demonstrated that the retina can be damaged by the effect of light in different animal models [12–14] showing various morphological patterns. Furthermore, these patterns may vary according to species and the severity of damage [15]. Retinal phototoxicity models in small rodents have been the subject of most studies, rather than in other animals. Primary damage occurring in the retinas of rats exposed to white light lies in the outer nuclear layer (ONL) [16] although some damage can be observed in the inner nuclear layer (INL) [17]. In rats and mice, the rods are more sensitive than cones to damage by light [18] while in chickens and pigeons cones are damaged first [19].

Although these models are widely used, they have several problems. One of them is the small eye size that makes them unsuitable for some experimental surgery procedures like cataract surgery and intraocular lens (IOL) implantation.

This is important since some of the most frequently used IOLs have yellow filters in order to protect the retina from harmful blue light, aimed at preventing oxidative stress related diseases such as AMD [12, 13]. Larger animals have also been used. Messner et al. [20] conducted a study in newborn monkeys (*Macaca arctoides*) continuously exposed to a fluorescent light source (400 foot-candles) for periods of 12 hours, 24 hours, 3 days, and 7 days. These authors were able to show structural damage in the retina of the exposed animals, especially evident in the ONL. Other authors have used pigs as experimental animals. Sisson et al. [21] showed how retinal newborn pigs exposed for 72 hours to a source of blue light also suffered extensive damage to retinal cytoarchitecture with vacuolization of photoreceptors and the presence of pyknotic nuclei in the ONL. However, maintenance expenses of these animals are high and availability is much lower.

We used rabbits given that they are more accessible, easier to handle than pigs or nonhuman primates, and their eye size allows performing therapeutic or surgical procedures such as the insertion of IOLs with protective filters [22]. Although studies in rabbits have shown histological changes and dysfunction of the RPE after light exposure [1, 23], photochemical retinal damage in rabbits has not been fully described. In this work, we investigate the effect of photochemical retinal damage on lipid peroxidation and structural modifications in the rabbit retina.

2. Methods

2.1. Animal Model. Animals were handled according to the rules of the Association for Research in Vision and Ophthalmology (ARVO) and all experiments were approved by the Ethical Committee for Animal Experimentation of the University of Navarra. We used 71 New Zealand white rabbits. Albino rabbits were chosen because of the absence of melanin in the RPE which was supposed to increase retinal susceptibility to phototoxic damage [16]. All specimens were adult females weighing between 2.5 and 3 kg at the beginning of the experiment.

2.2. Induction of Phototoxicity. To ensure the absence of basal retinal pathology, the fundus of all animals was explored using a Canon retinography camera (Canon 8 CF 604 retinography camera, Japan), after pupil dilation with tropicamide 1% (Alcon cusí, Barcelona, Spain) and phenylephrine 10% (Alcon cusí, Barcelona, Spain) eye drops. Before light exposure, rabbits were anesthetized by intramuscular injection of ketamine (1 mL/kg) and xylazine (0.5 mL/kg) which was maintained during all exposure. Eyes remained open by placing blepharostat and the cornea was irrigated with saline using an anterior chamber cannula. The phototoxicity model was created with a 150 W white light fibre optic halogen lamp (type 6423 FO. 150 W Philips) with two optical fiber sources through which the light was transmitted that were placed at a distance of 0.5–1 cm from the cornea. Thermal damage was ruled out given that the temperature measured at 0.5 cm from the light source after 30 minutes had only increased by 0.5°C.

Animals were divided into two interventional groups and one control group. Each animal was randomly assigned to different study groups. The 142 eyes of 71 rabbits were distributed into the following groups: control group (C), 120 minutes of light exposure and immediate sacrifice of the animal after exposure (LE), and 120 minutes of light exposure (LEW) with sacrifice of the animal one week after exposure. After anesthesia with 1 mL/kg body weight of ketamine and 0.5 mL/kg body weight of xylazine, rabbits were sacrificed by intravenous injection of T61 (Intervet Deutschland GmbH, Unterschleißheim, Germany) for histological evaluation.

2.3. Extraction and Processing of the Retina. Once the animal was sacrificed the eyes were enucleated. Briefly, a 360° peritomy was performed; extraocular muscles, the optic nerve, and vessels were cut. The ocular surface was cleaned of any traces of conjunctiva and washed with saline.

For biochemical analysis purposes, the eyeball was placed on blotting paper and an incision was made 5 mm behind the *limbus* to separate the anterior and posterior poles. Approximately 0.5 mL of vitreous was collected using a 1 mL syringe. The retina was then detached from EPR-choroid complex using forceps and a scalpel blade. Once the samples were removed, they were placed in a 1.5 mL microtube and homogenization was performed with an Ultra-Turrax (IKA T10basic, Staufen, Germany). The samples were divided into aliquots which were kept frozen at −80°C until use.

2.4. Preparation of Tissues for Light Microscopy and Conventional Hematoxylin-Eosin Staining. For histological purposes, the standard procedure was applied [24]. Prior to fixation, globes were marked with a suture as a landmark for trimming. The 12 o'clock position was marked with a suture and after enucleation, the eyeball was immersed for 48 hours in Davidson fixative (35% distilled water, 20% formol (4%), 10% glacial acetic acid, and 35% absolute ethanol). Then eyeballs were kept 24 hours in 4% formaldehyde and ethanol 70%. Dehydration was carried out through successive baths of ethanol at increasing concentrations until clearing with xylene in an automatic tissue processor. Samples were embedded in paraffin taking into account the sample orientation

and 4 μm slides were obtained using a microtome. Optic nerve appeared in all cuts, so that they would be comparable between one another. Sections were then stained with Harris's hematoxylin stain (Polysciences Inc., Warrington, PA) and eosin following the standard procedure.

2.5. ONL and Neurosensory Retina Thickness and Photoreceptor Counting. ONL and neurosensory retina thickness measurements and photoreceptor counting were performed on hematoxylin-eosin stained sections. Images of slides were captured digitally with standardized microscope and camera settings. For ONL and neurosensory retina thickness quantification, a screen associated photomicrograph system (DSL-1 Sight, Nikon) was used. Photoreceptor counting was performed manually in 1,000x digital photographs. In order to standardize all tissue sample locations, four measurements (two in the upper and two in the inferior retina) were performed in each preparation, 1,000 μm from the optic nerve for each study variable. Measurements were made by personnel unaware of the study groups.

2.6. Immunohistochemistry for Caspase-1 and Caspase-3. After pretreatment with antigen retrieval (DAKO) for 20 minutes at 95°C, paraffin-embedded sections were examined for immunohistochemical expression of caspase-3 and caspase-1. Caspase-3 antibody (Promega G7481) was used at a concentration of 1 : 100 and caspase-1 antibody (Millipore 92590) at a concentration of 1 : 250. Both antibodies were visualized with an anti-rabbit secondary antibody detection system (*Envision*, Dako). All reactions were revealed by diaminobenzidine (DAB) and counterstained with hematoxylin. As positive control for caspase-3 and caspase-1 detection, rabbit ovarian and lung samples were used, respectively. Negative control experiments included nonimmune serum of the same species as the primary antibody at the same protein concentration and incubation in buffer alone.

2.7. Determination of Oxidative Stress: TBARS and DHE. For lipid peroxidation (LPO) measurement, we slightly modified the method described by Conti et al. [25]. Thiobarbituric acid reactive substances (TBARS) were measured in retinal homogenates as indicators of lipid peroxidation [26, 27]. Diethylthiobarbituric acid-malondialdehyde (DETBA-MDA) complex was determined by fluorescence with 540/590 nm excitation/emission wavelength and all samples were measured in triplicate. The protein concentration was determined using a modified Bradford assay (Bio-Rad, Hercules, CA, USA) as we have used previously [26, 27]. As a second method to detect the presence of oxidative stress, in situ superoxide generation production was detected by fluorescence with dihydroethidium (DHE) (Molecular Probes). Dehydrated paraffin samples were incubated with DHE (125 mg) in a light-protected humidified chamber at 37°C for 30 minutes. The cell nuclei were labeled with TOPRO-3. The DHE images were obtained with a laser scanning confocal imaging system (Zeiss LSM-510 Meta) with a 585 nm long-pass filter.

2.8. Determination of the Total Antioxidant Capacity. The total antioxidant capacity (TAC) is a measurement in moles of antioxidant substances and determines the capacity of neutralization of free radicals. TAC is a sensitive and reproducible marker to detect changes in oxidative status, which often cannot be determined by measuring the antioxidants separately. For the present work we measured the TAC following the manufacturer's instructions (Total Antioxidant Power kit, Oxford Biomedical, Oxford, UK) to determine TAC based on the ability of antioxidants to reduce Cu^{++} into Cu^{+} in retinal homogenates.

2.9. Oxidative Ratio. In order to quantify the oxidative status of retinas, we calculated an oxidative ratio using data from oxidation (TBARS) and TAC using the following formula: TBARSx100/TAC.

2.10. Statistical Analysis. Values are reported throughout as the mean ± standard deviation (SD). Statistical significance was determined applying an analysis of variance (ANOVA) or a Kruskal-Wallis test to assess differences among groups. After a significant ANOVA, comparisons between groups were made with the following orthogonal contrasts: (1) control versus immediate sacrifice after exposure to light exposure; (2) control versus sacrifice one week after light exposure; and (3) immediate sacrifice after light exposure versus sacrifice one week after light exposure.

After a significant Kruskal-Wallis, a Mann-Whitney test was applied to analyze differences. Statistical significance was accepted at the 95% confidence level ($P < 0.05$), and analysis was performed by using the computer program SPSS (v. 15.0, SPSS Inc., Chicago, USA).

3. Results

3.1. Neurosensory Retina Thickness. ONL and neurosensory retina thickness measurements and photoreceptor counting were performed on hematoxylin-eosin stained retinal sections. We observed a significant decrease in neurosensory retina after light exposure. The thickness of the neurosensory retina in the LE group (95.37 ± 4.56 μm) and LEW group (90.81 ± 8.14 μm) was lower than the control group (100.25 ± 4.31 μm) ($P = 0.041$ and $P = 0.003$, resp.). Furthermore, in the LEW group the neurosensory retina thickness was lower than in the LE group ($P = 0.049$). Representative images from all study groups are shown in Figure 1(a).

All the histological findings are summarized in Table 1.

3.2. Thickness of Outer Nuclear Layer. We found a large data dispersion, particularly in the LEW group. However, thickness of the ONL in the LE group was significantly lower than in the control group (23.89 ± 1.31 μm versus 24.85 ± 8.4 μm, $P = 0.047$). Similarly, the LEW group showed significantly lower ONL thickness compared with the control group (22.56 ± 2.85 μm versus 24.85 ± 8.4 μm, $P = 0.007$). Although the differences between the LE and LEW groups did not reach statistical significance ($P = 0.076$), data suggest

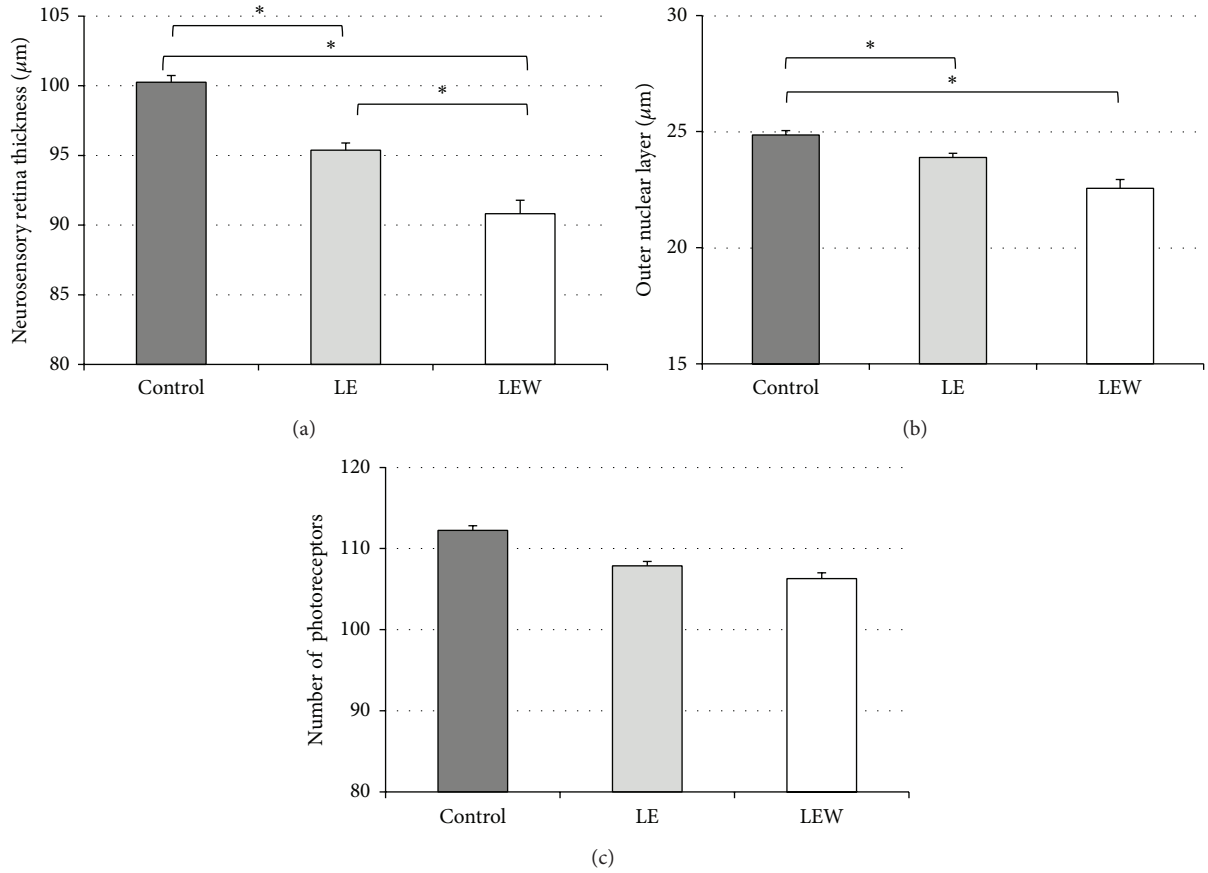

Figure 1: The effect of phototoxicity on The effect phototoxicity on retinal thickness and number of photorreceptors. (a) The thickness of the neurosensory retina in exposed animals is lower than the control group. Further, the LEW group neurosensory retina thickness is lower than in the LE group ($^{*}P < 0.05$). (b) Thickness of the outer nuclear layer in the exposed rabbits was significantly lower than in control group. (c) There were no significant differences in the number of photoreceptors in any group. Results are expressed as mean ± SEM.

Table 1: Histological results.

	N	Thickness of the outer nuclear layer (μm)	Neurosensory retina thickness (μm)	Number of photoreceptors
Control	6	24.85 (±0.84)	100.25 (±4.31)	112.24 (±10.30)
LE	18	23.89 (±1.31)*	95.37 (±4.56)*	107.87 (±9.55)
LEW	18	22.56 (±2.85)*†	90.81 (±8.14)*	106.30 (±12.08)

Data are expressed as mean ± S.D. Statistically significant differences from control are marked as $^{*}P < 0.05$ and differences from the LE group are marked as $^{\dagger}P < 0.05$. LE: 120 minutes of light exposure and immediate sacrifice of the animal, LEW: 120 minutes of light exposure with sacrifice of the animal one week after exposure.

that the thickness of the ONL decreases as the time between exposure to light and sacrifice increases (Figure 1(b)).

3.3. Number of Photoreceptors. Despite the decrease in ONL, we found no statistically significant differences in the number of photoreceptors between the groups ($P = 0.513$), Figure 1(c). However, animals from the LEW group showed an increase in vacuolization inside the outer segments of the photoreceptors which was absent in the other groups (Figures 2(a)–2(c)).

3.4. Immunohistochemistry. In order to detect the presence of apoptosis induced by light exposure, we assessed the presence of anti-caspase-3 by immunohistochemistry in retinal sections. The activity of caspase-3 was not detectable in the control group, Figure 3(a). We found caspase-3 activity in the inner nuclear layer in rabbit retinas from the LE group (Figure 3(b)). Caspase-3 activity then disappeared 1 week later in the LEW group (Figure 3(c)). On the other hand, caspase-1 activity, a protein involved in inflammatory processes, was not detectable in control animals (Figure 3(d)).

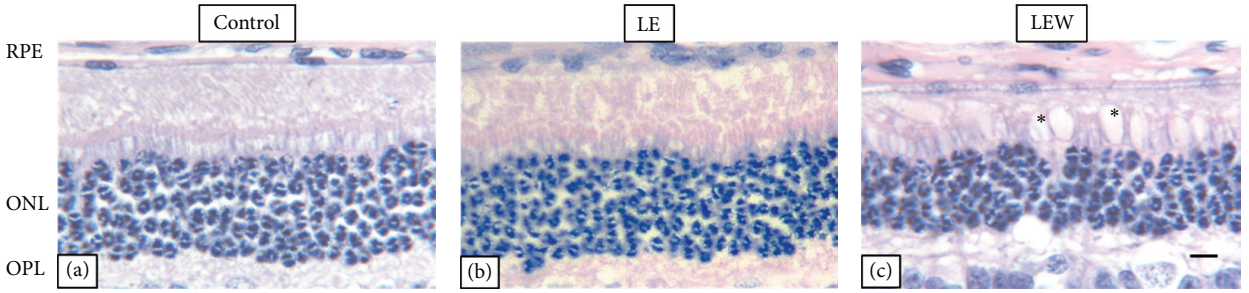

Figure 2: Hematoxylin-eosin stained retinal cross sections. (a) Control group, (b) LE group, and (c) LEWgroup. An increase in vacuolation inside the outer segments of the photoreceptors of rabbits sacrificed one week after light exposure was observed in this study ((c), asterisk, scale bar: 50 μm). (ONL) Outer nuclear layer, (OPL) outer plexiform layer, (RPE) retinal pigmented epithelium.

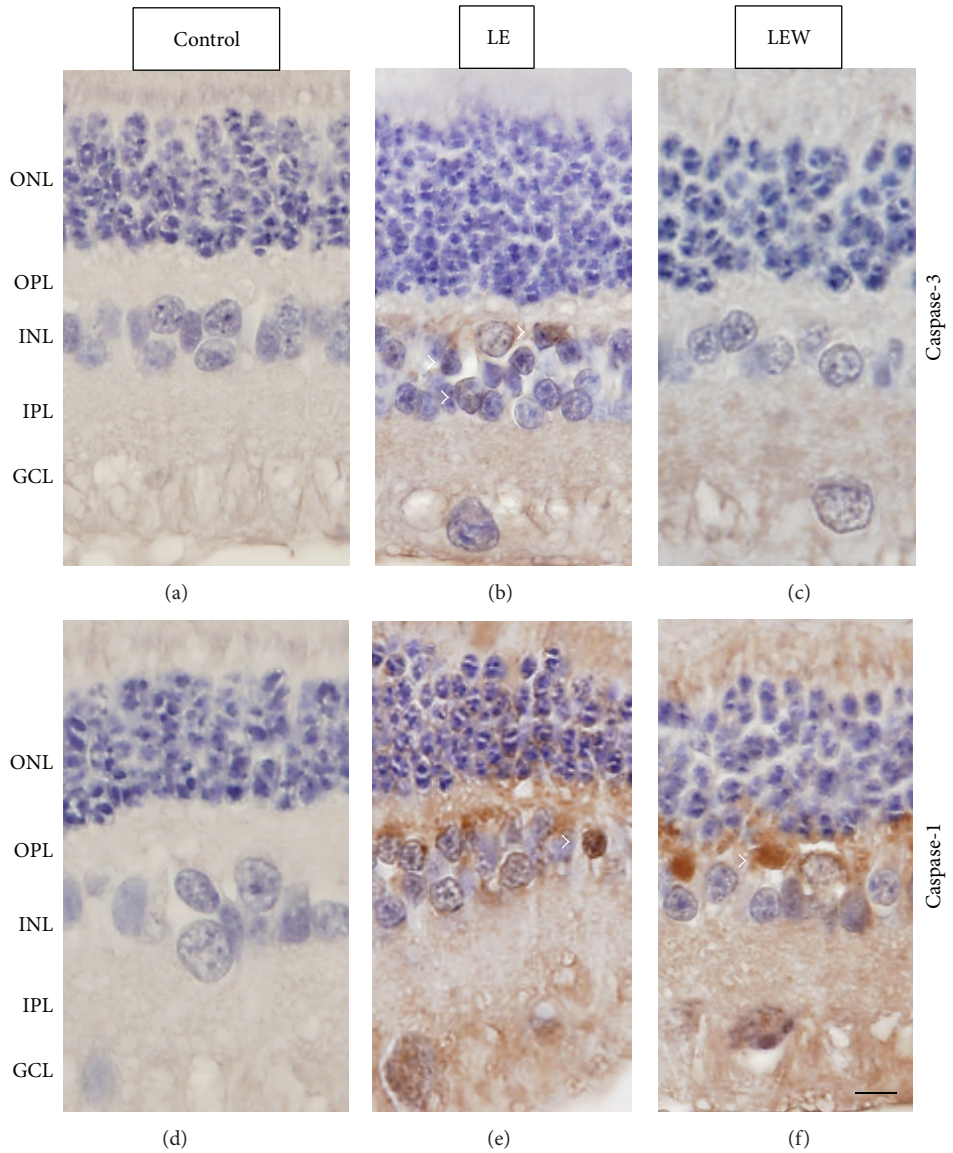

Figure 3: Phototoxicity induces apoptotic death and activation of inflammatory processes. ((a)–(c)) Caspase-3 immunohistochemistry showed no staining in control (a) and LEW rabbit retinas (c); however we found positive caspase-3 cells in the INL in LE rabbit retinas ((b) arrowheads). ((d)–(f)) Caspase-1 immunohistochemistry. Control rabbit retinas showed no caspase-1 staining (d). Labeling was seen (arrowheads) in LE (e) and LEW rat retinas (f). Scale bar: 50 μm. Retinas were contrasted with hematoxylin. (ONL) Outer nuclear layer, (OPL) outer plexiform layer, (INL) inner nuclear layer, (IPL) inner plexiform layer, and (GCL) ganglion cell layer.

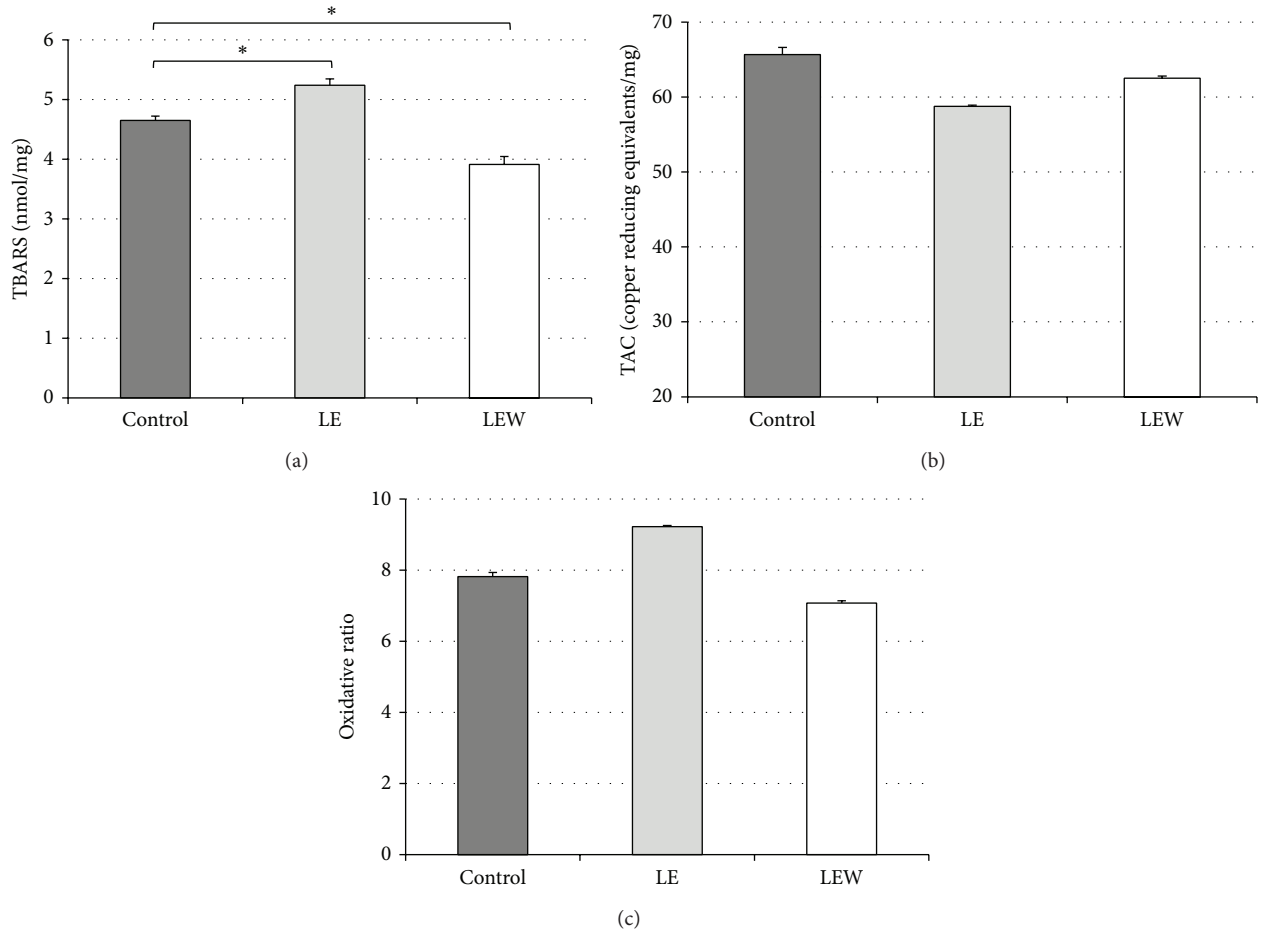

Figure 4: The effect of phototoxicity on TBARS, TAC, and oxidative ratio. (a) The LE group had a statistically significant increase of TBARS when compared with the control group (a). However, TBARS value of LEW group was lower than both the LE and the control group. $^{*}P < 0.05$. There were no significant differences in the TAC or oxidative ratio in any group ((b), (c)). Results are expressed as mean ± SEM.

However, cells in the inner nuclear layer were found to be caspase-1 positive in animals sacrificed immediately after exposure, LE (Figure 3(e)) and remained positive one week after light exposure, LEW (Figure 3(d)).

3.5. Biochemical Determinations

3.5.1. Lipid Peroxidation Measured by TBARS. We found a significant increase in lipid peroxidation immediately after light exposure. The LE group had a statistically significant increase of TBARS when compared with the control group (5.24 ± 1.25 nmol/mg versus 4.65 ± 0.45 nmol/mg, $P = 0.011$). On the other hand, the TBARS value of the LEW group was significantly lower than the control group (3.91 ± 1.36 nmol/mg versus 4.65 ± 0.45 nmol/mg, $P = 0.009$) and they were also lower than the LE group (3.91 ± 1.36 nmol/mg versus 5.24 ± 1.25 nmol/mg, $P < 0.001$). These results are represented in Table 2. Values from all the biochemical findings are summarized in Table 2.

3.5.2. Determination of the Total Antioxidant Capacity and Oxidative Ratio. TAC, a measurement of antioxidant substances, was performed to determine the capacity of neutralization of free radicals. Further, in order to quantify the oxidative status of retinas, we calculated an oxidative ratio using data from oxidation (TBARS) and TAC. However, there were no statistically significant differences in the TAC ($P = 0.635$) or in the oxidative ratio between the studied groups ($P = 0.635$). These results are shown in Figures 4(a)–4(c) and Table 2.

3.5.3. Detection of Superoxide Production by DHE. Likewise, retinal levels of superoxide were determined with DHE staining. DHE was absent in the control group (Figure 5(a)) but was strongly detected in the LE group, mainly in the outer nuclear layer, inner nuclear layer, and ganglion cell layer (Figure 5(b)). However, its presence was significantly lower in the LEW group in the aforementioned layers (Figure 5(c)),

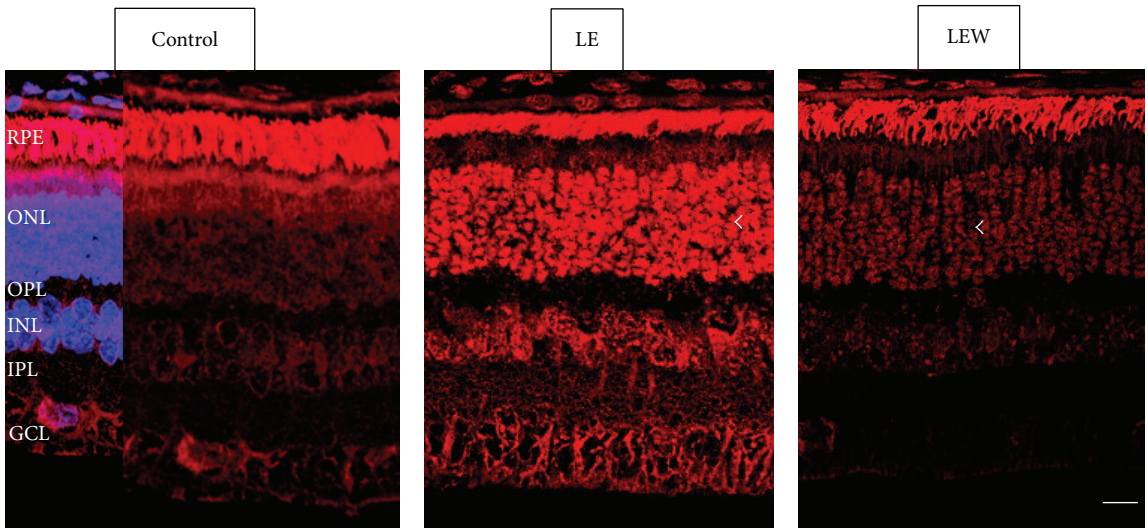

Figure 5: Superoxide generation was assessed in rabbit retinas with the fluorescent indicator DHE (red). (a) Confocal microscopic image of a rabbit retina from the control group. (b) DHE was detected in the LE group (arrowhead) at the ONL. (c) DHE fluorescence in the retinas from the LEW group was significantly lower. Staining of cell nuclei was observed for TO-PRO-3 (blue). Arrows indicate the ONL where the DHE was strong. Scale bar: 50 μm. (ONL) Outer nuclear layer, (OPL) outer plexiform layer, (INL) inner nuclear layer, (IPL) inner plexiform layer, and (GCL) ganglion cell layer.

Table 2: Biochemical results.

	N	TBARS (nmol/mg)	TAC (copper reducing equivalents/mg)	Oxidative ratio
Control	13	4.65 (±0.45)	65.65 (±6.20)	7.82 (±0.73)
LE	46	5.24 (±1.25)*	58.76 (±1.87)	9.22 (±0.38)
LEW	33	3.91 (±1.36)*†	62.50 (±2.91)	7.07 (±0.71)

Data are expressed as mean ± S.D. Statistically significant differences from control are marked as $^{*}P < 0.05$ and differences from the LE group are marked as $^{\dagger}P < 0.05$. LE: 120 minutes of light exposure and immediate sacrifice of the animal, LEW: 120 minutes of light exposure with sacrifice of the animal one week after exposure.

which confirms the initial increase of oxidative stress following light exposure that decreases when the animal is sacrificed one week after, observed with TBARS.

4. Discussion

In the present study, and for the first time to our knowledge, we describe some of the immediate and later biochemical changes associated with pathological exposure to light while developing a model of retinal phototoxicity in rabbits. Moreover, some histological changes observed were in accordance with previous data from other authors [28]. Retinas from rabbits exposed to light showed higher levels of lipid peroxidation and a decrease in ONL and neurosensory retina thickness.

Although some controversy exists over the role of phototoxicity in the pathogenesis of AMD, epidemiological evidence suggests a direct relationship between cumulative light exposure and the development and progression of this disease [9–13]. Consequently, there has been an increased interest in studying the pathologic effects of light on the retina and therapeutic strategies to prevent it, such as antioxidants and the use of blue light filtering IOLs [10, 13].

Retinal phototoxicity models in small rodents have been used in the majority of studies [12–14]. However, the use of larger animals, like rabbits, offers the advantage of having bigger eyes that enable the insertion of IOLs and studying the effect of this surgery in the retina as well as the possible effect of blocking blue and other visible light sources [22].

An increase in vacuolization inside the outer segments of the photoreceptors of rabbits sacrificed one week after light exposure was observed in this study. This finding was described by Grimm and Mukai and seems to be related with areas of minor and reversible damage, not sufficiently intense to activate the apoptotic cascade [29, 30].

Some studies demonstrate that retinal degeneration continues for several weeks after exposure to light [14, 16, 31] suggesting that an animal with a longer time period between the end of exposure and sacrifice will have more time to produce activation and operation of various mechanisms of damage as well as tissue regeneration. Our results agree with this hypothesis, given that histological damage continues after light exposure. We found a greater decrease in

neurosensory retina and ONL thickness in the group of rabbits sacrificed one week after the light exposure compared with the group immediately sacrificed. Further, caspase-1 remained active in the retinas one week after light exposure. Distinct inflammasomes may upregulate caspase-1 which, in the macrophage cytoplasm, cleaves pro-IL-1β to active IL-1β, increasing inflammation and expression of proinflammatory genes [32]. Our study shows high caspase-1 levels 1 week after exposure, which is in line with the observation that macrophages are observed in the retina weeks after phototoxic stimuli (McKechnie and Foulds). Both findings suggest a long-term response by the mononuclear phagocyte system. In contrast, caspase-3, an important effector of apoptosis, was only detectable in the animals sacrificed immediately after exposure.

Increased levels of retinal lipid peroxidation upon exposure to light are well documented in different animal models including rabbits [12–14, 33]. Dzhafarov exposed rabbits with diabetic retinopathy to bright light and observed an acute increase in retinal lipid peroxidation [33]. In our study, the level of oxidative damage measured by TBARS in animals sacrificed immediately after light exposure was 12% greater than the unexposed group. However, one week after light exposure, lipid peroxidation was recovered to levels even lower than the control group, suggesting that some restoring mechanisms could have been activated in response to light damage. We could not find any study in the literature describing retinal levels of lipid peroxidation after a long period from the acute exposure to light. In addition, DHE results in this study confirm the initial increase in oxidative stress that decreases one week after light exposure. Our results suggest a recovery in oxidative status, which could respond to an increase in the antioxidant defense mechanisms that counteract retinal oxidative stress. However, we have not been able to confirm this theory because the levels of TAC in the three groups were not significantly different. TAC measurement includes the activity of various antioxidants present in a tissue, but not all. In general, it measures primarily low molecular weight antioxidants and chain breakers, excluding antioxidant enzymes. Other authors have found increased levels of superoxide dismutase or glutathione peroxidase in eyes of various animals exposed to light [34]. It would be very interesting to measure these enzymes in the retinas of rabbits exposed to light and analyze their variation after one week of recovery from light exposure.

Along with other authors, we believe that the initial step resulting in retinal damage is an acute increase in lipid peroxidation following light exposure, which damages photoreceptors and other retinal cells that ultimately induce their own apoptosis [13, 35]. Our study supports this by the presence of caspase-3. After light exposure, we found initially high levels of lipid peroxidation that decreased over the course of one week relative to controls. During this time, either the cells may be destroyed via apoptosis or they remain alive but show signs of damage such as vacuolated outer segments, if oxidative damage is not intense enough. We hypothesize that lipid peroxidation may decrease following an oxidative insult as the cell overcompensates its antioxidant efforts to counteract such an insult. Despite this rigorous antioxidant effort by the cell, the damage may be too great and continue its course, and progressive destruction leads to the greatest loss of ONL thickness observed at one week after exposure.

As the role of melanin is controversial [36–38] and appears to depend on the intensity of light received, we decided to use albino animals. However, with high light intensity as we used in this study, melanin is able to generate oxygen free radicals [39]. It is possible that if we had used pigmented rabbits, melanin would have acted as another chromophore capable of causing more oxidative damage. It is also possible that we might have found increased retinal destruction if we would have used elderly animals, as antioxidative mechanisms decrease with age [40] and lipofuscin concentration in the retina increases [41, 42]. However, older animals might present a number of other conditions that may affect or alter the phototoxic retinal damage mechanisms [43].

5. Conclusions

In conclusion, in this study we demonstrate that light damage produces an increase in retinal oxidative stress immediately after light exposure that can be recovered by compensatory mechanisms. In spite of that recovery at a molecular level, some structural damage appears at a period of time after light exposure that could end in apoptosis phenomena. Oxidative stress and inflammation are crucial in degenerative diseases of the retina; this is particularly interesting for AMD, a disease in which these factors have been implicated as major players. Furthermore, this model may be useful in the future to study the protective effect against phototoxic damage of antioxidant substances or new IOLs with a yellow filter.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal

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Lipid peroxidation can be described generally as a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs). Over the last four decades, an extensive body of literature regarding lipid peroxidation has shown its important role in cell biology and human health. Since the early 1970s, the total published research articles on the topic of lipid peroxidation was 98 (1970–1974) and has been increasing at almost 135-fold, by up to 13165 in last 4 years (2010–2013). New discoveries about the involvement in cellular physiology and pathology, as well as the control of lipid peroxidation, continue to emerge every day. Given the enormity of this field, this review focuses on biochemical concepts of lipid peroxidation, production, metabolism, and signaling mechanisms of two main omega-6 fatty acids lipid peroxidation products: malondialdehyde (MDA) and, in particular, 4-hydroxy-2-nonenal (4-HNE), summarizing not only its physiological and protective function as signaling molecule stimulating gene expression and cell survival, but also its cytotoxic role inhibiting gene expression and promoting cell death. Finally, overviews of *in vivo* mammalian model systems used to study the lipid peroxidation process, and common pathological processes linked to MDA and 4-HNE are shown.

This review paper is dedicated to
Dr. Alberto Machado

1. Lipids Overview of Biological Functions

Lipids Are Classically Divided into Two Groups: Apolar and Polar. Triglycerides (apolar), stored in various cells, but especially in adipose (fat) tissue, are usually the main form of energy storage in mammals [1, 2]. Polar lipids are structural components of cell membranes, where they participate in the formation of the permeability barrier of cells and subcellular organelles in the form of a lipid bilayer. The major lipid type defining this bilayer in almost all membranes is glycerol-based phospholipid [3]. The importance of the membrane lipid physical (phase) state is evidenced by the fact that lipids may control the physiological state of a membrane organelle by modifying its biophysical aspects, such as the polarity and permeability. Lipids also have a key role in biology as signaling molecules.

Lipids as Signaling Molecules. The main enzymes that generate lipid signaling mediators are lipoxygenase, which mediate hydroperoxyeicosatetraenoic acids (HPETEs), lipoxins, leukotrienes, or hepoxilins biosynthesis after oxidation of arachidonic acid (AA) [4, 5], cyclooxygenase that produces prostaglandins [4], and cytochrome P-450 (CYP) which generates epoxyeicosatrienoic acids, leukotoxins, thromboxane, or prostacyclin [4]. Lipid signaling may occur via activation of a variety of receptors, including G protein-coupled and nuclear receptors. Members of several different lipid categories have been identified as potent intracellular signal transduction molecules. Examples of signaling lipids

include (i) two derived from the phosphatidylinositol phosphates, diacylglycerol (DAG) and inositol phosphates (IPs). DAG is a physiological activator of protein kinase C [6, 7] and transcription factor nuclear factor-kB (NF-κB), which promotes cell survival and proliferation. Diacylglycerol also interacts indirectly with other signalling molecules such as small G proteins [8]. IPs are a highly charged family of lipid-derived metabolites, involved in signal transduction that results in activation of Akt, mTOR [9], and calcium-homeostasis [10, 11]; (ii) sphingosine-1-phosphate, a sphingolipid derived from ceramide that is a potent messenger molecule involved in regulating calcium mobilization, migration, adhesion, and proliferation [12–14]; (iii) the prostaglandins, which are one type of fatty-acid derived eicosanoid involved in inflammation [15, 16] and immunity [17]; (iv) phosphatidylserine, a phospholipid that plays an important role in a number of signaling pathways, includes kinases, small GTPases, and fusogenic proteins [18]; (v) the steroid hormones such as estrogen, testosterone, and cortisol, which modulate a host of functions such as reproduction, metabolism, stress response, inflammation, blood pressure, and salt and water balance [19].

2. Lipids Damage by Reactive Oxygen Species

One of the consequences of uncontrolled oxidative stress (imbalance between the prooxidant and antioxidant levels in favor of prooxidants) is cells, tissues, and organs injury caused by oxidative damage. It has long been recognized that high levels of free radicals or reactive oxygen species (ROS) can inflict direct damage to lipids. The primary sources of endogenous ROS production are the mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes [20] through a variety of mechanisms including enzymatic reactions and/or autooxidation of several compounds, such as catecholamines and hydroquinone. Different exogenous stimuli, such as the ionizing radiation, ultraviolet rays, tobacco smoke, pathogen infections, environmental toxins, and exposure to herbicide/insecticides, are sources of *in vivo* ROS production.

The two most prevalent ROS that can affect profoundly the lipids are mainly hydroxyl radical ($HO^{\bullet}$) and hydroperoxyl ($HO^{\bullet}_2$). The hydroxyl radical ($HO^{\bullet}$) is a small, highly mobile, water-soluble, and chemically most reactive species of activated oxygen. This short-lived molecule can be produced from O_2 in cell metabolism and under a variety of stress conditions. A cell produces around 50 hydroxyl radicals every second. In a full day, each cell would generate 4 million hydroxyl radicals, which can be neutralized or attack biomolecules [21]. Hydroxyl radicals cause oxidative damage to cells because they unspecifically attack biomolecules [22] located less than a few nanometres from its site of generation and are involved in cellular disorders such as neurodegeneration [23, 24], cardiovascular disease [25], and cancer [26, 27]. It is generally assumed that $HO^{\bullet}$ in biological systems is formed through redox cycling by Fenton reaction, where free iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) and the Haber-Weiss reaction that results in the production of Fe^{2+} when superoxide reacts with ferric iron (Fe^{3+}). In addition to the iron redox cycling described above, also a number of other transition-metal including Cu, Ni, Co, and V can be responsible for $HO^{\bullet}$ formation in living cells (Figure 1).

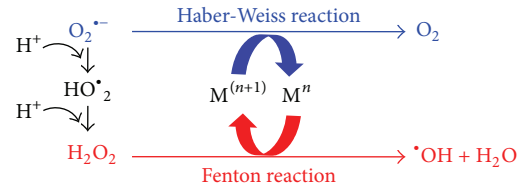

Figure 1: Fenton and Haber-Weiss reaction. Reduced form of transition-metals (M^n) reacts trough the Fenton reaction with hydrogen peroxide (H_2O_2), leading to the generation of $^{\bullet}OH$. Superoxide radical ($O_2^{\bullet-}$) can also react with oxidized form of transition metals ($M^{(n+1)}$) in the Haber-Weiss reaction leading to the production of M^n, which then again affects redox cycling.

The hydroperoxyl radical ($HO^{\bullet}_2$) plays an important role in the chemistry of lipid peroxidation. This protonated form of superoxide yields H_2O_2 which can react with redox active metals including iron or copper to further generate $HO^{\bullet}$ through Fenton or Haber-Weiss reactions. The $HO^{\bullet}_2$ is a much stronger oxidant than superoxide anion-radical and could initiate the chain oxidation of polyunsaturated phospholipids, thus leading to impairment of membrane function [28–30].

2.1. Lipid Peroxidation Process. Lipid peroxidation can be described generally as a process under which oxidants such as free radicals or nonradical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides as described previously [31]. Glycolipids, phospholipids (PLs), and cholesterol (Ch) are also well-known targets of damaging and potentially lethal peroxidative modification. Lipids also can be oxidized by enzymes like lipoxygenases, cyclooxygenases, and cytochrome P450 (*see above, lipid as signaling molecules*). In response to membrane lipid peroxidation, and according to specific cellular metabolic circumstances and repair capacities, the cells may promote cell survival or induce cell death. Under physiological or low lipid peroxidation rates (subtoxic conditions), the cells stimulate their maintenance and survival through constitutive antioxidants defense systems or signaling pathways activation that upregulate antioxidants proteins resulting in an adaptive stress response. By contrast, under medium or high lipid peroxidation rates (toxic conditions) the extent of oxidative damage overwhelms repair capacity, and the cells induce apoptosis or necrosis programmed cell death; both processes eventually lead to molecular cell damage which may facilitate development of various pathological states and accelerated aging. The impact of lipids oxidation in cell membrane and how these oxidative damages are involved in both physiological processes and major pathological conditions have been analysed in several reviews [32–35].

The overall process of lipid peroxidation consists of three steps: initiation, propagation, and termination [31, 36, 37]. In the lipid peroxidation initiation step, prooxidants like hydroxyl radical abstract the allylic hydrogen forming the carbon-centered lipid radical ($L^{\bullet}$). In the propagation phase, lipid radical ($L^{\bullet}$) rapidly reacts with oxygen to form a lipid peroxy radical ($LOO^{\bullet}$) which abstracts a hydrogen from another lipid molecule generating a new $L^{\bullet}$ (that continues the chain reaction) and lipid hydroperoxide (LOOH). In the termination reaction, antioxidants like vitamin E donate a hydrogen atom to the $LOO^{\bullet}$ species and form a corresponding vitamin E radical that reacts with another $LOO^{\bullet}$ forming nonradical products (Figure 2). Once lipid peroxidation is initiated, a propagation of chain reactions will take place until termination products are produced. Review with extensive information regarding the chemistry associated with each of these steps is available [31].

2.2. Lipid Peroxidation Products. Lipid peroxidation or reaction of oxygen with unsaturated lipids produces a wide variety of oxidation products. The main primary products of lipid peroxidation are lipid hydroperoxides (LOOH). Among the many different aldehydes which can be formed as secondary products during lipid peroxidation, malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) have been extensively studied by Esterbauer and his colleagues in the 80s [38–49]. MDA appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most toxic [50].

MDA has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its facile reaction with thiobarbituric acid (TBA) [48, 51]. The TBA test is predicated upon the reactivity of TBA toward MDA to yield an intensely colored chromogen fluorescent red adduct; this test was first used by food chemists to evaluate autoxidative degradation of fats and oils [52]. However, the thiobarbituric acid reacting substances test (TBARS) is notoriously nonspecific which has led to substantial controversy over its use for quantification of MDA from *in vivo* samples. Several technologies for the determination of free and total MDA, such gas chromatography-mass spectrometry (GC-MS/MS), liquid chromatography-mass spectrometry (LC-MS/MS), and several derivatization-based strategies, have been developed during the last decade [53]. Because MDA is one of the most popular and reliable markers that determine oxidative stress in clinical situations [53], and due to MDA's high reactivity and toxicity underlying the fact that this molecule is very relevant to biomedical research community.

4-HNE was first discovered in 60s [54]. Later, in 80s 4-HNE was reported as a cytotoxic product originating from the peroxidation of liver microsomal lipids [40]. 4-Hydroxyalkenals produced in the course of biomembrane lipids peroxidation, elicited either by free radicals or by chemicals, might exert a genotoxic effect in humans [55]. The 4-hydroxyalkenals are the most significant products because they are produced in relatively large amounts, and they are very reactive aldehydes that act as "second messengers of free radicals." In particular 4-HNE, which has been subjected to intense scientific scrutiny in 90s [49], is considered as "one of the major toxic products generated from lipid peroxides" [49]. 4-HNE high toxicity can be explained by its rapid reactions with thiols and amino groups [56]. Reactive aldehydes, especially 4-HNE, act both as signaling molecules (*see below 4-HNE as signaling molecule*) and as cytotoxic products of lipid peroxidation causing long-lasting biological consequences, in particular by covalent modification of macromolecules (*see below 4-HNE biomolecular adducts*). 4-HNE is considered as "second toxic messengers of free radicals," and also as "one of the most physiologically active lipid peroxides," "one of major generators of oxidative stress," "a chemotactic aldehydic end-product of lipid peroxidation," and a "major lipid peroxidation product" [57]. Thus, it is not a surprise that 4-HNE is nowadays considered as major bioactive marker of lipid peroxidation and a signaling molecule involved in regulation of several transcription factors sensible to stress such as nuclear factor erythroid 2-related factor 2 (Nrf2), activating protein-1 (AP-1), NF-κB, and peroxisome-proliferator-activated receptors (PPAR), in cell proliferation and/or differentiation, cell survival, autophagy, senescence, apoptosis, and necrosis (*see below 4-HNE as signaling molecule*).

Characteristics of various lipid peroxidation products as biomarkers have been reviewed on the basis of mechanisms and dynamics of their formation and metabolism and also on the methods of measurement, with an emphasis on the advantages and limitations [58].

2.3. Primary Lipid Peroxidation Product-Lipid Hydroperoxides. Hydroperoxides are produced during the propagation phase constituting the major primary product of lipid peroxidation process. The hydroperoxide group may be attached to various lipid structures, for example, free fatty acids, triacylglycerols, phospholipids, and sterols. Lipid hydroperoxide generation, turnover and effector action in biological systems have been reviewed [36]. In contrast to free radical, usually highly reactive and chemically unstable, at moderate reaction conditions, such as low temperature and absence of metal ions, lipid hydroperoxides are relatively more stable products. We found that lipid hydroperoxides in serum could be useful to predict the oxidative stress in tissues [59], and the levels of oxidative stress, including lipid peroxidation, increased throughout the day [60]. Once formed lipid hydroperoxides can be target of different reduction reactions, resulting in peroxidative damage inhibition or peroxidative damage induction.

Peroxidative Damage Inhibition. Hydroperoxides may decompose *in vivo* through two-electron reduction, which can inhibit the peroxidative damage. The enzymes mainly responsible for two-electron reduction of hydroperoxides are selenium-dependent glutathione peroxidases (GPx) and selenoprotein P (SeP). GPxs are known to catalyze the reduction of H_2O_2 or organic hydroperoxides to water or the corresponding alcohols, respectively, typically using glutathione (GSH) as reductant. Widely distributed in

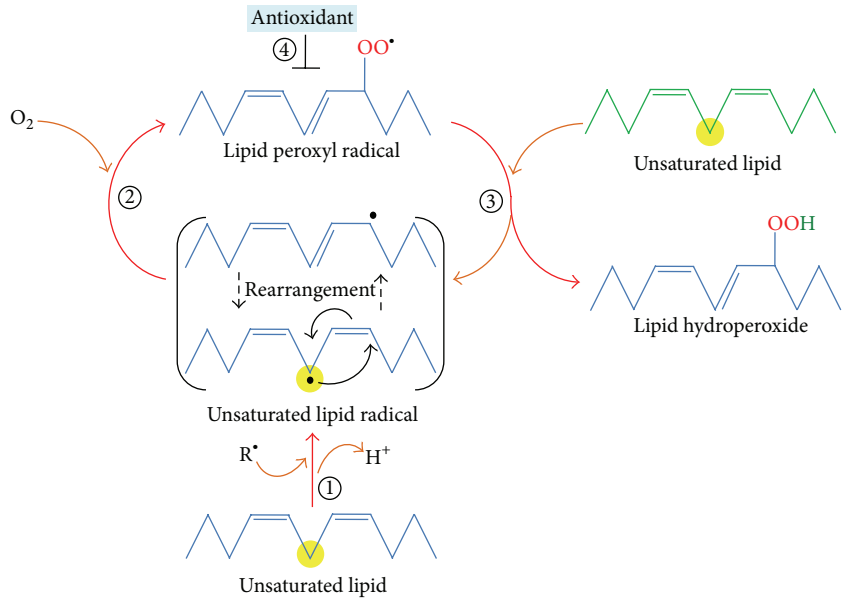

Figure 2: Lipid peroxidation process. In Initiation, prooxidants abstract the allylic hydrogen forming the carbon-centered lipid radical; the carbon radical tends to be stabilized by a molecular rearrangement to form a conjugated diene (step 1). In the propagation phase, lipid radical rapidly reacts with oxygen to form a lipid peroxy radical (step 2) which abstracts a hydrogen from another lipid molecule generating a new lipid radical and lipid hydroperoxide (step 3). In the termination reaction, antioxidants donate a hydrogen atom to the lipid peroxy radical species resulting in the formation of nonradical products (step 4).

mammalian tissues GPx can be found in the cytosol, nuclei, and mitochondria [61, 62]. The presence of selenocysteine (in the catalytic centre of glutathione peroxidases) as the catalytic moiety was suggested to guarantee a fast reaction with the hydroperoxide and a fast reducibility by GSH [61]. SeP is the major selenoprotein in human plasma that reduced phospholipid hydroperoxide using glutathione or thioredoxin as cosubstrate. It protected plasma proteins against peroxynitrite-induced oxidation and nitration or low-density-lipoproteins (LDL) from peroxidation [62].

Peroxidative Damage Induction. Hydroperoxides may also decompose *in vivo* through one-electron reduction and take part in initiation/propagation steps [31, 36, 37], induce new lipid hydroperoxides, and feed the lipid peroxidation process; all these mechanisms can contribute to peroxidative damage induction/expansion. Lipid hydroperoxides can be converted to oxygen radicals intermediates such as lipid peroxyl radical ($LOO^{\bullet}$) and/or alkoxyl ($LO^{\bullet}$) by redox cycling of transition metal (M), resulting in lipid hydroperoxide decomposition and the oxidized or reduced form of theses metal, respectively [63]. The lipid peroxyl and alkoxyl radicals can attack other lipids promoting the propagation of lipid peroxidation

$$LOOH + M^{n} \longrightarrow LO^{\bullet} + OH^{-} + M^{n+1} \quad (1)$$

$$LOOH + M^{n+1} \longrightarrow LOO^{\bullet} + H^{+} + M^{n}. \quad (2)$$

Lipid hydroperoxides can also react with peroxynitrite (a short-lived oxidant species that is a potent inducer of cell death [64] and is generated in cells or tissues by the reaction of nitric oxide with superoxide radical) or hypochlorous acid (a high reactive species produced enzymatically by myeloperoxidase [65, 66], which utilizes hydrogen peroxide to convert chloride to hypochlorous acid at sites of inflammation) yielding singlet molecular oxygen [67, 68]. Singlet oxygen (molecular oxygen in its first excited singlet state $^{1}\Delta_{g}$; $^{1}O_{2}$)[1] can react with amino acid, and proteins resulting in multiple effects including oxidation of side-chains, backbone fragmentation, dimerization/aggregation, unfolding or conformational changes, enzymatic inactivation, and alterations in cellular handling and turnover of proteins [69, 70].

Major substrates for lipid peroxidation are polyunsaturated fatty acids (PUFAs) [31, 36, 37], which are a family of lipids with two or more double bounds, that can be classified in omega-3 (*n*-3) and omega-6 (*n*-6) fatty acids according to the location of the last double bond relative to the terminal methyl end of the molecule. The predominant *n*-6 fatty acid is arachidonic acid (AA), which can be reduced (i) via enzymatic peroxidation to prostaglandins, leukotrienes, thromboxanes, and other cyclooxygenase, lipoxygenase or cytochrome P-450 derived products [4]; or (ii) via nonenzymatic peroxidation to MDA, 4-HNE, isoprostanes, and other lipid peroxidation end-products (more stables and toxic than hydroperoxides) through oxygen radical-dependent oxidative routes [49, 71]. The continued oxidation of fatty acid side-chains and released PUFAs, and the fragmentation of peroxides to produce aldehydes, eventually lead to loss of membrane integrity by alteration of its fluidity which finally triggers inactivation of membrane-bound proteins. Contrary to radicals that attack biomolecules located less than a few nanometres from its site of generation [22], the lipid peroxidation-derived aldehydes can easily diffuse across

membranes and can covalently modify any protein in the cytoplasm and nucleus, far from their site of origin [72].

2.4. Secondary Lipid Peroxidation Products: MDA. MDA is an end-product generated by decomposition of arachidonic acid and larger PUFAs [49], through enzymatic or nonenzymatic processes (Figure 3). MDA production by enzymatic processes is well known but its biological functions and its possible dose-dependent dual role have not been studied although MDA is more chemically stable and membrane-permeable than ROS and less toxic than 4-HNE and methylglyoxal (MG) [49]. So far, only few papers have reported that MDA may act as signaling messenger and regulating gene expression: (i) very recent research indicated that MDA acted as a signaling messenger and regulated islet glucose-stimulated insulin secretion (GSIS) mainly through Wnt pathway. The moderately high MDA levels (5 and 10 μM) promoted islet GSIS, elevated ATP/ADP ratio and cytosolic Ca^{2+} level, and affected the gene expression and protein/activity production of the key regulators of GSIS [73]; (ii) in hepatic stellate cells, MDA induced collagen-gene expression by upregulating specificity protein-1 (*Sp1*) gene expression and Sp1 and Sp3 protein levels [74]. Both Sp1 and Sp3 can interact with and recruit a large number of proteins including the transcription initiation complex, histone modifying enzymes, and chromatin remodeling complexes, which strongly suggest that Sp1 and Sp3 are important transcription factors in the remodeling chromatin and the regulation of gene expression [75]. On the other hand, MDA production by nonenzymatic processes remains poorly understood despite their potential therapeutic value, because this MDA is believed to originate under stress conditions and has high capability of reaction with multiple biomolecules such as proteins or DNA that leads to the formation of adducts [76–78], and excessive MDA production have been associated with different pathological states [79–85] (see Table 1). Identifying *in vivo* MDA production and its role in biology is important as indicated by the extensive literature on the compound (over 15 800 articles in the PubMed database using the keyword "malondialdehyde lipid peroxidation" in December 2013).

MDA Production by Enzymatic Processes. MDA can be generated *in vivo* as a side product by enzymatic processes during the biosynthesis of thromboxane A_2 (Figure 3) [86–90]. TXA_2 is a biologically active metabolite of arachidonic acid formed by the action of the thromboxane A2 synthase, on prostaglandin endoperoxide or prostaglandin H2 (PGH_2) [4, 91, 92]. PGH_2 previously is generated by the actions of cyclooxygenases on AA [4, 91, 93].

MDA Production by Nonenzymatic Processes. A mixture of lipid hydroperoxides is formed during lipid peroxidation process. The peroxyl radical of the hydroperoxides with a cis-double bond homoallylic to the peroxyl group permits their facile cyclization by intramolecular radical addition to the double bond and the formation of a new radical. The intermediate free radicals formed after cyclization can cyclize again to form bicycle endoperoxides, structurally related to prostaglandins, and undergo cleavage to produce MDA. Through nonenzymatic oxygen radical-dependent reaction, AA is the main precursor of bicyclic endoperoxide, which then undergoes further reactions with or without the participation of other compounds to form MDA (Figure 3) [31, 49, 94, 95]. However, it should be possible that other eicosanoids that can also be generated by nonenzymatic oxygen radical-dependent reaction [96–99] may be precursor of bicyclic endoperoxide and MDA. Recent review has addressed the pathways for the nonenzymatic formation of MDA under specific conditions [100].

MDA Metabolism. Once formed MDA can be enzymatically metabolized or can react on cellular and tissular proteins or DNA to form adducts resulting in biomolecular damages. Early studies showed that a probable biochemical route for MDA metabolism involves its oxidation by mitochondrial aldehyde dehydrogenase followed by decarboxylation to produce acetaldehyde, which is oxidized by aldehyde dehydrogenase to acetate and further to CO_2 and H_2O (Figure 3) [49, 101, 102]. On the other hand, phosphoglucose isomerase is probably responsible for metabolizing cytoplasmic MDA to methylglyoxal (MG) and further to D-lactate by enzymes of the glyoxalase system by using GSH as a cofactor [103]. A portion of MDA is excreted in the urine as various enaminals (RNH-CH–CH-CHO) such as N-epsilon-(2-propenal)lysine, or N-2-(propenal) serine [49].

2.4.1. MDA Biomolecules Adducts. As a bifunctional electrophile aldehyde, MDA reactivity is pH-dependent, which exists as enolate ion (conjugate bases having a negative charge on oxygen with adjacent C–C double bond) with low reactivity at physiological pH. When pH decreases MDA exists as beta-hydroxyacrolein and its reactivity increases [49]. MDA's high reactivity is mainly based on its electrophilicity making it strongly reactive toward nucleophiles, such as basic amino acid residues (i.e., lysine, histidine, or arginine). Initial reactions between MDA and free amino acids or protein generate Schiff-base adducts [49, 104, 175]. These adducts are also referred to as advanced lipid peroxidation end-products (ALEs). Acetaldehyde (product of MDA metabolism) under oxidative stress and in the presence of MDA further generates malondialdehyde acetaldehyde (MAA) adducts [157, 176]. MAA adducts are shown to be highly immunogenic [177–181]. MDA adducts are biologically important because they can participate in secondary deleterious reactions (e.g., crosslinking) by promoting intramolecular or intermolecular protein/DNA crosslinking that may induce profound alteration in the biochemical properties of biomolecules and accumulate during aging and in chronic diseases [72, 104, 182, 183]. Important proteins that can be modified by MDA adducts are as follows: (i) eElongation factor 2 (eEF2) catalyzes the movement of the ribosome along the mRNA in protein synthesis. MDA adducts with eEF2 could contribute to decline of protein synthesis, secondary to LP increase (*see below—cumene hydroperoxide-induced lipid peroxidation*); *(ii)* factor H (FH) is the main regulator of the alternative pathway in plasma that tightly controls the activation of complement to prevent attack against host cells. MDA

Table 1: Common pathological processes linked to MDA and 4-HNE.

Pathological processes	Aldehyde	References
Alzheimer's disease	MDA 4-HNE	[104–113] [81, 108, 114–121]
Cancer	MDA 4-HNE	[109, 122–130] [72, 126–128, 131–136]
Cardiovascular diseases	MDA 4-HNE	[72, 79, 109, 123, 135, 137–141] [72, 104, 109, 131, 135, 138, 139, 142–144]
Diabetes	MDA 4-HNE	[79, 109, 123, 140, 145–150] [131, 135, 142, 143, 151–156]
Liver disease	MDA 4-HNE	[123, 135, 157–164] [135, 160–163, 165–169]
Parkinson's disease	MDA 4-HNE	[81, 108, 114–121] [72, 114, 131, 135, 142, 170–174]

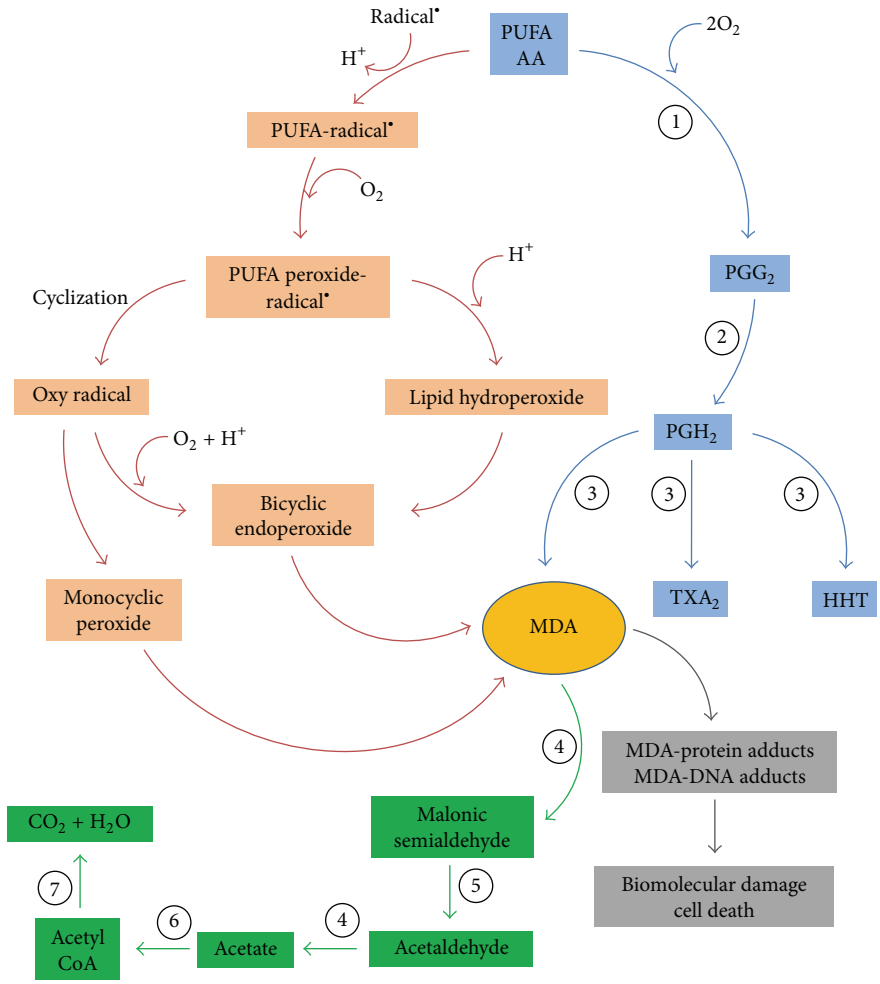

Figure 3: MDA formation and metabolism. MDA can be generated *in vivo* by decomposition of arachidonic acid (AA) and larger PUFAs as a side product by enzymatic processes during the biosynthesis of thromboxane A_2(TXA_2) and 12-l-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (blue pathway), or through nonenzymatic processes by bicyclic endoperoxides produced during lipid peroxidation (red pathway). One formed MDA can be enzymatically metabolized (green pathway). Key enzymes involved in the formation and metabolism of MDA: cyclooxygenases (1), prostacyclin hydroperoxidase (2), thromboxane synthase (3), aldehyde dehydrogenase (4), decarboxylase (5), acetyl CoA synthase (6), and tricarboxylic acid cycle (7).

adducts with FH can block both the uptake of MDA-modified proteins by macrophages and MDA-induced proinflammatory effects *in vivo* in mice [184]; MDA adducts or MAA adducts can promote binding of complement; (iii) anaphylatoxin C3a (proinflammatory complement components) with oxidatively modified low-density lipoproteins (Ox-LDL) and contributes to inflammatory processes involving activation of the complement system in atherosclerosis [185]; and (iv) protein kinase C (PKC) is known to play a major role in intracellular signal transduction affecting such processes as proliferation, differentiation, migration, inflammation, and cytoskeletal organization. BSA-MAA induces the activation of a specific isoform of PKC, PKC-α, in hepatic stellate cells (HSCs) and induces the increased secretion of urokinase-type plasminogen activator, a key component of the plasmin-generating system, thereby contributing to the progression of hepatic fibrosis [186]. A recent review shows a list of up to thirty-three proteins known to be modified by MDA and including enzymatic proteins, carrier proteins, cytoskeletal proteins, and mitochondrial and antioxidant proteins [76].

It has also been proposed that MDA could react physiologically with several nucleosides (deoxy-guanosine and cytidine) to form adducts to deoxyguanosine and deoxyadenosine, and the major product resulting is a pyrimidopurinone called pyrimido[1,2-a]purin-10(3H-)one (M1G or M1dG) [122, 123, 187, 188]. MDA is an important contributor to DNA damage and mutation [122, 124]. The main route for repair of M1dG residues in genomic DNA appears to be the nucleotide excision repair (NER) pathway [188, 189]. In the absence of repair, MDA-DNA adducts may lead to mutations (point and frameshift) [124], strand breaks [122, 190], cell cycle arrest [191], and induction of apoptosis [192]. M1dG is oxidized to 6-oxo-M1dG in rats and that xanthine oxidase (XO) and aldehyde oxidase (AO) are the likely enzymes responsible [193]. This MDA-induced DNA alteration may contribute significantly to cancer and other genetic diseases. Hypermethylated in cancer 1 (HIC1) is a tumor suppressor gene that cooperates with p53 to suppress cancer development. New funding has shown that highest HIC1 methylation levels in tobacco smokers were significantly correlated with oxidative DNA adducts M1dG [125]. Research also suggests that persistent M1dG adducts in mitochondrial DNA hinder the transcription of mitochondrial genes [194]. Dietary intake of certain antioxidants such as vitamins was associated with reduced levels of markers of DNA oxidation (M1dG and 8-oxodG) measured in peripheral white blood cells of healthy subjects, which could contribute to the protective role of vitamins on cancer risk [195].

2.5. Secondary Lipid Peroxidation Products: 4-HNE. 4-Hydroxynonenal (4-HNE), α, β-unsaturated electrophilic compounds, is the major type of 4-hydroxyalkenals end-product, generated by decomposition of arachidonic acid and larger PUFAs, through enzymatic or nonenzymatic processes [49]. 4-HNE is an extraordinarily reactive compound containing three functional groups: (i) C=C double bond that can be target to Michael additions to thiol, reduction or epoxidation, (ii) carbonyl group which can yield acetal/thio acetal or can be target to Schiff-base formation, oxidation, or reduction, and (iii) hydroxyl group which can be oxidized to a ketone [56].

4-HNE is the most intensively studied lipid peroxidation end-product, in relation not only to its physiological and protective function as signaling molecule stimulating gene expression, but also to its cytotoxic role inhibiting gene expression and promoting the development and progression of different pathological states. In the last three years, excellent reviews have been published summarizing both signaling and cytotoxic effects of this molecule in biology, for example, overview of mechanisms of 4-HNE formation and most common methods for detecting and analyzing 4-HNE and its protein adducts [196]. Review focuses on membrane proteins affected by lipid peroxidation-derived aldehydes, under physiological and pathological conditions [131]. Jaganjac and Co-workers have described the role of 4-HNE as second messengers of free radicals that act both as signaling molecules and as cytotoxic products of lipid peroxidation involvement in the pathogenesis of diabetes mellitus (DM) [151]. Chapple and Co-workers summarized the production, metabolism and consequences of 4-HNE synthesis within vascular endothelial, smooth muscle cells and targeted signaling within vasculature [142]. Review focuses on the role of 4-HNE and Ox-PLs affecting cell signaling pathways and endothelial barrier dysfunction through modulation of the activities of proteins/enzymes by Michael adducts formation, enhancing the level of protein tyrosine phosphorylation of the target proteins, and by reorganization of cytoskeletal, focal adhesion, and adherens junction proteins [197]. An overview of molecular mechanisms responsible for the overall chemopreventive effects of sulforaphane (SFN), focusing on the role of 4-HNE in these mechanisms, which may also contribute to its selective cytotoxicity to cancer cells [198]. Perluigi and Co-workers summarized the role of lipid peroxidation, particularly of 4-HNE-induced protein modification, in neurodegenerative diseases. In this review, the authors also discuss the hypothesis that altered energy metabolism, reduced antioxidant defense, and mitochondrial dysfunction are characteristic hallmarks of neurodegenerative [170]. Zimniak described the effects of 4-HNE and other endogenous electrophiles on longevity, and its possible molecular mechanisms. The role of electrophiles is discussed, both as destabilizing factors and as signals that induce protective responses [199]. Reed showed the relationship between lipid peroxidation/4-HNE and neurodegenerative diseases. It also demonstrates how findings in current research support the common themes of altered energy metabolism and mitochondrial dysfunction in neurodegenerative disorders [171]. Fritz and Petersen summarized the generation of reactive aldehydes via lipid peroxidation resulting in protein carbonylation, and pathophysiologic factors associated with 4-HNE-protein modification. Additionally, an overview of *in vitro* and *in vivo* model systems used to study the physiologic impact of protein carbonylation, and an update of the methods commonly used in characterizing protein modification by reactive aldehydes [200]. Butterfield and Co-workers showed that several important irreversible protein

modifications including protein nitration and 4-HNE modification, both which have been extensively investigated in research on the progression of Alzheimer's disease (AD) [201]. Balogh and Atkins described the cellular effects of 4-HNE, followed by a review of its GST-catalyzed detoxification, with an emphasis on the structural attributes that play an important role in the interactions with alpha-class GSTs. Additionally, a summary of the literature that examines the interplay between GSTs and 4-HNE in model systems relevant to oxidative stress is also discussed to demonstrate the magnitude of importance of GSTs in the overall detoxification scheme [202]. Like MDA, 4-HNE has high capability of reaction with multiple biomolecules such as proteins or DNA that lead to the formation of adducts [49].

4-HNE Production by Enzymatic Processes. 4-HNE is a lipid peroxidation end-product of enzymatic transformation of *n*-6 PUFAs (AA, linoleic acid, and other) by 15-lipoxygenases (15-LOX). Two different 15-LOX exist, (i) 15-LOX-1 (reticulocyte type) expressed in reticulocytes, eosinophils, and macrophages; (ii) and 15-LOX-2 (epidermis type) expressed in skin, cornea, prostate, lung, and esophagus [203–205]. Mice do not express 15-LOX and only express the leukocyte-derived 12-LOX. In plant enzymatic route to 4-HNE includes lipoxygenase (*LOX*), -hydroperoxide lyase (*HPL*), alkenal oxygenase (AKO), and peroxygenases (Figure 4) [206]. The main precursors of 4-HNE in human are 13-hydroperoxyoctadecadienoic acid (13-HPODE) produced by the oxidation of linoleic acid by 15-LOX-1 [207] and 15- hydroperoxyeicosatetraenoic acids (15-HPETE) produced by the oxidation of AA by 15-LOX-2 [208]. These compounds are short lived and are catabolised into various families of more stable compounds such as 15-HETEs, lipoxins, and leukotrienes [4]. 15-HPETE is associated with anti-inflammatory and proapoptotic functions (the release of cytochrome *c*, activation of caspase-3 and 8, PARP, and Bid cleavage) and DNA fragmentation [209, 210].

4-HNE Production by Nonenzymatic Processes. 4-HNE can be formed through several nonenzymatic oxygen radical-dependent routes involving the formation of hydroperoxides, alkoxyl radicals, epoxides, and fatty acyl crosslinking reactions. Spickett C [196] recently reviewed the mechanisms of formation of 4-HNE during lipid peroxidation and showed that the main processes leading to 4-HNE are likely beta-cleavage reaction of lipid alkoxy-radicals, which can be *summarized* into five generic mechanisms: (i) reduction of the hydroperoxide to a lipid alkoxy radical by transition metal ions, such as Fe^{2+} followed by b-scission; (ii) protonation of the lipid hydroperoxide yields an acidified lipid hydroperoxide that undergoes Hock rearrangement of a C–C to C–O bond followed by hydrolysis and Hock cleavage; (iii) the lipid peroxyl radical of the hydroperoxides permits their facile cyclization to dioxetane and ending with dioxetane cleavage; (iv) free radical attack to *ω*-6 PUFA on bis-allyl site yielding a free radical intermediate, that further reacts with molecular oxygen to generate hydroperoxide derivatives such as 13-HPODE or 15-HPETE. The abstraction of an allylic hydrogen

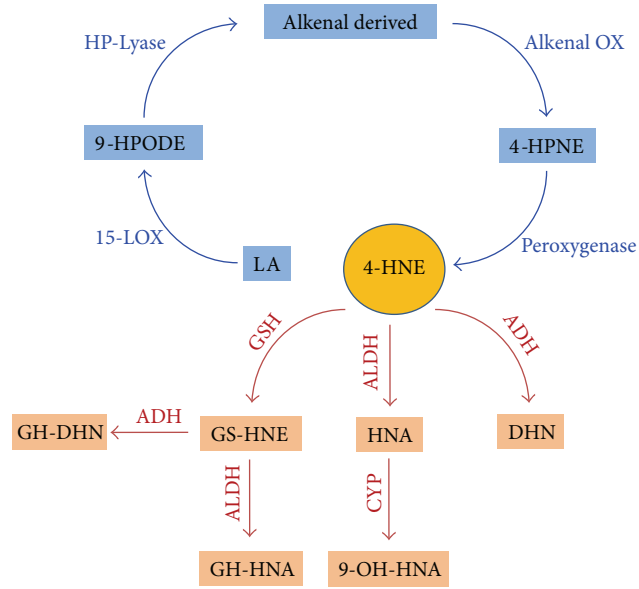

Figure 4: Enzymatic production of 4-HNE and metabolism. In plant enzymatic route to 4-HNE includes lipoxygenase (LOX), -hydroperoxide lyase (HPL), alkenal oxygenase (AKO), and peroxygenases. 4-HNE metabolism may lead to the formation of corresponding alcohol 1,4-dihydroxy-2-nonene (DHN), corresponding acid 4-hydroxy-2-nonenoic acid (HNA), and HNE–glutathione conjugate products. 4-HNE conjugation with glutathione s-transferase (GSH) produce glutathionyl-HNE (GS-HNE) followed by NADH-dependent alcohol dehydrogenase (ADH-)catalysed reduction to glutathionyl-DNH (GS-DNH) and/or aldehyde dehydrogenase (ALDH-)catalysed oxidation to glutathionyl-HNA (GS-HNA). 4-HNE is metabolized by ALDH yielding HNA, which is metabolized by cytochrome P450 (CYP) to form 9-hydroxy-HNA (9-OH-HNA). 4-HNE may be also metabolized by ADH to produce DNH.

of their structure produce another radical intermediate that after oxygenation step forms the corresponding dihydroperoxyde derivative (unstable), which after Hock rearrangement and cleavage produces 4-hydroperoxy-2E-nonenal (4S-HPNE), immediate precursor of HNE; and (v) the oxidation products generated after reaction of linoleate-derived hydroperoxy epoxide (13-Hp-Epo-Acid) with Fe^{+2} yields an alkolxyl radical, which undergo to di-epoxy-carbinyl radical and after beta-scission yield different aldehydes compounds including 4-HNE (Figure 5).

Once formed 4-HNE, and depending of cell type and cellular metabolic circumstances can promote cell survival or death. Cells expressing differentiated functions representative for the *in vivo* situation react more sensitively to 4-HNE than cell lines. The different response with respect to the endpoints of genotoxicity probably depends on the different metabolizing capacities and thus the action of different metabolites of 4-HNE [211]. 4-HNE can be enzymatically metabolized at physiological level and cells can survive; 4-HNE can play an important role as signaling molecule stimulating gene expression (mainly Nrf2) with protective functions that can enhance cellular antioxidant capacity and exert adaptive response when 4-HNE level is low; under this circumstances

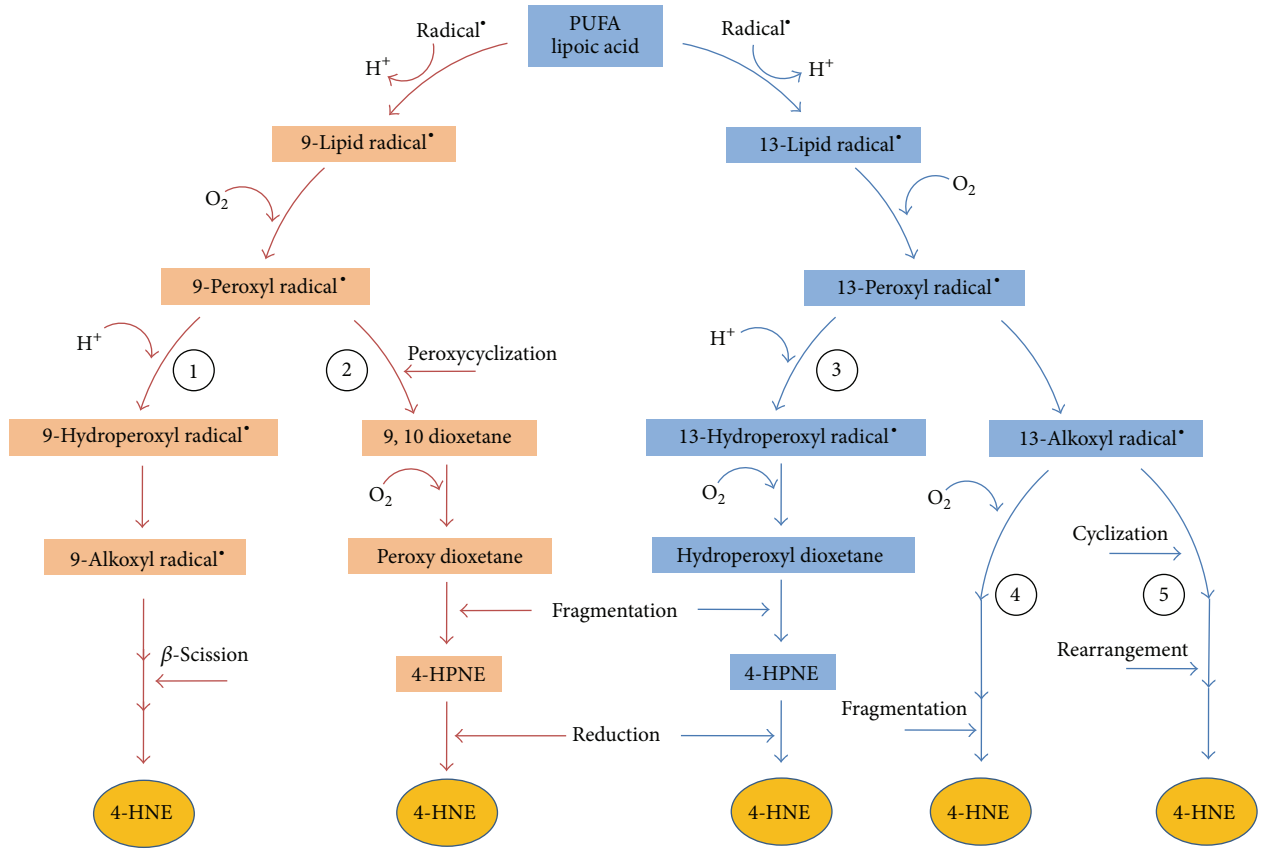

Figure 5: Nonenzymatic 4-HNE production. Initial abstraction of bisallylic hydrogen of lipoic acid (LA) produces fatty radicals. 4-HNE formation starting with 9- and 13-hydroperoxyoctadecadienoate (HPODE) (red and blue pathways, resp.). 4-HNE is generated by beta-scission of a hydroxyalkoxy radical that is produced after cyclization of alkoxy radical in the presence of transition metal ions and two molecules of oxygen; this reaction involves hydrogen abstraction (1). Peroxy radical cyclizes to form a dioxetane which is oxygenated to peroxy-dioxetane that is fragmented and after two hydrogen abstractions produce 4-HNE (2). Hydroperoxyl radical is oxygenated to dioxetane that is further fragmented to produce 4-hydroperoxy-2E-nonenal (4-HPNE), an immediate precursor of 4-HNE (3). Bicyclic endoperoxides react with reduced form of transition metal, such as iron (Fe^{2+}) to produce alkoxyl radicals which after reaction with oxygen (O_2), hydrogen abstraction (H^+), and fragmentation produce 4-HNE (4). Alkoxyl radical after cyclization, oxygenation, hydrogen abstraction, oxidation of transition metal, hydrolysis, and rearrangement yields 4-HNE (5). With arachidonic acid, 11- and 15- hydroperoxyeicosatetraenoic acids (HPETE) are the precursors to form 4-HNE via the analogous mechanisms.

cells can survive; 4-HNE can promote organelle and protein damage leading to induction of autophagy, senescence, or cell cycle arrest at 4-HNE medium level and cells can subsist; and finally 4-HNE induces apoptosis or necrosis programmed cell death at 4-HNE high or very high level, respectively, and cells die. These processes eventually lead to molecular cell damage which may facilitate development of various pathological states. High levels of 4-HNE can also react with proteins and/or DNA to form adducts resulting in a variety of cytotoxic and genotoxic consequences (Figure 6).

4-HNE Metabolism. The main goal of the rapid intracellular metabolism of 4-HNE in mammalian cells is to protect proteins from modification by aldehydic lipid peroxidation products [212]. The biochemical routes of 4-HNE metabolism that lead to the formation of corresponding alcohol 1,4-dihydroxy-2-nonene (DHN), corresponding acid 4-hydroxy-2-nonenoic acid (HNA), and HNE-glutathione conjugate products can be summarized according to stress levels: (i) under physiological or low stress levels the major 4-HNE detoxification step is conjugation with GSH to yield glutathionyl-HNE (GS-HNE) or glutathionyl-lactone (GS-)lactone (cyclic ester 4-HNE- form) followed by NADH-dependent alcohol dehydrogenase (ADH-)catalysed reduction to glutathionyl-DNH (GS-DNH) and/or aldehyde dehydrogenase (ALDH-)catalysed oxidation to glutathionyl-HNA (GS-HNA); (ii) at moderate stress levels, 4-HNE undergoes aldehyde dehydrogenase (ALDH-)catalysed oxidation yielding HNA, that may be further metabolized in mitochondria through beta-oxidation by cytochrome P450 to form 9-hydroxy-HNA; and (iii) at high stress levels, 4-HNE is metabolized by ADH (that belongs to the aldo-keto reductase (AKR) superfamily) to produce DNH [131, 196, 202, 212, 213] (Figure 4). By disrupting the *Gsta4* gene that encodes the alpha class glutathione s-transferase (GST) isozyme GSTA4-4 in mice showed that GSTA4-4 plays a major role in

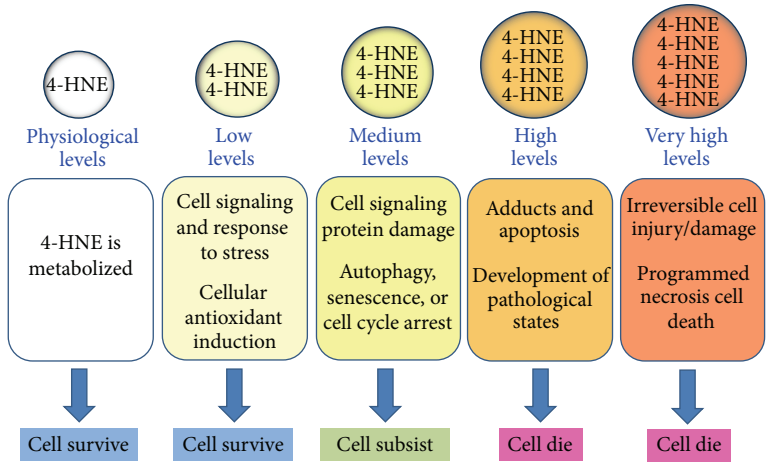

Figure 6: 4-HNE promotes cell survival or induces cell death. Depending on cell type, damage/repair capacities and cellular metabolic circumstances 4-HNE can promote cell survival or induce cell death. 4-HNE at physiological levels is enzymatically metabolized and at low levels plays an important role as signaling molecule stimulating gene expression, enhance cellular antioxidant capacity and exert adaptive response; at medium levels organelle and protein damage lead to induction of autophagy, senescence, or cell cycle arrest and at high or very high levels promote adducts formation and apoptosis or necrosis cell death, respectively.

protecting cells from the toxic effects of oxidant chemicals by attenuating the accumulation of 4-HNE [214]. Overexpression and inhibition of ALDH activity reduce and increase, respectively, the 4-HNE toxicity and 4-HNE-protein adducts levels in cell culture [215, 216].

2.5.1. 4-HNE as Signaling Molecule. At moderate concentration, when the basal level of antioxidant enzymes cannot be sufficient to neutralize 4-HNE, cells can survive due to 4-HNE may regulate several transcription factors sensible to stress such as nuclear factor erythroid 2-related factor 2 (Nrf2), activating protein-1 (AP-1), NF-κB, and peroxisome-proliferator-activated receptors (PPAR). It also activates stress response pathways such as mitogen-activated protein kinases (MAPK), EGFR/Akt pathways, and protein kinase C. Different labs demonstrated the 4-HNE-dependent induction of *Nrf2*, a primary sensor and oxidative stress regulator [217–221]. Also administration of the Nrf2-ARE activators protect from 4-HNE toxicity [222]. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by the repressor protein Keap1, but in response to oxidant stimuli Nrf2 is activated and translocated into the nucleus where mediate the transcription of antioxidant/cytoprotective genes by binding to the antioxidant-response element (ARE) within DNA [223]. The Nrf2-ARE pathway has essential role in different pathological states such as neurodegenerative diseases [223], cancer [224], diabetes [225], and infectious disease [226]. The main genes regulated by 4-HNE- induced Nrf2-ARE pathway are as follows: (i) HO-1, an antioxidant protein that catalyzes the degradation of heme to biliverdin, which is then degraded to bilirubin; both biliverdin and bilirubin have antioxidant properties [227]; 4-HNE can upregulate HO-1 [217, 220, 221, 228–230]; (ii) thioredoxin (Trx) and thioredoxin reductase (TrxR); Trx is a small (13 kDa) antioxidant ubiquitous protein with two redox-active cysteine residues (-Cys-Gly-Pro-Cys-) in its active center; oxidized Trx is reduced back to the active form of Trx by Trx reductase (TrxR) in the presence of NADPH [231]; 4-HNE can upregulate Trx/TrxR [220, 221, 232]; (iii) glutamate cystein ligase (GCL) is a major determinant enzyme in GSH synthesis [233, 234]. 4-HNE can upregulate GCL [235–239].

Involvement of *AP-1* transcription factor in 4-HNE-induced cell signaling has been demonstrated by several studies which showed an AP-1 upregulation by 4-HNE [240–243]. Activation of AP-1 binding may lead to the 4-HNE-induced increase in GSH content [239]. AP-1 is a dimer consisting of basic region-leucine zipper proteins from the Jun and Fos subfamilies. AP-1 transcription factors control cell proliferation, survival, and death. Growth factors, cytokines, cellular stress, and many other stimuli activate AP-1 [244, 245].

NF-κB is a dimeric transcription factor that regulates diverse biological processes, including immune responses, inflammation, cell proliferation, and apoptosis. The NF-κB protein complex is retained in an inactive state in the cytoplasm by binding to inhibitory proteins IκBs family [246]. Various cellular stimuli, such as oxidative stress, IκBs are phosphorylated, making them susceptible to degradation by the ubiquitin-proteasome system. This results in nuclear translocation of NF-κB complex where it can bind to various promoter areas of its target genes and induce gene transcription of the corresponding genes [246, 247], most of which are implicated in the regulation of inflammation. 4-HNE can activate or inhibit NF-κB depending on the type of cells used. For example, 4-HNE inhibited the activity of NF-κB in hepatocytes [165], cortical neurons [248], ARPE-19 human retinal pigment epithelial cells [249], Kupffer cells [250], human aortic endothelial cells [251], human colorectal carcinoma, and lung carcinoma cell [252]. On the

contrary, 4-HNE induced activity of NF-κB in macrophages [253], vascular smooth muscle cells [254], PC12 cells [255], optic nerve head astrocytes [256], human osteoarthritic chondrocytes [257], human fibroblasts [258], and human monocytic lineage cells [259].

PPARs comprise three subtypes (PPARα, β/δ, and γ) to form a nuclear receptor superfamily. PPARs act as key transcriptional regulators of lipid metabolism, mitochondrial biogenesis, and antioxidant defense [260, 261]. PPARs interaction/modulation with 4-HNE has been reviewed [262]. 4-HNE increased PPAR-γ gene expression and accelerated adiponectin protein degradation in adipocytes [263]; expression of PPAR-γ was induced in HL-60 and U937 cells by 4-HNE treatment [264], whereas in the colon cancer cell (CaCo-2) PPARγ protein expression was not induced after 4-HNE treatment [265]; 4-HNE increased PPARγ2 expression in C2C12 cells [266]. PPAR-β/δ is activated by 4-HNE in 3T3-L1 preadipocytes cells [267]. 4-HNE activates PPAR-δ and amplifies insulin secretion in INS-1E β-cells [152].

MAP kinases family can be activated in response to diverse stimuli such as oxidative stress, lipopolysaccharides, inflammatory cytokines, growth factors, or endoplasmic reticulum (ER) stress and are involved in several cellular responses like cell proliferation and/or differentiation, inflammation, proteasomal-mediated protein degradation, and apoptosis. Members of the major mitogen-activated protein kinase (MAPK) subfamilies are the extracellular signal-regulated kinase (ERK), p38, and Jun N-terminal kinase (JNK) subfamilies. The mechanism by which MAPK signaling cascades are activated by 4-HNE is not well known. For example, activation of different MAPK under various stimuli can affect both apoptotic and prosurvival signaling. In corneal epithelial cells, 4-HNE caused a time-dependent induction of HO-1 mRNA and protein via modification and activation of Erk1/2, JNK and p38 MAP kinases, as well as phosphoinositide-3-kinase (PI3)/Akt. Inhibition of p38 blocked 4-HNE-induced HO-1 expression; inhibition of Erk1/2 and, to a lesser extent, JNK and PI3 K/Akt suppressed 4-HNE-induced HO-1 [268]. 4-HNE also stimulated Erk1/2, JNK, p38, and PI3 kinase in keratinocyte, and the inhibitors of these enzymes suppressed 4-HNE-induced expression of HO-1 [269]. In PC12 cells, 4-HNE treatment induced ERK, JNK, and p38 MAPK activation as well as induced the expression of HO-1. Addition of p38 MAPK specific inhibitor SB203580 attenuated HO-1 upregulation; these results indicate that 4-HNE-induced transient p38 MAPK activation may serve as an upstream negative regulator of ER stress and confer adaptive cytoprotection against 4-HNE-mediated cell injury [228]. In rat liver epithelial RL34 cells, 4-HNE upregulates the cyclooxygenase-2 (COX-2, which plays a key role in conversion of free arachidonic acid to PGs) expression by the stabilization of COX-2 mRNA via activation of the p38 MAPK pathway [270]. In human hepatic stellate cells (hHSC), 4-HNE forms adducts with JNK and this event leads to JNK nuclear translocation and activation as well as to c-jun and AP-1 induction [271]. In human bronchial epithelial cells, 4-HNE downmodulates the protein-tyrosine phosphatase SH2 domain containing phosphatase-1 (SHP-1) which negatively regulates JNK activity [272]. We can also see the protective effects of MAPK activation via GSH induction because the activation of the ERK pathway is involved in GCL (the rate-limiting enzyme in de novo glutathione (GSH) synthesis) regulation in rat cells [273] while the JNK pathways appear to be involved in human HBE-1 cells [274].

In human monocytes, 4-HNE was shown to significantly inhibit p38 and ERK activity, which resulted in inhibition of TNF and interleukin-1beta production in response to LPS. The data suggest that 4-HNE, at nontoxic concentrations, has anti-inflammatory properties [275]. In human osteoarthritic osteoblasts, 4-HNE also showed a significant (approximately 70%) decrease of TNF-α-induced IL-6 mRNA expression via the NF-κB signaling pathway. However, only p38 MAPK and JNK1/2 were activated, but not ERK1/2 [276], while 4-HNE also induced COX-2 expression and prostaglandin E2 (PGE2) release [257, 276].

On the other hand, 4-HNE mediated depletion of intracellular thiols, protein tyrosine phosphorylation, MAPK (JNK, ERK, and p38) activation, and modulates integrin resulting in reorganization of cytoskeletal, focal adhesion proteins, and barrier dysfunction in lung microvascular endothelial cells [277]. Results suggest that activation and phosphorylation of MAP kinases (JNK, ERK, and p38) play an important role in 4-HNE mediated toxicity and cell death in mouse embryonic fibroblasts (MEF), and absence of GSTA4–4 potentiates the cytotoxic effects of 4-HNE. The increase of apoptosis in *Gsta*4 null MEF by 4-HNE was associated with the enhanced accumulation of 4-HNE-protein adducts, DNA damage, and the activation of caspases-3, -8, and -9 [214]. 4-HNE upregulates and phosphorylates cytosolic phospholipase A-2 (cPLA-2) in cultured microglial cell line (Ra2) via the ERK and p38 MAPK pathways [278]. cPLA is a proinflammatory enzyme that stimulate AA- release by hydrolyzes glycerophospholipids with AA in the *sn*-2 position.

Matrix metalloproteinases *(MMPs)* constitute a large group of endoproteases that are not only able to cleave all protein components of the extracellular matrix but also to activate or inactivate many other signaling molecules, such as receptors, adhesion molecules, and growth factors [279]. 4-HNE induced MMP-9 production in macrophages [280] and MMP-2 in vascular smooth muscle cells (VSMC) [281] via activation of ERK and p38 MAPK pathways, consequently leading to plaque instability in atherosclerosis. 4-HNE also enhances MMP-2 production in VSMC via mitochondrial ROS-mediated activation of the Akt/NF-kappaB signaling pathways [254]. In osteoarthritic (OA) synovial cells, 4-HNE induced MMP-13 mainly through activation of p38 MAPK [282].

Akt (a.k.a protein kinase B or PKB) comprises three closely related isoforms Akt1, Akt2, and Akt3 (or PKB$\alpha/\beta/\gamma$ resp.), which play a role in the regulation of cell proliferation, survival, and metabolism. Dysregulation of Akt leads to diseases such as cancer, diabetes, and cardiovascular and neurological diseases [283]. Under conditions of enhanced oxidative stress, a major cellular response is the activation of the Akt pathway that involves the oxidation and subsequent inactivation of PTEN (phosphatase and tensin homolog deleted on chromosome 10), a tumor suppressor

and primary regulator of Akt [284]. Recent studies have also demonstrated that activation of PI3 K/Akt signaling by 4-HNE occurs via modification and inhibition of PTEN, a regulatory protein that suppresses Akt2 activity, which is selectively phosphorylated by 4-HNE in both cellular human hepatocellular carcinoma cell line (HepG2) [285] and animal models (ethanol-fed mice) [286]. In HepG2 cells, 4-HNE inhibits H_2O_2-mediated activation of the Akt pathway in leading to phosphorylation of Akt1 but not Akt2, decreased cell proliferation, and decreased expression of cyclin D1 [287]. In retinal pigment epithelial (RPE) cells, at lower concentrations 4-HNE triggered phosphorylation of epidermal growth factor receptor (EGFR) and activation of its downstream signaling components ERK1/2 and Akt; this led to protective mechanism against oxidative stress [288]. Akt-induced activity by 4-HNE promotes cell survival through induction of HO-1 mRNA and protein in corneal epithelial cells [268], and in keratinocyte [269]. The inhibitors of Akt suppressed 4-HNE-induced expression of HO-1.

Protein kinases C (PKCs) are a family of multifunctional enzymes that play crucial roles in the transduction of many cellular signals such as control of cell proliferation, survival, and transformation by phosphorylating various targets. The PKC family consists of three different groups: conventional (α, β1, β2, and γ), novel (δ, ε, η, and θ), and atypical (ζ and λ/τ). Conventional and novel PKC isoforms are lipid-sensitive enzymes and calcium-dependent and are usually activated by growth factors through stimulation of phospholipase C (PLC) which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to generate inositol triphosphate (IP3) and DAG [6, 289]. Cells can express more than one PKC isoform, and individual PKCs can mediate different biological processes. For example, in human promyelocytic leukemia (HL-60) cells [290–292] and rat neutrophils [293] 4-HNE induced a significant increase of PLC activity, which should result in an increased production of IP3 and DAG, known to stimulate PKC [289]. Phagocytes, such as granulocytes and monocytes/macrophages which engulf microbial intruders and effectively kill and eradicate the foreign bodies, contain a membrane-associated NADPH oxidase that produces superoxide leading to other ROS with microbicidal, tumoricidal, and inflammatory activities [294]. In RAW 264.7 mouse macrophage cells, 4-HNE exhibited a concentration-dependent inhibition of ROS by adduction to PKC, a protein vital in the assembly and activation of NADPH oxidase [295]. In rat hepatocyte PKC- isoforms activity is differentially regulated by concentrations 4-HNE. For example, PKC-α activity was decreased in a dose-dependent manner by all concentrations of 4-HNE, while low concentrations of 4-HNE increased PKC βI and, to a much greater extent, PKC βII activities. By contrast, they were unaffected or even inhibited by higher concentrations of 4-HNE. This PKC-dependent- 4-HNE regulation could be involved in the traffic of secretory glycoproteins [296]. In NT2 neurons, low 4-HNE concentrations (similar to concentrations detected in AD brain tissue) induced a 2–6 fold increase of intracellular amyloid β-protein (Aβ) production that was concomitant with selective activation of βI and βII PKC isoforms [297, 298]. In macrophages, a marked and early upregulation of monocyte chemoattractant protein 1 (MCP-1) release occurs in response to low 4-HNE concentrations, most likely through of the increase in the activity of PKC-βI and βII classic isoforms, while the activation of PKC-δ appeared to be involved in LPS-stimulated cells [299]. Treatment of macrophages with 4-HNE, cell-permeable esters of glutathionyl-4-hydroxynonenal (GS-HNE) and glutathionyl-1,4-dihydroxynonane (GS-DHN) activated NF-κB and PLC/PKC. Aldolase reductase catalyzes the reduction of GS-HNE to GS-DHN. AR inhibition/ablation prevented PLC, PKC, and IKKalpha/beta, and NF-κB activation caused by 4-HNE and GS-HNE, but not by GS-DHN, suggests a novel role for a reduced glutathione-lipid aldehyde conjugate (such as GS-DHN) as an obligatory mediator of ROS-induced cytotoxicity [300].

2.5.2. Effect of 4-HNE on Autophagy. One of the most important processes for maintaining normal metabolic and redox signaling, through degradation of damaged proteins and organelles, is autophagy-lysosomal pathway [301]. 4-HNE can promote protein-adducts leading to protein damage and to induction of autophagy-lysosomal pathway [302], a process that is increased by treatment with an autophagy stimulator, rapamycin. If autophagy is blocked with a PI3 K inhibitor, 3-methyladenine, apoptotic cell death occurs [301, 302]. Several mechanisms by which 4-HNE induces autophagy have been reported. For example, 4-HNE promotes the formation of protein adducts that accumulate in the endoplasmic reticulum (ER) and led to autophagy in rat aortic smooth muscle cells, through selective activation of the PKR-like ER kinase (PERK) pathway accompanied by JNK activation, the upregulation of the HO-1, increased microtubule-associated protein 1 light chain 3 (LC3) formation, and maintenance of cell viability under conditions of excessive 4-HNE-protein adducts accumulation [303]. In differentiated SH-SY5Y neuroblastoma cells, glucose-dependent autophagy serves as a protective mechanism in response to 4-HNE because low 4-HNE-concentrations increased autophagy and induced concentration dependent CASP3/caspase-3 activation and cell death. Additionally inhibition of glucose metabolism by 2-deoxyglucose and glycolysis by koningic acid, a GAPDH, inhibitor, led to autophagy inhibition and increased CASP3 activation and cell death [304]. On the contrary, phagocytosis of 4-HNE- and MDA-modified photoreceptor outer segments (POS) induced a marked reduction of autophagic activity by 40% in retinal pigment epithelium (RPE) cells, which may contribute to RPE cell dysfunction and degeneration. In contrast, unmodified POS had no significant effect on autophagy [305].

2.5.3. Effect of 4-HNE on Senescence. Cellular senescence, defined as arrest during the cell cycle (G0), is involved in the complex process of the biological aging of tissues, organs, and organisms. Senescence is driven by many factors including oxidative stress, the DNA damage/repair response, inflammation, mitogenic signals, and telomere shortening. Telomeres are considered a "biological clock" of the cell and are shortened by each cell division until a critical

length is reached and dysfunction ensues. Rapid telomere shortening may indicate a very high cellular activity. DNA-repair pathways are then recruited and cells enter senescence, losing their capacity to proliferate. In addition to cell division, factors causing telomere shortening include DNA damage, inflammation, and oxidative stress [306]. Activation of a DNA damage response including formation of DNA damage foci containing activated H2A.X (γ-histone 2A.X) at either uncapped telomeres or persistent DNA strand breaks is the major trigger of cell senescence. γH2AX is a sensitive marker of DNA damage, particularly induction of DNA double-strand breaks [307]. The length of telomeres depends on the telomerase activity and the catalytic subunit of telomerase (hTERT) which is strongly upregulated in most human cancers [308], and the major consequence of the reactivation of telomerase activity is that tumor cells escape from senescence. The expression of c-*myc* (an activator), *mad-1* (a repressor) and *sp-1* (an activator/repressor), which have been shown to activate *hTERT* transcription. The formation of 4-HNE-proteins adducts in general increased as a function of age [309]. Quantitative evaluation showed that the majority of senescent hepatocytes (as measured by γ-H2A.X) were also positive for 4-HNE [310, 311]. 4-HNE can induce premature senescence by a direct suppression of telomerase activity affecting the expression of hTERT. In endothelial cells (EC) isolated and cultured from arterial segments of patients with severe coronary artery disease, chronic treatment with an antioxidant (that significantly decreased the levels of lipid peroxidation, that is, 4-HNE expression) N-acetyl-cystein, NAC, significantly delayed cellular senescence via decrease of DNA damage marker (γH2AX), decrease of nuclear p53, and increase in hTERT activity [312]. In three human leukemic cell lines (HL-60, U937, and ML-1) [313] and in colon cancer cells (Caco-2 and HT-29) [314], telomerase activity and hTERT expression were downregulated by 4-HNE, as a consequence of downregulation of c-*myc* mRNA expression and *c-Myc* DNA binding activity as well as upregulation of *mad-1* mRNA expression and *Mad-1* DNA binding activity. On the other hand, 4-HNE may induce cellular senescence through activation of critical cell cycle sentinels that mediate this process, such as the tumor suppressor proteins p53 (*see below*), which is well known to play a central role in senescence [315–320]. p53 protects cells of oxidative stress and promotes DNA repair. However, when in the cells the extent of damage overwhelms repair capacities, p53 induces cell death [315–319]. All these data thus confirmed a cell-specific association between senescence and 4-HNE.

2.5.4. Effect of 4-HNE on Cell Cycle and Proliferation. In cell cycle the transition of different phases is driven by several phase-specific cyclin-CDK (cyclin-dependent kinase) complexes which previously have been activated. In response to mitogens, cyclin D is activated and phosphorylate retinoblastoma protein (RB) which leads to activation of E2F proteins and the expression of E2F-responsive genes inducing cells to reenter the cell cycle from quiescence called G0, to G1. Activation of E2F leads to the transcription of cyclin E for transition from G1 to S phase. Subsequent expression of cyclin A leads to transition of S to G2 and cyclin B leads G2 to M phases [321, 322]. The promitotic factor Cdc25 stimulates cell cycle progression through the activation of cyclin A-Cdk1, cyclin B-Cdk1, and cyclin E-Cdk2 for entry into M phase by removing the inhibitory phosphorylation on Cdk1 and Cdk2. On the contrary, the anti-mitotic factor (p21, p27, p57) inhibit cell cycle progression through inhibition of cyclin A–Cdk1, cyclin B–Cdk1, cyclin E–Cdk2 and cyclin D–Cdk4/6 [321–323]. In response to 4-HNE, the expression of key components of cell cycle can be modulated and cells are arrested at G1 or G2. Several studies showed that in general 4-HNE may induce cell cycle arrest in malignant cell and inhibition or decrease of cell proliferation. For example, treatment of HL-60 cells with 4-HNE (1 μM) causes a p53-independent increase of p21 expression, RB dephosphorylation, progressive reduction in the amount of free E2F bound to DNA, and a relative increase in E2F complexes at higher molecular weights with repressive activity decrease of E2F complexes [324], and decrease of cyclin D1, cyclin D2, and cyclin A [325]. In human erythroleukemia cells (K562), 4-HNE treatment increased p53 and p21 expression and decreased expression of cyclin D2. The additional decrease of A- and B-cyclin suggests that the S- and G2-phase were also retarded contributing to the overall slowdown of the cycle [326]. In human breast cancer cells (MCF7) the increase in endogenous levels of 4-HNE caused by treatment with conjugated linoleic acid (CLA) resulted in the inhibition of cell proliferation through a p53-dependent mechanism [327]. In human osteosarcoma cells (HOS), 4-HNE treatment declined gradually the proportion of cells in mitosis, inhibited proliferation and differentiation, and increased apoptosis [328]. In malignant cells like hepatome cells, with a below-normal content of PUFAs and very high expression of aldehyde dehydrogenase-3 (ADH3) which metabolize *4-HNE* to DNH, the inhibitory effects of 4-HNE on cell proliferation are lower, but the inhibition of ADH3 resulted in an increase in the quantity of aldehyde in the cells and inhibit cell proliferation through the MAPK pathway by reduction of pRaf-1 and pERK1,2 [329, 330]. Moreover, 4-HNE has also antiproliferative/differentiative effect mainly in malignant cell, by affecting the expression of key genes, such as oncogenes (e.g., *c-myc* and c-*myb*) and cyclins. In three human leukemic cell lines (HL-60, U937, and ML-1) [313] and in colon cancer cells [265, 314], cell proliferation was inhibited by 4-HNE, as a consequence of downregulation of c-*myc* mRNA. 4-HNE mediated inhibition of cell proliferation in the HL-60 cell line by downregulation of Notch1, which is involved in expression of cyclin D1 and c-Myc [331]. In SK-N-BE human neuroblastoma cells, 4-HNE upregulated p53 family gene expression and p53 gene targets p21 and bax, and the consequent reduction in S-phase cells and the increased apoptotic cell proportion; 4-HNE also reduced cyclin D2 expression [332]. In HepG2 cells, 4-HNE decreased both cell survival and proliferation as evidenced by MTT assays and EdU incorporation as well as decreased expression of cyclin D1 and β-catenin [287]. In K562 cells [333], HL-60 human leukemic cell line [334], and murine erythroleukemia (MEL) cells [335], 4-HNE inhibited c-myc expression; a oncogene is involved in the regulation of cellular multiplication and transformation (see review of Barrera and co-workers [336]).

All these effects increased the proportion of G0/G1 cells, indicating cell cycle arrest at G1 [324, 325, 336, 337]. 4-HNE-induced G2/M cell cycle arrest was via p21 through a mechanism (s) that is independent of p53. The cell cycle arrest leads to apoptotic cell death [338]. *Enterococcus faecalis*—infected macrophages produce 4-HNE. This electrophile, when purified, mediated bystander effects in colonic epithelial cells by generating γH2AX foci and inducing G2/M cell cycle arrest. 4-HNE was also associated with mitotic spindle damage, activation of stathmin, cytokinesis failure, and the development of tetraploid [339]. In PC3 prostate cancer cell, 4-HNE induced G2/M cell cycle arrest by decreasing p-Cdc2 (entry into M phase is determined by activation of the Cdc2 protein kinase, which requires Cdc2 dephosphorylation); increased amount of p-H2A.X indicated that 4-HNE induced apoptotic cell death after a G2/M accumulation [340].

In an opposite way, different studies indicated that 4-HNE can promote cell proliferation in normal cells, mainly by upregulation of cyclin or E2F. In cultured primary cortical neurons, 4-HNE increased the protein levels of phospho-p53 and cell cycle-related proteins (cyclin D3, cyclin D1, and CDC25A), caspase-3 activation, PARP cleavage, calpain activation, serine/threonine kinase 3 (Stk3), and sphingosine phosphate lyase 1 (Sgpl1) upregulation. NAC decreased cell death [341]. In smooth muscle cells (SMCs), treatment with 4-HNE enhanced cyclin D1 expression and activation of the ERK signaling pathway, which were stronger in young SMCs compared with aged SMCs [342]. 4-HNE induced vascular smooth muscle cell proliferation [142, 343]. Aldose reductase (AR) efficiently reduces 4-HNE and GS-HNE. Inhibition of AR can arrest cell cycle at S phase. In VSMC cells, the inhibition of AR prevents high glucose (HG-) and/or TNF-alpha-induced VSMC proliferation by accumulating cells at the G1 phase of the cell cycle. Treatment of VSMC with 4-HNE or its glutathione conjugate (glutathionyl (GS-)HNE) or AR-catalyzed product of GS-HNE, GS-1,4-dihydroxynonane resulted in increased E2F-1 expression. Inhibition of AR prevented 4-HNE- or GS-HNE-induced upregulation of E2F-1. Collectively, these results show that AR could regulate HG- and TNF-alpha-induced VSMC proliferation by altering the activation of G1/S-phase proteins such as E2F-1, cdks, and cyclins [344]. In airway smooth muscle cells, 4-HNE is mitogenic by increasing cyclin D1 activity through ERK signaling pathway [345].

The differential effect of 4-HNE on cell proliferation in both malignant and nonmalignant cells may be the consequence of lower aldehyde-metabolizing enzymes, deregulation of antioxidant defenses, and mitochondrial metabolism alteration [132, 346], so that malignant cells are more vulnerable to further oxidative stress induced by exogenous ROS-generating agents or inhibitors of the antioxidant systems [347–349].

2.5.5. 4-HNE-Induced Apoptosis and Necrosis. Apoptosis is essential programmed cell death process for cells, and its dysregulation results in too little cell death which may contribute to carcinogenesis, or too much cell death which may be a component in the pathogenesis of several diseases. The alternative to apoptosis or programmed cell death is necrosis or nonprogrammed cell death, which is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death. Depending on the cell type, DNA damage/repair capacity or cellular metabolic circumstances 4-HNE can activate proliferative signaling for cell division and promote cell survival or "stop" cell division, and after prolonged arrest, cells die from apoptosis. 4-HNE may induce these processes by modulating several transcription factors sensible to stress such as Nrf2, AP-1, NF-κB, and PPAR or by modulating several signaling pathways, including MAPK (p38, Erk, and JNK), protein kinase B, protein kinase C isoforms, cell-cycle regulators, receptor tyrosine kinases, and caspases. Depending on 4-HNE concentrations the cells "end" their lives by apoptosis or necrosis. For example, the cytotoxicity of 4-HNE to HepG2 cells was evaluated by MTT assay. 4-HNE concentrations ranging from 10 to 100 μM gradually decreased cell viability corresponding to an IC_{50} value of $53 \pm 2.39\,\mu$M. 4-HNE concentrations of 5–40 μM caused apoptotic cell death (measured by flow cytometry, caspase-3 activation, and PARP cleavage). Finally, a significant increase in necrotic cell population, that is, 31.8% and 55.4%, was observed in cells treated with 80 and 100 μM of 4-HNE, respectively [350]. These results show that 4-HNE induces apoptosis at low concentration and necrosis at high concentration.

The two main pathways of apoptosis are extrinsic and intrinsic pathways. The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. This pathway is triggered by the binding of death ligands of the tumor necrosis factor (TNF) family to their appropriate death receptors (DRs) on the cell surface; best-characterized ligands and corresponding death receptors include FasL/FasR and TNF-α/TNFR1 [351, 352]. The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli. The proapoptotic member of the Bcl-2 family of proteins, such as Bax, permeabilizes the outer mitochondrial membrane. This allows redistribution of cytochrome c from the mitochondrial intermembrane space into the cytoplasm, where it causes activation of caspase proteases and, subsequently, cell death [352, 353]. Each apoptosis pathway requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9) which in turn will activate the executioner caspase-3 [352]. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies, and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages [352, 353]. A multitude of mechanisms are employed by p53 to ensure efficient induction of apoptosis in a stage-, tissue-, and stress-signal-specific manner [354]. 4-HNE-mediated activation of p53 may be one of the mechanisms responsible for 4-HNE-induced apoptosis reported in many cell types. For example, in SH-SY5Y cells 4-HNE-induced oxidative stress was associated with increased transcriptional and translational expressions of Bax and p53; these events trigger other processes, ending in cell death [355]. In RPE

cells, 4-HNE causes induction, phosphorylation, and nuclear accumulation of p53 which is accompanied with downregulation of MDM2, a negative regulator of the p53 by blocking p53 transcriptional activity directly and mediating in the p53-degradation. Associated proapoptotic genes Bax, p21, and JNK, which are all signaling components p53-mediated pathway of apoptosis, are activated in response to exposure to 4-HNE. The induction of p53 by 4-HNE can be inhibited by the overexpression of either *hGSTA4* (in RPE cells) or *mGsta4* (in mice) which accelerates disposition of 4-HNE [356]. In CRL25714 cell, 4-HNE induced dose-dependent increase in the expression of p53 in the cytoplasmic and nuclear compartments and increase in the expression of Bax [357]. In human osteoarthritic chondrocytes, 4-HNE treatment led to p53 upregulation, caspase-8, -9, and -3 activation, Bcl-2 downregulation, Bax upregulation, cytochrome c-induced release from mitochondria, poly (ADP-ribose) polymerase cleavage, DNA fragmentation, Fas/CD95 upregulation, Akt inhibition, and energy depletion. All these effects were inhibited by an antioxidant, N-acetyl-cysteine [358].

4-HNE can induce apoptosis through the death receptor Fas (CD95-)mediated extrinsic pathway as well as through the p53-dependent intrinsic pathway. For detailed information of the molecular mechanisms involved in 4-HNE-induced programmed cell death see review [359]. However, these mechanisms can be summarized in the following: (i) 4-HNE is diffusible and can interact with Fas (CD95/Apo1) on plasma membrane and upregulate and activate its expression to mediate the apoptotic signaling through activation of downstream kinases (apoptosis signal-regulating kinase 1 or ASK1 and JNK), which leads to activation of executioner caspase-3 and ending in apoptosis; (ii) 4-HNE interacts with cytoplasmic p53 which causes its induction, phosphorylation, and nuclear translocation. In the nucleus p53 inhibits transcription of antiapoptotic genes (Bcl2) and promotes transcription of proapoptotic genes (Bax) or cell cycle genes (p21) leading to activation of executioner caspase-3 and ending in apoptosis or cell cycle arrest, respectively; (iii) 4-HNE also activates a negative feedback on Fas activation, by a mechanism involving transcription repressor death domain-associated protein (Daxx), a nuclear protein which is associated with DNA-binding transcription factors involved in stress response. 4-HNE interacts with the Daxx, bound to heat shock factor-1 (HSF1), translocates Daxx from nucleus to cytoplasm where it binds to Fas, and inhibits activation of ASK1 to limit apoptosis.

2.5.6. 4-HNE-Biomolecules Adducts. The preference for amino acid modification by 4-HNE is Cys ≫ His > Lys resulting in covalent adducts with the protein nucleophilic side chain [104, 131, 360, 361]. The reaction between primary amines and 4-HNE carbonyl carbon groups yields a reversible Schiff base and the addition of thiol or amino compounds on 4-HNE β-carbon atom' (C of double bond) produces the corresponding Michael adduct [49]. 4-HNE-protein adducts can contribute to protein crosslinking and induce a carbonyl stress. Recently it has been shown that a membrane associated protein called regulator of G-protein signaling 4 (RGS4) can be modified by 4-HNE. RGS4, like other RGS proteins, is responsible for temporally regulating G-protein coupled receptor signaling by increasing the intrinsic GTPase activity of Gα subunit of the heterotrimeric signaling complex. 4-HNE modification of RGS4 at cysteine residues during oxidative stress can disrupt RGS4 activity and alter signaling from stressed cells. Possibly 4-HNE acts as an internal control for aberrant signaling due to excess RGS4 activity in a variety of pathologies where oxidative stress is a strong component [362]. Our lab has reported that 4-HNE can affect protein synthesis rates by forming adduct with eEF2 (*see below—cumene hydroperoxide-induced lipid peroxidation*). Large lists of peptides and proteins known to be modified by 4-HNE are given in the reviews [76, 104, 363] and including glutathione, carnosine, enzymatic proteins, carriers proteins, membrane transport proteins, receptor proteins, cytoskeletal proteins, chaperones, mitochondrial upcoupling proteins, transcription and protein synthesis factors, and antioxidant proteins.

It has been reported that 4-HNE also could react with deoxyguanosine to form two pairs of diastereomeres adducts (4-HNE-dG 1,2 and 3,4) that further induced DNA crosslink or DNA-protein conjugates. The mechanism involves a nucleophilic Michael addition of the NH_2-group of deoxyguanosine to the CC double bond of 4-HNE, which yields 6-(1-hydroxyhexanyl)-8-hydroxy-1,N(2)-propano-2′-deoxyguanosine (HNE-dG), an exocyclic adduct [49, 133, 134]. HNE-dG adducts have been detected in human and animal tissues. They are potentially mutagenic and carcinogenic and can be repaired by the nucleotide excision repair (NER) pathway [364, 365]. In the presence of peroxides a different reaction takes place, and the stable end-product found in the reaction of 4-HNE with DNA bases is etheno-DNA adducts because 4-HNE is converted by the peroxide to the corresponding epoxynonanal, which then reacts to the NH2-group of guanosine followed by cyclization reaction to form 1, N^6-etheno-2′-eoxyadenosine (εdA), and 3, N^4-etheno-2′-deoxycytidine (εdC). These ε-adducts are eliminated by the base excision repair (BER) pathway [49, 366]. Etheno-DNA adduct levels were found to be significantly elevated in the affected organs of subjects with chronic pancreatitis, ulcerative colitis, and Crohn's disease, which provide promising molecular signatures for risk prediction and potential targets and biomarkers for preventive measures [367, 368]. The 4-HNE-DNA adducts in tissue could serve as marker for the genetic damage produced by endogenous oxidation of omega-6-PUFAs.

3. The Use of Mammalian Model in Lipid Peroxidation Research: Compounds Induced Lipid Peroxidation

The use of mammalian model in lipid peroxidation research is ideal for studying the consequences of lipid peroxidation in the context of whole organism and also to analyze their influence on biomarkers to gain more insight into what controls the lipid peroxidation and how lipid peroxidation-related diseases occur. Animal models used to investigate the

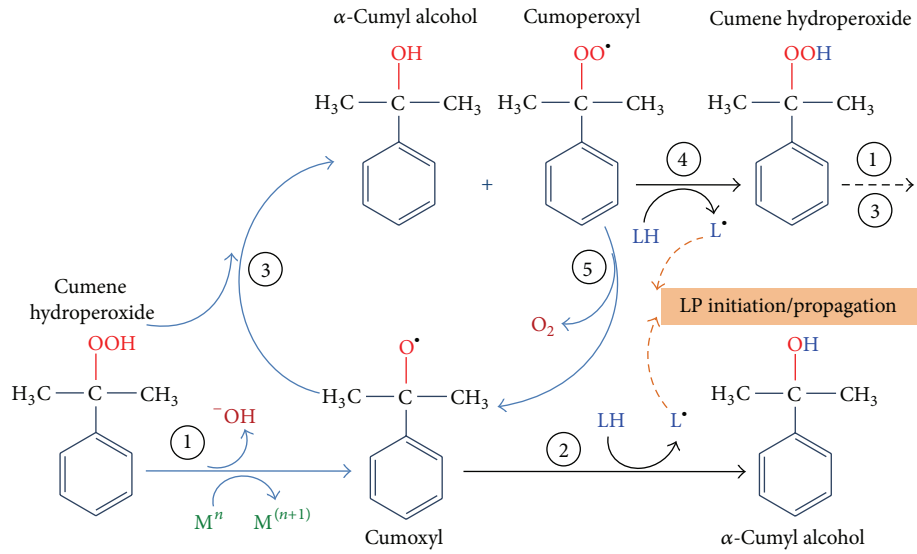

FIGURE 7: Mechanisms showing how cumene hydroperoxide produces lipophilic cumoxyl and cumoperoxyl radicals. Cumene hydroperoxide in presence of transition metal ions produces cumoxyl radical (step 1), which abstracts a hydrogen (H) from a lipid (PUFA) molecule (LH) generating cumyl alcohol and lipid radical ($L^{\bullet}$) that reacts readily with oxygen promoting the initiation or propagation of lipid peroxidation. (Step 2). Cumoxyl radical can also react with other cumene hydroperoxide molecules to yield cumyl alcohol and cumoperoxyl radical (step 3). Finally, cumoperoxil radical may abstract hydrogen (H) from the closest available lipid to produce a new cumene hydroperoxide and lipid radical ($L^{\bullet}$) which then again affects lipid peroxidation cycling (step 4). Cumoperoxyl radical may also react with oxygen to yield a new cumoxyl radical thus initiating a chain reaction (step 5).

genetic, physiological, or pathological consequences of lipid peroxidation should try to control the intrinsic and extrinsic influences. Genetic background, diet, environment, and health status can be strictly controlled in many model organisms. Compared with other model organisms, such as worms (*Caenorhabditis elegans*) and flies (*Drosophila melanogaster*), the mammalian model is highly comparable to the human in respect to organ systems, tissues, physiologic systems, and even behavioral traits. Finally, mammalian model in LP can be used as a first step toward possible development of drugs or interventions to control lipid peroxidation process and prevent disease progression in humans. Various mammalian models have been developed to study the lipid peroxidation process.

3.1. Cumene Hydroperoxide-Induced Lipid Peroxidation. Cumene hydroperoxide (CH) a catalyst used in chemical and pharmaceutical industry [369] is a stable organic oxidizing agent with the peroxy function group, –O–O–, which induces lipid peroxidation. On the existence of transition-metal, CH can be reduced to form an alkoxyl radical, which can attack adjacent fatty acid side-chains to produce lipid radical and cumyl alcohol. The resulting lipid radical reacts with oxygen to form a lipid peroxyl radical. And a lipid peroxyl radical reacts with other fatty acid side-chains to produce a new lipid radical and lipid hydroperoxide and this chain reaction continues. These lipid hydroperoxides may undergo transition-metal mediated one-electron reduction and oxygenation to give lipid peroxyl radicals, which trigger exacerbating rounds of free radical-mediated lipid peroxidation (Figure 7). In our lab we have made extensive use of membrane-soluble CH as a model compound for lipid hydroperoxides (LOOH), which are formed in the process of lipid peroxidation during oxidative stress. CH-induced lipid peroxidation in animals has been important to study the effect of lipid peroxidation on protein synthesis through mechanisms that involve regulation of eElongation Factor 2 (eEF2). It is known that eEF2 plays a key role as a cytoplasmic component of the protein synthesis machinery, where it is a fundamental regulatory protein of the translational elongation step that catalyzes the movement of the ribosome along the mRNA. One particularity of eEF2 is that it is quite sensitive to oxidative stress and is specifically affected by compounds that increase lipid peroxidation, such as cumene hydroperoxide (CH) [370–373]. We have previously reported that cytotoxic end-products of lipid peroxidation 4-HNE and MDA are able to form adducts with eEF2 *in vitro* [374] and *in vivo* [309], demonstrating, for the first time, that this alteration of eEF2 could contribute to decline of protein synthesis, secondary to LP increase. The formation of these peroxide-eEF2-adducts is a possible mechanism responsible of suboptimal hormone production from hypothalamic-hypophysis system (HHS) during oxidative stress and aging [375]. The protection of eEF2 alterations by end-products of lipid peroxidation must be specifically carried out by compounds with lipoperoxyl radical-scavenging features such as melatonin. We have reported the ability of melatonin to protect against the changes that occur in the eEF2 under conditions of lipid peroxidation induced by CH, as well as decline of protein synthesis rate caused by lipid peroxidation, demonstrating that melatonin can prevent the decrease of several hormones after exposure to LP [376]. *In vitro* studies carried out in

our lab also indicated that the antioxidants have different capacities to prevent eEF2 loss caused by CH [377, 378]. In rat hippocampal neurons and in response to lipid peroxidation induced by exposure to CH, eEF2 subcellular localization, abundance, and interaction with p53 were modified [379]. Finally, using CH-induced lipid peroxidation, we found that a unique eEF2 posttranslational modified derivative of histidine (H715) known as diphthamide plays a role in the protection of cells against the degradation of eEF2, and it is important to control the translation of IRES-dependent proteins XIAP and FGF2, two proteins that promote cell survival under conditions of oxidative stress [380]. Other labs have used cumene hydroperoxide as a model compound for lipid hydroperoxides *in vivo* [381–385].

3.2. Tert Butyl Hydroperoxide. It is an organic oxidizing agent containing a tertiary butyl group, commonly used in industry as prooxidizing, a bleaching agent, and an initiator of polymerization. Tert butyl hydroperoxide is a strong free radical source and has been utilized to induce lipid peroxidation *in vivo* mammalian model [386–392].

3.3. Carbon Tetrachloride (CCl_4). It is a toxic, carcinogenic organic compound which is used as a general solvent in industrial degreasing operations. It is also used as pesticides and a chemical intermediate in the production of refrigerants. Carbon tetrachloride has been utilized to induce lipid peroxidation *in vivo* mammalian model [90, 393–398].

3.4. Quinolinic Acid (QA). It is a neuroactive metabolite of the kynurenine pathway. It is normally presented in nanomolar concentrations in human brain and cerebrospinal fluid (CSF) and is often implicated in the pathogenesis of a variety of human neurological diseases [399]. QA has been used to induce lipid peroxidation mediated by hydroxyl radicals *in vivo* mammalian models [400–405].

3.5. Transition Metals Ions. They are essential elements which, under certain conditions, can have prooxidant effect. Redox active transition metals have ability to induce and initiate lipid peroxidation through the production of oxygen radicals, mainly hydroxyl radical, via Fenton's/Haber-Weiss reactions [63, 406]. Transition metal, including copper [407–410], chromium [411, 412], cadmium [413–416], nickel [417, 418], vanadium [419–421], manganese [59, 422–424], and iron [59, 407, 425–434] has been utilized to induce lipid peroxidation *in vivo* mammalian model.

4. Pathological Processes Linked to MDA and 4-HNE

The accumulation of lipid peroxidation by-product has been extensively studied and implicated in many toxic tissue injuries and in pathological processes. An increasing amount of literature has been published in the field. In particular, the measurement of free MDA and/or 4-HNE levels or its derived protein adducts in biological samples from subjects affected by several diseases has been widely utilized, indirectly implicating MDA and 4-HNE in the pathogenesis of these diseases. Table 1 shows a brief extract of studies presented in the literature in which MDA and 4-HNE have been found to be significantly modified in pathological contexts. The "big" challenge in the field of pathological processes is that it is often difficult to determine whether these lipid peroxidation-derived aldehydes are actually involved in causing the disease or are a consequence to it.

5. Conclusions

As conclusion, in this review we summarized the physiological and pathophysiological role of lipid peroxides. When oxidant compounds target lipids, they can initiate the lipid peroxidation process, a chain reaction that produces multiple breakdown molecules, such as MDA and 4-HNE. Among several substrates, proteins and DNA are particularly susceptible to modification caused by these aldehydes. MDA and 4-HNE adducts play a critical role in multiple cellular processes and can participate in secondary deleterious reactions (e.g., crosslinking) by promoting intramolecular or intermolecular protein/DNA crosslinking that may induce profound alteration in the biochemical properties of biomolecules, which may facilitate development of various pathological states. Identification of specific aldehyde-modified molecules has led to the determination of which selective cellular function is altered. For instance, results obtained in our lab suggest that lipid peroxidation affects protein synthesis in all tissues during aging through a mechanism involving the adduct formation of MDA and 4-HNE with elongation factor-2. However, these molecules seem to have a dual behavior, since cell response can tend to enhance survival or promote cell death, depending of their cellular level and the pathway activated by them.

Conflict of Interests

The authors declare no competing financial interests.

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Research Article

Resveratrol Counteracts Inflammation in Human M1 and M2 Macrophages upon Challenge with 7-Oxo-Cholesterol: Potential Therapeutic Implications in Atherosclerosis

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Macrophages consist of two main subsets: the proinflammatory M1 subset and the anti-inflammatory M2 one. 7-oxo-cholesterol, the most abundant cholesterol autoxidation product within atherosclerotic plaque, is able to skew the M1/M2 balance towards a proinflammatory profile. In the present study, we explored the ability of the polyphenolic compound resveratrol to counteract the 7-oxo-cholesterol-triggered proinflammatory signaling in macrophages. Resveratrol-pretreated human monocyte-derived M1 and M2 macrophages were challenged with 7-oxo-cholesterol and analyzed for phenotype and endocytic ability by flow cytometry, for metalloproteinase- (MMP-) 2 and MMP-9 by gelatin zymography, and for cytokine, chemokine, and growth factor secretome by a multiplex immunoassay. We also investigated the NF-κB signaling pathway. In the M1 subset, resveratrol prevented the downregulation of CD16 and the upregulation of MMP-2 in response to 7-oxo-cholesterol, whereas in M2 macrophages it prevented the upregulation of CD14, MMP-2, and MMP-9 and the downregulation of endocytosis. Resveratrol prevented the upregulation of several proinflammatory and proangiogenic molecules in both subsets. We identified modulation of NF-κB as a potential mechanism implicated in 7-oxo-cholesterol and resveratrol effects. Our results strengthen previous findings on the immunomodulatory ability of resveratrol and highlight its role as potential therapeutic or preventive compound, to counteract the proatherogenic oxysterol signaling within atherosclerotic plaque.

1. Introduction

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of immune cells within the atherosclerotic plaque [1, 2], including macrophages that are the main cellular components [3]. Human atherosclerotic plaque is composed of a large mixture of elements, predominantly lipids and oxidized lipids, lipid-loaded macrophages, and smooth muscle cells, forming foam cells. Plaque contents undergo dynamic changes during the plaque's progression, being in a constant interaction with the circulating blood [4]. The fate of atherosclerotic plaques is highly dependent upon the balance between recruitment and activation of monocyte-derived macrophages, upon their clearance from the vessel wall [5] and upon macrophage polarization state [6]. Macrophage M1 and M2 activation phenotypes represent two ends of a functional spectrum of macrophage polarization state [6], which may accelerate or decelerate atherosclerotic disease progression through igniting or cooling down inflammatory reactions. The proinflammatory M1, or classically activated subset, produces inflammatory cytokines and is a leading source of reactive oxygen species

in atherosclerotic lesions [7, 8]. M2, or alternatively polarized macrophages, are a heterogeneous group of cells that show an anti-inflammatory phenotype and appear to be critical for the resolution of inflammation [7, 9]. Plasticity is a hallmark of cells of the monocyte-macrophage lineage [10]. The molecules and mechanisms associated with plasticity and polarized activation of macrophages may provide a basis for innovative diagnostic and therapeutic approaches [10]. We have recently demonstrated that 7-oxo-cholesterol, the most abundant cholesterol autoxidation product within atherosclerosis lesions [11–14], is able to affect human macrophage polarization by skewing the M1/M2 balance towards a proinflammatory profile [15]. Because proinflammatory macrophages play a key role in atherogenesis, plaque rupture, and subsequent clinical events, the inhibition of this new 7-oxo-cholesterol-triggered proinflammatory pathway by the use of a therapeutic approach capable to modulate the M1/M2 macrophage balance within atherosclerotic plaque might provide interesting therapeutic prospects in reducing atherosclerosis and/or in the prevention of plaque rupture. There is emerging evidence that beside current Western therapies, many alternative and nutrition therapies have the ability to modulate the immune system and disrupt the proinflammatory cascade through a variety of mechanisms, including antioxidant effects, alterations in cell signaling, cytokines, and proinflammatory mediators [16]. Resveratrol, a polyphenolic compound found in red wine and grapes, plays a potentially important role in many disorders [17]. It possesses antioxidant, anti-inflammatory, antiproliferative, and antiangiogenic effects and many signaling pathways are among its molecular targets.

With regard to anti-inflammatory and immunomodulatory effects, the process activated by resveratrol has not been clearly established, even though it does not seem to be mechanically as simple as a nonspecific inhibition of inflammation [16].

In this study, we explored the ability of resveratrol to counteract the proinflammatory signaling triggered by 7-oxo-cholesterol in M1 and M2 macrophage subsets and we investigated a potential mechanism implicated in such prevention. By the use of flow cytometry, gelatin zymography, and a multiplex immunoassay we demonstrated that resveratrol was able to counteract oxysterol-induced proinflammatory phenotypical and functional changes in both M1 and M2 subsets.

2. Materials and Methods

2.1. Reagents. Recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) and rh macro phage colony-stimulating factor (M-CSF) were from R&D System (Minneapolis, MN). Foetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). Anti-CD14-coated microbeads were from Miltenyi Biotec (Gladbach, Germany). RPMI 1640 was from GIBCO (Paisley, UK). Phycoerythrin- (PE-) conjugated monoclonal antibodies (mAbs) to CD1a and human leukocyte antigen-D region-related (HLA-DR) and fluorescein isothiocyanate- (FITC-) conjugated mAbs to CD16 were from PharMingen (San Diego, CA); allophycocyanin- (APC-) conjugated mAbs to CD14 and CD163 (clone GHI/61) were from BioLegend (San Diego, CA). 7-oxo-cholesterol, resveratrol, and the other chemicals were from Sigma-Aldrich (Milan, Italy). Resveratrol was dissolved in ethanol at 50 mg/mL and aliquots were frozen at −80°C under sterile conditions.

2.2. Preparation of Human Monocyte-Derived M1 and M2 Macrophages. Blood samples from 4 healthy blood donors from the Transfusion Center at the Sapienza University of Rome were used to obtain peripheral blood mononuclear cells (PBMCs). The study was conducted in accordance with the Helsinki Declaration of 1975 and 1983.

Monocytes were obtained from PBMCs, as described previously [18]. In brief, PBMCs were isolated by density gradient (Lympholyte, Cedarlane, Oxford, UK). CD14+ monocytes were purified by incubating PBMCs with anti-CD14-coated microbeads, followed by sorting with a magnetic device (MiniMacs, Miltenyi Biotec). Monocytes were induced to differentiate for 6 days in cell culture dishes (100 mm) (BD-Biosciences, San Diego, CA), in the presence of either rhGM-CSF (10 ng/mL) to obtain M1 macrophages or rhM-CSF (10 ng/mL) to obtain M2 ones. Cells were cultured at 8×10^5 cells/mL in RPMI 1640—supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10% FBS.

2.3. Flow Cytometric Analysis of Monocyte-Derived M1 and M2 Macrophage Phenotype. Flow cytometric analysis was performed before any treatment to confirm M1 and M2 induction and was later used to evaluate the effect of resveratrol and 7-oxo-cholesterol on macrophage phenotype. The presence of characteristic phenotypic surface markers ($CD14^{high}$, $CD16^{high}$, $CD163^{low}$, and $HLA\text{-}DR^{high}$ for M1 and $CD14^{high}$, $CD16^{low}$, $CD163^{high}$, and $HLA\text{-}DR^{low}$ for M2) was analyzed on a FACSCanto and using CellDIVA software (BD-Biosciences). Macrophages were stained with PE-conjugated mAb to HLA-DR, FITC-conjugated mAb to CD16, APC-conjugated mAbs to CD14 and CD163 (clone GHI/61) or with isotype-matched control mAbs for 30 minutes at 4°C. All samples were analyzed by flow cytometry (FACSCanto, BD-Biosciences).

2.4. Treatment of M1 and M2 Macrophages with Resveratrol and Exposure to 7-Oxo-Cholesterol. On day 6, adherent macrophages were collected and 7×10^5 cells were cultured in 24 well plates (BD-Biosciences) and treated or not with resveratrol (30 μM) for 1 hour at 37°C, 5% CO_2. Resveratrol concentration was chosen on the basis of preliminary dose/response experiments using concentrations ranging from 3 to 80 μM. Then cells were stimulated with 7-oxo-cholesterol in ethanol (15 μM) for 20 hours. LPS-treated cells (100 ng/mL) were used as positive control. Macrophages pretreated or not with resveratrol and stimulated with 7-oxo-cholesterol were exposed to 0.2% trypan blue and then

counted in a hemocytometer to calculate cell viability and the percentage of dead cells.

2.5. Flow Cytometric Analysis of Macrophage Endocytosis. To deliver more information on M1/M2 macrophage discrimination, we investigated macrophage mannose receptor-mediated endocytosis as previously described [15]. In brief, macrophages treated or not with resveratrol for 1 hour at 37°C and then stimulated with 7-oxo-cholesterol (15 μM) at 37°C for 20 hours were incubated (2×10^5 cells/sample) with FITC-dextran (1 mg/mL; molecular mass 40.000, Sigma) for 30 min at 37°C. After incubation, macrophages were washed twice with PBS and fixed with 1% formaldehyde. At least 5×10^3 cells/sample were analyzed by flow cytometry (FACSCanto, BD-Biosciences).

2.6. Assessment of MMP-2 and MMP-9 by Gelatin Zymography. The effect of resveratrol and 7-oxo-cholesterol treatment on macrophage function was evaluated by determining metalloproteinases activity. Macrophage culture supernatants were collected after pretreatment with resveratrol and stimulation with 7-oxo-cholesterol (15 μM). MMP-2 and MMP-9 activity was measured by gelatin zymography as described previously [15]. Cell supernatants were subjected to polyacrylamide gel electrophoresis (SDS-PAGE). Gels (10.5%) were copolymerised with gelatin (0.9%). For each sample, 6 μL of cell supernatant in 6 μL of loading buffer (Bio-Rad) was loaded under native conditions. Electrophoresis was carried out using the mini-gel slab apparatus Mini Protean 3 (Bio-Rad, Milan, Italy) at a constant voltage of 150 V. Following electrophoresis, gels were washed in renaturating buffer (2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5) for 1 h in an orbital shaker. Then, the zymograms were incubated for 18 h at 37°C in Tris buffer pH 7.5 (0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 in 50 mM Tris-HCl). Gels were then stained with Coomassie blue and destained with 7% methanol and 5% acetic acid. Areas of enzymatic activity, which appeared as clear bands over the dark background, were quantified using ChemiDoc densitometer (Bio-Rad, Hercules, CA). For analysis purpose, the image was digitally inverted so that the integration of bands was reported as positive values. The pixel density was determined after background subtraction and used to calculate the integrated density of a selected band that was reported as the mean of three different measurements of the same gel for each sample run in triplicate.

2.7. Assessment of MMP-2 and MMP-9 by Western Blotting. The identification of macrophage-derived MMP-2 and -9 was performed by Western blotting. Supernatants were subjected to 10.5% SDS-PAGE and then blotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore, Tullagreen, Ireland). Blots were incubated with anti-human MMP-2 or -9 Abs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then with anti-goat HRP-coupled secondary Ab (Bio-Rad, Hercules, CA). Immunoreactivity was assessed by the chemiluminescence reaction with the ECL system (Amersham, Buckinghamshire, UK) and analyzed by ChemiDoc densitometer (Bio-Rad).

2.8. Secretome Profile of Cytokines, Chemokines, and Growth Factors in Macrophage Culture Supernatants. Conditioned media were harvested and processed for cytokine analysis in duplicate with a custom Bio-Rad Bio-Plex human cytokine reagent kit for IL-1 receptor antagonist (IL-1ra), IL-6, IL-8, IL-10, IL-12, granulocyte colony stimulating factor (G-CSF), GM-CSF, interferon-inducible protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-α (MIP-1α or CCL3), MIP-1β (CCL4), regulated and normal T cell expressed and secreted (RANTES), TNF-α, and vascular endothelial growth factor (VEGF) according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Data were acquired on the Bio-Rad Bio-Plex 200 reader equipped with a magnetic workstation and analyzed using Bio-Plex software version 6.0 (Bio-Rad). Values presenting a coefficient of variation beyond 10% were discarded before the final data analysis. Minimum levels of detection (pg/mL) were 4.89 for IL-1ra, 0.23 for IL-6, 0.58 for IL-8, 0.17 for IL-10, 0.26 for IL-12, 0.1 for G-CSF, 2.26 for GM-CSF, 1.83 for IP-10, 3.56 for MCP-1, 2.38 for MIP-1α, 2.69 for MIP-1β, 0.49 for RANTES, 8.84 for TNF-α, 3.54 for VEGF.

2.9. Nuclear Factor-κB (NF-κB) Translocation. The NF-κB (p65 and p50) transcription factor assay kit (Active Motive Carlsbad, CA, USA) was used to monitor NF-κB activation as previously described [19]. Macrophages treated or not with resveratrol for 30 min at 37°C and then stimulated with 7-oxo-cholesterol (15 μM) at 37°C for 1 hour were lysed. Protein content was quantified, and activated levels of p65 and p50 subunits were determined in equal amounts of lysates by the use of Abs directed against the subunits bound to the oligonucleotide containing the NF-κB consensus binding site.

2.10. Statistical Analysis. Mean values and standard deviations were calculated for each variable under study. All the statistical procedures were performed by GraphPad Prism software (San Diego, CA, USA). Data were tested for Gaussian distribution with the Kolmogorov-Smirnov test. Normally distributed data were analysed using one-way ANOVA with a Bonferroni *post hoc* test to evaluate the statistical significance of intergroup differences in all the tested variables. P values <0.05 were considered statistically significant.

3. Results

3.1. Resveratrol Prevents 7-Oxo-Cholesterol-Induced CD16 and CD14 Changes in M1 and M2 Macrophage Subsets. The impact of resveratrol on the 7-oxo-cholesterol-induced phenotypical changes in M1 and M2 macrophages was assessed by flow cytometric analysis of the differentiation and activation surface markers CD14, CD16, CD163, and HLA-DR (Figure 1, Table 1). A reduction in CD16 expression ($P < 0.001$) and an increase in HLA-DR expression ($P < 0.05$) were observed on the M1 subset, whilst M2 subset showed increased CD14 expression ($P < 0.001$). Treatment of cells with resveratrol before challenge with oxysterol prevented CD16 downregulation in M1 and CD14 upregulation in M2

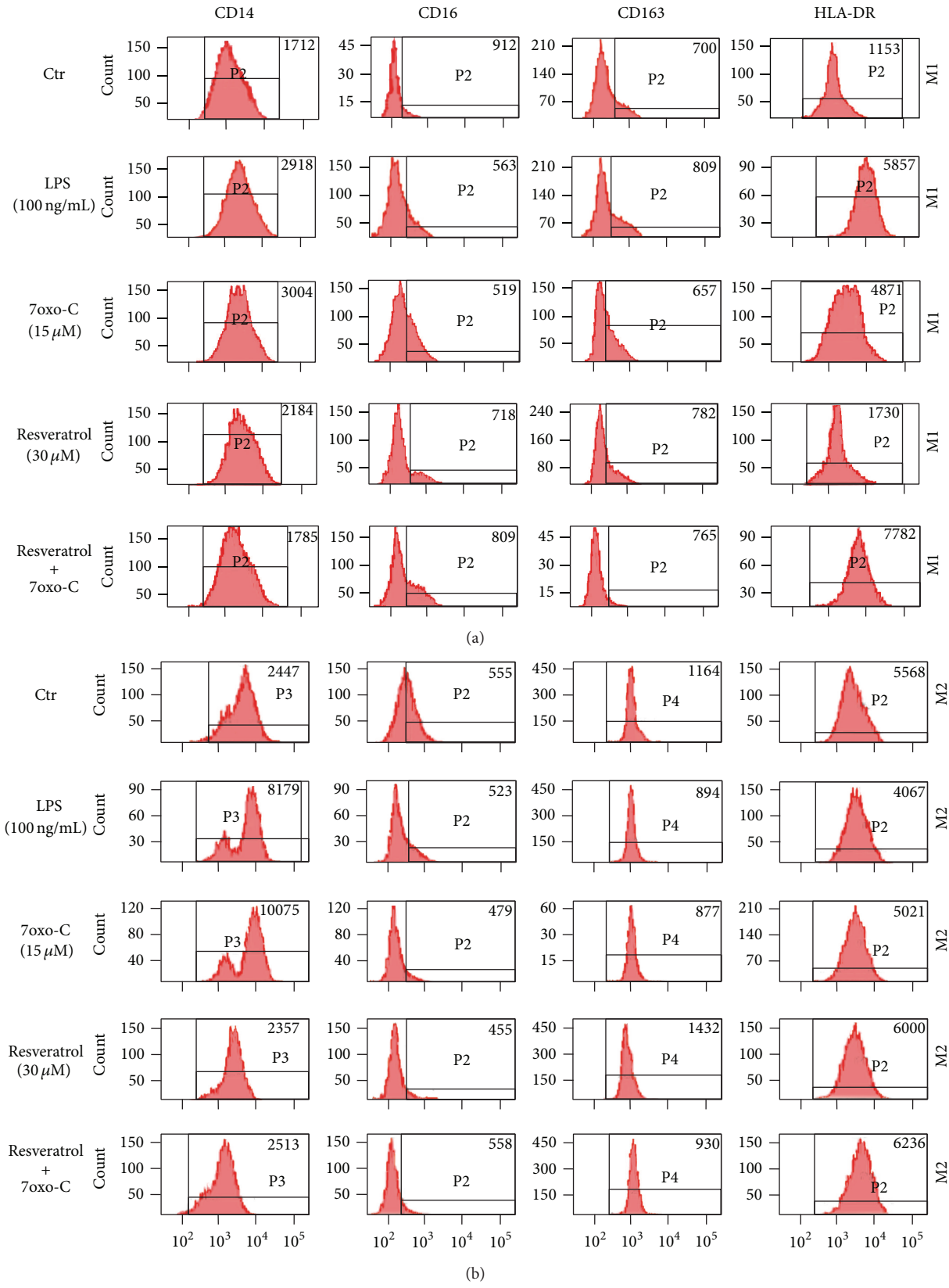


Figure 1: Flow cytometric analysis of differentiation and activation surface markers on M1 and M2 macrophage subsets. Resveratrol prevented 7-oxo-cholesterol (7oxo-C) induced CD16 and CD14 changes in M1 (a) and M2 (b) macrophage subsets. Polarized M1 and M2 macrophages pretreated or not with resveratrol for 1 hour were stimulated with 7oxo-C (15 μM) for 20 hours and then analyzed for surface molecule expression by flow cytometry. Macrophages stimulated with LPS (100 ng/mL) were used as positive control. The results of one representative experiment of three are shown. The number in the histograms shows the mean fluorescence intensity.

Table 1: Flow cytometric analysis of differentiation and activation surface markers on M1 and M2 macrophage subsets.

Surface markers		Ctr	LPS	7oxo-C	Resveratrol	Resveratrol + 7oxo-C	P value
				M1			
CD14	%	98.6 ± 1.1	100.0 ± 0.0	98.0 ± 3.5	99.3 ± 1.1	98.6 ± 1.1	NS
	MFI	1810.0 ± 569.1	3107.0 ± 1128.0	2457.0 ± 737.8	1743.0 ± 568.1	1837.0 ± 304.8	NS
CD16	%	30.7 ± 14.0	39.0 ± 6.0	35.0 ± 7.0	25.0 ± 15.0	22.7 ± 12.0	NS
	MFI	1001.0 ± 107.5*†	625.0 ± 52.0*	472.0 ± 148.0†‡	809.3 ± 121.1	977.0 ± 98.3‡	*‡<0.05 †<0.001
CD163	%	8.3 ± 0.6	35.7 ± 23.0	15.7 ± 5.8	8.7 ± 0.6	8.6 ± 0.6	NS
	MFI	1113 ± 554.1	1046.0 ± 461.4	999.7 ± 650.9	1061.0 ± 191.0	1015.0 ± 179.7	NS
HLA-DR	%	99.7 ± 0.6	98.3 ± 2.0	99.3 ± 0.6	99.3 ± 0.6	99.3 ± 0.6	NS
	MFI	1343.0 ± 239.8*†	5296.0 ± 1264.0	5601.0 ± 777.8*	1632.0 ± 178.1	5883.0 ± 2427.0†	<0.05
				M2			
CD14	%	93.3 ± 9.9	99.3 ± 1.1	98.0 ± 3.5	99.3 ± 1.1	99.3 ± 1.1	NS
	MFI	2103.0 ± 149.8*†	7277.0 ± 2699.0*	8199.0 ± 1501.0†‡§	2103.0 ± 952.6‡	2424.0 ± 479.8§	<0.001
CD16	%	43.0 ± 17.1	39.3 ± 16.0	35.3 ± 2.5	25.0 ± 8.0	27.0 ± 5.6	NS
	MFI	434.0 ± 86.1	421.7 ± 137.6	448.3 ± 159.9	402.0 ± 71.2	445.3 ± 115.8	NS
CD163	%	92.7 ± 11.9	96.3 ± 5.5	93.3 ± 10.7	93.7 ± 8.5	94.0 ± 10.4	NS
	MFI	1663.0 ± 409.5	1058.0 ± 208.8	1083.0 ± 195.6	1685.0 ± 223.1	1416.0 ± 480.0	NS
HLA-DR	%	99.0 ± 0.0	98.7 ± 0.6	99.3 ± 0.6	100.0 ± 0.0	99.3 ± 0.6	NS
	MFI	3143.0 ± 1630.0	3019.0 ± 994.5	4247.0 ± 947.0	4636.0 ± 1235.0	4718.0 ± 1747	NS

Results are expressed as percentage of positive cells (%) and mean fluorescence intensity (MFI) (mean ± SD; $n = 3$). P values were calculated by one-way ANOVA with a Bonferroni *post hoc* test. 7oxo-C: 7-oxo-cholesterol; NS: no significance. CD16 MFI: ctr M1 versus ctr M2, $P < 0.001$; CD163 %: ctr M1 versus ctr M2, $P < 0.05$.
*†§‡: indicate the statistical significant difference between numbers with the same symbol.

macrophages (7-oxo-cholesterol plus resveratrol versus 7-oxo-cholesterol: CD16, $P < 0.01$; CD14, $P < 0.001$). Resveratrol *per se* did not cause any surface marker changes.

3.2. Resveratrol Prevents the Impairment of Endocytosis in M2 Macrophages in Response to 7-Oxo-Cholesterol. Flow cytometric analysis showed that resveratrol pretreatment prevented the reduction of M2 macrophage ability to take up FITC-dextran in response to 7-oxo-cholesterol whereas it had no effect on 7-oxo-cholesterol-treated M1 macrophage endocytosis (Figure 2). Resveratrol *per se* did not change the endocytic ability of unstimulated M1 and M2 macrophages.

3.3. Resveratrol Prevents 7-Oxo-Cholesterol-Induced MMP-2 and MMP-9 Production in M1 and M2 Macrophage Subsets. The impact of resveratrol on macrophage functions was investigated by determining its ability to modulate the MMP-2 and MMP-9 production in response to 7-oxo-cholesterol. Analysis of zymograms for proteolytic activity of macrophage supernatants demonstrated that M1 and M2 macrophages constitutively express the pro-MMP-2 (72 kDa) and pro- and active forms of MMP-9 (92 and 84 kDa). 7-oxo-cholesterol upregulated the expression of MMP-2 in M1 and M2 subsets and of MMP-9 in the M2 subset (Figure 3). Pretreatment of cells with resveratrol prevented upregulation of MMP-2 in M1 and M2 subsets and of MMP-9 in M2 macrophages in response to 7-oxo-cholesterol ($P < 0.001$) (Figure 3). Resveratrol *per se* did not cause any change in metalloproteinase expression. Western blotting showed that the 72 kDa and the 92–84 kDa gelatinolytic activities observed in the zymograms corresponded to MMP-2 and MMP-9, respectively.

3.4. Resveratrol Prevents 7-Oxo-Cholesterol-Induced Proinflammatory and Proangiogenic Molecule Production by M1 and M2 Macrophage Subsets. To investigate the impact of resveratrol on proinflammatory macrophage activation in response to 7-oxo-cholesterol, we screened the secretome profile for cytokines, chemokines, and growth factors released in the culture supernatants by M1 and M2 macrophages treated or not with resveratrol before stimulation with the oxysterol (Figure 3). 7-oxo-cholesterol potentiated the proinflammatory capacity of M1 cells by triggering statistically significant upregulation of the cytokines TNF-α and IL-6 (Figure 4(a)), of the chemokines IL-8, CCL3, CCL4, RANTES, and IP-10 (Figure 4(b)), and of the growth factors G-CSF, GM-CSF, and VEGF (Figure 4(c)). It also skewed M2 cell polarization towards a M1-like phenotype by increasing the production of the cytokines TNF-α, IL-6, and particularly of IL-12, of the chemokines IL-8, MCP-1, CCL3, CCL4, and RANTES, and finally the production of the growth factors G-CSF and VEGF. It also increased the production of the anti-inflammatory cytokine IL-10.

In M1 macrophages, resveratrol pretreatment significantly prevented TNF-α and IL-6 upregulation observed in

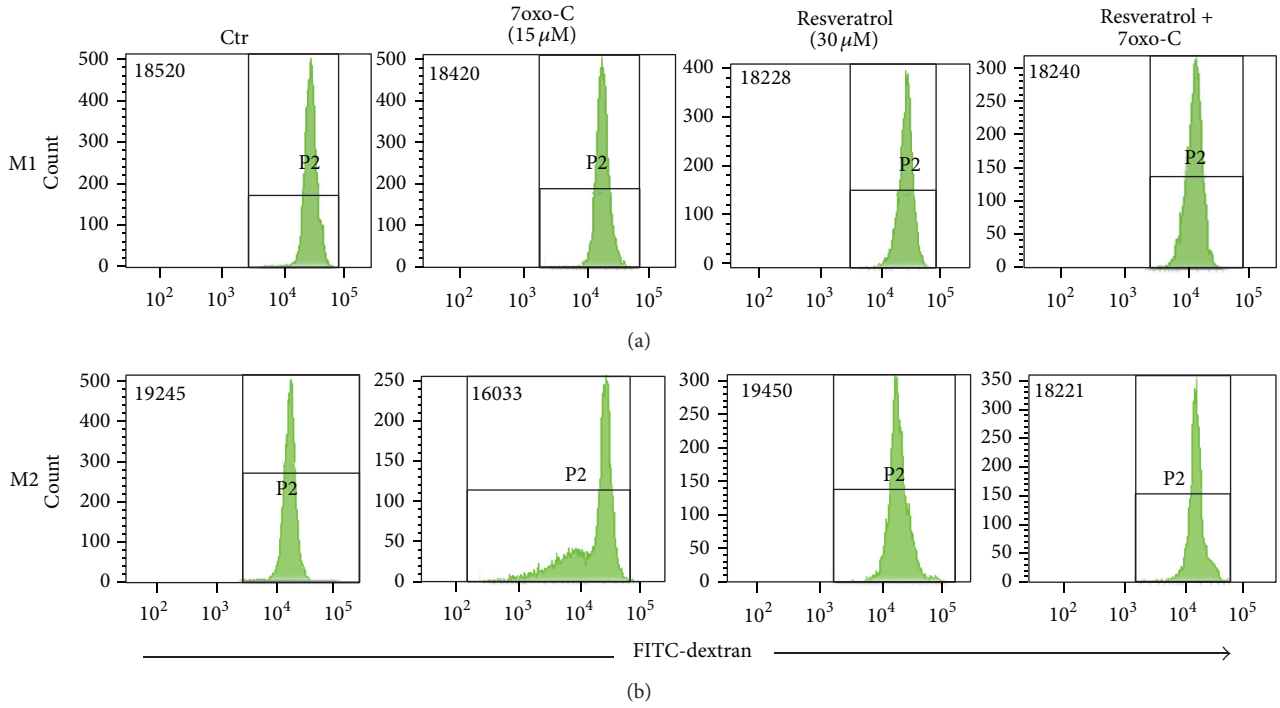


Figure 2: Analysis of macrophage endocytosis. Resveratrol prevented the impairment of endocytosis in M2 macrophages in response to 7-oxo-cholesterol (7oxo-C). M1 (a) and M2 (b) macrophages—pretreated or not with resveratrol (30 μM) for 1 hour and then incubated with 7oxo-C (15 μM) for 20 h or left unstimulated—were added with FITC-dextran (1 mg/mL) and incubated for 30 minutes at 37°C at 5% CO_2. The cellular uptake was analyzed by flow cytometry. The results of one representative experiment of three are shown. The number in the histograms shows the mean fluorescence intensity.

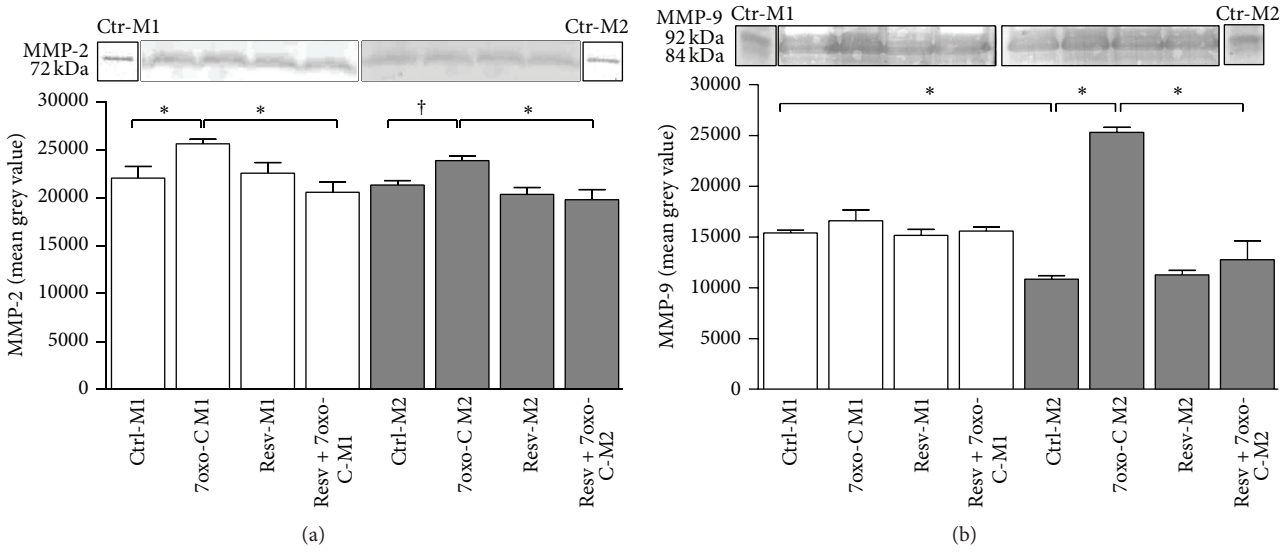
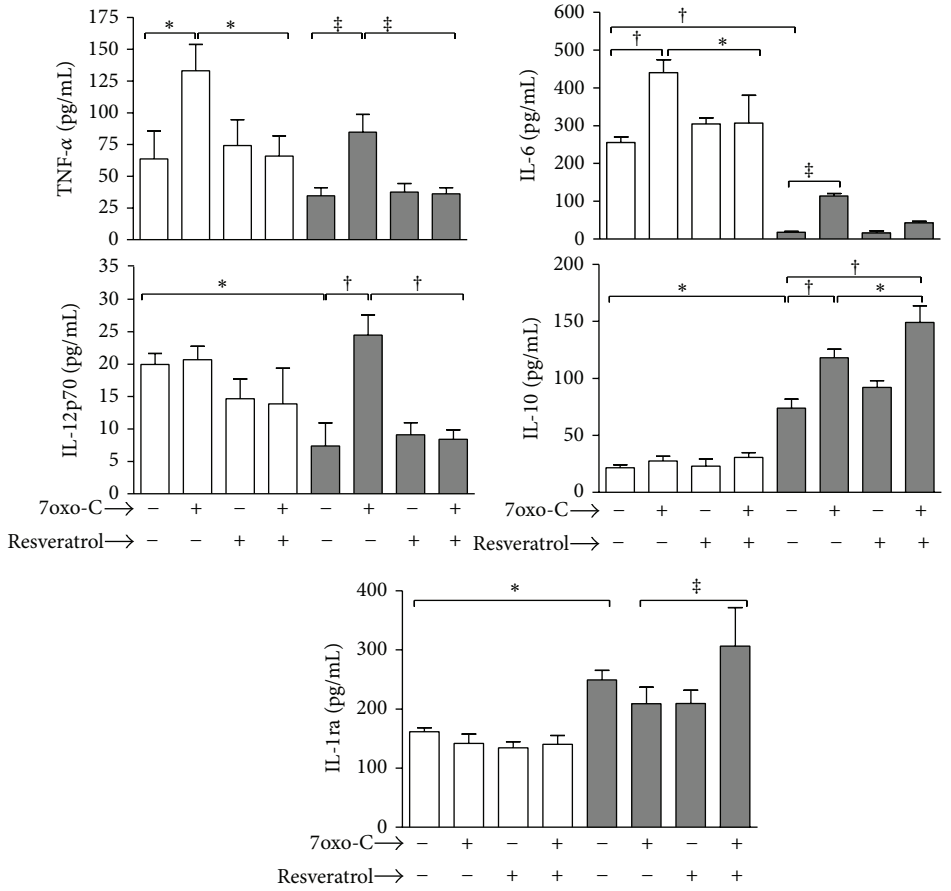


Figure 3: Gel zymography for MMP-2 and MMP-9 detection. Pretreatment of cells with resveratrol prevented upregulation of MMP-2 (a) in M1 and M2 subsets and of MMP-9 (b) in M2 macrophages in response to 7-oxo-cholesterol (7oxo-C). Culture supernatants of polarized M1 (□) and M2 (■) macrophages treated or not with resveratrol (Resv; 30 μM) for 1 hour and then stimulated with 15 μM 7oxo-C for 20 hours or left unstimulated were subjected to acrylamide gel electrophoresis and the gelatinolytic activity was determined by classical zymography as described in Section 2. Results are expressed as means ± SD of four independent experiments ($^{*}P < 0.001$; $^{\dagger}P < 0.05$). Representative Western blotting and zymograms are reported on the top of the bar plot.



(a)

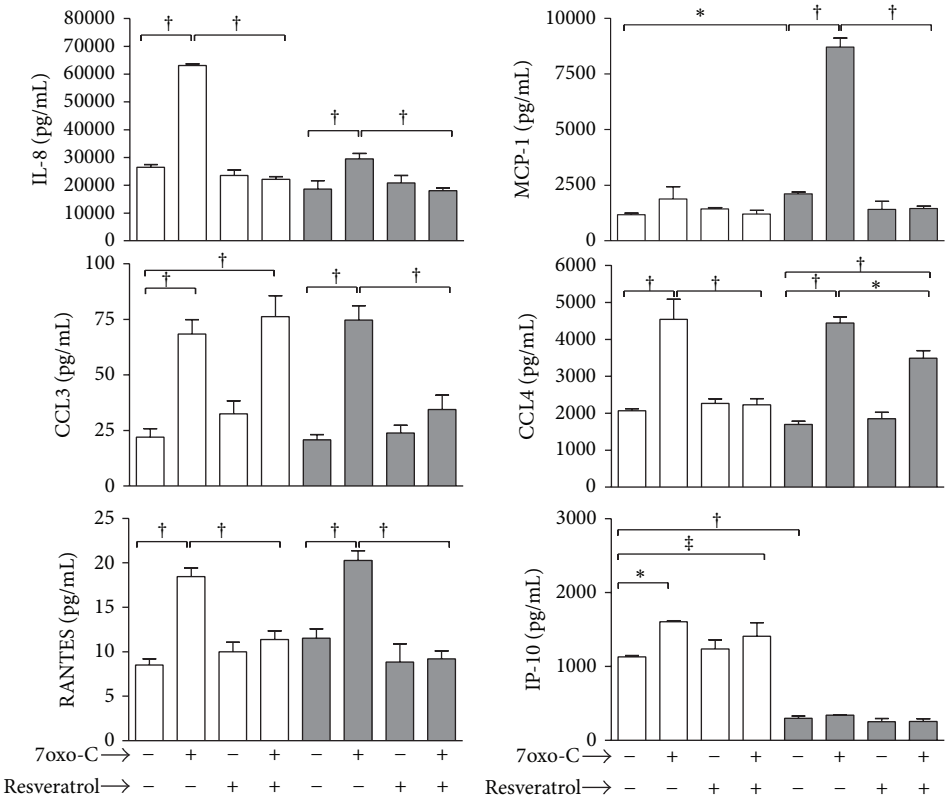


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Figure 4: Continued.

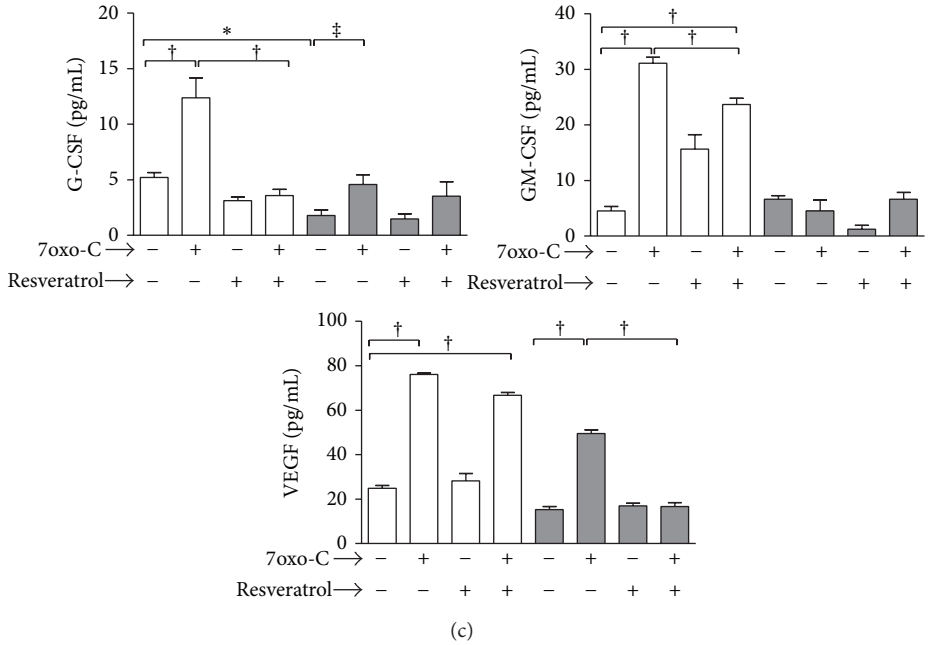

Figure 4: Secretome profile of cytokines, chemokines, and growth factors in M1 and M2 macrophages pretreated or not with resveratrol before stimulation with 7-oxo-cholesterol. Polarized M1 (□) and M2 (■) macrophages were stimulated with 15 μM 7-oxo-cholesterol (7oxo-C) for 20 hours after pretreatment or not with resveratrol (30 μM) for 1 hour at 37°C, 5% CO_2. At the end of incubation time, supernatants were analyzed for cytokines (a), chemokines (b), and growth factors (c) release using a commercially available multiplex bead-based sandwich immunoassay kit, as described in Section 2. Results are expressed as means ± SD of three independent experiments ($^{*}P < 0.01$; $^{\dagger}P < 0.001$; $^{\ddagger}P < 0.05$).

response to 7-oxo-cholesterol ($P < 0.01$). It also prevented the upregulation of the chemokines IL-8, CCL-4, and RANTES and of the growth factors G-CSF and GM-CSF ($P < 0.001$).

In the M2 macrophage subset, resveratrol pretreatment significantly prevented TNF-α ($P < 0.05$) and IL-12 ($P < 0.001$) upregulation in response to 7-oxo-cholesterol and increased IL-10 ($P < 0.01$) and IL-1ra production ($P < 0.05$). It also prevented IL-8, MCP-1, CCL3 ($P < 0.001$), CCL-4 ($P < 0.01$), RANTES, and VEGF upregulation ($P < 0.001$). Resveratrol *per se* did not cause any change in the secretome profile.

3.5. Resveratrol Prevents NF-κB Activation in Response to 7-Oxo-Cholesterol. 7-oxo-cholesterol treatment significantly increased active NF-κB p50 and p65 levels in M2 macrophages (Figure 5). It also tended to increase the p65 levels in the M1 subset, although not in a statistically significant way. Pretreatment of macrophages with resveratrol prevented the upregulation of active p50 and p65 in response to 7-oxo-cholesterol in the M2 subset.

4. Discussion

In the present study we demonstrated that resveratrol, a known antioxidant and anti-inflammatory natural phenolic compound [20], possesses immunomodulatory and anti-inflammatory activities in human M1 and M2 macrophages challenged with 7-oxo-cholesterol, a cholesterol autoxidation product.

In a recent study, we demonstrated that 7-oxo-cholesterol affects human macrophage biology by switching M2 macrophages from an anti- to a proinflammatory and proatherogenic M1-like phenotype [15]. We postulated that this new pathway may have implications in atherosclerotic disease where oxidative stress, which generates oxidized lipids, and cell-based inflammatory mechanisms are tightly connected. In this same study, we demonstrated by surface markers that 7-oxo-cholesterol-stimulated M1 macrophages exhibit an increased expression of the activation marker HLA-DR, even more pronounced than that one caused by LPS [15]. This points to an upregulation of macrophage function as antigen presenting cells that favour the activation of adaptive immune responses. In the same subset, we demonstrated that 7-oxo-cholesterol is able to downregulate CD16, a low affinity Fc receptor for IgG antibodies, thus likely impairing the phagocytosis of antibody-antigen complexes [21]. In our present study, we confirmed the effects of 7-oxo-cholesterol on M1 cell phenotype and demonstrated that resveratrol was able to prevent the oxysterol-induced phenotypical changes (Figure 1). In this way, resveratrol may exert an anti-inflammatory activity by limiting the activation of the immune system and preserving the anti-inflammatory clearance capacity of M1 cells [21]. Concerning the effects of 7-oxo-cholesterol on M2 subset, we confirmed its ability to increase surface expression of the monocyte differentiation

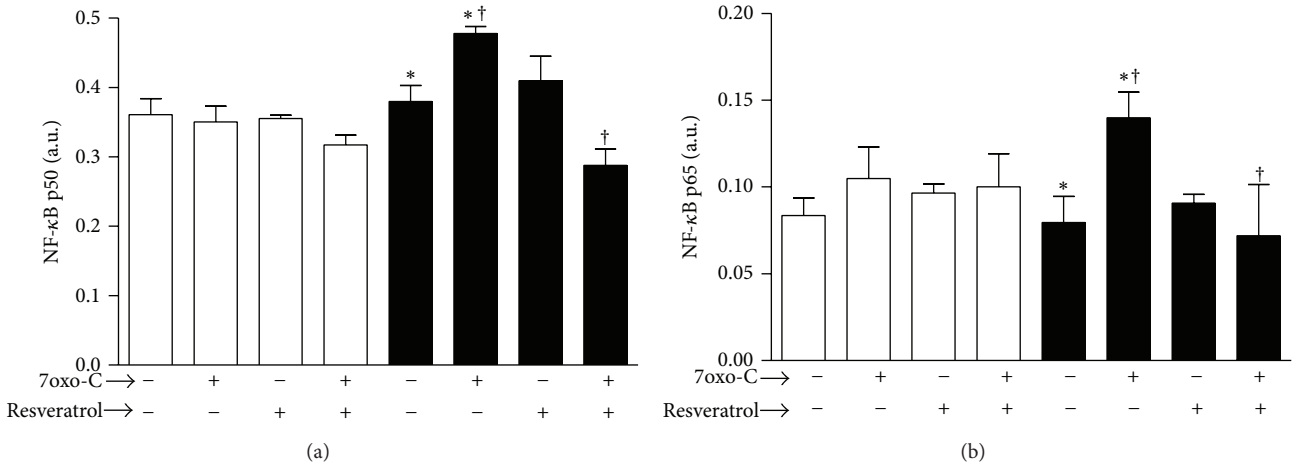

Figure 5: NF-κB activation in M1 and M2 macrophages. 7-oxo-cholesterol (7oxo-C) stimulation significantly increased active NF-κB p50 (a) and p65 (b) levels in M2 subset. Pretreatment of M2 with resveratrol prevented the upregulation of both active p50 and p65 in response to 7-oxo-cholesterol. M1 (□) and M2 (■) macrophages pretreated or not with resveratrol (30 μM) for 30 minutes were cultured for 1 hour with or without 7oxo-C (15 μM). Cells were then analyzed by NF-κB (p50 and p65) transcription factor assay to monitor NF-κB activation. The results are expressed as arbitrary units ($n = 3$, p50: $^{*}P < 0.01$; $^{\dagger}P < 0.001$; p65: $^{*}P < 0.05$; $^{\dagger}P < 0.01$).

antigen CD14, a pattern recognition coreceptor for bacterial LPS and cell-activating mediator of inflammatory responses [22]. Resveratrol was able to counteract the oxysterol-induced switch of the M2 subset to a more pronounced proinflammatory phenotype (Figure 1).

Numerous investigations indicate that—beside phenotype—a main difference between different polarized macrophage subsets lies in the production of key cytokines and chemokines, proteases, and other mediators [23]. Macrophages are major components of the innate immune system. The activation of macrophages has been shown to play a pivotal role during the initiation and development of inflammatory responses by producing numerous proinflammatory mediators [24].

Macrophages are a significant source of extracellular proteases, including MMPs, as well as of pro- and anti-inflammatory cytokines that regulate extracellular matrix remodelling, inflammatory cell recruitment and activation, and vascular smooth muscle cell proliferation and apoptosis. All these events play a role in the progression of atherosclerotic lesions and facilitate an unstable phenotype [15]. To better investigate the impact of resveratrol on M1 and M2 macrophage subsets, we analyzed the macrophage endocytic activity, the release of two key metalloproteinases, and the secretome profile of several cytokines, chemokines, and growth factors in oxysterol-stimulated macrophages pretreated or not with resveratrol. Under physiological conditions, macrophages promote tissue homeostasis by clearing debris and preventing excessive inflammation in response to environmental stress [25]. This represents a hallmark function of M2-like macrophages that usually express higher levels of surface scavenger, mannose, and galactose-type receptors that are involved in debris clearance as compared to M1 cells [26]. In the present study, we confirmed the ability of 7-oxo-cholesterol to decrease the high endocytic clearance capacity of M2 macrophages [19] and demonstrated the ability of resveratrol to preserve this fundamental anti-inflammatory property of the M2 subset.

It is known that proinflammatory M1 macrophages release higher amounts of MMPs than the anti-inflammatory M2 cells. We have previously demonstrated that 7-oxo-cholesterol increases the ability of M2 cells to secrete MMP-9 [15] and in the present study we demonstrated that it also upregulates the expression of MMP-2 in M1 and M2 subsets, supporting the concept that this oxysterol is able to polarize macrophages toward a proinflammatory state. As further evidence, we here provided a relevant outcome on the inhibitory effect of resveratrol upon MMP-2 and MMP-9 activity upregulation in macrophages pretreated with resveratrol before the challenge with 7-oxo-cholesterol. Our results agree with previous findings by Walker et al. [20] who demonstrated that resveratrol is able to downregulate PMA-mediated induction of MMP-9 activity in U-937 macrophages by inhibiting MMP-9 gene transcription.

The present study clearly showed that resveratrol is able to modulate the release of many cytokines, chemokines, and growth factors in M1 and M2 macrophages in response to 7-oxo-cholesterol.

In our previous study, analysis of cytokine, chemokine, and growth factor secretion profile by means of a multiplexed bead assay system showed that 7-oxo-cholesterol selectively activated in both macrophage subsets the production of many key proatherogenic mediators involved in proinflammatory, proinvasive, and proangiogenic mechanisms within the atherosclerotic plaque [15]. We had previously observed that 7-oxo-cholesterol in M1 cells raised the production of the proinflammatory cytokines TNF-α and IL-6, thus leading to incremental proinflammatory attitude of these cells. Interestingly, 7-oxo-cholesterol induced M2 subset to release TNF-α and IL-6 and the M1-polarizing cytokine IL-12 [27], thus

further confirming the ability of 7-oxo-cholesterol to skew M2 cell polarization towards an M1-like phenotype. Notably, in this present study, we investigated the anti-inflammatory effects of resveratrol on the secretion of the same panel of cytokines, chemokines, and growth factors by M1 and M2 macrophages. We found that resveratrol pretreatment significantly prevented TNF-α and IL-6 upregulation in response to 7-oxo-cholesterol in M1 cells and of TNF-α and IL-12 in M2 ones, thus confirming the ability of this compound to counteract the proinflammatory signaling of oxysterol in macrophages. The anti-inflammatory and immunomodulatory activities of resveratrol were further confirmed by the inhibition of many chemokines in both subsets, particularly IL-8, MCP-1, CCL3, CCL4, and RANTES and of the growth factors G-CSF and GM-CSF (in M1 cells) and VEGF (in M2 cells). These inflammatory mediators, beside their active role in recruiting leukocytes into inflammatory sites, may stimulate endothelial cell migration, spreading, and neovessel formation, thus promoting the angiogenesis associated with the progression of atherosclerotic plaque [28]. The inhibitory effects of resveratrol on TNF-α, IL-6, IL-8, MCP-1, CCL-4, RANTES, and G-CSF in the M1 macrophage subset and on TNF-α, IL-12, IL-8, MCP-1, CCL3, CCL-4, RANTES, and VEGF in M2 macrophages together with metalloproteinases inhibition may be added to a variety of resveratrol antiatherogenic actions, since these molecules are known to be involved in inflammatory responses in arterial walls during progression of atherosclerosis [29].

Our results are in accordance with the inhibitory effect of resveratrol on the release of proinflammatory mediators shown in various cell models after stimulation with lipopolysaccharides and in *in vivo* animal models [30–36]. Walker et al. [20], in U-937 cells stimulated with lipopolysaccharides from *Escherichia coli*, proved that 10 mM resveratrol completely inhibited the *E. coli*-LPS-induced release of IL-6 and reduced TNF-α release by 48.1%. In accordance with Walker et al. [20], Qureshi et al. [37] showed that 0.1 to 10 mM resveratrol inhibited the LPS-stimulated release of TNF-α and gene expression of TNF-α, IL-1β, IL-6, and iNOS from RAW 264.7 macrophages. Another study showed that pretreatment of RAW 264.7 macrophages with resveratrol ($\geq$25 μM) followed by LPS stimulation resulted in a reduction of the IL-6 and TNF-α release compared to the LPS treatment [30].

To investigate potential resveratrol mechanism(s) implicated in the prevention of macrophage proinflammatory activation in response to 7-oxo-cholesterol we analyzed the effects on the modulation of NF-κB, the prototypical transcription factor, which plays a central role in innate immune response [38]. In our study we confirmed previous findings on the ability of oxysterols to trigger NF-κB activation [39–41]. In particular we observed that 7-oxo-cholesterol enhanced nuclear binding activity of NF-κB p50 and p65 in M2 macrophages and that resveratrol completely prevented such signaling pathway activation. This observed effect of resveratrol is in agreement with previous investigations showing that resveratrol is able to downregulate inflammatory responses through this mechanism [19, 42]. Resveratrol exists as two isomers, cis- and trans-resveratrol [24]. The cis isomer is thought to be produced naturally during grape fermentation as a result of isomerization of the trans isomer by yeast isomerases; in addition, cis-resveratrol can be obtained by exposure of the trans isomer to sunlight [24]. Huang et al. [24] provided findings that cis-resveratrol produces anti-inflammatory effects by inhibiting both the canonical and noncanonical inflammasomes, and associated pathways in human macrophages.

The lack of strong clinical/scientific evidence prompted scepticism in many cardiologists regarding the cardioprotective effects through interventions with specific dietary molecules or food-derived concentrates [43]. However, in a number of studies with large cohorts, cardiologists began to consider that the percentage of decrease in deaths from coronary heart disease attributed to risk factor changes through the implementation of healthy lifestyles, including the diet, could be higher than the percentage attributed to specific treatments. To date, and according to the clinical trials conducted so far in cardiovascular disease-prevention patients, resveratrol may exert cardioprotection by improving inflammatory, fibrinolytic, and atherogenic profiles, as well as improving glucose metabolism and endothelial function. However, the specific mechanisms related to these effects and the doses needed to achieve an optimum benefit/risk ratio have not been unequivocally established so far. In addition, the actual metabolite(s) responsible for the effects is not known. It has to be taken into account that chemical instability and low resveratrol preparation yields have limited its biopharmaceutical application [44]. In an effort to overcome these problems and enhance the pharmacological activity of resveratrol, several groups have attempted to synthesize and derivatize resveratrol [44].

5. Conclusion

Our study is, to the best of our knowledge, the first study showing effects of resveratrol on phenotype and function of human M1 and M2 macrophages. Taken together, the results presented here strengthen previous findings on the immunomodulatory effects of resveratrol on innate immune cells and highlight the role of resveratrol as potential therapeutic compound to counteract the proatherogenic oxysterol signaling in the macrophage subsets within atherosclerotic plaque.

Ultimately, although our study does not provide evidence on the resveratrol mechanisms and metabolite(s) related to the observed immunomodulatory effects, it is nevertheless evident that our *in vitro* model could be useful to screen the immunomodulatory effects of pharmacologically active resveratrol derivatives that exhibit anti-inflammatory properties with higher chemical stability and lower cytotoxicity.

In addition, it proves to be useful when investigating the interaction of resveratrol and resveratrol derivatives with other anti-inflammatory and antiatherogenic compounds.

Conflict of Interests

The authors do not have competing financial interests with this study.

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Research Article

Role of Lipid Peroxidation Products, Plasma Total Antioxidant Status, and Cu-, Zn-Superoxide Dismutase Activity as Biomarkers of Oxidative Stress in Elderly Prediabetics

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The relationship between hyperglycemia and oxidative stress in diabetes is well known, but the influence of metabolic disturbances recognized as prediabetes, in elderly patients especially, awaits for an explanation. *Methods*. 52 elderly persons (65 years old and older) with no acute or severe chronic disorders were assessed: waist circumference (WC), body mass index (BMI), percentage of body fat (FAT), and arterial blood pressure. During an oral glucose tolerance test (OGTT) fasting ($0'$) and 120-minute ($120'$) glycemia and insulinemia were determined, and type 2 diabetics ($n = 6$) were excluded. Subjects were tested for glycated hemoglobin HbA1c, plasma lipids, total antioxidant status (TAS), thiobarbituric acid-reacting substances (TBARS), and activity of erythrocyte superoxide dismutase (SOD-1). According to OGTT results, patients were classified as normoglycemics, (NGT, $n = 18$) and prediabetics, (PRE, $n = 28$). *Results*. Both groups did not differ with their lipids, FAT, and TBARS. PRE group had higher WC ($P < 0.002$) and BMI ($P < 0.002$). Lower SOD-1 activity ($P < 0.04$) and TAS status ($P < 0.04$) were found in PRE versus NGT group. *Significance*. In elderly prediabetics, SOD-1 and TAS seem to reflect the first symptoms of oxidative stress, while TBARS are later biomarkers of oxidative stress.

1. Introduction

In the XXI century an elderly population (65 years old and older) will grow [1, 2]. Studies on obesity, hypertension, dyslipidemia, and hyperglycemia in elderly population are currently widely discussed [3, 4]. Patients with type 2 diabetes mellitus suffer from late diabetic complications—atherosclerosis, hypertension, and dyslipidemia [5–7]. Chronic hyperglycemia leads to oxidative stress, dyslipoproteinemia, glycation of proteins, and endothelial dysfunction [8–10].

Over 40% of those affected by carbohydrate metabolism disturbances are 65 or older [11]. Among elderly subjects the late diabetic complications are more common. Many studies have been carried out to evaluate markers of free radical-induced lipid peroxidation and antioxidant status in diabetic patients [12]. Thus, we know how diabetic hyperglycemia influences oxidant-antioxidant stress parameters [13], but still it is not clear in which way prediabetic hyperglycemia may influence metabolic balance in elderly patients. The oxidative stress may accompany and explain metabolic complications in hyperglycemic persons [14].

Hence, the present study has been undertaken, to evaluate the hypothesis that oxidative stress in elderly patients with increased risk for diabetes (prediabetes, impaired fasting glucose, IFG, and impaired glucose tolerance, IGT) is responsible, at least partially, for the clinical and metabolic complications. There is some evidence of early symptoms of cell damage caused by acute short-time elevated glucose concentration in medium, since changes of the NAD^+/NADH ratio, mitochondrial membrane potential, and reactive oxygen species production were observed in human hepatic carcinoma model exposed to hyperglycemia-like *in vitro* situation [15].

An incomplete reduction of oxygen to water during electron transport chain in mitochondria is a possible source of oxygen-free radicals, that is, superoxide radical $^{\bullet}O_2^-$, in the elementary model of oxygen-free radical production. The following oxidative modification of biomolecules is discussed in many pathologies. The human body presents natural defense against free radicals: antioxidants preventing the formation of free radicals (i.e., metal-binding proteins), antioxidants scavenging free radicals and derivates: enzymatic (i.e., superoxide dismutase, catalases, glutathione peroxidase, and paraoxonase) and nonenzymatic (including vitamins, uric acid, bilirubin, and proteins) systems, and repair enzymes (targeting DNA especially). Cooper and zinc-containing superoxide dismutase, Cu-, Zn-SOD (SOD-1), cytoplasmic enzyme, metabolizes superoxide radicals to molecular oxygen and hydrogen peroxide providing defence against oxygen toxicity [16].

The low-weight molecules are involved in the total plasma antioxidant status in the following proportions: 35–65% for uric acid, 10–50% for plasma proteins, 14% for vitamin C, and 7% for vitamin E [17], while other investigators assessed the detailed contribution of thiol groups (52.9%), uric acid (33.1%), vitamin C (4.7%), bilirubin (2.4%), vitamin E 1.7%, and others (5.2%) [18]. Researchers agree that due to the participation of many factors in the creation of plasma antioxidant defense and their possible variability, in pathological conditions of a significant share of oxidative stress, the total measurement could be more valuable [18]. Thus, intracellular antioxidant enzyme, the erythrocyte Cu-, Zn-superoxide dismutase (SOD-1), and the plasma total antioxidant status (TAS) as extracellular were chosen to describe the antioxidant potential. Thiobarbituric acid-reacting substances (TBARS) were to reflect plasma lipid peroxidation products.

2. Material and Methods

The study was performed under the permission from local ethics group in accordance with the Declaration of Helsinki of 1975 for Human Research and the study protocol was approved by the Bioethics Committee of Poznan University of Medical Sciences in Poznan, Poland (statements numbers 142/11 and 595/11). The subjects participating in the study gave informed consent to the study procedure.

2.1. Subjects. This study enrolled 313 elderly Caucasians (65 years old and older) with no complains, from Poznan metropolitan area (west of Poland). Nonsmoking elderly persons, using no medication, no special diet, no supplements, and no alcohol, without acute or chronic disease, were studied. The exclusion criteria were the positive history of stroke, coronary artery disease (accompanied by current steady-state electrocardiography), diabetes, neoplastic disease, and inflammatory disease. Additional biochemical exclusion criteria were albuminuria reflected by albumin/creatinine ratio >30 mg of albumin/1 g of creatinine in fresh morning urine sample and decreased eGFR (less than 60 mL/min) based on MDRD formula eGFR (mL/min/1.73 m^2) = {186 × [creatinine]$^{-1.154}$ × [age]$^{-0,203}$ × 0.742 [for women] × 1.210 [for Afro-American]}. Complete physical examination, including the measurement of waist circumference (WC), systolic (SBP) and diastolic (DBP) arterial blood pressure, percentage of body fat (FAT) measured by bioimpedance method using BodyStat equipment, and the calculation of body mass index (BMI = kg/m^2), was performed.

Finally 52 individuals were qualified for the 75 g oral glucose tolerance test (OGTT) due to WHO recommendations [19]. Results of OGTT allowed classifying subjects for normal glucose tolerance (NGT) (n = 18, mean age 69.0 years) and prediabetic (PRE) (n = 28, mean age 71.0 years) categories, while newly diagnosed type 2 diabetes mellitus (T2DM, n = 6) patients were excluded from the study. The interpretation of oral glucose tolerance test is presented in Table 1 [20].

2.2. Blood Sampling and Biochemical Analysis. Blood was collected from the ulnar vein twice: fasting at 0 min ($0'$) and at 120 min ($120'$) of the 75 g OGTT. Fasting blood sample was used to measure the level of glycated hemoglobin (HbA_{1c}) as well as glucose, insulin, and lipid concentrations in plasma samples without symptoms of hemolysis. Oxidant-antioxidant balance was measured in fasting blood samples. Blood collected at 120 min of OGTT was used for plasma glucose and insulin determinations.

2.2.1. Glucose, Lipid, Insulin, and HbA_{1c} Assays. Oral glucose tolerance test was performed according to WHO recommendations between 7.00 and 9.00 am. Glucose concentrations were determined at 0 minutes and 120 minutes of OGTT, following a standard dose of 75 g glucose load. Glucose and lipid parameters, including total cholesterol (T-C), high density lipoprotein cholesterol (HDL-C), and triacylglycerols (TAG) concentrations, were evaluated by enzymatic methods using bioMerieux reagent kit (Marcy-l'Etoile, France) and the UV-160A Shimadzu spectrophotometer (Shimadzu Co., Kyoto, Japan). Low density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula for lipid parameters expressed in mmol·L^{-1}: [LDL-C] = [T-C] − [HDL-C] − [0.45·TAG], if TAG <4.56 mmol·L^{-1}.

Insulin concentration was measured by an ELISA method (BioSOurce, Nivelles, Belgium) with sensitivity of 0.15 mU·L^{-1}, using microplate reader Sunrise (Tecan Group, Männedorf, Switzerland). The intra- and interassay coefficients of variation (CV) were 3.8% and 4.5%, respectively.

Glycated hemoglobin (HbA_{1c}) level was determined by ion exchange high performance liquid chromatography using

Table 1: The interpretation of oral glucose tolerance test (OGTT) adapted from [20].

Categories of glycemia during OGTT	Plasma glucose concentration	
	Fasting (at 0 min)	At 120 min
Normal glucose tolerance (NGT)	<5.6 mmol·L^{-1} <100 mg·L^{-1}	<7.8 mmol·L^{-1} <140 mg·L^{-1}
High risk of diabetes (prediabetes, PRE)		
Impaired fasting glycemia (IFG)	5.6–6.9 mmol·L^{-1} 100–125 mg·L^{-1}	<7.8 mmol·L^{-1} <140 mg·L^{-1}
Impaired glucose tolerance (IGT)	<7.0 mmol·L^{-1} <126 mg·L^{-1}	7.8–11.0 mmol·L^{-1} 140–199 mg·L^{-1}
Diabetes mellitus (DM)	<7.0 mmol·L^{-1} <126 mg·L^{-1}	≥11.1 mmol·L^{-1} ≥200 mg·L^{-1}

D-10 Instrumentation (BioRad, Heidelberg, Germany) due to the national glycohemoglobin standardization program (USA), with the sensitivity 0.05% of HbA_{1c}, and intra- and interassay CV for HbA_{1c} measurement were 5.0% and 6.8%, respectively.

The Reference Sera. RANDOX Assayed Human Multi-Sera Level 1 (as normal) and RANDOX Assayed Human Multi-Sera Level 2 (as pathological) (Randox, Crumlin, United Kingdom) were used for monitoring the accuracy of the determinations.

2.2.2. Oxidative Stress Markers. Concentration of plasma total antioxidant status (TAS) and activity of erythrocyte cytoplasmatic superoxide dismutase Cu-, Zn-SOD (EC: 1.15.1.1) (SOD-1) were measured spectrophotometrically by a colorimetric assay based on the decrease of the optical density of the blank produced by each sample in analogy to its antioxidant property using Randox reagent kits (Randox Laboratories Ltd., Crumlin, Co. Antrim, United Kingdom) and Stat Fax 1904 Plus spectrometer (Awareness Technology, Inc., Palm City, Florida, USA).

Total Antioxidant Status (TAS). Total antioxidant status was carried out using $ABTS^+$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical formation kinetics. The presence of antioxidants in plasma suppressed the bluish-green staining of the ABTS cation, which was proportional to the antioxidant concentration level. Kinetics was measured at 600 nm. The intra- and interassay CV for plasma TAS concentrations were 1.5% and 3.8%, respectively.

Red Blood Cell Cu-, Zn-Superoxide Dismutase (SOD-1) EC: 1.15.1.1.. The method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD-1 activity was measured by degree of inhibition of the reaction. Kinetics was measured at 505 nm. The intra- and interassay CV for SOD-1 were 1.6% and 2.7%, respectively.

Thiobarbituric Acid-Reacting Substances (TBARS). Concentration of plasma TBARS, reflecting plasma lipid peroxidation products, was determined by Okhawa method [21] using Sigma reagents (Germany) and Specord M40 spectrometer (Germany). The intra- and interassay CV for TBARS were 1.8% and 3.7%, respectively.

2.3. Statistical Analysis. Statistica 10.0 version for Windows was used for statistical analysis. The normality of value distribution was checked by Shapiro-Wilk test. Then, the results with a Gaussian distribution were analyzed with Student's t-test, and those with a non-Gaussian distribution were verified by a nonparametric Mann-Whitney U test to assess the differences between studied NGT and PRE groups. The Spearman rank correlation test was used to evaluate the strength of association between two variables. Multiple regression analysis was performed to evaluate the relationship between independent variables and SOD-1 activity and TAS and TBARS concentrations. A $P < 0.05$ was taken as indicative of significant differences. The results with a Gaussian distribution are expressed as mean and standard deviation (SD), and those with a non-Gaussian distribution are expressed as median and interquartile range.

The correlations between studied oxidative stress markers (TBARS, SOD-1, and TAS) and age, BMI, waist circumference, FAT, plasma lipids, HbA1c, G0′, and G120′ were tested with the use of multiple regression comparison. The analyzed models included the following:

(A) age, BMI, waist circumference (WC), and percentage of body fat (FAT) (Table 4);

(B) HbA1c, G0′, and G120′ (Table 5);

(C) SBP, DBP, age, BMI, FAT, and WC (Table 6);

(D) T-C, HDL-C, and TAG (LDL-C was not included as derivative of analyzed variables).

Analyzed subgroups of patients were as follows:

(i) females/males;

(ii) AH = 0/AH = 1, as with no arterial hypertension (AH = 0) and with arterial hypertension (AH = 1);

(iii) G0′ = 0/G0′ = 1, as with no fasting hyperglycemia (G0′ = 0) and with fasting hyperglycemia (G0′ = 1);

(iv) PRE = 0/PRE = 1 as with no prediabetes (PRE = 0) and with prediabetic states IFG or IGT (PRE = 1).

Table 2: The characteristics of the study groups. Data are presented as mean ± SD for Gaussian distribution and median with interquartile range for the non-Gaussian distribution.

	Total $n = 46$	NGT $n = 18$	PRE $n = 28$
Age (years)	70.0 (67.0–74.0)	69.0 (66.0–73.0)	71.0 (67.0–75.0)
BMI ($kg \cdot m^{-2}$)	28.0 (26.0–30.8)	26.0 (24.0–28.5)	29.3 (26.5–34.2)*
WC (cm)	92.0 (83.0–98.0)	84.5 (78.0–92.0)	94.0 (88.0–104.0)*
FAT (%)	37.2 +/− 15.2	34.4 +/− 18.4	38.6 +/− 12.8
SBP (mmHg)	140.0 (130.0–145.0)	132.5 (125.0–145.0)	140.0 (135.0–145.0)
DBP (mmHg)	80.0 (75.0–90.0)	85.0 (80.0–90.0)	80.0 (70.0–85.0)
G0′ ($mmol \cdot L^{-1}$)	5.71 (5.17–6.35)	5.13 (4.97–5.39)	6.19 (5.83–6.44)
G120′ ($mmol \cdot L^{-1}$)	6.67 (5.56–7.50)	5.62 (5.03–6.67)	7.22 (5.70–8.5)
Ins 0′ ($mU \cdot L^{-1}$)	30.83 (16.39–34.66)	29.25 (16.39–31.93)	32.85 (16.54–37.44)
Ins 120′ ($mU \cdot L^{-1}$)	60.41 (27.65–90.97)	62.68 (49.29–81.66)	56.38 (27.51–120.64)
HbA_{1c} (%)	5.99 (5.60–6.40)	5.80 (5.60–6.10)	6.20 (5.50–6.50)
T-C ($mmol \cdot L^{-1}$)	5.13 (4.64–5.70)	5.71 (4.79–5.78)	5.05 (4.61–5.67)
TAG ($mmol \cdot L^{-1}$)	0.94 +/− 0.75	1.24 +/− 0.59	1.32 +/− 0.85
HDL-C ($mmol \cdot L^{-1}$)	1.66 (1.41–1.79)	1.71 (1.48–1.81)	1.65 (1.29–1.78)
LDL-C ($mmol \cdot L^{-1}$)	2.90 (2.49–3.46)	3.11 (2.58–3.54)	2.79 (2.45–3.24)
SOD-1 ($U \cdot gHGB^{-1}$)	1049.9 (886.7–1257.9)	1208.6 (1002.4–1623.0)	980.4 (841.7–1137.2)*
TAS ($mmol \cdot L^{-1}$)	1.338 (1.228–1.510)	1.341 (1.280–1.717)	1.303 (1.154–1.401)*
TBARS ($\mu mol \cdot L^{-1}$)	2.082 (1.737–2.416)	1,902 (1.722–2.088)	2.189 (1.770–2.952)

*Significant difference, as compared to NGT group.
NGT: normal glucose tolerance, PRE: prediabetes states, FAT: percentage of body fat, BMI: body mass index, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, G0′: fasting glucose, G120′: glucose at 120 min of oral glucose tolerance test (OGTT), Ins 0′: fasting insulin, Ins 120′: insulin at 120 min of OGTT, HbA_{1c}: glycated hemoglobin, T-C: total cholesterol, TAG: triacylglycerols, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, SOD-1: Cu-, Zn-superoxide dismutase, TAS: total antioxidant status, and TBARS: thiobarbituric acid-reacting substances.

3. Results

The data in Table 2 show oxidative-antioxidative status and the clinical and biochemical characteristics of the groups. In the study population, prediabetes was diagnosed according to American Diabetes Association Standards for Medical Care 2013 [20] using OGTT and reflected 63.0% of IFG and 37.0% of IGT. Normoglycemic and prediabetes groups did not differ in lipid profile and percentage of body fat, but PRE group had higher waist circumference ($P < 0.002$) and BMI ($P < 0.002$). Concerning the oxidative stress markers, decreased SOD-1 ($P = 0.033$) and TAS ($P = 0.039$) and increased TBARS (no significance, $P = 0.062$) were observed in the elderly prediabetics.

Correlation analysis considering oxidative stress markers and other parameters, in both groups, was performed (Table 3). In normoglycemic elderly subjects highly positive correlation between TAS and SOD was observed, whereas such an association was not found in the prediabetic group. However, in prediabetic subjects, a positive correlation between TAS and WC and a negative correlation between TAS and HDL-C were found. In addition, in PRE group, TBARS correlated positively with fasting glucose and HbA1c and negatively with age and BMI, whereas we did not observe such a correlation in the normoglycemic elderly group.

4. Discussion

Oxidative stress and failure of protein repair are one of the most discussed abnormalities in the aging process—both at the cellular and tissue levels [22, 23].

In the present study we investigated only elderly persons with or without prediabetic states to find out that oxidative stress and its markers depend not only on aging but also on hyperglycemia and its complications. Antioxidant defense systems, both located in the intracellular and extracellular spaces, are actively involved against reactive oxygen species, which are continuously generated in the body due to normal metabolism and disease. Studies concerning patients with late diabetic complications [24] or without them [25] have revealed a decrease in antioxidant defenses and an increase in oxidative damage markers. The authors of the present study investigated antioxidant status at the very early stages of hyperglycemia and found lower SOD-1 activity and plasma TAS in prediabetic elderly persons in comparison with normoglycemic ones.

Nakhjavani and colleagues suggested that the chronicity of DM promotes lipid peroxidation and malondialdehyde production, independent of glycemic control and antioxidant activity [26]. In our study we did not find any differences in the TBARS (as investigated for lipid peroxidation products) between normoglycemic and prediabetic elderly people,

Table 3: The correlations between oxidative stress markers and clinical and biochemical parameters in the studied subjects.

Variables	NGT						PRE					
	SOD-1		TAS		TBARS		SOD-1		TAS		TBARS	
	R	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*
Age	−0.379	0.120	−0.258	0.301	0.341	0.166	0.245	0.218	0.024	0.907	**−0.659**	**<0.001**
BMI	0.229	0.360	−0.012	0.961	0.198	0.430	0.192	0.924	0.115	0.567	**−0.437**	**0.023**
WC	−0.174	0.491	−0.215	0.391	0.337	0.172	0.209	0.295	**0.398**	**0.040**	−0.164	0.413
FAT	−0.034	0.893	−0.106	0.675	0.242	0.332	0.200	0.585	0.042	0.835	−0.366	0.061
SBP	−0.028	0.912	−0.203	0.418	0.229	0.360	−0.159	0.429	0.113	0.574	0.057	0.777
DBP	0.017	0.946	−0.105	0.677	−0.044	0.863	−0.068	0.738	0.185	0.357	−0.204	0.307
G0′	0.189	0.453	0.130	0.607	0.032	0.900	−0.318	0.106	−0.080	0.693	**0.407**	**0.035**
G120′	−0.057	0.823	−0.118	0.639	0.358	0.144	0.062	0.759	−0.029	0.886	0.314	0.110
Ins 0′	−0.154	0.542	−0.349	0.155	0.120	0.636	−0.052	0.794	**0.422**	**0.025**	**−0.403**	**0.033**
Ins 120′	−0.135	0.593	−0.244	0.328	0.253	0.311	0.214	0.273	0.104	0.597	0.177	0.368
HbA_{1c}	−0.140	0.579	−0.003	0.990	0.351	0.153	−0.056	0.779	−0.093	0.645	**0.503**	**0.007**
T-C	0.320	0.195	0.272	0.275	−0.012	0.961	0.055	0.786	−0.200	0.317	0.186	0.354
TAG	0.020	0.936	0.041	0.872	0.118	0.642	−0.255	0.199	0.040	0.843	0.082	0.684
HDL-C	0.174	0.489	0.236	0.345	−0.170	0.499	−0.029	0.885	**−0.568**	**0.002**	−0.194	0.332
LDL-C	0.376	0.123	0.234	0.349	−0.141	0.576	0.173	0.387	−0.025	0.902	0.222	0.266
SOD-1	—	—	**0.864**	**<0.001**	−0.416	0.086	—	—	0.225	0.258	−0.052	0.795
TAS	**0.864**	**<0.001**	—	—	−0.178	0.478	0.225	0.258	—	—	−0.221	0.267
TBARS	−0.416	0.086	−0.178	0.478	—	—	−0.052	0.795	−0.221	0.267	—	—

NGT: normal glucose tolerance, PRE: prediabetes states, FAT: percentage of body fat, BMI: body mass index, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, G0′: fasting glucose, G120′: glucose at 120 min of oral glucose tolerance test (OGTT), Ins 0′: fasting insulin, Ins 120′: insulin at 120 min of OGTT, HbA_{1c}: glycated hemoglobin, T-C: total cholesterol, TAG: triacylglycerols, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, SOD-1: Cu-, Zn-superoxide dismutase, TAS: total antioxidant status, and TBARS: thiobarbituric acid-reacting substances.

Table 4: The significant correlations in the multiple regression analysis between oxidative stress markers and clinical and biochemical parameters in the model A, including age, BMI, waist, and FAT.

	B	Beta	*P*	*r*	R^2
TBARS and age All subjects	3.6400	−0.02746	0.0462	−0.3008	0.1175
TBARS and waist Males	2.4462	0.02728	0.0215	0.3240	0.5515
TBARS and age G0′ = 1	5.9268	−0.03448	0.0402	−0.4909	0.3053
TBARS and age Pre = 1	6.1224	−0.03550	0.0347	−0.5050	0.3379
SOD-1 and BMI G0′ = 0	3235.9562	59.4009	0.0405	0.2569	0.4653
SOD-1 and BMI PRE = 0	2522.5913	66.3857	0.0340	0.4116	0.4653
TAS and FAT G0′ = 0	3.0917	−0.008491	0.0433	−0.4332	0.4150

PRE = 0: no prediabetes states, PRE = 1: prediabetes states, G0′ = 0: no hyperglycemia, G0′ = 1: fasting hyperglycemia, FAT: percentage of body fat, BMI: body mass index, SOD-1: Cu-, Zn-superoxide dismutase, TAS: total antioxidant status, and TBARS: thiobarbituric acid-reacting substances.

whereas SOD-1 and TAS were lower in prediabetic ones. Thus, we suggest that antioxidant capacity is the first marker which declines in prediabetic elderly people. Kumawat and colleagues concluded that there is enhanced oxidative stress and decreased antioxidant defense in geriatrics as compared to younger counterparts [27]. However, their elderly group had highly increased total cholesterol, triacylglycerols, LDL-cholesterol, and decreased HDL-cholesterol. We investigated only elderly population with lipid profile within references but with or without hyperglycemia and thus we suggest that decreased antioxidant capacity is rather due to hyperglycemia than aging itself.

It is important to note that longer duration of hyperglycemia and chronic diabetes complications are associated with older age [28]. Our findings showed strong positive correlation between SOD-1 and TAS in elderly normoglycemic

Table 5: The significant correlations in the multiple regression analysis between oxidative-antioxidative status and clinical and biochemical parameters in the model B, including HbA_{1c}, G0′, and G120′.

	B	Beta	P	r	R^2
TBARS and HbA_{1c} All subjects	−2.4365	0.4511	0.0225	0.5422	0.4118
TBARS and HbA_{1c} Females	−2.6736	0.5289	0.0479	0.6294	0.4893
TBARS and HbA_{1c} AH = 1	−2.4637	0.4848	0.0369	0.4812	0.3533
TBARS and G0′ AH = 1	−2.4637	0.01756	0.0255	0.4844	0.3533
TBARS and G0′ All subjects	−2.4365	0.01504	0.0317	0.4959	0.4118
SOD-1 and G0′ Females	2473.4391	−15.0145	0.0293	−0.4508	0.2075
TAS and G0′ Females	1.8850	−0.01307	0.0159	−0.4636	0.2567
TAS and G0′ AH = 0	3.5926	−0.04342	0.0184	−0.7862	0.8063

AH = 0: no arterial hypertension, AH = 1: with arterial hypertension, G0′: fasting glycemia, HbA_{1c}: glycated hemoglobin, SOD-1: Cu-, Zn-superoxide dismutase, TAS: total antioxidant status, and TBARS: thiobarbituric acid-reacting substances.

Table 6: The significant correlations in the multiple regression analysis between oxidative-antioxidative status and the clinical and biochemical parameters in the model C including SBP, DBP, age, BMI, FAT, and waist.

	B	Beta	P	r	R^2
TBARS and SBP All subjects	3.7117	0.01727	0.0205	0.2325	0.3167
TBARS and SBP Females	3.4882	0.01664	0.0470	0.2276	0.3941
TBARS and SBP AH = 1	4.5396	0.01606	0.0479	0.2090	0.4041
TBARS and DBP All subjects	3.7117	−0.02932	0.0051	−0.2777	0.3167
TBARS and DBP Females	3.4882	−0.03155	0.0221	−0.3767	0.3941
TBARS and DBP AH = 1	4.5396	−0.02766	0.0108	−0.4208	0.4041
TBARS and age AH = 1	4.5396	−0.03200	0.0485	−0.3471	0.4041
SOD and BMI G0′ = 0	2678.6219	64.3659	0.0473	0.2569	0.4946

AH = 0: no arterial hypertension, AH = 1: with arterial hypertension, G0′ = 0: no fasting hyperglycemia, G0′ = 1: fasting hyperglycemia, BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, SOD-1: Cu-, Zn-superoxide dismutase, TAS: total antioxidant status, and TBARS: thiobarbituric acid-reacting substances.

subjects, whereas in prediabetic ones there is deactivation between intra- and extracellular antioxidative state (Table 3).

The authors of the present work found an interesting negative correlation between TAS and HDL-C in prediabetic elderly people, independent of other metabolic factors (Table 7). The linear changes of plasma HDL-C concentration may accompany or even supplement 28% of plasma TAS variability. This suggests the complementarity of these two important antioxidant factors in elderly patients with high risk for T2DM. It also supports the current suggestion about HDL function in humans, what was pointed out not only in hyper-LDL-C patients but in normal low density lipoprotein levels patients as well [29].

A very interesting work was published by Bandeira and colleagues, and they found correlation between lipid peroxidation and diabetes mellitus irrespective of the presence of hypertension [30]. In the present work the multiple regression analysis showed negative correlation between TAS and fasting glycemia in those without hypertension, what suggests that fasting glucose in 80% accompanies TAS in the preservation of development of hypertension and positive correlation between TBARS and fasting glycemia in

Table 7: The significant correlations in the multiple regression analysis between studied oxidative-antioxidative status and clinical and biochemical parameters in the model D including TC, HDL-C, and TAG (LDL-C not included as a derivative of analyzed variables).

	B	Beta	P	r	R^2
SOD and TC All subjects	811.6939	3.6717	0.0421	0.2094	0.1134
TAS and HDL G0′ = 1	1.8910	−0.009533	0.0245	−0.5293	0.2806
TAS and HDL PRE = 1	1.9046	−0.009167	0.0309	−0.5320	0.2839

G0′ = 0: no fasting hyperglycemia, G0′ = 1: fasting hyperglycemia, PRE = 0: no prediabetes, PRE = 1: prediabetes, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, SOD-1: Cu-, Zn-superoxide dismutase, and TAS: total antioxidant status.

those with developed hypertension, which is the next point of developing chronic complications in elderly prediabetic patients. Thus, we suggest that disturbances in the oxidative-antioxidative status may serve as very early markers of chronic complications of hyperglycemia.

Limitation of the Study. Although there is much that remains to be done, our work generates important findings in the field of antioxidant capacity among elderly population. We confirm that there are some limitations of this study. The main limitation is small elderly group, but it is hard to find elderly subjects without complaints, with no acute and/or chronic diseases, using no medication or supplements. Future research would have been more convincing if the researchers would have more elderly subjects with the very early hyperglycemia state both impaired glucose tolerance and impaired fasting glycemia.

5. Conclusions

In elderly patients metabolic factors differ among prediabetic and normoglycemic patients leading to disturbances in oxidative-antioxidative state. Erythrocyte SOD-1 activity and plasma TAS are lower in elderly prediabetics in comparison with normoglycemic cases revealing deactivation of antioxidative capacity by hyperglycemia in elderly patients. In elderly prediabetic subjects, TBARS did not differ significantly in comparison with control group, indicating early oxidative stress. Thus, SOD-1 and TAS are suggested to be the very early biomarkers in the course of hyperglycemic complication among prediabetic elderly people. Identification of pathomechanisms involved in disturbances of carbohydrate metabolism in the course of early diabetes stages enables the explanation of chronic diabetic complications leading to optimization of the treatment in elderly hyperglycemic cases.

Disclosure

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Conflict of Interests

All authors disclose no conflict of interests in relation to this work.

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Clinical Study

Intake of Red Wine in Different Meals Modulates Oxidized LDL Level, Oxidative and Inflammatory Gene Expression in Healthy People: A Randomized Crossover Trial

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Several studies have found that adherence to the Mediterranean Diet, including consumption of red wine, is associated with beneficial effects on oxidative and inflammatory conditions. We evaluate the outcome of consumption of a McDonald's Meal (McD) and a Mediterranean Meal (MM), with and without the additive effect of red wine, in order to ascertain whether the addition of the latter has a positive impact on oxidized (ox-) LDL and on expression of oxidative and inflammatory genes. A total of 24 subjects were analyzed for ox-LDL, CAT, GPX1, SOD2, SIRT2, and CCL5 gene expression levels, before and after consumption of the 4 different meal combinations with washout intervals between each meal. When red wine is associated with McD or MM, values of ox-LDL are lowered ($P < 0.05$) and expression of antioxidant genes is increased, while CCL5 expression is decreased ($P < 0.05$). SIRT2 expression after MM and fasting with red wine is significantly correlated with downregulation of CCL5 and upregulation of CAT ($P < 0.001$). GPX1 increased significantly in the comparison between baseline and all conditions with red wine. We highlighted for the first time the positive effect of red wine intake combined with different but widely consumed meal types on ox-LDL and gene expression. *Trial Registration*. This trial is registered with ClinicalTrials.gov NCT01890070.

1. Introduction

In the post genomic era, food is considered not only a reservoir of macronutrients, vital in the maintenance of cellular metabolism, but also a major factor capable of determining the quality of health. In fact, the close relationship that exists between micronutrients and gene expression may underlie the pathophysiologic phenomena or, conversely, may represent an early target in delaying the onset of chronic noncommunicable disease (CNCD) [1]. Inflammatory enzymes and oxidative stress are involved in the pathogenesis of numerous inflammatory diseases, including cardiovascular disease (CVD) [2].

The atherosclerotic processes underlying CVD are intimately connected with a state of chronic inflammation involving a variety of pathological changes including endothelial cell activation, low density lipoprotein (LDL) modification, macrophage chemotaxis, and vessel smooth muscle cell migration [3, 4]. Indeed small LDL particles themselves are easily oxidized to yield atherogenic oxidized LDL (ox-LDL) particles, also detectable in healthy subjects [5, 6]. Elevated levels of ox-LDL particles in blood stream have been reported to be associated with increased cardiovascular disease risk [6, 7].

Several epidemiological studies [8–12] that have examined the relationship between the extent of polyphenol-rich

food consumption (wine, fruit, tea, and cocoa) and chronic diseases support a protective effect of these antioxidant compounds from cardiovascular disease.

It is widely accepted that the consumption of fruits and vegetables prevents diseases related to the oxidative processes [13]. Several studies have found that adherence to the Mediterranean Diet, due to its unique combination of micro- and macronutrients, appears to have beneficial effects on risk of cardiovascular disease, metabolic syndrome, weight management, several types of cancer, and major chronic degenerative diseases, decreasing overall and cardiovascular mortality [14, 15].

This may be partially mediated through the action of polyphenols present in these foods in their apparent ability to potentiate the endogenous antioxidant system. Mediterranean red wine is an excellent source of polyphenolic compounds such as phenolic acids, flavonoids, stilbenes, and tannins, and a considerable body of research has focused on determining the chemical composition of wine and assaying its *in vitro* antioxidant properties [16].

Different studies have reported positive data on gene expression after feeding animals with phenolic rich extracts or normal food [17, 18]. Rodrigo et al. (2004) demonstrated that superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities, all integral to the correct functioning of the antioxidant defense system, were higher in rats after chronic consumption (10 weeks) of red wine as compared to the control group, thus demonstrating the attenuation of oxidative stress by red wine [19]. The processes and enzymatic reactions behind the endogenous response to antioxidant stress have previously been demonstrated [20].

Oxidative stress is closely related to atherosclerotic processes and is believed to be an important secondary consequence of the underlying inflammation which eventually manifests as cardiovascular disease and its complications [21, 22]. Moreover, inflammation is a complex biological process that leads to the coordinated regulation of diverse sets of genes such as chemokine C-C motif ligand 5 (CCL5). CCL5, a chemotactic cytokine (chemokines), usually called RANTES (regulated on activation, normal T cell expresser and secreted), plays diverse roles in the pathology of inflammatory disease [23, 24]. Sirtuins, silent information regulator (SIR), a class of proteins that possess deacetylase or monoribosyltransferase activity, are NAD+-dependent deacetylase regulators of several biological processes such as lifespan, aging, tumorigenesis, neurodegeneration, and metabolic diseases [25]. Seven types of SIRs have been identified in humans [26]. The only cytoplasmic sirtuin protein SIRT2 has been shown to increase in response to oxidative stress but promotes cell death through Forkhead Box proteins (FOXO) [27]. However, the biological function and mechanism of the SIRT2 protein in inflammation and oxidative stress are poorly understood.

In the present study, we evaluated the effect of the consumption of a McDonald Meal (McD) and a Mediterranean Meal (MM) with or without red wine intake on LDL oxidative status. Moreover, we investigated the effects of the two meals on the expression of oxidative stress (SIRT2, SOD, CAT, and GPx) and inflammation (CCL5) genes.

2. Materials and Methods

2.1. Participants and Study Design. A total of 30 healthy volunteers were recruited by the Clinical Nutrition and Nutrigenomic Section at the University of Rome Tor Vergata. To be eligible for the study, participants had to meet the following inclusion criteria: age between 18 and 65 years and a BMI $\geq 19\ Kg/m^2$. Exclusion criteria included active tobacco smoking, arterial hypertension (≥140/90 mm Hg), body mass index (BMI) $>30\ kg/m^2$, past history of ischaemic coronary artery disease, peripheral or cerebral vasculopathy, hepatic disease, diabetes mellitus, autoimmune disease HIV/AIDS, neoplastic disease, and use of the following medications: NSAIDS, lipid-lowering medications, oral antidiabetic medication or insulin, nitroglycerin, and corticosteroids.

At baseline, all participants were evaluated in terms of their health status. The clinical evaluation focused on nutritional status, blood pressure, clinical-biochemical analysis, quantification of ox-LDL, and a genomic evaluation with analysis of five genes belonging to the pathway of oxidative stress and inflammation.

The experimental study was conducted according to a randomized crossover trial with six arms (T1, T2, T3, T4, T5, and T6), as shown in the diagram presented in Figure 1.

During the study period volunteers consumed in a randomized order (a) baseline (B); (b) fasting + 250 mL red wine (FRW); (c) Mediterranean Meal (MM) [13] (carbohydrates 55–60% of total Kcal; protein 15–20% of total Kcal of which 50% are of vegetable derivation; total fats <30% of total Kcal; saturated fat <10% of total Kcal; polyunsaturated fatty acids (PUFA) 6–10% of total Kcal: 5-6% of total Kcal from n-6 PUFA, and 1-2% of total Kcal from n-3 PUFA; monounsaturated fatty acids (MUFA) about 15% of total Kcal; trans-fatty acids <1% of total Kcal; 30 g of fiber); (d) MM + 250 mL red wine (MMRW); (e) McDonald's Meal (McD) (n.1 sandwich Big Tasty Bacon and n.1 small French Fries package: carbohydrates 26.8% of total Kcal; protein, 18.2% of total Kcal (of which about 70% was comprised of animal proteins); total fat 55% of total Kcal McD; (f) McD + 250 mL red wine (McDRW).

Each intervention was followed by a 3-week washout period to avoid additive effects on treatments to follow.

The parameters of body composition were collected at baseline. Samples for the genomic and biochemical analysis were collected at baseline and 4 hours after each meal intervention.

Participants were not blinded to the type of diet they consumed.

The MM was prepared and distributed by the staff of the Clinical Nutrition and Nutrigenomic Section, Department of Biomedicine and Prevention, University of Rome Tor Vergata.

Subjects were asked to maintain their usual lifestyle habits and to report any illness or abnormality presented during the study period. At the end of each arm, a clinician assessed any adverse effects from the interventions by going through a checklist of symptoms including bloating, fullness, or indigestion, altered bowel habit, dizziness, and other symptoms

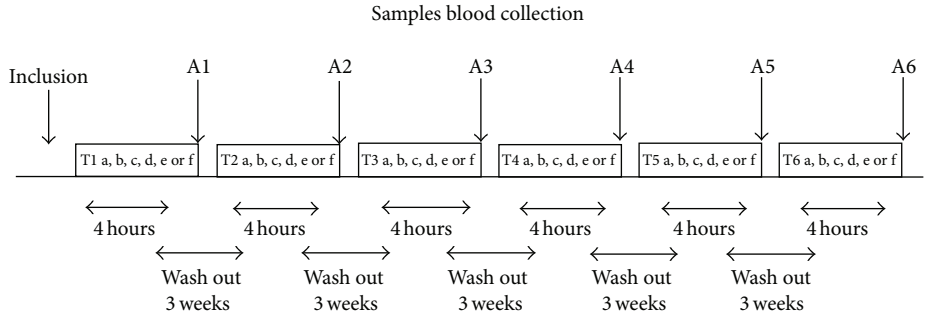

Figure 1: Study design and diets. This randomized crossover study was divided into six treatment interventions (T1, T2, T3, T4, T5, and T6) each lasting 4 hours, split by three 3-week washout periods with total study period of 18 weeks. In each treatment period (T1, T2, T3, T4, T5, and T6), volunteers consumed (a) baseline (fasting); (b) fasting + 250 mL red wine (FRW); (c) Mediterranean Meal (MM); (d) MM + 250 mL red wine (MMRW); (e) McDonald's Meal (McD); (f) McD + 250 mL red wine. The oxidative status of each volunteer was evaluated at baseline and at the end (A1, A2, A3, A4, A5, and A6) of each treatment period.

that were possibly associated with the interventions. All patients completed the study.

Nutritional status assessment and genomic analysis were performed at the Clinical Nutrition and Nutrigenomic Section, Department of Biomedicine and Prevention of University of Rome Tor Vergata.

2.2. Anthropometric Measurements. After a 12 h overnight fast, all subjects underwent anthropometric evaluation. Anthropometric parameters of all the participants were measured according to standard methods (body weight, height, and waist and hip circumferences) [28]. Subjects were instructed to take off their clothes and shoes before performing all the measurements. Body weight (Kg) was measured to the nearest 0.1 Kg, using a balance scale (Invernizzi, Rome, Italy). Height (cm) was measured using a stadiometer to the nearest 0.1 cm (Invernizzi, Rome, Italy). The waist (WC) and hip (HC) circumferences were measured with a flexible steel metric tape to the nearest 0.5 cm. WC was measured on the horizontal plane that corresponds with the narrowest point between the iliac crest and the bottom rib. HC was measured at the largest point when observed on a horizontal plane. BMI was calculated using the formula BMI = body weight (Kg)/height $(m)^2$.

The blood pressure was taken using a mercury sphygmomanometer on the right upper arm after the subject was seated quietly for at least 5 min (average of three measurements).

2.3. Bioelectrical Impedance Analysis (BIA). Resistance (R), reactance (X_c), impedance, and phase angle (PA) at 50 kHz frequency (single frequency, SF) were measured using BIA phase-sensitive system (BIA 101S, Akern/RJL Systems, Florence, Italy). Measurements were taken on the left side of the body with injection and sensor electrodes placed on the hand and foot in the reference position. TBW, extracellular water (ECW), intracellular water (ICW), Na/K ratio, PA, body cell mass (BCM), and body cell mass index (BCMI) were calculated from bioelectrical measurements and anthropometric data by applying the software provided by the manufacturer which incorporated validated predictive equations [29].

2.4. Dual-Energy X-Ray Absorptiometry (DXA). Body composition analysis was assessed by DXA (DXA, GE Medical Systems, Milwaukee, WI, USA) according to the previously described procedure for evaluating soft tissues, that is, TBFat and TBLean [29, 30]. The subjects were instructed not to exercise within 24 h of the test. The subjects were given complete instructions on the testing procedure. They wore a standard cotton t-shirt, shorts, and socks. They lay supine on the DXA scanner without moving for the duration of the scan. The average measurement time was 20 min. Radiation exposure was equivalent to 0.01 mSv. The intra- and intersubject coefficient of variation (CV% = 100 × s.d./mean) ranged from 1 to 5%. The coefficient of variation for bone mass measurements was ≤1%; the coefficients on this instrument for five subjects scanned six times over a 9-month period were 2.2% for TBFat and 1.1% for TBLean.

2.5. Sample Collection and RNA Extraction. A fasting blood sample was collected and stabilized in PAXgene Blood RNA Tubes (PreAnalytiX Qiagen, Hombrechtikon, Switzerland) and stored at −80°C until RNA extraction. The total RNA of each collected sample was purified using the PAXgene Blood miRNA Kit according to the manufacturer's instructions (PreAnalytix Qiagen, Hombrechtikon, Switzerland). Aliquots of total RNA were then quantified and assessed for quality by spectrophotometry (Nanodrop, Wilmington, USA) and agarose gel electrophoresis.

2.6. Quantitative Real Time PCR and Data Analysis. We used specific RT^2 Profiler PCR Arrays (Qiagen, Netherlands); for our study we focused on the Human Oxidative Stress (PAHS-065ZA) pathway, in particular SIRT2 CCL5SOD, CAT, and GPx. Each qRT-PCR experiment was performed in triplicate and repeated at least twice according to the manufacturer's instructions (Qiagen, Netherlands). The comparative threshold (CT) cycle was used to determine the

gene expression level relative to the calibrator RNA from the controls. Steady state mRNA levels were expressed relative to the calibrator as "*n*-fold" differences. The CT value for each gene was normalized using the formula ΔCT = CT (*gene*)-CT (*housekeeping gene*).

In particular we used the average of 4 housekeeping genes included in the plates actin beta *ACTB* (NM_001101), hypoxanthine phosphoribosyltransferase, HPRT1(NM_000-194), beta-2-microglobulin, BM2 (NM_00404080), and glyceraldehyde-3-phosphate dehydrogenase, GAPDH (NM_00-2046). The relative gene expression levels were determined according to the following formula: ΔΔCT = ΔCT sample −ΔCT calibrator.

The value used to plot relative gene expression was determined using the expression fold change (FC) = $2^{-\Delta\Delta CT}$. Raw data were filtered for genes that were significantly changed above factor 1.0 within the 95% confidence interval ($P \leq 0.05$) for each experiment. Finally, only genes with an absolute FC value of at least ±1.5 and P value ≤ 0.05 (indicating a statistical significance) were considered as differentially expressed genes.

2.7. Low Density Lipoprotein Oxidative Status. Blood samples were collected and stabilized in EDTA. Analysis of the level of oxidation of the organism was observed by the quantification of protein and oxidized LDL from the nutrigenomic study. An ELISA test was utilized for the study of LDL using the Mercodia oxidized LDL ELISA (Mercodia Diagnostic, Sweden) according to the customer protocol.

2.7.1. Red Wine Description. Masieri red wine (by Biancara of Angiolino & Alessandro Maule, Gambellara, Italy, 2012) was used in the study. This wine is made from a selection of mixed grapes including Merlot (75%), Tocai Rosso (10%), and Cabernet Sauvignon (15%) grown in volcanic soils using natural methods and is produced using the spontaneous fermentation method. The wine's characteristics are as follows: unfiltered wine, without added sulphites; total alcohol: 14.52% volume; relative density: 0.9 g/L; residual sugar: 0.7 g/L; total acidity: 5.9 g/L; dry extract: 30 g/L; volatile acidity: 0.59 g/L; total sulfur dioxide: 2 mg/L.

2.8. Statistical Analysis. A paired t-test or a nonparametric Wilcoxon test was performed to evaluate differences before and after nutritional intervention. All tests were considered significant at $P \leq 0.05$. Statistical analysis was performed using a computer software package SAS version 9.3 (SAS Institute, Cary, NC).

3. Results

3.1. Clinical Trial. Of the 30 initial participants initially enrolled, 24 subjects were eligible for the study. Three subjects declined to participate during the first phase, and another three did not meet the inclusion criteria: one of them measured a BMI < 19 Kg/m^2, one had diabetes mellitus, and one had a previous history of ischemic heart disease.

Table 1: Baseline characteristics of healthy volunteers.

Parameters	Median ± SE	Min–max (n = 15)
Age (y)	31,04 ± 5,88	25,00–46,00
SBP (mmHg)	110,22 ± 11,42	100,00–130,00
DBP (mmHg)	70,67 ± 8,83	60,00–88,00
Height (cm)	168,92 ± 10,03	157,00–183,00
Weight (Kg)	65,12 ± 9,85	48,00–79,00
BMI (Kg/m^2)	23,24 ± 2,32	20,00–27,10
WC (cm)	75,64 ± 5,59	68,00–88,00
HC (cm)	97,23 ± 6,44	88,00–108,00
W/H	0,78 ± 0,07	0,67–0,88
TW (%)	53,94 ± 3,80	47,80–59,70
IW (%)	44,35 ± 3,93	39,50–51,20
EW (%)	55,74 ± 4,00	48,80–60,50
BCMI	9,45 ± 1,28	7,80–11,80
BMD (g/cm^2)	1,14 ± 0,11	0,95–1,34
T-score	0,12 ± 0,91	−1,30–1,40
BMC (Kg)	2,57 ± 0,54	1,90–3,80
PBF (%)	28,79 ± 6,36	18,80–40,90
TBF (Kg)	18,58 ± 4,56	11,71–27,97
Lean arms (Kg)	5,02 ± 1,24	3,39–6,96
Lean legs (Kg)	14,57 ± 3,38	9,81–19,65
TBL (Kg)	43,80 ± 8,53	31,54–57,09
RMR (Kcal)	1517,69 ± 194,17	1175,00–1805,00
ASMMI (kg/m^2)	6,78 ± 0,97	5,33–8,35

Baseline characteristics of subjects at the beginning of the study. Results are expressed as median ± standard error and minimum (min) and maximum (max) for each parameter.
SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; WC: waist circumference; HC: hip circumference; W/H: waist hip ratio; TW: total water; IW: intracellular water; EW: extracellular water; BCMI: body cellular mass index; BMD: bone mineral density; BMC: bone mineral content; PBF: percentage of total body fat mass; TBFat: total body fat mass; TBLean: total body lean mass; RMR: resting metabolic rate; ASMMI: appendicular skeletal muscle mass index.

Table 1 shows the baseline characteristics of all 24 individuals. No subjects were obese on the basis of BMI classification; however 25% of the subjects were overweight. On the contrary, 25% of subjects had a PBF% > 35 and were therefore classified as obese. None of the subjects were osteoporotic, and, according to ASMMI, 4% of subjects were sarcopenic. No subjects were hypertensive.

The comparison of ox-LDL level in the intervention treatments was shown in Figure 2. A significant increase ($P \leq 0,05$) of ox-LDL in the B compared to McD (Δ% = 17.5%) was highlighted.

Ox-LDL levels significantly decreased ($P \leq 0.05$) under the following conditions: (i) McDM versus MM (Δ% = 18.2%); (ii) FRW versus MMRW (Δ% = 11.3%); (iii) McD versus McDRW (%Δ = 20.78%). No significant differences ($P > 0.05$) in the level of ox-LDL were observed under the following conditions: (i) B versus FRW (Δ% = 1.59%); (ii) B versus MM (Δ% = 3.87%); (iii) MM versus MMRW (Δ% = 6.23%); (iv) FRW versus McDRW (Δ% = 8.32%, $P > 0.05$);

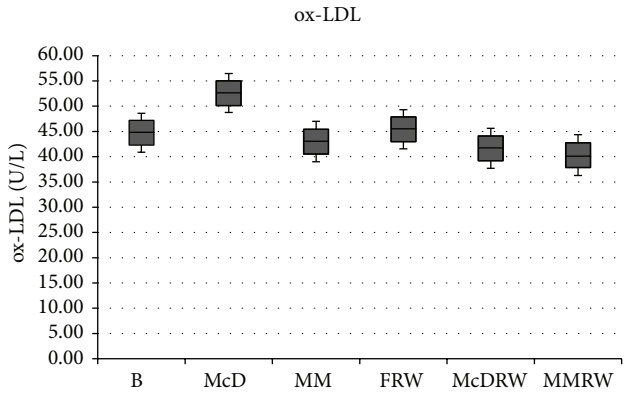

Figure 2: Oxidized LDL level. Comparative values of ox-LDL level for each treatment intervention. B: baseline; McD: McDonald Meal; MM: Mediterranean Meal; FRW: fasting + 250 mL red wine; McD RW: McDonald Meal + 250 mL red wine; MMRW: Mediterranean Meal + 250 mL red wine. The significant values are expressed as (a) $P \leq 0.05$ and (b) $P > 0.05$: (a) B versus McD; McD versus MM; FRW versus MMRW; McD versus McDRW and (b) B versus FRW; B versus MM; MM versus MMRW; FRW versus McD RW; McD RW versus MMRW; B versus McDRW; B versus MMRW.

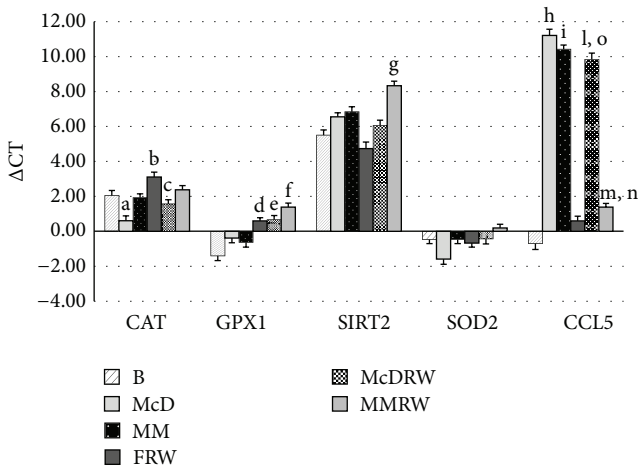

Figure 3: Variation in gene expression following each intervention. ΔCT value of gene expression of CAT, GPX1, SIRT2, SOD2, and CCL5 under the different conditions analyzed after each treatment intervention. B: baseline; MM: Mediterranean Meal; McD: McDonald Meal; MMRW: Mediterranean Meal with red wine; McDRW: McDonald Meal with red wine; CAT: catalase; GPX1: glutathione peroxidase 1; SOD2: superoxide dismutase 2; SIRT2: sirtuin 2; CCL5: chemokine ligand 5. The significant values are expressed as $P \leq 0.05$: (a) ΔCT value of gene expression of CAT: B versus McD; (b) ΔCT value of gene expression of CAT: B versus FRW; (c) ΔCT value of gene expression of CAT: McD versus McDRW; (d) ΔCT value of gene expression of GPX1: B versus FRW; (e) ΔCT value of gene expression of GPX1: B versus McDRW; (f) ΔCT value of gene expression of GPX1: B versus MMRW; (g) ΔCT value of gene expression of SIRT2: FRW versus MMRW; (h) ΔCT value of gene expression of CCL5: B versus McD; (i) ΔCT value of gene expression of CCL5: B versus MM; (l) ΔCT value of gene expression of CCL5: B versus McDRW; (m) ΔCT value of gene expression of CCL5: B versus MMRW; (n) ΔCT value of gene expression of CCL5: MM versus MMRW; (o) ΔCT value of gene expression of CCL5: FRW versus McDRW.

(v) McDRW versus MMRW (Δ% = 3.22%); (vi) B versus McDRW (Δ% = 6.86%); (vii) B versus MMRW (Δ% = 9.85%).

Moreover, we analyzed the variation of gene expression of five genes related to oxidative stress and inflammation depending on consumption of different meals with and without red wine (Figure 3).

CAT expression decreased significantly ($P \leq 0.05$) after McD. On the contrary, a significant increase ($P \leq 0.05$) of CAT expression was observed between B versus FRW and between McD versus McDRW.

GPX1 expression increased significantly ($P \leq 0.05$) in the comparison between (i) B versus FRW; (ii) B versus McDRW; and (iii) B versus MMRW.

SIRT2 expression increased significantly ($P \leq 0.05$) in comparison of FRW versus MMRW. No significant SOD expression was observed in all conditions.

CCL5 expression significantly increased ($P \leq 0.05$) in the comparison between (i) B versus McD; (ii) B versus MM; (iii) B versusboth meals with wine (MMRW and McDRW); and (iv) FRW versus McDRW. Meanwhile, CCL5 expression significantly decreased ($P \leq 0.05$) between MM versus MMRW.

The value of the Pearson coefficient of $R = 0.89$ shows a positive correlation ($P < 0.001$) between SIRT2 and CAT expression in McD and MMRW. The value of the Pearson coefficient of $R = -0.91$ shows a negative correlation ($P < 0.001$) between expression of SIRT2 and CCL5 in MM and McD.

4. Discussion

Interactions between genetic and environmental factors such as diet and lifestyle, particularly in the case of overnutrition and sedentary behavior, promote the progression and pathogenesis of polygenic diet-related diseases, the prevalence of which is increasing to epidemic proportions.

The effects of dietary compounds on metabolic pathways related to cardiovascular diseases, diabetes, and other CNCD are currently under investigation and are leading the traditional methods of nutritional counseling towards a more complex approach based on the modulation of gene expression by food.

The evidence connecting nutritional factors to the etiology of cardiovascular disorders is compelling [31]. CVDs have multiple causes, but the majority of CVD events originate from the complications of atherosclerosis, a pathophysiological process that can be prevented by nutritional interventions [32]. For a long time, based on the results of experimental studies carried out *in vitro*, the preventive effect of phenolics on age-related chronic diseases, such as CVD, was attributed to their antioxidant capacity [33]. The existing data indicate that the role of fruits and their associated nutrients in cardiovascular prevention may be more influential than that of vegetables alone; however, due to the disappointing

results of a number of large interventional studies performed with these micronutrients, showing no reduction in overall mortality and even an increased cardiovascular risk, scientists were led to consider other potentially beneficial compounds present in fruits and vegetables [12, 34, 35]. Many studies have emphasized their ability to protect various cellular constituents against oxidation [36].

Among many genetic and environmental causes, the accumulation of modified LDL [37], such as ox-LDL, and recruitment of monocyte-derived macrophages at the atrial subendothelial space [38] are the key factors leading to the development of an atherosclerotic lesion [39]. Indeed, small LDL particles are easily oxidized to yield the atherogenic ox-LDL particles that can accumulate in the foam cells of the atherosclerotic plaque [40].

Antioxidant flavonoids and polyphenols became the first substances present in red wine shown to have proven beneficial effects in various diseases, such as inhibition of LDL oxidation or attenuation of ischemia-reperfusion injury [41, 42]. Red wine exhibits higher antioxidant capacity and protective effect against LDL oxidation when compared to white wine [43–45].

We observed a significant reduction of ox-LDL depending on of the quality of meal consumed. In particular the values for ox-LDL were significantly decreased ($P \leq 0.05$) after the MM alone; MMRW increases the protective effect ($P \leq 0.05$). On the other hand, the consumption of McD increases the values of ox-LDL ($P \leq 0.05$), while McDRW brings the values for ox-LDL back towards baseline levels ($P > 0.05$).

Our findings correspond with the observation that moderate alcohol consumption, in particular red wine, is associated with a reduced risk for cardiovascular disease and an improved lipid profile [46–51]. In agreement with Tomè-Carneiro et al. (2012) [52] we observed that the consumption of red wine significantly decreases the level of ox-LDL ($P \leq 0.05$), resulting in a more efficient endogenous antioxidant defense system, with the better protection from oxidative and inflammatory damage [53]. Polyphenols appear to interact with molecular signaling pathways and related cellular machinery that regulate processes such as inflammation and consequently oxidative status [54].

We investigate, in human peripheral blood mononuclear cells (PBMC), the expression of genes related to antioxidant defenses (SIRT2, SOD, CAT, and GPx) and inflammation (CCL5) after consumption of MM and McD with or without red wine.

Particular attention was given to superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase 1 (GPX1) for their fundamental role in reducing intracellular reactive oxygen species (ROS) levels, thus protecting against cell/tissue damage [53, 55, 56]. We found that SOD expression did not significantly differ between intervention arms in our study, in agreement with Rodrigo et al. (2002) [18]. However, CAT expression was upregulated after MM, MMRW, and McDRW, probably due to the enrichment of the meals with antioxidant compounds.

SIRT2 modulates ROS production and increases resistance to its damaging effects [57, 58]. It is interesting to observe the different levels of SIRT2 expression after consumption of the MM and McD, with and without red wine, likely attributable to amplified expression of SIRT2 in response to the resveratrol present in red wine, as suggested by Lagouge et al. (2006), Schirmer et al. (2012), and Mukherjee et al. (2009) [58–60].

After McD and McDRW consumption, SIRT2 is expressed to a lesser extent than after MM, in agreement with Kim et al. (2013), where SIRT2 is upregulated in response to calorie restriction and oxidative stress, and promotes cell death under severe stress conditions via interaction with FOXO3a [56].

We observed a higher expression of SIRT2, after MM with red wine, which was negatively correlated ($P < 0.001$) with expression of CCL5, an important chemokine involved in inflammatory process. This result is in accordance with Lin et al. (2000) [61], who observed a protective effect of transduced PEP-1-SIRT2 against inflammation and oxidative stress in murine macrophages. Our results are probably due to the antioxidant characteristics of this meal in association with the polyphenols of red wine, possibly representing an optimal nutritional combination. On the other hand, lower expression of SIRT2 in the McD is correlated ($P < 0.001$) with a high level of CCL5 expression. Lin et al. (2000) [61] and Zheng et al. [62] suggested that antioxidants effectively suppressed CCL5 mRNA expression, indicating that oxidation may be involved in the induction of the CCL5 gene expression by dengue-2-virus infection. In our results, CCL5 expression is lower after MMRW consumption, which attests to the proposed antioxidant and anti-inflammatory activity of red wine and food polyphenols.

A positive correlation ($P < 0.001$) between SIRT2 and CAT was observed, in McD and MMRW, which may be due to SIRT2 increasing expression of CAT [62]. To summarize, modulation of SIRT2 through diet may have a significant impact on the inflammation underlying chronic noncommunicable diseases.

On the other hand, consumption of a McD was related to prooxidant and proinflammatory activity, as demonstrated by the increase of LDL oxidation and hyperexpression of the inflammatory CCL5 gene. Interestingly, associating red wine with the McD attenuates this effect.

5. Conclusion

The effect of red wine in association with McDonald's and a Mediterranean Meal on ox-LDL and gene expression was studied for the first time, with positive results indicating that the antioxidant potential of the nutrients found in red wine and the Mediterranean Diet may be an essential component of a holistic approach to combatting chronic noncommunicable diseases linked to inflammation.

However, prospective long-term data on consumption of a Mediterranean Diet as opposed to Western Diet (with or without red wine) on ox-LDL and gene expression is not yet available and deserves further research in order to verify changes on body composition related to gene expression. Although the number of subjects enrolled in our study is

acceptable in this instance [1], much greater numbers are required to definitively confirm these results.

In conclusion, this study provides an interesting insight into the possibility of preventing future illness through manipulation of environmental factors including diet, in line with the concept of "prospective health care," according to predictive, preventive, and personalized medicine [63].

Consent

A statement of informed consent was signed by all participants in accordance with principles of the Declaration of Helsinki.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

Laura Di Renzo designed the research and wrote the paper; Alberto Carraro, Roberto Valente, and Leonardo Iacopino conducted the research; Roberto Valente analyzed the data; Antonino De Lorenzo had primary responsibility for the final content. All authors read and approved the final paper. The authors would like to thank Francesca Sarlo, Daniela Minella, Guido Rillo, Anna Anzidei, Elaine Tyndall, and Nicoletta Del Duca for their contribution to the study. The authors have no financial or personal interests in any organization sponsoring the research at the time the research was conducted. This study was supported by Grants from the Ministry of Agriculture, Food and Forestry (DM 18829/7818/2009).

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Clinical Study

Lipid and Protein Oxidation in Newborn Infants after Lutein Administration

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Objectives. To test the hypothesis that neonatal supplementation with lutein in the first hours of life reduces neonatal oxidative stress (OS) in the immediate postpartum period. *Methods*. A randomized controlled, double-blinded clinical trial was conducted among 150 newborns divided into control group, not supplemented ($n = 47$), and test group, supplemented with lutein on the first day postpartum ($n = 103$). Blood Samples were collected at birth from cord and at 48 hrs postpartum while routine neonatal metabolic screenings were taking place. Total hydroperoxide (TH), advanced oxidation protein products (AOPP), and biological antioxidant potential (BAP) were measured by spectrophotometry and data were analyzed by Wilcoxon rank sum test and by multivariate logistic regression analysis. *Results*. Before lutein supplementation, the mean blood concentrations of AOPP, TH, and BAP were 36.10 umol/L, 156.75 mmol/H_2O_2, and 2361.04 umol/L in the test group. After lutein supplementation, significantly higher BAP increment (0.17 ± 0.22 versus 0.06 versus ± 0.46) and lower TH increment (0.46 ± 0.54 versus 0.34 ± 0.52) were observed in the test group compared to controls. *Conclusion*. Neonatal supplementation with lutein in the first hours of life increases BAP and reduces TH in supplemented babies compared to those untreated. The generation of free radical-induced damage at birth is reduced by lutein. This trial is registered with ClinicalTrials.gov NCT02068807.

1. Introduction

Protecting the newborn infant against perinatal oxidative stress (OS) is an healthcare priority, and therefore the search for new, safe, and efficacious antioxidants has been a major quest during the last decade.

Among the therapeutic antioxidant approaches, lutein, a compound belonging to the xanthophyll family of carotenoids, is one of the emerging strategies applied in newborns. Lutein is characterized by a hydroxyl group attached to either ends of the molecule, making it react more easily with singlet oxygen than other carotenoids [1–3] and neutralizing reactive oxygen species [4]. Previous experimental reports demonstrated that lutein has antiangiogenic and neuroprotective properties [5, 6] and studies *in vitro* proved its protective effect on macula and photoreceptors against phototoxicity and oxidative injury [7, 8]. Furthermore, this compound is able to ameliorate *in vitro* and *in vivo* inflammatory responses by suppressing nuclear factor kappa B (NF-κB) activation [9, 10]. Taken together, these findings support the role of lutein in modulating inflammatory processes by regulating cellular redox potential.

Human body does not synthesize lutein and the intake primarily depends on diet [11], since it is found in dark green leafy vegetables, such as kale and spinach [12, 13]. Particularly, in the neonatal period, fresh, nonprocessed human milk is the main dietary source of lutein and zeaxanthin, that is, its stereoisomer [14, 15], while infant formula is lacking it.

As of now few data are available about the effects of lutein supplementation in newborns [16–19].

In a preliminary pilot study we found that lutein supplementation to newborns infants in the first days of life reduced free radical formation and oxidative injury [20]. Considering these encouraging results, we therefore designed

this randomized, double-blind study to test the hypothesis that lutein acts as antioxidant *in vivo*.

2. Patients and Methods

2.1. Patients and Data Acquisition. A randomized controlled, double-blinded, hospital-based clinical trial was conducted at the Neonatology Unit of the Policlinico Santa Maria alle Scotte in Siena and at the Neonatal Division of the Clinical Hospital of Prato, Italy.

The local Ethics Committees approved the study protocol and the parents of the examined subjects gave informed consent.

Infant inclusion criteria were healthy singleton term newborns discharged on third day of life whose mothers had low obstetric risk and with normal adaptation to extrauterine life (clinical characteristics are reported in Table 1). The exclusion criteria included newborns with congenital malformations, suffering from perinatal hypoxia or born to mothers with mental disorders.

A computer-generated-randomization schedule was used to define test or control group. A significance level of 5% (u) and a power of 90% (v) were adopted. The sample group size was calculated by using the following formula: $n = [(u + v)^2\ (\mu_1 + \mu_0)]/(\mu_1 - \mu_0)^2$. The minimum sample size for test group was 80 newborns. To correct for inevitable cohort monitoring losses, 20 infants were added. The final cohort consisted of 150 newborns: 103 received lutein (test group) and 47 received an equivalent dose of the vehicle (control group).

The study intervention consisted of oral administration before breastfeeding of 0.28 mg of lutein or vehicle (0.5 mL of 5% glucose solution) in two doses: within 6 hours (hrs) after birth and at 36 hrs of life. In that period all babies were breast fed.

The lutein and placebo drops were produced by Neoox Laboratories (NEOOX Division of SOOFT Italia SpA, Montegiorgio, Italy). The placebo drops had the same consistency, coloration, and flavor as the lutein ones. The lutein drops were composed of a mixture containing 0.14 mg of lutein and 0.0006 mg of zeaxanthin (five drops equal to 0.5 mL of the product LuteinOfta gtt, Italy).

Clinical and research staff remained unaware of test group assignments until the completion of data analysis.

Plasma concentrations of total hydroperoxides (TH) (mmol/H_2O_2), advanced oxidative protein products (AOPP) (micromol/L), and BAP (biological antioxidant potential) (micromol/L) were determined in 200 μL of cord blood (baseline levels) and at 48 hours of life (after lutein supplementation), when 200 μL of blood was collected for neonatal metabolic screenings.

2.2. Methods. Plasma AOPP levels provide information regarding aspects of proteins involvement in free-radical (FR) reactions, namely, oxidized plasma proteins that have lost their oxidant properties. AOPP were measured as described by Witko-Sarsat et al. [21] using spectrophotometry on a microplate reader. The AOPP were calibrated with chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide. The absorbance of the reaction mixture was immediately read at 340 nm on a microplate reader. Because the absorbance of chloramine-T at 340 nm is linear up to 100 μM, AOPP concentrations were expressed as μmol/L chloramine-T equivalents (n.v. < 29 ± 0.49 μmol/L).

BAP test is based on the ability of colored solution, containing ferric (Fe^{3+}) ions adequately bound to special chromogenic substrate, to decolor when its Fe^{3+} ions are reduced to ferrous (Fe^{2+}) ions and it can be observed by adding a reducing system, that is, blood plasma as well. Plasma samples were then dissolved in a colored solution that has been previously obtained by mixing a source of ferric ions ($FeCl_3$) with a special chromogenic substrate (thiocyanate-derived compound). After 5 min of incubation, such a solution will decolor and the intensity of its change will be directly proportional to the ability of plasma to reduce, during the incubation, ferric ions, initially responsible for the color of solution, to ferrous ions. By assessing photometrically the intensity of decoloration, the amount of reduced ferric ions can be adequately calculated and the reducing ability or antioxidant power of blood plasma tested can be effectively measured. The range of standard curve was from 600 to 4,500 μmol/L and the detection limit was 587 μmol/L [22].

TH production was measured with a d-ROMs Kit (Diacron International, Italy) as described by Buonocore et al. [23]. This method makes it possible to estimate the total amount of ROMs (reactive oxygen metabolites), hydroperoxide primarily, present in a plasma sample by using a spectrophotometric procedure. The test is based on the ability of transition metals to catalyse in the presence of peroxides with formation of FR, which are trapped by an alchilamine, according to the Fenton reaction. The alchilamine (a chromogen) reacts forming colored radicals detectable at 505 nm. The intensity of developed color is directly proportional to the concentration of ROMs. The results were expressed in mg/dL of hydrogen peroxide.

2.3. Statistics. The data have been analyzed both raw and in the form of relative increments. The relative increment was calculated as the difference between the basal level of biomarkers in cord blood and the concentration observed at 48 hrs of life.

Data were expressed as median, mean, and SD and analyzed by means of the Wilcoxon rank sum test [24]. TH, BAP, and AOPP were analyzed by multivariate logistic regression model [25] using the Akaike information criterion (AIC) [26] and by the receiver operating characteristic (ROC) curve to identify the best predictor biomarker capable of distinguishing test and control groups.

The AIC was used to assess the best performing logistic regression model and chi square of the final model with respect to the null model.

The above analysis was carried out using R version 3.0.2 (2013-09-25) [27].

In the box plots the median and the interquartile ranges were reported together with the whiskers extending to the most extreme data point which is no more than 1.5 times

Table 1: Clinical characteristics of patients.

Clinical characteristic	Control group	Test group
Number of patients*	47 (100)	103 (100)
Sex*		
Male	22 (47)	57 (55)
Female	25 (53)	46 (45)
Gestational Age# (weeks)	38.18 ± 1.23	38.58 ± 1.33
Weight# (grams)	2964.37 ± 292.16	3237.73 ± 416.89
APGAR 1° minute#	9.44 ± 0.89	9.25 ± 1.11
APGAR 5° minute#	9.75 ± 0.58	9.78 ± 0.69
Type of delivery*		
Vaginal	12 (26)	37 (36)
Elective caesarean section	32 (68)	61 (59)
Emergency caesarean section	3 (6)	3 (5)
Premature rupture of membranes*		
<18 h	44 (94)	95 (92)
>18 h	3 (6)	8 (8)
Amniotic fluid*		
Clear	45 (96)	98 (95)
Stained	2 (4)	5 (5)
Vaginal swab*		
Negative	27 (57)	52 (51)
Remote or not performed	12 (26)	29 (28)
Positive	8 (17)	22 (21)
Maternal intrapartum prophylaxis*		
Not performed	32 (68)	80 (78)
Incomplete	9 (19)	12 (11)
Complete	6 (13)	11 (11)
C-reactive Protein# (mg/dL)		
24 hours of life	0.2 ± 0.09	0.21 ± 0.26
48 hours of life	0.26 ± 0.13	0.3 ± 0.41

#mean ± SD; *n (%).

the interquartile range from the box [28]. A black dot representing the mean value and an interval showing the standard error (SD/n) were superimposed to the box plot.

3. Results

Birth weight and gestational age were 3237 ± 416.89 grams and 38.58 ± 1.33 weeks, respectively, for the lutein supplemented infants (test group) and 2964 ± 292.16 grams and 38.18 ± 1.23 weeks for the vehicle treated infants (control group). No statistical differences exist in the body weight or in any other clinical characteristics of the two respective groups. Clinical characteristics of study population are reported in Table 1.

Data elaboration was carried out separately for each biomarker: TH, AOPP, and BAP; therefore a logistic multivariate analysis was done with the aim of validating the initial hypothesis and checking for important biomarkers and their interactions. Table 2 shows the statistics about the raw data.

Smaller TH and AOPP concentration increments were observed from cord blood to 48 hrs of life in treated newborns than controls. Table 3 shows the relative increments summary statistics for TH, AOPP, and BAP levels in cord blood and at 48 hrs of life.

A statistical significant difference between test and control groups relative increments in BAP from cord blood to 48 hrs of life was observed: control group 3353.78 ± 990.57 versus 3273.25 ± 937.92; test group 2361.04 ± 466.08 versus 2699.01 ± 284.25, P value = 0.0250) (Figure 1).

By using logistic regression model both TH and BAP showed statistical significant coefficients strictly related to the antioxidant effect of lutein administration. In Table 4 are reported the estimated coefficients and the relative standard errors and P value. The TH values resulted less important than BAP, which instead showed a more pronounced effect: the absolute value of the BAP standardized estimate was higher than the one of the TH. Furthermore, TH had a negative estimate, which means that subjects in test group have a lower TH relative increment compared to those in control group, while, on the opposite, subject in the test group have a larger relative increment of BAP with respect to the control group.

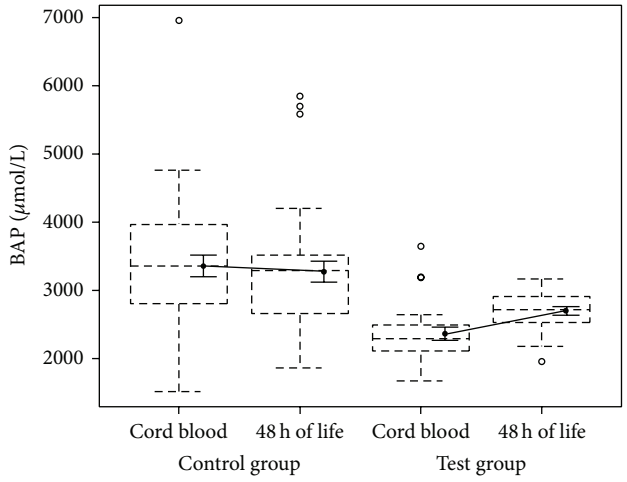

Figure 1: Plasma concentration of BAP in cord blood and at 48 hrs of life.

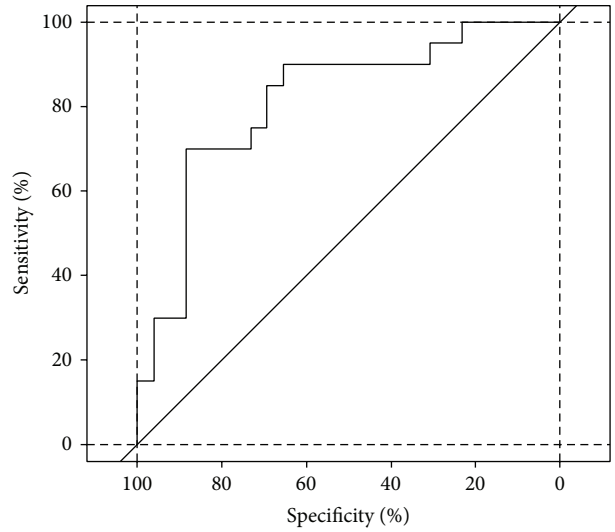

Figure 2: ROC curve for the multivariate logistic model (AUC = 81.3%, c.i. = 68.4%–94.3%).

By using a multivariate logistic model, ROC curve showed that a randomly selected normal newborn has a reduction in OS, when treated with lutein, in 81.3% of cases with the 95% confidence interval between 68.4% and 94.3%. (Figure 2).

No treatment-related adverse effect was documented in the lutein supplemented infants.

4. Discussion

The sharp increase in oxygen concentrations at birth is matter of concern for all newborns. Intrauterine life is characterized by a hypoxic environment with very low oxygen concentrations (arterial oxygen saturation around 24–30 mmHg) [29]. Thus birth represents a hyperoxic challenge for all newborns due to the high environmental oxygen availability. As consequence various reactive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen, and hydroxyl radicals are produced [30].

ROS generated through inflammatory reactions may attack DNA, RNA, proteins, and lipids in biological fluids and tissues. Moreover, ROS may act as a secondary messenger to activate various signaling pathways by inducing stress-response genes or proteins [31]. Several reports using animal models suggest that the administration of antioxidants reduces ROS damage and is effective for preventing or treating inflammatory diseases [32].

Lutein has been shown to be able to block paraquat and hydrogen peroxide-induced apoptosis in cultured retina photoreceptors [8]. Membrane bound lutein is considered able to scavenge the oxygen intermediates [33], whereby the numerous unconjugated double bonds in the lutein molecule allow the quenching of reactive oxygen intermediates.

Since newborns are exposed to hyperoxic challenge at birth, they are prone to OS-induced damage, a fact that has created a great deal of interest focusing on the protective role of lutein as antioxidant compound [34].

In the present study we found a significantly higher BAP increment and lower TH increment from cord blood to 48 hrs of life in lutein supplemented infants with respect to the control group.

Furthermore, in a pilot study we observed that lutein administration has antioxidant effects in healthy term newborns even at lower doses than those used by other authors [17, 35]. Together these results strongly support the hypothesis that lutein given orally may have protective effects on organs and tissues. Lutein seems to have not only antioxidant activity but also anti-inflammatory action as it has been recently reported [36]. Lutein inhibits arachidonic acid release from a macrophage cell line, blocking cytosolic phospholipase A2 activity [37]. Moreover lutein is thought to scavenge reactive oxygen species generated during the inflammatory cascade [38]. Lutein counteracts H_2O_2 effects and modifies the intracellular pathways leading to the expression of various proinflammatory molecules [10].

In a model of LPS stimulated macrophages, it has been found that intracellular lutein can reduce the level of intracellular H_2O_2 accumulation by scavenging H_2O_2 and superoxide anion, thereby inhibiting LPS-induced NF-κB activation [10]. Similar findings were observed using *in vitro* model of gastric epithelial cells [39].

It has been also reported that lutein treatment could diminish oxidative stress and apoptosis [40]. Lutein reduces PDGF-induced intracellular ROS production and attenuates ROS-induced ERK1/2 and p38 MAPK activation. Lutein may also lower the concentration of H_2O_2-induced PDGFR signaling, through an oxidative inhibition of protein tyrosine phosphatase [32, 41].

In line with the above reports, the results of the present randomized prospective study clearly show that even low doses of lutein have antioxidants effects. Lutein is shown to enhance BAP, thus reducing OS, as demonstrated by lower levels of TH in treated newborns. Higher doses may

Table 2: TH (total hydroperoxide, mmol/H_2O_2), AOPP (advanced oxidative protein products, micro-mol/L), and BAP (biological antioxidant potential, micro-mol/L) plasma levels in control and test groups.

	Control group (n = 47)		Test group (n = 103)	
	Cord blood	48 hrs of life	Cord blood	48 hrs of life
TH median (q25–q75)	**127.6 (99.1–160.6)**	**169.3 (132.5–263.5)**	**150.9 (112.5–185.7)**	**179.0 (140.5–244.0)**
TH mean (SD)	138.03 (±52.50)	191.43 (±82.32)	156.75 (±64.0)	195.0 (±77.54)
AOPP median (q25–q75)	**15.07 (12.7–55.42)**	**35.72 (24.64–68.82)**	**39.27 (14.54–56.14)**	**70.87 (41.34–81.48)**
AOPP mean (SD)	27.52 (±20.58)	48.40 (±33.68)	36.10 (±20.73)	64.84 (±31.23)
BAP median (q25–q75)	**3359.6 (2808.6–3966.7)**	**3287.2 (2660.3–3510.6)**	**2289.2 (2112.2–2485.3)**	**2717.1 (2528.7–2905.8)**
BAP mean (SD)	3353.7 (±990.5)	3273.2 (±937.9)	2361 (±466)	2699 (±284.2)

Table 3: Summary statistics for TH, AOPP, and BAP relative increments.

	Control group (n = 47)	Test group (n = 103)	P value
TH median (q25–q75)	**0.43 (0.12–0.82)**	**0.29 (−0.01–0.65)**	
TH mean (SD)	0.46 (±0.54)	0.34 (±0.52)	0.1344
AOPP median (q25–q75)	**0.73 (0.42–1.40)**	**0.51 (0.33–1.19)**	
AOPP mean (SD)	0.95 (±0.93)	0.83 (±0.76)	0.5034
BAP median (q25–q75)	**−0.04 (−0.20–−0.15)**	**0.16 (0.03–0.30)**	
BAP mean (SD)	0.06 (±0.46)	0.17 (±0.22)	0.0250

Table 4: Logistic regression model coefficients.

Parameters	Estimate std.	Std. error	P value
Intercept	0.2213	0.4233	0.6011
TH	−1.7214	0.7905	0.0294
BAP	3.4524	1.7111	0.0436
TH ∗ BAP	−4.0311	2.4005	0.0931

surely magnify the property of lutein to stop the increase of lipoprotein oxidation *in vivo*.

Few studies evaluated the effectiveness of lutein in reducing preterm and term infant morbidity with no results [16, 18].

The failure of lutein prophylaxis in these infants is probably related to the multifactorial nature of the pathological processes and to the need of higher doses of lutein than those used until now. The well-ascertained high safety of lutein in animals [42] and in humans [43] is a good support for studying the protective effects of large dose of lutein on organs and tissues. Our data, with their encouraging results, are powerful tools for medical research as well as for routine clinical purposes.

Further clinical trials with lutein at higher doses than those used in this study are needed to evaluate therapeutic effects of lutein on free-radical-mediated diseases of the newborn.

In conclusion lutein supplementation should be considered in all formula fed newborns and to integrate the nursing mother maternal diet, lacking an adequate dietary intake of lutein.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Isoprostanes and Neuroprostanes as Biomarkers of Oxidative Stress in Neurodegenerative Diseases

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Accumulating data shows that oxidative stress plays a crucial role in neurodegenerative disorders. The literature data indicate that *in vivo* or postmortem cerebrospinal fluid and brain tissue levels of F_2-isoprostanes (F_2-IsoPs) especially F_4-neuroprotanes (F_4-NPs) are significantly increased in some neurodegenerative diseases: multiple sclerosis, Alzheimer's disease, Huntington's disease, and Creutzfeldt-Jakob disease. Central nervous system is the most metabolically active organ of the body characterized by high requirement for oxygen and relatively low antioxidative activity, what makes neurons and glia highly susceptible to destruction by reactive oxygen/nitrogen species and neurodegeneration. The discovery of F_2-IsoPs and F_4-NPs as markers of lipid peroxidation caused by the free radicals has opened up new areas of investigation regarding the role of oxidative stress in the pathogenesis of human neurodegenerative diseases. This review focuses on the relationship between F_2-IsoPs and F_4-NPs as biomarkers of oxidative stress and neurodegenerative diseases. We summarize the knowledge of these novel biomarkers of oxidative stress and the advantages of monitoring their formation to better define the involvement of oxidative stress in neurological diseases.

1. Introduction

The CNS (central nervous system) is very vulnerable to oxidative injury due to its high oxygen demand, high level of polyunsaturated fatty acids (PUFAs), and weak antioxidant defenses. The vulnerability of the brain to oxidative damage increases with the age due to reduced integrity of the blood-brain barrier (BBB) and increased mitochondrial dysfunction [1–19]. Brain aging and neurodegeneration are characterized by chronic inflammation with persistent microglial activation and higher level of proinflammatory cytokines [4]. In addition, it promotes oxidative stress and neuronal damage. Neurons are particularly vulnerable to oxidative damage not only due to excitotoxicity but also to mitochondrial dysfunction. Moreover, neuronal membranes have plenty of unsaturated fatty acids. At higher concentrations, reactive oxygen/nitrogen species (ROS/RNS) cause neural membrane damage. Therefore, it can change not only membrane fluidity but also decreased activities of membrane-bound enzymes, ion channels, and receptors. The main sources of ROS/RNS are the mitochondrial respiratory chain, an uncontrolled arachidonic acid (AA) cascade, and NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) [5, 6].

It is known that both inflammation and oxidative stress contribute to the development of various neuropathologies including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) [5, 6, 20]. As discussed by Guest et al. [7] the cerebrospinal fluid (CSF) of participants aged over 45 years contained statistically higher amounts of the oxidative damage marker F_2-isoprostane (F_2-IsoPs) and the inflammatory cytokine IL-6.

Brain response to oxidative stress-mediated neurodegeneration is very complex. All brain structures are involved

in this multifactorial process. Astrocytes constitute approximately 90% of human brain and protect neurons from excitotoxicity through glutamate uptake system, and on the other side astrocytes contribute to the extracellular glutamate *via* reversed glutamate transporter [5]. Moreover, they may undergo astrocytosis after dopaminergic cell loss and are involved in the inflammatory processes. In general, inflammation is a protective response. The main mediators of neuroinflammation are microglial cells. Microglial cells consist mainly of macrophages and react to oxidative stress by transformation into activated microglia that are characterized by amoeboid morphology and rapid migration. Chronic activation of microglia may cause neuronal damage through the release of potentially toxic molecules such as proinflammatory cytokines, matrix metalloproteinases, ROS/RNS, proteinases, prostaglandin E_2, complement proteins, and growth factors and also leads to the DNA and RNA damage [5, 21]. These factors have neuroprotective properties but on the other hand they can be responsible for acceleration of oxidative stress and neurodegeneration.

Markers of lipid peroxidation include different molecules such as 4-hydroxy-trans-2-nonenal (4-HNE), 4-oxo-trans-2-nonenal (4-ONE), acrolein, isoprostanes, and isofurans. These markers are derived from AA, which is released from neural membrane glycerophospholipids through the activation of cytosolic phospholipases A_2 ($cPLA_2$) [22].

Lipid peroxidation is a hallmark of oxidative stress. High level of lipid peroxidation products is a characteristic for many human diseases, especially neurodegenerative diseases. It can cause damage to cellular membranes through changes of membrane organization and alteration of membrane integrity, fluidity, and permeability [23].

2. Biomarkers

Biomarkers are defined as the indicators of normal biological processes or pathologic processes that can be objectively measured and evaluated [1]. The well-characterized, appropriate biomarkers may be used for health examination, diagnosis of pathologic processes at early stage, assessment of treatment response, and prognosis. Noninvasive measurements of circulating levels of specific biomarker are useful along the whole spectrum of the disease process and before diagnosis biomarkers could be used for screening and risk assessment of the diseases [2]. Moreover, biomarkers may be used for precise measurement of oxidative stress status *in vivo* [3]. Among the biological molecules, lipids appear to be the most susceptible to the attack of ROS/RNS [24, 25] and lipid peroxidation has been implicated in the neurodegeneration [11]. Therefore, the levels of lipid peroxidation products may be used as a biomarker for the measurement of oxidative stress status *in vivo* in neurodegenerative diseases [3]. The levels of lipid peroxidation products in biological fluids and tissues of human subjects have been measured extensively [26]. Presently, various lipid peroxidation products are applied for assessment of lipid peroxidation and oxidative stress status *in vivo.*

The measurement of F_2-IsoPs is currently the best available biomarker of lipid peroxidation [8, 10, 22, 24].

3. The Isoprostanes Pathways

In the mid-1970s, it was shown that PG-like compounds could be formed *in vitro* by the nonenzymatic peroxidation of purified PUFAs. F_2-IsoPs have been discovered in 1990 by Milne et al. [27] and Roberts II and Morrow [28] and since then they collected a lot of evidence that these compounds might be biomarkers of lipid peroxidation and oxidative stress *in vivo* as well as *in vitro*.

F_2-IsoPs are a unique prostaglandin-like products, which are formed *via* nonenzymatic, free radical-mediated peroxidation of polyunsaturated fatty acids—for example, AA [28, 29]. The oxidation of AA proceeds by many competing reactions to give numerous products. IsoPs containing a variety of prostane ring structures are composed of various isomers including F_2-IsoPs, which are isomeric to $PGF_{2\alpha}$ [27, 30, 31] and D_2/E_2-IsoPs, which are isomers of PGD_2 and PGE_2, respectively [32]. The mechanism of F_2-IsoPs formation involves several steps. In the first stage, ROS reacts with the arachidonic acid and undergoes abstraction of an bisallylic hydrogen atom to yield an arachidonyl carbon-centered radical. What is more, there is insertion of oxygen, which leads to the formation of peroxyl radicals. Four different peroxyl radical isomers are formed depending on site of hydrogen abstraction and oxygen insertion. Peroxyl radicals isomers undergo 5-endocyclization and a second molecule of oxygen adds to the backbone of the compound to form four bicyclic endoperoxide intermediate regioisomers—PGH_2-like compounds. These unstable bicycloendoperoxide intermediates are reduced to the F_2-IsoPs and four F_2-IsoPs regioisomers are formed [30, 33, 34]. This regioisomers are reduced to four series of F-ring regioisomers (15-, 8-, 12-, 5-series), each consisting of eight racemic diastereoisomers. These regioisomers are depending on the carbon atom to which the side chain hydroxyl is attached [8].

To sum up, the biosynthetic steps of IsoPs include formation of the following:

(i) three arachidonyl radicals,

(ii) four peroxyl radical isomers with subsequent endocyclization; finally, formation of bicycloendoperoxide regioisomers, which are reduced to F_2-IsoPs; IsoPs are compounds that have F-type prostane rings isomeric to $PGF_{2\alpha}$.

The alternative ring structures, D/E-type and A/J-type prostane, are formed by the same mechanisms [32, 34]. So that E- or D-ring and thromboxane-ring compounds of IsoPs are formed during the rearrangement of isoprostane endoperoxides *in vivo*. E_2- and D_2-IsoPs are not terminal products of the IsoP pathway. These compounds are unstable and readily undergo dehydration *in vivo* to yield A_2/J_2-IsoPs. The cyclopentenone IsoPs might be neurotoxic products of the IsoPs pathway and might contribute to the pathogenesis of oxidative neurodegeneration. A_2-/J_2-IsoPs contain α,β-unsaturated carbonyls, which rapidly adduct cellular thiols and these cyclopentenone IsoPs induce neuronal apoptosis and promote neurodegeneration [27].

The other electrophilic lipid peroxidation products can also damage neurons. The γ-ketoaldehydes (e.g., isoketals,

isolevuglandins), highly reactive acyclic compounds, might be formed as a products of IsoPs endoperoxide rearrangement [35].

PUFAs are the most susceptible to free radical attack and, in general, oxidizability increases as the number of double bonds increases. So, the oxidizability of PUFAs can be estimated by the linear increase in the rate of oxidation with the increasing number of active methylene groups located between two bonds. From such correlation, the oxidizability of each PUFA is increased for about twofold for each active methylene group. Thus, the oxidizability of common fatty acids is as follows: linoleic acid (18 : 2) < arachidonic acid (20 : 4, $n-6$) < eicosapentaenoic acid (EPA, 20 : 5, $n-3$) < docosahexaenoic acid (DHA, 22 : 6, $n-3$) [27, 36].

The oxidation mechanisms of IsoPs are well known, but they are not the only substrate for the IsoPs pathway. The presence of at least three double bonds in fatty acid molecule allows the cyclization.

F2-dihomo-isoprostanes (F2-dihomo-IsoPs) are the peroxidation products from adrenic acid, which is the main component of myelin. The great amount of DHA is observed in brain but primarily found in white matter and is associated with myelin. White matter is commonly damaged by ischemic stroke and is uniformly damaged in MS. F2-dihomo-IsoPs are generated in significant amounts from adrenic acid and their levels are greatly increased in settings of oxidative stress occurring in the white matter portion of the human brains. Roberts II and Milne [8] demonstrate that, proportionally, levels of F2-dihomo-IsoPs in white matter undergoing oxidative injury increase to a greater extent than IsoPs and NeuroPs derived from AA and DHA, respectively. Their studies suggest that the quantification of F2-dihomo-IsoPs might be a selective marker of white matter injury *in vivo* [8].

F2-dihomo-IsoPs are also present in kidney, adrenal glands, and tissues and might be regarded as an early marker of lipid peroxidation in Rett syndrome—a disorder of the nervous system that leads to developmental reversals, especially in the areas of expressive language and hand use [37].

3.1. AA Is Not the Only One PUFA That Can Be Oxidized to Form IsoPs. By the peroxidation of the ω-3 PUFA, EPA and DHA, F-ring IsoPs have been generated. The IsoPs-like compounds generated from this acid are named NeuroPs [8].

F_3-IsoPs are formed in abundance *in vitro* and *in vivo* from EPA nonenzymatically peroxidation [38–40], while DHA may be oxidized nonenzymatically into F_4-, D_4-, E_4-, A_4-, and J_4-neuroprostanes (F_4-, D_4-, E_4-, A_4-, and J_4-NeuroPs) [38, 39]. AA is relatively evenly distributed in brain with similar concentrations in gray matter and white matter, and within glia and neurons. Unlike AA, DHA is highly concentrated in neuronal membranes to the exclusion of other cell types. Moreover, F_4-NeuroPs are by far the most abundant products of this pathway in the brain [32]. The quantification of F_4-NeuroPs provides a highly selective quantitative window for neuronal oxidative damage *in vivo*. Thus, F_2-IsoPs quantification is a reflection of oxidative damage to the brain in general and F_4-NeuroPs in particular [40, 41]. Roberts II and Milne [8] have found that the level of IsoPs produced from the oxidation of EPA significantly exceeds those of the F_2-IsoPs generated from AA. This is because EPA contains more double bonds, and therefore, it is more easily oxidizable. The authors have also observed that EPA supplementation markedly reduced levels of arachidonate-delivered F_2-IsoPs mouse heart tissues by over 60%. Such observations are crucial because F_2-IsoPs are generally considered as a proinflammatory molecules associated with the pathophysiological sequelae of oxidant stress. It is thus surprising to propose that the part of mechanism by which EPA prevents certain diseases is its ability to decrease F_2-IsoP generation [8].

3.2. IsoPs As Biomarkers of Lipid Peroxidation in Neurodegenerative Diseases. Oxidative stress is caused by an imbalance between free radicals production and antioxidant defenses in favor of the oxidation and leads to lipid peroxidation, membrane protein, and DNA damage and is thought to be important in the pathogenesis of a variety of neurological disorders, especially neurodegenerative diseases or atherosclerosis, cancer, and aging [42]. Lipid peroxidation is the most important source of free radical-mediated injury that directly damages neuronal membranes and yields a number of secondary products responsible for extensive cellular damage. Any specific repair process of lipid peroxidation does not exist as it does for proteins and DNA and this may explain why moderate levels of lipid peroxidation could have physiological significance for cell signaling and membrane remodeling [7]. One of the major targets of the lipid peroxidation process is the CNS. The brain is the most susceptible to oxidative damage because of the high oxygen consumption, the low levels of antioxidant enzymes (catalase and glutathione peroxidase), the elevated levels of iron (a potent catalyst for oxidant formation), and the ability to oxidize different substrates (e.g., membrane polyunsaturated fatty acids). Despite the fact that free radicals can attack many various critical biological molecules, such as DNA and cellular proteins, the high content of unsaturated lipids renders lipid peroxidation, the central feature of oxidant injury in the brain [43]. Peroxidation of membrane lipids affects neuronal homeostasis resulting in augmented membrane inflexibility, diminished activity of membrane-bound enzymes (e.g., sodium pump), destruction of membrane receptors, and changed permeability [44, 45]. One leading hypothesis is that the free radical-mediated oxidation of lipids contributes to the main pathological effects of oxidative stress in the brain. In support of this theory, increased levels of bioactive lipid peroxidation products have been identified in affected brain regions from humans with various neurodegenerative diseases [46, 47], as well as in corresponding animal models [48].

Due to the fact that free radicals are unstable and highly reactive, there are difficulties in direct measurement of their level. That is why elucidation of the importance of oxidative damage in neurological diseases is very hard. Because of their stability, the measurement of F_2-IsoPs by mass spectrometry has been extensively employed as a marker of oxidant stress and is widely considered to be the gold-standard index of lipid peroxidation *in vivo* [49, 50]. IsoPs can be relatively easily

Table 1: Isoprostanes as markers of oxidative stress in neurodegenerative diseases.

Classes of isoprostanes	Material	Disease	Study	Versus control	Reference
F2-IsoPs	CSF*, post mortem brain tissue, plasma, urinary	Alzheimer disease	*vivo*	High	[10–13, 32]
		Creutzfeldt-Jakob	*vivo*/*vitro*	High	[17, 18, 53]
		Huntington disease	*vivo*	High	[54]
8-iso PGF 2alfa	Urine	SPMS**	*vivo*	6-fold	[55]
	CSF	RRMS***	*vivo*	Higher	[9, 53, 56, 57]
		ALS****	*vivo*	Higher	[5, 19, 58]

CSF*: cerebrospinal fluid; SPMS**: secondary-progressive type of multiple sclerosis; RRMS***: relapsing-remitting type of multiple sclerosis. ALS****: amyotrophic lateral sclerosis.

quantified in body fluids because they are commonly found in urine, blood, and CSF and are also present in the exhaled air (Table 1). Their formation *in vivo* can be reliably monitored in every biological fluid by the noninvasive measurements of specific signals of lipid peroxidation, which tend to be sensitive and specific [51]. The measurement of F_2-IsoPs has emerged as one of the most reliable approaches to assess oxidative stress status *in vivo*, providing an important tool to explore the role of oxidative stress in the pathogenesis of human disease. In the oxidative tissue injury the level of F_2-IsoP is significantly increasing. The rapid development of analytical methods for IsoPs measurement helped clarify the role of the free radicals in human physiology and pathophysiology [52].

Measurement of F_4-NPs, the stable product of free radical damage to DHA, also provides valuable data in exploring the role of oxidative stress in neurodegenerative diseases. The products of the IsoP pathway were found to have strong biological actions and therefore may participate as physiological mediators of the disease [59]. Research on brain-derived IsoPs has begun only a few years ago, but it has already provided convincing evidence on the usefulness of these markers in understanding the role of oxidative damage in brain diseases [60]. IsoPs as active products of free-radical-mediated peroxidation of AA contained in phospholipids of cell membranes and lipoproteins have a potential relevance to human neurodegenerative and demyelinating diseases. The role of free radical-induced oxidative damage in the pathogenesis of neurodegenerative disorders has been definitely established [61–65]. The elevated formation of F_2-IsoPs has been observed in brain tissues and body fluids in numerous neurodegenerative diseases, including Alzheimer's disease [32], Parkinson's disease [6], Huntington's disease (HD) [66], Creutzfeldt-Jakob disease (CJD) [66], multiple sclerosis [55], and amyotrophic lateral sclerosis (ALS) [43].

The measurement of free F_2-IsoPs in plasma or urine can be utilized to assess the endogenous formation of IsoPs but not to reveal the organ in which they are formed. Determining the levels of IsoPs in the unique fluid compartment—CSF, which reflects the ongoing metabolic activity of the brain, provides a great opportunity to reveal the occurrence of oxidative stress and lipid peroxidation in the brain [10, 44].

4. Multiple Sclerosis

Multiple sclerosis is a multifactorial, heterogeneous disease with several pathophysiological components: inflammation, demyelination, redox, axonal damage, and repair processes. These components are not uniformly contributed in patient populations but can individually predominate [67, 68]. MS is a leading cause of neurological disabilities in young adults and affects up to 2.4‰ of population in USA and Canada and up to 1.9‰ in some European countries. It is considered to be autoimmune, or at least its etiopathogenesis involves intensive autoaggressive immune response [69]. MS is heterogeneous disease on several grounds. There are several different clinical courses of this disorder. The most usual (over 80%) is relapsing-remitting course (RRMS) in which relapse occurs from time to time followed up by complete or partial recovery [67]. This stage of disease is characterized with multifocal inflammation, oedema, and cytokines actions. About half of RRMS patients after 10–20 years of disease lasting accumulate irreversible neurological deficits [67, 70]. This type of MS is known as secondary progressive (SPMS) that is dominated by neurodegeneration processes and progression of clinical symptoms [71]. The next 20% of MS patients with progressive symptoms from the onset have primary-progressive (PPMS) type. For the transition from RRMS to progressive stage axonal injury is responsible [67]. Neurodegeneration of demyelinated axons is a major cause of irreversible neurological disability in MS. Disability levels in progressive forms of MS patients often worsen despite a stable MRI T(2) (magnetic resonance) lesion burden [67]. The presence of oxidative stress in the absence of measurable inflammation could help explain this phenomenon [3, 47].

Currently classifications of biomarkers of MS are connected with the pathophysiological processes. It has been divided into seven categories:

(1) alteration of the immune system (interleukins IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-23, interferon (IFNγ), tumor necrosis factor (TNFα), transforming growth factor (TGFβ), cytokines CXCR3/CXCL10—marker activated T cells; E-selectin, L-selectin, ICAM-1, VCAM-1, CD31, surface expression of LFA-1 and VLA-4 (adhesion molecules), CD40/CD40L, CD80, CD86, and heat shock proteins (hsp));

(2) axonal/neuronal damage (Tau protein, 24S-hydroxycholesterol, N-acetylaspartic acid);

(3) blood-brain barrier disruption (matrix metalloproteinases (MMPs): MMP-9 and their inhibitors (TIMP), platelet activating factor (PAF), and thrombomodulin);

(4) demyelination (MBP and MBP-like material, proteolytic enzymes);

(5) oxidative stress and excitotoxicity (nitric oxide derivatives, F_2-IsoPs, and uric acid);

(6) gliosis (glial fibrillary acid protein (GFAP), S-100 protein);

(7) remyelination and repair (NCAM (neural cell adhesion molecule), CNTF (ciliary neurotrophic factor), MAP-2 + 13 (microtubule-associated protein-2 exon 13), and CPK-BB (creatine phosphatase BB) [67].

IsoPs are the candidate biomarkers of lipid peroxidation in MS. In diseases with a complex pathogenesis an individual biomarker is reflected in only one of many ongoing pathogenic processes [5, 67]. The data presented in our studies indicate that lipid peroxidation and oxidative stress in patients with MS may occur. It was found that the urine IsoPs level was over 6-fold elevated in patients with SPMS than in control [3]. The increased level of 15-F_{2t}-IsoPs in CSF of MS patients has been described by Mattsson et al. [9]. To investigate the possible correlation between F_2-IsoPs and the disease inflammatory activity it has been observed that the CSF levels of 15-F_{2t}-IsoPs in patients with RRMS were not correlated with the clinical signs of the disease. These observations suggest that high levels of F_2-IsoPs (about 9-fold higher than control subjects) may represent an index of degenerative phenomena, which persist also in the lack of an ongoing inflammatory activity. In other researches, Minghetti et al. [10] have found that the CSF level of the reliable marker of oxidative stress *in vivo*, 15-F_{2t}-IsoP, is 3 times higher in patients with MS than in a benchmark group of subjects with other neurologic diseases. This increase was not correlated with the 15-F_{2t}-IsoP levels and was much lower in steroid-treated patients. Clearly, the levels of 15-F_{2t}-IsoP were associated with the degree of disability. What is more, in the spinal cord of mice during early progressive stages of experimental autoimmune encephalomyelitis (EAE) the elevated levels of F_2-IsoPs and F_4-NPs were observed [72, 73]. In white matter and myelin-forming oligodendrocytes the DHA levels are relatively low, which are affected in MS. So, F_2-IsoPs might be preferable to F_4-NPs as lipid peroxidation biomarkers in this demyelinating disease [66]. There are some studies which examine the correlation between levels of F_2-IsoPs in CSF of MS patients, their healthy siblings, and unrelated controls. The CSF concentrations of F_2-IsoPs in siblings of MS patients were significantly higher than in healthy controls. The F_2-IsoPs levels in patients suffering from MS were intermediate between siblings, as well as controls. In patients with MS and siblings, the levels of F_2-IsoPs were significantly correlated. These researches have been proved that siblings of MS patients have an increased oxidative stress response to the environmental and genetic factors that might be involved in MS pathogenesis [9].

5. Alzheimer's Disease

Alzheimer's disease (AD) is one of the major causes of dementia, which is characterized by the deposition of the amyloid β (Aβ) peptide and microtubule-associated protein tau in the brain [74, 75]. The critical role in the AD pathogenesis plays an abnormal tau phosphorylation. It has been proved that Aβ has capacity to interact with transition metals generating redox active ions, which precipitate in lipid peroxidation and cellular oxidative stress [76]. In other words, Aβ promotes cellular oxyradicals accumulation in neurons and glial cells in vulnerable regions of AD brain. Such oxidative stress may lead to many of the metabolic and neurodegenerative alterations observed in this disease [77]. Moreover, in tau phosphorylation, the mediation of oxidant toxicity by Aβ has been also implicated. Besides the oxidative stress, the mitochondrial dysfunction has been observed in AD [78]. A variety of markers of oxidative stress are increased, with a clear relationship with Aβ deposition and neurofibrillary degeneration has been observed in postmortem brain tissues from AD patients [79]. It has been reported that the activity and/or protein levels of several antioxidant enzymes were altered in AD brain regions, consistent with ongoing oxidative stress [11]. Increased F_2-IsoPs and F_4-NPs levels in the postmortem ventricular fluid from definite AD patients had been firstly demonstrated by Montine et al. [11]. The authors, given the partial overlap between CSF concentrations of F_2-IsoPs in AD patients and healthy subjects, suggested that the quantification of CSF F_2-IsoPs could not be utilized as an early marker of dementia. There was no correlation between CSF F_2-IsoPs and age or duration of disease. This study concerns the relative small group of AD patients and probably may not be fully representative of the AD population [11]. In an independent study, Musiek and colleagues [80] demonstrated the formation of F_4-NPs during peroxidation of DHA *in vitro* F_4-NPs may be used as a marker of lipid peroxidation in the pathogenesis of neurodegenerative diseases, because in these diseases the elevated levels of F_4-NPs is observed. Subsequently, they proved the presence of esterified F_4-NPs in the human brain and showed abnormally high levels in occipital and temporal lobes of AD brains. Interestingly, while *in vitro* oxidation of DHA yields 3.4-fold higher levels of F_4-NPs compared with F_2-IsoPs, the CSF levels of these two classes of compounds showed a very close correlation in a small number of AD patients [66].

In Yao et al.'s [12] and Praticò et al.'s [13] researches, found that the contents of 15-F_{2t}-IsoPs and IPF_2alpha-VI were markedly elevated in the frontal and temporal lobes of AD brains compared to the corresponding cerebella and to the same regions of control brains. Moreover, there was also a significant correlation between the levels of the two IsoPs measured in each AD brain. In postmortem ventricular CSF, IPF_2alpha-VI levels were higher in AD patients than in healthy people. In contrast, brains levels of 6-keto PGF_1alpha, an index of prostaglandin production, and ventricular CSF 15-F_{2t}-IsoP levels did not differ in AD and control subjects.

F_2-IsoPs were measured also in plasma and in urine of AD patients. It has been shown that plasma and urinary levels were higher than controls, but only in the case of plasma the difference was statistically significant. So, plasma or urine content of IsoP in patients with AD reflects a specific increase in oxidative stress within the brain or a more generalized

systemic oxidative stress remains to be determined. The authors also found that in the control group F_2-IsoPs levels in females were higher than in males and suggested that this could be related to an increase in oxidative stress associated with the loss of estrogens in the postmenopausal period [81]. Indeed, estrogens can be antioxidants because of their phenolic structure [82] or may upregulate apolipoprotein E, favoring the formation of the apolipoprotein E/A-complex, and thus the sequestration of Aβ. Consistent with this hypothesis, Praticò et al. [13] demonstrated markedly elevated F_2-IsoPs in the brains of aged apolipoprotein E-deficient mice compared with wild-type C5 [83].

6. Huntington's Disease

The abnormal expansions of an unstable cytosine-adenine-guanine repeat region at the 5′-end of a gene on chromosome 4 are the main cause of this disease. This genetic abnormality results in the expression of an expanded polyglutamine tract in huntingtin protein, which can aggregate in neuronal nuclei and dystrophic neuritis in Huntington's disease brains. The HD gene defect causing the death of specific populations of striatal neurons is still unknown. The elevated oxidative damage observed in areas of degeneration in patients' brains with HD and the increased free radical production in animal models indicate the involvement of oxidative stress either as a cause or as a consequence of the cell death cascade in the disease [66, 84]. There are a lot of studies suggesting that oxidative stress is prominent in the neostriatum of HD brains [85] and contributes to degeneration of the neostriatum. In patients suffering from HD, the mitochondrial dysfunction results in overproduction of ROS leading to oxidative and nitrosative stress [54, 86–88]. Such stress contributes to neuronal dysfunction by damaging the main structures: DNA, proteins, and lipids. It has been shown that the highly reactive product of nitric oxide and superoxide free radicals—peroxynitrite, which inhibits mitochondrial respiration and reduce antioxidant defenses in cells, is marked by increased of 3-nitrotyrosine (3-NT) levels [54, 87, 89]. The immunoreactivity of 3-NT is increased in postmortem HD brain tissue [85]. Also, increased levels of protein carbonyls in HD striatum and cerebral cortex have been observed [85]. It has been observed that 4-hydroxynonenal and malondialdehyde, lipid peroxidation products, are increased eightfold in HD human plasma [90] and also in postmortem brain tissue [84].

The measurement of the levels of F_2-IsoPs in the CSF of HD patients indicates the contribution of oxidative stress to the pathogenesis of HD. The level of F_2-IsoP in HD patients was significantly higher than in the control group. However, the overlap of levels between these groups suggested that the oxidative damage to the brain may not occur uniformly in the early phase of the disease. But like in AD, correlation between F_2-IsoPs and age or disease duration there was not found; moreover no difference between men and women was observed [66].

In addition, in HD plasma the glutathione levels are significantly reduced [91]. Browne and Beal [14] suggest that in transgenic HD mice, there are increased immunostaining for malondialdehyde, 4-hydroxynonenal, and 15-F_{2t}-IsoPs [54].

7. Creutzfeldt-Jakob Disease

Creutzfeldt-Jakob disease (CJD) is one of the most known human transmissible spongiform encephalopathies (TSEs) or prion diseases, a heterogeneous group of infectious, sporadic, and genetic disorders characterized by rapidly progressive dementia. The characteristic neuropathological hallmark of the disease is the amyloid deposition of the pathological form of a cellular protein (like in AD—Aβ or HD—huntingtin). The accumulation of the pathological prion protein is considered as a central event and is thought to trigger several pathogenetic mechanisms, eventually culminating in the typical spongiform degeneration [66].

The physiological functions of cellular prion protein are still unknown; however, due to its cooper binding ability it might play an important role in the oxidative homeostasis of the brain and could act as an antioxidant. These antioxidant properties may be related to its superoxide dismutase- (SOD-) like activity [15, 16]. Kralovicova et al. [15] have proved that these cells, which express higher levels of prion protein, are more resistant to oxidative stress. Wong et al. suggest [16] that the levels of several oxidative stress markers, protein carbonyl groups and products of lipid peroxidation, were increased in brain tissues of prion protein knockout mice [92]. In brains of mice infected with scrapie, the elevated levels of nitrotyrosine and heme oxygenase-1 had been found [93]. It has been suggested that the level of lipid peroxidation products is increased in brains of scrapie-infected mice and also prion proteins purified from brains of these animals possess a reduced SOD-like activity [94].

The increased levels of F_2-IsoP in CSF of Creutzfeldt-Jakob patients have been observed in Minghetti et al.'s [17] researches. Also, another product of lipid peroxidation has been found to be unchanged in CSF from patients suffering from CJD in comparison to controls [95]. Arlt et al. [18] found that CSF lipids from patients suffering from CJD were more susceptible to oxidation process than those from nondemented controls. Thus, they observed that in the CJD patients, the levels of antioxidants and the amount of PUFAs were reduced. Their researches indicate that oxidative stress is elevated in CJD patients and the oxidative mechanisms are correlated with pathogenesis of this disease.

It has been observed that in patients with sporadic and familial CJD, CSF levels of 15-F_{2t}-IsoP were about 2.5-fold higher than in patients with noninflammatory disorders. No correlation was found between 15-F_{2t}-IsoPs and PGE_2 and also 15-F_{2t}-IsoP levels and age of patients nor polymorphism at codon 129 of the prion protein gene, indicating that lipid peroxidation and prostaglandin synthesis are unrelated phenomena in this disease. PGE_2 concentrations, that were about 6.5-fold higher than in controls, were inversely correlated with patient survival; meanwhile, the levels of 15-F_{2t}-IsoP were not correlated with the clinical duration of the disease. It has been suggested that the inflammation might be more relevant than oxidative stress to the pathogenesis of this particular disease [53, 66].

In other studies, it has been proved that the increased level of PGE_2 in hippocampal is associated with a strong induction of COX-2 expression, which was elevated with

progression of disease and is localized to microglial cells [56]. In sporadic CJD patients the shorter survival was associated with higher levels of PGE_2 in CSF patients. PGE_2 may be an index of disease severity rather than progression, because PGE_2 levels were not dependent on the time of CSF sampling during the course of the disease [10]. PGE_2 can be associated with neuronal death, because in neuroblastoma cells, prion proteins peptides increase PGE_2 levels and COX-1 inhibitors protect against prion proteins toxicity [57]. Whether PGE_2 contributes to neuronal death in CJD, is a consequence of neuronal apoptosis, or is just an index of the disease state remains to be established.

8. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a multifactorial and complex disease, in which genetic, environmental, or genetic-environmental interactions lead to motor neuronal degeneration. The deposition of a misfolded protein in neural tissue, in this instance copper/zinc SOD, is characteristic for the ALS and other neurodegenerative diseases [96, 97]. Several neuroinflammatory changes, such as increased levels of proinflammatory molecules, astrogliosis, and also microglial activation, which are characteristic for many neurodegenerative diseases, have been also found in spinal cord tissue from patients who died of ALS. These processes suggest that inflammation might promote motor neuron death. In addition, in ALS, high CSF levels of glutamate and excitotoxicity have been reported [98]. It has been proved that in ALS, oxidative stress is closely associated with motor neuron degeneration. Several recent clinical researches suggest that there exist a number of biomarkers for oxidative stress in ALS. Mitsumoto et al. [19] have observed that the level of urinary 15-F_{2t}-IsoP and urinary 8-oxodG was higher among ALS patients than in control. No correlation has been found between age and urinary IsoPs. Protein carbonyl levels did not differ between patients suffering from ALS and controls, in contrast to urinary levels of IsoPs and urinary 8-oxodG, which are strongly correlated. This suggests that 15-F_{2t}-IsoPs and 8-oxodG are biomarkers of oxidative stress in patients with ALS. [58, 99].

What is more, it has been proved that the well-established role of COX-2 in inflammation and in glutamate-dependent neurotoxicity is a basic hypothesis of COX-2 involvement in ALS pathogenesis. The increased COX-2 mRNA and protein were found in postmortem spinal cord of ALS patients. Together with the increase of PGE_2 tissue levels, the elevated expression of COX-2 has been observed [100]. COX-2 is expressed in neurons in the spinal cord dorsal and ventral horns and also in dorsal root ganglia under normal conditions. The COX-2 expression was markedly evaluated and localized to both neurons and glial cells in postmortem spinal cord of ALS patients. It has been proved that COX-2 is associated with astrocytes and much lesser extent with glial cells [101]. Some studies suggest that inhibition of COX-2 may have therapeutic benefits by altering the cascade of events leading to the progressive neuronal death in ALS patients. But the efficacy of COX-2 inhibition in the presence of overt clinical signs of disease still remains unknown.

In addition, in spinal cords of sporadic ALS patients, the immunoreactivity of 15-deoxy-D12-14-PGJ_2 (15d-PGJ_2) has been found. 15d-PGJ_2, this bioactive prostanoid produced by dehydration and isomerization of PGD_2, activates the nuclear peroxisome proliferator—activated receptor γ (PPARy). PPARy is a critical transcription factor involved in adipocyte and monocyte differentiation.

This receptor can be considered as a potential therapeutic benefit of its activation in several inflammatory neurological diseases [31].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Lipid Peroxidation in Psychiatric Illness: Overview of Clinical Evidence

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The brain is known to be sensitive to oxidative stress and lipid peroxidation. While lipid peroxidation has been shown to contribute to many disease processes, its role in psychiatric illness has not been investigated until recently. In this paper, we provide an overview of lipid peroxidation in the central nervous system as well as clinical data supporting a link between lipid peroxidation and disorders such as schizophrenia, bipolar disorder, and major depressive disorder. These data support further investigation of lipid peroxidation in the effort to uncover therapeutic targets and biomarkers of psychiatric disease.

1. Introduction

Over the last century, few areas of clinical and biomedical inquiry have undergone as rapid a transformation as the neuroscience of psychiatric disorders. In the past several decades, increasingly sophisticated experimentation, investigational tools, and model systems have led to more nuanced approach to pharmacotherapy. Although different psychiatric disorders are currently thought to stem from unique abnormalities in neuronal biochemistry, circuitry, and/or brain architecture, emerging data indicates that oxidative stress is present and may play an active role in these psychiatric illnesses. In this paper, we will provide an appraisal of recent clinical findings on lipid peroxidation as it applies to the pathobiology of schizophrenia, bipolar disorder, major depressive disorder, and several other psychiatric disorders.

2. Lipid Peroxidation and the Central Nervous System

The degree of oxidative stress in the cellular milieu is a direct result of those processes that accelerate the production of reactive oxygen species and those that detoxify them (for a discussion on oxidative stress, see [1]). Reactive oxygen species such as superoxide anion and hydroxyl radicals are produced from a variety of cellular processes. These reactive oxygen species are neutralized by antioxidants such as vitamins C and E as well as enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. Low levels of reactive oxygen species are utilized by redox sensors to modulate cell function, but high levels of reactive oxygen species damage and oxidize nucleic acids, carbohydrates, and lipids. The brain is approximately 2% of total body weight but utilizes 20% of total oxygen, allowing ample metabolic substrates for free radical generation [2]. In light of this, and given the fact that lipids are the major components of neuronal membranes as well as the myelin sheaths that help conduct neuronal signaling, their peroxidation and dysfunction can dramatically compromise brain function globally.

Thus far, brain lipid peroxidation is not able to be directly assessed in living subjects. In lieu of this, markers of global oxidative stress can approximate lipid peroxidation and include antioxidant status and the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase peripherally [3]. More direct measures of lipid peroxidation *in vivo* include assessment of isoprostanes and malondialdehyde/thiobarbituric acid reactive species in a variety of biological fluids or alkanes such as pentane in

exhaled air [3, 4]. Although circuit level dysfunction and the dynamic pathophysiology of psychiatric disorders are poorly approximated by postmortem investigation of the brain, such tissue analysis can aid in localization of lipid peroxidation in regions known to be important to psychiatric disease.

3. Schizophrenia

Schizophrenia is characterized by the presence of positive symptoms such as delusions, hallucinations, disorganized thinking, and negative symptoms such as flat affect. In part due to the fact that the success of treating the positive symptoms of schizophrenia with dopamine receptor antagonists and that intoxication with substances that increase brain levels of dopamine such as amphetamine or cocaine can lead to episodes of psychosis, inappropriate activation of dopaminergic pathways was initially thought to underlie the disease. However, this dopamine hypothesis of schizophrenia is unable to account for several clinical and pharmacologic observations, and other pathologic mechanisms are likely to play a role (for a lengthier discussion, see [5]). Among these mechanisms, the role of toxic metabolites resulting in oxidative damage has been proposed since the mid-1950s [6]. In their 1954 manuscript, Hoffer, Osmond, and Smythies purported that a highly reactive "adrenochrome" was involved in the evolution of schizophrenia symptoms. Although this adrenochrome hypothesis was quickly shown to be inadequate, oxidative insults to the brain in schizophrenia have continued to be explored.

The largest body of evidence of lipid peroxidation in schizophrenia is from indirect markers such as activity of SOD, catalase, and glutathione peroxidase. Increased peripheral activity of SOD has been observed in patients with schizophrenia, correlating with positive symptoms (hallucinations, delusions, disorganized behavior), and is reduced upon administration of antipsychotic medications [7, 8]. However, several reports have suggested that increased SOD activity occurs late in the disease, with reduced SOD activity early and in younger patients, implying SOD function varies with disease progression [9, 10]. Recently, study of SOD polymorphism in patients with schizophrenia has shown an association between the Ala-9Val variant and poorer performance in neuropsychological assessment [11]. Catalase activity in schizophrenia has been reported as increased, unchanged, or reduced by different investigators, although it is likely that peripheral levels poorly approximate brain levels of catalase especially since catalase activity can be dramatically altered by antipsychotic medications [12–14]. Finally, glutathione peroxidase has been generally reported to be reduced peripherally as well as in postmortem brains. Overall, these findings imply that there is a prooxidative state in schizophrenia. Assessment of more direct markers of lipid peroxidation has generally supported the more indirect assessments—patients with schizophrenia have greater plasma and cerebrospinal levels of thiobarbituric acid reactive substances, increased exhaled pentane, and increased urinary isoprostane-8-epi-prostaglandin F2 alpha [15–18].

Clinical interventions attempting to mitigate the effects of lipid peroxidation have been attempted including administration of eicosapentaenoic acid, long-chain omega-3 fatty acid, vitamin antioxidant (primarily vitamins E and C), and N-acetyl cysteine [19–23]. However, no placebo-controlled study to date has demonstrated long-term symptom improvement following normalization of lipid peroxidation. While some have reported that antipsychotic medications used for schizophrenia have altered markers of oxidative stress, others have found no improvement [24–26]. Interestingly, electroconvulsive therapy in schizophrenia patients reduces plasma thiobarbituric acid reactive substances, which is only evident months after the initial therapy [13].

4. Bipolar Disorder

Bipolar disorder is a mood disorder characterized by periods of both mania and depression. While its underlying pathophysiology remains multifaceted and elusive, recent data have indicated that mitochondrial dysfunction and aberration in oxidation status are important components of bipolar disorder. Andreazza and colleagues have previously shown that thiobarbituric acid reactive substances are elevated in both episodes of mania and depression and are even elevated compared to controls when in remission [27, 28]. Beyond a general prooxidative state, oxidative damage appears to also be localized in bipolar disorder. Using postmortem brain samples, Andreazza and colleagues have noted increased carbonylation and nitration along with isoprostane-8-epi-prostaglandin F2 alpha levels in the prefrontal cortex of bipolar disorder patients [29]. Additionally, oxidation also appears to localize to dopamine transporter-positive and tyrosine-hydroxylase neurons in the prefrontal cortex [30]. In depressed or euthymic females patients with bipolar disorder, sleep cycle disruption positively correlates with measures of malondialdehyde levels, a relationship not found in age-matched healthy controls which suggests that lipid peroxidation follows disease course rather than occurring incidentally [31]. Catalase and glutathione peroxidase levels are also elevated in patients with bipolar disorder during depressive episodes [32]. Treatment with the mood-stabilizer lithium reduces thiobarbituric acid reactive substances in those patients presenting for an initial manic episode as well as in those patients with episodes of hypomania (bipolar disorder type II) [32, 33]. Lithium administration in bipolar disorder patients has been noted to increase the activity of the Na+ K+ ATPase, a cellular event which is independently associated with reduction in lipid peroxidation [34]. Correlation between peripherally collected markers of lipid peroxidation and magnetic resonance imaging of bipolar disorder patients also explains the majority of variance in white matter lesions on diffusion tensor imaging in their brains. Longitudinal prospective trials that reduce oxidative burden in patients in addition to treatment with mood stabilizers would be instructive to see how much of the disease burden can be attributable to lipid peroxidation in bipolar disorder.

5. Major Depressive Disorder

Major depressive disorder is characterized by depressed mood and symptoms that significantly impair normal function. The mainstay pharmacologic therapy in major depressive disorder involves usage of serotonergic and noradrenergic agents for symptomatic control, but several observations have led to consideration of lipid peroxidation targets in the disease. For example, individuals who are depressed as young adults show increased rate of cardiac morbidity and mortality later in life and meta-analyses have indicated that patients who are depressed after myocardial infarction have worse cardiac outcomes that non-depressed patients [35, 36]. Since a major component of cardiovascular risk stems from lipid oxidation and atherosclerotic plaque progression, commentators have conjectured that elevated oxidative burden in major depressive disorder could account for this finding. In a recent study of community-dwelling elderly subjects, plasma levels of isoprostane-8-epi-prostaglandin F2 alpha were higher in those with depressive symptoms than in nondepressed controls [37]. These results have been observed in a younger cohort and other work has also indicated that increased urinary isoprostane-8-epi-prostaglandin F2 alpha is associated with depressed mood, especially in men [38, 39]. Increased glutathione peroxidase and superoxide dismutase levels are found in depressed patients, particularly those with chronic disease and these elevations persist for months even after initiation of standard pharmacotherapy [40, 41]. Thus, lipid peroxidation appears to be significantly perturbed in major depression, and its attenuation may be beneficial in reducing coincidental medical disease. As with the data in schizophrenia and bipolar disorders, longer-term studies must be carried out to better understand the role of lipid peroxidation in major depression.

6. Other Psychiatric Disorders

Evidence of lipid peroxidation has also been recently discovered in other psychiatric disorders. In a small study of adults with attention deficit hyperactivity disorder, serum malondialdehyde levels were elevated compared to nondiseased controls; however no correlation was found between symptom severity on neuropsychiatric battery and levels of malondialdehyde [42]. In children with attention deficit hyperactivity disorder, lipid peroxidation products are also found to be increased in urine when compared to controls [43]. By contrast, serum levels of malondialdehyde were found to correlate with behavioral assessment of severity of obsessive compulsive disorder [44]. Several reports have also described an association between other anxiety disorders and oxidative stress, including generalized anxiety disorder, panic disorder, and posttraumatic stress disorder [45–47]. Elevated markers of oxidation have also been found in brain regions of patients with autism spectrum disorder, including in temporal cortex and cerebellum [48–50]. Investigation of oxidation stress and lipid peroxidation in other psychiatric illness such as personality disorders and eating disorders, however, has not been extensively conducted.

7. Conclusion: Therapeutic and Diagnostic Implications

In summary, recent clinical data has revealed oxidative damage and lipid peroxidation is seen in several psychiatric disorders. Larger longitudinal studies must be conducted to see if measures of lipid peroxidation and oxidative stress can be used to determine risk of developing various psychiatric diseases and to see if long-term disease outcomes can be modified by interventions that mitigate reactive oxygen species. Assessment of oxidative damage and lipid peroxidation in patients is currently limited to using indirect peripheral assessment of brain lipid peroxidation or postmortem analysis of brain samples. Since lipid peroxidation appears to be present in several psychiatric disorders, measures of lipid peroxidation are unable to be used as specific biomarkers for screening or monitoring disease progression. Development of more sophisticated methods of detecting lipid peroxidation would undoubtedly be useful in this endeavor. Regardless, based on current evidence, further investigation of lipid peroxidation in psychiatric illness is likely to reveal clinically-relevant information and should be carried out.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Physical Exercise Combined with Whole-Body Cryotherapy in Evaluating the Level of Lipid Peroxidation Products and Other Oxidant Stress Indicators in Kayakers

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The influence of exercise combined with whole-body cryotherapy (WBC) on the oxidant/antioxidant balance in healthy men was assessed. The study included 16 kayakers of the Polish National Team, aged 22.7 ± 2.6, subjected to WBC (−120°C–−145°C; 3 min) twice a day for the first 10 days of a 19-day physical training cycle: pre exercise morning stimulation and post exercise afternoon recovery. Blood samples were taken on Day 0 (baseline) and on Days 5, 11 and 19. The serum concentration of malondialdehyde (MDA), conjugated dienes (CD), thiobarbituric acid reactive substances (TBARS), protein carbonyls, vitamin E, urea, cortisol, and testosterone were determined, along with the glutathione peroxidase (GPx) activity, the total antioxidant capacity (TAC), and morphological blood parameters. On 5th day of exercise/WBC, the baseline GPx activity decreased by 15.1% ($P < 0.05$), while on 19th day, it increased by 19.7% ($P < 0.05$) versus Day 5. On Day 19 TBARS concentration decreased versus baseline and Day 5 (by 15.9% and 17.4%, resp.; $P < 0.01$). On 19 Day urea concentration also decreased versus 11 Day; however, on 5th and 11th days the level was higher versus baseline. Combining exercise during longer training cycles with WBC may be advantageous.

1. Introduction

Cryotherapy involves applying extremely low temperatures (between −100°C and −160°C) to the body surface for 1 to 3 minutes. Such low temperatures are applied uniformly to the entire body surface (whole-body cryotherapy, WBC) or locally and are generated using the vapour of liquid nitrogen or liquid synthetic air [1]. Cryotherapy has been used for many years in sports to treat injuries and prevent overtraining [2, 3], as well as in the treatment of many diseases due to its analgetic, antioedematous, and anti-inflammatory effect [4]. A rapid increase in body temperature after leaving the cryogenic chamber and intensive cutaneous blood flow (tissue overperfusion lasting for several hours) induce the removal of metabolites and inflammation mediators from damaged tissues. WBC also positively affects the central nervous system (CNS) by decreasing anxiety and stress while increasing the CNS resistance to exhaustion, which may be related to the increased level of beta-endorphins [1]. However, WBC may also induce oxidative stress [1, 5]. One of the sources of ROS during WBC is a reaction catalyzed by xanthine oxidase resulting in the initial ischaemia occurring during body exposure to extremely low temperatures, as well as hyperaemia occurring after leaving the cryogenic chamber [6]. During WBC, ROS may also be generated through the oxidation of catecholamines: even a single cryogenic chamber session increases the levels of adrenalin and noradrenalin in the blood serum of young men and women [1]. The higher ROS generation during WBC sessions may also be a result of stimulation of the metabolism of brown

adipose tissue which has a high content of mitochondria and cytochromes [1]. Exercise is another factor that stimulates oxygen metabolism and leads to an increased level of oxygen-derived free radicals [5]. The results of postexercise oxidative stress are, for example, microdamage of skeletal muscles and connective tissues (e.g., joint cartilages, ligaments) [7], as well as increased peroxidation of lipids forming muscle fibres and also as a result of post-WBC ROS activity, blood plasma lipids (LDL fraction), and erythrocyte membranes [5, 8]. Despite the disadvantageous effects of oxidative stress, a controlled increase in ROS concentration may cause adaptive changes involving improvements in the antioxidant capacity of the organism [9]. Many authors postulate positive adaptive changes as a result of using WBC during physical exercise and indicate WBC as a recovery or stimulation method that is being increasingly employed by athletes [1, 5, 9]. Thus, the aim of this study was to determine the effect of a total of 20 WBC sessions combined with physical exercise on the oxidant/antioxidant balance in high-level kayakers from the Polish National Team. In blood serum of sportsmen, the concentration of lipid peroxidation products (MDA, CD, and TBARS), protein carbonyls, vitamin E, urea, cortisol, and testosterone, as well as the activity of GPx and the TAC, were determined. Additionally, selected morphological blood parameters were measured: the count of red blood cells (RBC) and white blood cells (WBC) which were divided into neutrophils and lymphocytes, as well as the concentration of hemoglobin (HGB).

2. Material and Methods

2.1. Study Subject. The study involved 16 kayakers of the Polish National Team (age 22.7 ± 2.6 years, body height 184.3 ± 5.2 cm, and body weight 86.0 ± 4.9 kg). The athletes prepared for the World Championships in Duisburg (held on 22–31 August, 2013) between 22 July and 9 August, 2013 (19 days) in the Olympic Preparation Centre in Wałcz, Poland. During that period, the kayakers were subjected to strict control regarding their diet, exercise, and recovery by the team of trainers, that is, coaches, a physician, and two sport dieticians. The athletes performed specialized physical training two times a day—in the morning and afternoon. The intensity range of the exercise bouts was characterized by measuring lactate acid concentration using portable analyzer. The schedule of the training is presented in Table 1. The study was approved by the Bioethics Committee of Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland (number KB 370/2013). The athletes provided their written consent for the participation in the study.

2.2. Experimental Design. During the first 10 days of the training cycle, the kayakers combined their strict exercise schedule with two WBC sessions per day. The first session was conducted in the morning, after breakfast, immediately before the first exercise bout (preexercise morning stimulation), while the second session was conducted in the afternoon, following a short rest and supper after the second (last) exercise bout (postexercise afternoon recovery). The temperature in the cryogenic chamber was gradually decreased every day from −120 to −145°C. Before every entry into the chamber, the participants remained in an adaptive vestibule for 30 seconds at −60°C. Every WBC session was 3 minutes long (excluding the stay in the adaptive vestibule). Blood samples for analysis were taken from the basilic vein at 4 time points: on Day 0 (baseline—the day before the start of the training camp), Day 5 (twice a day WBC for 5 days), Day 11 (first day without WBC), and Day 19 (no WBC for 9 successive days of the training camp). Blood samples were collected every time at midday, between the first exercise bout and the dinner.

2.3. Determination of the Concentrations of MDA, CD, TBARS, and Vitamin E. The levels of MDA and vitamin E were determined using high-performance liquid chromatography (HPLC), while the TBARS level was determined using the spectrophotometric method by Buege and Aust [10] as modified by Esterbauer and Cheeseman [11]. The method was also used for the preparation of serum samples for MDA quantification. The CD level was determined using the spectrophotometric method described by Sergent et al. [12]. The analytical performance of the methods used for MDA and vitamin E assessment was satisfactory with the intra-assay coefficient of variation (CV) between 5.6% and 10.4% and the interassay CV between 4.6% and 13.2%. As regrads the CD and TBARS determination methods, the ranges of intra- and interassay CV were 7.5% to 11.2% and 3.6% to 12.2%, respectively.

Vitamin E quantification was conducted by mixing 20 μL working solution of internal standard (tocopheryl acetate, 186 μg/mL) with 200 μL serum. Protein denaturation was induced by shaking the investigated solution with 800 μL acetonitrile. Once centrifuged, the supernatant was filtered using an SPE system (Captiva 2 μm) into glass tubes and 4 mL hexane was added to perform extraction. Subsequently, the samples were shaken, centrifuged, and frozen at −80°C for approximately 45 min. The frozen hexane fraction containing vitamins was decanted into clean tubes and evaporated to dryness under nitrogen at 40°C. Then the sample was dissolved by adding 100 μL phase, mixed ultrasonically, and finally injected into an HPLC system using a syringe. The detection was conducted using a UV-Vis detector at the wavelength λ = 292 nm. The concentration of vitamin E was expressed as μg/L of serum.

Determination of the TBARS concentration was conducted by mixing 0.5 mL serum with 4.5 mL reaction mix consisting of 0.375% thiobarbituric acid (TBA) and 15% trichloroacetic acid (TCA) in 0.25 N HCl. The samples were incubated on a water bath for 20 min at 100°C to optimize the conditions for the MDA-TBA reaction. Subsequently, the samples were cooled down and centrifuged at 4°C for 15 min at 2000 ×g. After centrifugation, supernatant was collected. The detection was conducted at the wavelength λ = 532 nm. TBARS consist mainly of MDA; therefore, for the sake of simplicity, the TBARS level in serum was expressed as the MDA level (nmol/mL).

Table 1: The weekly course of the training cycle combining exercise and cryotherapy in the kayakers from the Polish National Team between 22 July and 9 August, 2013.

Day of week	Time of exercise	Type of exercise	Duration (min)	Intensity range*
Monday	10.00 AM	Strength training + specialized on-water training	140	III/V
	16.00 PM	Specialized on-water training	90	II
Tuesday	10.00 AM	Specialized on-water training + stretching	100	I/III
	16.00 PM	Specialized on-water training + 6 km running	110	II/III
Wednesday	10.00 AM	Specialized on-water training + stretching	110	I/III
	16.00 PM	Specialized on-water training + strength training	100	V/II
Thursday	10.00 AM	Ergometer + specialized on-water training + 6 km running	130	II/III
	16.00 PM	Recovery	—	—
Friday	10.00 AM	Strength training + specialized on-water training	120	V/III
	16.00 PM	Specialized on-water training + stretching	80	I/III
Saturday	10.00 AM	Ergometer + specialized on-water training	110	II/III
	16.00 PM	Specialized on-water training + 6 km running	90	II/III
Sunday	10.00 AM	Specialized on-water training	110	III
	16.00 PM	Recovery		

*Lactate acid concentration in blood: I < 2 mmol/L, II < 4 mmol/L, III = 4 mmol/L (lactate threshold), IV > 4 < 6 mmol/L, and V > 6 < 8 mmol/L.

Properly prepared serum samples for MDA quantification were separated on an HPLC system using a C18 (250 mm) column. The detection was conducted using a UV-Vis detector. The concentration of the investigated compound was determined using the WorkStation Polaris software. The MDA level in blood serum was expressed as nmol/mL.CD are generated in the process of lipid peroxidation as a result of double bond regrouping after a hydrogen atom is removed from a residue of a polyunsaturated fatty acid. They form a characteristic absorbance peak at the wavelength $\lambda = 233$ nm. The CD level was expressed as absorbance units per mL of serum (10^{-1} Abs./mL).

2.4. Determination of TAC, Protein Carbonyls Concentration, and GPx Activity. The intra-assay and interassay control imprecision, as CV% obtained for the methods used in the determination of TAC and protein carbonyls, was 6.5–10.2% and 8.6–11.9%, respectively. For the GPx determination method, the interassay CV was between 6.7% and 9.1%, and the intra-assay CV was between 3.3% and 10.3%. TAC and protein carbonyls were determined using commercial ELISA kits by Cell Biolabs, Inc. The TAC test involved the reduction of Cu^{2+} ions to Cu^{+} ions by the antioxidants present in the sample. The quantity of antioxidants in the sample was directly proportional to the concentration of the newly formed Cu^{+} ions that reacted with a chromogen, forming coloured products. The absorbance of the solution was then measured at the wavelength $\lambda = 490$ nm and compared with the absorbance values on the calibration curve, which enabled the estimation of the antioxidant levels in the investigated sample. The calibration curve was generated based on the same procedure but using known concentrations of uric acid as an antioxidant. TAC in the serum sample was expressed as the concentration of copper-reducing equivalents (μM CRE).

The quantification of protein carbonyls in the sample was based on their binding on a 96-well plate in 2-hour incubation at 37°C, followed by their detection using appropriate antibodies and estimation of their quantity from a standard curve based on the oxidized and reduced bovine serum albumin (BSA) standards. The concentration of protein carbonyls in blood serum was expressed as μmol/mg.

The activity of GPx was determined using the method described by Paglia and Valentine [13]. The method is based on the decomposition of hydrogen peroxide by GPx at 20°C with the concurrent oxidation of reduced glutathione. Oxidized glutathione is then reduced in a reaction catalyzed by glutathione reductase. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is a coenzyme in this reaction and turns into an oxidized form, which causes a change in light absorbance at the wavelength $\lambda = 340$ nm. The GPx activity was expressed as U/L of serum.

2.5. Morphological Blood Parameters, Urea, and Hormones. An additional analysis of blood cell count (RBC, WBC) and HGB concentration, as well as the levels of urea, cortisol, and testosterone, was conducted. The morphological parameters were determined using the Sysmex XS 800i hematology analyzer. The concentrations of testosterone and cortisol were measured via the competition method using commercial kits by Roche. In turn, the urea concentration was assayed via the kinetic method with urease using commercial reagents by Roche as well. The assays had the intrarun CV ranging from 5.7% to 7.3% and the interrun CV ranging from 4.6% to 8.3%.

2.6. Statistical Analysis. The obtained results were tested for the normality of their distribution (the Kolmogorov-Smirnov test) and the homogeneity of variance (Levene's test). The main statistical analysis was represented by the ANOVA

Table 2: Oxidative stress indicators and the concentration of urea, cortisol, and testosterone in the blood serum of kayakers from the Polish National Team during a 19-day physical activity including WBC sessions twice a day for the first 10 days.

	Baseline (Day 0)	Day 5	Day 11	Day 19
MDA [10^{-2} nmol/mL]	26.3 ± 2.5	27.7 ± 2.4	26.0 ± 1.8	25.5 ± 1.8
CD [10^{-1} Abs./mL]	4.6 ± 2.5	4.6 ± 1.2	4.4 ± 1.0	3.6 ± 0.8
TBARS [10^{-2} nmol MDA/mL]	50.3 ± 5.0	51.2 ± 7.0	45.9 ± 5.3	42.3 ± 4.4^{aabb}
Protein carbonyls [μmol/mg]	538.4 ± 23.3	534.8 ± 136.1	535.4 ± 36.2	535.8 ± 25.5
Vit. E [μg/L]	17.7 ± 8.2	14.6 ± 3.7	13.6 ± 3.3	13.1 ± 2.9
GPx [U/L]	225.9 ± 26.4	191.6 ± 27.8^{a}	212.9 ± 42.6	229.4 ± 30.3^{b}
TAC [μM CRE]	813.6 ± 67.7	769.6 ± 78.4	774.1 ± 102.0	845.3 ± 114.0
Urea [mg/dL]	33.1 ± 6.3	39.9 ± 6.3^{a}	45.2 ± 5.5^{aa}	37.2 ± 8.4^{c}
Cortisol [nmol/L]	628.2 ± 62.9	661.3 ± 83.1	688.7 ± 73.4	638.4 ± 109.2
Testosterone [nmol/L]	23.8 ± 5.9	22.8 ± 4.9	24.4 ± 4.7	24.9 ± 5.6

The results are expressed as the mean ± standard deviation; [a]statistically significant difference versus baseline ([a]$P < 0.05$, [aa]$P < 0.01$), [b]statistically significant versus Day 5th ([b]$P < 0.05$, [bb]$P < 0.01$), and [c]statistically significant versus Day 11 ($P < 0.05$).

Table 3: Selected morphological parameters of the blood of kayakers during a 19-day training cycle with two WBC sessions per day for the first 10 days, one before an exercise bout (morning stimulation) and one after an exercise bout (afternoon recovery). The results are expressed as the mean ± standard deviation. No statistically significant differences were observed.

Morphological blood variable	Term of the study			
	Baseline (Day 0)	Day 5	Day 11	Day 19
RBC (mln/mm^3)	5.0 ± 0.3	5.2 ± 0.2	5.1 ± 0.2	5.1 ± 0.3
WBC (thous./mm^3)	5.5 ± 1.1	5.9 ± 1.0	5.6 ± 0.8	5.5 ± 1.1
NEUT (%)	41.6 ± 5.0	44.6 ± 8.1	43.1 ± 4.9	42.4 ± 6.9
LYMPH (%)	44.2 ± 5.0	42.4 ± 7.6	42.7 ± 5.1	44.0 ± 5.9
HGB (g/dL)	15.3 ± 0.7	15.9 ± 0.6	15.4 ± 0.5	15.5 ± 0.8

WBC: white blood cells, RBC: red blood cells, NEUT: neutrophils, LYMF: lymphocytes, and HGB: haemoglobin.

test with post hoc analysis (Tukey's range test). Moreover, Pearson's product-moment correlation coefficients between the investigated parameters were evaluated. The results were expressed as the mean ± standard deviation and differences at a significance level $P < 0.05$ were considered as statistically significant.

3. Results

The study demonstrated a statistically significant decrease in the baseline GPx activity by 15.1% as compared with the activity measured on 5th day of the training cycle involving physical exercise and WBC ($P < 0.05$), while after Day 19, it increased by 19.7% ($P < 0.05$) versus Day 5. Moreover, on 19th day, 9 days after the discontinuation of cryotherapy, a statistically significant decrease in the TBARS level was found as compared with the baseline value and the level detected on 5th day of exercise combined with WBC (by 15.9% and 17.4%, resp.; $P < 0.01$). On 19th day, significantly lower urea level versus 11th day was also found ($P < 0.05$), whereas on 5th and 11th days, the values were higher than baseline ($P < 0.05$ and $P < 0.01$, resp.; Table 2).

Throughout the experiment, no statistically significant changes in the levels of MDA, CD, protein carbonyls, vitamin E, cortisol, and testosterone were found in the blood serum of the study subjects. No TAC changes in the blood serum of the athletes (Table 2) and no changes in the hematological parameters were observed either (Table 3) ($P > 0.05$).

Moreover, the study indicated many statistically significant linear correlations through the whole experiment: at the baseline time point of the study, between TAC and GPx ($r = -0.697$, $P < 0.05$); on Day 5 between vitamin E and MDA ($r = -0.645$, $P < 0.05$), TBARS and vitamin E ($r = -0.608$, $P < 0.05$), and between TBARS and TAC ($r = -0.710$, $P < 0.01$); on Day 19 between MDA and TAC ($r = -0.634$, $P < 0.05$), as well as between TBARS and TAC ($r = -0.683$, $P < 0.01$).

4. Discussion

The obtained results show a possible profitable effect of exercise combined with WBC. Combining exercise and WBC may potentially improve sports performance because of the prolongation of exercise duration or intensity. Probably, it may result from maintenance of oxidant/antioxidant balance.

In the study, on the 5th day of physical exercise combined with WBC sessions, a decrease in the GPx activity by 15.1% versus baseline was observed ($P < 0.05$). The lower

activity of GPx is a manifestation of the decreased activity of antioxidant mechanisms in the studied sportsmen. In turn, on 19th day of the training cycle (the 9th day of the exercise bouts without WBC), the GPx activity increased by 19.7% versus 5th day ($P < 0.05$), but concurrently it did not change in a statistically significant manner relative to baseline (Table 2). It demonstrates a certain degree of disturbance in oxidant/antioxidant balance during the first ten days of the training cycle associated with WBC and its recovery at the end of the cycle, that is, during nine successive days of exercise bouts following the discontinuation of WBC sessions. Both the physical exercise undertaken by the athletes and the whole-body effect of extremely low temperatures may be the source of the increased generation of ROS. During aerobic exercise, the main source of ROS is the respiratory chain, whose natural by-products are free radicals [7]. Physical exercise intensifies the metabolism of oxygen. Endurance exercise increases the demand of oxygen in the organism between 10 and 20 times. At the same time, oxygen consumption in skeletal muscles increases 100–200-fold [7]. During anaerobic exercise, that is, above the lactate threshold, the main source of ROS is xanthine oxidase produced from xanthine dehydrogenase in vascular endothelium under ischemic conditions [14]. A similar occurrence may also be observed during WBC sessions, along with the subsequent hyperemia [6]. The physical exercise undertaken by the kayakers during the training cycle was at a variable level of intensity—from aerobic to anaerobic (Table 1). The TBARS level on the 9th day of the post-WBC training cycle decreased in a statistically significant manner compared to the values measured either before the training cycle or on Day 5 of the exercise/WBC combination. Therefore, the lower level of lipid peroxidation products demonstrates the decreased level of oxidative tissue damage in kayakers through the action of these two stressors. However, cryotherapy may improve the efficiency of the TBARS elimination mechanism, whose main component is MDA, which in turn is metabolized in the liver and probably also in the skeletal muscles of physically well-trained people [5]. In turn, the results of changes in serum urea concentration in kayakers indicate that WBC also intensifies metabolism of proteins. The obtained results of GPx activity and TBARS concentration suggest that adding the effect of extremely low temperatures to physical exercise helps to maintain the balance in oxidoreduction processes. It may be explained by adaptive changes in the organism, which are described by hormesis theory. A stressor can have a tempering effect if it is used regularly for longer period at optimal intensity [15]. Such effect of WBC is also indicated by other authors, who all in all highlight the antioxidant effect of whole-body cryotherapy. In a study involving multiple WBC sessions but no physical exercise (10 WBC sessions, −130°C/3 min, once a day), in both men ($n = 24$) and women ($n = 22$), a statistically significant increase in the total antioxidant status (TAS) and the plasma level of uric acid as compared with the control group not subjected to WBC (men: $n = 22$, women: $n = 26$) was observed [16]. The results demonstrating the antioxidant properties of WBC have also been presented by other authors. Woźniak et al. [1], for example, conducted a study involving a group of professional kayakers ($n = 20$) who were subjected to a 10-day physical activity with WBC sessions conducted three times a day (1 WBC session before the first exercise bout and 2 WBC sessions before the second exercise bout, with temperatures decreasing from −120 to −140°C, 3 min) and a similar 10-day control physical activity without WBC. The study showed that the GPx activity after the 10th day of the physical activity including WBC was lower than that observed after the 10th day of the physical activity without WBC [1]. The activity of the enzyme after Day 10 of the latter physical training cycle was higher in a statistically significant manner than before this physical activity, whereas after the 10th day of the exercise bouts including three WBC sessions a day, the activity showed no statistically significant difference from that measured before the study. The article by Woźniak et al. [1] also indicates lower levels of plasma TBARS/CD and erythrocyte CD with a lower activity of the erythrocytic superoxide dismutase (SOD) after the 6th day of exercise bouts including WBC, as compared with the values obtained on the same day of the physical activity without the cryogenic chamber stimulation sessions. The authors claim that the oxidative stress induced by extremely low temperatures causes changes in the cells of the organism which may protect them against the disruption of the oxidant/antioxidant balance during physical exercise [1]. Other data indicate an anti-inflammatory effect of cryotherapy (5 sessions/week, once a day, −110°C/2 min) [17]. The authors demonstrated a statistically significant decrease in the levels of proinflammatory cytokines and an increase in the levels of anti-inflammatory cytokines/chemokines in the blood of the Italian national rugby team members, where $n = 10$ [17]. The same authors also claim not to have observed any changes in the values of selected immune system parameters: antibodies (IgA, IgM, IgG), C-reactive protein, prostaglandin E2 (PGE2), and muscle enzymes: creatine kinase (CK) and lactate dehydrogenase (LAD) [17].

The effect of a single WBC session on the oxidation and reduction processes in the human organism has also been described. Mila-Kierzenkowska et al. [18] designed an experiment in which professional volleyball players ($n = 18$) were subjected to a single WBC session (−130°C, 2 min) immediately followed by a 40-min submaximal physical exercise on a cycloergometer and then a control exercise bout excluding WBC, conducted 2 weeks later. The authors demonstrated the antioxidant and anti-inflammatory effect of WBC: higher catalase (CAT) and SOD activity was observed after the control exercise bout than after the exercise bout preceded by a WBC session. The levels of proinflammatory cytokines, interleukin 6, and 1β were also higher after the control exercise bout [18]. A single WBC session (−130°C, 3 min) with no exercise involved was also administered to healthy nonathletes ($n = 10$, 21.0 ± 0.9) in whom an increase in the activity of GPx and erythrocytic glutathione reductase (R-GSSG) was observed, along with an increase in the levels of nonenzymatic plasma antioxidants (glutathione, uric acid, albumins, and extra-erythrocyte hemoglobin) [19]. The authors indicated WBC as a source of ROS but also considered cryotherapy as a factor stimulating the antioxidant defense mechanisms of the organism [19]. The only

nonenzymatic antioxidant that level was determined in this study was vitamin E. No statistically significant changes in its level were observed. However, it was also demonstrated that on Day 5 of the training cycle, combined with WBC, the levels of vitamin E and TBARS correlated in a statistically significant manner ($r = -0.608$, $P < 0.05$). This demonstrates the role of vitamin E in the removal of ROS generated by physical exercise and whole-body cryotherapy. Moreover, statistically significant correlations that were found are evidence for correct physiological functions in kayakers during whole experiment: baseline—TAC versus GPx ($r = -0.697$), Day 5—vitamin E versus MDA ($r = -0.645$) and TBARS versus TAC ($r = -0.710$), and Day 19—MDA versus TAC ($r = -0.634$) and TBARS versus TAC ($r = -0.683$).

Despite the ambiguous effect of WBC on the oxidant/antioxidant balance, which depends on the conditions of the study and the characteristics of the investigated group, the authors of most papers conclude that the exposure to extremely low temperatures increases the antioxidant capacity of the organism, although it is a source of ROS at the same time. The authors unanimously emphasize that WBC does not generate any significant oxidant/antioxidant imbalance and, in the long term, according to hormesis theory, may induce adaptive changes. Therefore, the WBC sessions are beneficial for health and improve the speed of postexercise recovery [1, 15, 16, 19]. It can be supposed that the results of this study confirm this hypothesis because on 5th day a clear normalization of the observed changes was noticed—the oxidant/antioxidant balance in kayakers was recovering despite the continuance of both intensive exercise bouts and, until 10th day, WBC sessions.

5. Conclusions

Combining exercise with whole-body cryotherapy sessions may have a positive effect on the oxidant/antioxidant balance during physical effort.

Possible profitable effect of combining exercise with cryotherapy could extend exercise duration or intensity, thus improving sports performance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

In Vitro Antioxidant and Cytotoxic Activities of *Arnebia benthamii* (Wall ex. G. Don): A Critically Endangered Medicinal Plant of Kashmir Valley

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Arnebia benthamii is a major ingredient of the commercial drug available under the name Gaozaban, which has antibacterial, antifungal, anti-inflammatory, and wound-healing properties. In the present study, *in vitro* antioxidant and anticancer activity of different extracts of *Arnebia benthamii* were investigated. Antioxidant potential of plant extracts was evaluated by means of total phenolics, DPPH, reducing power, microsomal lipid peroxidation, and hydroxyl radical scavenging activity. The highest phenolic content (TPC) of 780 mg GAE/g was observed in ethyl acetate, while the lowest TPC of 462 mg GAE/g was achieved in aqueous extract. At concentration of 700 μg/mL, DPPH radical scavenging activity was found to be highest in ethyl acetate extract (87.99%) and lowest in aqueous extract (73%). The reducing power of extracts increased in a concentration dependent manner. We also observed its inhibition on Fe^{2+}/ascorbic acid-induced lipid peroxidation (LPO) on rat liver microsomes *in vitro*. In addition, *Arnebia benthamii* extracts exhibited antioxidant effects on Calf thymus DNA damage induced by Fenton reaction. Cytotoxicity of the extracts (10–100 μg/mL) was tested on five human cancer cell lines (lung, prostate, leukemia, colon, and pancreatic cell lines) using the Sulphorhodamine B assay.

1. Introduction

Research on relationships between antioxidants and prevention of noncommunicable disease, such as cardiovascular disease, cancer, and diabetes has been increasing sharply in recent years. Free radicals have been claimed to play a key role in affecting human health by causing severe diseases, such as cancer and cardiovascular diseases by cell degeneration. These free radicals can be generated during normal body function and can be acquired from the environment. Oxygen radicals can cause damage to biomolecules (lipids, proteins, and DNA), eventually leading to many chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, postischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging, and other degenerative diseases in humans [1]. Human body has multiple mechanisms especially enzymatic and nonenzymatic antioxidant systems to protect the cellular molecules against reactive oxygen species (ROS) induced damage [2]. However the innate defense may not be enough for severe or continued oxidative stress. Hence,certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS in human body. There are several types of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydrotoluene (BHT), and tertiary-butylhydroquinone (TBHQ) which have been widely used in foods to prevent oxidation. The use of synthetic antioxidants in food, however, is discouraged because of their toxicity and carcinogenicity [3]. Hence, compounds

especially from natural sources capable of protecting against ROS mediated damage may have potential application in prevention and/or curing of diseases.

Arnebia benthamii is a monocarpic perennial and reaches reproductive maturity in 3-4 years. The basal part of the root, leaves, and flowering stalk can be utilized for consumption and for trade. *Arnebia benthamii* is a major ingredient of the commercial drug available under the name Gaozaban, which has antifungal, anti-inflammatory, and wound-healing properties. The roots yield a red pigment, Shikonin (a dye), which has several medicinal properties and is marketed under the trade name Ratanjot and alkanin, a lipophilic red pigment which is the main active constituent of this plant and is responsible for its colour and therapeutic efficacy. On folklore levels the plant is used for curing various diseases of tongue, throat, fever, and cardiac disorders and has wound healing properties. The root has anthelmintic, antipyretic, and antiseptic property. *Arnebia benthamii* is used for imparting pleasing red colour to foodstuff, oils, and fats. The plant also possesses stimulant, tonic, diuretic, and expectorant properties. The flowering shoots are used in preparation of sherbet (syrup) and jam useful in various diseases of tongue, throat, fever, and cardiac disorders. No work has been done so far on this endemic plant of Kashmir valley to determine its antioxidant and antibacterial activities. The main objective of this preliminary investigation was to evaluate the protective effects of different extracts of *Arnebia benthamii* against free radical mediated damages under *in vitro* situations. *In vitro* assays were carried on DPPH radical scavenging activity, total phenolic content (TPC), reducing power, microsomal lipid peroxidation, hydroxyl radical scavenging activity, and Calf thymus DNA damage. In addition, the anticancer effect of different extracts on five human cancer cell lines was also investigated by Sulphorhodamine B (SRB) assay. Results from this study provide a better understanding of the nutritional and health benefits of this medicinal plant of Kashmir valley.

2. Materials and Methods

2.1. Chemicals. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, Folin-Ciocalteu reagent, ascorbic acid, and SRB were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and obtained from Himedia Company.

2.2. Plant Material. The *Arnebia benthamii* was collected from higher altitudes of Gulmarg, Jammu, and Kashmir state, India, in the months of September and October 2012, identified by the Centre of Plant Taxonomy, Department of Botany, University of Kashmir, and authenticated by Dr. Irshad Ahmad Nawchoo (Department of Botany) and Mr. Akhter Hussain Malik (Curator, Centre for Plant Taxonomy, University of Kashmir). A reference specimen has been retained in the herbarium of the Department of Botany at the University of Kashmir under reference number KASH-bot/Ku/AB-702-SAG.

2.3. Extract Preparation. The whole plant material was dried in the shade at 30 ± 2°C. The dried material was ground into a powder using mortar and pestle and passed through a sieve of 0.3 mm mesh size. The powder obtained was extracted with different solvents like methanol, ethanol, ethyl acetate, and water for 48 hrs using a Soxhlet extractor (60–80°C) (Figure 1). The extract was then concentrated with the help of rotary evaporator under reduced pressure and the solid extract was stored in refrigerator for further use.

2.4. Determination of Total Phenolic Content. The TPC of the extracts of *Arnebia benthamii* was measured by the Folin-Ciocalteu method described with some modifications [4]. Briefly, an aliquot of 0.5 mL of sample solution (with appropriate dilution to obtain absorbance in the range of the prepared calibration curve) was mixed with 1.0 mL Folin-Ciocalteu reagent (10 times dilution before use) and allowed to react at 30°C for 5 min in the dark. Then 2.0 mL of saturated Na_2CO_3 solution was added and the mixture was allowed to stand for 1 h before the absorbance of the reaction mixture was read at 747 nm. A calibration curve, using gallic acid with a concentration range of 0.01–0.10 mg/mL, was prepared. The TPC of the samples was standardized against gallic acid and expressed as mg gallic acid equivalent (GAE) per gram of sample on a dry weight basis.

2.5. DPPH Radical Scavenging Activity. DPPH method was carried out according to the method modified by Kim et al. [5]. An aliquot of the radical formed from DPPH was left to react with 100–700 μg/mL of the extract for 30 min. The absorbance was read at 517 nm. Catechin was used as the standard (10 mg/10 mL). The percentage of radical inhibition was calculated by the following formula:

$$\% \text{ inhibition} = \left[1 - \left(\frac{A_e}{A_0}\right)\right] \times 100, \quad (1)$$

where A_0 is the absorbance without sample, and A_e is absorbance with sample.

2.6. Reducing Power Test. The reducing power test based on Fe (III) to Fe (II) transformation in the presence of the solvent fractions was carried out by using the method of Oyaizu [6]. The Fe (II) can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Various concentrations of the sample (2 mL) were mixed with 2 mL of phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferricyanide (10 mg/mL). The mixture was incubated at 50°C for 20 min followed by addition of 2 mL of trichloroacetic acid (100 mg/L). The mixture was centrifuged at 1500 ×g for 10 min to collect the upper layer of the solution. A volume of 2 mL from each of the mixture earlier mentioned was mixed with 2 mL of distilled water and 0.4 mL of 0.1% (w/v) fresh ferric chloride. After 10 min reaction, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates a higher reducing power.

2.7. Microsomal Lipid Peroxidation. Liver was washed in ice cold 1.15% KCl and homogenized in a homogenizing buffer (50 mM Tris-HCl, 1.15% KCl pH 7.4) using Teflon

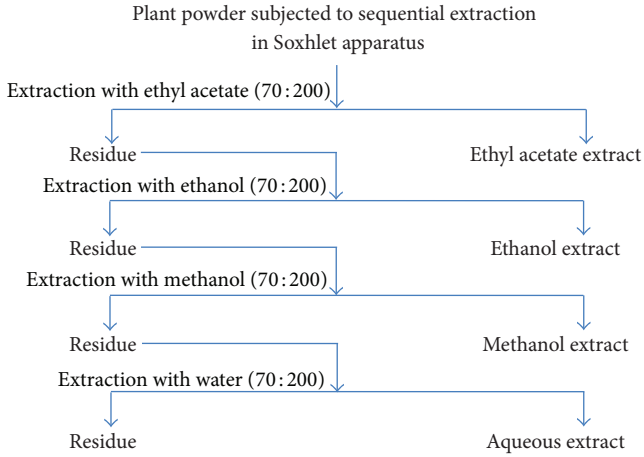

Figure 1: Systematic representation of preparation of different solvent extracts of *Arnebia benthamii* by sequential extraction method.

homogenizer. The homogenate was centrifuged at 9,000 ×g for 20 minutes to remove debris. The supernatant so obtained was further centrifuged at 15,000 rpm for 20 minutes at 4°C to get post mitochondrial supernatant (PMS). Microsomes were obtained by centrifuging the portion of prepared PMS by using Sorvall Ultracentrifuge at 105,000 ×g for 1 hr at 4°C to obtain the microsomal fraction. This fraction was resuspended in 0.25 M sucrose and stored frozen until use.

Rat liver microsomal lipid peroxidation was carried out according to the method of Urata et al. [7] with little modifications. The test sample (20–100 μg/mL) was added to 1 mL of liver microsomes. Lipid peroxidation was induced by adding 100 μL of ferric nitrate (20 mM) and 100 μL of ascorbic acid (100 mM). After incubation for 1 hr at 37°C, the reaction was stopped by the addition of 1 mL of TCA (10%) and 1 mL of (1.67%) TBA was added and the reaction mixture was boiled for 15 min, cooled, and centrifuged and the absorbance of the supernatant was measured at 532 nm.

2.8. Hydroxyl Radical Scavenging Assay. Hydroxyl radical scavenging activity was measured by the ability of the different concentrations of *Arnebia benthamii* extract to scavenge the hydroxyl radicals generated by the Fe^{3+}-ascorbate-H_2O_2 system (Fenton reaction) [8]. The reaction mixture contained; 500 μL of 2-deoxyribose (2.8 mM) in phosphate buffer (50 mM, pH 7.4), 200 μL of premixed ferric chloride (100 mM), 100 μL of H_2O_2 (200 mM) with or without the extract solution (100–500 μg/mL). The reaction was triggered by adding 100 μL of 300 mM ascorbate and incubated for 1 h at 37°C. 0.5 mL of the reaction mixture was added to 1 mL of TCA (10%), then 1 mL of 1% TBA was added to the reaction mixture. The mixture was heated for 15 min on a boiling water bath. After the mixture being cooled, the absorbance at 532 nm was noted against a blank (the same solution but without reagent). The scavenging activity on hydroxyl radical was calculated as follows:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100. \quad (2)$$

2.9. Antioxidant Activity against Oxidative Damage to DNA. Hydroxyl radicals generated by Fenton reaction were used to induce oxidative damage to DNA [9]. The reaction mixture (15 μL) contained 25 mg of calf thymus DNA in 20 mM phosphate buffer saline (pH 7.4) and different concentrations of plant extract (10, 30, 50 and 80 μg) were added and incubated with DNA for 15 min at room temperature. The oxidation was induced by treating DNA with 20 mM ferric nitrate and 100 mM ascorbic acid and incubated them for 1 h at 37°C. The reaction was terminated by the addition of loading buffer bromophenol blue (0.25%) and glycerol (30%) and the mixture was subjected to gel electrophoresis in 0.7% agarose/TAE buffer run at 100 V. DNA was visualized and photographed by gel doc.

2.10. Cell Lines and Culture. Human cancer cell lines lung (HOP-62, A549), prostate (PC-3), leukemia (THP-1), colon (HCT-116), and pancreatic (MIA-Pa-Ca) were obtained from IIIM Jammu. These cell lines were grown and maintained in a high glucose concentration (4.5 g/L) Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (100 IU–100 mg/mL) in a humidified incubator at 37°C and in 5% CO_2 atmosphere.

2.11. Cytotoxicity Assay. This assay was carried out as described by Sun et al. [10]. SRB assay is a rapid, sensitive, and inexpensive method for measuring the cytotoxic potential of test substances, based on the cellular protein content of adhered suspension cultures in 96 well plates. This method is suitable for ordinary laboratory purposes and for large-scale applications like high through put *in vitro* screening in anticancer drug discovery. The anticancer activity was determined by the cytotoxic potential of the test material using human cancer cell line, which was allowed to grow on tissue culture plate in the presence of test material. The cell growth was measured on ELISA reader after staining with Sulphorhodamine B (SRB) dye which binds to basic amino acid residues in trichloroacetic acid (TCA) fixed cells.

2.12. Statistical Analysis. The values are expressed as mean ± standard deviation (SD). The results were evaluated by using the SPSS (version 12.0) and Origin 6 software and evaluated by one-way ANOVA. The IC_{50} values were calculated by using Origin 6.0 version by plotting the percentage inhibition versus the concentrations. The quality of the radical scavenging property of the extracts was determined by calculating the IC_{50}. The IC_{50} value is the concentration of each extract required to scavenge the free radical to 50% of the control.

3. Results and Discussion

3.1. Total Phenolic Content. Phenolic compounds in plants are powerful free radical scavengers that can inhibit lipid peroxidation by neutralizing peroxyl radicals generated during the oxidation of lipids [11]. The TPC of the different extracts of *Arnebia benthamii* was assayed by the Folin-Ciocalteu method using gallic acid as standard. It was found that the TPC of different extracts was in the descending order of ethyl

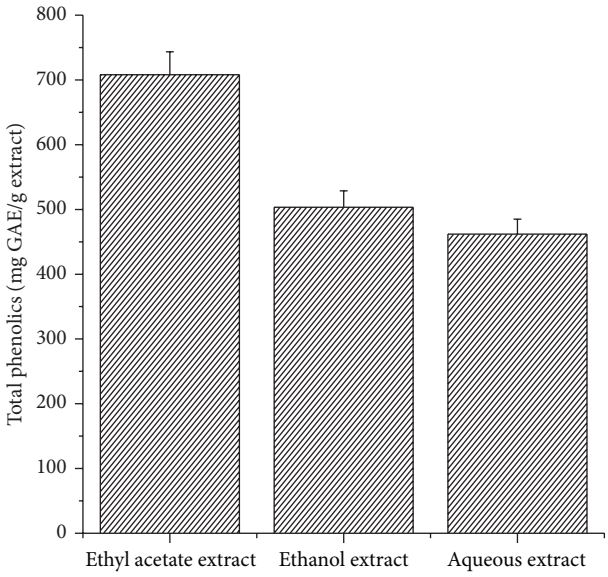

Figure 2: Total phenolic content of ethyl acetate, ethanol, and aqueous extracts of *Arnebia benthamii*. Data are presented as the mean value ± standard deviation of 3 separate experiments.

acetate > ethanol extract > aqueous extract (Figure 2). The highest TPC of 780 mg GAE/g was obtained in ethyl acetate, whereas the lowest TPC of 462 mg GAE/g was achieved in aqueous extract. It is worthwhile to mention that the TPC of ethanol extract was lower than that of ethyl acetate extract, but higher than that of aqueous extract, which may be the result of enrichment of the phenolic components in the extracts.

3.2. DPPH Radical Scavenging Activity. DPPH radical scavenging assay is one of the most commonly used methods to evaluate the radical scavenging activity of antioxidants because of its quickness, reliability, and reproducibility. This method depends on the reduction of the purple DPPH by accepting an electron or hydrogen radical to become a stable diamagnetic molecule with discoloration. The degree of discoloration indicates the free radical scavenging potentials of the antioxidant compounds or extracts in terms of hydrogen-donating ability [12, 13]. DPPH free radical scavenging activities of the different extracts of *Arnebia benthamii* are shown in Figure 3. For each sample, seven concentrations (100–700 μg/mL) of the plant extract were tested. All tested extracts showed a promising DPPH scavenging effect in a concentration-dependent manner. Ethyl acetate extract exhibited considerably higher DPPH radical scavenging activity than other two extracts, and the lowest DPPH radical scavenging rate was found in aqueous extract. The free radical scavenging activities of different extracts decreased in the order of ethyl acetate extract > ethanol extract > aqueous extract. The DPPH radical scavenging activity of these extracts positively correlated with the total phenolic content. The results are considered to be noteworthy when compared to our previous findings that ethyl acetate extract of *Podophyllum hexandrum* rhizome showed a maximum percentage inhibition of 85.77% on DPPH [14]. The IC_{50} values were also calculated to further evaluate the antioxidant activity, as shown in Table 1. The lower the IC_{50} value is, the greater the free radical scavenging activity is. The highest DPPH radical scavenging effect was obtained in ethyl acetate extract with the lowest IC_{50} of 250 μg/mL, followed by ethanol extract (300 μg/mL) and aqueous extract (335 μg/mL). As we have taken catechin as a standard, it showed higher radical scavenging ability with IC_{50} of 230 μg/mL once compared with different extracts. Results of our study suggest that the plant extracts with higher concentration of phytochemical constituents have increased capability of donating hydrogen atom to scavenging free radicals.

3.3. Reducing Power. In reducing power assay, potential antioxidants reduce the Fe^{3+}/ferricyanide complex to its ferrous form which can then be monitored spectrophotometrically at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power. The antioxidant activities of natural components may have a reciprocal correlation with their reducing powers. Figure 4 shows the dose-response curves for the reducing power of all the extracts of *Arnebia benthamii*. The reducing power values were found to be correlated with the concentration of each extract. The highest reducing power among the extracts was found in ethyl acetate extract, followed by ethanol and aqueous extract. Significantly higher reducing power (0.859) was observed for ethyl acetate extract at 300 μg/mL, while as it was 0.802, 0.759, and 0.901 for ethanol extract, aqueous extrac,t and catechin, respectively. In our earlier studies, we observed similar results with aqueous extracts of *Podophyllum hexandrum* that the reducing power activity increased with the increase in the extract concentration [15].

3.4. Lipid Peroxidation. Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon that can lead to various pathological consequences [16]. Fe^{2+}-ascorbic acid mixture is well known to stimulate lipid peroxidation in rat liver *in vivo* and in microsomes and mitochondria of rat liver *in vitro* [17]. Since it is believed that lipid peroxidation is one of the causes of the occurrence of cardiovascular disease [18] and cancer [19], its high inhibition by extracts of plants may represent an indicator of their high therapeutic potential. Furthermore, it has been shown that flavonoids have the capacity to terminate the chain reaction of lipid peroxidation by scavenging the peroxyl radical LOH [20].

The inhibitory effect of *Arnebia benthamii* extracts and catechin on TBARS production in rat liver microsomes induced by ferric nitrate-ascorbic acid/H_2O_2 is shown in Figure 5. Results showed that the inhibition of TBARS formation increased with increasing concentrations of *Arnebia benthamii* extracts and catechin. At concentrations of 20–140 μg/mL, all the three extracts displayed a different potency of antilipid peroxidation activity, with an inhibition rate for aqueous extract that varies from 12.35% to 83.94%; for ethanol extract the inhibition rate was found to be 30.98% to 86.10 and

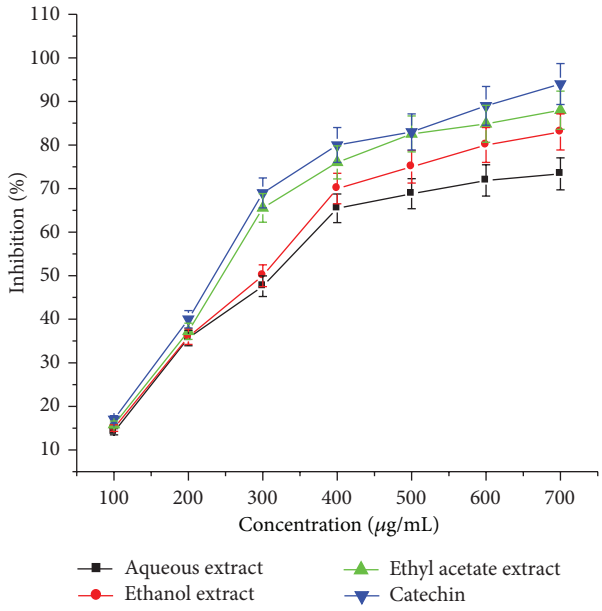

Figure 3: DPPH radical scavenging activity of aqueous, ethyl acetate, and ethanol crude extract *Arnebia benthamii*. Data are presented as the mean value ± standard deviation of 3 separate experiments. Absorbance at 517 nm.

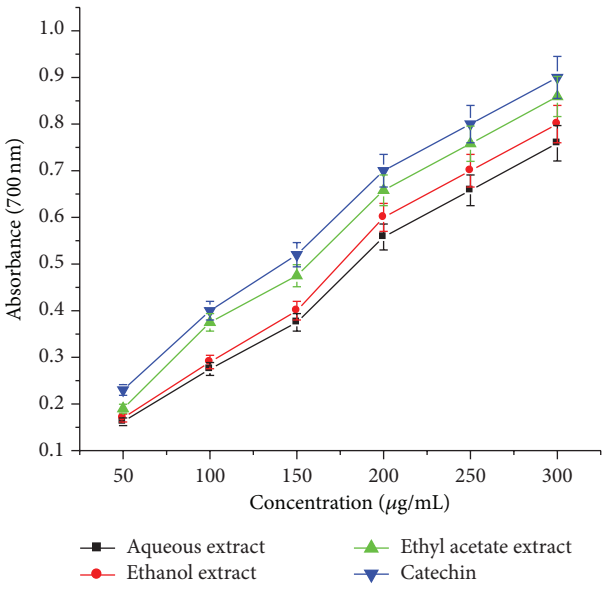

Figure 4: Reducing power of aqueous, ethyl acetate, and ethanol crude extracts *Arnebia benthamii*. Data are presented as the mean value ± standard deviation of 3 separate experiments. Absorbance at 700 nm.

for ethyl acetate extract the percentage inhibition varies from 35% to 95%, respectively. The IC_{50} values were also calculated as shown in Table 1. The highest antilipid peroxidation effect was again obtained in ethyl acetate extract with the lowest IC_{50} of 60 μg/mL, followed by ethanol extract (82 μg/mL) and aqueous extract (85 μg/mL).

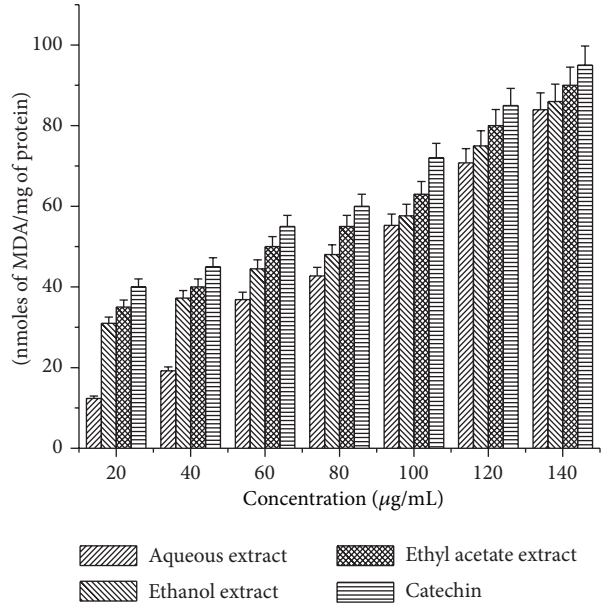

Figure 5: Microsomal lipid peroxidation of aqueous, ethyl acetate, and ethanol crude extracts *Arnebia benthamii*. Data are presented as the mean value ± standard deviation of 3 separate experiments. Absorbance at 532 nm.

3.5. Hydroxyl Radical Scavenging Activity Radical. The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells [16]. This radical has the capacity to react with nucleotides of DNA and cause strand breakage, which leads to carcinogenesis, mutagenesis, and cytotoxicity. In addition, this species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids. The hydroxyl radical scavenging activity of the three different extracts of *Arnebia benthamii* at the concentration range of (100–500 μg/mL) can be ranked as ethyl acetate > ethanol > aqueous extract (Figure 6). All the extracts exhibited good hydroxyl radical scavenging activity with 63.75% for aqueous extract, 67% for ethanol extract, and 71.42% for ethyl acetate extract at the highest concentration used (500 μg/mL). The ability of the above mentioned extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of reactive oxygen species.

3.6. Antioxidant Activity against Oxidative Damage to DNA. The protective effect of *Arnebia benthamii* extracts on calf thymus DNA is shown in the Figure 7. Hydroxyl radicals generated by Fenton reaction were found to induce DNA strand breaks in calf thymus DNA. H_2O_2 alone did not cause DNA strand cleavage. However in presence of ferric nitrate and ascorbic acid, H_2O_2 leads to high DNA damage (lane 20). *Arnebia benthamii* extracts at 10–80 μg offered complete protection to DNA damage induced by hydroxyl radicals in

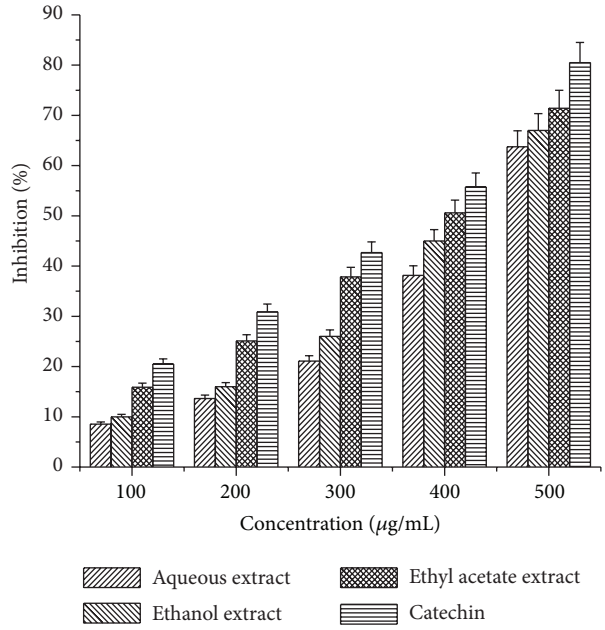

Figure 6: Hydroxyl radical scavenging activity of aqueous, ethyl acetate, and ethanol crude extracts *Arnebia benthamii*. The results represent mean ± S.D of 3 separate experiments. Results are reported as the percentage of the maximum formation of $OH^{\bullet}$ radical (100% deoxyribose oxidized): in absorbency, 100% is 1.270 ± 0.007 (control). Absorbance at 532 nm.

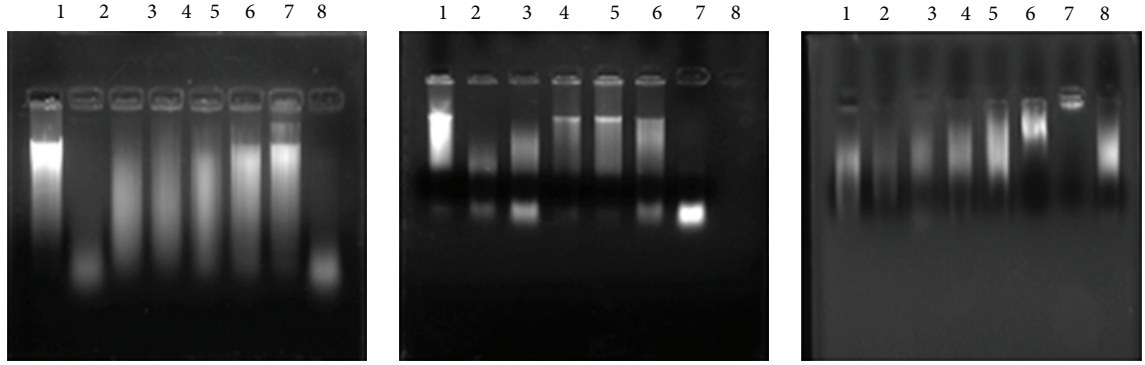

Figure 7: Protective effect of ethyl acetate, ethanol, and aqueous extracts of *Arnebia benthamii* on oxidative damage to calf thymus DNA. Lane 1: native calf thymus DNA, lane 2: DNA + 20 mM ferric nitrate + 100 mM ascorbic acid + 30 mM H_2O_2, lane 3: DNA + 20 mM ferric nitrate + 100 mM ascorbic acid + 30 mM H_2O_2 + 10 μg of plant extract, lane 4: DNA + 20 mM ferric nitrate + 100 mM ascorbic acid + 30 mM H_2O_2 + 20 μg of plant extract, lane 5: DNA + 20 mM ferric nitrate + 100 mM ascorbic acid + 30 mM H_2O_2 + 30 μg of plant extract, lane 6: DNA + 20 mM ferric nitrate + 100 mM ascorbic Acid + 30 mM H_2O_2 + 50 μg of plant extract, lane 7: DNA + 20 mM ferric nitrate + 100 mM ascorbic acid + 30 mM H_2O_2 + 80 μg of plant extract, and lane 8: DNA + 20 mM ferric nitrate + 100 mM ascorbic acid + 30 mM H_2O_2 + 10 μg of catechin.

calf thymus DNA (lanes 3–7). Again our results indicate that ethyl acetate extracts showed strong DNA damage protection once compared with that of ethanol and aqueous extract (Figure 7). Thus, the hydroxyl radical quenching ability of polyphenolic compounds of *Arnebia benthamii* could be responsible for the protection against oxidative damage to DNA.

3.7. Cytotoxic Activity. The growth inhibitory effects of different solvent extracts of *Arnebia benthamii* on six human cancer cell lines (HOP-62, A549, PC-3, THP-1, HCT-116, and MIA-Pa-Ca) were tested with the SRB assay. Cells were treated with various concentrations (10–100 μg/mL) of the extracts for 48 h. All the extracts did not exhibit significant effect on cells viability at the concentration of 10 μg/mL. However, at the concentrations of 50 and 100 μg/mL the extracts inhibited cell proliferation in a concentration-dependent manner on most of the cell lines, Table 2. Among those cancer cells tested, HOP-62, A549, THP-1, and MIA-Pa-Ca were the most sensitive cancer cells when treated with methanol extract of *Arnebia benthamii* with percentage inhibition of 100, 100, 90, and 100% at the concentration of 100 μg/mL, Table 2. The most resistant cancer cell to the extracts-induced growth inhibition was found to be PC-3 (prostrate) with

Table 1: Antioxidant assays (IC_{50} values) of different extracts of *Arnebia benthamii*.

Extracts	IC_{50}/DPPH	IC_{50}/reducing power	IC_{50}/lipid peroxidation	IC_{50}/DNA damage
Aqueous extract	335	195	85	415
Ethanol extract	300	185	82	425
Ethyl acetate extract	250	165	60	400
Catechin	230	145	45	325

Table 2: Cytotoxicity of the different crude extracts of *Arnebia benthamii* and 5-fluorouracil (5-FU) and Paclitaxel on six human cancer cell lines.

	Cell line type		HOP-62	A549	PC-3	THP-1	HCT-116	MIA-Pa-Ca
	Tissue type		Lung	Lung	Prostate	Leukemia	Colon	Pancreatic
S. no.	Code	Conc. (μg/mL)	%Age growth inhibition					
1	EB-MET	100	100	100	0	90	63	100
2	EB-MET	50	46	13	0	45	49	47
3	EB-MET	10	33	18	0	48	40	25
4	EB-ETH	100	55	42	0	65	57	55
5	EB-ETH	50	49	24	0	62	57	44
6	EB-ETH	10	43	0	0	57	47	40
7	EB-EA	100	39	21	35	68	72	66
8	EB-EA	50	24	0	6	7	8	23
9	EB-EA	10	0	0	0	0	0	0
10	EB-PE	100	47	54	39	29	19	0
11	EB-PE	50	47	53	10	18	14	0
12	EB-PE	10	39	15	0	1	12	0
13	EB-AQ	100	35	36	0	56	44	40
14	EB-AQ	50	32	24	0	28	42	30
15	EB-AQ	10	24	17	0	9	10	12
16	5-FU	20 μM	—	—	—	**67**	**67**	—
17	Paclitaxel	1 μM	**72**	**70**	—		—	—

Data are means ± SD of three independent experiments.

0% for methanol, aqueous, and ethanol extracts, 35% with ethyl acetate extract, and 39% with petroleum ether extract at the concentration of 100 μg/mL. As shown in Table 2, ethyl acetate extract comparably showed stronger growth inhibition on all the cell lines but less than the methanolic extract at the 100 μg/mL, indicating that the active anticancer compounds were mainly concentrated in the methanol and ethyl acetate extracts of *Arnebia benthamii*. Interestingly, the antiproliferation effect of methanolic extract was higher on HOP-62 and A549 than the known anticancer drug Paclitaxel. Ethyl acetate extract showed comparable inhibition on THP-1, MIA-Pa-ca, and HCT-116 cell lines. Similar results were observed in our previous study, with the 70% ethanolic and methanolic extracts of *Podophyllum hexandrum*, where both the extracts showed strong anticancer activities against different human cancer cells [21].

4. Conclusion

The results of the present study provide an evidence that antioxidant properties of *Arnebia benthamii* extracts showed mainly the ethyl acetate and ethanol extracts to be the potent source of antioxidants which positively correlates with their total phenolic content. Furthermore, the ethyl acetate and methanol extracts also showed the potent cytotoxic activity on six human cancer cell lines. Therefore, *Arnebia benthamii* extracts especially ethyl acetate, methanol, and ethanol deserves further investigation in active compounds responsible for the antioxidant and anticancer properties as it might be used in the field of pharmaceutical products and functional foods for the preservation and treatment of cancers.

Conflict of Interests

The authors of this paper report no conflict of interest regarding the publication of this paper.

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Research Article

Oxidative Stress Induced in Nurses by Exposure to Preparation and Handling of Antineoplastic Drugs in Mexican Hospitals: A Multicentric Study

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The impact of involuntary exposure to antineoplastic drugs (AD) was studied in a group of nurses in diverse hospitals in Mexico. The results were compared with a group of unexposed nurses. Anthropometric characteristics and the biochemical analysis were analyzed in both groups. Also, lipid peroxidation level (LPX), protein carbonyl content (PCC), and activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were evaluated in blood of study participants as oxidative stress (OS) biomarkers. The group of occupationally exposed (OE) nurses consisted of 30 individuals ranging in age from 25 to 35 years. The control group included 30 nurses who were not occupationally exposed to the preparation and handling of AD and whose anthropometric and biochemical characteristics were similar to those of the OE group. All biomarkers evaluated were significantly increased ($P < 0.5$) in OE nurses compared to the control group. Results show that the assessment of OS biomarkers is advisable in order to evaluate exposure to AD in nurses.

1. Introduction

AD have been reported to induce OS as a mechanism of toxicity. Free radicals formed during this process interaction with macromolecules to induce LPX, as well as oxidation of proteins and of puric and pyrimidine bases of deoxyribonucleic acid (DNA) [1–7]. However, there is a group of antioxidant enzymes such as SOD, CAT, GPx, and glutathione reductase which inhibit oxyradical formation thus aiding in the process of detoxification of these substances in the body [1, 2].

AD should be prepared in a biological safety cabinet designed and operated to ensure protection of the product being handled as well as of nurses and the environment. In all cases, health care workers should receive formal training so that, besides being aware of the risk involved, they can minimize it with appropriate work methods. Exposure of health professionals to this type of pharmaceuticals depends not only on the number of preparations performed each day but also on individual work procedures as well as the precautions taken in handling these agents. The lack of a centralized unit for formal training in the preparation and handling of AD implies a lower level of protection against the potential toxicity of these agents.

Diverse pathologies have been reported in nurses and pharmacy personnel who handle and prepare AD, among others; these pathologies include leukemia, impaired reproductive activity, spontaneous abortion, genotoxicity, cytotoxicity, carcinogenicity, and lymphocyte DNA damage [8–18].

It is important to mention that health professionals in Mexico in charge of preparing and handling AD do not for the most part receive formal training nor are they provided special areas equipped for handling these agents.

Some studies have evaluated OS parameters in nurses. Ulas et al., in 2012, assessed changes in OS parameters, anxiety indexes, and metabolic activities of the nurses in day and night shifts. These parameters were measured in ordinary service and intensive care unit. They found that in ordinary service and intensive care unit nurses, OS parameters, anxiety indexes, and metabolic activities were not different and all nurses suffer the similar effects of the shifts both in day and night. However, there are no reports in the literature indicating the evaluation of OS biomarkers in nurses occupationally exposed to AD preparation and handling [19, 20].

The goal of this study was to evaluate OS by means of LPX, PCC, SOD, CAT, and GPx activities in OE nurses regarding the preparation and handling of AD in different hospitals in Mexico and to determine if OS is a potentially reliable early warning biochemical marker for toxicity assessment in these health care professionals.

2. Material and Methods

2.1. Selection of Subjects. The transversal and multicentric study was conducted on OE nurses regarding preparation and handling of AD and nurses unexposed to these conditions, who work in different hospitals in the state of Mexico including the Centro Oncológico Estatal ISSEMyM, DIF Children's Hospital, Clinic 220 of the Instituto Mexicano del Seguro Social (IMSS), and ISSEMyM Mother and Child Hospital in the city of Toluca, as well as the IMSS Family Medicine Unit 231 in Metepec.

The research protocol used complies with guidelines of ethical principles in the Declaration of Helsinki (particularly in those aspects involved in noninvasive procedures for human studies) and was approved by the Ethics in Research Committee of the Centro Oncológico Estatal ISSEMyM, the hospital where the project for the present study was submitted for evaluation and to which nurses from the various hospitals and clinics participating in the study were directed.

The initial selection criteria were based on the face-to-face questionnaire. From the started selected group, subsequent inclusion/exclusion criteria were applied (detailed below).

Questionnaire data were collected by two staff members who were trained by the study investigation in participant recruitment, interview content and techniques, the safe handling of the biological samples, and ethical issues related to the study. Each interview was carried out on the day when the blood extractions were performed and required approximately 40 min. The questionnaire includes information on their lifestyle (age, place of residence, birthplace, sleep and rest habits, diet, and physical activities) and employment history (years in an AD preparation-related job and use of protective equipment).

Just before extraction of the sample a complete medical interview was carried out in both selected groups. All the nurses included in this study were free from neoplasias, osteoarticular degenerative diseases, any kind of autoimmune diseases, chronic infections of any etiology (viral, bacterial, or fungal), allergy in any degree, nutritional disorder (such as dislipemias and malnutrition), neurodegenerative diseases, heart diseases under pharmacological treatments, and endocrine illnesses. Excessive smokers (more than 10 cigarettes per day) and alcohol consumers were excluded.

Sampling was nonprobabilistic, opportunistic, sequential, consecutive, and by intact groups. The sample size for each group was 30 individuals, taking into account OE nurses regarding AD preparation and handling and nurses unexposed to these conditions for a total study population of 60 nurses.

Nurses evaluated were invited to participate in the study. They were informed of the characteristics of the study and of the need to take a blood sample from each. Individuals agreeing to take part in the study signed an informed consent letter.

2.2. Study Groups. Based on questionnaire responses and inclusion and exclusion criteria, study participants were divided into two groups: OE and unexposed or control.

Nurses in the OE group were selected according to the following criteria: more than two years in an AD preparation-related job and 25 to 35 years of age. Individuals receiving radiation treatment or chemotherapy were excluded from the study.

The control group was formed by nurses who did not come into contact with AD, were similar in socioeconomic characteristics and age to OE participants, and whose work activity did not involve the preparation or handling of AD. These volunteers were initially contacted at the Centro Oncológico Estatal ISSEMyM.

2.3. Baseline Definitions and Measurements. Anthropometric measurements were performed according to a standard protocol. Blood pressure (BP) was measured in the morning after 10 min of rest in the sitting position. Abdominal circumference was measured horizontally at the umbilical level at the end of normal expiration. Body mass index (BMI) was calculated by body weight (kg/height (m^2).

Information on their lifestyle, including age, place of residence, birthplace, sleep and rest habits, diet and physical activities, and employment history, years in an AD preparation-related job, and use of protective equipment was obtained by self-reported questionnaires.

2.4. Sample Collection. Morning fasting (8 am) blood samples were collected in both groups on the same day using heparin as an anticoagulant (10 UI/mL) in graduated ice-cold polypropylene test tubes. Plasmas were immediately separated by centrifugation (4000 ×g, 10 min) and stored at −80°C until analyzed. The serum was stored at −80°C. All samples were coded at the time of preparation. The following biomarkers were evaluated: LPX and PCC in order to evaluate oxidized protein content and activity of the antioxidant enzymes SOD, CAT, and GPx.

Other blood samples were collected using EDTA (5.0 mmol/L) as an anticoagulant for use in hemoglobin determination. Hemoglobin level was used to express results of OS markers.

2.5. Biochemical Analysis. The activity of aspartate aminotransferase (AST), alanine aminotransferases (ALT), alkaline phosphatase (ALP), and total bilirubin were determined to evaluate hepatic performance. Renal function was evaluated by plasma creatinine and urea concentrations. Also serum glucose and triglycerides were determined. These determinations were performed using commercial kits from Fluka-Sigma-Aldrich, Toluca.

2.6. Determination of OS Status

2.6.1. Determination of LPX. LPX was determined using the thiobarbituric acid-reactive substances method (Büege and Aust, 1978) [21]. To 500 μL blood was added Tris-HCl buffer solution with pH 7.4 (Sigma-Aldrich, St. Louis) until a 1-mL volume was attained. Samples were incubated at 37°C for 30 min; 2 mL TBA-TCA reagent (0.375% thiobarbituric acid (Fluka-Sigma-Aldrich, Toluca) in 15% trichloroacetic acid (Sigma-Aldrich, St. Louis)) was added and samples were shaken in a vortex. They were then heated to boiling for 45 min, allowed to cool, and the precipitate removed by centrifugation at 3,000 ×g for 5 min. Absorbance was read at 535 nm against a reaction blank. Malondialdehyde (MDA) content was calculated using the molar extinction coefficient (MEC) of MDA (1.56×10^5 M/cm). Results were expressed as μmol MDA/mg hemoglobin.

2.6.2. Determination of PCC. PCC was determined using the method of Levine et al. [22]. To 100 μL of supernatant was added 150 μL of 10 mM DNPH in 2 M HCl and the resulting solution was incubated at room temperature for 1 h in the dark. Next, 500 μL of 20% trichloroacetic acid was added and the solution was allowed to rest for 15 min at 4°C. The precipitate was centrifuged at 16,000 ×g for 5 min. The bud was washed several times with 1 : 1 ethanol : ethyl acetate, then dissolved in 1 mL of 6 M guanidine solution (pH 2.3) and incubated at 37°C for 30 min. All reagents were obtained from Sigma-Aldrich, St. Louis. Absorbance was read at 366 nm. Results were expressed as μmol reactive carbonyls formed (C=O)/mg hemoglobin, using the MEC of 21,000 M/cm.

2.6.3. Determination of SOD Activity. SOD activity was determined by the Misra and Fridovich (1972) method [23], which is based on inhibition of adrenaline autoxidation at pH 10.2 in erythrocyte lysates free of hemoglobin and other proteins. In a quartz cuvette were placed 150-μL aliquots of homogenate (obtained from 500 μL total blood in 2 mL distilled water, sonicated for 15 min and then supplemented with 2.5 mL of 1 : 1 ethanol : chloroform). Addition was then made of 750 μL of carbonate buffer solution with pH 10.2 (50 mM sodium bicarbonate, 0.1 mM EDTA, adjusted to pH 10.2 with Na_2CO_3 in powdered form) and 600 μL adrenaline (30 mM) in 0.05% acetic acid. All reagents were from Sigma-Aldrich, St. Louis. Absorbance was read at 0 s, 30 s, and 5 min, at 480 nm. Absorbance was read at 480 nm after 30 s and 5 min. Results were expressed as UI/mg Hb. Estimates were derived by the formula $[\text{SOD}] = (A_{30\,\text{s}} - A_{5\,\text{min}}) * (A_0/\text{MEC})$, where the MEC of adrenaline is 21/M/cm.

2.6.4. Determination of CAT Activity. CAT activity was quantified by the Radi et al. method [24], which is based on disappearance of H_2O_2 as a result of CAT action through change in absorbance per minute. To 20 μL erythrocyte homogenate plus 1 mL of isolation buffer solution (0.3 M sucrose; 1 mM HEPES; 5 mM KH_2PO_4 adjusted to pH 7.4) (Vetec-Sigma-Aldrich, St. Louis) was added 200 μL H_2O_2 (20 mM) (Vetec-Sigma-Aldrich, St. Louis), reading absorbance at 0 and 60 s, at 240 nm in quartz cuvettes. Results were expressed as mM H_2O_2/mg hemoglobin. Estimates were obtained using the formula $[H_2O_2] = (A_{0\,\text{s}} - A_{60\,\text{s}})/\text{MEC}$, where the MEC of H_2O_2 is 0.043/mM/cm.

2.6.5. Determination of GPx Activity. GPx activity was determined by the Gunzler and Flohe-Clairborne method [25]. To 100 μL of supernatant was added 10 μL glutathione reductase (2 U glutathione reductase, Sigma-Aldrich) plus 290 μL reaction buffer (50 mM K_2HPO_4 (Vetec), 50 mM KH_2PO_4 (Vetec) with pH 7.0, 3.5 mM reduced glutathione (Sigma-Aldrich), 1 mM sodium azide (Sigma-Aldrich), and 0.12 mM NADPH (Sigma-Aldrich)) and 100 μL H_2O_2 (0.8 mM, Vetec), prior to reading absorbance at 340 nm at 0 and 60 s. Enzyme activity was estimated using the equation GPx concentration $= (A_0 - A_{60})/\text{MEC}$, where the MEC of NADPH is 6.2 mM/cm. Results were expressed as mM NADPH/g hemoglobin.

2.7. Determination of Hemoglobin. Hemoglobin was determined using a Beckman Coulter AcT Diff hematology analyzer.

2.8. Statistical Analysis. This was a transversal study designed to compare analytical data between two samples. Processing and scoring of the samples from exposed and control groups were immediately performed blind and concurrently. At the end of the study, the analytical data and the results obtained from the questionnaire were linked for statistical analyses. All data were expressed as mean ± standard deviation (SD). Student's t-test or the χ^2 test (depending on the type of variable tested) was used for analyzing the results. However, due to the fact that some biochemical parameters may not follow a normal distribution (as judged by Kolmógorov-Smirnov test) the nonparametric Wilcoxon-Mann-Whitney test was also employed (although with equivalent final conclusions). A probability value of $P < 0.05$ was considered to be statistically significant. All analyses were performed using Statistical Package of SPSS version 17.0 for Windows (SPSS, Chicago, IL, USA).

3. Results

3.1. General Characteristics of the Study Population. The total number of OE nurses was 30; 100% were women, with a mean age of 32 years (range 25–35 years). Control group individuals

Table 1: Demographical and anthropometric characteristics and the biochemical analysis in control and occupationally exposed groups.

Parameter	Control group	Occupationally exposed group	RV
Age (years)	32 (25–35)	34 (25–35)	
BMI (Kg/m^2)	21.6 ± 2.1	22.1 ± 2.3	18.5–22.9
Systolic blood pressure (mmHg)	125 ± 12	121.6 ± 10.3	<120
Diastolic blood pressure (mmHg)	77 ± 8	80.5 ± 13.2	<80
Triglycerides (nmol/L)	2.1 ± 0.6	2.6 ± 0.9	<2.82
Serum glucose (nmol/L)	6.1 ± 0.9	5.8 ± 0.7	<7.8
AST (UI/mL)	7.6 ± 0.8	8.3 ± 1.1	<12
ALT (UI/mL)	9.2 ± 0.9	9.8 ± 1.3	<12
ALP (UI/L)	110.3 ± 8.3	122.5 ± 9.6	68–240
Total bilirubin (mg/L)	4.5 ± 0.6	5.1 ± 0.8	<10
Urea (g/L)	0.4 ± 0.06	0.35 ± 0.08	0.20–0.45
Creatinine (mg/L)	11.2 ± 0.9	12.9 ± 1.3	8–14

Data were expressed as the mean ± SD. Results were obtained using commercial kits as detailed in Section *Biochemical analysis*. Reference values (RV) are those established for the World Health Organization and the kits.
BMI: body mass index; AST: aspartate amino transferase; ALT: alanine amino transferase; ALP: alkaline phosphatase.

Table 2: Oxidative stress markers in control and occupationally exposed nurses groups.

Biochemical marker	Control group	Occupationally exposed group	P value
LPX (μmol MDA/mg Hb)	1.9 ± 0.05	4.8 ± 0.14*	$P < 0.05$
PCC (μmol carbonyls/mg Hb)	1.6 ± 0.07	3.5 ± 0.08*	$P < 0.05$
SOD (UI/mg Hb)	4.5 ± 0.09	7.9 ± 0.07*	$P < 0.05$
CAT (mM H_2O_2/mg Hb)	1.2 ± 0.02	2.0 ± 0.05*	$P < 0.05$
GPx (mM NADPH/mg Hb)	5.1 ± 0.06	18.75 ± 0.09*	$P < 0.05$

Data were expressed as the mean ± SD. Values significantly different compared to control group were indicated with *($P < 0.05$). LPX: lipid peroxidation level; MDA: malondialdehyde; Hb: hemoglobin; PCC: protein carbonyl content; SOD: superoxide dismutase activity; CAT: catalase activity; GPx: glutathione peroxidase activity.

number was 30; 100% were women, with a mean age of 34 years (range 25–35 years) (Table 1).

Mean time in an AD-related job for OE participants was 4 years (range 2–9 years), suggesting chronic exposure to a wide spectrum of AD including cisplatin, etoposide, gemcitabine, doxorubicin, docetaxel, paclitaxel, vinorelbine, and carboplatin. As regards the use of protective equipment during work, 100% of OE participants said they did not use facemasks, gloves, surgical caps, and protective eyewear or lab coats.

Since none of the nurses in the OE group use protective equipment, they come in greater contact with diverse AD via any one of the potential absorption routes (dermal, inhalatory, digestive, or through the mucosa) which, combined with different temperature gradients and lack of adequate ventilation, poses increased risks to their health.

It is worth noting that in the lifestyle questionnaire, 16 OE group nurses reported working a second shift in private hospitals, where they performed similar activities but with fewer safety measures.

The control group did not carry out any activities associated with AD preparation or handling.

3.2. Baseline Definitions and Biochemical Markers. Table 1 shows the main anthropometric characteristics of the study subjects. No significant differences ($P > 0.05$) were observed between OE and unexposed nurses concerning age, BMI, and systolic and diastolic blood pressure.

The biochemical markers, triglycerides, serum glucose, AST, ALT, ALP, total bilirubin, urea, and creatinine also were evaluated. The results in both OE and unexposed groups were within the range of reference values established for the World Health Organization and the kits. No significant differences were observed between OE and unexposed nurses ($P > 0.05$).

3.3. Oxidative Stress Markers. In order to assess the exposure degree to AD, the OS markers were measured as typical OS biomarkers. Table 2 shows the results of LPX obtained in blood samples of the study population. A significant increase ($P < 0.05$) in the OE group (252.6%) compared to the control group was observed in this biomarker. PCC results in the OE group show a significant 218.8% increase compared to the control group ($P < 0.05$). The results of antioxidant status were also significantly altered. A marked increase in SOD activity was found in nurses in the OE group (75.5%) compared to control group individuals ($P < 0.05$). A 166.6% increase in CAT activity occurred in the OE group with respect to the control group ($P < 0.05$) and was statistically significant. Finally, GPx results (Table 2) in the group of OE nurses show a significant 367.7% increase compared to the control group ($P < 0.05$).

4. Discussion

Health parameters and OS markers were compared between OE nurses and unexposed or control. The results in OE nurses

of anthropometric characteristics, such as age, BMI, and systolic and diastolic blood pressure, as well as the biochemical markers, triglycerides, serum glucose, AST, ALT, ALP, total bilirubin, urea, and creatinine showed not significant differences compared with unexposed group.

Referring to the results of OS status in the present study, they show increases in LPX and PCC in the group of OE nurses regarding the preparation and handing of AD, with respect to the control group ($P < 0.05$). Neoplastic disease studies reveal that treatment with AD increases OS and reduces plasma levels of vitamins C and E as well as of glutathione peroxidase [26].

Diverse AD have been associated with OS. For example, cisplatin induces formation of reactive oxygen species (ROS) in mitochondria, eliciting oxidative alterations in lipids, proteins, and DNA of this organelle [27], while doxorubicin-induced cytotoxicity has been associated with ROS production and in particular to presence of the superoxide anion radical and of hydrogen peroxide [28, 29]. This pharmaceutical is also able to produce reactive nitrogen species (RNS) such as peroxynitrite [30]. The oxidant peroxynitrite is known to induce protein oxidation and nitration in the absence of GSH, eliciting mitochondrial dysfunction and eventually leading to irreversible damage and severe loss of cellular ATP [31]. It is worth noting that both medications are prepared, handled, and administered by nursing personnel in hospitals participating in the present study.

The increases in LPX and PCC found in our study may be explained by an increase in the number of radical species produced by the biotransformation of AD in OE nurses, such as superoxide anion and hydrogen peroxide, which are known to attach to membrane lipids, inducing their lipid peroxidation. Similarly, increased peroxynitrite concentrations may oxidize directly the prosthetic protein group or else react directly with the peptide chain, leading to conformational and functional changes with severe biological consequences for the individual [32].

Paradoxically, oxidative stress induced by oxidative metabolism of antineoplastic drugs interferes with the tumoral growth produced in different types of cancer, since one of the indicators of this process—increased lipid peroxides—favors the prolongation of cell quiescence (G 0 phase). The problem lies in the fact that cytostatic or chemotherapeutic agents act while malignant cells are in constant replication, not when they are quiescent [33–36].

Likewise, antioxidant capability has been reported to be greater in tumoral cells than in normal cells [34], but this effect is surpassed by the OS induced by AD. Short-lived cells or cells with higher renewal rates which are constantly being regenerated are the most affected, in addition to the fact that there are other undesirable effects associated with free radical generation, such as doxorubicin-induced cardiac toxicity (rapid heartbeat, heart failure), bleomycin-induced pulmonary fibrosis, and cisplatin-induced ototoxicity [37–39].

During a person's lifetime, a sophisticated antioxidant network counteracts the deleterious action of ROS on macromolecules [40]. Cells synthesize some of their own antioxidants, as do also SOD, CAT, and GPx as well as peptides with thiol groups, such as glutathione (GSH) and the thioredoxin family. These systems play a major role in the ability of the body to respond to the oxidative challenge of using molecular oxygen to drive reactions that yield the necessary energy.

Increased ROS production is known to be associated with increases in antioxidant enzyme activity. A marked increase in SOD activity occurred in our study in the OE group (75.5%) compared to the control group ($P < 0.05$). Comparison of CAT activity results between study groups found a 166.6% increase of this activity in the OE group, which differed significantly from activity in the control group ($P < 0.05$). Finally, GPx results in the OE group showed a significant 367.6% increase compared to the control group ($P < 0.05$).

SOD is the first mechanism of antioxidant defense and the main enzyme responsible for offsetting toxic effects is induced by the presence of ROS, particularly the superoxide ion, which is formed as a minor product of mitochondrial respiration. Increased SOD activity in our study may be explained by high levels of the superoxide anion radical, which can stimulate this activity. It is well known that the enzyme SOD is known to transform $O_2^{\bullet}$ to H_2O_2.

Subsequently, the enzyme CAT takes part in the catalytic reaction that decomposes two molecules of the hydrogen peroxide—formed by dismutation of superoxide—into water and oxygen, without the use of cofactors. This function is shared with GPx which uses GSH as a reducing agent [41].

The increase in CAT and GPx may be due to higher levels of hydrogen peroxide, since the oxidative metabolism of AD, such as doxorubicin, to which nurses in our study were exposed, is known to increase the levels of peroxide, which is a specific substrate of GPx.

Similar results of our study were found by Ulas et al. in 2012; they observed that in ordinary service and intensive care unit, the nurses in day and night shifts presented values of total antioxidant status of 0.95–1.01 μmol H_2O_2. These values were similar to those found in the activity of catalase in nurses unexposed to AD (1.2 mM H_2O_2/mg Hb) [19, 20]. However, OE nurses showed a significant increase from baseline of unexposed nurses to AD.

The increases in HPC, LPX, and PCC in the present study may explain the increases observed in the activity of antioxidant enzymes, as a mechanism of defense against oxidative damage.

Our results showed that OE nurses were more susceptible to oxidative stress than unexposed nurses. No significant differences were found in both groups with respect to biochemical markers evaluated, to explain OS induced in OE nurses. Neither anthropometric characteristic explain OS induced in the exposed group. For these reasons, we believe that OS induced in OE nurses may be explained by exposure to AD.

5. Limitations

Certain limitations of the present study should be considered. First, a kinetic used several times must be performed for the different biomarkers of OS to be evaluated. Second, determine AD concentrations in blood of OE nurses and

perform a correlation between AD concentration and OS parameters in OE nurses. Third, the sample size was relatively small. Therefore, these results should be verified with large-scale, multicenter prospective cohort studies.

6. Conclusions

OE nurses to AD preparation and handling are at potential risk of increasing their levels of OS by not taking preventive and protective measures. Determination of a set of OS biomarkers is important for early detection of their toxic effects in order to prevent health damage in the exposed population.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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