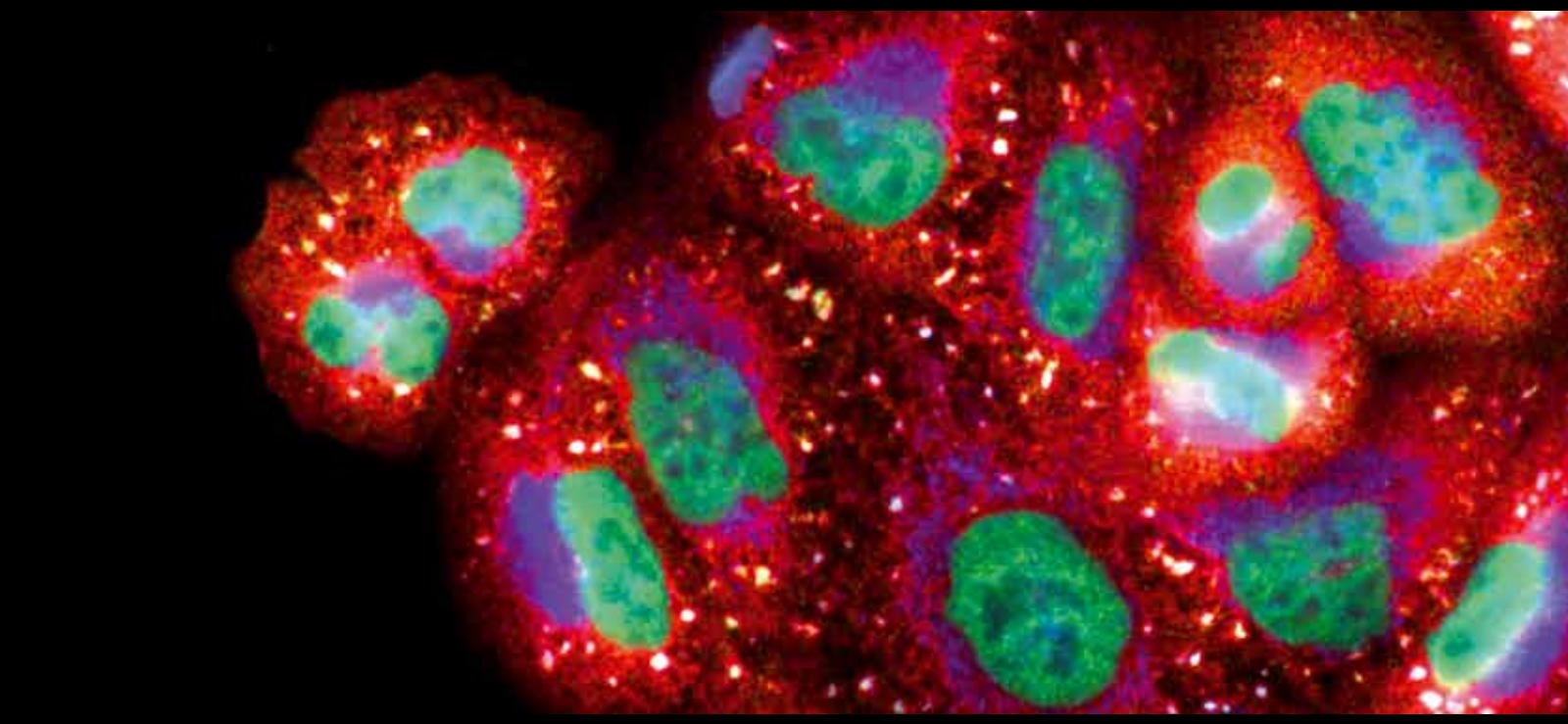
Oxidative Medicine and Cellular Longevity

Antioxidants in Longevity and Medicine

Guest Editors: Nilanjana Maulik, David Mcfadden, Hajime Otani, Mahesh Thirunavukkarasu, and Narasimham L. Parinandi



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Editorial Board

Contents

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Editorial

Antioxidants in Longevity and Medicine

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Oxidative stress has emerged as a critical player in the pathogenesis and pathophysiology of many diseases in experimental animal models and humans. The oxidative stress theory of aging emphasizes that a progressive and irreversible escalation of oxidative damage caused by reactive oxygen species (ROSs) exerts severe influence on the critical aspects of the biology of aging and contributes to impaired physiological functions, increased incidence of diseases, and shortening of life span. The focus of this special issue is to identify avenues that affect both the life span and age-related diseases in humans. In order to accomplish this, experts in the field have contributed original research articles and reviews focusing on the current state of understanding of the molecular pathology of oxidative stress underlying the biology of aging and age-related diseases and development of strategies to alleviate or treat these conditions.

S. Gazman-Beltran et al. demonstrate that a natural antioxidant, nordihydroguaiaretic acid (NDGA) attenuates the oxidative stress-induced decrease of CD33 expression in human monocytes, which is decreased in diabetic patients under oxidative stress. The antioxidant mechanism of NDGA appears to be multifactorial and includes the increased availability of glutathione and activation of nuclear factor E2-related factor-2 (Nrf2), which is known to orchestrate antioxidative and cytoprotective responses to oxidative stress. NDGA appears to be an effective antioxidant to prevent chronic inflammation induced by monocytes.

Recently, studies conducted with dietary polyphenols on neurological disorders have shown promising results. K. S. Bhullar and H. P. V. Rupasinghe have reviewed the effect of polyphenols on age-related neurological disorders and discussed the role of polyphenols in multiple signaling pathways, including PI3K/Akt, Nrf2/HO1 PPAR, STAT, NF-κB, HIF, and MAPK. All of the pathways can alleviate oxidative damage and inflammation. This review article offers insights into the complex interactions of polyphenols with intracellular signaling pathways in neuronal cells and provides novel approach to neurological disorders.

Resveratrol is a calorie restriction (CR) mimetic agent. CR reverses many of the obesity-related increases in inflammatory cytokines. I. Zagotta et al. have reported that PAI-1 gene expression is increased in adipose tissue in obese subjects, and this is critically involved in chronic inflammation of blood vessels that leads to atherosclerosis and cardiovascular disease. PAI-1 gene is found to be upregulated due to inflammatory conditions associated with obesity. Resveratrol treatment has been shown to suppress the PAI-1 gene in human inflamed adipose tissue. Interestingly, the effect of resveratrol on PAI-1 is not mediated by modulation of PI3 K/Akt, SIRT1, AMPK, and Nrf2 or reactive oxygen species (ROS) but proceeds through the inhibition of the NF-κB pathway. This suggests that resveratrol inhibits the generation of inflammatory cytokines in the adipose tissue via a novel anti-inflammatory pathway.

Advanced glycation end products (AGEs) play a crucial role in senescence. B. Buttari et al., have demonstrated that resveratrol may be effective in treating autoimmune diseases that are intimately related to the activation of dendritic cells

(DCs) with the AGEs. B. Buttari et al. have also shown that resveratrol pretreatment of human monocyte-derived DCs prevents the activation of DCs in response to the AGE-albumin. The mechanism is by inhibiting the DC maturation and proinflammatory cytokine expression associated with the inhibition of NF-κB activation. Because the specific receptor for AGE (RAGE) is down-regulated in the resveratrol-pretreated DCs, resveratrol inhibits oxidative stress and inflammatory response at the level of RAGE upstream of PI3K/Akt, MAPK, or NF-κB. This finding is interesting since the NF-κB pathway is modulated by resveratrol in other biological systems. This unique pharmacological action of resveratrol on DCs that are activated by AGEs is a subject for further investigation.

ROS derived from the mitochondria activates the redox-sensitive transcriptional factor, nuclear respiration factor-2 (NRF2). NRF2-induced mitochondrial biogenesis improves the function of electoral transfer chain (ETC) and prevents ROS generation in mitochondria. Thus, the agents that can activate NRF2 are attractive in ameliorating oxidative stress-induced pathology. X. Miao et al. have demonstrated that an NRF2 activator MG132 has prevented the aortic injury in type-1 diabetic mice, suggesting that activation of NRF2 represents a promising approach for hyperglycemia-induced vascular complications.

Aging is related to circadian rhythm disruption. The concept of chrononutrition raised by M. Garrido et al. has prompted development of a strategy that takes timing of tryptophan-enriched food intake into account when delivering antioxidative medicine. Chrononutrition studies suggest that along with the content of food, the time of ingestion is essential for the natural functioning of the circadian system, which stimulates melatonin secretion at night. Melatonin produces circadian rhythm and acts as a potent antioxidant and ROS scavenger. It also inhibits ROS generation by preventing the electron leakage from mitochondria, thereby stimulating the antioxidative defense system. Thus, it is anticipated that administration of melatonin either directly or indirectly from food enhances one's antioxidant status.

A review article described by H. Otani opens a new avenue for antioxidative medicine. Although ROS is involved in many pathological phenomena in humans with advanced age, antioxidative medicine is not always successful in alleviating human disease. The limitation of general antioxidants in preventing oxidative stress-induced diseases is attributed to ROS playing a pivotal role in generating redox signaling. This is necessary for maintaining homeostasis and generating adaptive response to lethal oxidative damage. Thus, antioxidants must be more site specific to eliminate only harmful ROS and leave beneficial ROS. Recent new drugs used for treating lifestyle-related diseases such as hypertension, hyperlipidemia, and hyperuricemia possess a pleiotropic effect that is designated as the site-specific antioxidant against endothelial NADPH oxidase and xanthine oxidase. Antioxidants that specifically reduce mitochondrial ROS generation will also be putative drugs to ameliorate age-associated disease and prolong lifespan.

Mitochondrial generation of ROS is intimately related to aging. Eliminating the harmful production of ROS from mitochondria represents a powerful strategy to prevent cellular senescence. The review article authored by K. Shinmura highlights the importance of preventing mitochondrial generation of ROS. Age-related mitochondrial dysfunction is due to oxidative stress-mediated accumulation of somation mutation in mitochondrial DNA. A growing body of evidence suggests that caloric restriction (CR) mediates attenuation of mitochondrial oxidative damage via decreasing the production of mitochondrial ROS rather than by enhancing the antioxidant defense. K. Shinmura suggests that sirtuin might be involved in the CR-induced attenuation of mitochondrial oxidative damage. This review article concludes that CR-mimetic agents, such as resveratrol and sirtuin activators, are promising agents to improve the mitochondrial function and prolong lifespan, although their exact mechanism(s) of action should be carefully examined.

Myocardial reperfusion injury is mediated at least in part by oxidative stress. Yet, there have been no antioxidants that unequivocally confer benefit in the postischemic heart in the clinical setting, although numerous agents have been shown to exert cardioprotective effects on animal models of ischemia/reperfusion injury. Angelos et al. have demonstrated that the neutrophil esterase inhibitor sivelestat reduces the infarct size and improves cardiac function in an isolated rat heart that lacks neutrophils. The cardioprotective effect of sivelestat appears to be mediated by nitric oxide, which has consistently been implicated in the cardioprotection against myocardial ischemia/reperfusion injury. Future clinical trials using sivelestat are warranted to test whether this clinically available drug is indeed effective in ameliorating myocardial reperfusion injury in humans.

G. Grosso et al. have advocated in favor of the health benefits of red orange on the basis of research on experimental models and epidemiological evidence. The authors argue that the beneficial effects of the red orange fruit may be exerted through the synergistic actions of the natural products present in the fruit. These natural products possess antioxidant actions, which may protect against oxidative damage, and the total beneficial actions of all the natural products (antioxidants) present in the red orange fruit could be more effective than an individual antioxidant.

The lack of mitochondrial superoxide dismutase (Mn-SOD) leading to the loss of hearing during aging has been clearly demonstrated by M. Kinoshita et al. With the use of Mn-SOD heterozygous knockout mice as the experimental model of mitochondria-derived oxidative stress, the authors have shown that loss of Mn-SOD by half appears to elevate the oxidative stress in the cochlea to a certain degree, but that may not be adequate to speed up the age-induced damage of the cochlea. Nevertheless, the role of Mn-SOD in the mitochondria that scavenges the superoxide radical in the oxidative stress that leads to loss of hearing during aging has been attempted in this study.

Z. Makpol et al. have studied the gene expression-modulating actions of gamma-tocotrienol in the senescent human diploid fibroblasts by utilizing the microarray analysis. This study has emphasized on the lipid-soluble antioxidant (gamma-tocotrienol) actions on the modulation of genes associated with the cellular aging and oxidative stress.

This study has demonstrated that gamma-tocotrienol appears to block cellular aging of human fibroblasts through the modulation of gene expression in the cells. In addition, the role of lipid peroxidation in cellular aging and associated gene expression is apparent from this study.

D. McCormack and D. McFadden have discussed in depth the antioxidant and disease-modifying actions of pterostilbene. The antioxidant actions of pterostilbene have been connected with cancer protection, treatment of neurological diseases, suppression/treatment of inflammation, attenuation of vascular diseases, and improvement of diabetes. The authors have provided an excellent account on the clinical use of pterostilbene in prevention/treatment of several disorders and diseases.

A. B. Rodriguez et al. showed that fish oils containing the omega-3 polyunsaturated fatty acids (n3-PUFA) are capable of attenuating the levels of inflammatory cytokines and oxidative stress markers in the serum of multiple sclerosis patients. From this study, the authors have concluded that the fish oil supplementation to the multiple sclerosis patients effectively lowers the levels of inflammatory cytokines and nitric oxide (oxidant), thus leading to the protection against multiple sclerosis.

J. Rosado-Perez et al. have shown that physical exercise like Tai Chi offers beneficial effects in lowering oxidative stress in humans. In their study on Mexican adults, the authors have revealed that the subjects who have practiced Tai Chi have exhibited lower extent of lipid peroxidation and elevated superoxide dismutase activity as compared to the control individuals. From this study, the authors have concluded that Tai Chi is more effective in elevating the antioxidant status in humans as compared to walking.

S. Dande et al. have discussed on the natural antioxidant actions of the commonly consumed foods of plant origin in India. In this review, the authors have emphasized on the domestic processing of plant foods that could affect the quality of the plant foods that are consumed. The authors discussed the importance of polyphenols present in the plant foods that could act as natural antioxidants. The authors have stated that the domestic processing of plant foods may not alter the content of polyphenols and antioxidant activities in the plant foods.

In a study on the hypoxia-induced neuroinflammation, Z. Wu et al. have shown that the Brazilian green propolis inhibits the NF-κB activation in the microglia. The authors have demonstrated that the mitochondrial ROS formation is crucial for the activation of NF-κB activation in the microglia. Therefore, it appears that propolis could have also acted as an antioxidant in attenuating the hypoxia-induced neuroinflammation in the microglia.

A. L. Zagayko et al. have shown that the grape polyphenols increase the activity of high-density lipoprotein (HDL) enzymes in old and obese rats. The authors have revealed that the activity of paraoxonase, lecithin: cholesterol acyltransferase (LCAT) in plasma have been lowered and the cholesterol ester transfer protein (CETP) activity has been elevated in old rats. This study emphasizes the actions of grape polyphenols on the elevation of HDL enzymes during aging.

These papers represent an exciting and insightful snapshot of current antioxidant research. State-of-the-art, exciting challenges, which still present many new challenges, are highlighted in this special issue, which may inspire and help advance antioxidant research. We would like to thank all authors, reviewers, and the editors for producing this special issue.

Antioxidative medicine has become a practical tool to treat age-associated disease and to prolong the lifespan since oxidative stress is critically involved in human aging. The purpose of the topics covered in this special issue is to provide additional opportunities to all the investigators in the field in order to identify avenues that impact both the life span and age-related diseases in humans. Therefore, the contributions made by the experts in this issue as original research articles and reviews will hopefully stimulate the continuing efforts of the investigators to understand and establish the molecular pathology of oxidative stress underlying the biology of aging and age-related diseases and development of strategies to alleviate or treat these conditions.

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Erratum

Erratum to "Site-Specific Antioxidative Therapy for Prevention of Atherosclerosis and Cardiovascular Disease"

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Research Article

Brazilian Green Propolis Suppresses the Hypoxia-Induced Neuroinflammatory Responses by Inhibiting NF-κB Activation in Microglia

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Hypoxia has been recently proposed as a neuroinflammatogen, which drives microglia to produce proinflammatory cytokines, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-6. Considering the fact that propolis has hepatoprotective, antitumor, antioxidative, and anti-inflammatory effects, propolis may have protective effects against the hypoxia-induced neuroinflammatory responses. In this study, propolis (50 μg/mL) was found to significantly inhibit the hypoxia-induced cytotoxicity and the release of proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, by MG6 microglia following hypoxic exposure (1% O_2, 24 h). Furthermore, propolis significantly inhibited the hypoxia-induced generation of reactive oxygen species (ROS) from mitochondria and the activation of nuclear factor-κB (NF-κB) in microglia. Moreover, systemic treatment with propolis (8.33 mg/kg, 2 times/day, i.p.) for 7 days significantly suppressed the microglial expression of IL-1β, TNF-α, IL-6, and 8-oxo-deoxyguanosine, a biomarker for oxidative damaged DNA, in the somatosensory cortex of mice subjected to hypoxia exposure (10% O_2, 4 h). These observations indicate that propolis suppresses the hypoxia-induced neuroinflammatory responses through inhibition of the NF-κB activation in microglia. Furthermore, increased generation of ROS from the mitochondria is responsible for the NF-κB activation. Therefore, propolis may be beneficial in preventing hypoxia-induced neuroinflammation.

1. Introduction

The brain is highly susceptible to being damaged by hypoxia because of its high demand for oxygen supply [1]. Microglia are resident innate immune cells in the brain, constituting the first line of defense against brain insults [2, 3]. It is generally accepted that hypoxia is one of the neuroinflammatogens in the brain, because hypoxia activates microglia to provoke excessive secretion of proinflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [4–7]. It is also known that proinflammatory cytokines secreted by microglia promote cognitive deficits in aged people and Alzheimer's disease (AD) patients [8, 9]. In our previous studies, we have found that enhanced production of reactive oxygen species (ROS) due to the increased mitochondrial DNA damage in microglia is responsible for exaggerated inflammatory responses in aged animals after treatment with lipopolysaccharide, because the increased intracellular ROS level activates nuclear factor-κB (NF-κB) which regulates the expression of several proinflammatory cytokines [10]. Hypoxia can drive microglia to generate ROS [11–14]. Therefore, it is reasonable to consider that hypoxia activates NF-κB to induce exaggerated inflammatory response by microglia through enhanced production of ROS due to the mitochondrial DNA damage.

Propolis is a resinous substance produced by honeybees as a defense against intruders. It has relevant therapeutic properties that have been used since ancient times. The chemical composition of propolis depends on the local floral at the site of collection [15, 16]. Considering the fact that

propolis has hepatoprotective, antitumor, antioxidative, and anti-inflammatory effects [17–20], propolis may have protective effects against the hypoxia-induced neuroinflammatory responses. In this study, we provide the first evidence that propolis can significantly inhibits the secretion of IL-1β, TNF-α, and interleukin-6 (IL-6) by microglia through inhibition of the NF-κB activation in microglia. Furthermore, propolis significantly inhibit the increased generation of ROS from the mitochondria that is responsible for the NF-κB activation. These observations suggest that propolis may be useful to prevent hypoxia-induced neuroinflammation.

2. Material and Methods

2.1. Reagents. The Brazilian green propolis ethanol extract (propolis) was purchased from Yamada Apiculture Center, Inc Ltd. (Okayama, Japan). The suitable concentration of ethanol for cell culture was titrated in order to prevent the interference induced by the ethanol solvent. Goat anti-mature IL-1β (mIL-1β), goat anti-TNF-α, goat anti-IL-6, and mouse anti-phospho-IκBα, rabbit anti-IκBα, mouse anti-phospho-p65 were purchased from Santa Cruz Biotechnology (Delaware Avenue Santa Cruz, CA). Rabbit polyclonal anti-Iba1 antibody was purchased from Wako Pure Chemicals (Sapporo, Japan), and mouse monoclonal anti-8-oxo-deoxyguanosine (8-oxo-dG) was purchased from NOF Corporation (Kyoto, Japan).

2.2. Microglial Cell Culture. The c-myc-immortalized mouse microglial cell line, MG6 (RIKEN Cell Bank, Tsukuba, Japan), was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (ICN Biomedicals, Inc.) supplemented with 100 μM of β-mercaptoethanol, 10 μg/mL of insulin, 100 μg/mL of streptomycin, and 100 U/mL of penicillin (BD Falcon, Franklin Lakes, NJ) [21, 22].

2.3. Assay for Cell Survival. Relative cell viability was measured using CellQuanti-MTT Cell Viability Assay Kits (BioAssay Systems, Hayward, USA). The assay was performed according to the protocol provided by manufacturer. The absorbency at 570 nm was performed by using a microplate reader.

2.4. Hypoxic Exposure. For *in vitro* studies, MG6 microglia (cell density of 2×10^4 cells/mL) were plated overnight and then cultivated under the normoxia (20% O_2, 5% CO_2) or hypoxia (1% O_2, 5% CO_2, and 92% N_2) at 37°C for the indicated periods using a chamber (Model: MCO 18M; Sanyo Biomedical Electrical Co., Ltd., Tokyo, Japan). For *in vivo* studies, eighteen C57B/6N mice (4-week old) with or without treatment of propolis (9 mice each, 8.33 mg/kg, 2 times/day, i.p.) for 7 days were exposed to hypoxia by placing in a chamber (Model: MCO 18 M) filled with a gas mixture of 10% oxygen and 95% nitrogen for 4 h. The mice were then allowed to recover under normoxic conditions for 24 h before killing. Another group of eighteen mice kept outside the chamber were used as matched controls. This study was approved by the Institutional Animal Care and Use Committee of Kyushu University.

2.5. Tissue Preparation. Mice were exposed to normoxia or hypoxia with pretreatment of propoplis (8.33 mg/kg, 2 times/day). Mice were exposed to hypoxia with pretreatment of 0.01 M phosphate-buffered saline (PBS, pH 7.4, 2 times/day) as control. The mice were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and then were perfused intracardially with PBS (pH 7.4) and periodate lysine paraformaldehyde (PLP) fixative containing 0.01 M sodium metaperiodate, 0.075 M L-lysine-HCl, 2% paraformaldehyde, and 0.03% phosphate buffer (pH 6.2). The brains were removed and immersed in the same fixative for 6 h at 4°C. The specimens were cryoprotected for 2 days in 30% sucrose in PBS and then were embedded in an optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Serial coronal frozen sections (14 μm) of the somatosensory cortex for staining with immunohistochemistry and double-immunofluorescent staining were prepared as previously reported [23, 24].

2.6. Detection of Mitochondrial ROS. Mitochondrial ROS was measured using MitoSOX Red (Invitrogen, USA), which is a live-cell permeant and is rapidly and selectively targeted to mitochondria [25]. Once in the mitochondria, MitoSOX Red reagent is oxidized by superoxide and exhibits red fluorescence (with excitation at 510 nm and emission at 580 nm). The cultured MG6 microglia (cell density of 1×10^5 cells/mL) were exposed to nomoxia or hypoxia in the presence or absence of propolis (50 μg/mL). The cells were collected at 24 h after treatment and then incubated in Hank's balanced salt solution (HBSS) containing 5 mM MitoSOX Red for 10 min at 37°C. After incubation, the cells were washed with PBS twice the cells were then mounted in a warm buffer for imaging. Images were collected using a ×20 objective lens (NA = 0.50, 200x magnification, yielding a frame of 0.575 mm^2). The procedure resulted in arbitrary optical density values on a scale of 0 (background staining) to 255.

2.7. Immunofluorescence Imaging. MG6 microglia exposed to normoxia or hypoxia for 60 min using a chamber (Model: MCO 18 M) in the presence or absence of propolis (50 μg/mL) were fixed with 4% paraformaldehyde and then incubated with the mouse anti-NF-κB p65 (1 : 500, F-6) overnight at 4°C. After incubated with anti-mouse Alexa 488 (1 : 2000, Jackson Immunoresearch Lab. Inc.) at 4°C for 2 h, they were then incubated with Hoechst (1 : 2000, Sigma-Aldrich, Japan) and mounted in the antifading medium Vectashield. Fluorescence images were taken using a confocal laser scanning microscope (CLSM; C2si, Nikon, Japan).

The sections were hydrated and treated with 10% donkey serum for 1 h at 25°C and then were incubated with each primary antibody overnight at 4°C. The primary antibodies were goat polyclonal anti-IL-1β antibody (1 : 500), goat polyclonal anti-TNF-α (1 : 500), goat polyclonal anti-IL-6 (1 : 500), and mouse monoclonal anti-8-oxo-dG (1 : 500) antibodies mixed with rabbit polyclonal anti-Iba1 antibody (1 : 5000). The sections were washed with PBS and incubated with a mixture of FITC-conjugated and rhodamine-conjugated secondary antibodies for 2 h at 25°C. The sections were washed, mounted in the antifading medium Vectashield

(Vector Laboratory), and then were examined by a confocal laser scanning microscope (CLSM, C2si, Nikon, Japan). CLSM images of individual sections were taken as a stack at 1 μm step size along the z-direction with a 20x objective (Numerical Aperture = 0.5), zoom factor 1.0. A rectangle (1024 × 1024 pixels) corresponding to the size of 450 × 450 μm was used as the counting frame. CLSM images were shown as the middle of the stacked images.

2.8. Enzyme-Linked Immunosorbant Assay (ELISA). The cultured MG6 microglia (density of 5×10^5 cells/mL) with propolis (50 μg/mL) were subjected to hypoxia in a chamber filled with a mixture of gases containing 1% O_2, 5% CO_2, and 92% nitrogen at 37°C. MG6 microglia were incubated in 21% O_2, and 5% CO_2 at as 37°C as the normoxic control. The condition medium was collected at 6, 12, 24, and 48 hours after the above incubation, and the amounts of IL-1β, TNF-α, and IL-6 released from microglia were measured using the enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) following the protocol provided by the manufacturer. The absorbency at 450 nm was measured using a microplate reader.

2.9. Electrophoresis and Immunoblotting. MG6 microglia were cultured at a density of 5×10^5 cells/mL, and the cytosolic samples were collected at the time points 15, 30, and 60 min after hypoxic exposed hypoxia (1% O_2). The samples were electrophoresed in 15% or 12% SDS-polyacrylamide gels, and the proteins on SDS gels were transferred electrophoretically to nitrocellulose membranes. Following blocking, the membranes were incubated at 4°C overnight under gentle agitation with each primary antibody: mouse anti-phosphorylated IκBα (1 : 1000) and rabbit anti-IκBα (1 : 1000) antibodies overnight at 4°C. After washing, the membranes were incubated with horseradish-peroxidase- (HRP-) labeled anti-mouse (1 : 2000, Beckman Coulter) and anti-rabbit (1 : 2000, Beckman Coulter) antibodies for 2 hours at 24°C and then detected using an enhanced chemiluminescence detection system (ECK kit, Amersham Pharmacia Biotech) with an image analyzer (LAS-4010, GE health care, Uppsala, Sweden).

2.10. Statistical Analysis. The data are represented as the means ± SEM. The statistical analyses were performed using a one-way or two-way analysis of variance (ANOVA) with a post hoc Tukey's test using the GraphPad Prism software package. A value of $P < 0.05$ was considered to indicate statistical significance (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effects of Propolis on the Hypoxia-Induced Reduction of Microglia Viability and Hypoxia-Induced Mitochondria-Derived ROS by Microglia. We first investigated the effects of propolis on the cell viability of MG6 microglia using MTT assay. The mean cell viability was not significantly changed after treatment with propolis with the final concentrations of 5 or 50 μg/mL (Figure 1(a)). On the other hand, the mean cell viability was significantly reduced after treatment with propolis with the final concentration of 500 mg/mL. Therefore, we used propolis with the concentration of 50 μg/mL to examine its effects on hypoxia-induced reduction of microglial cell viability. As shown in Figure 1(b), hypoxic exposure (1% O_2, 24 h) significantly reduced the mean cell viability of MG6 microglia. Treatment with propolis (50 μg/mL) significantly restored the hypoxia-induced reduction of microglial cell viability (Figure 1(b)).

Hypoxia drives microglia to generate ROS. In our previous study, the mitochondria in microglial were found to be most susceptible to oxidative damage [10, 26, 27]. These facts prompted us to examine hypoxia-induced mitochondrial oxidant generation in microglia using oxidation of the MitoSOX Red probe, a mitochondrially targeted hydroethidine derivative [25]. The mean immunofluorescence intensity of MitoSOX Red oxidation was significantly increased in MG6 microglia at 24 h after hypoxia (Figures 1(c) and 1(d)). Propolis (50 μg/mL) significantly inhibited the hypoxia-induced increase in the mean fluorescent intensity of MitoSOX Red probe in microglia (Figures 1(c) and 1(d)). These results demonstrate that propolis inhibits the hypoxia-induced ROS generation from mitochondria in microglia.

3.2. Effects of Propolis on the Hypoxia-Induced Secretion of Proinflammatory Cytokines by Cultured Microglia. Next, the effects of propolis on the hypoxia-induced secretion of proinflammatory cytokines by microglia were examined. IL-1β, TNF-α, and IL-6 secreted by MG6 microglia into the culture medium were measure by ELISA after hypoxic exposure (1% O_2, 24 h). The mean concentrations of IL-1β, TNF-α, and IL-6 in the culture medium of microglia by microglial cells were significantly increased at 24 h after exposure to hypoxia (Figure 2). Propolis (50 μg/mL) significantly inhibited the hypoxia-induced secretion of IL-1β, TNF-α, and IL-6 by microglia (Figure 2).

3.3. Effects of Propolis on Hypoxia-Induced Activation of NF-κB by Microglia. The effects of propolis on the NF-κB activation after exposure to hypoxia were next examined, because NF-κB regulates the expression of several proinflammatory cytokines, including IL-1β, TNF-α, and IL-6. The expression of IκBα phosphorylation in MG6 microglia was significantly increased after hypoxia (Figures 3(a) and 3(b)). Propolis (50 μg/mL) significantly inhibited the hypoxia-induced phosphorylation of IκBα in microglia (Figures 3(a) and 3(b)). Furthermore, the nuclear translocation of p65 was induced in MG6 microglia at 60 min after hypoxia (Figure 3(c)). Propolis (50 μg/mL) markedly inhibited the hypoxia-induced nuclear translocation of p65 in microglia (Figure 3(c)). These results demonstrate that propolis suppresses the hypoxia-induced neuroinflammatory responses by inhibiting NF-κB activation in microglia.

3.4. Effects of Propolis on the Neuroinflammatory Responses in the Somatosensory Cortex of Mice Exposed to Hypoxia. Finally, the effects of propolis on the cortical microglia in mice exposed to hypoxia were exposed. Under the normoxic

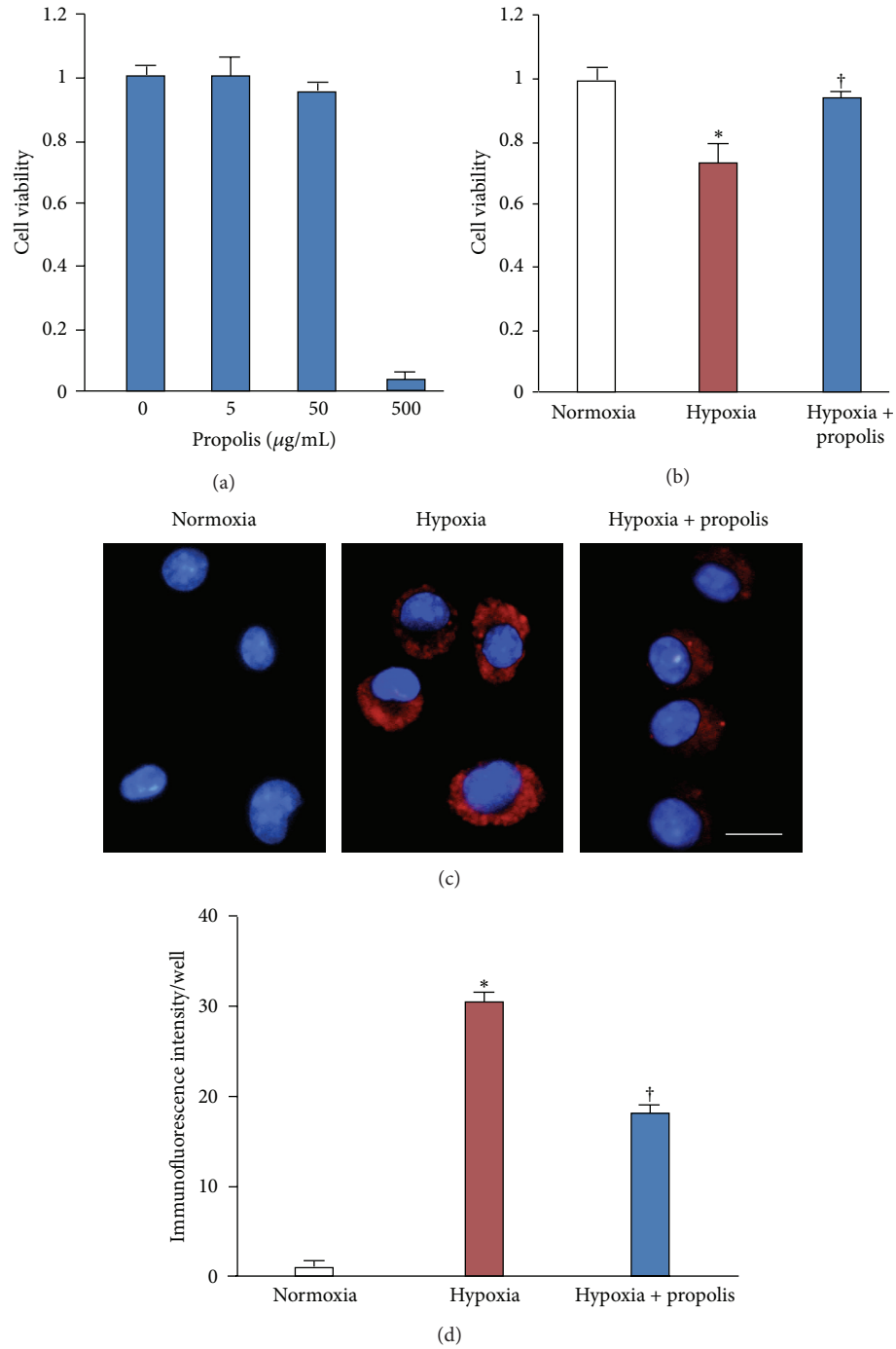

Figure 1: Effect of propolis on hypoxia-induced mitochondria ROS associated cytotoxicity in cultured microglia. (a) Cell viability in MG6 microglia in the presence of propolis with different three concentrations. (b) Cell viability of MG6 microglia exposed to normoxia (20% O_2) or hypoxia (1% O_2) in the presence or absence of propolis (50 μg/mL) for 24 h. Each column and bar represent mean ± SEM ($n = 4$ each). An asterisk indicates a statistically significant difference from the value in Normoxia ($^*P < 0.05$). A sword indicates a statistically significant difference from the value in hypoxia ($^\dagger P < 0.05$). (c) Fluorescent mages of MitoSOX Red fluorescence signals in MG6 microglia exposed to normoxia (20% O_2) or hypoxia (1% O_2) in the presence or absence of propolis (50 μg/mL) for 24 h. Scale bar = 10 μm. (d) The quantitative analyses of MitoSOX Red fluorescence signal intensity in (c). Each column and bar represent the mean ± SEM ($n = 4$ each). An asterisk indicates a statistically significant difference from the value in normoxia ($^*P < 0.05$). A sword indicates a statistically significant difference from the value in hypoxia ($^\dagger P < 0.05$).

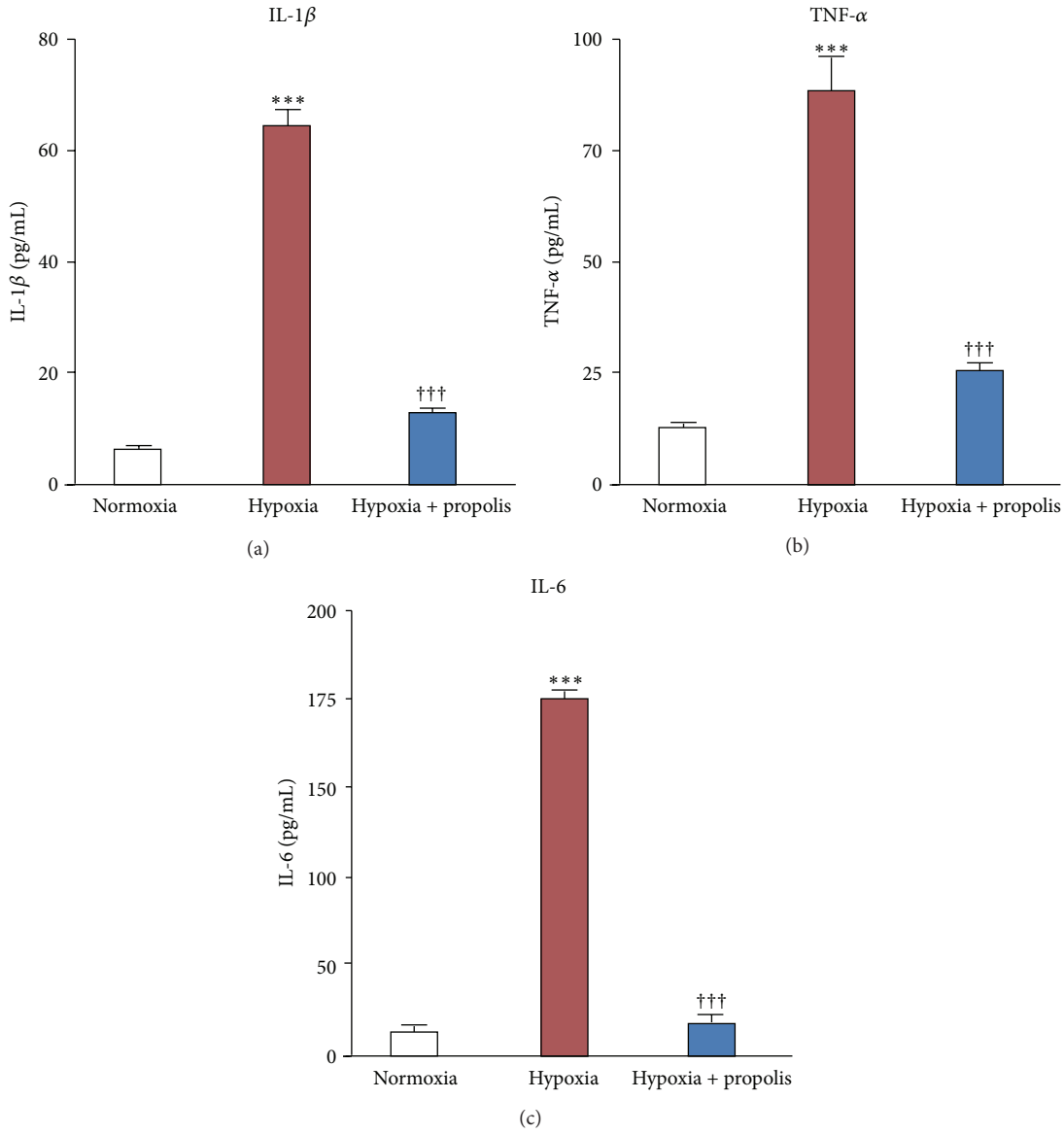

Figure 2: Inhibitory effects of propolis on the hypoxia-induced proinflammatory cytokine secretion by cultured microglia. The mean concentration of IL-1β, TNF-α, and IL-6 in the culture medium of MG6 microglia exposed to normoxia (20% O_2) or hypoxia (1% O_2) in the presence or absence of propolis (50 μg/mL) for 24 h was measured by ELISA. Each column and bar represent the mean ± SEM ($n = 4$ each). Asterisks indicate a statistically significant difference from the value in normoxia ($^{***}P < 0.001$). Swords indicate a statistically significant difference from the value in hypoxia ($^{\dagger\dagger\dagger}P < 0.001$).

condition, the cortical microglia exhibited ramified morphology (Figures 4(a)–4(d)). In contrast, the cortical microglia showed hyperactivated morphology, which was characterized by enlarged cell bodies with short processes at 4 h after hypoxia which recover under normoxic conditions for 24 h (Figures 4(e)–4(h)). Furthermore, the mean cell numbers of cortical microglia positive for immunoreactivities of IL-1β, TNF-α, IL-6, and 8-oxo-dG were significantly increased in mice at 4 h after hypoxia (Figures 4(e)–4(h), and 4(m)–4(p)). When mice were chronically treated with propolis (8.33 mg/kg, 2 times/day, i.p.) for 7 days before exposure to hypoxia, the morphology of the cortical microglia was characterized by small cell bodies with long processes, similar to the ramified microglia under the normoxic condition (Figures 4(i)–4(l)). At the same time, the mean cell numbers of cortical microglia positive for the immunoreactivities of IL-1β, TNF-α, IL-6, and 8-oxo-dG were significantly reduced (Figures 4(i)–4(l), and 4(m)–4(p)). However, the pretreatment of PBS (controls) did not show inhibition of the activated morphology and the immunoreactivities of IL-1β, TNF-α, IL-6, and 8-oxo-dG in microglia of hypoxia exposed mice (data not shown).

4. Discussion

The major finding of this study is that propolis significantly inhibits the hypoxia-induced activation of NF-κB-dependent neuroinflammatory pathway in microglia. NF-κB is a transcription factor that encodes genes of the proinflammatory cytokines, including IL-1β, TNF-α and IL-6 [28]. It is also

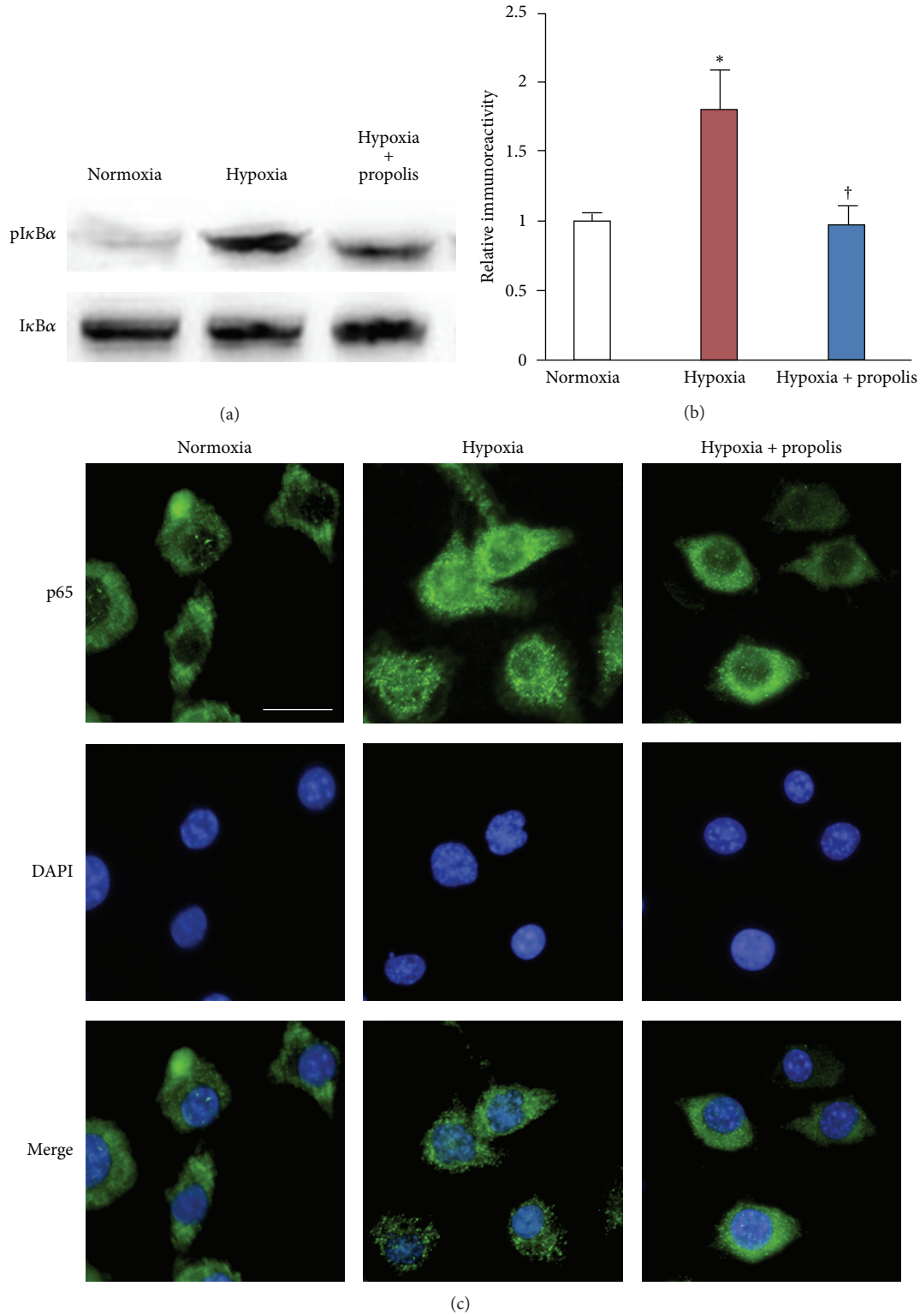

Figure 3: Inhibitory effects of propolis on the hypoxia-induced NF-κB activation in cultured microglia. (a) Phosphorylation of IκBα in MG6 microglia exposed to normoxia (20% O_2) or hypoxia (1% O_2) in the presence or absence of propolis (50 μg/mL) for 24 h. (b) The quantitative analyses of immunoblots in (a). Each column and bar represent the mean ± SEM ($n = 4$ each). An asterisk indicates a statistically significant difference from the value in Normoxia ($^*P < 0.05$). A sword indicates a statistically significant difference from the value in hypoxia ($^\dagger P < 0.05$). (c) Immunofluorescent CLMS images of p65 (green) with Hoechst-stained nuclei (blue) in MG6 microglia exposed to normoxia (20% O_2) or hypoxia (1% O_2) in the presence or absence of propolis (50 μg/mL) for 24 h.

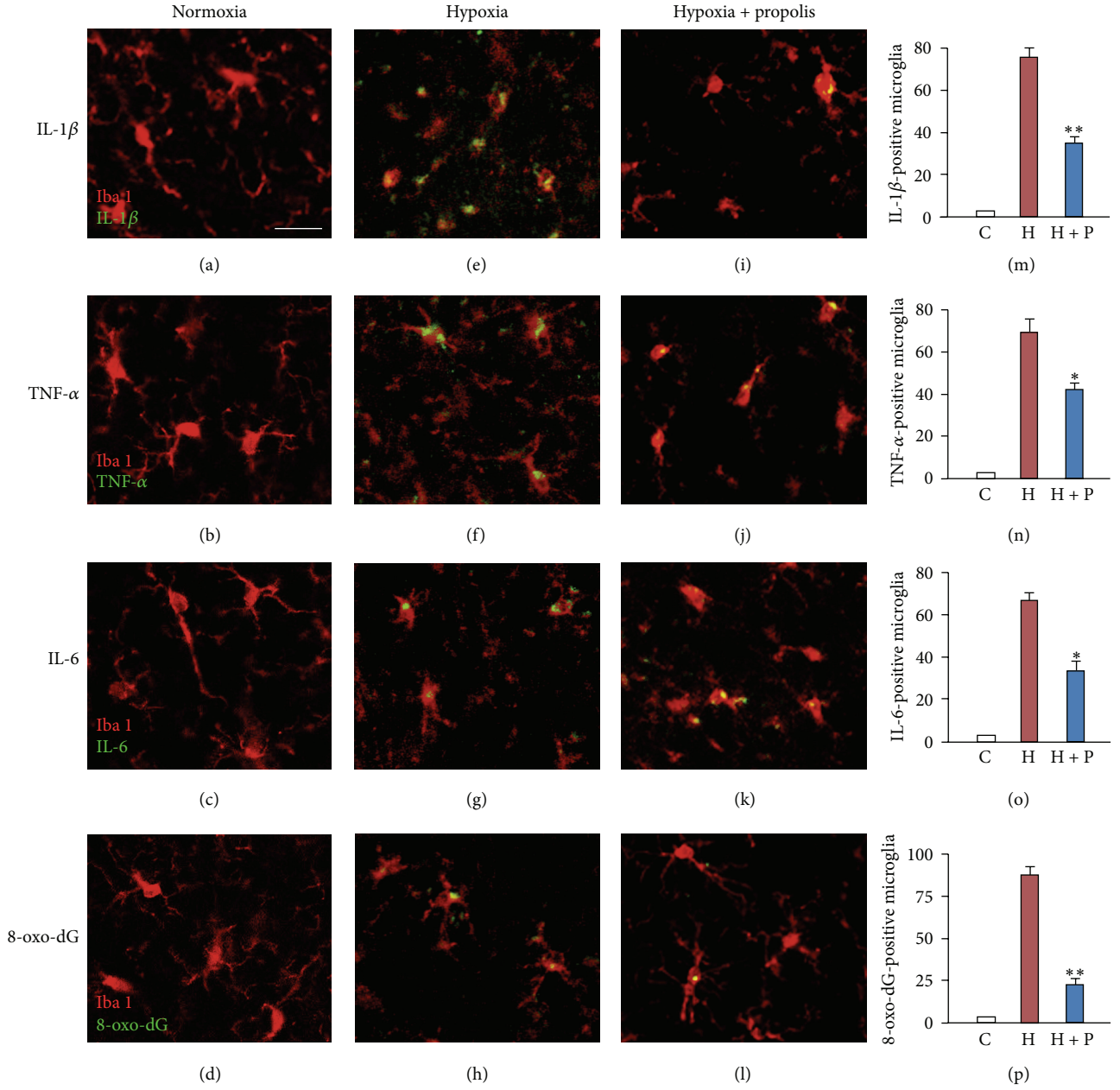

FIGURE 4: Inhibitory effects of propolis on the expression of IL-1β, TNF-α, IL-6, and 8-oxo-dG in the cortical microglia of mice exposed to hypoxia. Immunofluorescent CLMS images of IL-1β (a, e, i), TNF-α (b, f, j), IL-6 (c, g, k), and 8-oxo-dG (d, h, l) in the Iba1-positve cortical microglia of mice exposed to normoxia (20% O_2) or hypoxia (10% O_2) for 4 h with or without pretreatment of propolis (8.33 mg/kg, 2 times/day). (m–p) The mean cell number of IL-1β-positive (m), TNF-α-positive (n), IL-6-positive (o) and 8-oxo-dG-positive (p) Iba1-positive microglia in the somatosensory cortex per 0.15 mm^2. Each column and bar represent the mean ± SEM ($n = 3$ each). Asterisks indicate a statistically significant difference from the value in normoxia ($^*P < 0.05$, $^{**}P < 0.01$). Scale bar = 20 μm.

known that NF-κB activation is facilitated by conditions associated with an increased intracellular redox state [29]. In the present study, the mean fluorescent intensity of MitoSOX Red probe, a marker for mitochondria-derived ROS generation, was found to be significantly increased in microglia following hypoxia. Furthermore, the increased expression of 8-oxo-dG, a biomarker for oxidative damaged DNA [30], was observed mainly in the cytosol of microglia after hypoxia, suggesting the mitochondrial origin of damaged DNA. We have previously found that ROS damages the mitochondrial DNA and the damaged mitochondria DNA, in turn, impairs the respiratory chain, forming a vicious cycle to promote the ROS generation [10]. Taken together, the mitochondrial DNA damage after hypoxia is considered to be a major causative factor for the increased ROS production, which activates the NF-κB-dependent neuroinflammatory pathway. Therefore, propolis may protect the mitochondrial DNA against hypoxia-induced oxidative stress dependently on its antioxidant properties [18, 19, 31].

Stroke is the most common form of hypoxia-ischemic brain injury and remains a major challenge to public health due to its high incidence and life-threatening nature [32]. In

the Western world, over 70% of individuals experiencing a stroke are over 65 years of age. Since life expectancy continues to grow, the absolute number of individuals with stroke will further increase in the future [33]. Oxidative stress and neuroninflammation are known as the two important pathophysiological mechanisms involved during hypoxia-ischemic brain injury [33], because mice lacking the p50 subunit of NF-κB develop significantly smaller infarcts after transient focal ischemia [34] and antioxidants reduce infarct volume and improve behavior deficits [35]. More evidence further explores the critical role of microglia in stroke progression, because the experimental and postmortem studies reveal the presence of activated microglia in the brain of stroke patients [36, 37] and microglia are clarified as the major cell population that leads to NF-κB-dependent upregulation of proinflammatory molecules, such as TNF-α and IL-1β during stroke. Therefore, propolis efficiently attenuates the hypoxia-induced activation of NF-κB-dependent neuroinflammatory pathway in microglia and may be beneficial in the prevention and treatment of stroke, because blockade of microglia activation prevents hypoxia-ischemic brain injury [38].

Recently, much attention has been paid to the association of hypoxia with cognitive deficits. It is well known that hypoxia suffering mountaineers have demonstrably poorer memory and concentration, and the effect of hypoxia is sustained for significant periods of time after returning from altitude [39, 40]. In addition, we have previously found that healthy individuals who are living in high altitude in excursions are with cognitive defects [41]. A similar decline in memory arising from brief hypoxic exposure has been reported in experimental animals [42]. Brain oxygen levels are largely dependent on cerebral blood flow [43, 44], which declines with aging [44]. Furthermore, the cerebral blood flow was 20% lower in AD than in the age-matched nondemented control group [45]. Older adults with low cerebral oxygen levels showed more cognitive dysfunction than those with normal levels [46]. Therefore, the chronic hypoxia may contribute to the cognitive decline in aging and aging-related neurodegenerative diseases, such as AD [47, 48]. Recently, we have reported that the exaggerated neuroinflammatory responses evoked by microglia are associated with an impairment of the hippocampal long-term potentiation (LTP), a cellular basis for memory and learning, because minocycline, a known inhibitor of microglial activation, significantly improved systemic inflammation-induced impairment of LTP in the middle-aged animals [49]. Therefore, it is reasonable to consider that propolis efficiently attenuates hypoxia-induced NF-κB-dependent neuroinflammatory pathway in microglia and may be beneficial in preventing of neurodegenerative diseases-related cognitive deficits.

People living in high altitudes are daily exposed to hypoxia. Our preliminary human experiments in the high altitudes show that the mean level of proinflammatory mediators in the blood of a propolis-treated elderly group is significantly lower than that of a nontreated group. Furthermore, the propolis-treated elderly group shows significantly higher scores of cognitive tests than the nontreated elderly group (unpublished data), which suggests that propolis may also helpful to prevent the aging-related cognitive deficits.

5. Conclusion

The present study provides the first evidence of potential protective effects of propolis on the hypoxia-induced neuroinflammatory responses. The protective effects may involve a reduction in oxidative stress and NF-κB-dependent pathway in microglia. Thus making it beneficial in the prevention and treatment of hypoxia/ischemia-induced cognitive deficits. Our ongoing investigation is to clarify the synergistic and additive effects of individual propolis components in anti-neuroinflammation.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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Research Article

Grape Polyphenols Increase the Activity of HDL Enzymes in Old and Obese Rats

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HDL particles are protein-rich particles that act as a vehicle for reverse cholesterol transport from tissues to the liver. The purpose of this study was to investigate age-dependent changes in the functional activity of HDL and the effect of high-energy diet on this index, as well as to correct it under the influence of grape polyphenols from "Enoant" obtained from *Vitis vinifera* grapes. We observed the age-dependent composition changes in HDL particle. It was shown that total lipids and triacylglycerol (TG) levels were higher in 24-month-old animals. In obese rats, HDL total lipids and TG levels were higher in 24-month-old than in the 3-month-old and 12-month-old groups but did not differ from 24-month-old group. The plasma HDL paraoxonase (PON) and lecithin:cholesterol acyltransferase (LCAT) activity levels were decreased in old-aged rats, and cholesteryl ester transfer protein (CETP) activity was higher in old rats. Keeping 12-month-old animals on high-fructose diet completely leveled the age differences in the data that have been measured between 12-month-old and 24-month-old rats. After "Enoant" administration, an increase of HDL PON and LCAT activity levels and a reduction of CETP activity were found in 24-month-old and obese rats.

1. Introduction

Coronary vascular diseases (CVDs) are the leading cause of morbidity and mortality in adults [1]. Major risk factors for CVD in addition to a sedentary lifestyle and a diet high in saturated fat and cholesterol include family history, age, sex, elevated low-density-lipoprotein cholesterol (LDL-C), decreased high-density-lipoprotein cholesterol (HDL-C), hypertension, cigarette smoking, and diabetes mellitus [2–4].

HDLs are synthesized in the liver and intestine and are responsible for transporting 20–30% of the total plasma cholesterol. Apolipoprotein A-I is the most abundant protein in HDL. The remaining protein mass is made up of minor amphipathic proteins, such as apoC, apoE, apoD, apoM and apoA-IV, enzymes, such as paraoxonase (PON) 1 and platelet-activating factor acetylhydrolase, and glutathionehlt peroxidase, and lipid transfer proteins, such as lecithin : cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) [5].

CETP is physically associated with HDL particles and facilitates the transport of cholesteryl ester from HDL to apoB-containing lipoproteins. A decrease of CETP activity may increase HDL-C and decrease VLDL-C and LDL-C. Thus, CETP simultaneously affects the composition and concentration of apoA−/− and apoB-containing lipoproteins. Clinical studies demonstrate a low prevalence of coronary heart disease among subjects with CETP deficiency [6]. Although CETP deficiency might prevent atherogenesis by increasing HDL-cholesterol levels, their effect has been controversial. Studies performed in Japan indicate that CETP deficiency might increase cardiovascular disease risks [7]. Davidson et al. reported on a CETP vaccine that induces autoantibodies that specifically bind and inhibit endogenous CETP, with the intention of increasing HDL and reducing the development of atherosclerosis [8]. LCAT is a key enzyme necessary for extracellular cholesterol metabolism [9]. LCAT may facilitate the uptake of cholesterol from peripheral tissues into HDL particles by maintaining a concentration gradient for the efflux of free cholesterol [10]. If LCAT is impaired, mature HDL generation would presumably be decreased, resulting in augmentation of atherosclerosis [11].

There are data suggesting that the direct antioxidant effect of HDL on LDL oxidation, measured as a reduction in lipid

peroxides, is likely mediated by PON1 [12]. High serum cholesterol and insulin resistance are, for example, associated with decreased PON1 activity [12]. Aging is associated with alteration PON1 activities as a consequence of a higher content of PON1 molecules in the blood, which probably corresponds to the need to respond to the negative effects of senescence. PON1 decreases in older people and with the beginning of menopause [13].

Serum PON1 activity and concentration have also been shown to be modulated by lifestyle and dietary factors [14]. Polyphenols (present in wine, tea, and fruit juice) also increase PON1 activity in both humans and mice [15, 16].

In earlier, studies it was shown that consumption of red wine or its polyphenols quercetin or catechin by apolipoprotein E-deficient mice (whose plasma PON1 activity is lower than controls), was associated with an increase in serum PON activity [17]. Administration of a mixture of red wine polyphenols increased hepatic PON1 activity in mice, while a higher dosage levels had an opposite effect [18]. The low dose of polyphenols was also capable of reversing the decrease of plasma and hepatic PON1 activities and of liver mRNA levels present in mice with hyperhomocysteinemia.

One of the richest sources of polyphenols is *Vitis vinifera,* and products of its processing, including flavonoids and other polyphenols of grape, wine, and grape seeds, are of a great interest due to their antioxidant properties and the ability to scavenge free radicals [19].

Studies in vitro have shown that grape products inhibit the oxidation of LDL. The activity of those substances as oxidation inhibitors in wine diluted 1,000 times markedly exceeded the analogous values for vitamins C and E [20]. In our research, we have used polyphenolic concentrate "Enoant" obtained from *Vitis vinifera* grapes and manufactured by Ressfood Company (Yalta, Ukraine).

The purpose of this study was to investigate age-dependent changes in the functional activity of HDL and the effect of high-energy diet on this index, as well as a way to correct it under the influence of grape polyphenols from "Enoant."

2. Materials and Methods

2.1. Animals. 3-, 12-, and 24-month-old Wistar male rats which had a free access to food and water and were kept at 24°C on a cycle of 12 h light/12 h darkness were used for experiments. Polyphenol concentrate was given os daily during 21 day (at a dose of 9 mg in recalculation on polyphenols/100 g body weight) [21]. Control animals were given the corresponding volume of physiological solution.

Rats from "Obese" group, 12-month-old rat, were kept on a diet that consisted of 60% fructose. Both diets contained equal percentage of carbohydrates (70%), proteins (20%), and lipids (10%) [22].

The animals were decapitated under chloralose-urethane anesthesia. All procedures were approved by National University of Pharmacy Institutional Animal Care and Use Committee and are in accordance with PHS guidelines.

2.2. Isolation of HDL. HDL (d 1.085–1.190 g/mL) was isolated from the rat blood plasma by sequential ultracentrifugation and was washed at its lower density limit. Lipoproteins were extensively dialyzed against 10 mM Tris-HC1 buffer (pH 7.4), containing 150 mM NaCl, 0.01% (w/v) sodium azide, and 0.25 mM EDTA (Tris-NaC1 buffer). To distribute the plasma lipoproteins, samples were centrifuged at 65,000 rpm (342,000 g) for 4 h at 4°C in an Optima XL-100 K ultracentrifuge (Beckman Coulter) set at slow acceleration and deceleration. Samples were fractionated within 1 h of centrifugation.

2.3. Determination of Lipid Levels. Lipids were extracted with chloroform and methanol (1 : 2 v/v) 2 times as described [23], and the supernatant were collected for the determination of triglycerides (TG) and cholesterol. TG and cholesterol were determined by enzymatic colorimetric methods with commercial kits (Zhongsheng, Beijing, China). Total cholesterol content was detected with the help of standard enzymatic cholesterol oxidase kits (Biocon Diagnostik GmbH, Germany). Total lipid concentration was determined with the help of standard kit (Eagle Diagnostics, USA) reaction with the vanillin reagent.

2.4. Determination of Lipid Peroxide Product Quantity. Lipids from various samples were extracted by chloroform : methanol (2 : 1, v/v) [23]. Lipids in the chloroform phase were recovered by evaporating the chloroform under oxygen-free N_2 (ultrapure carrier, Grade 99.99% N_2). The final total lipid extract was dissolved in cyclohexane (1 mg/mL) and then scanned against a cyclohexane blank in a Beckman Model 25 spectrophotometer. Optical density was measured at wavelength of 220 nm (for compounds with isolated double bonds), 232 nm (for diene conjugates), and 278 nm—for ketodienes and conjugated trienes.

2.5. Measuring of α-Tocopherol. A modified version of the high-performance liquid chromatography (HPLC) procedure [24] was used to measure vitamins E. The HPLC system included a 150 × 3.9 mm Nova-Pak C18 (4 microns) column with a Guard-Pak precolumn (both from Waters, Milford, MA, USA), Waters Millipore TCM column heater, Waters 490 multiwavelength detector, Hitachi 655-61 processor, Hitachi 655A-11 liquid chromatography, and Bio-Rad autosampler AS-100.

2.6. Assay of PON1. PON1 activity was assayed using synthetic paraoxon (diethyl-p-nitrophenyl phosphate) as substrate. PON1 activity was determined by measuring the initial rate of substrate hydrolysis to p-nitrophenol, which absorbance was monitored at 412 nm in the assay mixture containing 2.0 mM paraoxon, 2 mM $CaCl_2$, and 20 μL of plasma in 100 mM Tris-HCI buffer (pH 8.0). The blank sample containing incubation mixture without plasma was run simultaneously to correct for spontaneous substrate breakdown. The enzyme activity was calculated from E412 of p-nitrophenol (18.290 perM/cm) and was expressed as U/mL [25].

2.7. CETP Activity. CETP activity in duplicate 10 μL aliquots of plasma was determined after incubations with ^{3}H-cholesterol ester-labeled HDL_3 and LDL. Radioactivity transferred from ^{3}H-HDL_3 to LDL (measured in the supernatant after precipitation with heparin/$MnCl^{2+}$) was used to calculate CETP activity (expressed as the percentage of radioactivity transferred from ^{3}H-HDL_3 to LDL per 16 h of incubation) [26].

2.8. LCAT Activity. LCAT activity measured by determination of the amount of radioactivity in each spot permits the calculation of the free cholesterol/total cholesterol ratio in each plasma sample before and after the LCAT reaction and thus the estimation of the esterification rate [27]. The fractional esterification rate (%, h′) expressed the percentage of free cholesterol esterified in the plasma sample per hour.

2.9. Statistical Analysis. The differences between the results were evaluated using analysis of variance (ANOVA) and Student's t-test. In all of the analyses, a difference of $P < 0.05$ was considered statistically significant. The data are expressed as the means ± standard deviations.

3. Results

Table 1 shows the plasma lipid and apolipoprotein concentrations of the experimental animals. Total cholesterol level was higher in 24-month-old than in the 3-month-old and 12-month-old groups. The cholesterol level was greater in the obese rats than in the middle-aged and young animals ($P < 0.05$) but did not differ between the 3-month-old and 12-month-old groups. Triglycerides, apo A1, and apo B levels were not different among these three groups. HDL level was higher in 24-month-old than in the 3-month-old and 12-month-old groups. LDL level was higher in the obese rats ($P < 0.05$).

In our experiment, we also observed that the age-dependent composition changes in HDL particle (Table 2). It was shown that total lipids and TG levels were higher in 24-month-old than in the 3-month-old and 12-month-old groups. In obese rats, HDL total lipids and TG levels were higher in 24-month-old than in the 3-month-old and 12-month-old groups but did not differ between 24-month-old group. Total HDL cholesterol level was significantly lower in 24-month-old and obese animals than in the 3-month-old and 12-month-old groups. There was no difference between old-aged and obese rats. It was shown that diene conjugates, ketodienes + conjugated, and total hydroperoxides levels were higher in 24-month-old than in the 3-month-old and 12-month-old groups. Isolated double bonds level was significantly lower in old-aged and obese rats.

Table 3 shows the plasma HDL PON, LCAT, and CETP activity in experimental rats of different age and obese rats. The plasma HDL PON and LCAT activity levels were decreased in old-aged rats, and CETP activity level was higher in old rats.

Figure 1 shows body weight of different ages and obesity groups of experimental animals. These data suggest that keeping rats on a fructose-rich diet resulted in a significant increase in body weight. "Enoant" administration decreased this index, which, however, did not reach the control values.

"Enoant" administration decreased HDL diene conjugates, ketodienes + conjugated, and total hydroperoxides levels in 24-month-old and obese rats groups and increased HDL total lipids and TG levels (Table 4). It was shown that α-tocopherol level was significantly higher in HLD under "Enoant" administration.

The effect of "Enoant" on the plasma HDL PON, LCAT, and CETP activity levels in experimental is shown in Table 4. After "Enoant" administration, it was found an increase of HDL PON and LCAT activity levels and a reduction of CETP activity in 24-month-old and obese rats.

4. Discussion

We have carried out a series of experiments to study the age-dependent changes in the functional activity of HDL, the effect of high-energy diet on this index.

We found that in older animals, there was a significant increase in both HDL and LDL (Table 1). The increase of lipid peroxidation and the reduction of endogenous antioxidant α-tocopherol level and the increase of TG levels and key enzymes of HDL activity (Tables 2 and 3) could indicate that the functional activity of HDL in old animals is lower than that in younger ones.

Data from the Prospective Cardiovascular Münster (PROCAM) Study have shown that having a low HDL cholesterol level was the predominant characteristic of subjects older than 60 years with a history of myocardial infarction compared to subjects older than 60 years without previous cardiovascular events [28].

The HDL fraction is composed of heterogeneous particles with sizes ranging from 7 to 14 nm [2, 12]. In addition to its roles in reverse cholesterol transport and cholesterol esterification, HDL has antioxidant activity that is mostly due to its association with PON1 and anti-inflammatory, antithrombotic, and vasodilation activities that may account for the antiatherogenic action of the lipoprotein [12, 13].

Currently, in the literature, there are contradictory data on the content and activity of PON age-dependent changes [28, 29]. While a major determinant of PON1 activity is represented by genetic polymorphisms, additional factors, not discussed in this paper, should also be mentioned. Age plays the most relevant role, as PON1 activity is very low before birth and gradually increases during the first year or two of life in humans [30]. PON1 activity may also decline with aging, possibly because of the development of oxidative stress conditions [31].

The inhibition of LDL oxidation is a major antiatherogenic property of HDL [32]. This activity is, partially due, to HDL associated proteins and enzymes. However, whether these proteins interact in the antioxidant activity of HDL is unknown. In an impressive study conducted to understand this situation, LDL was incubated with apolipoprotein A1, LCAT, and PON1 in the presence or absence of HDL under

Table 1: Plasma biochemical parameters of the investigated rats, in each group $n = 10$.

Parameters	3 months	12 months	24 months	Obese
HDL (mg/dL)	41.56 ± 6.37	43.49 ± 7.19	50.67 ± 3.15*	52.99 ± 6.31*
LDL (mg/dL)	95.66 ± 7.29	102.45 ± 7.93	104.53 ± 9.78	143.84 ± 10.11*
Cholesterol (mg/dL)	113.56 ± 9.96	155.67 ± 4.33	190.78 ± 9.87*	201.93 ± 15.13*
TG (mg/dL)	86.45 ± 1.56	100.83 ± 2.08	101 ± 3.56	119 ± 4.61*
Apo A1 (mg/dL)	128.56 ± 3.76	135.67 ± 1.89	141.28 ± 3.78	131.56 ± 4.12
Apo B (mg/dL)	70.56 ± 1.82	88.23 ± 2.57	80.36 ± 1.93	87.89 ± 4.32

The data are expressed as means ± SD; * $P < 0.05$ when compared to the young and middle-aged groups.

Table 2: The plasma HDL composition of the investigated rats, in each group $n = 10$.

Parameters	Group			
	3 months	12 months	24 months	Obese
Total lipids, % of the total HDL composition	49.45 ± 1.35	51.45 ± 2.32	57.31 ± 1.93*	55.31 ± 1.93*
Total cholesterol, % of the total HDL composition	14.97 ± 0.23	14.56 ± 0.41	11.21 ± 0.76*	10.23 ± 0.66*
TG, % of the total HDL composition	1.75 ± 0.07	2.15 ± 0.08	2.75 ± 0.14*	2.98 ± 0.15*
α-Tocopherol, mmol/L	8.02 ± 0.39	7.88 ± 0.49	6.71 ± 0.45*	5.70 ± 0.49*
Isolated double bonds, U/mL	8.64 ± 0.59	8.33 ± 0.58	7.31 ± 0.17*	7.48 ± 0.25*
Diene conjugates, mmol/L	18.88 ± 1.14	21.78 ± 2.10	29.63 ± 2.35*	31.05 ± 1.65*
Ketodienes + conjugated trienes, U/mL	1.15 ± 0.08	1.21 ± 0.07	1.38 ± 0.06*	1.39 ± 0.07*
Total hydroperoxides, mmol/L	69.04 ± 2.88	69.37 ± 3.46	73.31 ± 1.57*	74.31 ± 1.43*

The data are presented as mean ± SD or percentage.
* $P \leq 0.05$ versus intact 3-month-old animals.

Table 3: Plasma HDL PON, LCAT, and CETP activity levels in the investigated rats, in each group $n = 10$.

	3 months	12 months	24 months	Obese
PON 1 (nmol min^{-1} mL^{-1})	97.42 ± 4.42	85.15 ± 4.01	71.45 ± 2.21*	76.23 ± 3.12**
LCAT, mkmol/L/h	54.92 ± 1.58	44.86 ± 1.99	36.25 ± 1.28*	20.25 ± 2.28**
CETP, mkmol/L/h	20.42 ± 1.76	33.51 ± 1.98	68.32 ± 8.54*	86.88 ± 9.43**

The data are expressed as means ± SD; * $P < 0.05$ when compared to the young and middle-aged groups; ** $P < 0.05$ when compared to 24-month-old group.

oxidizing conditions. When LDL lipid peroxide concentrations were determined, ApoA-1, LCAT, and PON1 were all found to inhibit LDL oxidation in the absence of HDL and enhance the ability of HDL to inhibit LDL oxidation [33].

Age-related loss of PON activity was not found in elderly patients, which, however, associated with the heterogeneity of patient population [34]. It is known that one of the main functions of PON is to protect the HDL from lipid peroxidation processes. In this study, we found a correlation between the decrease in PON activity (Table 3) and an increase in lipid peroxidation of HDL (Table 4).

Thus, a decrease in LCAT activity in 24-month-old animals may be one of the causes of increased oxidized LDL levels in old age [35], which in turn, may increase the risk of cardiovascular disease.

One of the major cardiovascular risk factors is obesity. Keeping 12-month-old animals on a high-fructose diet is accompanied not only by an increase in body weight and lipid metabolism disorders, but also the exchange of lipoproteins. It should also be noted that high-calorie diet completely eliminates the age differences between the studied parameters in 12-month-old animals with obesity and 24-month-old animals. Thus, animals with obesity tend to have significantly more intense lipid peroxidation, lower levels of antioxidants, and reduced activity levels of LCAT and PON.

Previous researches in our laboratory using experimental model of metabolic syndrome have shown that the introduction to animals of grape polyphenols complex inhibited LDL oxidation, HDL lipid peroxidation, normalized PON and CETP activity levels [36].

Therefore, the next stage of our research was to study the influence of grape polyphenols on the composition and functional state of HDL. We have found that "Enoant" significantly inhibits peroxidation processes in HDL and increases the activity of HDL enzymes that indicates an improvement in the functional activity of HDL (Table 4).

PON-1 has also been shown to protect against CVD: (a) it prevents the formation of oxidized HDLs and low-density lipoproteins (LDLs) [12]; (b) it hydrolyses the thiolactone form of homocysteine, which alters proteins in the arterial wall [37]; and (c) it hydrolyses platelet-activating factor, a bioactive phospholipid which is involved in vascular disease development [38]. Several studies suggest that a low-level plasma PON-1 activity is associated with increased

Table 4: The effect of "Enoant" on the plasma HDL composition and the plasma HDL paraoxonase, LCAT, and CETP activity levels in male 24-month-old and obese rat groups, in each group $n = 10$.

Parameter	Group	
	24-month-old + "Enoant"	Obese + "Enoant"
Total lipids, % of the total ApoB-LP composition	52.01 ± 1.87*	52.34 ± 1.88**
Total cholesterol, % of the total ApoB-LP composition	13.34 ± 0.79*	12.56 ± 0.673**
TAG, % of the total ApoB-LP composition	2.05 ± 0.21*	1.98 ± 0.31**
α-Tocopherol, mmol/L	7.15 ± 0.55*	6.70 ± 0.52*
Isolated double bonds, U/mL	7.83 ± 0.27*	7.99 ± 0.31**
Diene conjugates, mmol/L	22.63 ± 1.55*	22.54 ± 1.85**
Ketodienes + conjugated trienes, U/mL	1.28 ± 0.08*	1.25 ± 0.09**
Total hydroperoxides, mmol/L	70.32 ± 1.47*	71.54 ± 1.33**
PON 1 (nmol min^{-1} mL^{-1})	79.31 ± 3.29*	81.64 ± 3.04**
LCAT, mkmol/L/h	33.81 ± 1.95*	42.04 ± 3.57**
CETP, mkmol/L/h	58.52 ± 4.13*	77.88 ± 5.64**

The data are expressed as the means ± SD; * $P < 0.05$ when compared to 24-month-old group; ** $P < 0.05$ when compared to obese group.

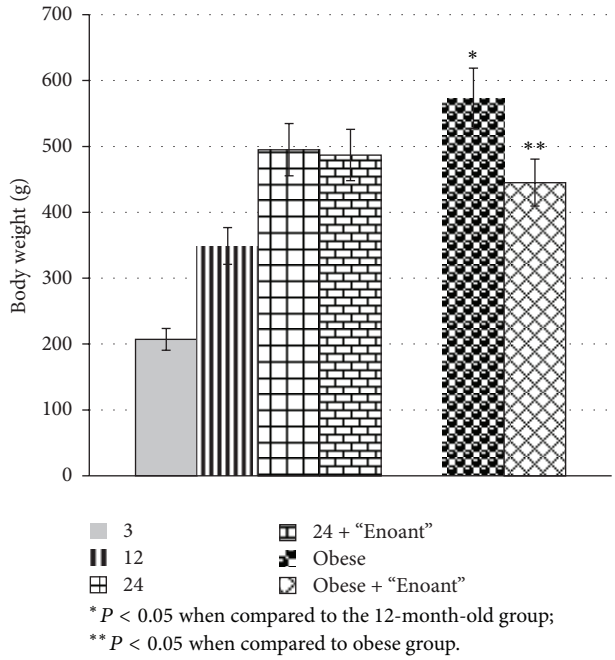

Figure 1: The effect of "Enoant" on the body weight of investigated rats, in each group $n = 10$.

prevalence of atherosclerosis and could be an independent risk factor for coronary events [12, 39].

Several dietary polyphenols, and in particularly quercetin, have been shown to upregulate PON1 [15]. Resveratrol, a polyphenolic phytoalexin found in grapes and wine, was shown to increase PON1 gene expression in human hepatocyte primary cultures and in HuH7 cells [40]. This effect appeared to be dependent upon activation of the Ah receptor and to involve an XRE-like element in PON1's promoter within the −126 and −106 region. Grape seed extracts have also been reported to increase serum PON activity in control, and particularly in streptozotocin-induced diabetic rats [41]. Polyphenols may upregulate PON1 expression and release by sequential activation of protein kinase A and PPAR-γ [42]. Except these, quercetin administration to mice lacking the LDL receptor (LDL−/−) for four weeks was reported to increase liver PON1 mRNA and serum PON1 activity by 40–90%, depending on the dose [16].

5. Conclusions

Thus, results of our investigations showed an age-dependent increase in HDL lipid peroxidation and a decrease in the PON activity in obese and 24-month-old animals, which may indicate a decrease of the functional activity of HDL. Keeping 12-month-old animals on high-fructose diet completely leveled age differences in the test indices between 12-month-old rats and 24-month-old rats. "Enoant" administration restored blood indices in rats with obesity and reduced peroxidation processes and normalized the PON and LCAT activity levels in old rats.

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Research Article

Resveratrol Prevents Dendritic Cell Maturation in Response to Advanced Glycation End Products

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Advanced glycation end products (AGEs), generated through nonenzymatic glycosylation of proteins, lipids, and nucleic acids, accumulate in the body by age thus being considered as biomarkers of senescence. Senescence is characterized by a breakdown of immunological self-tolerance, resulting in increased reactivity to self-antigens. Previous findings suggest that AGE and its receptor RAGE may be involved in the pathogenesis of autoimmune reactions through dendritic cell (DC) activation. The aim of this study was to investigate whether resveratrol, a polyphenolic antioxidant compound with tolerogenic effects on DCs, was able to counteract the mechanisms triggered by AGE/RAGE interaction on DCs. By immunochemical and cytofluorimetric assays, we demonstrated that *in vitro* pretreatment of human monocyte-derived DCs with resveratrol prevents DC activation in response to glucose-treated albumin (AGE-albumin). We found that resveratrol exerts an inhibitory effect on DC surface maturation marker and RAGE upregulation in response to AGE-albumin. It also inhibited proinflammatory cytokine expression, allostimulatory ability upregulation, mitogen-activated protein (MAP) kinases, and NF-κB activation in AGE-albumin-stimulated DCs. We suggest that resveratrol, by dismantling AGE/RAGE signaling on DCs may prevent or reduce increased reactivity to self-molecules in aging.

1. Introduction

Advanced glycation end products (AGEs) are a heterogenous group of molecules that are generated through nonenzymatic glycosylation (glycation) and oxidation of proteins, lipids, and nucleic acids [1]. Even though glycation is physiologically present and is modulated by several factors, disorders of glucose metabolism and systemic autoimmune diseases associated with inflammation and oxidative stress may favour the formation and accumulation of these products [2–6]. It is noteworthy that AGEs are normally produced in the body and they accumulate by age thus being considered as biomarkers of senescence [7]. Further, AGE adverse effects on cellular and tissue functions arise from their potential to cross-link intracellular and extracellular proteins thus altering their structure and function and triggering the development of different age-associated diseases, such as neurodegenerative and cardiovascular diseases [8, 9]. Proteins modified by glycation have been shown to become antigenic thus inducing activation of immune responses [10–12]. Six receptors that recognize and bind AGEs have been identified [13, 14], among which the best characterized and most extensively studied receptor being RAGE, a 46-kDa protein, mainly expressed on the surface of endothelial cells, smooth muscle cells, and monocyte-derived dendritic cells (DCs) [15, 16]. Although there is accumulating evidence that AGEs are involved in senescence [9], nevertheless further investigations are needed to clarify the role of glycation in aging and aging-related diseases. It is known that the immune system undergoes continuous morphological and functional changes throughout the lifetime and gradually declines with age [17]. The decline in protective immune responses to

exogenous and infectious agents is accompanied by an increased reactivity towards self- or endogenous antigens [17]. The mechanisms underlying the breakdown of immunological self-tolerance are not yet fully understood. Dendritic cells, the most potent antigen-presenting cells (APCs), have a pivotal role in the onset and regulation of adaptive immune response and in the induction of autoimmunity [18]. Previous studies demonstrated that AGE-modified serum molecules induced maturation of DCs and augmented their capacity to stimulate T-cell proliferation and cytokine secretion possibly through upregulation of RAGE [11, 12]. These findings suggest that AGE and its receptor (RAGE) axis may be involved in the pathogenesis of age-related autoimmune reactions through DC activation. Disrupting this axis may have a beneficial effect in longevity because many pathological mechanisms triggered by AGE/RAGE interaction can be prevented. Previous studies demonstrated that resveratrol, a natural polyphenol phytoalexin, prevents the AGE-induced acceleration of macrophage lipid accumulation through suppression of RAGE [19] and renders DCs tolerogenic upon activation [20]. Considering this previous evidence, the aim of our study was to investigate whether resveratrol exerts an inhibitory effect on AGE-induced activation of DCs. By immunochemical and flow cytometric analysis we determined the effects of resveratrol on phenotype and functions of human monocyte-derived DCs *in vitro* stimulated with AGE-albumin.

2. Materials and Methods

2.1. Reagents. A highly purified preparation of bovine serum albumin (Sigma-Aldrich, Milan, Italy) was dissolved in glycation buffer solution (GB) (0.144 g/l KH_2PO_4, 0.426 g/l Na_2HPO_4) pH 7.4, at 10 μg/mL final concentration, and immediately frozen at −80°C, under sterile conditions. Then albumin aliquots were incubated, under sterile conditions, in the dark, at 37°C for 10, 30, or 60 days, in sealed vials containing either the same concentrations (250 mM) of D-glucose (Sigma-Aldrich) or the nonreducing sugar D-mannitol (Sigma-Aldrich) used as iso-osmotic control, as described [21].

Endotoxin contamination in albumin, determined by the quantitative chromogenic limulus amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD, USA), was less than 0.05 EU/mL of protein. Polymyxin B was added to the cell culture medium at 10 μg/mL, concentration that completely neutralizes the activity of these amounts of lipopolysaccharide (LPS) in all experiments involving albumin.

Resveratrol was purchased from Sigma. All the chemicals used were of the highest available purity.

2.2. Characterization of AGE-Albumin

2.2.1. SDS-PAGE Analysis. Aliquots (50 μg) of glucose-treated albumin, mannitol-treated albumin, and native albumin were subjected to SDS-PAGE using 8–12% acrylamide gradient gels prepared as described [22]. Nonspecific binding of glycated proteins was minimized by saturation of plastic surfaces and check of the protein recovery after each manipulation. The protein molecular weight standards were provided by Novex (sharp standard molecular weight, Invitrogen, Carlsbad, CA, USA).

2.2.2. AGE Fluorescence Studies. Fluorescence studies were carried out as reported [12]. Briefly, albumin (20 μg/200 μL final volume in GB) was incubated at 37°C in the presence or absence of glucose or mannitol for increasing time in the dark under sterile conditions and constant temperature (21°C), and steady-state fluorescence emission spectra were collected with a FluoroMax-2 spectrofluorometer (Jobin Yvon-Spex, Edison, NJ, USA) using an excitation wavelength of 370 nm and collecting the emission data between 400 and 650 nm at 20°C (equal band widths for excitation and emission 5/5). The emission peak was recorded at 440 nm as reported [23].

2.2.3. Size Exclusion Chromatography. Fast protein liquid chromatography (FPLC) (Pharmacia, Uppsala, Sweden) analysis was carried out to evaluate under native conditions the albumin and AGE-albumin molecular size. After the incubation with sugars, aliquots of 50 μL (1 mg/mL protein concentration) were injected onto a Superose S12 Pharmacia column equilibrated in Phosphate buffer saline without calcium and magnesium $PBS^{-/-}$, pH 7.4. Elution was carried out with a 0.4 mL/min flow rate at room temperature, and protein peaks were detected under UV recording (optical density at 280 nm). The column was calibrated with a mixture of protein molecular weight standards (Pharmacia) according to the manufacturer's instructions.

2.2.4. Bioinformatic Analysis of Albumin Glycation. The potentiality of albumin (Bos taurus, accession number AAA 51411), to be glycated and to generate AGE product, was studied by means of a web source (NetGlycate 1.0 server) able to predict glycation of ε amino groups of lysines in proteins. Functional sites were analyzed by means of bioinformatic tools [24]. Structural analyses and computer-assisted molecular simulations were carried out as described [25].

2.3. Generation of DCs and T Lymphocytes. Blood samples from 5 healthy blood donors from the Transfusion Center at La 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 and immature dendritic cells (iDCs) were obtained from PBMCs, as previously described [12]. Immature DCs were stimulated with 0.2 μg/mL LPS (strain 0111:B4 *Escherichia coli*, Sigma-Aldrich) for 18 hours to obtain LPS-matured DCs. The purity of iDCs was found higher than 95%, as assessed by flow cytometric analysis (FACSCanto using CellDIVA, BD-Biosciences, San Diego, CA, USA) of cells stained with a mixture of CD14-fluorescein isothiocyanate (FITC) and CD1a-phycoerytrin (PE) monoclonal antibodies (mAbs) (PharMingen, San Diego, CA, USA). $CD4^+$ T cells were purified from PBMCs by magnetic selection using the anti-$CD4^+$ microbeads (Miltenyi Biotec Belgish, Gladbach, Germany), according to the manufacturer's instructions. The

purity of positively selected $CD4^+$ T cells was higher than 95%, as assessed by flow cytometric analysis.

2.4. Flow Cytometric Analysis of Phenotypic DC Maturation. Preliminary dose-response experiments (0–200 μg/mL) established that AGE-albumin effects on DC phenotypic maturation were dose dependent: we determined 30 μg/mL as the optimal reagent concentration for DC stimulation. Five-day human iDCs were stimulated with AGE-albumin (30 μg/mL), albumin (30 μg/mL), and LPS (0.2 μg/mL) for 18 hours or left unstimulated. AGE-albumin-stimulated DCs had been pretreated or not with resveratrol for 1 hour at 37°C, 5% CO_2 at concentrations ranging from 3 to 80 μM. After stimulation, DCs were collected, washed and stained with PE-conjugated mAbs to CD1a, CD80, and human leukocyte antigen-D region related (HLA-DR), and FITC-conjugated mAbs to CD83, CD86, and CD40 (PharMingen) and with the mouse anti-human RAGE mAb (Chemicon International, Inc. Temecula, CA, USA) or with isotype-matched control mAb for 30 minutes at 4°C. To assess RAGE surface expression, cells were then washed and stained with FITC-conjugated goat anti-mouse Ab (Sigma-Aldrich) for 30 minutes on ice. All samples were analyzed by flow cytometry on a FACSCanto using CellDIVA software (BD-Biosciences).

2.5. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot. Cell lysates of 1×10^6 unstimulated or stimulated DCs were mixed with loading buffer (Roth, Karlsruhe, Germany), heated for 5 min at 95°C, and subjected to SDS-PAGE on a 10% polyacrylamide gel with 0.1% SDS using standard procedures (constant voltage at 200 V; 50 μg protein/lane). Proteins were blotted onto polyvinylidenfluoride membrane (Millipore, Bedford, MA, USA) using a semidry blotting unit (Trans-Blot SD; Bio-Rad, München, Germany) in a Tris/Glycin buffer. After transfer, the membrane was blocked in blocking buffer (PBS containing 0.1% Tween-20 and 5% non-fat-dry milk powder) for 1 hour. For detection of RAGE, the membrane was incubated with rabbit anti-human RAGE polyclonal Ab (Chemicon International) at a dilution of 1 : 1000 in blocking buffer overnight. Bound antibodies were visualized with horseradish peroxidase- (HRP-) conjugated goat anti-rabbit immunoglobulin G Ab (1 : 5000; BioRad), and immunoreactivity was assessed by the chemiluminescence reaction using the enhanced chemoluminescence (ECL) Western blotting system (Amersham Life Science). Densitometric analysis was performed using an IMAGEJ 1.43 software.

2.6. Cytokine Production. Culture supernatants were collected at 18 hours after DC stimulation with AGE-albumin (30 μg/mL), albumin (30 μg/mL), or LPS (0.2 μg/mL). AGE-albumin-treated DCs had been pretreated or not with resveratrol (50 μM). Levels of IL-12p70, TNF-α, IL-10, and IL-1β were determined by ELISA (OptEIA kits; BD-Biosciences) following the manufacturer's instructions. The limits of detection were as follows: IL-10, TNF-α, and IL-1β: 16 pg/mL; IL-12p70 : 7.8 pg/mL.

2.7. Mixed Lymphocyte Reaction and IFN-γ^+ $CD4^+$ T-Cell Proliferation. Because features of DC function *in vivo* are critical for antigen presentation and T-cell activation, we evaluated the allostimulatory ability of unstimulated or stimulated DCs in a standard mixed lymphocyte reaction (MLR). LPS-stimulated DCs were used as positive controls. Allogeneic T cells (1×10^5 cells/well) were incubated with irradiated DCs (30 Gy) for 3 days at different responder/stimulator ratios (1 : 4 to 1 : 64 DC : T) in a 96-well round bottom plate. On day 2, 0.5 μCi/well of [^{3}H]-methyl-thymidine (Amersham) was added to each well. After additional 18 hours at 37°C, cells were harvested on a glass fiber filter paper (Wallac, EG&G Company, Turku, Finland), using an automatic cell harvester (Harvester 96, MACH III M, TOMTEC Orange, CT, USA). [^{3}H]-methyl-thymidine uptake into cell DNA was measured by reading samples in a β counter (1450 Microbeta Plus, Wallac). Net counts per minute (cpm) of triplicate cultures were measured.

To determine proliferating IFN-γ^+ $CD4^+$ T cells, allogenic $CD4^+$ T cells were stained with 5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE, Invitrogen, Milan, Italy). Briefly, cells were extensively washed and resuspended at final concentration of 10^7/mL in PBS. CFDA-SE was added at a final concentration of 2.5 μM and incubated for 4 min at room temperature. The reaction was stopped by washing the cells with RPMI 1640, containing 10% heat-inactivated FBS. CFDA-SE-labeled $CD4^+$ T cells were incubated with irradiated stimulated and unstimulated DCs for 3 days at 1 : 32 DC : T cell ratio in RPMI medium containing 10% FBS serum. Cells were monitored on day 3 for CFDA-SE content and IFN-γ cytokine expression by flow cytometry. In brief, 10^6 cells were stimulated with 10^{-7} M phorbol 12-myristate 13-acetate (PMA) plus 1 μg/mL ionomycin for 4 hours in the presence of 10 μg/mL brefeldin A (all reagents from Sigma-Aldrich). Cells were labeled with anti-CD4 peridinin-chlorophyll-protein (PerCP) (BD-Biosciences) (5 μL/10^4 cells, 30 minutes on ice) and treated with FACS lysing solution and then with FACS permeabilizing solution (BD-Pharmingen Biosciences). Cells were then stained with a predetermined optimal concentration of anti-IFN-γ mAb or of the appropriate isotype control mAb (BD-Biosciences) and analyzed on FACSCanto. The percentage of $CD4^+$ CFDA-SE^+ T cells (proliferating cells) on the IFN-γ positive cell gate was evaluated. Cells were gated according to their light scatter properties to exclude cell debris. A minimum of 10,000 viable cells was analyzed for each sample.

2.8. Mitogen-Activated Protein (MAP) Kinase p38 and ERK Assay. The fast activated cell-based ELISA MAPK assay kits were used to monitor p38 and ERK activation according to manufacturer's recommendation (Active Motive, Rixensart, Belgium). In brief, iDCs were cultured and seeded in 96-well plates at 5×10^4 cells/well. Cells were stimulated for different times (0–60 minutes) with AGE-albumin (30 μg/mL) after pretreatment or not with resveratrol (50 μM), with albumin (5 mM), LPS (0.2 μg/mL), or PMA (0.2 μg/mL). The number of cells in each well was counted and normalized using the

crystal violet solution. The results were expressed as arbitrary units.

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. Unstimulated DCs and DCs stimulated for 45 min at 37°C, in 5% CO_2 with AGE-albumin (30 μg/mL), after pretreatment or not with resveratrol (50 μM) and with albumin (30 μg/mL), 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. As a positive control we used a HeLa cell extract and NF-κB wild-type and mutated consensus oligonucleotides to monitor the specificity of the assay, according to manufacturer's instructions.

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 analyzed with the Kolmogorov-Smirnov test to verify Gaussian distribution. 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

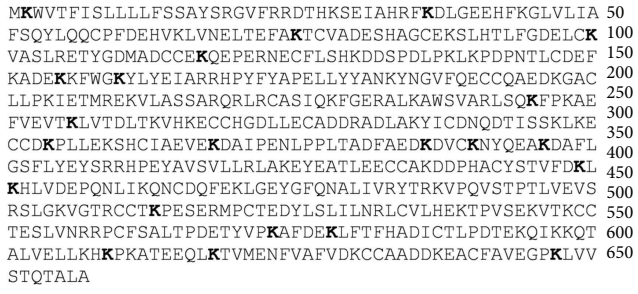
3.1. Characterization of Glucose-Treated Albumin. Preliminary evaluation of potential glycation sites within serum albumin sequence was assessed through a bioinformatic analysis showing that 22 out of 60 lysine residues have a significant probability to be nonenzymatically glycosylated (Figure 1(a)). Serum albumin was incubated for increasing time in the presence of D-mannitol or D-glucose as described in Material and Method section. In order to achieve a preliminary molecular characterization of the AGE-albumin preparations, fluorescence, denaturing SDS-PAGE, and native size exclusion chromatographic analyses were performed (Figure 1). The fluorescence at 440 nm, specific for AGE formation, was measured and confirmed the creation of time-dependent AGE products (Figure 1(b)). Of note, doubling the time of sugar exposure (60 *versus* 30 days) induced a 2-fold increase of fluorescence, as expected for a nonsaturated reaction. This result also indicated that the grade of sugar-induced albumin modification at 30-day exposure was not very high (moderately AGE-modified albumin). SDS-PAGE experiments showed only a slight band shift corresponding to a possible 1-2 kDa molecular size increase, likely due to some adducts formation, involving only a few lysine groups (Figure 1(c)). In order to verify whether the AGE-albumin might undergo a significant molecular size modification, a size exclusion chromatographic separation was carried out as described in Section 2, showing the formation of a molecular size increase likely due to some adduct formation (Figure 1(d)). The left shift of the monomeric peak of AGE-albumin sample (Figure 1(d)) has been determined to correspond to about the 11% of protein modification, confirming the low grade of protein AGE modification.

In all the experiments reporting DC maturation and pathways analyses the moderately AGE-modified albumin preparation (30 days with 250 mM glucose, marked by the black star in Figure 1) was used.

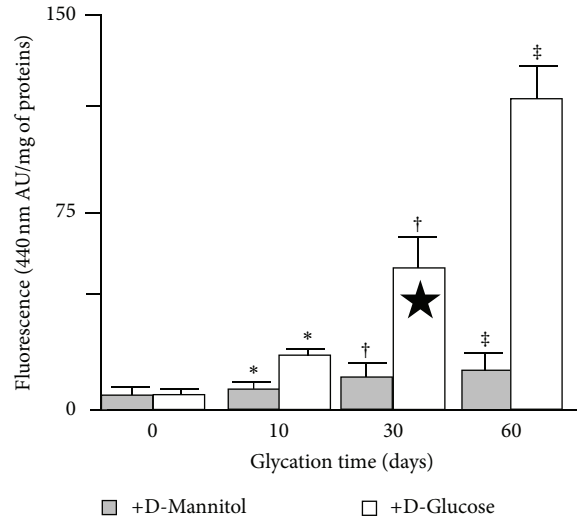
3.2. Resveratrol Prevents Phenotypical DC Maturation and RAGE Upregulation on Cell Surface in Response to AGE-Albumin. Unstimulated DCs showed an immature phenotype (HLA-DR^{low} and $CD83^{-}$) and were weakly immunoreactive for CD80, CD86, CD40, and RAGE. As expected, after 18 hours of incubation, LPS caused DCs to mature, so that CD83 appeared (Figure 2(a)) and HLA-DR, CD80, CD86, and CD40 expression increased (Figure 2(b)). RAGE expression on DC surface remained unchanged (Figure 3(a)). Similarly to LPS, AGE-albumin, but not albumin, induced DC maturation (CD83 and HLA-DR: $P < 0.001$; CD40, CD80, and CD86: $P < 0.01$; Figures 2(a) and 2(b)). AGE-albumin induced also a statistically significant upregulation of RAGE ($P < 0.001$; Figure 3). Of note, RAGE expression on AGE-albumin-stimulated DCs remained elevated until 60 hours ($P < 0.01$; Figure 3(a), panel (ii)). Resveratrol concentration of 50 μM was chosen on the basis of dose-response experiments as the optimal one to modulate DC phenotypic surface marker without affecting cell viability (data not shown). Pretreatment of iDC with resveratrol prevented the appearance of CD83 ($P < 0.001$; Figure 2(a)) and the upregulation of HLA-DR, CD40, CD86, and CD80 ($P < 0.01$, Figure 2(b)). This pretreatment also prevented the upregulation of RAGE after stimulation with AGE-albumin (Figure 3). Western blotting followed by densitometric analysis confirmed cytofluorimetric results (AGE-albumin *versus* AGE-albumin + resveratrol: $P < 0.001$; Figure 3(b)). Interestingly, resveratrol treatment did not affect cell viability, as assessed by trypan blue staining (data not shown).

3.3. Resveratrol Prevents Upregulation of Cytokine Production by DCs Stimulated with AGE-Albumin. After 18 hours of culture, AGE-albumin, similarly to LPS, triggered a statistically significant upregulation of IL-12p70, TNF-α, IL-10, and IL-1β expression ($P < 0.001$; Figure 4). Pretreatment of iDCs with resveratrol (50 μM) prevented the upregulation of all proinflammatory cytokines in response to AGE-albumin (IL-12: $P < 0.001$; TNF-α and IL-1β: $P < 0.05$; Figure 4), whereas it left IL-10 production expression unchanged. Control albumin and resveratrol alone left cytokine expression unmodified.

3.4. Resveratrol Prevents the Allostimulatory Function of DCs Stimulated with AGE-Albumin. When irradiated DCs, prestimulated with AGE-albumin, were tested in MLR, the relatively low proliferative ability (mean cpm) of resting allogenic T cells achievable with unstimulated DCs significantly increased, starting from a DC/T cell ratio of 1 : 4 (DC/T cell ratio of 1 : 16: unstimulated versus AGE-albumin-stimulated: $P < 0.001$; Figure 5(a)). This result is similar to that



(a)



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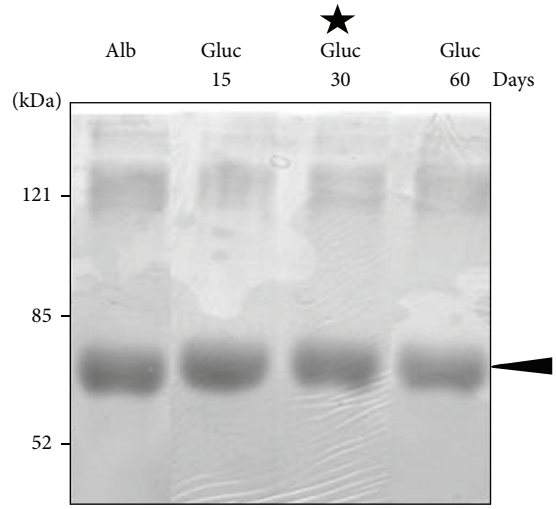

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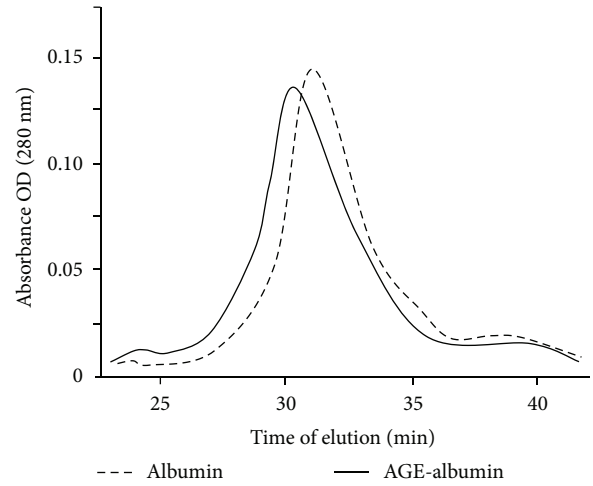

(d)

Figure 1: Molecular characterization of glycated albumin. (a) Structural analysis of the amino acid sequence of albumin. The analysis indicates that 22 (in bold) out of 60 lysine residues (K) are potential glycation sites. (b) Fluorescent AGE formation in an albumin solution incubated in the presence of D-glucose or D-mannitol (250 mM) after 10, 30, or 60 days. Data are expressed as means of arbitrary unit/mg of proteins ± SD ($n = 3$). $^{*}P < 0.05$; $^{\dagger\ddagger}P < 0.001$. The black star indicates the moderately modified AGE-albumin used in the subsequent cellular studies. (c) SDS-PAGE analysis of albumin preparations (50 μg per lane) incubated or not with 250 mM glucose for the reported times, followed by Coomassie staining, according to standard protocol. (d) Size exclusion chromatography of albumin and AGE-albumin. Albumin was incubated for 30 days with 250 mM D-mannitol (albumin) or D-glucose (AGE-albumin). One representative experiment out of three is reported.

obtained in response to LPS. We observed that pretreatment with resveratrol significantly impaired the allostimulatory function of DCs stimulated with AGE-albumin (at 1 : 16 DC/T cells ratio: $P < 0.001$). Resveratrol alone, when added *in vitro* during DC maturation, did not alter the degree of alloantigen-induced T-cell proliferation observed in response to iDCs (data not shown).

As a function of T-cell activation, we also measured the percentage of proliferating IFN-γ-producing $CD4^+$ T cells. Most of IFN-γ^+ $CD4^+$ T cells primed by LPS-matured DCs were proliferating cells (72%) (Figure 5(b)). A high percentage of proliferating IFN-γ-producing $CD4^+$ T cells was detected also in response to AGE-albumin-matured DCs (56%). In contrast, when $CD4^+$ T cells were cultured with

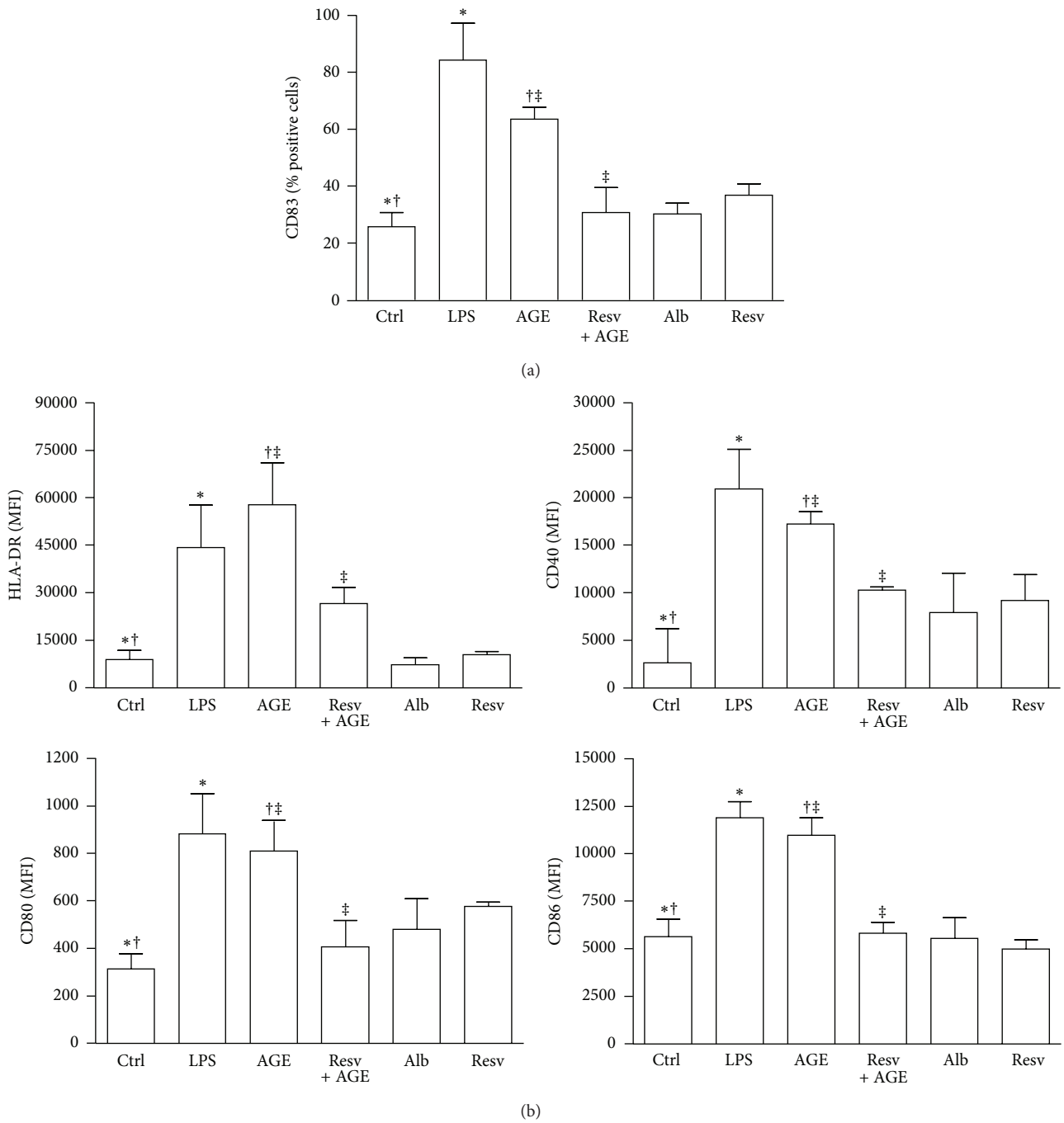

Figure 2: Flow cytometric analysis of phenotypic dendritic cell (DC) maturation. Five-day human DCs pretreated or not with resveratrol (Resv, 50 μM) were cultured for 18 hours with or without AGE-albumin (AGE, 30 μg/mL). DCs treated with LPS (0.2 μg/mL), albumin (Alb; 30 μg/mL), and resveratrol (Resv; 50 μM) were used as controls. Expression of surface molecules was analyzed by flow cytometry as described in Section 2. Phenotypic maturation of DCs was detected by the appearance of CD83 (a) and by the expression of surface molecules (b). After 18 hours of incubation LPS and AGE-albumin induced almost similar DC maturation. Pretreatment of iDCs with resveratrol prevented the phenotypic maturation of DCs induced by AGE-albumin (CD83 and HLA-DR: $^{*\dagger\ddagger}P < 0.001$; CD40 and CD80: $^{*\dagger}P < 0.001$, $^{\ddagger}P < 0.01$; CD86: $^{*}P < 0.001$, $^{\dagger\ddagger}P < 0.01$). Results are expressed as positive cell percentages (a) and mean fluorescence intensity (MFI, B) (means ± SD, $n = 4$). Samples were analyzed on a FACSCanto cytofluorimeter using CellDIVA (BD-Biosciences). P values by one-way ANOVA with a Bonferroni *post hoc* test.

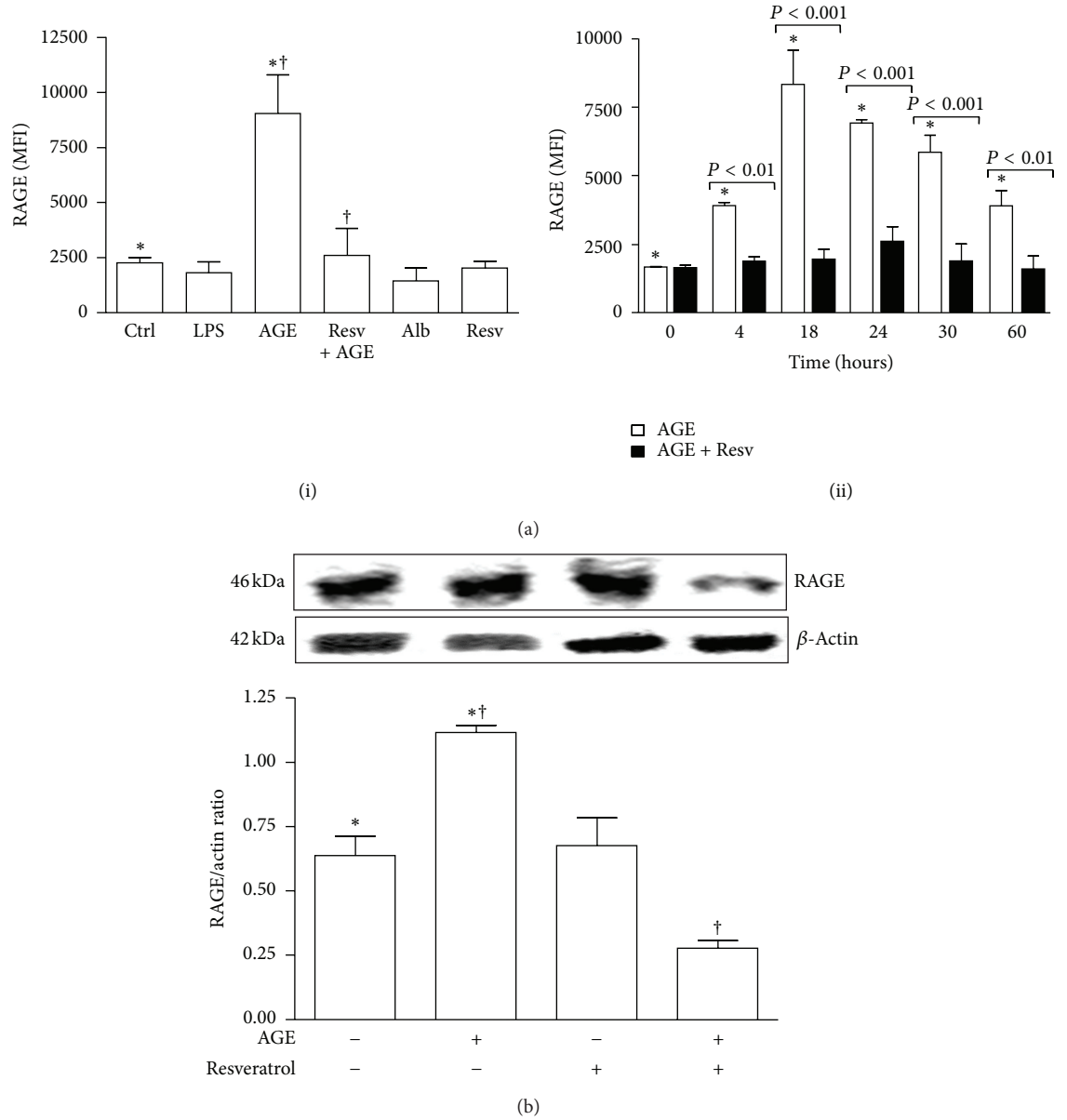

Figure 3: Analysis of RAGE expression on dendritic cells (DCs). Five-day human DCs pretreated or not with resveratrol (Resv, 50 μM) were cultured for 18 hours with or without AGE-albumin (AGE, 30 μg/mL). DCs treated with LPS (0.2 μg/mL), albumin (Alb; 30 μg/mL) and resveratrol (Resv; 50 μM) were used as controls. (a) Flow cytometric analysis of RAGE expression. AGE-albumin (AGE) induced a statistically significant upregulation of RAGE (panel (i)) whose expression remained elevated until 60 hours (panel (ii)). Pretreatment of iDCs with resveratrol prevented RAGE upregulation in response to AGE-albumin at all-time points investigated (panel (ii)). Results are expressed as mean fluorescence intensity (MFI) (means ± SD, $n = 3$). $^{*}P < 0.001$. $^{\dagger}P < 0.05$. (b) Western blotting analysis of RAGE expression on DCs. Western blotting followed by densitometric analysis confirmed RAGE downregulation on AGE-albumin-stimulated DCs by resveratrol (means ± SD, $n = 3$). $^{*\dagger}P < 0.001$.

resveratrol-pretreated DCs matured in the presence of AGE-albumin, the percentage of proliferating IFN-γ^{+} CD4^{+} T cells resulted lower (56% *versus* 36%).

3.5. Resveratrol Prevents MAPK and NF-κB Activation in Response to AGE-Albumin. Increased phosphorylation of p38 and ERK, which peaked at 45 minutes, was observed in AGE-albumin-stimulated DCs in comparison to the unstimulated ones ($n = 4$, p38: $P < 0.01$ and ERK: $P < 0.05$; Figure 6(a)). Pretreatment of iDC with resveratrol prevented the upregulation of both MAPKs in response to AGE-albumin ($P < 0.001$; Figure 6(a)).

In AGE-albumin-stimulated DCs, active p65 and p50 levels significantly increased in comparison to iDCs ($n = 4$, $P < 0.05$; Figure 6(b)). Pretreatment of iDCs with resveratrol prevented the upregulation of active p50 and p65 in response to AGE-albumin ($P < 0.001$).

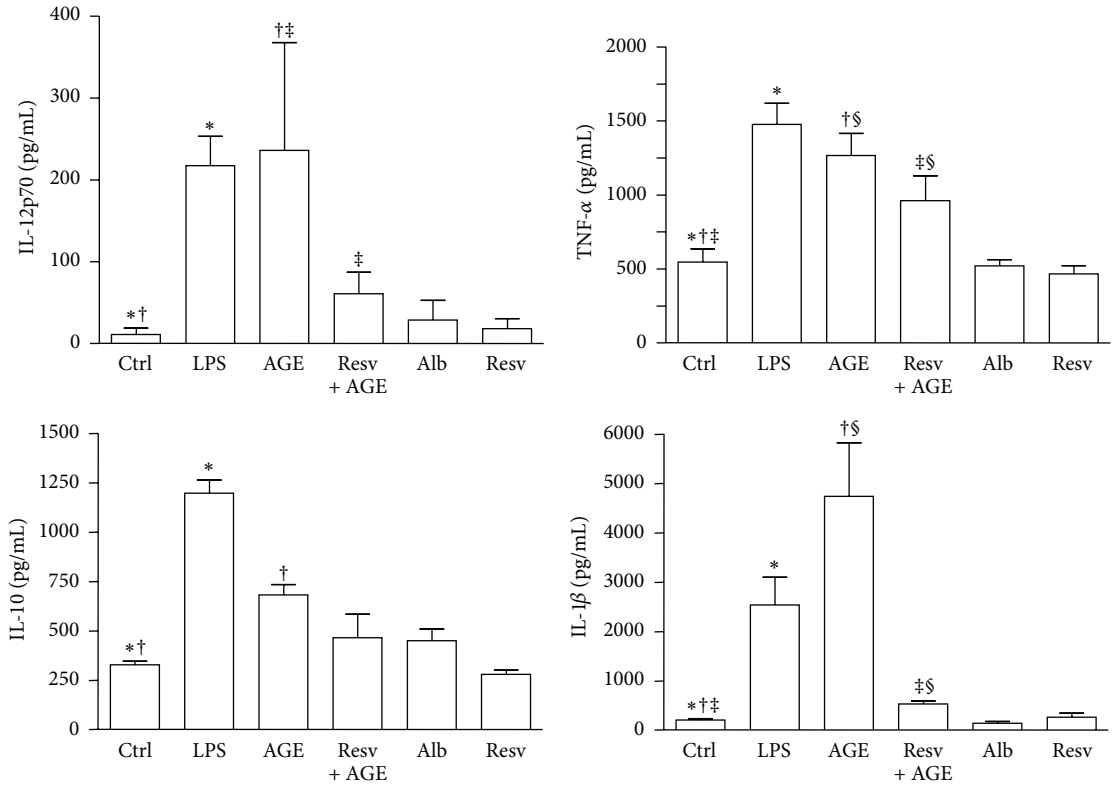

Figure 4: Cytokine production in dendritic cell (DC) culture supernatants. Five-day human DCs pretreated or not with resveratrol (Resv, 50 μM) were cultured for 18 hours with or without AGE-albumin (AGE, 30 μg/mL). DCs treated with LPS (0.2 μg/mL), albumin (Alb; 30 μg/mL), and resveratrol (Resv; 50 μM) were used as controls. Supernatants were collected after 18 hours to measure IL-12p70, TNF-α, IL-10, and IL-1β by specific ELISA experiments. LPS and AGE-albumin (AGE) triggered a statistically significant upregulation of all cytokine secretions. Pretreatment of iDC with resveratrol prevented the upregulation of all proinflammatory cytokines in response to AGE-albumin. Results are expressed as means ± SD of four independent experiments (IL-12p70: $^{*}P < 0.01$, $^{\dagger\ddagger}P < 0.001$; TNF-$\alpha$ and IL-1β: $^{*\dagger\ddagger}P < 0.001$, $^{\S}P < 0.05$; IL-10: $^{*\dagger}P < 0.001$).

4. Discussion

In this study we demonstrated that resveratrol exerts *in vitro* an inhibitory effect on the maturation of human monocyte-derived DCs in response to AGE-albumin by a mechanism that involves reduction of RAGE surface expression, proinflammatory cytokine production, and NF-κB signaling. To the best of our knowledge, this is the first evidence that resveratrol affects DC full maturation in response to an AGE product thus leading to an immature/semimature DC phenotype.

Resveratrol, a polyphenolic compound found in red wine and grapes, plays a potentially important role in many disorders [26]. It possesses antioxidant, anti-inflammatory, antiproliferative, and antiangiogenic effects, and many signaling pathways are among its molecular targets. Resveratrol is also believed to be beneficial in increasing the lifespan and healthy aging [26]. Aging is characterized by an erosion of tolerance, resulting in increased reactivity to self-antigens [27, 28]. The knowledge on the underlying mechanisms of resveratrol action on aging, particularly on immunosenescence and autoimmunity, remains incomplete.

DCs are the most potent APCs and have a pivotal role in the onset and regulation of adaptive immune response. They control Th1/Th2 and Th17/Treg polarization and the state of tolerance to self-antigens. Immature DCs induce regulatory T cells, thus promoting tolerance, whereas mature DCs stimulate effector T cells that support immunity [29–32].

DCs also have the ability to regulate inflammatory responses by secreting cytokines and chemokines [30, 31].

A previous study demonstrated that AGE-albumin induced maturation of DCs and augmented their capacity to stimulate T-cell proliferation and cytokine secretion possibly through upregulation of RAGE and scavenger receptor A [11]. On the basis of this previous evidence we designed the present study to examine the attenuating effects of *in vitro* resveratrol on AGE-albumin-matured DCs. Our bioinformatic analyses of the albumin primary structure indicated that several potential glycation sites are present within the molecule. We verified the occurrence of glycation in glucose-treated albumin by the increase of albumin's molecular dimension and by the formation of a fluorescent AGE. Our preliminary AGE-albumin molecular characterization also indicated that the preparation used in our *in vitro* study was a moderately AGE-modified albumin and that the protein folding modifications likely involved new antigenic features due to the AGE adducts. When we analyzed the phenotypic characteristics of DCs after stimulation with AGE-albumin, we confirmed previous findings on the ability of AGE-albumin to

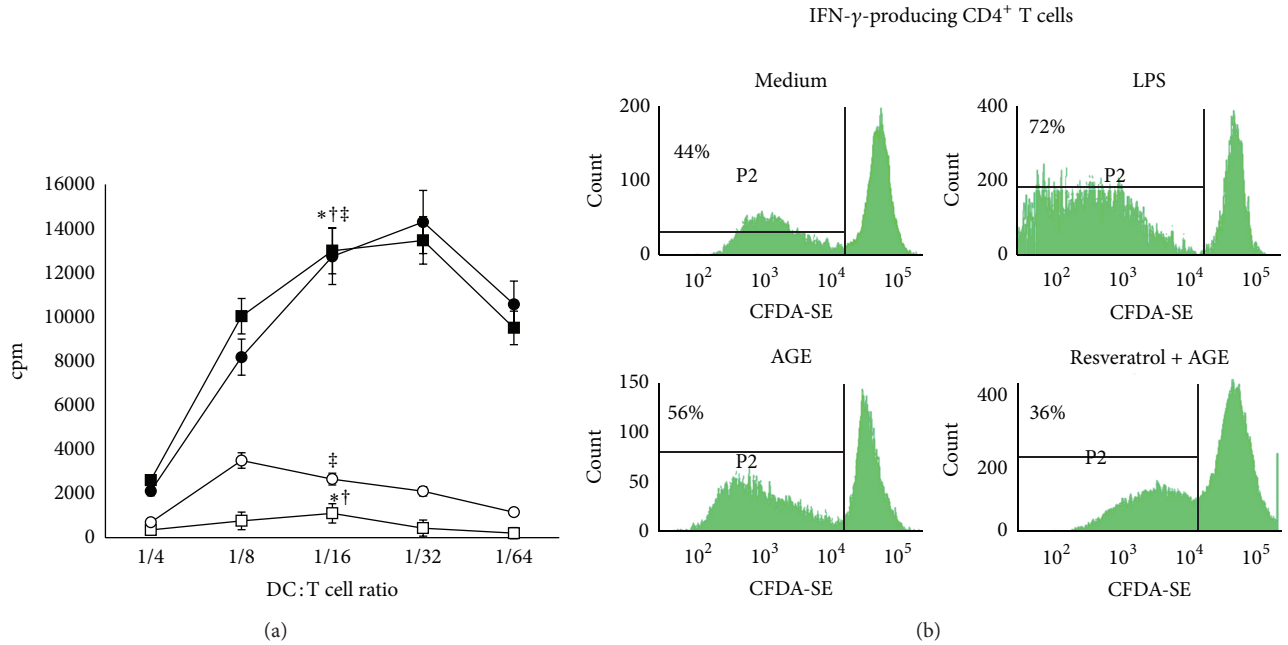

Figure 5: Allostimulatory ability of dendritic cells (DCs). Five-day human DCs were stimulated for 18 hours with LPS (0.2 μg/mL) (■), and AGE-albumin (AGE; 30 μg/mL) (●), AGE-albumin plus resveratrol (Resv, 50 μM) (◯) or left unstimulated (Ctrl) (□). After 18 hours, DCs were extensively washed and cultured with allogeneic T lymphocytes (1×10^5 cells/well) for 3 days at various stimulator-responder ratios (1 : 4 to 1 : 64 DC/T). (a) Proliferation of allogeneic T cells was measured by [^{3}H]-methyl-thymidine incorporation. Data are presented as mean cpm ± SD of four independent experiments (at 1/16 DC/T cells ratio: AGE-albumin and LPS versus unstimulated: $^{*\dagger}P < 0.001$; AGE-albumin + resveratrol *versus* AGE-albumin: $^{\ddagger}P < 0.001$). *P* values by the one-way ANOVA with a Bonferroni *post hoc* test. (b) Proliferation of IFN-γ-producing CD4$^+$ T cells was determined by staining allogeneic CD4$^+$ T cells with CFDA-SE (2.5 μM) and culturing them with irradiated AGE-albumin-stimulated or unstimulated DCs at 1 : 32 DC : T cell ratio. CD4$^+$ T-cell proliferative activity (CFDA-SE content), as well as their ability to produce IFN-γ was measured on day 3 by flow cytometry as described in Section 2. Figure shows a representative experiment out of 3 with similar results. The numbers show the percentage of proliferating IFN-γ-producing CD4$^+$ T cells. Samples were analyzed on a FACSCanto cytofluorimeter using CellDiva software (BD-Biosciences).

induce DC maturation. Our experiments also demonstrated that the pretreatment of iDCs with resveratrol prevented a complete phenotypic and functional DC maturation in response to AGE-albumin and induced a typical immature/semimature DC phenotype. This phenotype is characterized by a reduced CD83, HLA-DR, CD40, CD80, and CD86 expression associated with low IL-12, TNF-α, and IL-1β production. In line with their semimature phenotype, AGE-albumin-stimulated DCs pretreated with resveratrol did not increase the low proliferative ability of resting allogenic T lymphocytes in a standard mixed lymphocyte reaction. Further information about the resveratrol inhibitory activity on DCs comes from our experiments investigating RAGE expression on DC surface in response to AGE-albumin. Our results demonstrated that RAGE, the specific receptor for AGEs [13–15], remained down-regulated in AGE-albumin-stimulated iDCs pretreated with resveratrol for more than 48 hours. This last finding could explain how resveratrol may impair maturation of DCs in response to AGE-albumin. It is known that the binding of AGEs to RAGE activates multiple signaling cascades, including Erk1/2 MAPKs, and the generation of reactive oxygen species [33]. These cellular signals may induce activation of downstream effectors such as NF-κB [33]. Under our experimental conditions, resveratrol interferes with RAGE signaling cascade activated by AGE-albumin on DCs through silencing both MAPK p38 and ERK pathways and NF-κB translocation thus leading to the observed immature/semimature phenotype. These findings are in line with the downregulation of MAPK cascade observed on vascular smooth muscle cells [34]. Our results on the inhibitory effect of resveratrol on the NF-κB pathway support previous findings on LPS-matured DCs [20].

Our *in vitro* findings help to explain why self-proteins become immunogenic *in vivo*.

Nowadays, it is generally agreed that some autoimmune diseases are associated with abnormal presentation of cryptic or neoepitopes of self-antigens by DCs. Because epitope dominance is influenced by protein structure, glycation and glycoxidation events may change the molecular context of protein epitopes (for altered secondary or tertiary structure), thus permitting the efficient presentation of cryptic and neodeterminants. This is supported by our observations in which we reported that oxidatively modified proteins increase their immunogenicity [12, 35–39]. Although enhanced in diabetes, AGEs accumulation also occurs in euglycemia, aging [40], and systemic autoimmune diseases [41, 42], albeit to lower degrees, driven by oxidative stress and inflammation or simply by diet-induced postprandial hyperglycaemic peaks.

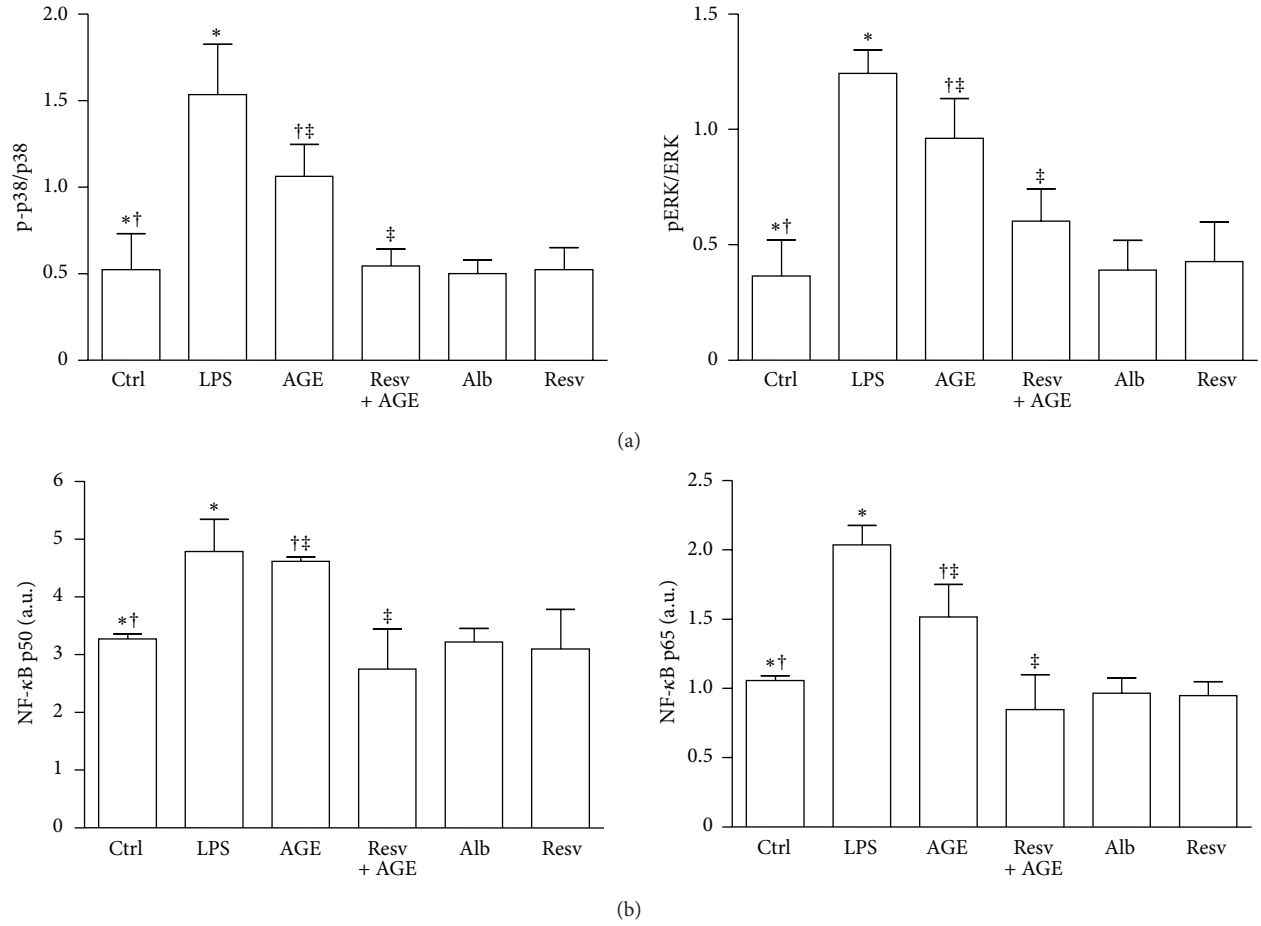

Figure 6: MAPK and NF-κB activation in dendritic cells (DCs). Five-day human DCs pretreated or not with resveratrol (Resv, 50 μM) were cultured for 45 minutes with or without AGE-albumin (AGE, 30 μg/mL). DCs treated with albumin (Alb; 30 μg/mL), resveratrol (Resv; 50 μM), LPS (0.2 μg/mL), or PMA (0.2 μg/mL) were used as controls. Cells were then analyzed by cell-based ELISA MAPK assay to monitor p38 and ERK activation and by NF-κB (p65 and p50) transcription factor assay to monitor NF-κB activation. (a) AGE-albumin stimulation induced the activation of both MAPK p38 and ERK pathways in DCs. Pretreatment of DCs with resveratrol prevented the upregulation of both MAPKs in response to AGE-albumin ($n = 4$; p-p38/p38: $^{*\ddagger}P < 0.001$, $^{\dagger}P < 0.01$; pERK/ERK: $^{*\ddagger}P < 0.001$, $^{\dagger}P < 0.05$). (b) AGE-albumin stimulation significantly increased active p65 and p50 levels in DCs. Pretreatment of DCs with resveratrol prevented the upregulation of both active p50 and p65 in response to AGE-albumin. The results are expressed as arbitrary units ($n = 4$, p50 and p65: $^{*}P < 0.05$; $^{\dagger}P < 0.001$).

Increased oxidative stress and AGE accumulation may result in the overexpression of RAGE [43, 44]. RAGE expression is increased in an inflammatory milieu and present in aging subjects, who in turn may particularly be exposed to the deleterious effect of AGEs. The AGE-RAGE interaction might act as a proinflammatory loop in these subjects, thus contributing to a chronic low-grade inflammation which is a precursor of aging-related diseases [43].

5. Conclusions

Our *in vitro* findings may help to explain the detrimental effects of AGE accumulation during aging, particularly the increased reactivity towards self- or endogenous antigens observed in aged individuals. A possibility is that chronic oxidative stress conditions in aged individuals cause AGE accumulation in the body. The generation of AGEs and augmentation of proinflammatory mechanisms provide a powerful feedback loop for sustained oxidative stress, ongoing generation of AGEs, and autoimmunity. Increased AGE-associated modifications in existing self-molecules may in fact enhance their immunogenic potential and may initiate a local autoimmune process in aged subjects with consequent development of different age-associated diseases.

Our *in vitro* findings now call for studies in aged individuals to verify the pathogenetic role of glycated proteins, as trigger of specific humoral and cellular immune reactions.

Our results suggest that an antioxidant therapy or a prevented diet with resveratrol, besides inhibiting glycation and glyco-oxidation reactions, may also directly act by dismantling AGE/RAGE signaling, thus preventing or reducing increased reactivity to self-molecules in aging.

Conflict of Interests

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

Acknowledgments

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Research Article

Effect of Tai Chi versus Walking on Oxidative Stress in Mexican Older Adults

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It has recently been reported that the practice of Tai Chi reduces oxidative stress (OxS), but it is not clear whether walking or Tai Chi produces a greater antioxidant effect. The aim of the present study was to evaluate the effect of the practice of Tai Chi and walking on markers for OxS. We carried out a quasi-experimental study with 106 older adults between 60 and 74 years of age who were clinically healthy and divided into the following groups: (i) control group ($n = 23$), (ii) walking group ($n = 43$), and (iii) Tai Chi group ($n = 31$). We measured the levels of lipoperoxides (LPO), antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), and total antioxidant status (TAS) pre- and post-intervention in all subjects. The data were subjected to a covariant analysis. We found lower levels of LPO in the Tai Chi group compared with the walking group (Tai Chi, 0.261 ± 0.02; walking, 0.331 ± 0.02; control, 0.304 ± 0.023 μmol/L; $P = 0.05$). Likewise, we observed significantly higher SOD activity and lower OxS-score in the Tai Chi group ($P < 0.05$). Our findings suggest that the practice of Tai Chi produces a more effective antioxidant effect than walking.

1. Introduction

Aging is a gradual and adaptive process characterized by a relative reduction of the homeostatic response due to the modifications produced by changes inherent to aging and the accumulated impact of the challenges confronted by the organism over the course of its life [1, 2]. In addition to genetic and environmental factors, lifestyles are determining factors for the process and type of individual aging [1]. Although aging is multifactorial and individualized, it has been linked to oxidative stress (OxS), a biochemical alteration that increases the risk of chronic degenerative disease [2, 3].

Physical exercise minimizes the biological changes associated with normal aging, such as reduction in muscle mass, strength, and rapidity of muscle contraction. It also has a positive effect on mitochondrial function, oxidizing enzymatic capacity, aerobic capacity, cardiac contractibility, rapidity of nerve conduction, and glucose intolerance. There is consistent evidence that exercise affects psychological wellbeing, increases longevity, and diminishes the risk of chronic degenerative diseases in old age [4–6].

On the other hand, moderate exercise is indicated for maintaining health and preventing chronic diseases in old age. In this respect, walking is one of the most recommended exercises, and it has been shown to have positive effects on various biochemical markers, including those relating to oxidative stress [7–11]. Tai Chi is another modality of exercise that is broadly recommended for the elderly, particularly for preventing falls and psychological wellbeing [12, 13]. In addition, it has recently been observed that Tai Chi stimulates the endogenous antioxidant enzymes activity and decrease the oxidative stress in middle-aged adults and pre- and

postmenopausal women [14, 15]. On the other hand, our research group found that Tai Chi diminishes the oxidative stress in older adults [16]. However, it is unknown whether Tai Chi or walking produces a more efficient antioxidant effect. The aim of the present study was to determine the effect of walking, in comparison with Tai Chi, on oxidative stress (OxS) in older Mexican adults.

2. Methods

2.1. Design and Subjects. We carried out a quasiexperimental study on a sample of 106 seniors who were divided into the following groups: (i) control group ($n = 23$), (ii) Tai Chi group ($n = 31$), and (iii) walking group ($n = 43$). In Figure 1, we outline the study. The criteria for inclusion were as follows: age greater than 60 but less than 74 years, no participation in any physical training program in the 6 months prior to the intervention, free of chronic diseases, normal resting electrocardiogram, and no consumption of antioxidants. The Tai Chi and walking were performed for 6 months in 1-hour daily sessions. The hour included 10 minutes of warmup, 40 minutes of exercise, and 10 minutes of cooldown. All participants exercised with their assigned modalities under the supervision of an expert instructor and personal doctor. Prior to the start of the 6-month study period, the Tai Chi group participated in a three-week period of physical training to work on the basic movements of Tai Chi in order to master forms 8 and 16 of the Yang style [17].

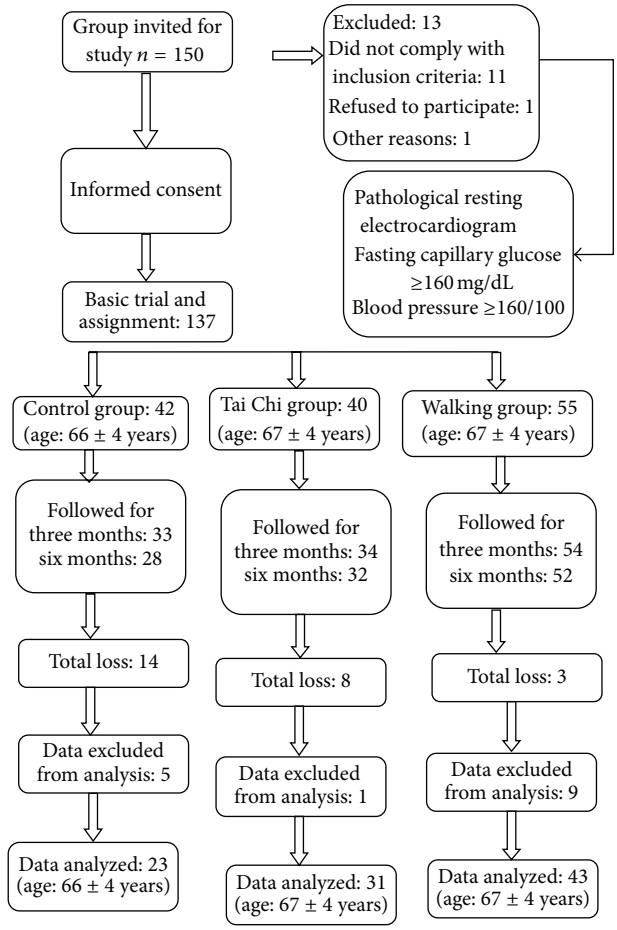

Figure 1: General scheme for study tracking.

2.2. Anthropometric and Blood Pressure Measurements. After clinical history and physical examination were conducted, we performed the following anthropometric measurements: weight was measured while the subject was wearing underwear and a clinical smock and in a fasted state (after evacuation). A Torino scale (Tecno Lógica, Mexicana, México, TLM) was used, calibrated before each weight measurement. Height was obtained with an aluminum cursor stadiometer graduated in millimeters. The subject was barefoot, back, and head in contact with the stadiometer in Frankfurt horizontal plane. Body mass index (BMI) was calculated by dividing weight (in kilograms) by height (in squared meters). Waist circumference was measured with a tape measure to the nearest 0.5 cm at umbilical scar level in centimeters.

Blood pressure was measured in both arms 3 times in the morning, in a fasting condition or 2 hours after breakfast, in sitting and standing positions. A mercurial manometer was used to measure the blood pressure. Subjects with pseudohypertension were identified by applying the Osler technique, which is, feeling the radial pulse when the manometer registered values above the true systolic pressure. Blood pressure was taken by medical technicians who had attended training sessions to standardize the procedures. The technicians were supervised to avoid possible biases in measurement.

2.3. Biochemical Analysis. For the biochemical tests, we sampled blood from all participants via venipuncture before the beginning of the exercise program (basal) and after six months of intervention. The samples were obtained between 8 and 9 AM with a previous fasting period of 8 hours and collected in vacuum tubes (Becton-Dickinson, México), without anticoagulant for the biochemical tests (glucose and lipid profile) and with heparin for the tests for OxS. Biochemical analyses (glucose and lipid profile) were conducted using a colorimetric technique in an Autoanalyzer Vitalab Eclipse Merck (Dieren, The Netherlands) DL concentration was calculated using the Friedewald formula.

2.4. Plasma TBARS. The TBARS assay was prepared as described by Jentzsch et al. (1996) [18]. In the TBARS assay, one molecule of malondialdehyde reacts with two molecules of thiobarbituric acid (TBA) and thereby produces a pink pigment with absorption peak at 535 nm. Amplification of peroxidation during the assay is prevented by the addition of the chain-breaking antioxidant, butylated hydroxytolueno toluene (BHT).

2.5. Plasma Total Antioxidant Status (TAS). Antioxidant quantification was done using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^+$) radical formation

kinetics (Randox Laboratories, Ltd., Crumlin Co., UK). The antioxidants present in plasma suppressed the bluish-green staining of the $ABTS^+$ cation, which was proportional to the antioxidant concentration level. The kinetics were measured at 600 nm with UV-spectrophotometer Shimadzu UV-1601 (Kyoto, Japan).

2.6. Red Blood Cell Superoxide Dismutase (SOD). The method uses xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolim chloride to form a red formazan dye. SOD activity was measured by degree of inhibition of the reaction (Randox Laboratories Ltd., Crumlin Co., UK). The kinetics were measured at 505 nm with UV-spectrophotometer Shimadzu UV-1601 (Kyoto, Japan).

2.7. Red Blood Cell Glutathione Peroxidase (GPx). In the presence of glutathione reductase and NADPH, the oxidation of glutathione (GSH) by cumene hydroperoxide is catalyzed by GPx. Oxidized glutathione (GSSG) is immediately converted into the reduced form with a subsequent oxidation of NADPH to NADP+ (Randox Laboratories, Ltd., Crumlin Co., UK). Decrease in absorbance was measured at 340 nm with UV-spectrophotometer Shimadzu UV-1601 (Kyoto, Japan).

2.8. Oxidative Stress Score. Alternative cut-off values of each parameter were defined on the basis of the 90th percentile of young healthy subjects: lipid peroxidation (LPO) $\geq$ 0.340 mmol/L, superoxide dismutase (SOD) $\leq$ 170 IU/L, glutathione peroxidase (GPx) $\leq$ 5500 IU/L, total antioxidant status (TAS) $\leq$ 0.9 mmol/L, SOD to GPx ratio (SOD/GPx) $\geq$ 0.023, and antioxidant GAP (GAP) $\leq$ 190 mmol/L. A stress score (SS) ranging from 1 to 6, representing the severity of biomarkers modifications; a score 1 was given to each value higher or lower than the cutoff. We categorize subjects as follows: according to their scale in SS: without OxS if SS was 0, Slight OxS if SS was 1-2, moderate OxS if SS was 3-4, and severe OxS if SS was 5-6. We have two categories: the subjects without OxS when a stress score (SS) are ranging from 0–2 and subjects with OxS when a stress score (SS) are ranging from 3–6 [19].

2.9. Statistical Analysis. The data were analyzed using the SPSS 16.0 (SPSS Inc., Chicago, IL, USA) statistical package. We used descriptive measures, average and standard deviation, and pre- and postintervention data. The data were analyzed using ANOVA, and parameters that displayed significant pre- and postintervention were subjected to multiple covariant analysis using the basal parameter conditions as covariables.

3. Results

3.1. Blood Pressure and Biochemical Characteristics. A statistically significant decrease in systolic blood pressure in the subjects who practiced Tai Chi was observed with respect to the control and walking groups ($P < 0.001$) (Table 1).

Table 1: Age and blood pressure and body mass index by group.

	Control $n = 23$	Tai Chi $n = 31$	Walking $n = 43$
Age (years)	66.4 ± 4	66.7 ± 3.8	66.8 ± 3.9
SBP (mm/Hg)			
Baseline	121 ± 23	117 ± 12	127 ± 15
Postintervention	124 ± 10	111 ± 13*	123 ± 13
DBP (mm/Hg)			
Baseline	77 ± 12	74 ± 8	76 ± 9
Postintervention	75 ± 5	74 ± 10	75 ± 8
BMI (kg/m^2)			
Baseline	27 ± 3	28 ± 5	28 ± 5
Postintervention	27 ± 4	27 ± 5	27 ± 6

SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index. ANOVA test, one time, $^*P = 0.001$.

Table 2: Biochemical parameters of the study population by group.

	Control $n = 23$	Tai Chi $n = 31$	Walking $n = 43$
Glucose (mg/dL)			
Baseline	119 ± 50	107 ± 20	112 ± 21
Postintervention	104 ± 29	92 ± 12	90 ± 19
Cholesterol (mg/dL)			
Baseline	207 ± 44	201 ± 48	220 ± 44
Postintervention	219 ± 48	180 ± 41*	218 ± 40
Triglycerides (mg/dL)			
Baseline	144 ± 60	170 ± 115	171 ± 72
Postintervention	146 ± 51	155 ± 93	168 ± 63
HDL (mg/dL)			
Baseline	55 ± 19	50 ± 12	49 ± 8
Postintervention	53 ± 11	49 ± 12	50 ± 11
LDL (mg/dL)			
Baseline	122 ± 38	116 ± 45	137 ± 40
Postintervention	137 ± 43	99 ± 37**	135 ± 65

ANOVA test, one time. $^*P < 0.05$, $^{**}P < 0.001$ HDL: High-Density Lipoproteins, LDL: Low-Density Lipoproteins.

With regard to biochemical parameters, we found a significant reduction in total cholesterol (baseline, 201 ± 48 versus postintervention, 180 ± 41 mg/dL; $P < 0.05$) and LDL cholesterol (baseline, 116 ± 45 versus postintervention, 99 ± 37 mg/dL; $P < 0.001$) in the subjects who practiced Tai Chi with respect to the control and walking groups (Table 2).

3.2. Oxidative Stress Markers. In LPO levels, we found a significant decrease in the Tai Chi group (baseline, 0.287 ± 0.01 versus postintervention, 0.257 ± 0.09 μmol/L; $P < 0.05$); in contrast, there was a significant increase in the

Table 3: Oxidative stress markers baseline and postintervention by group.

	Control $n = 23$	Tai Chi $n = 31$	Walking $n = 43$
Lipoperoxides (μmol/L)			
Baseline	0.280 ± 0.07	0.287 ± 0.01	0.244 ± 0.01
Postintervention	0.297 ± 0.10	0.257 ± 0.09*	0.330 ± 0.01**
SOD (U/mL)			
Baseline	171 ± 15	169 ± 8	176 ± 7
Postintervention	171 ± 8	178 ± 9*	177 ± 9
GPx (U/L)			
Baseline	7381 ± 2694	6225 ± 2869	7756 ± 2697
Postintervention	8154 ± 4208	8410 ± 4084	11600 ± 6779**
TAS (mmol/L)			
Baseline	1.07 ± 0.23	0.82 ± 0.28	0.95 ± 0.3
Postintervention	1.03 ± 0.19	1.04 ± 0.16	1.01 ± 0.13
SOD/GPx			
Baseline	25 ± 12	34 ± 20	26 ± 13
Postintervention	37 ± 28	37 ± 27	16 ± 15
OxS-score			
Baseline	1.1 ± 0.1	2.6 ± 1.1	1.8 ± 1.4
Postintervention	2.0 ± 0.9	0.9 ± 0.8**	1.1 ± 0.8**

ANOVA test, one time. *$P < 0.05$, **$P < 0.001$, SOD: superoxide dismutase, GPx: glutathione peroxidase, TAS: Total antioxidant status, GAP: Antioxidant Gap, OxS-score: Oxidative Stress score.

walking group (baseline, 0.244±0.01 versus postintervention, 0.330 ± 0.01 μmol/L; $P < 0.001$). Likewise, an increase of SOD activity in the Tai Chi group was observed (baseline, 169 ± 80 versus postintervention, 178 ± 90 U/mL; $P < 0{,}05$); nevertheless, walking group did not show a significant change postintervention. With regard to GPx activity, the walking group showed a significant increase (baseline, 7756 ± 2697 versus postintervention, 11600 ± 6779 U/L, $P < 0.001$); in contrast there was not a significant increase in the Tai Chi group (baseline, 6225 ± 2869 versus postintervention, 8410 ± 4084 U/L, $P > 0.05$). On the other hand, we found a significant decrease in the OxS-score in the Tai Chi and walking groups, although the change was more evident in the Tai Chi group (Table 3).

3.3. Changes in Oxidative Markers by Intervention. In Figure 2, we show the particular markers of OxS adjusted to baseline conditions. Lipoperoxides (LPO) levels were lower in the subjects who practiced Tai Chi compared to the controls, with a borderline statistical significance ($P = 0.08$). Likewise, LPO levels were significantly higher in the walking group compared to the Tai Chi group ($P < 0.05$). Superoxide dismutase activity was significantly higher in the subjects who practiced Tai Chi compared to the controls ($P < 0.05$), while GPx activity was higher in the walking group compared to the control group with a borderline statistical significance ($P < 0.07$). The OxS-score was significantly lower in the Tai Chi and walking groups in comparison with control group ($P < 0.001$), although the change was more evident in the Tai Chi group.

4. Discussion

Physical exercise has been recognized as part of a healthy lifestyle since ancient times, and the influence of physical exercise on the biology of aging is complex. It has also been demonstrated that the practice of moderate intensity physical exercise has an impact on various aspects of aging, including the notable development of an antioxidant response achieved by an adaptive process through mechanisms such as hormesis, in which continuous exposure to low quantities of stressors activates a controlled and beneficial response [8, 20–22].

Walking and Tai Chi are two moderate physical exercise modalities that are widely recommended for the aged. Walking has been suggested as a popular exercise that is accessible, can be performed freely, and can be incorporated into the activities of daily life and be continued into old age [23, 24]. Both the American College of Sports Medicine and the American Heart Association have indicated walking as an adequate alternative for older people because it is a safe activity that does not require special equipment and whose speed can be adjusted as a function of the individual physical condition in order to gradually achieve the required conditioning [9].

Numerous systematic reviews have demonstrated that walking programs have a positive influence on various health indicators including blood pressure, HDL cholesterol, proportion of body fat, aerobic capacity, mental health, and bone density [25–27].

On the other hand, Tai Chi is a traditional Chinese form of exercise, based on modifications of different martial arts. Tai Chi is classified as a moderate physical exercise, as its intensity does not exceed 55% of maximum oxygen consumption and 60% of maximum individual maximum heart rate. Recently, this exercise has increased in popularity because of evidence of the beneficial aspects of this discipline on the various aspects of health. The health benefits are particularly notable in older adults, and its practice does not pose risks, as it is a series of gentle and continuous motions that require control of position, deep breathing, and coordination of movements [10, 28].

The results of the present study, particularly the examination of routine biochemical parameters, indicate that Tai Chi has a positive effect on lipid metabolism. We observed a significant reduction in total cholesterol and in the LDL cholesterol fraction compared with the controls. These results are in accordance with earlier reports that have noted a reduction in total cholesterol, the LDL fraction, and an increase in HDL in various populations after the practice of Tai Chi [29, 30].

The physiological mechanisms that lead to these effects have not been entirely explained; however, the most accepted model suggests that it may be a consequence of the increase in metabolism produced by a chronic increase in muscular activity, as the movements, despite being gentle and low

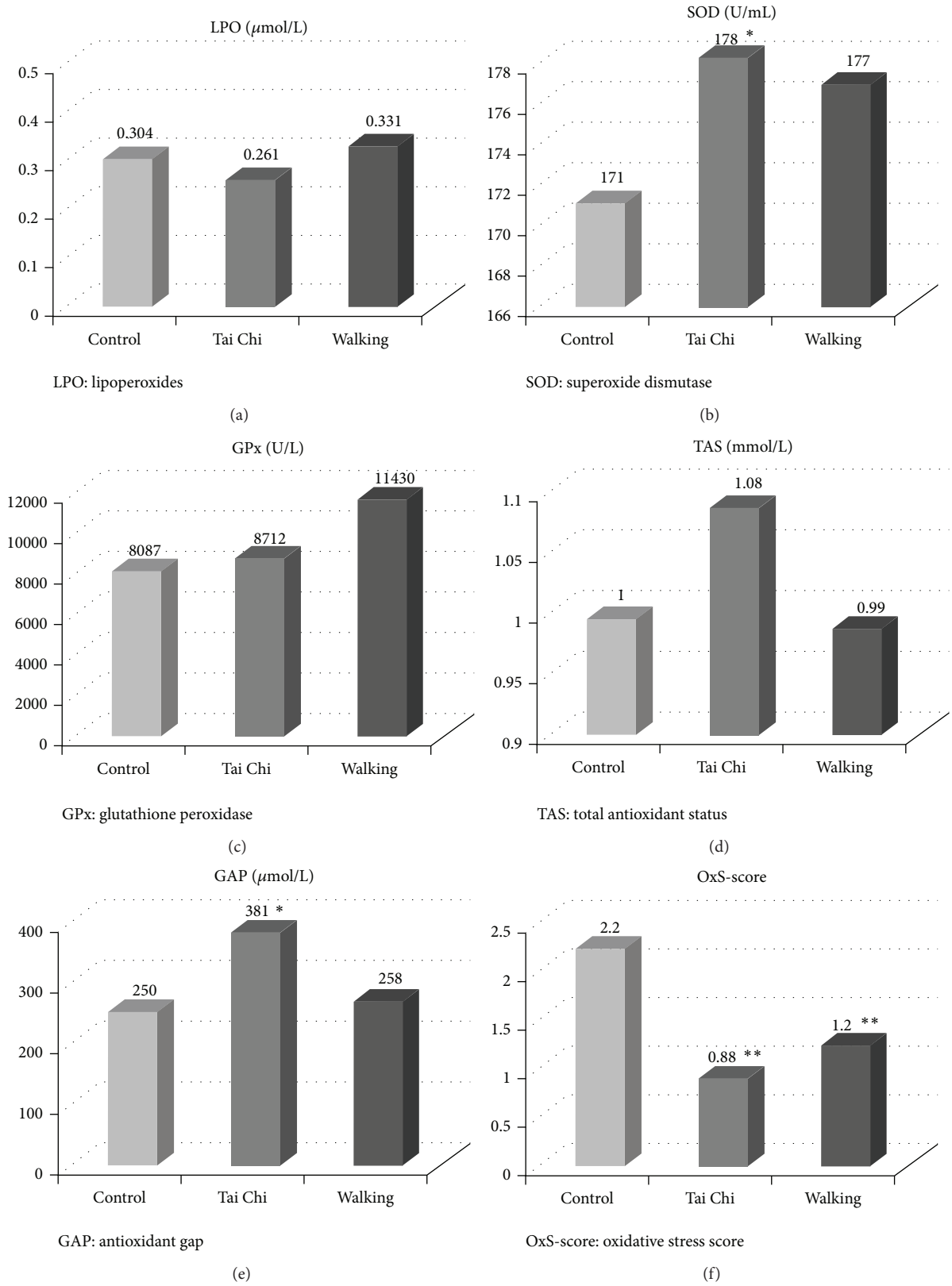

Figure 2: Bars show mean adjusted for initial conditions (ANCOVA Test). (a) LPO: control 0.304 ± 0.023, Tai Chi 0.261 ± 0.02, and walking 0.331 ± 0.02 μmol/L, $P = 0.08$; (b) SOD: control 171 ± 2, Tai Chi 178 ± 2, and walking 177 ± 1 U/mL,$^*P < 0.05$; (c) GPx: control 8087 ± 1113, Tai Chi 8712 ± 923, and walking 11430 ± 711 U/L, $P = 0.07$; (d) TAS: control 1.0 ± 0.035, Tai Chi 1.08 ± 0.029, and walking 0.99 ± 0.22 mmol/L, $P > 0.05$; (e) GAP: control 250 ± 46, Tai Chi 381 ± 37, and walking 258 ± 31 μmol/L, $P < 0.05$; (f) OxS-score: control 2.2 ± 0.18, Tai Chi 0.88 ± 0.15, and walking 1.2 ± 0.12, $P < 0.001$.

impact, require the holding of particular positions, which strengthen the muscle. In addition, these movements are combined with the demand for the expenditure of energy and the degradation of substrates produced by respiration [29–31].

Also, an effect synergy between the process of physical adaptation and the reduction of the dominance of the sympathetic nervous system given the increase in the vagus nerve response has been proposed as physiological mechanisms. The stimulus of the vagus nerve response is considered to be a result of the physiological and psychological relaxation associated with the practice of Tai Chi [28–30].

In the walking group, we did not observe any significant change in the routine biochemical parameters, which could be due to the length of the intervention. Some studies have noted beneficial effects on blood pressure and blood lipids following a longer intervention period [23, 31].

When considering the OxS markers, we observed a significant increase in SOD activity in the Tai Chi group in comparison with the control group, as well as a increase in the GPx activity in the walking group. These results are consistent with results reported by other studies, in which an increase in the antioxidant defense system after the regular performance of moderate physical exercise is often noted [8, 20, 32].

We observed an increase in GPx activity in the walking group versus the control group. Again, these results are consistent with what has been previously reported in other studies. The increase in GPx activity represents a modification that has been noted to be a component of the response of the glutathione system, which is indispensable in maintaining the redox state of the cell [33–35]. In this case, the redox state was altered by a substantial increase in reactive species (evident in this group through the increase of LPO) as a result of the increase in basic aerobic physical exercise such as walking, but it was not observed in the other study group, in which the exercise was aerobic, which could be part of a global response induced by the moderate exercise performed [36].

In the Tai Chi group, SOD activity increased significantly, which may be attributed to the stimulus involved in the constant performance of the exercise. A similar result has been previously reported after 12 months of the Tai Chi practice in adult subjects. Obtaining this response after only six months in our study may be due to the fact that Tai Chi was performed at least four times a week, while in the other studies, it was practiced less frequently [14].

It is important to note that OxS is a dynamic process in which observed modifications should be considered in the context of the entire response [4]. Accordingly, we examined the OxS index in the study subjects and found that value was significantly higher in controls versus the two intervention groups. These results suggest that moderate physical exercise *per se* has an antioxidant effect. Nevertheless, this effect is significantly enhanced when Tai Chi is practiced. The measured LPO levels in the Tai Chi group were significantly lower, and GAP levels were higher, which indicates an increase in the effectiveness of the antioxidant system to control the oxidative challenge represented by Tai Chi. Ultimately, this effectiveness was reflected in the index value of OxS, which was lower in this group.

It has been suggested that the mechanism that explains these findings may relate to an adaptive process influenced by the change in the body's redox balance in favor of more alkaline conditions in the cell. The reactive species generated during physical exercise act as the signal that is necessary for the activation of the MAPK proteins (p38 and ERK1/ERK2), which in turn activate the transcription factor sensitive to the redox state, NF-$\kappa\beta$, via activation of the kinase that phosphorylates the inhibitor of this factor (I$\kappa\beta$). Once freed of its inhibitor, NF-$\kappa\beta$ migrates toward the nucleus where it can promote the synthesis of various antioxidant enzymes such as MnSOD, iNOS, and glutamylcysteine synthetase (GCS) [34]. These enzymes possess binding sites for this factor in the promoter region of their respective genes. An increase in promoter binding is globally manifested as an increase in the antioxidant response consequent to the moderate oxidative stimulus caused by the intensity of physical exercise [34, 37, 38].

The positive effects of Tai Chi may be due to a combination of various mechanisms, including the signaling mentioned previously. In addition to being a moderate form of exercise, it provides a relaxing psychological effect, similar to that reported for transcendental meditation. The practice of deep meditation (which is an essential characteristic of Tai Chi) is associated with lower levels of LPO, which has been explained as an effect of lower activity in the sympathetic nervous system. It has been demonstrated that the contrary effect, psychosocial stress, is accompanied by an increase in catecholamines and prostaglandins, which have been associated with an increase in LPO. Another hypothesis suggests that profound meditation increases levels of the hormone dehydroepiandrosterone, a marker for aging that has been linked with an increase in the activity of antioxidant enzymes. These possibilities, independent of but along with the mechanisms induced by the physical activity directly, allow us to suggest that the practice of Tai Chi promotes an antioxidant response [39–42].

5. Conclusions

The findings of our study suggest that the practice of Tai Chi generates a more intense antioxidant effect than walking, which could be linked with delaying the process of aging. However, these results need to be corroborated by long-term cohort studies.

Conflict of Interests

No financial conflict interests exists.

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Research Article

Age-Related Hearing Loss in Mn-SOD Heterozygous Knockout Mice

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Age-related hearing loss (AHL) reduces the quality of life for many elderly individuals. Manganese superoxide dismutase (Mn-SOD), one of the antioxidant enzymes acting within the mitochondria, plays a crucial role in scavenging reactive oxygen species (ROS). To determine whether reduction in Mn-SOD accelerates AHL, we evaluated auditory function in Mn-SOD heterozygous knockout (HET) mice and their littermate wild-type (WT) C57BL/6 mice by means of auditory brainstem response (ABR). Mean ABR thresholds were significantly increased at 16 months when compared to those at 4 months in both WT and HET mice, but they did not significantly differ between them at either age. The extent of hair cell loss, spiral ganglion cell density, and thickness of the stria vascularis also did not differ between WT and HET mice at either age. At 16 months, immunoreactivity of 8-hydroxydeoxyguanosine was significantly greater in the SGC and SV in HET mice compared to WT mice, but that of 4-hydroxynonenal did not differ between them. These findings suggest that, although decrease of Mn-SOD by half may increase oxidative stress in the cochlea to some extent, it may not be sufficient to accelerate age-related cochlear damage under physiological aging process.

1. Introduction

Age-related hearing loss (AHL), also referred to as presbycusis, is the most common cause of hearing loss in aged people. It occurs in 25–45% of people aged 65 years or older; the prevalence rises with age, ranging from 40% to 66% in people older than 75 years and more than 80% in people older than 85 years [1]. The number of people suffering from this disorder is dramatically growing as the population of older people increases in developed countries such as the USA and Japan. AHL is defined as progressive, bilateral, and symmetrical hearing impairment. It generally begins at high-frequency region and progresses toward the lower-frequency region. AHL is often associated with difficulty in speech discrimination and sound detection and localization. Ultimately, it can affect the cognitive, emotional, and social function. The pathophysiology of AHL therefore needs to be clarified to develop new therapeutic modalities.

AHL is histologically characterized by structural changes in inner ear, such as degeneration of sensory cells, auditory neurons, and cells of the stria vascularis (SV) [2–8]. Nevertheless, AHL does not occur uniformly in humans, and the participation of multiple pathological processes has been assumed. One of the causes of the age-related cochlear degeneration has been attributed to the accumulated effects of numerous insults, including exposure to intense noise and ototoxic drugs. However, age-related cochlear degeneration can occur in the absence of such insults. Hellstrom and Schmiedt [9] reported that mammals reared in quiet environments without exposure to ototoxins showed progressive hearing loss with aging, suggesting the role of genetic and intrinsic factors in addition to environmental factors.

Accumulating evidence has shown that the damage of cochlear hair cell (HC) induced by intense noise and exposure to ototoxic agents such as aminoglycosides and cisplatin is mediated through the generation of reactive oxygen species (ROS) [10–14]. The free radical theory of aging has obtained consensus also in terms of AHL by several lines of evidence [15–17]. Mitochondria are considered to play a key role in aging and AHL as a main source of ROS, and the bulk of mitochondrial ROS is generated at the electron transport chain [18, 19]. A small amount of the electrons leaking from the electron transport chain causes one-electron reduction of oxygen and produces superoxide anion (O_2^-) which is a short-lived free radical [20, 21]. O_2^- is dismutated into hydrogen peroxide (H_2O_2). In the situation of respiratory chain dysfunction due to mutations, increase of O_2^- production can lead to the accumulation of H_2O_2 and other ROS. Once produced, ROS react with macromolecules such as lipid, DNA, and proteins. In particular, mitochondrial DNA (mtDNA) is vulnerable [22–24] to oxidative stress because it is located in the site of oxidative phosphorylation. Damage of mtDNA causes further dysfunction of mitochondria and augments oxidative stress. In the cochlea, cells of the SV [25], spiral ganglion cells (SGCs) [26, 27], and HCs [28] all contain numerous mitochondria and are considered to be susceptible to ROS-induced damage. Moreover, mtDNA damage has been reported to induce apoptosis of the important cochlear structures such as the SGCs [29]. These suggest that mtDNA damage induced by excess generation of ROS is one of the leading causes of AHL [29].

There are several enzymatic antioxidant defense systems, including copper/zinc superoxide dismutase (Cu/Zn-SOD, SOD1), manganese superoxide dismutase (Mn-SOD), catalase, and peroxidase, which convert ROS to neutral and nonreactive molecules. These antioxidant enzymes are considered to be important contributors to cellular homeostasis. Nevertheless, the roles of the antioxidant enzymes in the free radical theory of aging are controversial because of the inconsistent findings. For example, mice heterozygous for Mn-SOD have reduced activity of the enzyme, increased oxidative damage, but normal life span [30]. Overexpression of antioxidant enzymes Cu/Zn-SOD does not extend lifespan in mice [31]. The median lifespan of mice heterozygous of glutathione peroxidase 4 is significantly longer than that of wild-type mice in spite of increased sensitivity to oxidative stress-induced apoptosis [32].

Several previous studies assessed the importance of antioxidant enzymes in AHL. In Cu/Zn-SOD transgenic mice, absence of Cu/Zn-SOD resulted in a very large loss of auditory neurons and HCs and an early onset of hearing loss [33–35]. Conversely, heterozygous Cu/Zn-SOD knockout mouse, which had reduced expression of the enzyme, maintained hearing and normal cochlear morphology [33]. These results suggest that even half as much Cu/Zn-SOD is sufficient to maintain cochlear function and morphology under normal physiological condition.

To date, only a few studies have been available which examined the role of Mn-SOD in AHL. Mn-SOD, one of the antioxidant enzymes located in the mitochondrial matrix, plays an important role to protect mtDNA from oxidative stress. Le and Keithley [36] evaluated the hearing function in Mn-SOD heterozygous knockout mice and found no difference in the extent of hearing loss when compared to the background strain, although they did not examine the histological and immunohistochemical findings in the cochlea. We hypothesize that histological and immunohistochemical evaluation for oxidative markers would detect subtle changes reflecting the reduction of Mn-SOD even when functional assessments fail to detect the differences between Mn-SOD heterozygous knockout mice and the background strain.

In the present study, we conducted histological evaluation and immunostaining using anti-Mn-SOD, anti-4-HNE, and 8-OHdG antibody of the cochlea, as well as functional assessment by auditory brainstem response (ABR) in Mn-SOD heterozygous knockout mice and compared them with those in the background strain C57BL/6 mice.

2. Materials and Methods

2.1. Animals. Mn-SOD lox/lox mice were generated by one of the authors (Takahiko Shimizu) at the Molecular Gerontology Laboratory in Tokyo Metropolitan Institute of Gerontology, as described previously [37]. These mice were backcrossed with C57BL/6NCrSlc mice for five or six generations. The crossbreeding of homozygous Mn-SOD lox/lox mice with the chicken actin promoter (CAG)-Cre transgenic mice [38] of a C57BL/6 background gave rise to systemic heterozygous Mn-SOD-deficient (HET) mice. These mice presented systemically only half as much Mn-SOD. As a control, their littermate wild-type (WT) C57BL/6 mice were used. All animals were kept at 22 ± 1°C under a 12-hour light/12-hour dark cycle and had free access to water and regular mouse diet (MF, Oriental Yeast Co., Tokyo, Japan).

We employed a total of 22 mice in the current study: 4-month-old WT group ($n = 6$), a 16-month-old WT group ($n = 5$), a 4-month-old HET group ($n = 6$), and a 16-month-old HET group ($n = 5$). All animals underwent ABR assessment, after which they were euthanized for evaluation of cochlear pathology and immunohistochemistry.

Experiment protocol was approved by the Institutional Review Board of the Faculty of Medicine, University of Tokyo. All the procedures were performed in accordance with the guidelines of the University Committee for the Use and Care of Animals, University of Tokyo, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Assessment of Hearing Function. Detailed protocols for ABR measurements have been described elsewhere [39]. Briefly, two examiners who were blind to the experiment measured ABRs with a tone burst stimulus (4, 8, 16, and 32 kHz) using an ABR recording system (Neuropack Σ MEB5504, Nihon Kohden, Tokyo, Japan). Mice were anesthetized with a mixture of xylazine hydrochloride (10 mg/kg, i.m.) and ketamine hydrochloride (40 mg/kg, i.m.). Needle electrodes were placed subcutaneously at the vertex (active electrode), beneath the left pinna (reference electrode), and beneath the right ear (ground). The stimulus duration was 15 ms. Responses of 1024 sweeps were averaged at each intensity level (5 dB steps) to assess threshold. Threshold was

defined as the lowest intensity level at which a clear reproducible waveform was visible in the trace. ABR thresholds were measured for WT and HET mice at 4 or 16 months of age. To obtain ABR input/output (I/O) functions, the wave I peak amplitude was identified by visual inspection at each stimulus level. All data were reported as mean ± SD.

2.3. Histological Evaluation and Immunostaining. After ABR measurements, all animals were euthanized under deep anesthesia with xylazine hydrochloride and ketamine hydrochloride, and the cochleae were dissected out. They were immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) overnight at 4°C and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution. The specimens were then dehydrated through a graded alcohol series and embedded in paraffin. The embedded tissues were cut into 5 μm thick sections parallel to the modiolus, and every three sections were mounted on glass slides and deparaffinized. Every three slides containing 5 sections were stained with hematoxylin and eosin and observed under a light microscope (Nikon Eclipse E800M, 40x obj.) for evaluating HC survival rates, SGC densities, and strial thicknesses. The other slides were used for immunohistochemical evaluation. We used samples from 6 mice from 4-month-old groups and 5 mice from 16-month-old groups. All data were reported as mean ± SD.

2.3.1. Hair Cell Survival Rate. HCs were counted as present if the cell body and cuticular plate looked intact. The number of remaining HCs at the lower-basal, upper-basal, and lower-middle turns was counted at least in 10 sections per animal. We calculated the inner and outer hair cell (IHC and OHC) survival rates of these three turns in each animal by using the following formulae: IHC survival rate% = 100 × [(the number of present IHCs of examined specimens)/the number of examined specimens]; OHC survival rate% = 100 × [(the number of present OHCs of examined specimens)/the number of examined specimens/3].

2.3.2. Spiral Ganglion Cell Density. An unbiased investigator inspected the collection of mid-modiolar sections generated for each cochlea and selected the slides that had high-quality sections. From those selected, three sections from each cochlea were randomly chosen for counting. The number of SGCs and the area of Rosenthal's canal of the basal turns were measured in digital photomicrographs (40x obj.) using Photoshop CS4 software, and SGC density (SGC number/mm^2) was calculated, as previously reported [40]. SGCs in each profile were counted on the computers monitor. The area (mm^2) of the Rosenthal's canal profile was measured in each photomicrograph by outlining the margin of bony canal with a calibrated computer mouse. The area of the outline was calculated using Photoshop CS4 software. The density of SGC was calculated for each profile of the ganglion.

2.3.3. Stria Vascularis Thickness. The thicknesses of the SV in radial sections of the basal turns were measured in digital photomicrographs (40x obj.) using Photoshop CS4 software. A line was drawn from the strial margin to the spiral ligament junction half way between the attachment of Reissner's membrane and the spiral prominence using a calibrated computer mouse, and the length of the line was calculated by the computer. Three sections from each turn in each cochlea were measured.

2.3.4. Immunohistochemistry. The cochleae were fixed in PBS-buffered 4% paraformaldehyde for 24 hrs and decalcified with 10% EDTA solution (pH 7.0). After embedding in paraffin, 5 μm sections were cut and mounted on silane-coated slides. Deparaffinized sections were autoclaved in citrate-buffered saline (PH 4.0) for 20 minutes for antigen retrieval. Immunohistochemistry was performed using either of the following antibodies: anti-Mn-SOD antibody (rabbit monoclonal antibody, Epitomics Inc., San Francisco CA, USA; 1 : 100 dilution), anti-8-OHdG antibody (goat polyclonal antibody, Alpha Diagnostic International Inc., San Antonio, TX, USA; 1 : 100 dilution), and anti-4-HNE antibody (rabbit polyclonal antibody, Alpha Diagnostic International Inc., San Antonio, TX, USA; 1 : 100 dilution). Immunoreaction was detected using the following secondary antibody systems: Histofine Simple Stain MAX-PO (G) (Nichirei Corp., Tokyo, Japan). Ten randomly selected high-power fields (×400) from three section prepared from each mouse were examined under light microscope. The labeling index of each antibody was obtained by a modified Photoshop-based image analysis as described previously [41].

2.4. Statistical Analysis. SigmaStat statistical software was used and all data were expressed as mean ± SD. ABR thresholds, HC survival rates, SGC densities, and SV thicknesses were compared among groups by two-way analysis of variance (ANOVA), and then pairwise comparisons were performed by using Scheffe's test.

3. Results

3.1. Systemic Findings. Generally, WT and HET mice looked similar at 4 months and 16 months of age. The mean body weights of 4-month-old WT and HET mice and 16-month-old WT and HET mice were 24.7 ± 1.03 g (range 23 to 26 g), 24.5 ± 1.52 g (range 23 to 27 g), 30.6 ± 1.52 g (range 29 to 33 g), and 31.4 ± 1.67 g (range 30 to 34 g), respectively. The body weights did not significantly differ between HET and WT mice at either age.

3.2. Hearing Function. HET and WT mice showed nearly normal ABR thresholds at 4, 8, and 16 kHz and only slightly increased thresholds at 32 kHz at 4 months of age (Figure 1). At 16 months, ABR thresholds were significantly increased at all frequencies tested in both HET and WT mice, but they did not significantly differ between HET and WT mice at either age at any frequency tested.

We also employed ABR wave I amplitude I/O functions to assess the gross activity of the mouse auditory nerve. As shown in Figures 2(a)–2(d), the slopes of the I/O functions were similar between WT and HET mice at all frequencies

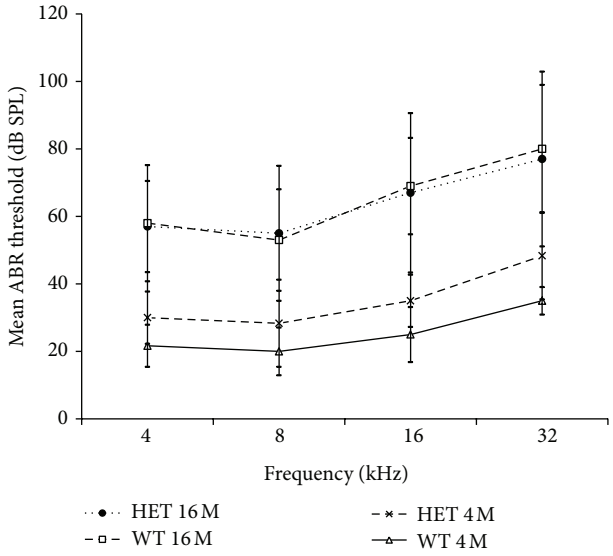

Figure 1: ABR thresholds (mean ± SD) of HET and WT mice at 4 and 16 months of age.

tested at either 4 or 16 months of age. The maximum wave I amplitudes were reduced significantly at all frequencies in 16-month-old WT and HET mice compared to 4-month-old WT and HET mice, but they did not significantly differ between HET and WT mice at either age.

3.3. Hair Cell Survival Rate. Both 16-month-old WT and HET mice displayed loss of IHCs in the basal turn, more significantly in the lower-basal turn (Figures 3(g), 3(j)), whereas 4-month-old WT and HET mice displayed no or only a little loss of IHCs in all turns (Figures 3(a)–3(f), 3(h), 3(i), 3(k), and 3(l)). The IHC survival rates in 4-month-old WT and HET mice and 16-month-old WT and HET mice were 96.7 ± 8.16%, 100 ± 0%, 77.0 ± 17.9%, and 80.4 ± 14.2% in the lower-basal turn, respectively; 100 ± 0%, 100 ± 0%, 89.3 ± 15.4%, and 81.3 ± 13.2% in the upper-basal turn, respectively; and 100 ± 0%, 100 ± 0%, 100 ± 0%, and 96 ± 8.9% in the lower-middle turn, respectively. The IHC survival rates were significantly reduced at the lower-basal and upper-basal turns in 16-month-old HET mice when compared to 4-month-old HET mice. They were also significantly reduced at the lower-basal turn in 16-month-old WT mice compared to 4-month-old WT mice. However, the extent of IHC loss did not significantly differ between HET and WT mice at either age in any of the turns (Figure 4(a)).

Similar trends were also observed in the extent of OHC loss. Both 16-month-old WT and HET mice displayed severe loss of OHCs (Figures 3(g)–3(l)), whereas 4-month-old WT and HET mice displayed no or only a little loss of OHCs (Figures 3(a)–3(f)). The OHC survival rates in 4-month-old WT and HET mice and 16-month-old WT and HET mice were 94.5 ± 4.5%, 86.6 ± 9.9%, 51.0 ± 18.3%, and 34.2 ± 13.4% in the lower-basal turn, respectively; 94.5 ± 4.5%, 89.4 ± 9.4%, 56.4 ± 13.8%, and 42.6 ± 14.4% in the upper-basal turn, respectively; and 95.9 ± 4.8%, 93.5 ± 7.4%, 81.3 ± 7.3%, and 68.0 ± 11.0% in the lower-middle turn, respectively. In either HET or WT mice, the OHC survival rates were significantly reduced in all turns at 16 months when compared to those at 4 months. However, they were not significantly different between HET and WT mice at either age in all turns (Figure 4(b)).

3.4. Spiral Ganglion Cell Density. At 4 months of age, the mean SGC densities of the basal turn were 4,263 ± 1,360/mm^2 and 4,157 ± 259/mm^2, and those of the middle turn were 5,431 ± 117/mm^2 and 5269 ± 417/mm^2 in the HET and WT mice, respectively. At 16 months, the mean SGC densities of the basal turn were 2,407 ± 1,595/mm^2, and 2,993 ± 1,554/mm^2, and those of the middle turn were 5,269 ± 417/mm^2 and 4,134 ± 583/mm^2 in the HET and WT mice, respectively. In either HET or WT mice, the SGC densities were significantly lower in both the basal and apical turns at 16 months compared to those at 4 months; however, they were not significantly different between HET and WT mice at either age (Figures 5(a) and 5(b)).

3.5. Stria Vascularis Thickness. At 4 months of age, the strial thickness in the basal turn was 16.4 ± 2.8 μm in the HET mice and 18.1 ± 2.1 μm in the WT mice, and that in the middle turn was 18.5 ± 3.4 μm in the HET mice and 18.8 ± 1.6 μm in the WT mice. At 16 months, the strial thickness in the basal turn was 13.2 ± 2.3 μm in the HET mice and 13.5 ± 1.7 μm in the WT mice, and that in the middle turn was 15.2 ± 1.8 μm in the HET mice and 17.2 ± 3.1 μm in the WT mice. While the strial thickness was significantly ($P < 0.05$) decreased from 4 months to 16 months in both HET and WT mice, it was not significantly different between HET and WT mice at either age (Figures 6(a) and 6(b)).

3.6. Expression of Mn-SOD. In anti-Mn-SOD immunostaining, immunopositive cells were more abundant in the SGC in the basal turn in WT mice compared to HET mice at both ages (Figure 7(a)). The labeling indices of Mn-SOD at 4 months were 6.61 ± 3.69 in WT mice and 3.01 ± 0.64 in HET mice, and those at 16 months were 5.27 ± 2.50 in WT mice and 1.18 ± 0.17 in HET mice (Figure 7(b)). In HET mice, the labeling indices of Mn-SOD were 45.6% and 22.5% of those in WT mice at 4 and 16 months of age, respectively, with statistically significant differences between them at either age ($P < 0.05$). From 4 months to 16 months of age, the labeling indices of Mn-SOD showed 20.3% decrease in WT mice and 60.8% decrease in HET mice; the labeling indices of Mn-SOD differed significantly ($P < 0.05$) between 4 and 16 months in both WT and HET mice.

In the SV in the basal turn, the immunoreactivities of Mn-SOD were markedly greater in WT mice compared to HET mice at both ages (Figure 8(a)). The labeling indices of Mn-SOD at 4 months were 3.50 ± 1.58 in WT mice and 1.52 ± 0.24 in HET mice, and those at 16 months were 2.97 ± 1.27 in WT mice and 1.54 ± 0.22 in HET mice (Figure 8(b)). In HET mice, the labeling indices of Mn-SOD were 43.4% and 51.9% of those in WT mice at 4 and 16 months of age, respectively, with statistically significant differences between

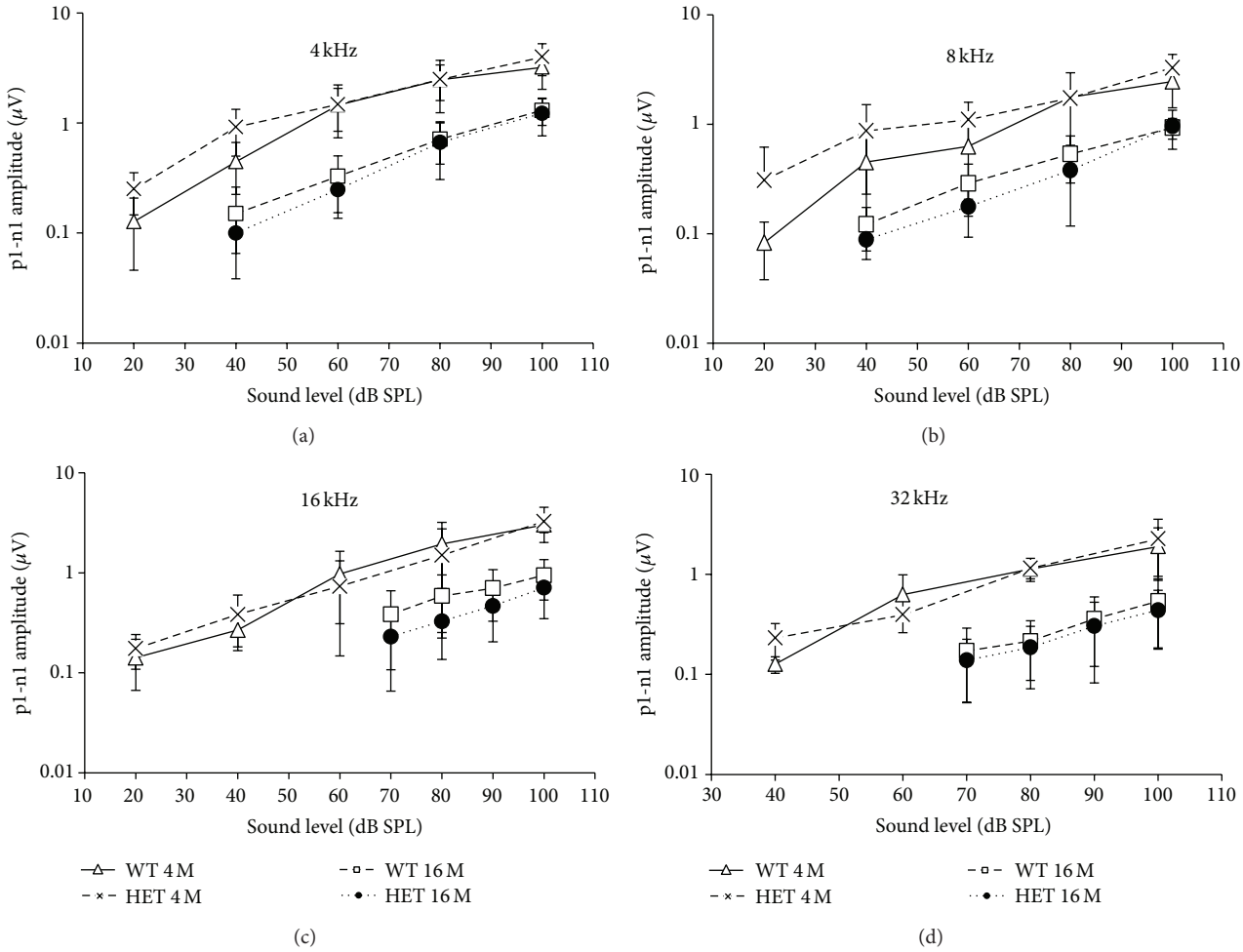

Figure 2: ABR wave I amplitude I/O functions (mean ± SD) at 4, 8, 16, and 32 kHz in HET and WT mice at 4 and 16 months of age.

them at either age ($P < 0.05$). Different from the SGC, the labeling indices of Mn-SOD in SV did not significantly change from 4 months to 16 months in either WT or HET mice.

3.7. Expression of 8-OHdG. In anti-8-OHdG immunostaining, in the SGC in the basal turn, the immunopositive cells were more abundant at 16 months compared to 4 months in both mice (Figure 9(a)). The labeling indices of 8-OHdG in WT and HET mice at 4 months were 23.7 ± 7.3 and 22.6 ± 3.7, respectively, and those at 16 months were 33.8 ± 6.3 and 44.0 ± 6.6, respectively (Figure 9(b)). The labeling indices of 8-OHdG at 16 months were significantly ($P < 0.05$) greater compared to those at 4 months in both mice. Also, the labeling indices of 8-OHdG showed a significant difference ($P < 0.05$) between HET and WT mice at 16 months of age.

In the SV in the basal turn, the immunoreactivity was the strongest in 16-month-old HET mice, while the immunoreactivity was almost the same among WT mice at both 4 and 16 months and HET mice at 4 months (Figure 10(a)). In the SV in the basal turn, the labeling indices of 8-OHdG of WT and HET mice were 13.4 ± 6.9 and 13.0 ± 4.9 at 4 months, respectively, and were 13.0 ± 4.2 and 20.2 ± 2.0 at 16 months, respectively (Figure 10(b)). The labeling indices of 8-OHdG in HET mice at 16 months were significantly ($P < 0.05$) greater compared to those at 4 months. Also, the labeling indices of 8-OHdG were significantly different ($P < 0.05$) between HET and WT mice at 16 months.

3.8. Expression of 4-HNE. In anti-4-HNE immunostaining, in the SGC in the basal turn, immunopositive cells were more abundant at 16 months of age compared to 4 months of age in both WT and HET mice (Figure 11(a)). The labeling indices of 4-HNE in WT and HET mice at 4 months were 11.8 ± 3.1 and 11.4 ± 4.5, respectively, and those at 16 months were 22.6 ± 6.4 and 25.2 ± 6.7, respectively (Figure 11(b)). The labeling indices of 4-HNE at 16 months in both WT and HET mice were significantly ($P < 0.05$) greater than those at 4 months, but there were no significant differences between HET and WT mice at either age.

In the SV in the basal turn, the labeling indices of 4-HNE in WT and HET at 4 months were 8.70 ± 3.2 and 10.4 ± 2.2, respectively, and those at 16 months were 11.5 ± 3.6 and 13.1 ± 5.4, respectively (Figure 12(b)). The labeling indices of 4-HNE tended to be greater in HET mice than in WT mice at either age and tended to increase with age in both HET and WT

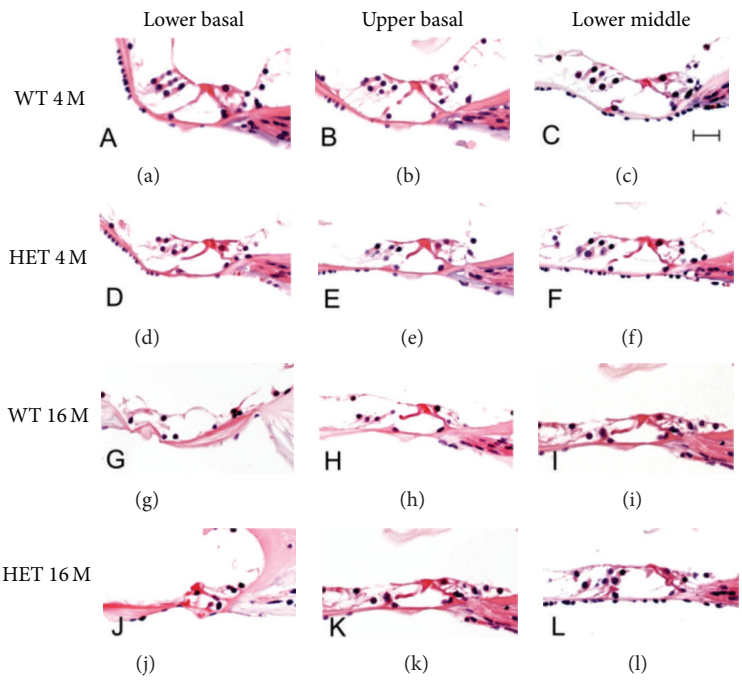

Figure 3: Representative photomicrographs of the organ of Corti in the lower-basal, upper-basal, and lower-middle turns from 4- and 16-month-old WT and HET mice. Scale bar: 25 μm.

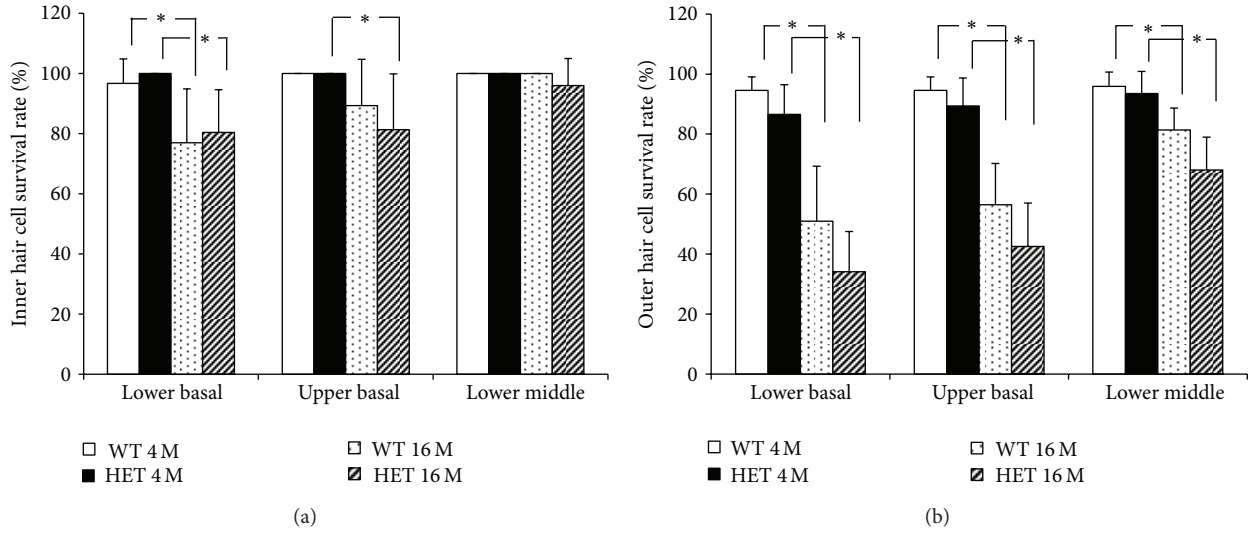

Figure 4: The survival rates (mean ± SD) of the inner (a) and outer (b) hair cells in the lower-basal, upper-basal, and lower-middle turns from 4- and 16-month-old WT and HET mice from each experimental group. *: $P < 0.05$.

mice, but they were not significantly different between WT and HET mice at either age or between 4 and 16 months of age in either of the mice.

4. Discussion

In the current study, HET mice did not show acceleration in ABR threshold shifts with aging when compared to WT mice. This physiological finding was confirmed by histological analysis, which revealed no significant differences in HC survival rates, SGC density, and SV thickness between HET and WT mice at either 4 or 16 months of age. The HET mice had reduced Mn-SOD activity (~50%) in the SGC and SV at either age when compared to WT mice. The expression of 8-OHdG, a marker of DNA oxidation, was increased in the SGC with aging in both WT and HET mice, and the expression of 8-OHdG in the SGC and SV at 16 months was significantly greater in HET mice compared to WT mice. The expression of 4-HNE, a marker of lipid peroxidation, was increased in the SGC with aging and tended to increase in the SV in both HET

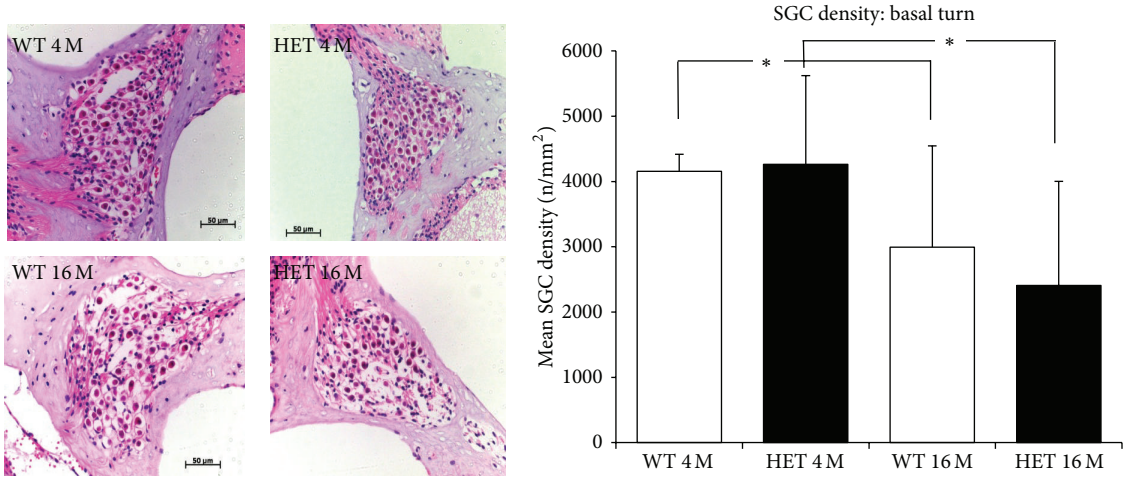

Figure 5: (a) Representative photomicrographs of Rosenthal's canal in the basal turn from 4- and 16-month-old WT and HET mice. Scale bar: 50 μm. (b) SGC densities (mean ± SD) in the modiolar sections from the basal turn in 4- and 16-month-old WT and HET mice. *: $P < 0.05$.

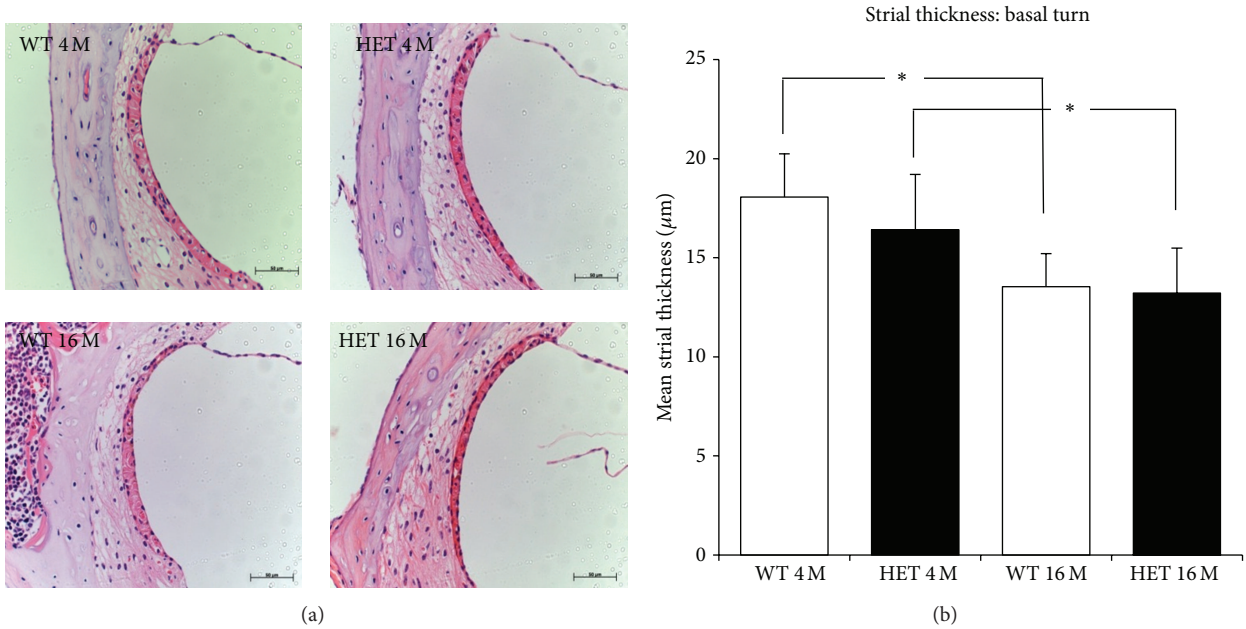

Figure 6: (a) Representative photomicrographs of SV in the basal turn from 4- and 16-month-old WT and HET mice. Scale bar: 50 μm. (b) Strial thickness (mean ± SD) in the modiolar sections from the basal turn in 4- and 16-month-old WT and HET mice. *: $P < 0.05$.

and WT, although it was not significantly different between HET and WT mice. These findings suggest that half reduction of Mn-SOD may accelerate oxidative stress, predominantly to DNA, to some extent, but may not be sufficient to increase the damage to the cochlear tissues under normal aging process. Since both WT and HET mice carry a specific mutation in the cadherin 23 gene, it is possible that the effect of Mn-SOD might be masked by C57 genetic pathology. However, we enrolled wild-type C57BL/6 littermates as a control, which we consider made the influence of the C57 background minimal.

It has been reported that mice lacking Cu/Zn-SOD exhibited 30% decrease in life expectancy [42], whereas overexpression of Cu/Zn-SOD and catalase extended lifespan in *Drosophila* [43]. Further, small synthetic mimetics of SOD/catalase increased lifespan in *C. elegans* [44]. Collectively, these results imply that interplay between ROS and protective responses by antioxidant enzymes is an important factor in determining aging and lifespan. Nevertheless, the role of these antioxidant enzymes in aging is still controversial. *Sod2*+/− mice have been reported to have reduced Mn-SOD activity (~50%) in all tissues throughout life, increased oxidative damage as demonstrated by elevation of 8-OHdG in all tissues (significantly higher compared with WT mice), and increase in tumor incidence. However, the lifespans of *Sod2*+/− mice were identical to those of WT mice and biomarkers of aging, such as cataract formation, immune

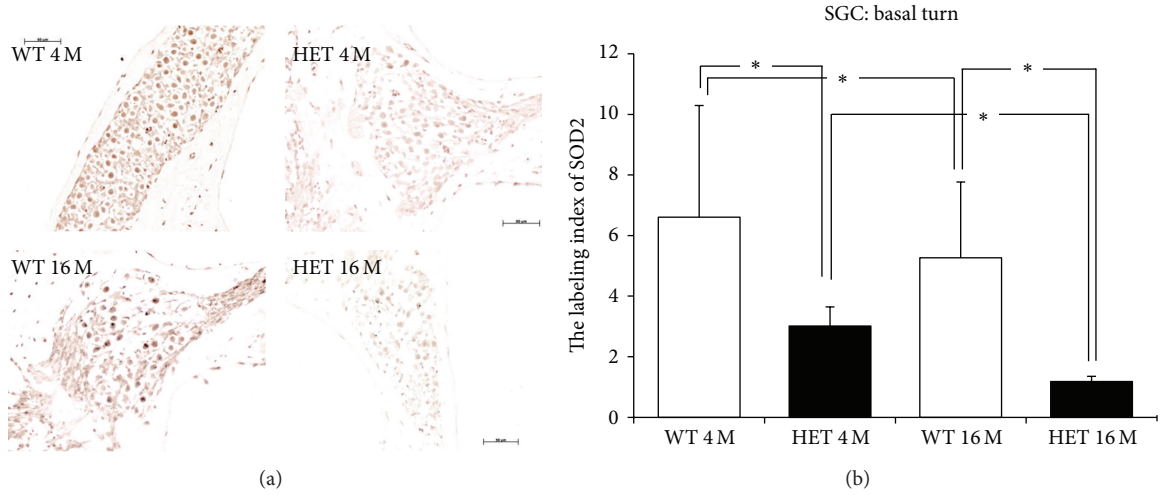

Figure 7: (a) Representative photomicrographs of immunostaining with anti-Mn-SOD antibody of the SGC in the basal turn from 4- and 16-month-old WT and HET mice. Scale bar: 50 μm. (b) SGC labeling indices (mean ± SD) of Mn-SOD in the basal turn in 4- and 16-month-old WT and HET mice. *: $P < 0.05$.

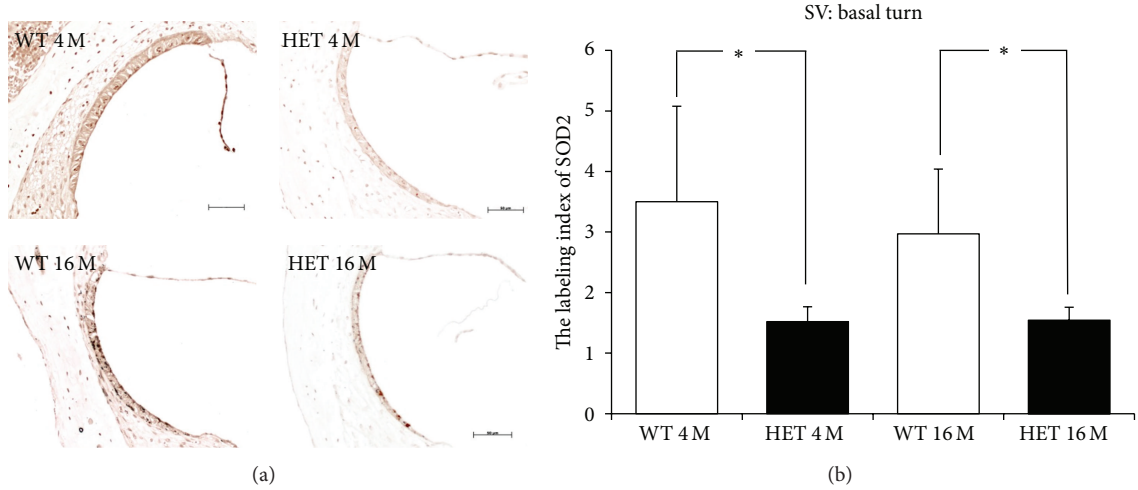

Figure 8: (a) Representative photomicrographs of immunostaining with anti-Mn-SOD antibody of the SV in the basal turn from 4- and 16-month-old WT and HET mice. Scale bar: 50 μm. (b) SV labeling indices (mean ± SD) of Mn-SOD in the basal turn in 4- and 16-month-old WT and HET mice. *: $P < 0.05$.

response, and formation of glycoxidation products carboxymethyl lysine and pentosidine in skin collagen changed with age to the same extent in both WT and *Sod2*+/− mice [30], indicating that life-long reduction of Mn-SOD activity leads to increased levels of oxidative damage to DNA and increased cancer incidence but does not appear to affect aging. Overexpression of antioxidant enzymes in mice, such as Cu/Zn-SOD or catalase, did not extend lifespan [31, 45]. The median lifespan of mice heterozygous of glutathione peroxidase 4 was significantly longer than that of wild-type mice, even though they showed increased sensitivity to oxidative stress-induced apoptosis [32]. These results pose a question in terms of the importance of antioxidant enzymes in preventing aging process.

As of age-related degeneration in the cochlea, it has been reported that overexpression of Cu/Zn-SOD did not prevent or slow AHL, whereas Cu/Zn-SOD KO mice exhibited acceleration of AHL due to massive loss of HCs and auditory neurons in an earlier onset [34]. Interestingly, half-expressed Cu/Zn-SOD did not accelerate AHL in mice [33]. Similarly, Le and Keithley [36] reported that Mn-SOD heterozygous transgenic mice showed no deterioration in the extent of ABR threshold shifts compared to the background strain but did not address the histological findings. Although it is ideal to investigate Mn-SOD homozygous knockout mice to assess the importance of Mn-SOD in AHL, systemic Mn-SOD-deficient mice are known to die at an early stage after birth. Because of this reason, we are forced to use Mn-SOD heterozygous knockout mice, but we examined not only their auditory function but also cochlear histology and immunohistology. The present study demonstrated no significant difference in ABR thresholds between HET and WT mice

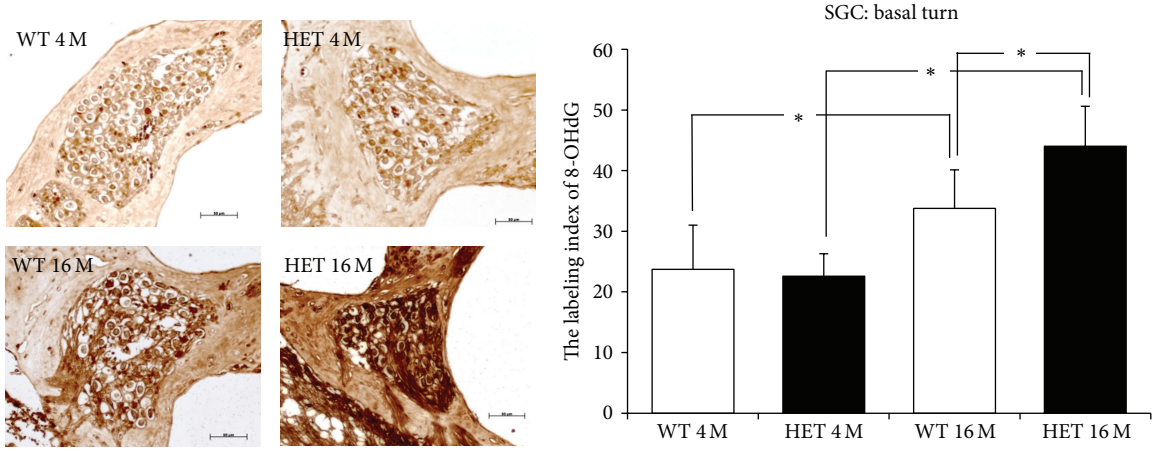

Figure 9: (a) Representative photomicrographs of immunostaining with anti-8-OHdG antibody of the SGC in the basal turn from 4- and 16-month-old WT and HET mice. Scale bar: 50 μm. (b) SGC labeling indices (mean ± SD) of 8-OHdG for the basal turn in 4- and 16-month-old WT and HET mice. *: $P < 0.05$.

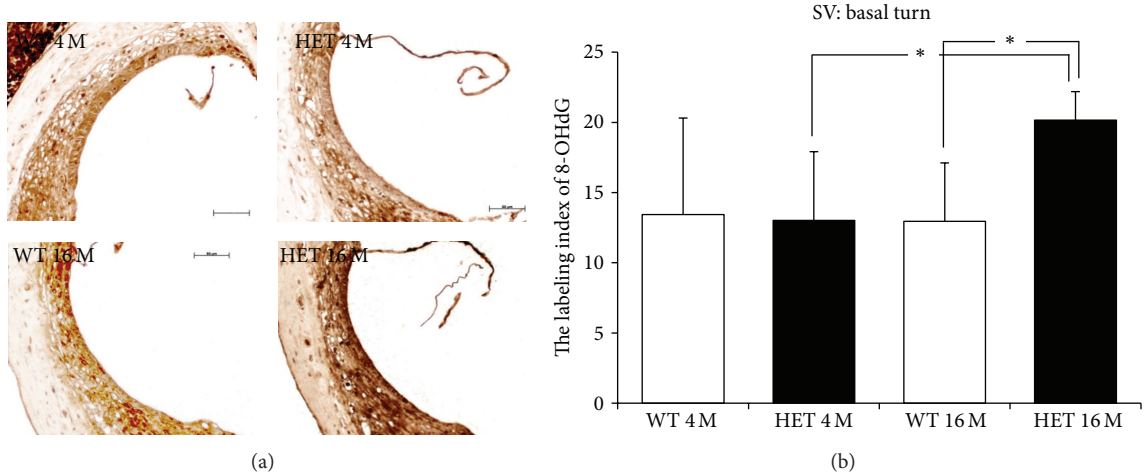

Figure 10: (a) Representative photomicrographs of immunostaining with anti-8-OHdG antibody of the SV in the basal turn from 4- and 16-month-old WT and HET mice. Scale bar: 50 μm. (b) SV labeling indices (mean ± SD) of 8-OHdG for the basal turn in 4- and 16-month-old WT and HET mice. *: $P < 0.05$.

at either 4 or 16 months of age, which is consistent with the report by Le and Keithley [36]. We also found no significant differences in SGC densities or SV thickness between HET and WT mice at either 4 or 16 months, supporting that reduction of Mn-SOD by half did not accelerate age-related damage in the cochlea.

In the present study, the labeling indices of Mn-SOD in the SGC and SV in HET mice were reduced to be ~50% of those of WT mice at either 4 or 16 months of age. The expression of Mn-SOD was declined with aging in the SGC in both WT and HET mice, whereas it was unchanged with aging in the SV. These results are in harmony with the report by Jiang et al. [46] that immunoreactivity of Mn-SOD was decreased with aging in the SGC but not in the SV in CBA/J mice. These suggest that age-related decline of Mn-SOD expression differs among tissues even in the same organ, namely, the cochlea.

In the SGC, the expression of 8-OHdG was significantly increased with aging in both WT and HET mice and was significantly greater in HET mice at 16 months compared to WT mice. On the other hand, in the SV, the expression of 8-OHdG was significantly increased with aging only in HET mice, being significantly greater at 16 months in HET mice compared to WT mice. These findings suggest that, under normal condition, 8-OHdG accumulates steadily in the SGC but not significantly in the SV. This appears reasonable, considering that Mn-SOD is decreased with aging in the SGC but not in the SV. Under pathological situation that Mn-SOD is decreased by half, 8-OHdG may accumulate with aging more significantly in the SGC and even in the SV.

The expression of 4-HNE was increased with aging in the SGC in both WT and HET mice, and there was no significant difference in the expression level between these mice. The expression of 4-HNE did not significantly differ in the SV

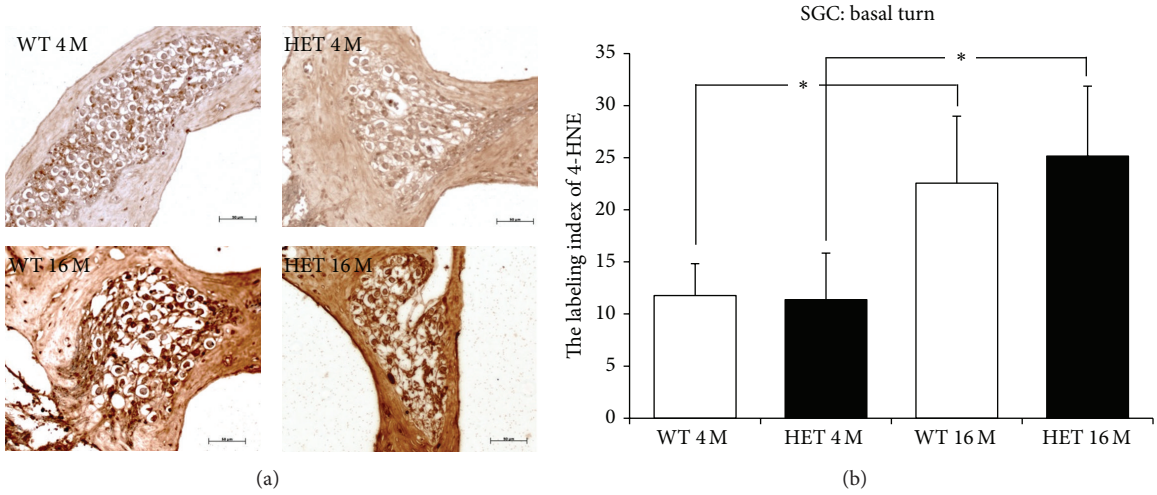

Figure 11: (a) Representative photomicrographs of immunostaining with anti-4-HNE antibody of the SGC in the basal turn from 4- and 16-month-old WT and HET mice. Scale bar: 50 μm. (b) SGC labeling indices of 4-HNE for the basal turn in 4- and 16-month-old WT and HET mice. *: $P < 0.05$.

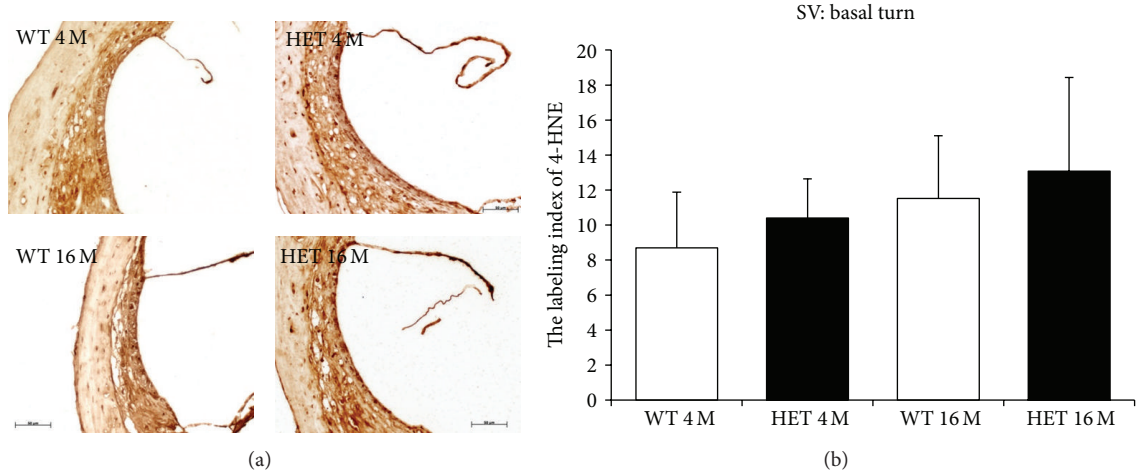

Figure 12: (a) Representative photomicrographs of immunostaining with anti-4-HNE antibody of the SV in the basal turn from 4- and 16-month-old WT and HET mice. Scale bar: 50 μm. (b) SV labeling indices of 4-HNE in the basal turn in 4- and 16-month-old WT and HET mice.

between WT and HET mice at any age, although it was slightly greater in HET mice than in WT mice at either 4 or 16 months of age and slightly increased with aging in both HET and WT mice. These findings suggest that, under normal condition, 4-HNE accumulates with aging in the SGC but not significantly so in the SV. It is unclear why reduction of Mn-SOD by half increased the expression of 8-OHdG but not 4-HNE in the SGC with aging. Downregulation of Mn-SOD might contribute to the augmentation of oxidative DNA stress more significantly than lipid peroxidation in the cochlear tissues.

Although we did not find significant differences of ABR threshold shift or the extent of degeneration of the SGC and SV between HET and WT mice under normal physiological condition, it is possible that reduction of Mn-SOD by half accelerates age-related cochlear damage under pathological condition. It has been reported that, when Mn-SOD heterozygote mice were administered hepatotoxic agent that promoted ROS generation, liver damage became prominent [47], although liver-specific Mn-SOD homozygous knockout mice presented no obvious morphological or biochemical damages under normal environmental stress [37]. This result implies that, even if tissue damage is not evident under normal condition, it can become prominent under the burden of oxidative stress. It is well known that inner ear damage by intense noise and ototoxic drugs is mediated through excessive generation of ROS [48]. To verify the hypothesis, functional and morphological assessment should be carried out in Mn-SOD homozygous knockout mice under pathological oxidative stress. We are now conducting

an experiment to investigate whether HET mice will exhibit acceleration of AHL compared to WT mice when they are raised in noisy environment.

5. Conclusion

The ABR thresholds were significantly increased from 4 months to 16 months in both WT and HET mice, but they did not significantly differ between WT and HET mice at either age. The HC survival rates, SGC density, and strial thickness did not differ between WT and HET mice at either age. At 16 months, immunoreactivity of 8-hydroxydeoxyguanosine was significantly greater in the SGC and SV in HET mice compared to WT mice, but that of 4-hydroxynonenal did not differ between them. These findings suggest that, although decrease of Mn-SOD by half may increase oxidative stress in the cochlea to some extent, it may not be sufficient to accelerate age-related cochlear damage under physiological aging process. Further study is needed to examine if reduction of Mn-SOD may accelerate AHL under pathological condition such as in a noisy environment.

Acknowledgments

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Review Article

Chrononutrition against Oxidative Stress in Aging

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Free radicals and oxidative stress have been recognized as important factors in the biology of aging and in many age-associated degenerative diseases. Antioxidant systems deteriorate during aging. It is, thus, considered that one way to reduce the rate of aging and the risk of chronic disease is to avoid the formation of free radicals and reduce oxidative stress by strengthening antioxidant defences. Phytochemicals present in fruits, vegetables, grains, and other foodstuffs have been linked to reducing the risk of major oxidative stress-induced diseases. Some dietary components of foods possess biological activities which influence circadian rhythms in humans. Chrononutrition studies have shown that not only the content of food, but also the time of ingestion contributes to the natural functioning of the circadian system. Dietary interventions with antioxidant-enriched foods taking into account the principles of chrononutrition are of particular interest for the elderly since they may help amplify the already powerful benefits of phytochemicals as natural instruments with which to prevent or delay the onset of common age-related diseases.

1. Introduction

Understanding the aging process has gained in importance with people's increasing life expectancy. Chronological age is the strongest predictor of chronic diseases, and the scientific community is searching for protective agents that can contribute to preventing or delaying the onset of many age-related diseases.

The complexity of the holistic systems of which living cells form a part makes it difficult to distinguish between the causes and consequences of aging. Indeed, it is still unknown whether this process derives from a single or multiple causes [1]. Theories of aging are mainly divided into those assuming that aging is genetically encoded and those assuming that it is due to a decline in maintenance mechanisms and exponential accumulation of molecular damage resulting in degeneration and dysfunction at the cellular level [2]. Among the latter theories, the free radical theory of aging (also known as oxidative stress theory) put forward by Harman in 1956 [3] has received extensive support. It posits that the organism's deterioration resulting from increasing longevity is above all a consequence of the persistent accumulation of free radical mediated damage to essential molecules. This accumulation gradually compromises cell and tissue function, and eventually the entire function of the organism itself [4]. Within this theory, some authors argue that aging results from damage caused by free radicals to nuclear DNA, while others argue that it is a result of alterations to, and progressive loss of, mitochondria as a result of the mutilation of their DNA, thus reducing their biological effectiveness [1]. Indeed, mitochondrial DNA lacks polyamines or protective histones and, thus, is more susceptible than nuclear DNA to oxidative damage. Mutations are, therefore, more likely to take place in the mitochondrial genome of differentiated cells [5]. Curiously, healthy elderly individuals can have oxidative stress levels that are similar to those of young adults [6], or at least comparable in terms of antioxidant defences [7]. This suggests that oxidation is not inevitable in aging.

It is commonly argued that aging is not a genetically controlled process but an interaction between environment and genes [8]. Psychological stress and lifestyle factors appear to have an impact on the level of oxidation [9, 10]. Stress has been the most extensively studied negative factor in the brain's vulnerability to aging. In contrast, positive environmental factors such as a healthy diet can lead to improvements in aging [11]. Indeed, it is estimated that a third

of all cancer deaths in the United States could be avoided through appropriate dietary modification [12]. Changes in dietary behaviour, such as increased consumption of fruits, vegetables, and grains, are a practical strategy for significantly reducing the incidence of chronic diseases.

Phytochemicals are bioactive nonnutrient compounds present in fruits, vegetables, grains, and other plant foods. They have been linked to reductions in the risk of major oxidative stress-induced diseases [13]. Numerous investigations have shown a strong link between dietary intake of phytochemicals and reduced risk of cancer and cardiovascular disease worldwide. Thus, a prospective study in Finland involving 9959 men and women (ages 15–99 years) found an inverse association between the intake of flavonoids and the incidence of cancer [14]. After a 24-year follow-up, the risk of lung cancer was reduced by 50% in the highest quartile of flavonol intake. As well as flavonoids, other phenylpropanoids, isoprenoids, and indoleamines, particularly the indole melatonin, merit particular attention due to their biological activities [15].

Melatonin is the principal neurohormone secreted at night by the vertebrate pineal gland. It is an important component of the body's internal timekeeping system [16]. In particular, it is a signal of darkness that encodes time of day and length of day information for the brain [17]. A conceptual difficulty in melatonin research is that, while it is a signal of darkness, it has different functional consequences depending on the given species' time of peak activity. In nocturnal species, it is associated with arousal and physical activity. In diurnal species, it is associated with sleep and rest [16].

In diurnal animals, the onset of melatonin secretion is closely associated with the timing of sleep propensity. It also coincides with decreases in core body temperature, alertness, and performance [18]. For this reason, it is believed to play a part in sleep initiation as the trigger for opening the circadian "sleep gate," thus acting as a sleep regulator [19]. In this respect, the efficacy of melatonin supplementation to combat sleep disorders is well known, especially in the elderly with their marked reduction in melatonin production [16]. This hormone has a broad spectrum of physiological effects [20]. These include, but are not limited to, chronobiological, immunomodulatory, neuroendocrine, and antioxidant activities (Figure 1). All of these may contribute to the observed anti-aging potency of this natural agent [21].

Related to its antioxidant activities, melatonin acts as a potent antioxidant and free radical scavenger [22–25]. It not only scavenges the especially toxic hydroxyl radicals, but also performs indirect antioxidant actions via its ability to stimulate antioxidative enzymes [26]. Melatonin diminishes free radical formation at the mitochondrial level by reducing the leakage of electrons from the electron transport chain [27]. Increasing the levels of circulating melatonin, either directly by exogenous administration or indirectly by including vegetables rich in this compound in the diet, enhances the individual's antioxidant status [28–31]. It also stimulates a number of antioxidative enzymes which metabolize reactive products to innocuous agents. As well as for diseases in which there is an elevated production of free radicals, this may have implications for aging since production of this pineal indole wanes with increasing age. Indeed, some authors speculate that its loss contributes to the aging process [4].

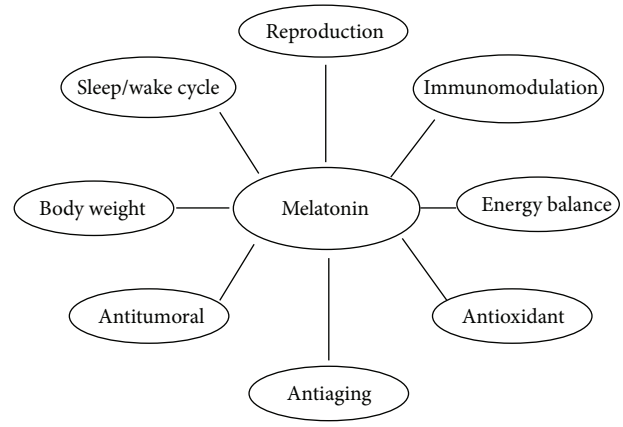

Figure 1: Physiological functions modulated by the indole melatonin.

Some components of foods possess biological activities which influence circadian rhythms in humans. But also "when" the food is consumed influences the normal functioning of biological rhythms. A central target of current chronobiological research is how nutrients can alleviate or even prevent diseases. In the present review, we shall focus on the potential use of chrononutrition as a novel dietary strategy to counteract the deleterious actions of free radicals and reactive species on physiological systems during aging.

2. Chronobiology: At the Cutting Edge of Health Sciences

We humans are immersed in an environment characterized by repetitive rhythmic cycles [32]. Conditions that are modified by different temporal cycles include organic efficiency, pathologies and the pharmacokinetics, pharmacodynamics, and efficacy of drugs. These cycles also entrain various processes, both physiological (body temperature, hormone and neurotransmitter secretion, and immune, cardiovascular, and digestive functions) and behavioural (sleep/wake cycle, activity/rest periods, digestion and excretion, sensory perception, learning, and memory), to a well-defined rhythm [33]. This phenomenon of rhythmicity extends to all classes of living beings, whether animals or plants, and at all levels of organization (molecules, cells, tissues, and organs).

Chronobiology is the discipline that studies the nature and function of biological rhythms, defined as the recurrence of any event within a biological system at roughly regular intervals [34]. The human body's biological clocks, for example, are controlled by synchronization with signals from the external environment [35].

The entire spectrum of biological rhythms covers an extremely wide range of frequencies [36]. All organisms, however, present circadian rhythms. These are biological processes that have an endogenous, entrainable oscillation

of about 24 hours. They affect most of the organism's physiological functions—the sleep/wake cycle, immune function, melatonin secretion, and the production and secretion of numerous neurotransmitters [37, 38]. The internal synchronization provided by circadian clocks may be altered by many factors, one of which is aging. The aging process leads to a situation of chronobiological imbalance that results in shortening the period and reducing the amplitude of the oscillator, loss of the circadian rhythm itself, appearance of an ultradian pattern, and internal desynchronization [39, 40]. During aging, any disturbance or imbalance in the relationship between the circadian and homeostatic systems may lead to the impairment of numerous physiological processes.

Antioxidant systems also deteriorate during aging. Elderly individuals, therefore, become more vulnerable to pathological conditions related to oxidative stress and may require an extra supply of dietary antioxidants to combat free radicals [41]. Previous studies by our research group and by other workers have shown that neuroendocrine and immune disorders due to the aging process may be ameliorated by supplementation with melatonin, serotonin, and/or their precursor, the amino acid tryptophan [42–46]. These are all compounds with powerful antioxidant properties [23, 29, 47]. The administration of tryptophan increases the availability of serotonin in the brain [46, 48]. Melatonin levels are consequently elevated as well [49]. Besides being an antioxidant, melatonin also has oncostatic, immunomodulatory, anti-inflammatory, and chronobiotic properties [50–52]. Given this context, the administration of tryptophan and melatonin in accordance with the needs of elderly subjects may contribute to readjusting any disturbances they have in their circadian rhythms.

Knowledge of the nature and function of biological rhythms is of practical as well as theoretical interest. This is reflected in the growing number of applications of chronobiology published in the recent health sciences literature. One such novel area of research is chronopharmacology. This is focused on the design and evaluation of drug delivery systems that release a bioactive agent at a rhythm matching the biological requirements of the treatment of a given disease [53]. Applying the knowledge of circadian function and regulation to the relevance of disease has enabled a chronobiology-based approach in the timing of administration of conventional drugs in order to synchronize the rhythms in disease activity with the efficacy of a particular drug, thus allowing for its optimal efficacy in the patient. Other recent applications of chronobiology include chronopathology, chronotherapy, and chrononutrition, all aimed at reducing the need for invasive methods in therapeutic interventions, and therefore are of unquestionable importance (Figure 2).

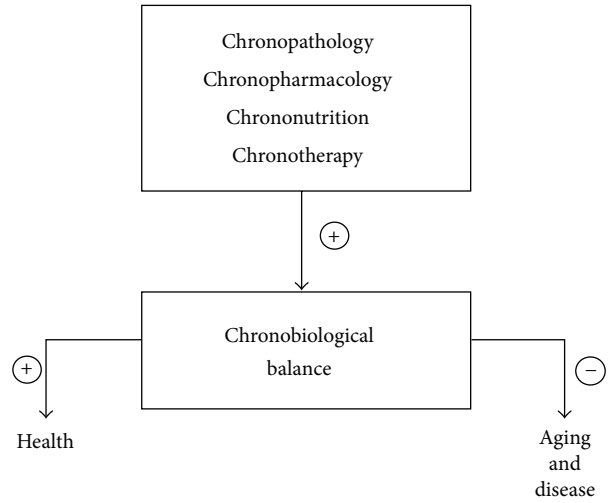

Figure 2: Influence of chronobiology applications on aging and disease.

3. Basis of Chrononutrition: Health Benefits

Our eating schedules are dictated not only by food supply, hunger, and satiety, but also by convenience and social habits and pressures. Feeding behaviour is the first element to consider in an organism's nutritional process. The vast majority of studies have focused on examining the homeostatic regulation of the quantity and quality of food ingested. The hypothalamus is the main neural structure involved. It acts in close correlation with the release of such hormones as cholecystokinin (CCK), leptin, ghrelin, and insulin. Temporal aspects of this regulation have been far less studied [54]. Meal timing can affect many physiological processes. The sleep/wake cycle, core body temperature, performance, alertness, and secretion of many hormones are examples. Since these are functions with a circadian rhythmicity, they deteriorate during aging with the weakening of overt circadian patterns [20]. That meal timing has major effects on the body has led to the conviction that, in choosing food, it is not only convenient to consider its nutritional value, but also its capacity to promote or hinder the normal functioning of the circadian cycle's control systems (Table 1). In humans, alterations have been detected in the overall expression of daily rhythms when food intake is limited to the usual period of rest (i.e., at night), as occurs during Ramadan [54].

Nutrients and phytochemicals play an essential part in the regulation of such circadian functions as sleep. Compared with a control diet high in carbohydrates and low in fat, a very low carbohydrate, fat-rich diet has been found to reduce the proportion of rapid eye movement (REM) sleep recorded by polysomnography. But it also increased the percentage of deep slow-wave (NREM) sleep [55]. While the total amount of carbohydrates may influence the architecture of sleep, it does not affect the duration. However, the evidence on whether carbohydrates positively impact sleep quality is not completely consistent, since consuming carbohydrate meals with high or low glycaemic loads seems not to affect any polysomnographically determined sleep index [55]. On the contrary, some components of the typical human diet, such as vitamin B12, improve alertness and concentration and reduce the daytime sleepiness phase [56]. Since some nutrients can entrain the circadian rhythm, diet design must

Table 1: Examples of antioxidant requirements according to chrononutrition principles.

	Which?	Where?	When?
Antioxidants	Vitamins (C and E)	Kiwi (*Actinidia chinensis*); broccoli (*Brassica oleracea*)	Daytime
	Carotenoids (lycopene)	Watermelon (*Citrullus lanatus*); tomato (*Lycopersicon esculentum*)	Daytime
	Phytoalexin (resveratrol)	Grape (*Vitis vinífera*); almond (*Prunus amygdalus*)	Daytime
	Tryptophan	Apple (*Malus domestica*); lentil (*Lens culinaris*)	Daytime/nighttime
	Serotonin	Banana (*Musa sapientum*); coffee (*Coffea arabica*)	Daytime/nighttime
	Melatonin	Cherry (*Prunus avium*); nut (*Juglans regia*)	Nighttime

take meal timing into account as well as the quantity and quality of the foods. In particular, the time of day at which food is consumed directly influences certain metabolic and hormonal factors—glucose, free fatty acids, glucocorticoids, and thyroid hormones, among others [57]. These ideas can be subsumed under the concept of chrononutrition. This concept reflects that it is not only the content of food, but also the time of ingestion and the interactions of its nutritional components which naturally contribute to the proper functioning of the circadian system.

3.1. Clinical Applications of Chrononutrition in Oxidative Stress. Harman's free radical theory of aging posits that oxidized macromolecules accumulate with age, resulting in decreased function and shortened life span [3]. Indeed, reduction of oxidative stress has been found to be associated with prolongation of life expectancy in many organisms [58–60]. Avoiding the formation of free radicals and reducing oxidative stress, thereby strengthening the body's antioxidant defences, can reduce the rate of aging and the risk of age-associated diseases [61]. In this sense, the primary prevention of chronic diseases through dietary modification may be just as effective as the secondary treatments that are commonly employed and less costly [62].

The possibility that mammalian life span could be significantly extended by diet modification was first demonstrated in a rodent study published by McCay and coworkers in 1935. This seminal experiment showed that life span can be extended by diet restriction without malnutrition, as opposed to diet restriction with malnutrition which can have the opposite effect [63]. Not only diet restriction, but also dietary patterns can have important long-term benefits for health. The abundance of bioactive phytochemical compounds provided by the fruits, vegetables, wine, and olive oil that make up the typical Mediterranean dietary pattern has proven to be effective in ameliorating some aging conditions associated with oxidative stress [64]. The tocopherols, carotenoids, and vitamin C present in pigmented and citrus fruits, and in such vegetables as carrots, tomatoes, broccoli, and red peppers, are positively correlated with a lower incidence of coronary heart disease [65]. Olive oil contains ubiquinol and tocotrienols which inhibit low-density lipoprotein (LDL) oxidation and reduce the risk of cancer [66]. Other dietary components, such as vitamin E or specific forms of fatty acids, especially ($n-3$) polyunsaturated fatty acids (PUFAs), contribute to the modulation of the immune and inflammatory systems, helping to prevent infectious and inflammatory diseases in the elderly [67]. Finally, in 1997 the most significant phenol in red wine, trans-resveratrol, was shown to prevent carcinogenesis in mice [68]. Since then, this phytochemical has been shown to have various pharmacological properties, namely, antioxidative [69], anti-inflammatory [70], anti-diabetic [71], anti-asthmatic [72], and antalgic [73], observed both *in vitro* and *in vivo* [74]. The doses applied in human trials examining specific health benefits of resveratrol range from 5 mg to 5 g, and some have considered additional compounds with putative synergistic effects [75]. A major critical limitation of most of the clinical research on resveratrol has been the lack of trials examining the longer-term health effects of this compound. The examples we have briefly discussed above are just a few of the more than several thousands of bioactive compounds that have been identified in foodstuffs from all over the world [76].

The vast majority of studies on the biological functions of phytochemicals have focused on analysing their antioxidant and/or anti-inflammatory properties. Few of them have taken into account the chrononutritional properties responsible for the effectiveness of such compounds. In humans, the type of food (macronutrient) is a temporally controlled variable. Once the nutrient enters the bloodstream, it may resemble the behaviour of a drug. It is thus subject to the principles of chronopharmacology, the most important of which is that the time of day influences both beneficial and unwanted effects. All the processes involved in nutrition display circadian and ultradian patterns which include rhythms of food intake, gut motility, secretion of digestive juices, absorption of digested foods, production of key metabolic enzymes, and energy expenditure. Consequently, different nutrients tend to be absorbed in different proportions, depending on the time of day [54].

Given their potent antioxidant activity, the tryptophan, serotonin, and/or melatonin content of foodstuffs and foodstuff-type beverages such as almonds, nuts, sweet

cherries, apples, corn, beer, and olive oil [77–82] may have major implications for animal and human health. This is particularly the case in combating a variety of disorders and diseases in which there is an elevated production of free radicals [83–85]. The antioxidant effects of these compounds have been demonstrated both in animal models and in humans. In animals, a Jerte Valley cherry-based beverage, rich in tryptophan, serotonin, and melatonin, has proven to be an efficient antioxidant-enriched product. Delgado et al. [86] showed that consumption of this product augmented the circulating levels of both melatonin and serotonin. This augmentation was positively correlated with increases in serum antioxidant capacity in both rats (*Rattus norvegicus*, a nocturnal animal) and ringdove (*Streptopelia risoria*, a diurnal animal) and in both young and old age groups. Its consumption also modulated the balance of pro- and anti-inflammatory cytokines, especially in the old animals, by downregulating the levels of proinflammatory cytokines and upregulating those of anti-inflammatory cytokines [87]. The clear importance of this type of finding is that nutritional interventions could delay or even prevent the functional deterioration of the immune system that accompanies aging [88]. The therapeutic properties shown by this cherry-based beverage may be attributed to the high melatonin content of the Jerte Valley cherry, although the involvement of other antioxidants, such as polyphenols, cannot be ruled out.

Given these results with animal models, tests were conducted with humans. It was found that both a diet enriched with Jerte Valley cherries and the ingestion of a Jerte Valley cherry-based nutraceutical product improve the antioxidant status of young, middle-aged, and elderly individuals [31, 89]. Particularly noteworthy was that not only the substantial amount of melatonin, serotonin [78], and tryptophan [80] contained in these Jerte Valley cherries, but also the timing of the meal, were critical to achieving the beneficial effects of these dietary interventions. Since serotonin and melatonin have opposite circadian rhythms, for example, serotonin levels peak during the day while those of melatonin peak at night (Figure 3), the cherries and the cherry-based product were consumed twice a day, once at lunch and once as supper desserts. The lunchtime consumption of cherries or the cherry-based product was designed both to directly increase the diurnal circulating serotonin concentration and to indirectly increase the nocturnal circulating melatonin concentration by enhancing the amount of serotonin available to be converted into melatonin at night [31, 90]. Hence, together with the ingestion of cherries (or the cherry-based product) at supper time, this boosts the total nighttime circulating melatonin levels [31, 91]. These interventions succeeded in improving the subjects' antioxidant status. The likelihood that this was indeed due to increases in the melatonin and serotonin concentrations was confirmed indirectly by determining the urine 6-sulfatoxymelatonin (aMT6-s) and 5-hydroxyindoleacetic acid (5-HIAA) levels. Most importantly, both of these nutritional interventions showed sleep-promoting and mood-enhancing actions in the young, middle-aged, and elderly subjects, which correlated with the increments in melatonin and serotonin levels, respectively [31, 90, 91].

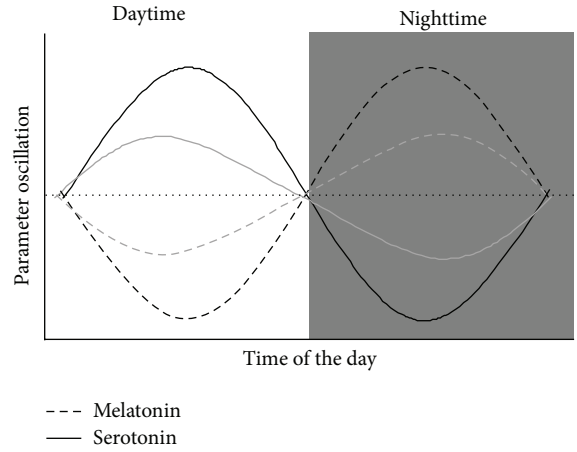

Figure 3: Circadian rhythms of melatonin and serotonin. Black lines represent normal circadian patterns of melatonin and serotonin secretion, while grey lines show a disturbed pattern due to aging process.

3.2. Clinical Implications of Chrononutrition in Aging. Aging is a complex process. It is related to circadian rhythm disruption with the resulting sleep disturbances [92] and other physiological and psychological dysfunctions [93]. These include impaired nutrient absorption [94, 95], immunosenescence [96–98], decreased hormone levels [99], and neuronal death [100, 101]. The age-associated disruption in the sleep/wake rhythm has been linked to the conventional decline in melatonin levels in advancing age (Figure 3). It may be corrected by supplementation with melatonin or with foodstuffs rich in melatonin (or in melatonin's precursors). In this respect, Bravo et al. [102] have shown that the consumption of tryptophan-enriched cereals increases aMT6-s and 5-HIAA levels, with the consequent improved mood and sleep quality in elderly subjects. Given the mood-enhancing and wakefulness-promoting properties of serotonin during the daytime, and the sleep-regulating functions of tryptophan, serotonin, and melatonin at night, these cereals were consumed twice a day (at breakfast and supper).

There is clear evidence that aging is associated with elevated basal morning levels of circulating glucocorticoids, such as cortisol [103]. It is commonly believed that altered HPA activity, for example, increased glucocorticoid activity or decreased brain serotonin concentration, is associated with several age-related pathologies [104]. In this sense, the consumption of the aforementioned Jerte Valley cherry product was effective in diminishing cortisol levels in young, middle-aged, and elderly subjects, the decline being especially more pronounced with advancing age. Increases in plasma tryptophan availability have been shown to enhance positive mood and dampen the cortisol response after an acute exposure to experimental stress. The mechanism is via enhancement of the brain serotonin functions that are involved in the adaptation to stress [105, 106]. Consequently, the tryptophan and serotonin present in the Jerte Valley cherry product may contribute to reducing cortisol levels, particularly in the elderly who are more vulnerable to stress.

It is noteworthy that many studies of the health-promoting effects of the antioxidant-rich fruits and foodstuff-type beverages present in the Mediterranean diet report better outcomes in advanced ages. Thus, González-Flores et al. [107, 108] find that the consumption of both plums (*Prunus salicina* Lindl. cv. Crimson Globe) and grape juice elevated the aMT6-s levels and the antioxidant capacity determined from the urine in young, middle-aged, and elderly individuals, with the effects being greater with advancing age. Likewise, Garrido et al. [109] reported that the inclusion of lycopene (the most potent *in vitro* antioxidant of the carotenoids) in a virgin olive oil enhanced the antioxidant effects of its ingestion. Again, the increases in this antioxidant capacity were positively correlated with age. The explanation may be that while energy requirements decrease with advancing age, nutritional requirements increase because of the greater demand for maintenance of the functionality of physiological systems [110].

4. Concluding Remarks

Just as research has provided a solid foundation for the overall relationship between food and health, the links between specific food components and particular health risks are now also being confirmed. This has had a direct impact on the food processing industry which has begun to turn its attention to the development of nutraceutical products. The marketing of the first infant milk formula that took into account the circadian variations of the different components present in breast milk clearly contributed to bringing science and industry together. This artificial infant milk (Blemil Plus®) is a dissociated formula consisting of different nutritional components that promote wakefulness, such as vitamins A, C, E, and B12 (Blemil 1 Plus day; N° WO2006/034955; PCT/EP2005/054516), and others that help to improve the effectiveness and quality of sleep, such as tryptophan, medium-chain triglycerides, and the nucleotides uridine and adenosine, among other constituents (Blemil 1 Plus night; N° WO2006/034955; PCT/EP2005/054516). A ground-breaking clinical study in the field of nutrition was the demonstration that the implementation of these chrono-biologically adapted formulas in the diet indeed contributes to consolidating the sleep/wake cycle in the newborn [111].

In general, dietary interventions with antioxidant-enriched foods based on the principles of chrononutrition are particularly relevant for the elderly, since this population commonly experiences progressive deterioration in physiological functions and metabolic processes. The aforementioned tryptophan-enriched cereals and the Jerte Valley cherry product have been shown to have especially beneficial effects on sleep quality and antioxidant status in the elderly. These nutritional strategies may contribute to taking full advantage of the potential benefits of phytochemicals as natural instruments with which to prevent or delay the onset of common age-related diseases.

Most clinical studies conducted in the field of chrononutrition have so far focused their attention on small groups of the population with no marked health problems. Clearly therefore, larger-scale studies are required on priority groups of the population, for example, cancer or obesity patients, to examine the clinical relevance of the supplementation of diets with chronobiotically enriched foodstuffs. Such studies would also help adapt the elaboration of diets and foodstuffs to the needs of specific populations according to age, sex, health goals, lifestyle, and genetic predisposition to certain diseases.

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Clinical Study

Efficacy of Fish Oil on Serum of TNFα, IL-1β, and IL-6 Oxidative Stress Markers in Multiple Sclerosis Treated with Interferon Beta-1b

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Multiple sclerosis (MS) is a chronic inflammatory disease, which leads to focal plaques of demyelination and tissue injury in the central nervous system. Oxidative stress is also thought to promote tissue damage in multiple sclerosis. Current research findings suggest that omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapenta-enoic acid (EPA) and docosahexaenoic acid (DHA) contained in fish oil may have anti-inflammatory, antioxidant, and neuroprotective effects. The aim of the present work was to evaluate the efficacy of fish oil supplementation on serum proinflammatory cytokine levels, oxidative stress markers, and disease progression in MS. 50 patients with relapsing-remitting MS were enrolled. The experimental group received orally 4 g/day of fish oil for 12 months. The primary outcome was serum TNFα levels; secondary outcomes were IL-1β 1b, IL-6, nitric oxide catabolites, lipoperoxides, progression on the expanded disability status scale (EDSS), and annualized relapses rate (ARR). Fish oil treatment decreased the serum levels of TNFα, IL-1β, IL-6, and nitric oxide metabolites compared with placebo group ($P \leq 0.001$). There was no significant difference in serum lipoperoxide levels during the study. No differences in EDSS and ARR were found. *Conclusion.* Fish oil supplementation is highly effective in reducing the levels of cytokines and nitric oxide catabolites in patients with relapsing-remitting MS.

1. Introduction

Multiple sclerosis (MS) is a chronic, inflammatory condition of the central nervous system, which affects approximately 2.5 million people worldwide [1]. Nutrition is commonly accepted as one of the possible environmental factors involved in the pathogenesis of MS. Western diet has dramatically decreased the intake of omega-3 essential fatty acids during the last decades [2, 3]. Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosahexanoic acid (EPA) and docosahexaenoic acid (DHA) are fatty acids that possess more than two carbon-carbon double bonds. A diet supplemented with PUFAs has clinical and biochemical effects in patients with autoimmune diseases such as MS [4]. EPA and DHA are found in high proportion in fish oil, and it has been proposed that these molecules may have anti-inflammatory, antithrombotic, antioxidant, and immunomodulatory functions and neuroprotective effects on the synaptogenesis and biogenesis

of the neuronal membrane [2, 3, 5–9]. Oxidative stress that is characterized by excessive production of reactive oxygen species and reduction of antioxidant defense mechanisms has been implicated in the pathogenesis of MS [10]. Increased cytokines and oxidative status have been related with the disease progression; therefore, a reduction of proinflammatory cytokines and oxidative stress could be beneficial for MS patients [11]. Interferon beta therapy has been a first-line treatment for relapsing-remitting multiple sclerosis (R-R MS); however, patients continue with inflammation and neurodegeneration [12, 13]. Over the past 60 years, there have been numerous studies on diet and MS, but there is no clear evidence about the efficacy of omega-3 PUFAs as complementary MS treatment [14–17]. The aim of this study was to evaluate the efficacy of fish oil on serum cytokines levels (TNFα, IL-1β, and IL-6), nitric oxide catabolites, lipoperoxides, expanded disability status scale (EDSS), and annualized relapse rate (ARR) in relapsing-remitting multiple sclerosis.

2. Methods

2.1. Participants. This study was a clinical trial on a randomized, double blind placebo-controlled group. Patients were recruited exclusively from the multiple sclerosis clinic of the Neurology Department of the Unidad Médica de Alta Especialidad (UMAE), Hospital de Especialidades (HE), Centro Médico Nacional de Occidente (CMNO), IMSS, Guadalajara, JAL, Mexico. Age of participants was 18–55 years. Patients had clinically definite and magnetic resonance image supported MS, at least one relapse in the year before entry into the study, and a baseline EDSS score of 0–5 and were treated with subcutaneous 250 μg interferon beta-1b (Betaseron, Bayer) every other day at least one year before the trial [18, 19].

Patients were excluded if they were taking another supplement; had progressive forms of MS; had history of severe depression; had history of acute liver or renal dysfunction; had history of tobacco, drug, or alcohol abuse; had intolerance, contraindication, or allergy to fish oil; and had customary antioxidant intake. Patients were followed up for at least 1 year. Patients were evaluated at the clinic every 3 months until each patient had reached the 1 year endpoint.

This study was conducted in accordance with the updated Declaration of Helsinki [20] and was approved by the Research Committee of the Social Security Institute of Mexico (Protocol number: R-2010-1301-8). Informed consent was obtained from all patients prior to study enrollment, according to the ethical code of the institution. Identification numbers were assigned to assure patient confidentiality.

2.2. Randomization and Blinding. Patients were randomly assigned in a 1 : 1 ratio to receive oral fish oil (4 g/day) or placebo, with a computer-generated randomization sequence (blocks of 2–4). To ensure masking between the fish oil and the placebo, capsules were identical in appearance, packaging, and labeling. Physicians and patients were blind to the intervention. An independent physician evaluated the EDSS score and collected the samples at each clinical visit.

2.3. Intervention. Patients received 4 g/day Omega Rx capsules (Dr. Sears zone diet) containing 0.8 g EPA, 1.6 g DHA, and excipient (glycerin, water purified, tocopherol, sunflower oil, and titanium dioxide) or placebo (glycerin, purified water, tocopherol, sunflower oil, and titanium dioxide), orally (4 capsules per day).

Clinic visits were scheduled every 3 months to assess serum levels of TNFα, IL-1β, and IL-6, nitric oxide catabolites, lipoperoxides, number of relapses, EDSS, safety, and tolerability. Fasting blood samples were taken at 0, 3, 6, 9, and 12 months. An independent physician assessed the occurrence of side effect at the Neurology Department. In this study, a relapse was defined as new or recurrent neurological abnormalities that were separated by at least 30 days from the onset of the preceding event, lasted at least 24 hours, and occurred without fever or infection.

2.4. Outcomes Measurements. Peripheral venous blood (10 mL) was collected into sampling tubes without EDTA. Blood was centrifuged at 3500 rpm for 5 minutes to separate the serum. Serum was stored at −80°C until analysis.

All assays were performed in a blinded fashion on coded samples. Serum levels of TNFα, IL-1β, IL-6, and NO were measured in duplicate by a sandwich-type enzyme-linked immunosorbent assay (ELISA) technique by using Kits from R&D Systems, TNF alpha DTA00C (range of detection: 15.6–1,000 pg/mL), IL-1b DLB50 (range of detection: 3.9–250 pg/mL), IL-6 D6050, and NO KGE001 (range of detection: 3.12–200 μmol/L).

A colorimetric assay was used to measure in duplicate the products of lipid peroxidation in serum; a standard curve was run on each assay plate using 1,1,3-trimethoxypropane as standard in serial dilutions. We measured malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) as lipid peroxidation products. The Bioxytech LPO-586 method is designed to assay MDA and HAE using methanesulfonic acid [21].

EDSS progression was measured as a one-point increase sustained for at least 3 months. A relapse was deemed if it was associated with an increase in EDSS by the treating neurologic physician. If any patient had a relapse, the treating physicians indicated intravenous methylprednisolone 1 g/day, for 3 days. We waited one month after the last dose of methylprednisolone for blood sampling. If a patient during the intervention had an infection that required antibiotic treatment, we waited one day after the last dose of antibiotic for blood sampling. The adverse effects of the fish oil treatment were recorded during the clinic visit. A severe adverse effect was defined as any event that causes death and requires hospitalization or prolonged hospital stay.

At study entry and every three months after enrollment, blood samples were collected to ascertain liver function (aspartate aminotransferase, and alanine aminotransferase and alkaline phosphatase); kidney function (urea, creatinine, and uric acid); blood lipids (total cholesterol, high density lipoproteins, low density lipoproteins, and very low density lipoproteins); hemoglobin; leukocytes; platelets; and glucose (data not shown).

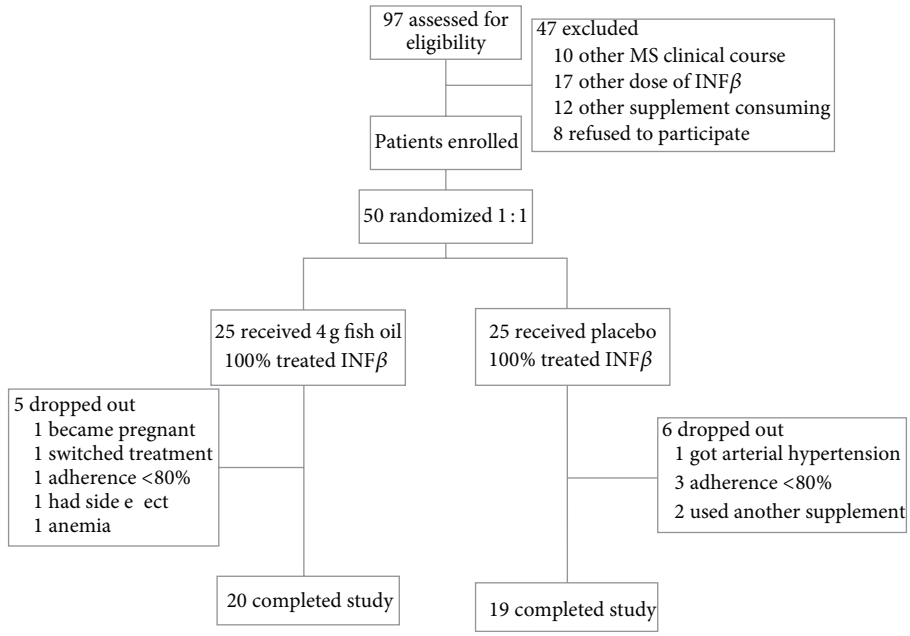

Figure 1: Trial profile.

The habitual dietary intake, including the essential fatty acids (EPA and DHA), was recorded for all participants. All the patients maintained their diet style and their exercise activity.

2.5. Adhesion and Safety. Participants reported daily consumption of the supplement in a consumption posting sheet. The percentage adherence for each subject was determined by the following formula: (number of pills consumed)/(number of pills returned to the physician) × 100.

We considered it as an optimal adherence if the percentage was higher than 80%.

2.6. Statistical Analysis. For power calculations, we assumed a decrease of 30% serum TNFα levels; if at least 21 patients were assigned to each group, this would give a power of more than 80% to detect differences of these magnitudes among groups. We analyzed changes in inflammatory mediators and oxidative stress markers outcomes with respect to each clinic visit with nonparametric and multivariate analysis of variance for repeated measurements (ANOVA) to determine whether there were time differences in each group. Also, we compared the differences between two treatment groups at the same visit with the Mann-Whitney *U* test. Adjustments for multiple comparisons were done with sequential test of Bonferroni. Statistical analyses were done on SPSS version 21 for windows.

3. Results

97 patients were screened, and 50 patients were enrolled and randomly assigned to receive 4 g fish oil (25 patients) or placebo (25 patients). 39 patients completed the trial. One subject in the fish oil group discontinued study participation because of the development of secondary progressive form of multiple sclerosis. 49 patients were treated with interferon beta-1b for at least 12 months. Figure 1 shows the trial scheme.

Table 1: Baseline characteristics of all randomized patients.

	Fish oil $n = 25$	Placebo $n = 25$	P value
Gender, women, %	16.6%	17.7%	0.89
Age, years	35.1 ± 7.6	34.7 ± 7.8	0.85
BMI, Kg/m^2	25.29 ± 3.9	24 ± 3.5	0.23
EDSS score	2.1 ± 0.98	2.06 ± 0.84	0.87
Evolution of disease, years	7.14 ± 4.79	6.68 ± 5.69	0.75
Total relapse before the study	5.44 ± 4.30	5.80 ± 6.93	0.82

Data are expressed in *n*, percentage (%) or mean and standard deviation (SD). Index of body mass (IBM), expanded disability status scale (EDSS), and Mann-Whitney *U* test.

Patients in the two groups had similar demographic and clinical characteristics at baseline (Table 1). In addition, no significant differences in body mass index (BMI), breathing rate, cardiac rate, and blood pressure rate were found during the course of the study. Baseline values of TNFα, IL-1β, and IL-6, nitric oxide catabolites, lipoperoxides, ARR, and EDSS score were similar in both groups. There was no measurable difference between groups and within subjects in serum levels of lipoperoxides during the study.

3.1. TNFα Levels. A significant decrease in serum TNFα levels was seen at 3, 6, 9, and 12 months for patients in fish oil group, compared with the placebo group (Table 2). After 12 months of fish oil supplementation, there was a decrease of percentage 42.9% compared with 0.7% in placebo. Figure 2(a) shows the reductions at each time point. Using ANOVA, there

Table 2: Effect of fish oil and placebo on cytokines, stress oxidative markers, and clinical outcomes.

	Baseline		*P* value	3 months		*P* value	6 months		*P* value	9 months		*P* value	12 months		*P* value
	Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
TNFα, pg/mL															
Fish oil	39.8	4.7	0.66	37.4	3.9	0.01	30.8	3.1	<0.001	26.6	1.8	<0.001	22.7	2.4	<0.001
Placebo	39.4	5.6		39.5	2.6		40.2	1.5		39.9	1.1		39.1	3.1	
IL-1b, pg/mL															
Fish oil	44.6	4.2	0.46	40.9	3.8	0.27	28.8	1.6	<0.001	25.2	2.4	<0.001	22.2	1.8	<0.001
Placebo	44.1	4.3		40.5	2.5		40.0	1.4		40.0	1.5		37.4	8.1	
IL-6, pg/mL															
Fish oil	689.8	48.9	0.21	657.6	50.7	0.1	558.8	38.0	<0.001	407.6	48.7	<0.001	356.7	31.7	<0.001
Placebo	669.9	61.9		672.4	44.2		711.2	31.8		671.2	42.4		644.6	57.3	
NO, μmol/L															
Fish oil	12.5	1.7	0.68	12.2	0.9	<0.001	9.4	0.8	<0.001	8.3	0.8	<0.001	8.0	0.9	<0.001
Placebo	12.8	1.5		13.7	0.7		14.2	0.7		13.9	0.7		13.7	0.8	
Lpo, μmol/L															
Fish oil	2.5	0.98	0.64	3.1	0.90	0.96	2.9	1.1	0.25	2.6	0.8	0.25	2.1	0.9	0.79
Placebo	2.4	1.0		2.8	1.1		2.6	1.1		2.3	1.2		1.9	0.8	
EDSS, score															
Fish oil	2.1	0.98	0.66	—	—	—	2.1	0.9	0.73	—	—	—	2.2	1.0	0.66
Placebo	2.0	0.84		—	—		2.0	0.8		—	—		2.2	0.8	
Relapses rate, number															
Fish oil	—	—	—	—	—	—	—	—	—	—	—	—	0.84	0.9	0.79
Placebo	—	—		—	—		—	—		—	—		1	1	

Data expressed in mean and standard deviation (SD), Mann-Whitney *U* test.

was significant change in the differences within subjects in the fish oil group, the difference between 9 months of baseline, 12 months of baseline, and 9 months to 6 months of baseline. TNFα levels were different, $P \leq 0.001$. Within subjects in placebo group show significant differences between 3 months of baseline.

3.2. IL-1β Levels. A significant decrease in the level of serum IL-1β was observed at 6, 9, and 12 months in the fish oil group compared with the placebo group ($P < 0.001$). At 12 months, there was a reduction of serum IL-1β level of about 50.3% in the fish oil group, compared with 15.2% in the placebo group (Figure 2(b)). In addition, the serum IL-1β levels at 6, 9, and 12 months were significantly reduced in the placebo group compared with the baseline values.

3.3. IL-6 Levels. Serum IL-6 levels decreased at 6, 9, and 12 months, in the fish oil group, compared with placebo ($P \leq 0.001$). After 12 months, there was a significant reduction in IL-6 levels, and the fish oil decreased to 48.3% compared with 3.8% placebo. There was a significant change in the differences within subjects in the fish oil group, the difference between 3 months of baseline, 6 months of baseline, 9 months of baseline IL-1β levels were different, $P \leq 0.001$. Within subjects in placebo group show no significant differences (Figure 2(c)).

3.4. Nitric Oxide Catabolites. Nitric oxide catabolites showed a significant decrease after 6 months of fish oil supplementation compared with placebo; this reduction continued until 12 months. The percentage of reduction after 12 months in the fish oil was 36.2% compared with −7% in placebo. The differences within subjects in fish oil group were significant between 3, 6, 9, and 12 months of baseline NO levels, $P < 0.001$. Within subjects in placebo group show no significant differences (Figure 2(d)).

3.5. Clinical Outcomes. There were no differences in the EDSS. The mean and standard deviation in fish oil treatment was 2.2 ± 1 points compared to 2.2 ± 0.8 points in placebo. The annualized relapses rate did not change after 12 months in each group; in the fish oil group it was 0.84 ± 0.94 versus placebo group 1 ± 1, $P = 0.79$.

Fish oil supplementation was well tolerated, and there were no serious adverse effects among the groups. No changes were found in the liver and renal test during the 12 months of the trial (data not shown). After 3 months of treatment, the two groups reported fishy taste. Less than 5% of all patients presented nausea, stomach pain, and diarrhea in the first clinic visit (at 3 months). Platelets, blood count, and bleeding did not change during the trial (not shown). Adherence to study treatment was slightly high in the fish oil group; however, this difference was not significant.

4. Discussion

Although it is commonly accepted that nutrition is one of the environmental factors involved in the pathogenesis of MS, its therapy does not involve a particular diet or supplement. In addition, the use of PUFAs as complementary MS treatment

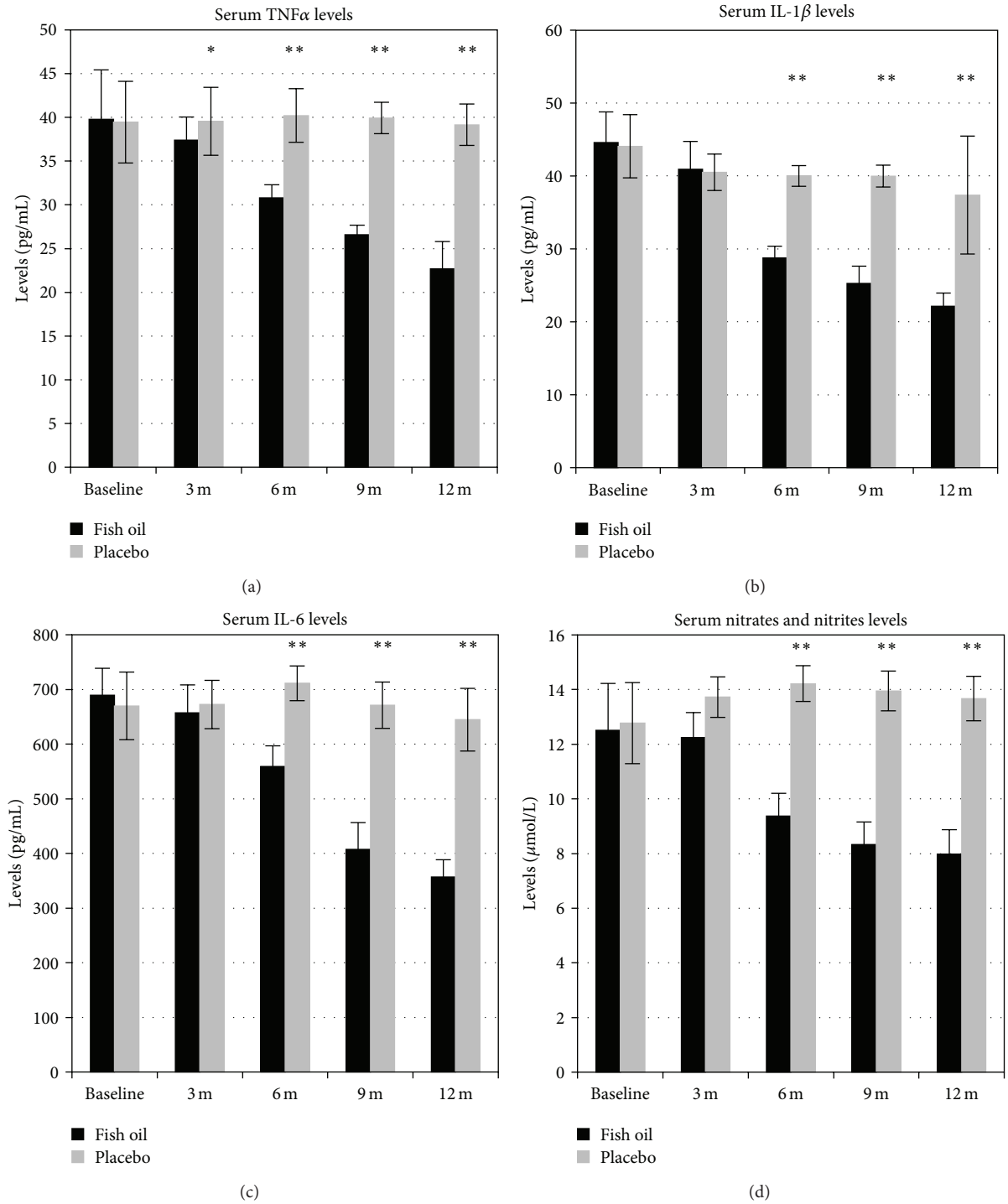

FIGURE 2: Outcomes at each timepoint in both groups. Data expressed in mean and standard deviation (SD), Mann-Whitney U test. Months (m), $^{*}P \leq 0.05$, $^{**}P \leq 0.001$.

is not approved. At present, in R-R MS patients (west of Mexico), there is no evidence about the efficacy of PUFAs contained in fish oil; instead of this, 72% intakes a supplement and ~40% intakes any n-3 PUFAs without medical prescription. The relationship between dietary PUFAs intake and progression of MS remains unclear, but our findings showed anti-inflammatory and antioxidants effects. This evidence is similar to the results of several *in vitro*, *in vivo*, and *postmortem* studies that are relevant to MS [22–26].

This clinical trial showed the efficacy of 4 g fish oil on TNFα, IL-1β, IL-6, and nitric oxide catabolites levels. Moreover, there were no differences between fish oil and placebo

with respect to the lipoperoxides and clinical efficacy outcome (progression on the EDSS and annualized relapse rate). In previous studies, TNFα, IL-1β, and IL-6 have been implicated as mediators of multiple sclerosis pathology. Increased cytokines have biological actions that lead to axon injury; particularly levels of TNFα in cerebrospinal and serum had been associated with disease progression; cerebrospinal fluid levels correlated with the degree of disability ($r = 0.83$, $P \leq 0.001$) [11]. Bertolotto et al. show an increase of 44% in RNAm levels of TNFα in relapsing phases and 22% in remitting phases. PUFAs have anti-inflammatory properties; Caughey et al. reported that 1.62 g/day of EPA and 1.08 g/day of DHA as encapsulated fish oil after 8 weeks inhibited TNFα and IL-1β synthesis by 70–80%. We observed that 0.8 g/day EPA and 1.6 g/day DHA decreased by 43% in TNFα, 50% in IL-1β, and 48% in IL-6 levels after 12 months of supplementation [26, 27].

The mechanisms for cytokines decrease by omega-3 fatty acids involve eicosanoid mediators; prostaglandin E_1 and E_2 are considered to be inhibitors of TNFα and IL-1β. The anti-inflammatory effects of EPA and DHA might include competitive inhibition of arachidonic acid, the metabolites of which are involved in promoting inflammation and might also inhibit the migratory activity of leucocytes, via alteration of cytoskeletal components [5, 26, 28–32].

Resolvins and protectins are lipid mediators derived from EPA and DHA via lipoxygenase action [33]. Recently, increasing attention is towards the PUFAs function as ligands for peroxisome proliferator-activated receptors (PPARs) that regulate genes involved in lipid metabolism and anti-inflammatory response; PUFAs might be agonist of PPARs [34, 35]. The inhibition of expression of nuclear factor κb, a transcription factor that is important for the synthesis of inflammatory cytokines and adhesion molecules, has been involved with the intake of omega 3 PUFAs. PUFAs might also reduce the production of matrix metalloproteinases-9 (MMP-9) that have been implicated in disruption of the blood-brain barrier in MS [36]. The initial phase of MS lesion formation is mainly characterized by a cluster of activated microglia and increased cytokines levels without evident signs of demyelination, whereas in the active phase of MS lesion development monocytes invade the lesion and initiate the demyelination process in time, active lesions gradually convert into chronic active lesions, where reactive oxygen species (ROS) and nitric oxide are present. In later stages of MS pathogenesis when inflammation has abates other mechanisms, such as mitochondrial dysfunction, this contributes to the formation of ROS. Normally, local antioxidant enzymes counteract the amount of ROS produced; in MS, ROS production is markedly increased, and the local antioxidant is not responding. In our study, there were no significant differences in the lipid peroxidation products, and we only observed a significant decrease in products of nitric oxide by 36.2% after 12 months in the fish oil group [37].

In addition, TNFα, IL-1β and INFγ can induce another cytotoxic effector molecule, inducible nitric oxide synthase (iNOS). This enzyme is present in actively demyelinating lesions, and stable reaction product of nitric oxide such as nitrates and nitrites is increased in the CSF and serum of patients with MS [38–42]. Several studies have suggested a role for nitric oxide and its oxidizing molecules, such as peroxynitrite in the immunopathogenesis of MS. In this study, we found that PUFAs contained in fish oil have antioxidant function since this decreases the nitric oxide products, and this reduction could be beneficial for MS patients [43].

Previously, Swank studied PUFAs intake contribution to risk and progressions in MS; however, their study had some deficiencies. Our study tried to control some confounding outcomes; there were no changes in the daily diet intake and physical exercise [14–17]. However, in this study, no differences in clinical outcomes were found. In others studies there was evidence about the efficacy on EDSS and the relapses number; MS patients ($n = 12$) who intaked EPA and DHA for 4 months showed minimal change in disability; only 5/12 of patients had R-R disease; they decreased 3.30 to 2.7 of EDSS score [44]. Another study with 47 patients showed decrease in the frequency and severity of exacerbations, mostly after the first year of observation. In a similar trial, 16 patients of R-R MS received DHA and EPA dose for 2 years, but the investigators also reduced the daily saturated fat intake and increased weekly fish consumption; after 2 years the patient showed a significant reduction in the annual relapse rate and EDSS score ($P \leq 0.001$) [45]. It is important to remark that those studies and their methodology could affect the results. In our trial, there was a minimal decrease in the number of relapses in the fish oil group, but this reduction was not a significant difference [14–17]. Recently, Torkildsen et al. in a clinical trial double-blinded, randomized, and multicentered study showed in 46 MS patients that 1.3 g/day EPA and 0.85 g/day DHA during 2 years had no significant difference on the annual relapse rate and EDSS score. Omega-3 PUFAs supplementation given as monotherapy or in combination with interferon beta had no effect on the progression outcome. We used similar doses of EPA (0.8 g/day) and DHA (1.6 g/day), and we also did not find change in the clinical outcomes [6]. However, in this trial the primary outcome was not to evaluate the efficacy on the clinical outcome, and the primary outcome was on the TNFα levels; this could be that the sample size did not have sufficient power to detect small and medium treatment effects on ARR and EDSS. There were limitations in this study; one was the sample size to evaluate the clinical outcomes, and the other was that we did not evaluate the severity on each relapse and the time between each one.

5. Conclusions

We show efficacy of 4 g/day fish oil, orally, for the reduction of TNFα, IL-1β, IL-6, and NO levels compared with placebo. Therefore, 4 g fish oil seems to have efficacy to decrease inflammatory cytokines and nitric oxide catabolites in relapsing-remitting multiple sclerosis. However, no differences in clinical efficacy were seen after 12 months of supplementation.

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Review Article

Natural Antioxidant Activity of Commonly Consumed Plant Foods in India: Effect of Domestic Processing

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Phytochemicals protect against oxidative stress which in turn helps in maintaining the balance between oxidants and antioxidants. In recent times natural antioxidants are gaining considerable interest among nutritionists, food manufacturers, and consumers because of their perceived safety, potential therapeutic value, and long shelf life. Plant foods are known to protect against degenerative diseases and ageing due to their antioxidant activity (AOA) attributed to their high polyphenolic content (PC). Data on AOA and PC of Indian plant foods is scanty. Therefore we have determined the antioxidant activity in 107 commonly consumed Indian plant foods and assessed their relation to their PC. Antioxidant activity is presented as the range of values for each of the food groups. The foods studied had good amounts of PC and AOA although they belonged to different food groups. Interestingly, significant correlation was observed between AOA (DPPH and FRAP) and PC in most of the foods, corroborating the literature that polyphenols are potent antioxidants and that they may be important contributors to the AOA of the plant foods. We have also observed that common domestic methods of processing may not affect the PC and AOA of the foods studied in general. To the best of our knowledge, these are the first results of the kind in commonly consumed Indian plant foods.

1. Introduction

Reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydroxyl radical, and hydrogen peroxide (H_2O_2) are often generated as byproducts of biological reactions or from exogenous factors [1]. These reactive species exert oxidative damage by reacting with nearly every molecule found in living cells including DNA [2]. Excess ROS, if not eliminated by antioxidant system, result in high levels of free radicals and lipid peroxides which underlie the pathogenesis of degenerative diseases like atherosclerosis, carcinogenesis, diabetes, cataract, ageing, and so forth [3].

Experimental and epidemiological evidence suggests a significant role of diet in the prevention of degenerative diseases [4]. Plant derived antioxidants, such as flavonoids and related phenolic compounds, have multiple biological effects, including antioxidant activity. Phytochemicals present in plant foods exert health beneficial effects, as they combat oxidative stress in the body by maintaining a balance between oxidants and antioxidants [5]. Although more than 8000 phytochemicals have been identified in plant foods, a large percentage remains to be identified. Further, data on the polyphenolic content antioxidant activity in Indian plant foods is scanty, and the effects of domestic processing on the AOA (antioxidant activity) in Indian plant foods are not reported yet [6].

Among plant foods, green leafy vegetables and grains are a rich source of antioxidants apart from energy, protein, and selected micronutrients in Indian diets [7]. Traditionally grains and GLVs have played a major role in providing nutrition particularly in the Indian Subcontinent and in other developing countries [8]. Since plant foods are often consumed in one or the other cooked forms, polyphenol and AOA intakes calculated on the basis of their content in raw

foods are likely to be inaccurate. Therefore it was considered pertinent to study the effect of domestic processing on the natural antioxidant activity and phenolic content of commonly consumed plant foods rich in these activities. Hence the effect of domestic processing (cooking) was determined on antioxidant activity and polyphenol content in some commonly consumed green leafy vegetables (GLVs) and food grains.

2. Sampling Procedures

The literature on antioxidant activity and phenolic content (PC) of plant foods is limited from India as well as other parts of the world. Available literature, mostly from other parts of the world, indicates that different researchers have adopted different sampling methods to get representative value of AOA and PC. Velioglu et al. [9] collected market samples and estimated AOA and PC in 200 mgs to 1 g of fruit, vegetable, and grain products. In another study, Al-Farsi et al. [10] took 1 g of sun-dried dates to estimate antioxidant parameters whereas Arcan and Yemenicioğlu [11] took about 20 g of fresh and dry nuts for the extraction. Sampling procedures followed in some Indian studies are as follows. Gupta and Prakash [12] used one gram of green leafy Vegetable for extraction whereas Nair et al. [13] have collected fresh food samples from local market, and five grams of cleaned food sample was taken to quantify PC and flavonoids in a few Indian plant foods. Keeping in view the differences in the sampling methods used and the quantities of samples extracted by different workers to analyse antioxidants in plant foods, it appears to be a good practice to take a higher quantity of food sample for the processing specially to get reproducible results and adopt ideal sampling practices for the quantification of AOA in food and herbal samples [6].

Commonly consumed cereals, pulses, legumes, and GLVs analyzed in this study were chosen based on NNMB survey [14]. Samples were collected from market outlets located in three different locations of the twin cities of Hyderabad and Secunderabad, India. The market samples were pooled and analyzed in triplicates, and the results are presented as mean values on fresh weight basis.

To determine the effect of different types of domestic processing of grains, edible portion of the sample was sorted out and divided into four aliquots of 25 grams each [11]. First portion was processed as such to know its natural (raw) antioxidant activity, while the 2nd, 3rd, and 4th portions of the sample were subjected to conventional, pressure, and microwave methods of cooking, respectively. We have mimicked consumer's habits of food procurement from market to household. In case of GLVs 10 g edible portions were taken and processed as above.

3. Extraction Procedure

To determine the antioxidant activity in plant foods several solvent extraction procedures have been used by different researchers. There is no single satisfactory solvent extraction method suitable for the extraction of all classes of food antioxidants and phenolics. This probably is due to the differences in the chemical nature of antioxidants and phenolics, namely, simple to highly polymerized chemical substances present in plant foods. Oki et al. [15] used six different polar solvents to extract milled rice: n-hexane, diethyl ether, ethyl acetate, acetone, methanol and deionized water and found that the extracts with highly polar solvents like methanol, and deionized water shown the highest radical-scavenging activity. Al-Farsi et al. [10] used seven different solvents to extract sun-dried dates: water-phosphate buffer (40 : 60 ratio), methanol containing 0.1% formic acid (88 : 12 v/v), methanol/HCl (99.9 : 0.1% v/v), acetone containing 0.7% cyclodextrin, water (50 : 50 v/v), methanol/water (50 : 50 v/v), and water alone. They reported that most antioxidants present in dates were water-soluble (hydrophilic). On the other hand extraction with 50% methanol yielded the highest recovery of phenolics in the same study. This could be due to the solubility differences of phenolic acids in methanol and water. Therefore they used phosphate buffer for extracting antioxidant activity and 50% methanol to estimate total phenolic content in dates.

Rochfort and Panozzo [7] used four different solvents to extract cereal grains: (i) acetone-water (80 : 10 v/v), (ii) ethanol-water (80 : 10 v/v), (iii) methanol-water (80 : 10 v/v), and (iv) water. Found that water and 80% methanol showed higher extraction than other solvents. In another study, Chidambara Murthy et al. [16] reported that methanol extracts of grape pomace protected the activities of hepatic enzymes and could thus be important in combating reactive oxygen species. In another study using *in vitro* models, Singh et al. [17] observed that methanol extracts of pomegranate peel and seeds had high antioxidant activity, and similar findings were reported by others [18]. Several workers used acidified 80% methanol extraction to assess antioxidant contents in plant foods, and the reasons for it could be that methanol extraction not only gives a higher yield but also gives high antioxidant activity as compared to that with other polar solvents. Hence we have used acidic, 80% methanol (with 0.1% HCl) for extraction of phenolics and AOA from foods in our studies. Methanol extracts were also used to know the effect of domestic cooking. Domestic cooking was done with normal tap water.

Briefly, 10 grams of GLV or 25 grams of the grain sample was cooked in 100 mL of water for 10–15 minutes (in case of conventional cooking it took about 15 minutes; pressure cooking was done at 120°C for 10–12 minutes, and microwave cooking was done for 5–8 minutes, resp.). Cooking was done with the sample covered with lid except in conventional cooking. To estimate natural (raw) antioxidant content, the first portion of 10 or 25 g of the edible portion of the sample was ground in a domestic blender and extracted as such in 80% methanol containing 0.1% HCl, and final volumes of GLVs and grain samples were made to 50 and 100 mL extracts, respectively, with 80% methanol.

4. Various Antioxidant Methods in Use

Determination of AOA in plant extracts is still an unresolved problem. It is not possible to evaluate multifunctional biological antioxidants in plant foods by a single antioxidant

method. Hence, batteries of tests are used; about twenty different biochemical methods are in practice to asses AOA [19]. The exact comparison of the results obtained by different methods and their general interpretation may be practically impossible due to the variability of experimental conditions and differences in physicochemical properties of oxidizable substrates. Many other factors including colloidal properties of substrate, experimental conditions, reaction medium, oxidation state, and antioxidant localization in different phases may influence antioxidant activity. Among the different antioxidant parameters in use, ABTS (Trolox equivalent antioxidant assay TEAC/ABTS) and DPPH (2,20-Diphenyl-1-picryl hydrazyl) are widely used due to their simplicity, stability, accuracy, and reproducibility [20]. In a review on the AOA methods Huang et al. [21] suggested that FRAP (ferric reducing antioxidant power) and DPPH are the two most commonly accepted assays for the estimation of AOA in plant foods. In another study Ozgen et al. [22] evaluated the three most commonly used AOA methods and suggested that FRAP < DPPH < ABTS in fruits. A study carried out by Siddhuraju and Becker [20] suggested that DPPH < ABTS < FRAP showed better antioxidant and free radical-scavenging activities in processed cow pea and its seed extracts. Several new analytical approaches have suggested investigating antioxidant power of food extracts on the basis of their electron-donating ability. One such recently suggested assay is CAAP (chemiluminescence analysis of antioxidant power) which is a chemiluminescence based method. The rapid CAAP assay is said to be convenient to investigate the antioxidant power of herbal extracts. CAAP method showed positive correlation with FRAP ($r = 0.959$) [23]. Nevertheless, FRAP and DPPH assays are the most widely used methods. Since these assays are electron transfer based assays and often show excellent correlation with phenolic contents, and they are carried out in acidic conditions; pH values have an important effect on the reducing capacity of antioxidants. In acidic conditions reducing capacity tends to be suppressed due to protonation on antioxidant compounds, whereas in basic conditions proton dissociation of phenolic compounds would enhance the sample reducing capacity [24].

4.1. Phenolic Content. Soluble and hydrolysable phenolic contents (free phenols) were estimated as per the procedure described by Singh et al. [17] and Singleton and Rossi [25]. Values are expressed as Gallic acid equivalents. Colorimetric method was adopted in the present study, since sensitive chromatographic method in quantification of phenols is often limited to single class of phenolics and is often limited to low-molecular weight compounds that are available as standards. It is, therefore, necessary to use colorimetric assays such as the Folin-Ciocalteu assay which rely on the reducing ability of phenols to quantify the amount of total phenolics in a sample [26].

4.2. DPPH Radical-Scavenging Activity. DPPH radical-scavenging activity was determined according to Aoshima et al. [27]. This method is based on the ability of the antioxidant to scavenge the DPPH cation radical. Briefly, to 100 μL of sample extract or standard, 2.9 mL of DPPH reagent (0.1 mM in methanol) was added and vortexed vigorously. This was allowed to stand in dark for 30 min at room temperature, and the discoloration of DPPH was measured against a suitable blank at 517 nm. Percentage inhibition of the discoloration of DPPH by the sample extract was expressed as Trolox equivalents (mg/100 g).

4.3. FRAP Assay. Ferric reducing antioxidant power (FRAP) was determined according to Benzie and Strain [28]. In the presence of TPTZ, the Fe^{+2}-TPTZ complex exhibits blue color which is read at 593 nm. Briefly, 3.0 mL of working FRAP reagent was added to an appropriate volume/concentration of the sample extract, incubated for 6 min at room temperature, and the absorbance was measured at 593 nm against $FeSO_4$ standard.

5. Over View

Current life style is one of the major causes in the overproduction of free radicals and reactive oxygen species and decreasing physiological antioxidant capacity [29]. Food provides not only nutrients essential for life but also other bioactive compounds for health promotion and disease prevention. Epidemiological studies have consistently shown that regular consumption of plant foods is associated with reduced risk in developing chronic degenerative diseases and biological ageing [30]. Phytochemicals are the bioactive nonnutrient compounds present in plant foods which have been suggested to be responsible for their bioactivity linked to the reduced risk of major chronic diseases. It has indeed been estimated that a healthy diet could prevent approximately 30% of all cancers [31]. So far, published data from other parts of the world and India account only for a minor fraction of total polyphenols and AOA of plant foods. Therefore it was suggested to have food composition tables on antioxidant activity and polyphenolic content of commonly consumed plant foods from developing countries [32]. Hence we have attempted for the first time to get representative values of AOA in 107 commonly consumed plant foods in India. Purposive samples were purchased from three different local markets of the twin cities of Hyderabad and Secundrabad (India). They were analyzed separately and data presented on fresh weight basis, to mimic natural practice of consumption. PC and AOA were assessd in the methanol extracts of the foods by Folin-Ciocalteu method and DPPH/FRAP methods, respectively, and the results are expressed as Gallic acid and Trolox/$FeSO_4$ equivalents, respectively. It has been observed in our studies that the foods studied had good amount of polyphenols and antioxidant activity, despite the fact that they belonged to different food groups. Also, a good correlation was observed between the natural AOA of the food and it's PC (Table 2) in many of the food groups studied. Part of natural antioxidant data was published by us as full length articles; hence range of values are given. Data on the effect of domestic processing on PC and AOA has been elaborated here since it is not yet published.

Table 1: Natural content of AOA and TPC.

S. no.	Group of foods	*n* (107)	Antioxidant content (mg/100 g)		PC (mg/100 g G A equ.)
			DPPH (Trol. equ.)	FRAP ($FeSO_4$ equ.)	
1	Cereals and millets	9	24–173	450–13093	47–373
2	Dry fruits	10	271–1541	1174–32416	99–959
3	Edible oils and sugars	11	3–208	11–11674	0.72–336
4	Fresh fruits	14	32–891	22–496*	26–374
5	Green leafy vegetables	11	21–1020	1380–27827	77–1077
6	Nuts and oil seeds	12	20–28622	220–4220341	10–10841
7	Pulses and legumes	11	26–107	1469–10362	62–418
8	Roots and tubers	10	11–125	256–6308	22–169
9	Vegetables	19	12–466	243–10510	27–339

Values are expressed on fresh weight basis. *ABTS: range of values are given.

Table 2: Correlation between PC versus DPPH, FRAP.

S. no.	Group of Foods	*n* (107)	PC versus DPPH (*r*)	PC versus FRAP (*r*)	DPPH Versus FRAP (*r*)
1	Cereals and millets	9	0.45	0.91	0.84
2	Dry fruits	10	0.97	0.87*	0.81*
3	Edible oils and sugars	14	0.93	0.93	0.99
4	Fresh fruits	14	0.77	0.84*	0.94
5	Green leafy vegetables	11	0.94	0.95	0.96
6	Nuts and oil seeds	12	0.99	0.99	0.99
7	Pulses and legumes	11	0.16	0.44	0.78
8	Roots and tubers	10	0.76	0.85	0.97
9	Vegetables	19	0.79	0.85	0.75

*ABTS: correlations are in natural form.

6. Natural DPPH Activity in Group of Plant Foods

The range of DPPH activities in different food groups are presented in Table 1, and the values are expressed as mg/100 g on fresh weight basis. Among all the food groups analysed, the highest DPPH scavenging activity was observed in areca nut (28622 mg/100 g) while the activity was the least in carrots 11.06. The DPPH activity in cereals and millets ranged from 24–173 mg/100 g, with the highest activity being found in finger millet and the lowest in Semolina. The activity in legumes and pulses ranged 26–107 mg/100 g, with the highest in rajma and the lowest in roasted Bengal gram dhal. Among the nuts and oil seeds studied, the DPPH values ranged from 20–28622 mg/100, with areca nut showing the highest and coconut water having the least activities. Among the dry fruits DPPH activity ranged 271–1541 mg/100 g, with the highest activity being in walnuts and the lowest in piyal seeds. On the other hand among fresh fruits, the values ranged 32–891 mg/100 g, with the highest in guava and the lowest in watermelon. In green leafy vegetables the values ranged 21–1021 mg/100 g, with Curry leaves having the highest whereas spinach had the least activity. In roots and tubers category the DPPH activity ranged 11–125 mg/100 g, with the highest activity being found in red beet root and the lowest in carrot. Among the vegetables studied, the DPPH values ranged 12–466 mg/100 g. The highest activity was found in okra and the lowest was in ridge gourd. Due to scanty data available in the literature on DPPH activity in Indian plant foods it was not possible for us to compare these DPPH findings with the literature.

7. Natural FRAP Activity in Group of Plant Foods

The range of FRAP activities in different food groups are presented in Table 1, and the values are expressed as mg/100 g on fresh weight basis. Among all the food groups analyzed, the highest FRAP activity was observed in areca nut 4220341 mg/100 g while the activity was the least in sunflower oil 36.10. Salient findings are as follows. Cereals and millets ranged 450–13093 mg/100 g, highest activity was found in finger millet, and the lowest was in Semolina. Among the dry fruits, activity ranged 1174–32416 mg/100 g, with the highest being in walnuts and the lowest in cashew nuts, whereas in fresh fruits, ABTS activity ranged 22–496 mg/100 g, with the highest in guava and the lowest was in pineapple. Some of these findings are in agreement with the literature values of fresh fruits [32]. The AOA determined by ABTS in fresh fruits and FRAP in dry fruits showed that both had reasonably good AOA. Interestingly dry fruits had higher activity than fresh fruits probably due to their low moisture content. It was pertinent to assess whether these observations made by two different methods in fresh and dry fruits could be validated by a common, third method.

Therefore, we determined the AOA in fresh and dry fruits by the DPPH scavenging method, another most commonly used antioxidant biochemical parameter. FRAP activity in green leafy vegetables ranges 1380–27827 mg/100 g, mint leaves had highest activity, and the lowest was in spinach. Edible oils and sugars range 36–11674 mg/100 g, the highest activity found in jaggery and lowest in groundnut oil (unrefined). Among the nuts and oil seeds studied, FRAP ranges 220–4220341 mg/100 g; the areca nut showed the highest activity and lowest was in coconut water. In pulses and legumes FRAP ranged 1469–10362 mg/100 g, with the highest in rajma and the lowest in green gram dhal. The roots and tubers showed a wide range 256–6308 mg/100 g, and beet root had the highest and carrot the least. Among the vegetables studied, FRAP ranges 243–10510 mg/100 g. The highest activity was found in red cabbage and the lowest in pumpkin. Due to scanty data it was not possible for us to compare our FRAP finding with the literature.

8. Natural Phenolic Content in Group of Plant Foods

The soluble total phenolic content (PC) data is presented in Table 1. Values are presented as mg Gallic acid equivalent/100 g on fresh weight basis. Among all the food groups analyzed, the highest PC was observed in areca nut 10841 GAE/100 g and was the least in coconut water 10.00. Coming to different food groups, in cereals/millets PC values ranged 47–373 mg/100 g; finger millets (Ragi) had the highest (373 mg/100 g), while milled rice had the lowest (47 mg/100 g). Dry fruits values range from 99 to 959 mg/100 g of which walnuts (959 mg/100 g) and piyal seeds (99 mg/100 g) had the highest and the lowest PC, respectively. PC of fresh fruits ranged from 26 to 374 mg/100 g, with the highest PC being in guava (374) and the least in watermelon (26 mg/100 g). PC of Papaya and sapota observed here are in agreement with reported data from other parts of the world [33], orange [34], pineapple [35], and apple [36]. Among the GLVs, PC was ranging 77–1077 mg/100 g; curry leaves have the highest (1077 mg/100 g) and the lowest was in spinach (77 mg/100 g). To compare our findings on natural phenolic contents of GLVs, as such there is very little or no published data available from India. However, Gupta and Prakash [12] analyzed phenolic content in 4 GLV samples, of which phenolic contents of fenugreek leaves values are comparable with our finding 158 versus 163 mg/100 g, whereas Amaranth and Curry leaves data is remarkably different from our findings 253 and 1077 mg/100 g, respectively, while the reported values are 150 and 387 mg/100 g. This variation could be due to the fact that they used tannic acid as standard whereas we used Gallic acid. However, it is not clear from Gupta and Prakash study [12] whether the data presented by them was on dry … or … on fresh weight basis was given. Coming to edible oils and sugars, the PC values ranged 0.72–336 mg/100 g. Jaggery had the highest PC (336 mg/100 g) while the lowest was in Vanaspati (0.72 mg/100 g). Nuts and oil seeds ranged from 10 to 10841 mg/100 g; areca nut had the highest phenolic content (10841 mg/100 g) and coconuts water the least (10 mg/100 g). Among the pulses and legumes, values ranged from 62–418 mg/100 g; black gram dhal had the highest (418 mg/100 g) while green gram dhal had the least (62 mg/100 g). Roots and tubers showed a wide range (22–169 mg/100 g), and beet root had the highest and carrot the least. Phenolic content of vegetables ranged from 27 to 339 mg/100 g, and red cabbage had the highest and ridge gourd the lowest. Very little published data is available on PC of Indian plant foods; some findings are in agreement with our data [37]. However, phenolic contents of plant foods can significantly vary due to various other factors, like plant genetics and cultivar, soil composition and growing conditions, maturity state and postharvest conditions, and so forth [38].

9. Correlation between PC, DPPH, and FRAP (in a Group of Natural Plant Foods)

Our observations on correlation between DPPH, FRAP, and PC of cereals, pulses, and legumes are in agreement with an earlier report [18] in that no significant correlation was observed between these two parameters among these food grains. Interestingly, no correlation was observed among PC, DPPH, and ABTS in wheat extracts [20]. The lack of correlation in cereal and legume grains could be due to the differences in the bound and free forms of phytochemicals present in them. It was observed that there was a possibility of underestimation of phenolic compounds in cereal/legume grains due to the differences in bound and free phenolics present in them. The bound phenolics contribute about 62% in rice to 85% in corn [5]. Another possibility could be due to the different responses of different phenolic compounds in different assay systems. Since the molecular antioxidant responses of phenolic compounds vary remarkably, depending on their chemical structure, their AOA does not necessarily correlate with the PC in grains [39].

However, both in dry and fresh fruits, there was a good correlation between PC and AOA, and our findings are in agreement with the available literature on the phenolic content of fresh and dry fruits [10]. However the discordance in phenolic content of different groups of foods studied could be due to varietal, seasonal, agronomical, and genomic differences, moisture content, method of extraction and standards used, and so forth [40]. Among GLVs, there was a good correlation among the PC and antioxidant parameters studied (Table 2). However, little information is available in the literature on the AOA and PC correlations in GLVs [12].

Although edible oils and sugars, belong to different food groups, there was a good correlation among their PC and AOA parameters in that the "r" value was 0.93 between both the AOA parameters and PC. Among nuts and oil seeds, a significant correlation was observed between AOA (both DPPH and FRAP) and PC. The "r" value between PC and AOA was 0.99, indicating the importance of PC to their AOA as assessed by these two methods. These findings are in agreement with earlier reports of this nature [10].

Correlations between the antioxidant activity and phenolic content of roots, tubers, and vegetables are given in

Table 2. In general, there was a good correlation between the PC and AOA among the vegetables, roots, and tubers studied. A significant correlation ($P < 0.01$) was observed between PC and AOA both in roots and tubers (r values being 0.76 and 0.85, resp. with DPPH and FRAP) and other vegetables ($r = 0.79$ and 0.85 with DPPH and FRAP). These findings suggest that PC may be an important contributor to the AOA of roots, tubers, and vegetables; our observations are in agreement with the literature from other parts of the world [34].

10. Effect of Domestic Cooking on PC and AOA in Green Leafy Vegetables

Plant foods are often consumed in one or the other processed forms. Therefore, it was considered pertinent to study the effect of common domestic processing (cooking) methods on the natural antioxidant activity and phenolic content of a few commonly consumed plant foods. Since oxidants and antioxidants have different chemical and physical characteristics, different types of cooking may bring different type of alterations in antioxidant activities of different foods. Further, if polyphenol intakes are calculated based on raw plant foods, the intake values computed may not be accurate. Hence effect of cooking was determined on phenolic content and antioxidant activity in commonly consumed green leafy vegetables (GLVs) and food grains.

Effect of cooking on PC and AOA of GLVs is presented in Tables 3–6. Phenolic content and antioxidant activity of foods cooked by different methods were compared with its natural (raw) activity from same portion of subsample. In general different cooking methods used in this study did not affect the AOA and phenolic contents in most of the GLVs. Percent change in the phenolic content (PC) or antioxidant activity (AOA) on cooking is given in parentheses with respective raw GLV value (Tables 3–6). Differences were considered significant at a P value at least <0.05.

Out of the eleven GLVs studied, only two GLVs, namely, Ambat Chukka and Ponnaganti showed a small decrease of 10–20% in their PC content on cooking. While Gogu showed very little or no change on cooking. The other eight GLVs showed an increase in PC during different types of cooking (Table 3). Among them six GLVs, namely, Amaranth, Curry leaves, Fennel, Fenugreek, Purslane and Sorrel leaves showed an increase in PC, ranging 108–146% on cooking. Coriander, Mint, and spinach showed a significant increase in PC in the above cooking methods, and the increase was ranging 125–211% (Table 1). As such there is very little data of this kind reported in the literature. Kuti and Konuru [41] demonstrated in spinach leaves a similar increasing trend in PC on cooking. Contrary to this, Faller and Fialho [42] showed cooking loss in PC in vegetables. The possible explanation for the increasing or decreasing trends of phenolic contents during various cooking methods could be that the phenolics are stored in pectin or cellulose networks of plant foods and can be released during thermal processing. In turn individual phenolics may sometime increase because heat can break supramolecular structure which might make the phenolic compounds react better with the reagents [43].

Effect of cooking on DPPH scavenging activity is given in Table 4. The increase or decrease activity in different GLVs during different cooking methods was compared with its natural DPPH activity of the raw GLV. Our findings on changes in DPPH are in line with those in PC. In general, an increasing trend in DPPH activity on cooking was seen in most of the GLVs studied. Out of eleven GLVs, marginal effect of 1–10% was seen only in Ambat Chukka. Most of the GLVs showed an increasing trend in all the three methods of cooking. Among them Purslane and Ponnaganti showed 10–20% increase, whereas Amaranth and Mint showed 17–50% increase. Coriander and Curry leaves showed an increase of 38–133%. During conventional cooking, curry leaves showed little effect (<7%) while spinach showed an enormous increase of 221–381%. Remaining three GLVs namely, Fennel, Fenugreek, and Gogu leaves did not show any effect in conventional and pressure cooking but in microwave cooking alone showed about 31–36% increase (Table 4). This could be due to effect of high temperature as compared to the above two methods of heat treatment. Considering that no data of similar type is available from other parts of the world, we are unable to compare our findings with literature reports. Data available on other vegetables (not GLVs) reported a mixed trend, which is in agreement with our results. Indeed an increasing trend was observed in potatoes [44], while a decreasing trend was reported in other vegetables [42].

Effect of cooking on FRAP is presented in Table 5. This biochemical indicator was chosen as the second most commonly used antioxidant biochemical parameter. Again, similar increasing trends were seen in FRAP activity on cooking. Most of the GLVs (nine out of eleven) showed an increase, ranging 119–181%. While Coriander and spinach leaves showed an enormous increase, two other GLVs, Ambat Chukka and Ponnaganti, showed a decrease (maximum of 10%). This type of complex trend during cooking requires further research [45]. However, the present data on natural antioxidant content in commonly consumed GLVs is the first data of its kind from India. Secondly our findings on the effect of different methods of cooking in above GLVs, most of them, show an increase in AOA; it could be due to better availability of bound phenolics. Correlation among the three biochemical parameters and effect of different methods of cooking were assessed next, and these correlations were compared by using rank correlations (Table 6). Phenolics versus antioxidant parameters in different cooking methods are highly correlated. Findings of this study suggest that although different cooking methods showed changes (highly significant in some cases) in the phenolic content and AOA of the GLVs, there was no effect of domestic cooking on the correlation between the PC and AOA. This observation confirms that PC may be important contributor to the AOA of GLVs both in raw and cooked forms.

11. Effect of Domestic Cooking (Food Grains)

PC and AOA of the food grains (raw and cooked by different methods) are presented in Tables 7–9. The PC of raw whole green gram (with peel) was the highest (284 mg/100 g) followed by black rajma (146 mg/100 g). Green gram dhal

Table 3: Effect of domestic processing on polyphenol content of commonly consumed green leafy vegetables.

Sl. no.	Common name	Botanical name	Phenolic content (mg/100 g Gallic acid Eq.)			
			Raw	Conventional	Pressure	Microwave
1	Amaranth	*Amaranthus gangeticus*	253.0[a] (100)	275[b] (108)	355[c] (140)	312[d] (123)
2	Ambat chukka	*Rumex vesicarius*	100.3 (100)	90 (89)	93 (92)	91 (91)
3	Coriander leaves	*Coriandrum sativum*	239.6[a] (100)	417[b] (174)	451[c] (188)	506[d] (211)
4	Curry leaves	*Murraya koenigii*	1077.0[a] (100)	1434[b] (133)	1184[c] (109)	1377[d] (127)
5	Fennel leaves	*Foeniculum vulgare*	251.3 (100)	268 (106)	265 (105)	312 (124)
6	Fenugreek leaves	*Trigonella foenum-graecum*	163.3[a] (100)	180[a] (110)	176[a] (107)	220[b] (134)
7	Purslane leaves	*Portulaca oleracea*	94.6[a] (100)	128[b] (135)	138[b] (146)	128[b] (135)
8	Sorrel leaves	*Hibiscus cannabinus*	191.3 (100)	194 (101)	211 (107)	213 (111)
9	Mint	*Mentha spicata*	440.3[a] (100)	657[b] (149)	796[c] (180)	761[c] (172)
10	Water amaranth	*Alternanthera sessilis*	136.3 (100)	122 (89)	110 (80)	123 (90)
11	Spinach	*Spinacia oleracea*	77.0[a] (100)	96[b] (125)	125[c] (162)	117[c] (152)

Mean values were compared ($n = 3$) by nonparametric Kruskal Wallis one way ANOVA. Differences in alphabets are significantly different at $P < 0.05$. Percent gain or loss calculated when raw value taken as 100%. Percent recovery values are given in parentheses. Decimal points are not given due to higher numbers.

Table 4: Effect of domestic processing on DPPH activity of commonly consumed green leafy vegetables.

Sl. no.	Common name	Botanical name	DPPH (mg/100 g Trolox Eq.)			
			Raw	Conventional	Pressure	Microwave
1	Amaranth	*Amaranthus gangeticus*	405.6[a] (100)	520[b] (128)	527[b] (129)	476[b] (117)
2	Ambat chukka	*Rumex vesicarius*	85.3 (100)	87 (101)	83 (97)	94 (110)
3	Coriander leaves	*Coriandrum sativum*	471.0[a] (100)	886[b] (181)	948[b] (201)	1100[c] (233)
4	Curry leaves	*Murraya koenigii*	1020.6[a] (100)	950[b] (93)	1724[c] (168)	1418[d] (138)
5	Fennel leaves	*Foeniculum vulgare*	545.3 (100)	592 (108)	540 (99)	746 (136)
6	Fenugreek leaves	*Trigonella foenum-graecum*	144.3 (100)	142 (98)	127 (87)	193 (134)
7	Purslane leaves	*Portulaca oleracea*	138.3 (100)	162 (117)	165 (119)	151 (109)
8	Gogu	*Hibiscus cannabinus*	346.0 (100)	365 (105)	334 (96)	456 (131)
9	Mint	*Mentha spicata*	1368.6 (100)	2055 (150)	1856 (135)	2020 (147)
10	Ponnaganti	*Alternanthera sessilis*	173.0 (100)	172 (99)	203 (117)	198 (114)
11	Spinach	*Spinacia oleracea*	21.6[a] (100)	69[b] (321)	85[c] (393)	104[d] (481)

Mean values were compared ($n = 3$) by nonparametric Kruskal wallis one way ANOVA. Differences in alphabets are significantly different at $P < 0.05$. Percent gain or loss calculated when raw value taken as 100%. Percent recovery values are given in parentheses. Decimal points are not given due to higher numbers.

Table 5: Effect of domestic processing on FRAP activity of commonly consumed green leafy vegetables.

Sl. no.	Common name	Botanical name	FRAP (mg/100 g $FeSO_4$ Eq.)			
			Raw	Conventional	Pressure	Microwave
1	Amaranth	*Amaranthus gangeticus*	8237.6[a] (100)	11370[b] (138)	12102[b] (146)	11786[b] (143)
2	Ambat chukka	*Rumex vesicarius*	3511.6(100)	3270(93)	2946(83)	3243(92)
3	Coriander leaves	*Coriandrum sativum*	7125.6[a] (100)	18636[b] (261)	16123[c] (226)	19802[d] (277)
4	Curry leaves	*Murraya koenigii*	20275.0[a] (100)	18533[b] (91)	24213[c] (119)	27392[d] (135)
5	Fennel leaves	*Foeniculum vulgare*	9238.6[a] (100)	10128[a] (109)	9970[a] (107)	13362[b] (144)
6	Fenugreek leaves	*Trigonella foenum-graecum*	3409.6[a] (100)	3919[b] (114)	4799[c] (140)	5429[d] (159)
7	Purslane leaves	*Portulaca oleracea*	2863.3[a] (100)	4327[b] (151)	4800[c] (167)	4030[b] (140)
8	Gogu	*Hibiscus cannabinus*	5254.0(100)	7274(138)	6921(131)	7107(135)
9	Mint	*Mentha spicata*	27827.6[a] (100)	42562[b] (152)	48909[b,c] (175)	50401[c] (181)
10	Ponnaganti	*Alternanthera sessilis*	5068.3(100)	4280(84)	4837(95)	4327 (85)
11	Spinach	*Spinacia oleracea*	1380.6[a] (100)	3196[b] (231)	3471[b] (251)	3502[b] (253)

Mean values were compared ($n = 3$) by nonparametric Kruskal wallis one way ANOVA. Differences in alphabets are significantly different at $P < 0.05$. Percent gain or loss calculated when raw value taken as 100%. Percent recovery values are given in parentheses. Decimal points are not given due to higher numbers.

Table 6: Rank correlation between phenolic content versus DPPH and FRAP in different cooking methods of GLV.

TPC versus AOA	Raw	Traditional	Pressure	Microwave	Homogeneity
TPC versus DPPH	0.945	0.936	0.918	0.945	$\chi^2 = 0.23$, $P = 0.97$
TPC versus FRAP	0.955	0.936	0.927	0.973	$\chi^2 = 1.23$, $P = 0.74$
DPPH versus FRAP	0.964	0.973	0.991	0.991	$\chi^2 = 3.23$, $P = 0.36$

All correlations are significant at $P < 0.001$ ($n = 11$).

without peel had the least phenolic content (41 mg/100 g) (Table 7). This difference in phenolic content of green gram whole and dhal could be due to the peel component, known to contribute high phenolic contents in grains. DPPH scavenging activity was the highest in black rajma (160 mg/100 g) followed by whole green gram (113 mg/100 g), and the lowest was in green gram dhal (without peel) (21 mg/100 g) (Table 8). FRAP content was the highest in black rajma followed by soya bean and the lowest was in green gram dhal. The FRAP values were 6852, 3778, and 1066 mg/100 g, respectively (Table 9).

Effect of different cooking methods on antioxidant activity of each food grain was compared with its AOA and phenolic contents of the raw sample. (Tables 7–9). Percentage change in the PC and antioxidant activity on cooking is given in parentheses in Tables 7–9. Overall, different cooking methods did not show any significant cooking losses but showed mixed results of increasing and/or decreasing trends (Tables 7–9), the changes being significant in most of the whole grains as compared to grains without seed coat.

Effects of cooking on PC are presented in Table 7. Nine out of 11 legumes samples showed the maximum of 20% increase or decrease in their PC during different types of domestic cooking. Interestingly, during conventional and pressure cooking, whole Bengal gram and rajma have shown 27 and 54% increase. Other studies showed similar effects on AOA in potatoes upon cooking [44] and in other vegetables [38]. The possible mechanism for the increase or decrease in AOA during various cooking methods could be that the phenolics were stored in pectin or cellulose networks of plant foods and were released during thermal processing [39].

DPPH scavenging activity in legumes cooked by different cooking methods also showed a mixed/inconsistent trend (Table 8). Nine out of eleven food grains studied showed less than 20% increase or decrease during cooking. It is however interesting that whole green gram (with peal) showed a higher increase in DPPH activity in all cooking methods studied, with the increase ranging 40–62% as compared to its content in the unprocessed form. Indeed some literature says that this type of complex trend on cooking is unexplainable and requires further research [45].

Effect of cooking on FRAP activity is given in Table 9. Findings are in line with DPPH, showing a mixed trend. Nine out of eleven legumes showed less than 20% variation in FRAP values. While whole green gram and dry green peas showed higher increase in FRAP ranging 41–102% in different methods of cooking; lentil and red gram dhal showed 34–73% increase *albeit* during pressure cooking only. It was however of interest that over all the percent increase or decrease found vis-à-vis their content in unprocessed food showed similar trend in different cooking methods in a given food grain. Such increasing or decreasing trends were reported in few vegetables from other parts of the world [46]. The possible explanation given for this type of finding was summarized by few workers as follows. Cooking could have resulted in liberation of high amounts of antioxidant compounds due to thermal destruction of cell wall and subcellular compartments [47, 48]. Another possibility might be the production of stronger radical-scavenging antioxidants by thermal or chemical reactions [49]. There can be a production of new nonnutritional antioxidants or formation of novel compounds such as Millard reaction products with

Table 7: Effect of domestic processing on polyphenol content of commonly consumed pulses and legumes in India.

Sl. No.	Common name	Botanical name	Phenolic content (mg/100 g Gallic acid Eq.)				P value
			Raw	Conventional	Pressure	Microwave	
1	Bengal gram dhal	*Cicer arietinum*	92.6 ± 5.5^a (100)	90.6 ± 6.5^a (98)	98.6 ± 4.0^a (106)	86.0 ± 5.5^a (93)	NS
2	Bengal gram dhal (roasted)	*Cicer arietinum*	116.3 ± 7.7^a (100)	105.6 ± 6.1^a (91)	108.6 ± 5.6^a (93)	102.0 ± 10.5^a (88)	NS
3	Bengal gram (whole grains)	*Cicer arietinum*	114.0 ± 10.4^a (100)	154.6 ± 7.0^b (136)	176.3 ± 4.5^c (154)	113.3 ± 6.0^d (99)	0.024
4	Black gram dhal (without peel)	*Phaseolus mungo Roxb*	69.3 ± 4.5^a (100)	58.6 ± 3.0^b (85)	60.0 ± 2.6^b (86)	51.3 ± 3.2^c (74)	0.022
5	Green gram dhal	*Phaseolus aureus Roxb*	41.3 ± 2.5^a (100)	43.6 ± 1.1^a (106)	43.0 ± 3.6^a (104)	34.0 ± 3.0^c (82)	NS
6	Green gram dhal (whole)	*Phaseollus aureus Roxb*	284.3 ± 6.5^a (100)	249.3 ± 3.0^b (88)	269.3 ± 4.5^c (95)	243.6 ± 4.0^b (86)	0.019
7	Lentil	*Lens esculenta*	64.3 ± 2.5^a (100)	64.6 ± 3.5^a (100)	59.0 ± 6.0^a (92)	56.0 ± 2.6^a (87)	NS
8	Peas green (dry)	*Pisum sativum*	82.3 ± 2.0^a (100)	84.0 ± 2.6^a (102)	103.3 ± 5.5^b (126)	75.6 ± 3.5^c (92)	0.024
9	Red gram dhal (without peel)	*Cajanus cajan*	70.0 ± 6.5^a (100)	83.6 ± 4.6^b (119)	81.6 ± 1.5^b (117)	74.0 ± 4.5^a (106)	0.035
10	Rajma (Black)	*Phaseolus Vulgaris*	146.6 ± 7.0^a (100)	186.0 ± 4.5^b (127)	195.6 ± 9.7^c (133)	159.3 ± 2.5^c (109)	0.020
11	Soya bean	*Glycine max* Merr.	81.6 ± 3.5^a (100)	82.0 ± 7.5^a (100)	98.3 ± 5.0^a (121)	94.3 ± 6.0^a (116)	NS

Pooled samples were analyzed in triplicates. Data is presented as mean ± SD. Mean values were compared by nonparametric Kruskal Wallies H test of one way ANOVA. Differences in alphabets are significantly different at $P < 0.05$. Percent gain or loss calculated when raw value taken as 100%. Percent recovery values are given in parenthesis. Decimal points are not given due to higher numbers.

Table 8: Effect of domestic processing on DPPH activity of commonly consumed Pulses and Legumes in India.

Sl. no.	Common name	Botanical name	DPPH (mg/100g Trolox Eq.)				P value
			Raw	Conventional	Pressure	Microwave	
1	Bengal gram dhal	*Cicer arietinum*	42.6 ± 2.5^a (100)	43.3 ± 1.5^a (102)	43.6 ± 4.0^a (102)	40.0 ± 3.6^a (94)	NS
2	Bengal gram dhal (roasted)	*Cicer arietinum*	31.3 ± 2.5^a (100)	34.3 ± 3.7^a (110)	31.3 ± 3.5^a (100)	25.6 ± 2.5^a (82)	NS
3	Bengal gram (whole grains)	*Cicer arietinum*	68.6 ± 4.5^a (100)	100.0 ± 7.5^b (146)	95.3 ± 3.5^b (139)	60.3 ± 2.5^c (88)	0.022
4	Black gram dhal (with out peel)	*Phaseolus mungo Roxb*	35.0 ± 3.0^a (100)	29.0 ± 1.0^a (83)	30.0 ± 7.2^a (86)	24.6 ± .0^a (70)	NS
5	Green gram dhal	*Phaselus aureus Roxb*	21.3 ± 4.5^a (100)	19.3 ± 4.5^a (91)	17.6 ± 3.5^a (83)	18.6 ± 3.6^a (87)	NS
6	Green gram dhal (whole)	*Phaseolus aureus Roxb*	113.6 ± 9.2^a (100)	184.3 ± 9.0^b (162)	159.3 ± 13.7^c (140)	171.3 ± 9.0^{b,c} (151)	0.027
7	Lentil	*Lens esculenta*	35.6 ± 3.5^a (100)	38.0 ± 4.0^a (107)	35.3 ± 3.7^a (99)	36.6 ± 6.5^a (103)	NS
8	Peas green (dry)	*Pisum sativum*	51.0 ± 3.0^a (100)	55.3 ± 3.0^a (108)	56.0 ± 4.0^a (110)	42.0 ± 5.5^b (82)	0.040
9	Red gram dhal (without peel)	*Cajanus cajan*	42.0 ± 4.0^a (100)	49.3 ± 7.5^a (117)	56.3 ± 4.7^a (134)	42.0 ± 4.0^a (100)	NS
10	Rajma (Black)	*Phaseolus Vulgaris*	160.0 ± 8.1^a (100)	182.3 ± 4.5^a (114)	170.3 ± 6.0^a (106)	174.0 ± 9.5^a (109)	NS
11	Soya been	*Glycine max* Merr.	75.6 ± 7.5^a (100)	61.3 ± 2.3^b (81)	59.3 ± 4.1^c (78)	71.6 ± 2.0^a (95)	0.023

Pooled samples were analysed in triplicates. Data is presented as mean ± SD. Mean values were compared by non-parametric Kruskal Wallies H test of one way ANOVA. Differences in alphabets are significantly different at $P < 0.05$. Percent gain or loss calculated when raw value taken as 100%. Percent recovery values are given in parenthesis. Decimal points are not given due to higher numbers.

Table 9: Effect of domestic processing on FRAP activity of commonly consumed Pulses and Legumes in India.

S. no.	Common name	Botanical name	FRAP (mg/100 g $FeSO_4$ Eq)				P Value
			Raw	Conventional	Pressure	Microwave	
1	Bengal gram dhal	*Cicer arietinum*	1679 ± 53.2[a] (100)	1909 ± 64.7[a] (114)	1968 ± 44.1[a] (117)	1973 ± 46.6[a] (118)	NS
2	Bengal gram dhal (roasted)	*Cicer arietinum*	1466 ± 125.2[a] (100)	1711 ± 109.5[a] (117)	1359 ± 114.5[a] (93)	1367 ± 103.5[a] (93)	NS
3	Bengal gram (whole grains)	*Cicer arietinum*	2283 ± 132.8[a] (100)	2560 ± 131.0[b] (112)	2676 ± 170.0[b] (117)	2177 ± 102.1[a] (95)	0.033
4	Black gram dhal (without peel)	*Phaseolus mungo Roxb*	1515 ± 41.4[a] (100)	1420 ± 80.1[a] (94)	1470 ± 46.5[a] (97)	1265 ± 47.8[a] (83)	NS
5	Green gram dhal	*Phaseolus aureus Roxb*	1066 ± 128.6[a] (100)	1371 ± 58.3[a] (128)	1042 ± 99.8[a] (98)	938 ± 85.7[a] (88)	NS
6	Green gram dhal (whole)	*Phaseolus aureus Roxb*	3098 ± 22.4[a] (100)	5490 ± 101.0[b] (177)	5785 ± 184.6[c] (187)	5505 ± 81.1[b] (178)	0.025
7	Lentil	*Lens esculenta*	1534 ± 54.0[a] (100)	1652 ± 121.0[a] (108)	2058 ± 109.0[a] (134)	1625 ± 107.9[a] (105)	NS
8	Peas green (dry)	*Pisum sativum*	1846 ± 80.8[a] (100)	3027 ± 93.7[a] (164)	3734 ± 71.0[b] (202)	2609 ± 64.5[c] (141)	0.016
9	Red gram dhal (without peel)	*Cajanus cajan*	2446 ± 84.9[a] (100)	3133 ± 81.6[b] (128)	4251 ± 106.6[c] (173)	2646 ± 84.8[b] (108)	0.016
10	Rajma (Black)	*Phaseolus Vulgaris*	6852 ± 66.4[a] (100)	6809 ± 125.2[a] (99)	7171 ± 81.4[b] (105)	7915 ± 130.5[c] (115)	0.025
11	Soya been	*Glycine max* Merr.	3778 ± 162.5[a] (100)	3504 ± 128.0[a] (93)	3714 ± 125.5[a] (98)	3502 ± 149.0[a] (93)	NS

Pooled samples were analysed in triplicates. Data is presented as mean ± SD. Mean values were compared by nonparametric Kruskal Wallies H test of one way ANOVA. Differences in alphabets are significantly different at $P < 0.05$. Percent gain or loss calculated when raw value taken as 100%. Percent recovery values are given in parenthesis. Decimal points are not given due to higher numbers.

Table 10: Rank correlation between phenolic content and AOA (DPPH and FRAP) in raw and cooked pulses and legumes.

TPC versus AOA	Raw	Traditional	Pressure	Microwave	Homogeneity
TPC versus DPPH	0.689	0.801	0.793	0.780	$\chi^2 = 1.23$, $P = 0.746$
TPC versus FRAP	0.573	0.701	0.619	0.706	$\chi^2 = 1.12$, $P = 0.772$
DPPH versus FRAP	0.918	0.909	0.895	0.916	$\chi^2 = 0.31$, $P = 0.959$

All correlations are significant at $P < 0.01$ ($n = 11$), and correlations are comparable across the methods. Between the methods, all the parameters are significantly correlated (TPC versus DPPH, TPC versus FRAP, and DPPH versus FRAP).

antioxidant activity during cooking. However these findings are first of their kind in commonly consumed pulses and legumes.

Correlations among the PC and AOA were determined in the legumes in unprocessed as well as during the three different types of domestic cooking. For this purpose rank correlations were used, and the data is presented in Table 10. Correlations between PC and AOA were significant in different cooking methods, and they were comparable across the methods. Although different cooking methods showed changes (highly significant in some cases) in the phenolic content and AOA of the food grains, the finding that they did not affect the correlation between the PC and AOA suggests that PC may be important contributor to the AOA even in pulses and legumes, both in raw and cooked forms.

12. Conclusions

To the best of our knowledge, findings observed in this review are first of their kind from India, this review mainly dealt with two aspects, and natural antioxidant content of commonly consumed plant foods in India was assessed and correlated with its phenolic content. And the second aspect is assessing the effect of domestic cooking on PC and antioxidant activity for the first time from India in the most commonly consumed GLVs and grains. Our findings demonstrate that antioxidant contents did not get affected in most of the foods studied; on the other hand most of them shown a higher AOA in different method of domestical processing. This overview would be useful to researchers, nutritionists, and consumers to assess AOA and/or formulate antioxidant-rich therapeutic

diets as well as commercial antioxidant-rich preparations from plant foods. In addition, they will be a valuable addition to the scanty knowledge on antioxidant activity of commonly consumed foods in India.

Limitation of the Present Findings. Purposive samples were collected from three local markets to provide first hand information on antioxidant activity of plant foods commonly consumed in India. Hence, findings cannot be considered as Indian plant foods data base.

Abbreviations

AOA: Antioxidant activity
DPPH: 2,2′-Diphenyl-1-picryl hydrazyl
FRAP: Ferric reducing antioxidant power
GLV: Green leafy vegetables
TPTZ: 2,4,6-Tripyridyl-s-triazine
PC: Phenolic content.

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Review Article

Polyphenols: Multipotent Therapeutic Agents in Neurodegenerative Diseases

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Aging leads to numerous transitions in brain physiology including synaptic dysfunction and disturbances in cognition and memory. With a few clinically relevant drugs, a substantial portion of aging population at risk for age-related neurodegenerative disorders require nutritional intervention. Dietary intake of polyphenols is known to attenuate oxidative stress and reduce the risk for related neurodegenerative diseases such as Alzheimer's disease (AD), stroke, multiple sclerosis (MS), Parkinson's disease (PD), and Huntington's disease (HD). Polyphenols exhibit strong potential to address the etiology of neurological disorders as they attenuate their complex physiology by modulating several therapeutic targets at once. Firstly, we review the advances in the therapeutic role of polyphenols in cell and animal models of AD, PD, MS, and HD and activation of drug targets for controlling pathological manifestations. Secondly, we present principle pathways in which polyphenol intake translates into therapeutic outcomes. In particular, signaling pathways like PPAR, Nrf2, STAT, HIF, and MAPK along with modulation of immune response by polyphenols are discussed. Although current polyphenol researches have limited impact on clinical practice, they have strong evidence and testable hypothesis to contribute clinical advances and drug discovery towards age-related neurological disorders.

1. Introduction

Neurodegenerative disorders such as Alzheimer's disease (AD), stroke, and Parkinson's disease (PD) represent a major clinical problem in the developed countries [1, 2] and are major economic burdens for health care systems [3]. Dietary [4], genetic, and molecular factors [5] are important determinants in progression and intervention of neurodegenerative diseases. AD is a common cause of dementia and mortality in the United States. Total numbers of reported deaths due to AD have increased in past years, and it is among 10 leading causes of deaths in the United States [6]. Amyloid-β (Aβ) peptides derived from amyloid precursor protein (APP) via γ-secretase and β-secretase cleavage are hallmarks of AD [7]. Cellular prion protein (PrP(C)) [8] and oxidative stress [9] mediate Aβ neurotoxicity, and the latter contributes to neuronal death by lowering intracellular glutathione. Along with Aβ, tau protein alteration in neuronal microtubules also contributes to the pathology of AD [10]. Abnormal phosphorylation and aggregation of tau protein leads to neural dysfunction and leads to pathological events which cause neuronal dysfunction in AD [11]. Failed clearance of Aβ aggregates resulting from impaired autophagy may also contribute to AD [12]. AD is also characterized by elevated peripheral blood cytokine concentrations for interleukin- (IL-) 6, tumor necrosis factor alpha (TNF-α), IL-1β, transforming growth factor beta (TGF-β), IL-12, and IL-18 suggestive of a pro-inflammatory response in AD pathology [13].

Multiple sclerosis (MS) is another neurodegenerative disease characterized by chronic inflammation accompanied by demyelination of neurons in brain [14]. MS is characterized by symptoms like mood disorder, fatigue, vision changes, muscle weakness, and motor changes [15]. Chemokines like IL-17, chemokine (C-C motif) ligand 17 (CCL17), and CCL20 are suggested as major mediators in MS neuroinflammation and pathology [16].

Stroke is the third leading cause of mortality, loss of cognitive functions, and heavy socioeconomic burden in the United States [17]. Similar to MS, stroke or cerebral ischemia is a pathological condition accompanied by inflammation

and immune system disease [18]. One minute of cerebral ischemia is estimated to destroy approximately 2 million neurons and 14 million synapses [19]. Like AD and MS, inflammatory cytokines including TNF-α and IL-1 and IL-6 play modulatory role in stroke pathology [20]. Transcription factor, nuclear factor kappa B (NFκB), is an important regulator in pathology of inflammation and neuronal cell survival, as its activation leads to cell death in cerebral ischemia [21].

PD is a progressive neurodegenerative disease; its familial forms are characterized by mutations of six genes including clinically important ATP13A3 resulting in cognitive impairment and depression [22]. Some PD cases also involve changes in micro-RNA and α-synuclein level in patients [23]. Like other neurodegenerative diseases, PD also involves elevated levels of proinflammatory cytokines monocyte chemoattractant protein-1 (MCP-1), CCL-5, macrophage inflammatory protein-1α (MIP-1α), IL-8, interferon-gamma (IFNγ), IL-1β, and TNFα [24]. After examining cytokines in 52 PD patients, researchers [25] suggested involvement of TNF-α in production and maintenance of nonmotor symptoms. Mitochondrial dysfunction also plays an important role in pathogenesis of PD [26] similar to AD [27], MS [28], and stroke (CI) [29].

Huntington's disease (HD) is another neurological disorder causing cognitive impairment, accompanied by oxidative stress and mitochondrial dysfunction. It results from increased number of CAG triplet nucleotide repeats and expanded polyglutamine region of huntingtin protein [30]. HD pathology leads to elevated levels of chemokines like eotaxin-3, MIP-1β, eotaxin, MCP-1, and MCP-4 [31].

The pathophysiology of neurological disorders is also accompanied by alterations in electrical activity of neurons at cellular level. The voltage gated ion channels are required for action potential generation and its propagation in neurons, and their dysfunction contributes to pathology of neurodegenerative diseases. The brain electrical activity is significantly changed in AD and dementia leading to impaired verbal memory and cognitive skills [32]. The Kv3 subfamily of K^+ channel subunits, which possess ability of fast repolarization of action potential [33], are compromised and slowed down in AD [34]. The upregulation of the K(v)1.3 potassium channel also plays important role in immunopathogenesis of multiple sclerosis and presents therapeutic option by blocking Kv channels [35]. As sodium channels ($Na_v1.8$, $Na_v1.5$) play an important role in electrical activity of neurons, their overloading is thus an important mediator in axonal degeneration in MS [36, 37]. Such electric disturbances are also found in PD [38] which impose energy burden by Ca^{2+} entry through L type voltage-dependent channels [39]. Similarly, sodium and potassium channel abnormalities are proposed to contribute to HD pathogenesis [40, 41].

There are a few clinically relevant medicines and therapies available for AD, HD, MS, PD, and stroke. A few clinically active, yet expensive, options such as acetylcholinesterase inhibitors, interferon β-1a, levodopa, tetrabenazine, and tissue type plasminogen activator (tPA) are available for AD [42], MS [43], PD [44], HD [45], and stroke [46], respectively. In wake of these pathologies and limited clinical treatments, alternative and preventive therapeutics are required which can control the occurrence and progression of neurodegenerative diseases. All the neurodegenerative diseases discussed above have the common features of pathogenesis which include cytokine changes, genetic alterations, immunomodulation, inflammation, mitochondrial dysfunction, oxidative stress, prions, and protein dysfunction. Recent research has shown that dietary polyphenols target the pathological manifestations of neurological disorders with their ability to cross blood-brain barrier [47] as they control neuronal disease pathogenesis at a molecular and symptomatic level by targeting these common features of neurodegeneration pathology. Polyphenols are naturally occurring phytochemicals found in fruits and vegetables, exhibiting strong neuroprotective properties [48]. Important dietary sources of polyphenols include apples, berries, cocoa, herbs, red wines, seeds, onions, and tea [49]. Dietary polyphenols have also been implicated in prevention of oxidative damage and LDL oxidation [50–52]. This review briefly outlines the pharmacological role of polyphenols in preventing neurodegenerative diseases based on the most recent scientific literature (Figure 1).

2. Polyphenols and Pharmacological Properties

2.1. Alzheimer's Disease and Dementia. Polyphenols exhibit neuroprotective properties including therapeutic action in AD and dementia. Green and white tea extracts have been shown to inhibit acetylcholinesterase which indicates their potential in treatment of age-related disorders such as AD [53]. Green tea polyphenols protect primary rat cortical neurons against Aβ-induced cytotoxicity [54]. In mouse model studies [55], polyphenols of grapes improved cognitive functions in mouse model of AD. As well, epicatechin metabolite 3′-*O*-methyl-epicatechin-5-*O*-β-glucuronide had improved synaptic transmission through cyclic adenosine monophosphate (cAMP) response element binding protein. In transgenic mice model studies, grape seed polymeric polyphenol extract has been shown to inhibit oligomerization of Aβ peptides and contributed to reduction in cognitive impairments in transgenic mice [56]. A similar study showed that polyphenols of grapes exhibited potential in neutralizing abnormal folding of tau proteins [57]. Earlier studies using animal models [58, 59] also confirm anti-Aβ action of grape seed polyphenols.

Resveratrol, a polyphenol abundant in grapes and red wines, inhibited Aβ 42 fibril formation [60] and protected from Aβ neurotoxicity by inhibiting inducible nitric oxide synthase inhibition [61]. Resveratrol, with possibly high bioavailability in lipid core nanocapsules, exhibited therapeutic action in AD [62]. Flavonoid fisetin and its analogues also inhibited Aβ fibril formation and have emerged as new drug candidates for AD treatment [63]. Morin (2′,3,4′,5,7-pentahydroxyflavone) has shown to prevent neuronal cell death by protecting neurons against tau hyperphosphorylation induced by Aβ [64]. Similarly, in transgenic mouse model studies [65], tannic acid has displayed the attenuation of Aβ deposition by decreasing cleavage of β-carboxyl terminal amyloid precursor protein (APP) fragment and controlled

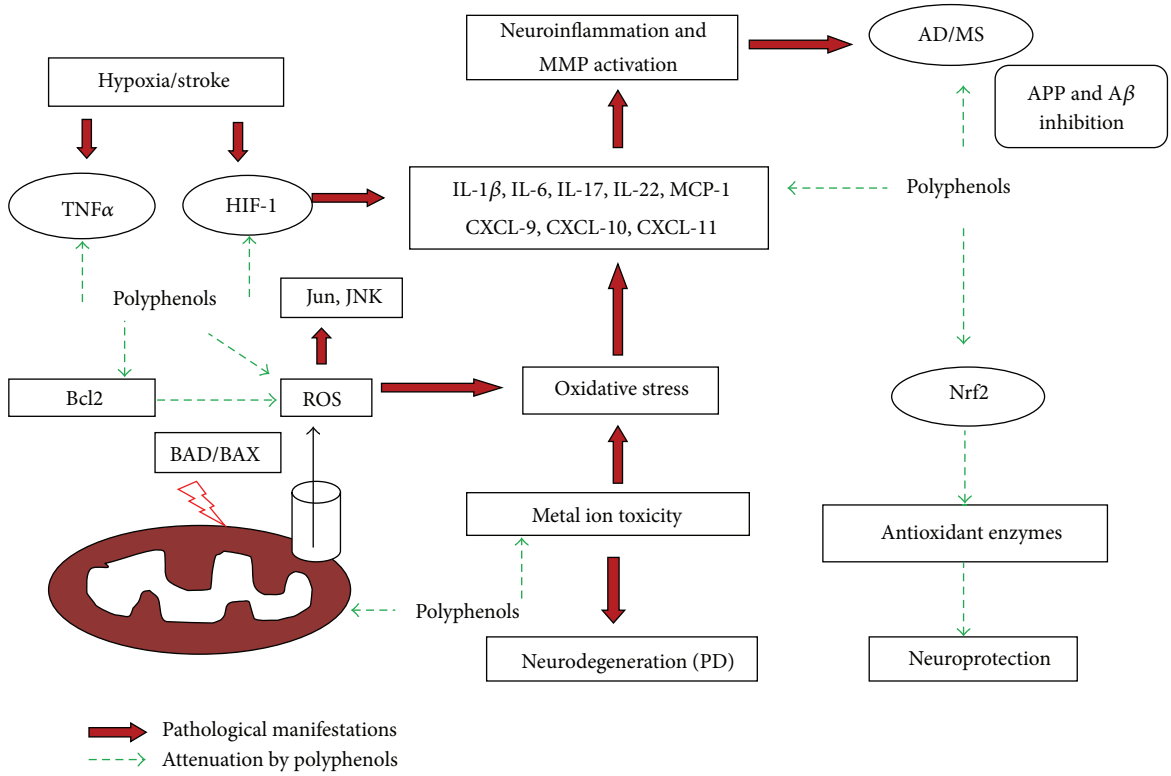

Figure 1: Neuroprotection by polyphenols against neurological disorders.

neural inflammation. A flavonoid, 7,8-dihydroxyflavone, has been shown to improve cognitive abilities in 5XFAD transgenic mouse model of AD by activation of tyrosine receptor kinase B leading to reduction in β-secretase enzyme levels and amyloid beta (Aβ) synthesis [66]. Similarly, liquiritigenin improved memory in Tg2576 mice model of AD, as it attenuated astrocytosis and decreased the Notch-2 expression as the latter can contribute to neuronal decay [67]. Unlike resveratrol, quercetin and rutin not only inhibited Aβ formation but also disaggregated Aβ fibrils in AD studies [68]. Both compounds also prevented scopolamine-induced amnesia in animal model systems [69]; however, resveratrol did not reverse scopolamine-induced deficit [70]. Rutin has been found to control oxidative stress, malondialdehyde, and glutathione disulfide formation in SH-SY5Y neuroblastoma cells. Rutin has also attenuated the inflammatory cascade by decreasing cytokines like TNF-α and IL-1β [71]. Ferulic acid, a phenolic acid, has also exhibited higher neuroprotection against Aβ toxicity than quercetin [72]. Recent research findings have shown that polyphenols have therapeutic relevance in both cell and animal model studies. The ability of polyphenols to improve synaptic transmission by elevating cAMP, target multiple signaling pathways, and reduce Aβ toxicity suggests their therapeutic utility for age-related disorders like AD and dementia.

2.2. Multiple Sclerosis. Multiple sclerosis is a neurodegenerative disease characterized by autoimmune-mediated demyelination in the CNS resulting in paralysis and cognitive deficits. MS therapies can reduce inflammation and downregulate immune function [73]. Resveratrol, a silent mating type information regulation 2 homolog1 (SIRT1) activator, has exhibited prevention of neural loss without immunosuppression in experimental autoimmune encephalomyelitis (EAE) model of MS [74]. Pharmaceutical grade formulation of resveratrol SRT501 was found to attenuate neural damage in EAE through SIRT1 activation [75]. Cell culture studies [76] have also shown SIRT1-mediated neuroprotection by resveratrol. Quercetin was found to control immune response via modulation of IL-1β and TNF-α and reduced the proliferation of peripheral blood mononuclear cells isolated from multiple sclerosis patients [77]. Epigallocatechin-3-gallate (EGCG) exhibited neuroprotective effects by modulating neuroinflammation and attenuating neural damage [78]. Quercetin [79], apple polyphenols [80], myricetin, and piceatannol [81] have also activated SIRT1, thus exhibiting potential in MS treatment. Earlier studies have also shown [82] that flavonoids limit demyelination in MS suggesting their potential against neuro-inflammation and related disorders. Preclinical data has shown that polyphenols exhibit potential to block neural inflammation and damage by activation of SIRT1 pathway along with modulation of inflammatory cytokines. The potential of polyphenols on limiting demyelination makes them prospective therapeutics in age-related MS and amyotrophic lateral sclerosis (ALS).

2.3. Ischemic Stroke. Various epidemiological studies suggest that diet rich in polyphenols can extend neuroprotection

and lower the risk and severity of stroke, the third leading cause of mortality [83]. Experimental evidence using rodent and cellular models also indicates neuroprotective potential of dietary polyphenols in cerebral ischemia. Green tea polyphenol, EGCG, has exhibited neuroprotective action by downregulation of matrix metalloproteinases (MMP) in mice model of cerebral ischemia [84]. Green tea polyphenols have also been found to protect neurons against hypoxia-induced ischemic injury by controlling inflammation cascade and attenuating decline in transmembrane potential [85]. Quercetin has been found to attenuate ischemic injury by controlling acid-sensing ion channel led calcium dysregulation and lipid peroxidation in neurons [86]. Another study with similar experimentation has supported neuroprotective role of quercetin, based on its ability to block sodium channels [87]. Quercetin with similar antioxidant therapy to green tea polyphenols has reduced the level of MMP-9 and attenuated blood-brain barrier disruption in cerebral ischemia (CI) studies [88]. Researchers have also hypothesized neuroprotective action of quercetin in CI to be based on its inhibitory action against MMP [89]. Rutin has been found to control neural damage in CI through downregulation of p53, a protein which leads to necrosis in stroke [90]. It has also shown the attenuation of glutathione peroxidase, glutathione reductase, and inflammatory cytokines in rodent model of ischemic stroke [91]. In addition, resveratrol has been found to extend protection against ischemic injury by improving brain energy metabolism and controlling oxidative stress during ischemia injury in animal model studies [92], along with the modulation of release of multiple therapeutic neurotransmitters and neuromodulators during ischemic injury [93]. The flavonoid fisetin has shown neuroprotective action during cerebral ischemia as it stopped infiltration of macrophages and dendritic cells into ischemic hemisphere, thus controlling neural inflammation and damage [94]. Another flavonoid baicalin has been shown to reduce ischemic stroke damage by targeting multiple therapeutic targets like MMP-9 [95], caspase-3, oxidative stress [96], and p38 mitogen-activated protein kinase (MAPK) [97] and by downregulating toll-like receptor (TLR2/4) pathway [98]. The experimental data reveals that polyphenols may prevent, attenuate, or slow down, via multiple mechanisms, the course of stroke and age-related neural disorders. Since the risk for stroke increases with age, consumption of polyphenol rich diet seems to be an important preventive strategy.

2.4. Parkinson's Disease (PD). PD is a neurodegenerative disease accompanied by inflammation and oxidative stress resulting in loss of dopaminergic neurons in the substantia nigra [137]. Polyphenols with their ability to attenuate oxidative stress and inflammation present therapeutic option in neurodegenerative disease. Resveratrol has been shown to inhibit the loss of dopaminergic neurons in rat model of PD [76]. Resveratrol has also been shown to reduce neural inflammation in PD by lowering mRNA levels of cyclooxygenase-2 (COX-2) and TNF-α mRNA in the substantia nigra [100] along with attenuation of oxidative stress, lipid peroxidation, and protein carbonyl (PC) in rat model of PD [138]. Oxyresveratrol has demonstrated attenuation of neural damage in SH-SY5Y cells by elevating levels of SIRT1 and downregulating expression of caspase-3, c-Jun N-terminal kinase (JNK), and c-Jun transcription factors [139]. Ferulic acid, like oxyresveratrol, has demonstrated neuroprotective effect via downregulation of JNK pathway [140]. Quercetin administration to neurons attenuated 1-methyl-4-phenylpyridinium (MMP) evoked microglia activation, which is a precursor for PD pathogenesis [124]. Studies have also shown that quercetin promises neuroprotection in PD mice model by stimulating glutathione peroxidase (GPx), superoxide dismutase (SOD), $Na(^{+})$, and $K(^{+})$-ATPase [141]. Quercetin suppressed cell death in PD cell model while its metabolite quercetin-3-*O*-β-glucuronide, due to its low absorption, did not affect cell viability [142]. Another study showed that conversion of quercetin metabolites to its aglycone in neural cells is essential for neuroprotective activity [143]. These studies had shown consistent results as compared to a contradictory report [144] which showed that quercetin had no neuroprotective role in PD cells and rat models. Other polyphenols such as baicalein [145], kaempferol [146], caffeic acid [147], and EGCG [148] have been shown to extend neuroprotection in PD studies. Similarly, polyphenolic extracts from various plants have also exhibited pharmacological role in PD studies. For instance, polyphenols-rich mulberry fruit extracts have shown antioxidant and antiapoptotic effect in SH-SY5Y cells by modulating caspase-3, B-cell lymphoma (Bcl-2), and BCL2-associated X protein (Bax) [149].

2.5. Huntington's Disease. CAG triplet nucleotide repeats and expanded polyglutamine region of huntingtin protein form basis of HD [30]. Polyphenols hold pharmacological relevance, as they are associated with numerous benefits including antiaging, anti-inflammatory, and anticancer effects. Resveratrol has been found to exhibit positive effects in transgenic mouse model of HD via SIRT1 activation of peroxisome proliferative activated receptor, gamma, and coactivator 1 alpha (PGC-1α) signaling pathway [76]. Studies have further demonstrated the Ras-extracellular signal-regulated kinase activation by resveratrol and fisetin as the basis for neuroprotection in models of HD [150]. Likewise, hesperidin and naringenin, abundant in citrus fruits, induced neuroprotection in rats possibly via nitric oxide synthase (NOS) inhibition [151]. Curcumin has been shown to control Huntington aggregates and improve various transgene-dependent parameters, thereby promising therapeutic action in HD [152]. Grape and green tea polyphenols have also exhibited potential for treating/preventing HD disease pathogenesis [153, 154]. The overall preclinical data suggests that polyphenols extend strong neuroprotection through genetic and immunological modulation, thus promising clinical prevention or delay of neurological disorders like PD and HD.

3. Polyphenols and Oxidative Stress

A large body of literature supports the antioxidant potential of polyphenols against oxidative stress. Resveratrol is a potent antioxidant *in vitro* [155] and *in vivo* as it attenuates oxidative stress in both animal [156] and various cell model studies

[157]. Resveratrol has been shown to extend antioxidant effect by reducing the production of reactive oxygen species (ROS) and superoxide ions [158]. Similarly, quercetin has also shown protection against oxidative stress and related disorders [159]. In a variety of cell and disease models, quercetin has been shown to engage in various signaling pathways to attenuate oxidative stress and exhibit pharmacological properties [51, 114]. Polyphenol-rich green tea and its principal constituent EGCG were found to ameliorate oxidative stress in various studies [160, 161]. Other polyphenols such as puerarin [162], baicalin [163], and phloridzin [136] also attenuated oxidative stress in various disease models. Apart from *in vitro* and *in vivo* evidence, sufficient clinical evidence also suggests the antioxidant potential of polyphenols. A clinical study [164] showed that polyphenol-rich diet reduced LDL oxidation and modulated cluster of differentiation 40-ligand (CD40L) gene expression, thus controlling atherogenesis and inflammation in humans. Polyphenol-rich fruit extracts have been shown to control free radicals and ROS. Polyphenol-rich bilberry juice was found to decrease oxidative stress and inflammatory markers in humans [165]. A 13-year long clinical study indicated that higher intake of antioxidant polyphenols including flavonoids and phenolic acids helps in improving memory and has potential for inhibiting brain aging [166]. Antioxidant-rich polyphenol supplementation as beverage has also been found to decrease plasma total homocysteine, thus contributing to attenuation of AD pathology [167]. It can be concluded that polyphenols are strong antioxidants *in vitro* and *in vivo* in both animal models and humans. Clinical translation of polyphenols as antioxidant therapy is a promising approach to attenuate oxidative damage due to aging and age-related disorders.

4. Polyphenols and Signal Transduction Pathways

4.1. Akt/PI3K/mTOR Pathway. Resveratrol has exhibited neuroprotection against brain ischemia through P13K/Akt pathway by downregulating the expression of glycogen synthase kinase 3 (GSK-3β) and cAMP response element binding (CREB) proteins [99]. Resveratrol increased cAMP and modulated Akt pathway in cell model studies [144]. Baicalein also protects against ischemia through P13K/Akt pathway [126]. The scientific evidence suggests therapeutic intervention by polyphenols via P13K/Akt pathway (Table 1).

4.2. NFκB Pathway. NFκB is an important mediator in inflammatory process and contributes to Aβ toxicity. Flavonoids and other dietary polyphenols have shown neuroprotective effects in neuronal ischemia through NFκB pathway. Flavonoids, including kaempferol, quercetin, acacetin, apigenin, and luteolin, inhibit Aβ1-40 and Aβ1-42 via NFκB pathway downregulation [101]. Similarly, soybean isoflavone had reversed memory impairment in rats through decrease in NFκB expression [102]. Other flavonoids such as resveratrol and baicalin inhibit Aβ-induced neural inflammation via a mechanism involving downregulation of NFκB signaling pathway [103, 168]. Activation of NFκB is an important event in ischemic injury and contributes to both inflammation and cell death [21]. Silymarin, a flavonolignan from milk thistle (*Silybum marianum*), has protected against cerebral ischemia by inhibiting signal transducer and activating transcription (STAT-1) pathway and NFκB [105]. Tetrahydroxystilbene glucoside from *Polygonum* multiforum has protected neurons from cerebral ischemia by activating SIRT1 and inhibiting NFκB signaling pathway in neurons [58]. Quercetin administration has also protected rat brain against oxidative stress and hypoxia-induced damage through NFκB inhibition [106]. Similarly, catechin hydrate and fisetin have been shown to protect rat brain against ischemic injury and oxidative damage by inhibiting expression of NFκB and proinflammatory cytokines such as IL-1β and TNF-α [83, 94]. It is observed that recent research advances confirm an immunomodulatory role of polyphenols as they control inflammatory response by inhibiting NFκB expression.

Table 1: Neuroprotective signal transduction by polyphenols.

Pathway	Polyphenol	References
P13K/AkT pathway	Resveratrol	[99, 100]
	Baicalein	[97]
NFκB pathway	Kaempferol, acacetin, apigenin, luteolin	[101]
	Soybean isoflavones	[102]
	Fisetin	[94]
	Resveratrol	[103]
	Baicalein	[104]
	Silymarin	[105]
	Tetrahydroxystilbene	[58]
	Quercetin	[101, 106]
	Catechin hydrate	[94]
STAT pathway	Silymarin	[105]
PPAR pathway	Baicalein	[104]
	Resveratrol	[107]
	Pterostilbene	[108]
Nrf2/HO1/ARE pathway	Epicatechin	[109]
	Resveratrol	[110]
HIF-1α	Xanthohumol	[111]
	Resveratrol	[112]
MAPK	Flavone glycoside	[113]
	Quercetin	[114]

4.3. PPAR Pathway. Peroxisome proliferator activated receptor gamma (PPAR gamma) plays a role of a biomarker in cerebral ischemia as CI results in its upregulation and translocation to nucleus from the cytosol. Baicalein has reversed PPAR gamma expression and also suppresses its translocation to nucleus [104]. Resveratrol has attenuated the MMP-9 by modulating the peroxisome proliferator activated receptor (PPAR) alpha expression in hypoxia model of neurons [107]. Pterostilbene, a resveratrol derivative, has significant effect on the downregulation and normalization

of PPAR-α expression in SAMP8 mouse model studies [108]. The scientific evidence shows that benefits associated with polyphenols in age-related and other disorders are associated with downregulation of PPAR pathway.

4.4. Nrf2/ARE/HO1 and HIF-1 Pathway. Nuclear factor (erythroid-derived 2-) like 2 (Nrf2) pathway is also involved in Sestrin2 (Sesn2), also known as Hi95, a p53 target gene expression which leads to encoding of antioxidant proteins [169]. Epicatechin has protected neurons against stroke and oxidative stress by upregulation of Nrf2 cascade and heme oxygenase-1 (HO1) enzyme [109]. Similarly, resveratrol has also been shown to protecte brain through increased expression of Nrf2, HO-1 expression, and downregulation of apoptotic enzymes like caspase-3 [110]. Xanthohumol, a prenylated chalcone, has demonstrated neuroprotective action by inhibiting HIF-1 pathway and it further stopped signal transduction pathway leading to apoptosis by caspases [111]. Resveratrol, apart from Nrf2 and HO-1 expression, has protected against ischemic injury in cells by downregulation of mRNA expression of hypoxia inducible factors-1α (HIF-1α) [112]. Upregulation of Nrf2 and HO-1 pathways by polyphenols in response to oxidative insult shows protective role of these compounds in brain health and oxidative damage.

5. Polyphenols and Immune Response

Proinflammatory cytokines and genes contribute to inflammation and neuronal death in various neurological disorders. Most of the therapeutics target cytokines and other immune responses for therapeutic intervention. Polyphenols are well known for their anti-inflammatory activities and thus control neuroinflammation and neural death. EGCG has been found to inhibit expression of monocyte chemotactic protein (MCP-1/CCL2) and IL-1β, thus protecting blood-brain barrier (BBB) integrity during pathological inflammation [170]. In another study, EGCG inhibited cytokine and chemokines including IL-1β, IL-6, and MCP-1 [116]. Resveratrol has also controlled hippocampal inflammation by reducing expression of MCP-1 mRNA levels [118]. Polyphenols, that is, catechin, caffeic acid, and transresveratrol, reduced production of inflammatory markers MCP-1, MIP-1α, MIP-1β, chemokine receptor-1 (CCR1), and CCR2 in vascular wall [119]. Polyphenol-rich olive oil controlled inflammation by inhibiting proinflammatory CD40, a costimulatory protein found on antigen presenting cells, gene expression [164]. EGCG reduced expression of inflammatory cytokines and chemokines such as chemokine (C-X-C motif) ligand (CXCL10), CCL22, CCL 17, and TGF-β, promising strong neuroprotection in AD and stroke [117]. Quercetin has been shown to inhibit proinflammatory cytokines like IL-1β, IL-6, COX-2, CD40, and TNFα receptor-associated factor-1 (TRAF1) [120]. A similar study [121] showed that quercetin exhibits neuroprotective effect in PC12 cells and zebrafish possibly by downregulating expression of proinflammatory genes like IL-1β and COX-2. Resveratrol also reduced neuroinflammation and improved memory along with IL-1β inhibition [115]. Resveratrol has exhibited restoration of BBB integrity and inhibited rising levels of IL-17A, T-helper 17 lymphocytes, and MMP [171]. Apple polyphenols have also reduced expression of a wide range of neuroinflammatory markers including IL-1β, IL-6, IL-17, 1L-22, CXCL9, CXCL10, CXCL11, and IFN-γ, thus providing immune-modulatory effects against inflammation [122]. Studies have also shown that blueberry and apple polyphenols can attenuate neuroinflammation and improve cognitive impairment, possibly by lowering the expression of IL-1β and TNFα in rat hippocampus [172, 173]. About 20 structurally related flavonoids have been shown to inhibit hypoxia-induced STAT3 tyrosine phosphorylation promoting cell survival [174]. Fisetin and quercetin protected neurons against LPS-induced inflammation by inhibiting TNFα production and JNK/Jun phosphorylation [120, 139]. Resveratrol administration during ischemic stroke inhibits neuronal damage along with reduced expression of IL-1β and TNFα [175]. Overall, polyphenols modulate immune response in neurodegenerative diseases as they induce expression of antiapoptotic factors, control neuroinflammation, and modulate cell signaling under stress (Table 2). These features of polyphenols make them strong neuroprotective candidates and support their translation from laboratory to clinical trials.

Table 2: Modulation of cytokines and inflammatory targets by polyphenols.

Polyphenol	Target	References
EGCG	IL-1β	[115]
	IL-6	[116]
	MCP-1	[115]
	CXCL 10,	[116]
	CCL22, CCL 17	[117]
	TGF β	[117]
Resveratrol	MCP-1	[118]
Catechin	MCP-1 (α and β)	[119]
Caffeic acid	CCR1, CCR2	[119]
Quercetin	IL-1β, IL-6	[120]
	COX-2	[120]
	COX-20, TRAF1	[121]
Apple polyphenols	IL-1β, IL-6, IL-17, IL-22 CXCL-9, CXCL-10, CXCL-11,	[122, 123]

6. Polyphenols and Metal Ion Chelation

Iron and copper play important roles in the generation of ROS through redox cycling and subsequent neurodegeneration. Metal accumulation in brain contributes to pathology of diseases like AD, MS, PD, and HD [176, 177]. EGCG exhibited iron chelating ability in SH-SY5Y neuroblastoma cells along with the inhibition of apoptotic factors like BCL2-associated agonist of cell death (Bad), Bax, and caspase-3 [178]. EGCG has exhibited stronger chelation of iron compared to desferrioxamine and increased transferrin receptor protein along with the elevation in mRNA levels in SH-SY5Y neuroblastoma cells [179]. Electron paramagnetic

resonance studies [180] demonstrated the interaction of EGCG and gallic acid as ligands to copper coordination sphere, thus demonstrating Cu modulatory potential of these polyphenols. Also, iron modulation by curcumin in rat brain homogenate has been observed, thus warranting that curcumin-based therapy in AD and PD disease models [181]. Curcumin has extended neuroprotection in a rat model of PD against 6-hydroxydopamine treatment through its iron chelating activity and reduced degeneration of neurons [182]. Similarly, curcumin's ability to reverse neurodegeneration in hemi-Parkinson's mice model has been shown [183]. Apart from iron chelation, NFκB modulation by curcumin has also contributed to the reduction in 6-OHDA-induced neurodegeneration [184]. Rosmarinic acid, a phenolic acid found in Lamiaceae herbs, protected neurons against 6-OHDA treatment by lowering the expression of Bax/Bcl-2 at gene level and decreasing iron level in both MES23.5 dopaminergic cells and rat model of PD [185, 186]. It is evident that polyphenols are potent metal chelators and extend neuroprotection against iron- and copper-induced oxidative stress and neurotoxicity via metal chelation, modulation of signal transduction, oxidative stress, and inflammation.

7. Polyphenols and Prions

Prion proteins are involved in neurodegenerative diseases, and their conformational transition forms basis of prion diseases. The pathology of prion proteins has been inhibited by EGCG and ECG, thus exhibiting neuroprotective potential [187]. Studies have also confirmed the antiprion activity of resveratrol through autophagy activation in neuroblastoma cells [188]. Resveratrol also protected mouse neurons against PG14-PrP (mutant prion protein) expression [189]. Curcumin also downregulated the prion pathology in neuroblastoma cells [190]. Therefore, polyphenols seem to protect neurons against prion diseases by controlling prion mutation and pathology.

8. Polyphenol and Anti Acetylcholinesterase Activity

Pathology of neurodegenerative diseases including AD includes deficiency of neuromediator acetylcholine, thus making acetylcholinesterase (AChE) inhibitors as important clinically relevant drugs in AD and other dementia [191]. Black chokeberry extract, a rich source of polyphenols, in combination with lemon juice inhibited AChE [192]. The prenylated flavonols from paper mulberry (*Broussonetia papyrifera*) were potent inhibitors of AChE, thus exhibiting neuroprotective potential [193]. Studies have shown that a polyphenol-rich blueberry extract also inhibited AChE activity *in vitro* [194]. Polyphenols extracted from *Paulownia tomentosa* fruits exhibited inhibitory action against both AChE and butyrylcholinesterase (BChE) [195]. Quercetin was found to improve cognitive ability and exhibit neuroprotection against trimethyltin-induced neurotoxicity by inhibiting AChE [196]. A report has also shown that quercetin inhibited AChE activity and improved cognitive abilities in streptozotocin-treated mice [197]. Quercetin and maclurax-anthone, from *Maclura tinctoria* and Dyer's mulberry, respectively, inhibited both AChE and BChE *in vitro* by competitive and noncompetitive inhibition, respectively [191]. Molecular docking studies [191] have indicated the hydrophobic interactions and strong hydrogen bonding of both flavonoids with enzymes as basis of their inhibitory activity. Polyphenols from *Cistus laurifolius* L. also exhibited cholinesterase inhibitory effects against AChE and BChE, supporting a neuroprotective role of polyphenols [198]. A herbal tea from *Paulownia barbatus* leaves reduced AChE activity by 40% and its principal constituent, natural polyphenol rosmarinic acid, reduces AChE activity by 25% [199]. Galangin, a flavonol isolated from rhizome of *Alpiniae officinarum,* has also exhibited strong AChE inhibition [200]. EGCG enhances huperzine A's (acetylcholinesterase inhibitor) effects against AChE as its supplementation leads to 88–91% inhibition [201]. Later reports have supported that EGCG supplementation with huperzine A improves cognitive abilities in AD [202]. Linarin, a flavonoid found in *Linaria* species, inhibited AChE activity in neuronal PC12 cells and extended potential for neuroprotection in AD and related disorders [203]. All these pieces of evidences suggest that polyphenols are potent AChE and BChE inhibitors, thus warranting neuroprotection and improved cognitive functions in AD and related dementia.

9. Polyphenols and Autophagy-Related Proteins

Flavonoids such as hesperetin and hesperidin inhibited Aβ-induced glucose metabolism impairment in neurons and downregulated Aβ stimulated autophagy, resulting in improved cognitive functions [204]. Kaempferol also protected SH-SY5Y and primary neurons from rotenone toxicity through induction of autophagy [154]. Resveratrol exhibited neuroprotective effect by activating AMPK-SIRT1 autophagy pathway in PD cell model studies [205]. Brain-related autophagy studies have a wide research gap, and polyphenols have strong potential for inducing neuroprotection via autophagy and its related pathways.

10. Polyphenols as Neuronal Mitochondria Medicine

Polyphenols from wine are known to reduce oxidative stress and increase the expression of antioxidant enzymes like catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase [131]. Resveratrol upregulates antiapoptotic Bcl-2 protein and downregulates Bax protein expression [206]. Resveratrol also acted as mitochondrial antioxidant by elevating the levels of antioxidants trioredoxin-2 (TRX2) and X-chromosome-linked inhibitor of apoptosis protein [207]. Another study has shown that resveratrol increased expression of Bcl-2, thus preventing neuronal apoptosis [100]. Similarly, resveratrol controlled oxidative stress in PC12 cells and inhibited mitochondria-mediated apoptosis by downregulating Bax and upregulating Bcl-2 [112]. Similarly, lutein has shown protection of mice against ischemic

Table 3: Modulation of mitochondrial targets by polyphenols.

Target	Polyphenol	Effect	References
AP-1	EGCG	Downregulation	[116]
Bad/Bax	Resveratrol	Downregulation	[112]
	Ferulic acid	Downregulation	[124]
Bcl-2	Lutein	Upregulation	[125]
	Baicalein	Upregulation	[126]
Cox-2	Lutein	Downregulation	[125]
	Hesperidin	Downregulation	[127]
	Hibiscus sabdariffa polyphenols	Downregulation	[128]
GCLM	Chrysin, apigenin, luteolin	Upregulation	[129]
GCLC	Chrysin, apigenin, luteolin	Upregulation	[129]
GPX	EGCG	Upregulation	[130]
	Red wine polyphenols	Upregulation	[131, 132]
	3,3′,5,5′-tetra-t-butyl-biphenyl-4,4′-diol	Upregulation	[133]
HO-1	Butein, apigenin	Upregulation	[129]
	Luteolin	Upregulation	[134]
IFN-γ	Quercetin	Downregulation	[135]
JNK	Quercetin	Downregulation	[135]
	EGCG	Downregulation	[116]
	Hibiscus sabdariffa polyphenols	Downregulation	[128]
JUN	Quercetin	Downregulation	[135]
SOD	Phloridzin	Upregulation	[136]

injury by enhancing the Bcl-2 levels and downregulated Cox-2 and pancreatic ER kinase (PERK) [125]. Baicalein also regulated Bcl-2 and antagonized cytochrome c release in cytosol [126]. Similarly, ferulic acid, a phenolic acid, attenuates mitochondria apoptosis by inhibiting Bax, tBid expression, and elevating Bcl-2-like proteins [124]. Important transcription factors of ERK/Nrf2 pathway like glutamate cysteine ligase catalytic (GCLC) and glutamate-cysteine ligase, modifier subunit (GCLM), are upregulated by flavones like chrysin, apigenin, and luteolin to combat oxidative stress [129]. Glutathione peroxidase (GPx) levels were modulated by red wine polyphenols resulting in combat of oxidative stress [132]. Similarly, phenolic antioxidant 3,3′,5,5′-tetra-t-butyl-biphenyl-4,4′-diol also controlled expression of GPx and HIF-1α in hypoxia studies [133]. Various polyphenols like butein, phloretin, chrysin, apigenin, and luteolin activated HO-1 (HMOX1), GCLC, and GCLM through expression of ERK/Nrf2 pathway [129, 134]. Quercetin has also downregulated inflammatory cascade by lowering the expression of JNK, c-Jun, and interferon-γ inducible protein [135]. Similarly, p-JNK and COX-2 were downregulated by polyphenols from *Hibiscus sabdariffa* L. providing relief from oxidative stress and pathological inflammation [128]. EGCG controlled mitochondria lead inflammation by lowering transcription of JNK and activator protein-1 (AP-1) [116]. Neuroprotection through the phosphatidylinositol 3-kinase (P13K) and MAPK has also been shown by flavone glycoside [113]. Hesperidin carsonic acid, a major rosemary polyphenol, exhibited strong anti-inflammatory action in neurons under hypoxia stress by inhibiting ROS, MAPKs, caspase-3, and COX-2 [127]. Lowering of JNK serves as mitochondrial therapy not only in stroke but also in AD as JNK activation in AD brain leads to tau hyperphosphorylation and Aβ pathogenesis [208]. Curcumin and resveratrol exhibited neuroprotection through increased activity of NAD(P)H quinone oxidoreductase (NQO1) via Nrf2 pathway in astrocytes [209]. Similarly, structurally modified isomers of resveratrol also elevated the NQO1 activity, thus promising antioxidant effects through NRf2 pathway [210]. ECG modulated endophilin-B1, also known as SH3GLB1, which is required for maintaining mitochondrial morphology and plays important role in apoptosis [211, 212]. EGCG has also increased expression of mitochondrial antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPX1) [130]. Flavonoid-enriched fraction (AF4) isolated from the peel of "Northern Spy" apples has been shown to suppress the expression of IL-1β, TNF-α, and IL-6 in a mouse model of hypoxic-ischemic (HI) brain damage [123]. Phloridzin, also an apple polyphenol, has been shown to increase expression of SOD1 and SOD2 genes, thus protecting mitochondria against oxidative stress [80]. Polyphenols are important mitochondrial therapeutics as they play a role in mitochondrial biochemistry by modulating apoptosis, antioxidant action, signal transduction, and inflammation (Table 3).

11. Polyphenols and Ion Channels

The neuroprotective benefits of polyphenols are often attributed to their antioxidant activity and their ability to modulate the cell signaling pathways [105, 124, 155]. Sodium channels (Na_v 1.5) involved in pathology of MS were found to be blocked by red grape polyphenols like quercetin,

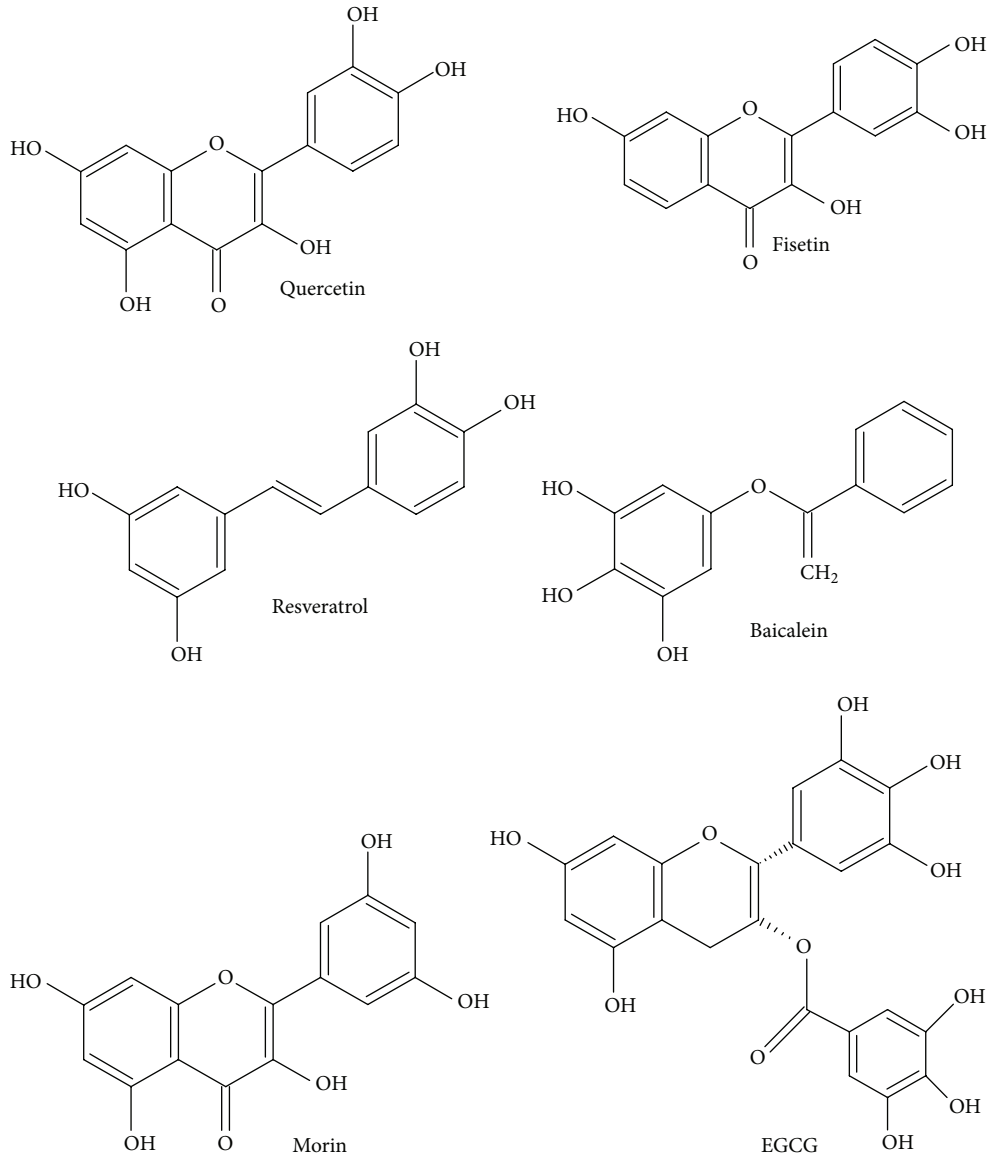

Figure 2: Chemical structure of polyphenols with therapeutic use in age-related neurological diseases.

catechin, and resveratrol in rodent and cell model studies [87, 213]. G protein-coupled inwardly rectifying potassium ($K_{IR}3$) channels, involved in neuron signaling and membrane excitability [214], are activated by naringin (flavonoid glycoside), thus exhibiting potential for improving cognition in AD [215]. EGCG's neuroprotective effect was proposed to occur through inhibition of high-voltage-activated calcium currents (I_{HVA}) and NMDA-induced inward currents (I_{NMDA}) along with elevation of Ca^{2+} through PLC-IP_3 pathway [216]. Similarly, curcumin exhibited modulation of a wide range of ionic channels including Ca^{2+}-release-activated Ca^{2+} channels (I_{CRAC}), voltage-gated K^+ channel (I_{Kv}), intermediate-conductance Ca^{2+}-activated K^+ channel (I_{SK4}), and the cytoplasmic Ca^{2+} concentration $\Delta[Ca^{2+}]_C$ in Jurkat-T cells [217]. However, the current literature has a research gap of specific ion channel study (Kv3 subfamily of K^+ channel subunits) in disease-specific conditions. Overall, the ability of polyphenols to modulate ion channels and action potential [218] complements their ability to protect neurons from disorders and thus supports the growing evidence that polyphenols may act as neuroprotectants in several neuropathological conditions.

12. Concluding Remarks

In conclusion, recent scientific evidence suggests that neurodegenerative diseases are accompanied by oxidative stress, inflammation, metal accumulation, and mitochondrial dysfunctions. Various physiological mechanisms are altered by these pathological changes which contribute to etiology of neurodegenerative diseases like stroke, MS, PD, AD, and HD. The prevention and treatment of these disorders with complex mechanisms need novel therapeutic strategies targeted for multiple genes and proteins. Polyphenols are natural plant

secondary metabolites which exhibit remarkable multipotent ability to control and modulate ROS, metal toxicity, inflammation, apoptosis, signal transduction, ion channels, and neurotransmitters. Polyphenolic dietary antioxidants, particularly resveratrol, EGCG, quercetin, and other fruit polyphenols, are potent neuroprotectants (Figure 2). Their direct usage and dietary supplementation could act as antioxidant and neuroprotective therapy for treatment of these diseases. Most of experimental and epidemiological studies suggest that dietary polyphenols activate antioxidant pathways such as Nrf2/HO1 and downregulate NFκB, MMPs, PPAR, HIF-1, and STAT pathways. Polyphenols also modulate immune response by inhibiting proinflammatory biomarkers such as CCL17, CCL22, CCR1, CCR2, MIP1α, MIP 1β, CXCL (9, 10, 11), IFN-γ, TNF-α, and IL(1β, 6, 17A, 22). These salient properties of polyphenols help to reduce two hallmarks of neurodegeneration, that is, oxidative damage and inflammation.

Polyphenols also protect mitochondria from pathological events by triggering prosurvival cell signaling. Polyphenols increase antioxidant enzymes, that is, catalase, superoxide dismutase (SOD1, SOD2), and prosurvival Bcl-2 and PERK pathways. Downregulation of Bad/Bax, c-jun, JNK, COX-2, AP-1, and caspase-3 also contributes to the survival of neurons. Polyphenols also help in improving cognitive abilities by inhibiting AChE and BChE. The inhibition of these enzymes plays an important role in clinical medicine of AD. Apart from their anti-AChE activity, polyphenols also induce metal chelation and modulate autophagy and prion proteins. These features along with reduction of Aβ toxicity, reduction of neural lesions, and activation of cell survival genes are of particular relevance to neurodegenerative diseases. The activation of novel spectrum of these molecular targets forms underlying mechanism of neuroprotection by polyphenols. The lack of toxic effect and availability from natural sources makes polyphenols as clinically relevant therapeutics in neurodegeneration.

The future of polyphenol research needs to aim towards clinical acceptance of health claims from preclinical *in vitro* and animal model studies. Therefore, future studies focusing on human clinical trials of several potent polyphenols and their combinations should be carried out. Furthermore, polyphenols must be investigated for the risk assessment and safety evaluation to observe any undesirable effects. The success in clinical research of polyphenols will decide their pharmacological relevance for humans.

Abbreviations

AAP: Amyloid precursor protein
AChE: Acetylcholinesterase
AD: Alzheimer's disease
AP-1: Activator protein-1
Aβ: Amyloid beta
Bad: BCL2-associated agonist of cell death
BAX: BCL2-associated X protein
BBB: Blood-brain barrier
BChE: Butyrylcholinesterase
Bcl: B-cell lymphoma
Bcl2: B-cell lymphoma 2
cAMP: Cyclic adenosine monophosphate
CCL: Chemokine (C-C motif) ligand
CCR: Chemokine receptor
CD: Cluster of differentiation
CI: Cerebral ischemia
COX: Cyclooxygenase
CREB: cAMP response element binding protein
CXCL: Chemokine (C-X-C motif) ligand
EAE: Experimental autoimmune encephalomyelitis
ECG: Epicatechin gallate
EGCG: Epigallocatechin gallate
GCLC: Glutamate cysteine ligase catalytic
GCLM: Glutamate-cysteine ligase, modifier subunit
GPx: Glutathione peroxidase
GSK: Glycogen synthase kinase
HD: Huntington's disease
HIF: Hypoxia inducible factor
HIF-1: Hypoxia inducible factor-1
HO: Heme oxygenase
IFNγ: Interferon-gamma
IL: Interleukin
JNK: c-Jun N-terminal kinase
MAPK: Mitogen activated kinase-like protein
MCP-1: Monocyte chemoattractant protein-1
MIP: Macrophage inflammatory protein
MMP: Metalloproteinases
MS: Multiple sclerosis
NFκB: Nuclear factor-kappa B
NOS: Nitric oxide synthase
NQO1: NAD(P)H quinone oxidoreductase
Nrf2: Nuclear factor- (erythroid-derived 2-) like 2
PC: Protein carbonyl
PD: Parkinson's disease
PERK: Pancreatic ER kinase
PGC-1α: Peroxisome proliferative activated receptor, coactivator 1 alpha
PI3K: Phosphatidylinositol 3-kinase
PPAR: Peroxisome proliferator activated receptor
PrP(c): Cellular prion protein
ROS: Reactive oxygen species
SIRT-1: Silent mating type information regulation 2 homolog1
SOD: Superoxide dismutase
STAT: Signal transducer and activation of transcription
TGFβ: Transforming growth factors β
TLR: Toll-like receptor
TNFα: Tumor necrosis factor-alpha
TRAF: TNF receptor associated factor
TRX: Thioredoxin.

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Research Article

Resveratrol Suppresses PAI-1 Gene Expression in a Human *In Vitro* Model of Inflamed Adipose Tissue

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Increased plasminogen activator inhibitor-1 (PAI-1) levels are associated with a number of pathophysiological complications; among them is obesity. Resveratrol was proposed to improve obesity-related health problems, but the effect of resveratrol on PAI-1 gene expression in obesity is not completely understood. In this study, we used SGBS adipocytes and a model of human adipose tissue inflammation to examine the effects of resveratrol on the production of PAI-1. Treatment of SGBS adipocytes with resveratrol reduced PAI-1 mRNA and protein in a time- and concentration-dependent manner. Further experiments showed that obesity-associated inflammatory conditions lead to the upregulation of PAI-1 gene expression which was antagonized by resveratrol. Although signaling via PI3K, Sirt1, AMPK, ROS, and Nrf2 appeared to play a significant role in the modulation of PAI-1 gene expression under noninflammatory conditions, those signaling components were not involved in mediating the resveratrol effects on PAI-1 production under inflammatory conditions. Instead, we demonstrate that the resveratrol effects on PAI-1 induction under inflammatory conditions were mediated via inhibition of the NFκB pathway. Together, resveratrol can act as NFκB inhibitor in adipocytes and thus the subsequently reduced PAI-1 expression in inflamed adipose tissue might provide a new insight towards novel treatment options of obesity.

1. Introduction

Obesity is becoming an increasing public health problem worldwide. The excessive accumulation of adipose tissue leads to the development of dyslipidemia, impaired glucose metabolism, hypertension, and proinflammation, processes playing an essential role in the pathogenesis of cardiovascular disease, type 2 diabetes, the metabolic syndrome, and various cancers (reviewed by [1]). Many of those obesity-related pathophysiological conditions are associated with increased plasminogen activator inhibitor-1 (PAI-1) levels [2–6]. PAI-1 is the primary, fast-acting inhibitor of both tissue-type and urokinase-type plasminogen activators and therefore controls the regulation of the fibrinolytic system in blood [7, 8]. In addition, PAI-1 is an important regulator of extracellular matrix turnover, tissue remodeling, and fibrosis [9]. PAI-1 levels can be increased in response to hypoxia [10, 11], hormones like insulin [12, 13], coagulation factors, and cytokines (discussed by [14]). More recently PAI-1 levels have been considered as one of the biomarkers used to predict obesity-associated diseases [15]. Elevated PAI-1 mRNA levels have been found in adipose tissues from obese ob/ob mice [16] and also in human obesity with higher expression levels in visceral compared to subcutaneous adipose tissue depots [17]. Thus, high plasma PAI-1 levels are a common finding in obesity in both mice and humans [18–25]. Most importantly, the obesity-induced PAI-1 increase is reversible by lifestyle intervention. Weight loss due to calorie restriction decreased plasma PAI-1 concentrations in obese individuals [26, 27]. These data imply that substances that potentially mimic calorie restriction may be used as modulators of PAI-1 levels in the treatment of obesity and obesity-related diseases.

From a number of natural compounds mimicking calorie restriction by targeting various metabolic pathways, resveratrol gained special interest. Resveratrol is a polyphenol produced by plants in response to environmental stress and found in red grape skin, peanuts, a variety of berries, and medical plants [28]. It has been suggested to act as a calorie restriction mimetic based on data from rodents. When mice and/or rats were fed a high-fat diet, resveratrol treatment improved glucose homeostasis, mitochondrial function, lipid parameters, body weight, and survival [29–39]. While the resveratrol effects are intensively studied in animal models only few clinical trials were conducted so far to study the effects of resveratrol supplementation in the context of human obesity [40] and coronary artery disease [41]; yet there exists some controversy [42] and the effect of resveratrol in obese human individuals remains to be further investigated.

Although obesity and obesity-associated diseases seem to be positively influenced by resveratrol, not much is known about the effect of resveratrol on PAI-1 in obesity. Therefore, it was the aim of this study to investigate the effects of resveratrol on the production of PAI-1 in human adipocytes and in an *in vitro* model of human adipose tissue inflammation. We found that resveratrol reduces PAI-1 levels in adipocytes especially under inflammatory conditions. Thus, our data support the concept that resveratrol can alleviate obesity-induced upregulation of PAI-1 in human adipose tissue.

2. Materials and Methods

2.1. Reagents and Cell Culture. All biochemicals were of analytical grade and were purchased from commercial suppliers. Resveratrol, sirtinol, and LY204002 were obtained from Sigma (Deisenhofen, Germany). SC-514 was from Merck Millipore (Darmstadt, Germany). Small molecule inhibitors were diluted in DMSO which alone was also used as vehicle control. The following concentrations of resveratrol and inhibitors were used in experiments: resveratrol 10, 50, 100 μM, sirtinol 10 μM; LY204002 20 μM, SC-514 100 μM.

Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes were cultured as previously described [43]. Human primary preadipocytes were prepared by collagenase digestion from subcutaneous adipose tissue of 3 healthy women using a previously described protocol [44]. Adipogenic differentiation of SGBS and human primary and SGBS preadipocytes was induced in serum-free DMEM/F12 medium supplemented with 10 μg/mL iron-poor transferrin, 10 nM insulin, 200 pM thyroid hormone, and 0.1 μM cortisol and for the first four days 2 μM rosiglitazone, 250 μM isobutylmethylxanthine, and 25 nM dexamethasone. Cells were used for experiments on day 8 of adipogenic differentiation.

THP-1 cells (ATCC, Wesel, Germany) were cultured as described earlier [45]. Differentiation into macrophages was induced by 125 ng/mL phorbol myristate acetate for 48 h. Macrophage-conditioned medium (MacCM) was collected after additional 48 h of incubation in serum-free basal medium containing 0.5% BSA and cleared by centrifugation. MacCM from 5 independently performed productions was pooled and then used for experiments.

Mouse embryonic fibroblasts (MEFs) were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany), 1% nonessential amino acids (Invitrogen), and 0.5% antibiotics in an atmosphere of 16% O_2, 5% CO_2, and 97% humidity at 37°C in a cell culture incubator. Mouse embryonic fibroblasts Sirt1$^{+/+}$ and Sirt1$^{-/-}$ were a generous gift from Dr. Michael McBurney (Ottawa Hospital Research Institute, Canada). We obtained AMPKα1,2$^{+/+}$ and AMPKα1,2$^{-/-}$ MEFs [46] from Dr. Benoit Viollet (Institut Cochin, Paris, France). Nrf2 wild-type and Nrf2 knockdown MEFs were provided by Dr. Stephan Immenschuh (Hannover Medical School, Germany).

2.2. RNA Preparation and Quantitative Real-Time PCR. Isolation of total RNA was performed using the peqGOLD HP Total RNA kit (Peqlab, Erlangen, Germany) following the manufacturer's instructions. One μg of total RNA was used for cDNA synthesis with using SuperScript II Reverse Transcriptase (Invitrogen, Darmstadt, Germany). Quantitative real-time PCR was performed with a LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) using a LightCycler FastStart DNA Master PLUS SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). The quantitative real-time PCR results were normalized using hypoxanthine phosphoribosyltransferase (HPRT) as a housekeeping gene. The following primer sets were used: human PAI-1-F (5′-ACA AGT TCA ACT ATA CTG AGT TCA CCA CGC CC-3′), human PAI-1-R sequence (5′-TGA AAC TGT CTG AAC ATG TCG GTC ATT CCC-3′), human HPRT-F (5′-GAG ATG GGA GGC CAT CAC ATT GTA GCC CTC-3′), and human HPRT-R (5′-CTC CAC CAA TTA CTT TTA TGT CCC CTG TTG ACT GGT C-3′). The experiments for each data point were carried out in triplicate. The relative quantification of gene expression was determined using the ΔΔCt method [47]. In some experiments conventional RT-PCR was performed using Sp1 as a reference gene (PAI-1-F: 5′-GTC TGC TGT GCA CCA TCC CCC-3′; PAI-1-R: 5′-GAA CAG CCT GAA GAA GTG GGG C-3′, Sp1-F: 5′-ACT ACC AGT GGA TCA TCA GGG-3′; Sp1-R: 5′-CTG ACA ATG GTG CTG CTT GGA-3′).

2.3. ELISA. SGBS adipocytes were treated for 48 h with 10% MacCM, 100 μM resveratrol, and 100 μM SC-514 alone or in combination. The ELISA was performed using the Platinum ELISA kit for human PAI-1 (eBioscience, Vienna, Austria). Absorbance was measured on a spectrophotometer using 450 nm wavelength (ELx800 Absorbance Microplate Reader, BioTek, Bad Friedrichshall, Germany).

2.4. Western Blot Analyses. Western blot analyses were performed as previously described [10]. In brief, 24 h after treatment with vehicle or resveratrol cell culture medium (for PAI-1) or total cell lysates were collected and 100 μg of protein was subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. The following primary antibodies were used: PAI-1 (polyclonal 1 : 100) (American Diagnostics, Pfungstadt, Germany), AMPKβ1/2 (polyclonal, 1 : 1000) (Cell Signaling, Hamburg, Germany), Nrf2 (polyclonal, Nrf2 1 : 200) (Santa

Cruz, Heidelberg, Germany), and Sirt1 (polyclonal, 1 : 1000) (Santa Cruz, Heidelberg, Germany). The secondary antibody was anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase IgG (1 : 5000) (Biorad, Munich, Germany). The enhanced chemiluminescence (ECL) system (Amersham, Freiburg, Germany) was used for detection. Blots were quantified by using the Fiji program (NCBI).

2.5. ROS Measurement. To determine ROS production, SGBS adipocytes were incubated with 2.5 μM CM-H_2DCFDA (Molecular Probes Europe BV, The Netherlands) for 30 min at 37°C. After three washes with PBS, cells were treated with 100 μM H_2O_2 or 10% MacCM for 15 min and analyzed by flow cytometry.

2.6. Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). SGBS adipocytes were treated with 100 μM resveratrol, 100 μM SC-514, and 10% MacCM alone or in combination. TNFα (10 ng/mL) was used as a positive control. Cells were collected from 6 cm dishes by scraping and centrifugation (10,000 g for 5 min at 4°C). After washing once with ice-cold PBS, cell pellets were resuspended in 200 μL low-salt buffer (10 mM HEPES-KOH pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl) and incubated for 10 min on ice. After addition of 12.5 μL of a 10% Nonidet P-40 solution, samples were mixed vigorously for 30 s. Nuclei were collected by centrifugation and resuspended in 25 μL high-salt buffer (20 mM HEPES-KOH pH 7.9; 1.5 mM $MgCl_2$; 420 mM NaCl, 0.2 mM EDTA; 25% glycerol). Both buffers were supplemented with a protease-inhibitor cocktail (Sigma), 0.2 mM PMSF, 0.5 mM dithiothreitol (DTT), and 1 mM sodium-orthovanadate before use. Nuclei were incubated 15 min on ice and vortexed periodically. Nuclear extracts were obtained by centrifugation at 12,500 g for 10 min at 4°C and stored at −80°C. Protein concentration was determined with the BCA Protein Assay Reagent kit (Pierce, Rockford, IL), according to manufacturer's instructions. Single-stranded oligonucleotides were purchased from Biomers.net (Ulm, Germany): standard NFκB probe: sense, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; antisense, 5′-GCC TGG GAA AGT CCC CTC AAC T-3′. The sense oligonucleotide was labeled with γ-^{32}P-ATP (Amersham, Freiburg, Germany) using T4-polynucleotide kinase (MBI Fermentas, St. Leon-Rot, Germany). A 2-fold molar excess of unlabeled antisense oligonucleotide was annealed, and the labeled double-stranded oligonucleotide was purified with a spin column (Micro Bio-Spin P30; Bio-Rad, Munich, Germany). Binding reactions were performed for 30 min on ice in 20 μL buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5; 4% glycerol) containing 5 μg nuclear extract protein, 1 μg poly (dI:dC) (Sigma), and 10,000 cpm-labeled oligonucleotide.

2.7. Statistics. Data represents mean ± standard error of means (SEM) of 3 independent experiments unless otherwise stated. Statistics: statistical significance was evaluated using one-way analysis of variants (ANOVA) considering $P < 0.05$ as statistically significant.

3. Results

3.1. Concentration- and Time-Dependent Downregulation of Human PAI-1 mRNA and Protein Levels by Resveratrol in SGBS Adipocytes. To determine how resveratrol modulates PAI-1 gene expression in SGBS adipocytes, we examined PAI-1 mRNA and protein levels after treatment with increasing concentrations of resveratrol at different time points (Figure 1). Treatment of cells for 12 h, 24 h, and 48 h with different concentrations of resveratrol resulted in a reduction of PAI-1 mRNA levels in a dose-dependent manner (data not shown); 100 μM resveratrol reduced PAI-1 mRNA levels by about 40% after 12 h and by about 60% after 48 h (Figures 1(a) and 1(b)). The resveratrol-mediated decrease of PAI-1 mRNA was followed by a decrease of PAI-1 protein levels. A resveratrol concentration of 50 μM or 100 μM diminished PAI-1 protein levels in the medium by about 50% after 24 h and by about 75% after 48 h (Figure 1(c)). Thus, resveratrol reduced PAI-1 mRNA and PAI-1 protein levels in a time- and concentration-dependent manner.

3.2. PAI-1 Gene Expression Is Upregulated in an In Vitro Model of Inflamed Human Adipose Tissue as well as in Primary Human Adipocytes. Obesity is associated with low-grade chronic inflammation [48] and increased circulating PAI-1 levels [6]. Therefore, we mimicked human adipose tissue inflammation by using our previously described *in vitro* model system [49] where we incubated SGBS adipocytes with medium supplemented with increasing doses of macrophage-conditioned medium (MacCM) for 48 h. As shown in Figure 2(a), the presence of MacCM increased PAI-1 mRNA in SGBS adipocytes; already 5% MacCM induced PAI-1 mRNA by about 2-fold. In line with these findings, treatment of primary human *ex vivo* differentiated adipocytes obtained from healthy donors with MacCM increasing PAI-1 mRNA by about 1.6-fold (Figure 2(b)). Thus, these data suggested that obesity mimicking inflammatory conditions lead to an upregulation of PAI-1.

3.3. Resveratrol Reduces Upregulation of PAI-1 Gene Expression in an In Vitro Model of Inflamed Human Adipose Tissue. To determine the effect of resveratrol on the elevated PAI-1 mRNA and protein levels under inflammatory conditions, SGBS adipocytes were cultured in the absence or presence of different concentrations of resveratrol, 10% MacCM, or a combination of both for 48 h. Treatment of SGBS adipocytes with increasing doses of resveratrol alone resulted in a concentration-dependent reduction of PAI-1 mRNA and protein levels (Figures 3(a), 3(b) and 3(c)). Incubation of cells with MacCM induced PAI-1 mRNA levels and PAI-1 protein levels by about 3-fold (Figures 3(a), 3(b) and 3(c)). The MacCM-dependent induction of PAI-1 mRNA and protein levels was abolished in the presence of 100 μM resveratrol (Figures 3(a), 3(b) and 3(c)). Together, these data suggested that PAI-1 gene expression is enhanced under inflammatory conditions and that this induction is antagonized by the action of resveratrol.

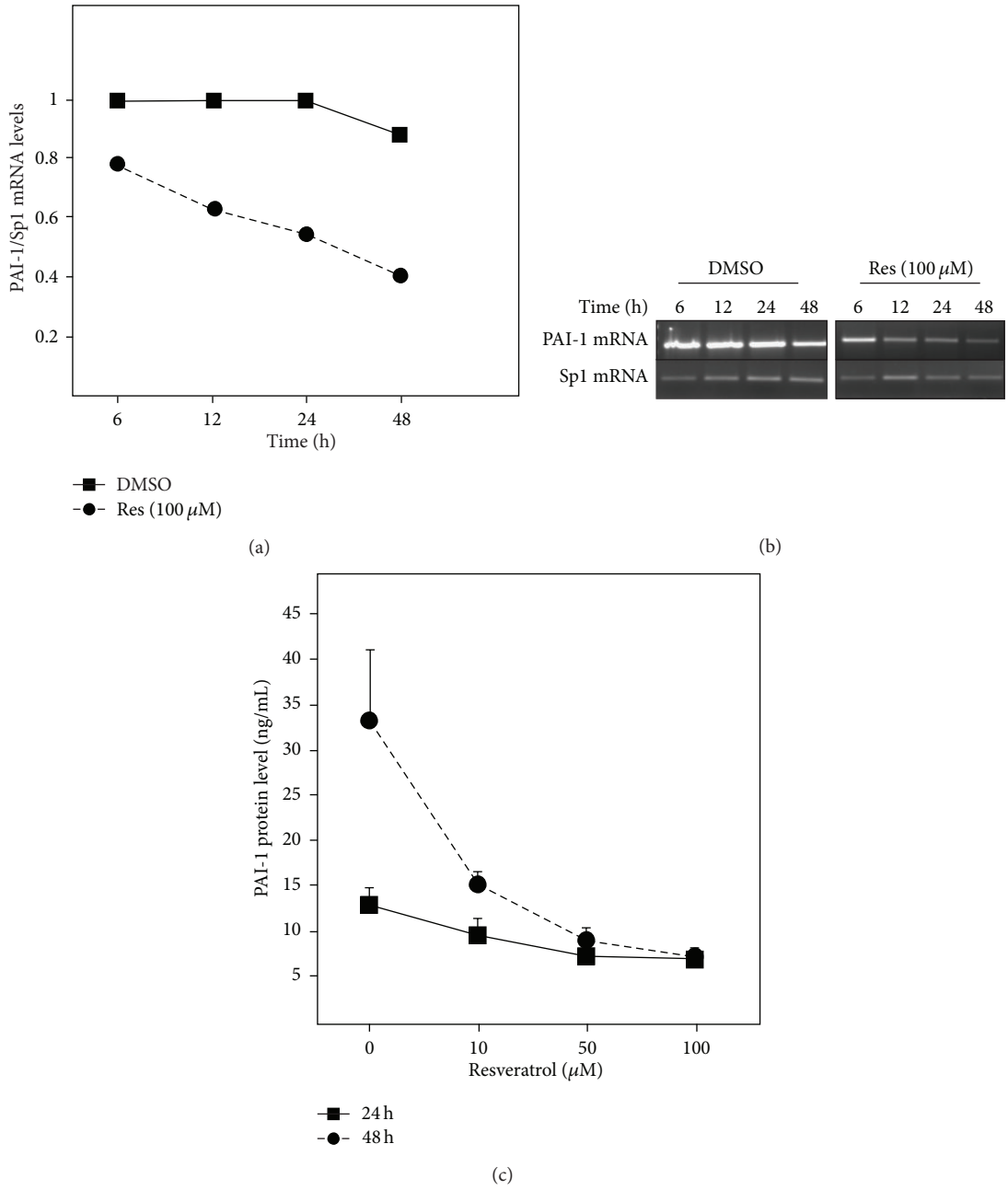

Figure 1: Resveratrol-dependent downregulation of PAI-1 mRNA and protein levels in SGBS adipocytes. SGBS adipocytes were incubated in adipogenic media with vehicle control (DMSO) or 100 μM resveratrol (Res) for the indicated time points. (a) PAI-1 mRNA levels were measured by semiquantitative RT-PCR. Sp1 was used as a reference gene. (b) A representative RT-PCR of PAI-1 and Sp1 mRNA levels after treatment with DMSO or 100 μM Res. (c) The accumulation of PAI-1 in the media was measured by ELISA after treatment with increasing doses of Res for 24 or 48 h.

3.4. The Effects of Resveratrol on PAI-1 Expression Are Not Mediated via Sirt1, AMPK, or PI3K. Resveratrol has been shown to modulate several key signaling molecules in adipocytes, including Sirt1 [50, 51], AMPK [52–54], and PI3K/Akt [52, 55–57]. To examine whether Sirt1, AMPK, and/or PI3K are involved in the resveratrol-dependent downregulation of PAI-1 gene expression, we used specific inhibitors of these signaling pathways as well as knockout cells. Concerning the inhibitor studies, SGBS adipocytes were incubated with DMSO as a vehicle control, the Sirt1 inhibitor sirtinol or PI3K inhibitor LY294002 along with resveratrol, MacCM, or combinations, and the PAI-1 mRNA levels were determined 48 h after treatment.

In line with the above mentioned results, resveratrol decreased PAI-1 mRNA levels in SGBS adipocytes cultured either with or without MacCM. Sirtinol treatment alone slightly reduced the basal expression of PAI-1 mRNA in SGBS adipocytes (Figure 4(a)). However, sirtinol had no

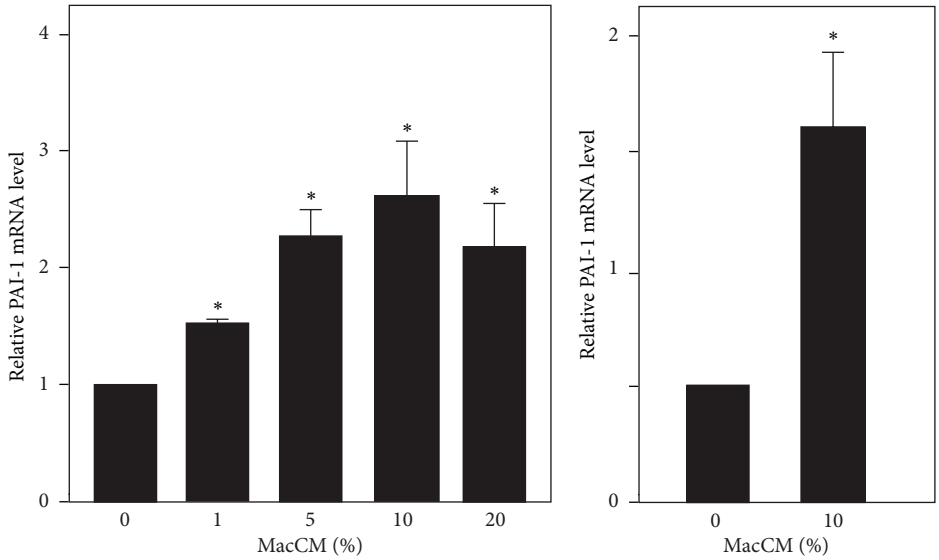

Figure 2: PAI-1 gene expression is upregulated in an *in vitro* model of inflamed human adipose tissue. (a) SGBS adipocytes were incubated with increasing doses of macrophage-conditioned media (MacCM) or vehicle for 48 h. PAI-1 mRNA levels were analyzed by qPCR and results were normalized to HPRT. * significant difference control versus MacCM. (b) Primary human *ex vivo* differentiated adipocytes isolated from 3 patients were treated with 10% macrophage-conditioned media (MacCM) or vehicle for 48 h. PAI-1 mRNA levels were analyzed with qPCR and normalized to HPRT. * significant difference control versus MacCM.

significant effect on the resveratrol-dependent downregulation of the PAI-1 mRNA in SGBS adipocytes incubated with MacCM (Figure 4(a)). To further rule out the role of Sirt1 in resveratrol-dependent regulation of PAI-1 expression, we examined the effect of resveratrol on the PAI-1 protein levels in Sirt1-deficient ($Sirt1^{-/-}$) mouse embryonic fibroblasts (MEFs). Although the basal PAI-1 protein levels were lower in $Sirt1^{-/-}$ MEFs, resveratrol treatment decreased the PAI-1 levels in both wild-type ($Sirt1^{+/+}$) and the $Sirt1^{-/-}$ MEFs by about 50% (Figures 4(b) and 4(c)). Together, these results indicate that although Sirt1 *per se* might be involved in the regulation of PAI-1 gene expression, the resveratrol-dependent modulation of PAI-1 gene expression is independent of Sirt1.

Next we investigated the role of AMPK in the resveratrol-dependent regulation of PAI-1 expression. For this purpose we used wild-type AMPK$\alpha 1/2^{+/+}$ (AMPK$\beta 1/2^{+/+}$) and AMPKα1/2-deficient (AMPK$\alpha 1/2^{-/-}$) MEFs and measured PAI-1 protein levels after treatment with resveratrol by Western blot. The basal PAI-1 protein levels were significantly lower in AMPK$\alpha 1/2^{-/-}$ MEFs, but again resveratrol treatment resulted in a significant decrease (by about 65%) of the PAI-1 protein levels in both wild-type and the AMPK$\alpha 1/2^{-/-}$ MEFs (Figures 4(d) and 4(e)). Together, these data show that even though AMPK itself might be involved in the regulation of PAI-1 gene expression, the resveratrol-dependent downregulation of PAI-1 is mediated by an AMPK-independent mechanism.

We further studied whether the PI3K/Akt pathway is involved in resveratrol-dependent downregulation of PAI-1 and used the PI3K inhibitor LY294002. While resveratrol treatment reduced PAI-1 mRNA levels by about 50% in both untreated and MacCM-treated SGBS adipocytes, LY294002 treatment did not change the basal PAI-1 mRNA levels (Figure 4(f)). Furthermore, incubation with LY294002 did not block the decline of PAI-1 mRNA levels by resveratrol in both untreated and MacCM-treated SGBS adipocytes, implicating that the PI3K/Akt pathway is not involved in the resveratrol-modulated downregulation of PAI-1.

3.5. ROS Formation and the Antioxidant Transcription Factor Nrf2 Do Not Contribute to the Effects of Resveratrol on PAI-1 Gene Expression. Obesity and inflammation are associated with increased ROS formation [58, 59] and ROS-mediated signaling has been reported to regulate PAI-1 gene expression [60, 61]. Resveratrol is well known for its antioxidant potential and therefore we aimed to determine whether the observed MacCM-dependent induction of PAI-1 gene expression and hence the effects of resveratrol were dependent on ROS generation. To address this issue, ROS levels were examined in SGBS adipocytes treated with MacCM or for the purpose of a positive control with H_2O_2. Intracellular ROS levels increased upon treatment with H_2O_2. By contrast, no changes in ROS generation were detected in MacCM-treated SGBS adipocytes (Figures 5(a) and 5(b)) implying that MacCM-dependent PAI-1 induction is independent of ROS.

The NFE2-related factor 2 (Nrf2) is a key transcription factor, involved in the primary cellular defense against the cytotoxic effects of oxidative stress [62]. To further exclude the possibility that the effects of MacCM and resveratrol on PAI-1 gene expression are independent of ROS, we used wild-type and Nrf2 knockdown MEFs. Interestingly, the knockdown of Nrf2 increased PAI-1 protein levels compared

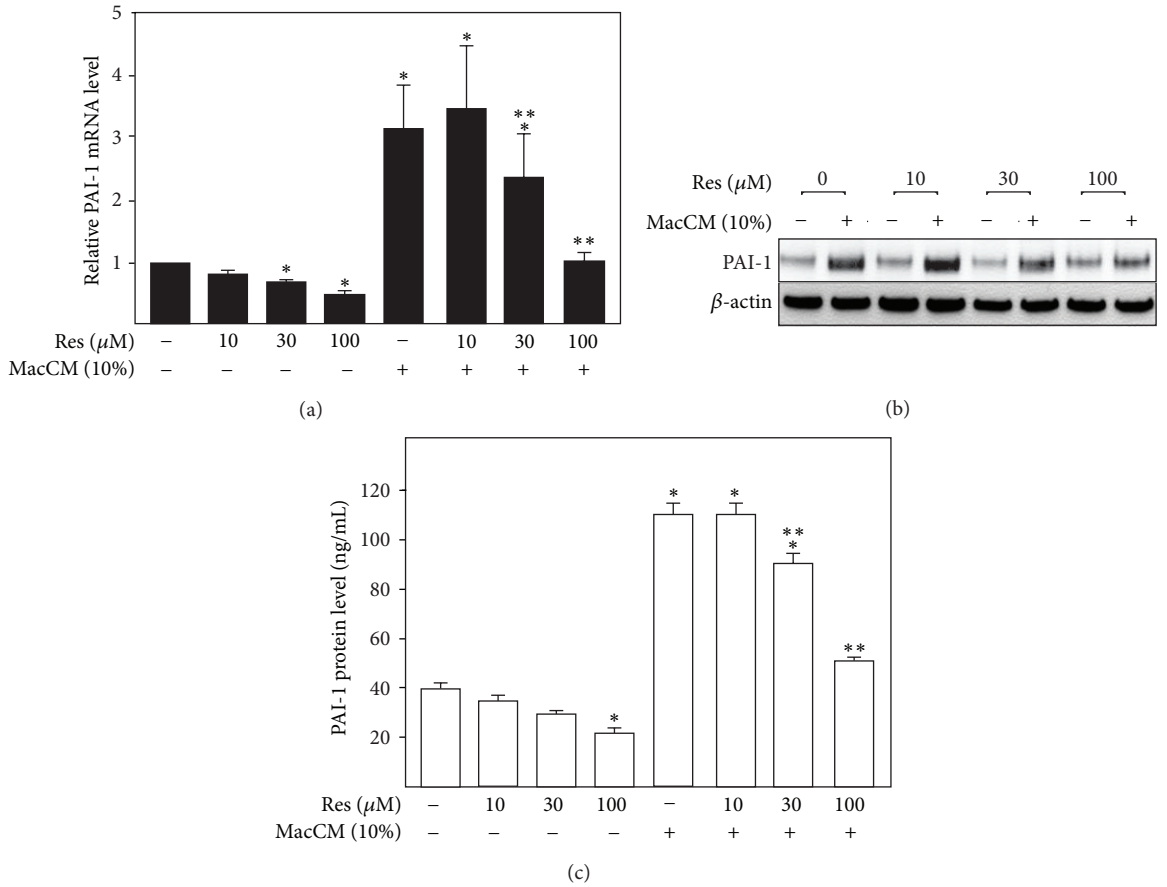

Figure 3: Resveratrol abolished the MacCM-dependent PAI-1 induction in SGBS adipocytes. SGBS adipocytes were treated with the indicated doses of resveratrol (Res), 10% MacCM, or a combination of Res and 10% MacCM for 48 h. (a) PAI-1 mRNA levels were analyzed by qPCR and results were normalized to HPRT. *Significant difference untreated versus Res or MacCM, **significant difference MacCM treated versus MacCM + Res. (b) Total cell protein lysates were isolated and subjected to Western blot analysis using an antibody against PAI-1 and β-actin as a loading control. (c) Accumulation of PAI-1 protein in media was measured by ELISA. *Significant difference untreated versus Res or MacCM, **Significant difference MacCM treated versus MacCM + Res.

to the wild-type cells (Figures 5(c) and 5(d)) but the addition of resveratrol caused a decrease in PAI-1 levels by about 80% in wild-type cells and by about 35% in Nrf2 knockdown cells (Figures 5(c) and 5(d)). Thus, the antioxidant transcription factor Nrf2 is not involved in mediating the resveratrol effects on PAI-1 expression.

3.6. The Effects of Resveratrol on PAI-1 Gene Expression in an In Vitro Model of Inflamed Adipose Tissue Are NFκB Dependent. The reduction of PAI-1 expression by resveratrol under inflammatory conditions may be partially explained by the ability of resveratrol to suppress the activity of NFκB, a transcription factor critically involved in inflammation. Therefore, we examined the effect of resveratrol on the DNA-binding activity of NFκB in the model of inflamed adipose tissue. By performing EMSA, we found that an oligonucleotide with a NFκB binding site was able to form a single DNA-protein complex (Figure 6(a)) when incubated with nuclear extracts from SGBS adipocytes treated with either MacCM or the established NFκB activator, TNF-α. The NFκB DNA-binding activity was significantly reduced in nuclear extracts from cells treated with MacCM and resveratrol or MacCM and SC-514 (Figure 6(a)). These data demonstrate that resveratrol can lead to a suppression of NFκB DNA-binding activity under inflammatory conditions in SGBS adipocytes. Based on the above findings, demonstrating the suppressive effect of resveratrol on NFκB DNA-binding, we expected that inhibition of NFκB by resveratrol would reduce PAI-1 gene expression. Accordingly, SGBS adipocytes were treated with MacCM, resveratrol, and SC-514 alone or in combination, and PAI-1 protein levels were measured by ELISA. In line, resveratrol and SC-514 reduced MacCM-dependent PAI-1 protein induction (Figure 6(b)), though the effects of resveratrol were much more pronounced than the effects of the NFκB inhibitor SC-514 alone. These data strongly suggest that the effects of resveratrol on PAI-1 gene expression in SGBS adipocytes are NFκB dependent.

4. Discussion

In this study we investigated the human PAI-1 expression in response to resveratrol in human SGBS adipocytes and

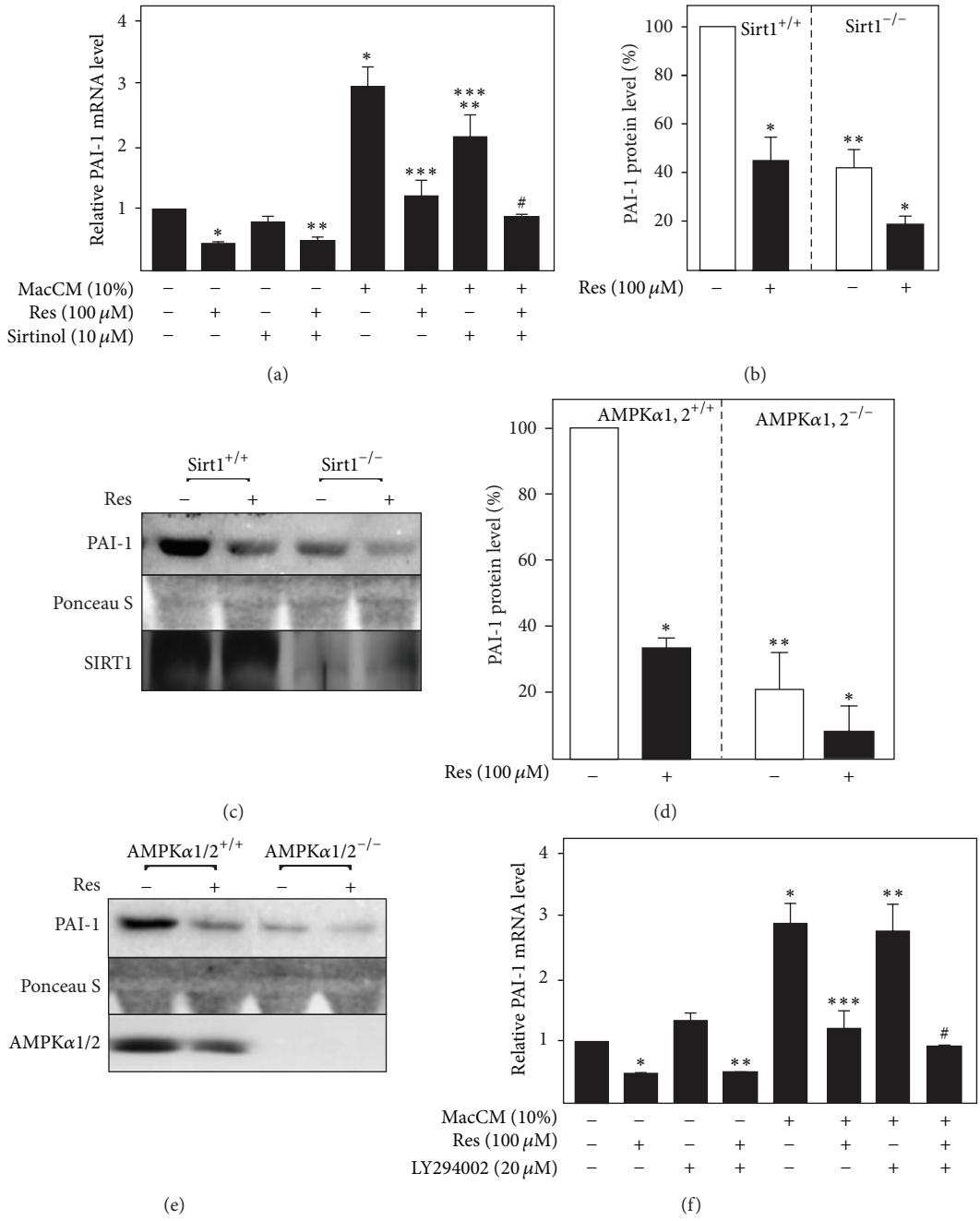

Figure 4: The effects of resveratrol on PAI-1 gene expression in SGBS adipocytes are not mediated via SIRT1, AMPK and PI3K. (a) Where indicated SGBS adipocytes were treated with 10 μM sirtinol, resveratrol (Res, 100 μM), and MacCM (10%) for 48 h. (a) PAI-1 mRNA levels were analyzed with qPCR and results were normalized to HPRT. *significant difference untreated versus Res, sirtinol, or MacCM; **significant difference untreated versus Res + sirtinol or Res + MacCM; ***significant difference MacCM versus MacCM + Res or MacCM + sirtinol; $^{\#}$significant difference MacCM versus MacCM + Res + sirtinol. (b) SIRT1$^{+/+}$ and SIRT1$^{-/-}$ mouse embryonic fibroblasts were treated with 100 μM resveratrol (Res) or vehicle control (DMSO) for 24 h. The PAI-1 and SIRT1 protein levels were measured by Western blot. *Significant difference untreated versus Res, **significant difference wild-type versus knockout cells. (c) Representative Western blot. (d) AMPKα1/2$^{+/+}$ and AMPKα1/2$^{-/-}$ mouse embryonic fibroblasts were treated with 100 μM resveratrol (Res) or vehicle control (DMSO) for 24 h. The PAI-1 and AMPKα1/2 protein levels were measured by Western blot. *significant difference untreated versus Res, **significant difference wild type versus knockout cells. (e) Representative Western blot. (f) Where indicated SGBS adipocytes were treated with 20 μM LY294002, 100 μM resveratrol (Res), and 10% MacCM for 48 h. The PAI-1 mRNA levels were measured by qPCR and results were normalized to HPRT. *Significant difference untreated versus Res, LY294002, or MacCM; **significant difference untreated versus Res + LY294002 or Res + MacCM; ***significant difference MacCM versus MacCM + Res or MacCM + LY204002; $^{\#}$significant difference MacCM versus MacCM + Res + LY294002.

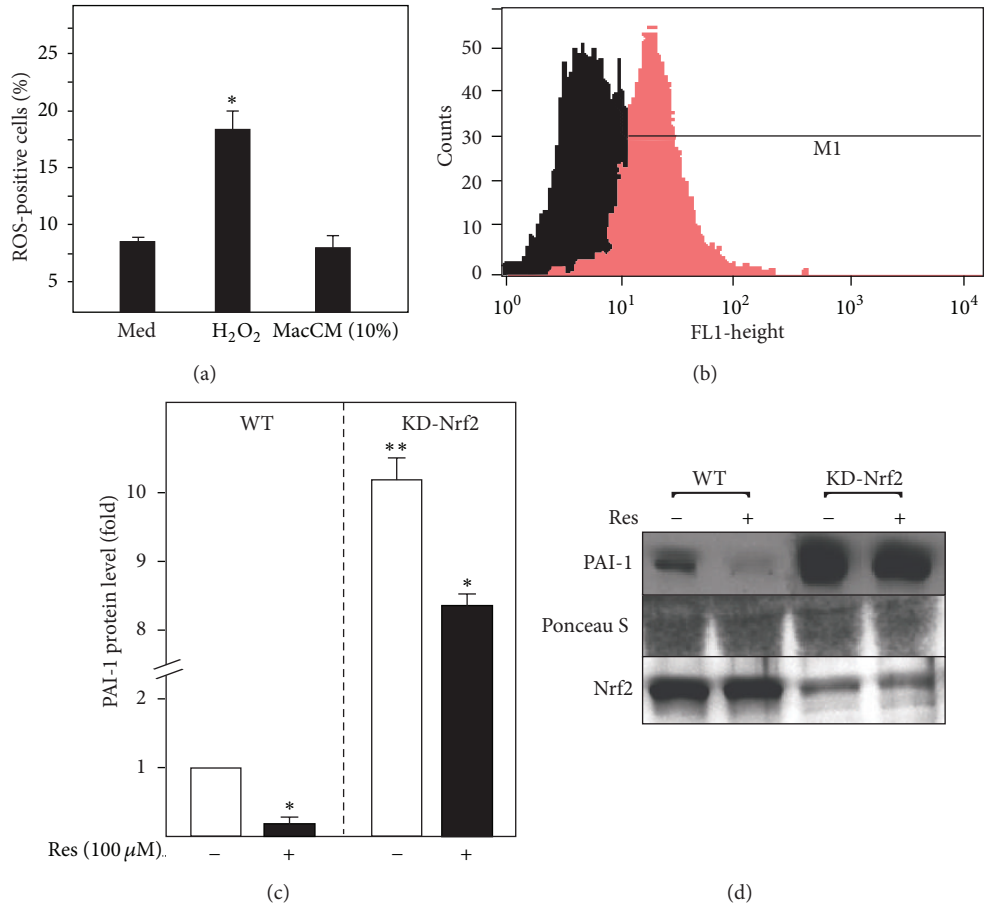

FIGURE 5: Macrophage-conditioned media do not induce ROS formation in human adipocytes and the antioxidant transcription factor Nrf2 does not contribute to the resveratrol effects on PAI-1 gene expression. (a), (b) SGBS adipocytes were labelled with 2.5 μM CM-H_2DCFDA and then treated with 50 μM H_2O_2 and 10% MacCM for 15 min. ROS production was analyzed by flow cytometry. (a) ROS-positive adipocytes after treatment with H_2O_2 and MacCM; *significant difference untreated versus H_2O_2. (b) Histograms of ROS-positive cell percentage in cells cultured in medium or treated with H_2O_2 for 15 min. (c) $Nrf2^{+/+}$ and Nrf2 knock-down mouse embryonic fibroblasts were treated with 100 μM resveratrol (Res) or corresponding vehicle control (DMSO) for 24 h. The PAI-1 and Nrf2 protein levels were measured by Western blot. *Significant difference untreated versus Res, **significant difference wild type versus knockout cells. (d) Representative Western blot.

in a model of inflamed human adipose tissue. Our data demonstrated several new findings with respect to resveratrol and human PAI-1 regulation under obesity-mimicking conditions. First, it was found that resveratrol downregulated PAI-1 mRNA and protein levels in a time- and concentration-dependent manner in human SGBS adipocytes. Second, the inhibitory effect of resveratrol on PAI-1 was even stronger on the obesity-associated and inflammation-dependent induction of PAI-1. Third, while resveratrol exerted its effects on inflammatory-dependent PAI-1 gene expression mainly via inhibition of NFκB, signaling via Sirt1, AMPK, PI3K, ROS, and Nrf2 did not mediate the effect of resveratrol on PAI-1 production.

Obesity represents a risk factor for the development of diseases like type 2 diabetes, hypertension, atherosclerosis and myocardial infarction. Intriguingly, obesity is also associated with a state of chronic low-grade inflammation characterized by elevated plasma concentrations of proinflammatory cytokines (IL-6, IL-1 and TNFα), chemokines (monocyte chemotactic protein 1, MCP-1), and adipokines (haptoglobin, PAI-1, leptin, visfatin, resistin and VEGF) [63]. Plasma PAI-1 levels are considerably enhanced in obese humans and in patients with insulin resistance, type 2 diabetes, and cardiovascular diseases [23, 64]. The adipose tissue appears to be the major source of elevated PAI-1 levels observed in obesity [65, 66] maybe as a result of its increased capacity to produce PAI-1 and/or as an effect of direct stimulation of adipocytes by hormones and cytokines upregulated in obesity [67]. Resveratrol is capable of attenuating obesity-associated inflammatory responses by inducing changes in the secretion profile of adipocytes [68–71]. In particular resveratrol inhibited TNFα-dependent PAI-1 upregulation in 3T3-L1 adipocytes [68, 70], IL1β-stimulated PAI-1 secretion [69], and PAI-1 production in human SGBS adipocytes [71]. These data are very much in line with the results from the present study where we have shown that resveratrol not only downregulated PAI-1 expression (Figure 1) but even exerted a stronger effect on PAI-1 in a model of inflamed

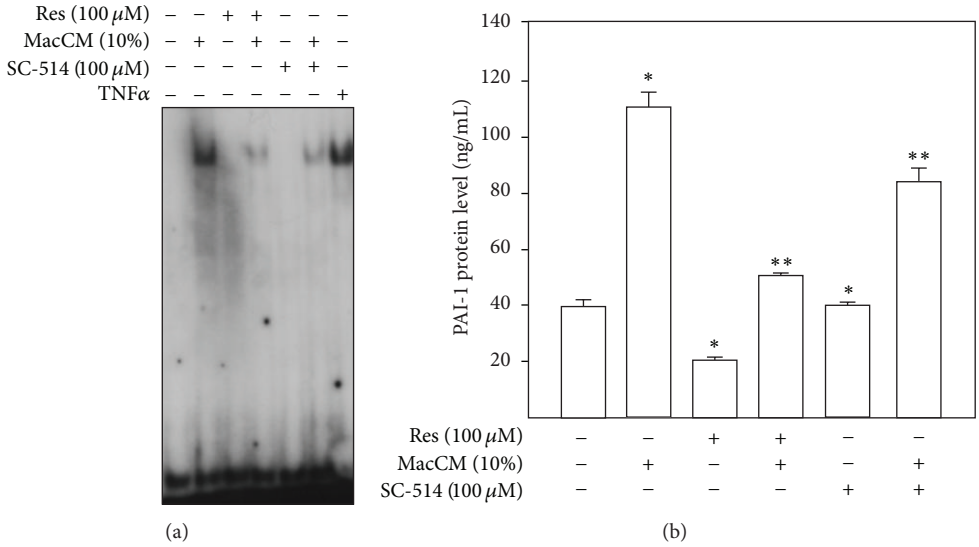

Figure 6: Resveratrol-mediated suppression of NFκB DNA binding activity did not abrogate PAI-1 gene expression. (a) Electrophoretic mobility shift assay using a 5′-end-labelled consensus oligonucleotide for NFκB binding and nuclear extracts from SGBS adipocytes. SGBS adipocytes were treated with 10% MacCM, 100 μM resveratrol, 100 μM SC-514, or combination of them and then incubated for 1 h. The DNA-protein complexes were separated by electrophoresis on 5% native polyacrilamide gels and visualized by phosphoimaging. (b) SGBS adipocytes were treated with 10% MacCM, 100 μM resveratrol (Res), 100 μM SC-514, or a combination of both Res and 10% MacCM or SC-514 and MacCM for 48 h. Accumulation of PAI-1 protein in media was measured by ELISA. *Significant difference untreated versus MacCM, Res or SC-514; **significant difference MacCM treated versus MacCM + Res or MacCM + SC-514.

human adipose tissue (Figures 2 and 3). Although all these data indicate that resveratrol can alleviate obesity-induced upregulation of PAI-1 in adipose tissue, it has not been fully elucidated by which molecular mechanisms resveratrol exerts its effect on PAI-1 under inflammatory conditions.

Calorie restriction is considered to be one of the most effective nutritional interventions protecting against obesity, diabetes, and cardiovascular disease [72]. The obesity-related enhancement of PAI-1 levels also appeared to be reversible by calorie restriction diet or calorie restriction mimetics [26, 27]. Several signaling pathways have been implicated in mediating the calorie restriction effect—the sirtuin pathway, the adenosine monophosphate (AMP) activated protein kinase (AMPK) pathway, and the insulin-like growth factor (IGF-1)/insulin signaling pathway (as discussed by [73]). In rodents calorie restriction and calorie restriction mimetics seem to extend the life span and are linked to silent mating type information regulation 2 homolog 1 (Sirt1) activation (references in [74]). Resveratrol was identified as a Sirt1 activator [75] and gained interest in a number of pathological settings—among them obesity. In line, the anti-inflammatory effects of resveratrol in adipocytes as well as in human adipose tissue were shown to be mainly dependent on Sirt1 activation [69, 76, 77]. However, in our study, neither inhibition of Sirt1 with sirtinol nor deficiency of Sirt1 was able to abrogate the resveratrol effects on PAI-1 (Figures 4(a), 4(b) and 4(c)) implicating that Sirt1 activation is not necessary to mediate the action of resveratrol on PAI-1 synthesis under inflammatory conditions.

Resveratrol is known to exert pleiotropic effects on cells and Sirt1 activation is not the only effect via which resveratrol exerts its beneficial actions on obesity-associated pathological consequences [29, 30, 78]. Therefore, the inhibitory effect of resveratrol on PAI-1 production in obesity may result from modulation of different signaling pathways. Some of the beneficial effects of resveratrol against diet-induced obesity and insulin resistance were mediated via AMPK activation [29, 35, 36, 38, 78, 79]. In addition, increasing evidence suggests that AMPK has anti-inflammatory actions [80, 81]. Therefore, we have tested whether the effects of resveratrol on PAI-1 expression are mediated via AMPK. Our results demonstrated that resveratrol-dependent downregulation of PAI-1 was still preserved in AMPK-deficient cells (Figures 4(d) and 4(e)) pointing out that resveratrol acts on PAI-1 in an AMPK-independent mechanism.

A number of experimental observations have demonstrated that the PI3K/Akt pathway represents an important signaling cascade in the initiation of the inflammatory response. Although we showed in an earlier study that resveratrol inhibits PI3K-driven Akt phosphorylation in SGBS cells [55] the PI3K inhibitor, LY294002, could not abrogate the resveratrol-dependent downregulation of PAI-1 (Figure 4(f)) implicating that the PI3K/Akt pathway is also not involved in the modulation of PAI-1 expression by resveratrol.

Inflammation is well known to exist in combination with oxidative stress which in turn is a potent modulator of PAI-1 gene expression in different systems [60, 82] as well as in this study. In this context, an important transcription

factor mediating responses to oxidative stress is Nrf-2 [83]. Resveratrol supplementation has been shown significantly to increase Nrf2 activity in humans after a meal [84]. However, the conditions of our inflammatory model did not induce ROS generation (Figures 5(a) and 5(b)). In line with that, the knockdown of Nrf2 did not impair the resveratrol effect on PAI-1 secretion (Figures 5(c) and 5(d)).

An increase in plasma PAI-1 levels observed in obesity can also be the result of a cytokine-dependent induction of PAI-1 transcription where the proinflammatory cytokines such as IL-1, IL-6, and TNFα play the major role [85–87]. Interestingly, no STAT3 binding element participating in the IL-6 response could be mapped in the PAI-1 promoter whereas the so-called NFκB-like sites within the PAI-1 promoter and a TNFα-responsive enhancer located 15 kb upstream of the transcription start site were shown to participate in response to IL-1 and TNFα (references in [14]).

Nuclear factor (NF)κB is a transcription factor with a central role in the induction of a chronic inflammatory state associated with obesity, development of type 2 diabetes, cardiovascular risk, and insulin resistance [88]. Previous reports established resveratrol as an inhibitor of NFκB [41, 89] and resveratrol treatment of TNFα-stimulated adipocytes reduced the expression of proinflammatory cytokines [88]. Therefore, our results showing that the resveratrol effects on PAI-1 gene expression were NFκB-dependent (Figure 6) are in line with those findings.

Interestingly a number of *in vivo* and *in vitro* studies showed an inhibitory role of the resveratrol target Sirt1 on NFκB signaling [76, 77, 90, 91]. Similarly, AMPK signaling has been shown to inhibit the inflammatory responses induced by NFκB via several downstream targets of AMPK (references in [92]). Moreover, several previous findings have demonstrated that the PI3K/Akt pathway has a crucial role in the activation of the NFκB pathway [93, 94]. Based on these studies and the role of resveratrol as a Sirt and AMPK activator, PI3K inhibitor as well as ROS scavenger, and we were expecting that Sirt, AMPK, PI3K, or ROS would be involved in the resveratrol effects. Surprisingly, none of these upstream NFκB modulators contributed to the effects of resveratrol; however, in line with previous studies [77, 88, 95–100] our findings show that resveratrol can act as an NFκB inhibitor, most likely via so far not a characterized pathway.

5. Conclusions

Together, our study showing that resveratrol mediates an inhibitory effect on PAI-1 may be useful to further establish PAI-1 as a marker for obesity-associated inflammatory conditions. In addition, we add at least one novel aspect to the pleiotropy of the resveratrol action by showing that it can act as an NFκB inhibitor without involving Sirt1, AMPK, PI3K or ROS.

Authors' Contribution

Ivana Zagotta and Elitsa Y. Dimova contributed equally to this work.

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Research Article

Sivelestat Attenuates Myocardial Reperfusion Injury during Brief Low Flow Postischemic Infusion

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The neutrophil elastase inhibitor sivelestat (ONO-5046) possesses unknown mechanisms of cardioprotection when infused following global ischemia, even in the absence of neutrophils. Since myocardial ischemia-reperfusion injury is strongly associated with endothelial dysfunction and reactive oxygen species (ROS) generation during reperfusion, we have tested the hypothesis that infusion of sivelestat during postischemic low flow would preserve endothelial and contractile function and reduce infarct size through an ROS-mediated mechanism. Isolated male rat hearts, subjected to global ischemia of 25 minutes, were reperfused with low flow with or without sivelestat followed by a full flow reperfusion. Hearts treated with sivelestat showed a significant improvement of LV contractile function and a reduction in infarct size. Infusion of L-NAME (nonspecific blocker of endothelial nitric oxide synthase (eNOS)) along with sivelestat during reperfusion reversed the preservation of contractile function and infarct size. *In vitro* EPR spin trapping experiments showed that sivelestat treatment decreased superoxide adduct formation in bovine aortic endothelial cells (BAECs) subjected to hypoxia-reoxygenation. Similarly, dihydroethidine (DHE) staining showed decreased superoxide production in LV sections from sivelestat-treated hearts. Taken together, these results indicate that sivelestat infusion during postischemic low flow reduces infarct size and preserves vasoreactivity in association with decreased ROS formation and the preservation of nitric oxide.

1. Introduction

Reperfusion of the ischemic myocardium occurs in nearly 2 million people annually in the United States in patients experiencing cardiac arrest, myocardial infarction, or undergoing cardioplegic arrest during cardiac surgery [1]. Various levels of low flow are induced following such ischemic events, most notably in the early moments of reperfusion [2–4]. In the case of cardiac arrest, which affects over 300,000 people annually in the USA, cardiopulmonary resuscitation (CPR) is used by first responders to initiate reperfusion [5]. Currently this low flow reperfusion generated by CPR [6] results in a survival-to-hospital discharge rate for out-of-hospital cardiac arrest patients of <10% [7]. Periods of low coronary flow following myocardial ischemia may present early opportunities for pharmacologic treatment of ischemic myocardium prior to full flow reperfusion [8].

There is currently no approved drug on the US market for postischemic reduction of myocardial infarction [9]. The neutrophil elastase inhibitor sivelestat (ONO-5046) [10] has recently been shown to be cardioprotective in several animal studies and in at least one study in humans [11–13]. It has been recently demonstrated that sivelestat preserves LV function when infused after global ischemia in the Langendorf buffer-perfused heart, a model of the functioning heart, that is, bereft of neutrophils [14]. The neutrophil-independent cardioprotective mechanisms of sivelestat in the setting of ischemia-reperfusion (IR) are unknown.

Reactive oxygen species (ROS) appear to play an important role in reperfusion-induced injury [15], specifically

superoxide, hydroxyl radical, and hydrogen peroxide [16]. The release of oxygen-derived free radicals concurrent with the reintroduction of oxygen contributes to the pathophysiology of IR injury [17, 18]. In addition to cellular and mitochondrial injury through oxygen radical-mediated damage, reactive oxygen species signal neutrophil infiltration and are implicated in endothelial dysfunction and smooth muscle injury [19–22]. Specifically, ischemia-reperfusion impairs endothelium-dependent vasorelaxation of coronary microvessels through production of ROS [23]. Excess hydrogen peroxide production during reperfusion damages vascular smooth muscle cells. Conversely reduction of ROS during reperfusion attenuates myocyte and endothelial cell injury following IR [24].

In this study, we tested the hypothesis that infusion of sivelestat during postischemic low flow would reduce infarct size and preserve endothelial and contractile function through an ROS-mediated mechanism. We used the isolated perfused rat heart model of IR and fluorescent microscopy in tissue slices, and electron paramagnetic resonance (EPR) imaging in cultured endothelial cells, to detect the formation of reactive oxygen species. Our results demonstrate a neutrophil-independent mechanism of sivelestat to reduce infarct size and preserve cardiac performance while reducing early ROS formation and preserving endothelial function.

2. Methods

2.1. Isolated Buffer-Perfused Rat Hearts. Male Sprague-Dawley rats (400–500 g) purchased from Harlan Laboratories (Indianapolis, IN, USA) were cared for in accordance with the National Institute of Health (NIH) guidelines and the approval of the Institutional Animal Care and Use Committee. Hearts were isolated and perfused with buffer as previously described [25]. Rats were anesthetized with intraperitoneal sodium pentobarbital (70 mg/kg) and heparin (1,000 U/kg). The trachea was cannulated with a 16-gauge angiocath attached to a rodent ventilator (Harvard Apparatus, South Natick, MA, USA). Animals were ventilated with room air at 70 respirations per min with a 2.5 mL stroke volume. A midsternal thoracotomy was performed to expose the heart and isolate the aorta. The aorta was cannulated in situ and hearts were excised. Retrograde perfusion of the coronary arteries was immediately initiated with warmed (37°C) modified Krebs-Henseleit buffer (1.25 mM $CaCl_2$, 11 mM glucose, 112 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM K_2PO_4, and 0.2 mM octanoic acid, bubbled with 95% O_2/5% CO_2, pH 7.4) at a constant perfusion pressure of 75 mm Hg. A saline-filled latex balloon attached to a pressure transducer was inflated to 5–10 mm Hg in the left ventricle (LV) for measurements of LV contractile function. The heart was positioned inside a temperature-controlled glass chamber at 37°C.

2.2. Experimental Protocol. Global ischemia was induced by completely occluding perfusion flow to the heart. Hearts were randomly assigned to 3 ischemia groups ($n = 8$ per group): group 1: 25 min of global ischemia; group 2: 25 min of ischemia followed by 3 min of low flow with perfusion buffer at 4 mL per min; group 3: 25 min of ischemia followed by 3 min of low flow with sivelestat (100 μg per mL) at 4 mL per min, administered through a side port directly above the aorta by a PHD 2000 Programmable pump (Harvard Apparatus, Holliston, MA, USA). All hearts were subsequently reperfused for 60 min at 75 mm Hg.

2.3. Coronary Flow and Left Ventricular Function. Coronary flow was continuously monitored by the use of an inline small animal flow meter (Model T206, Transonic Systems Inc., Ithaca, NY, USA) and recorded using an analog-to-digital converter box (Digi-Med ASA-400a, Micro-Med, Inc., Louisville, Kentucky, USA). LV pressure was continuously sampled at 30 Hz and digitally processed with a Digi-Med Heart Performance Analyzer (HPA-210a, Micro-Med, Inc.). Heart rate, dP/dt_{max}, LV systolic pressure, and LV end diastolic pressure were derived by computer algorithm. Developed pressure was calculated as the difference between systolic and end diastolic pressures. Rate pressure product (RPP) was calculated as the product of heart rate and developed pressure. Hearts that did not achieve an average RPP of at least 20,000 and an average dP/dt_{max} of at least 2,500 in the preischemic baseline stabilization period were excluded from further experimentation. Metrics of LV functional recovery were calculated as percent recovery at the end of experimentation relative to baseline (preischemic) values.

2.4. Acetylcholine-Induced Change in Coronary Flow. Following 60 min of reperfusion, hearts were infused with 1.0 μM acetylcholine chloride for one minute and then switched back to normal perfusion fluid. At this point the recovery in coronary flow was recorded as the maximum coronary flow value within one minute of reflow with normal perfusion fluid. Since perfusion pressure was maintained constant at 75 mm Hg, increases and decreases in coronary flow were a reflection of endothelial relaxation and constriction, respectively.

2.5. Infarct Size. Following IR, hearts were stained with triphenyltetrazolium chloride (TTC) for the measurement of infarct size, using the method of Ferrera et al. [26]. Under TTC staining, living tissue appears brick red and infarcted tissue appears pale pink or white. Hearts were frozen at −80°C, sliced in 2 mm sections, incubated in 1% TTC at 37°C for 10 min per side to allow mitochondrial uptake of TTC, and then fixed in formalin prior to photomicrography. Infarct size was measured using MetaVue imaging software (version 6.2r6, Universal Imaging Corp., Downingtown, PA, USA) and reported as a percent of the total left ventricular area.

2.6. Creatine Kinase Release. Coronary effluent was collected from all hearts before ischemia (i.e. in baseline) and at 10, 30, and 60 minutes of full reperfusion. The effluent was assayed for creatine kinase (CK) content using a standard spectrophotometric assay kit (Stanbio Laboratory, Boerne, TX). Values of CK activity are reported in $U \cdot L^{-1}$ [27].

2.7. Measurement of Superoxide in LV Sections by DHE Staining. In another block of experiments using duplicate groups,

hearts were collected at the end of the 10 min of reperfusion for measurements of ROS production under fluorescent microscopy, as previously reported [28]. After reperfusion, hearts were embedded in optimal cutting temperature gel, sliced to 5 μm thick in a cryotome, and placed on glass slides. After application of 10 μM dihydroethidium (DHE), tissue sections were incubated in a light-impermeable chamber at 37°C for 30 min. In the presence of superoxide DHE is converted to the red fluorescent hydroxyethidium molecule [29]. Slides were costained with 4′,6-diamidino-2-phenylindole (DAPI) and photographed using a Nikon Eclipse TE 2000-U microscope (Tokyo, Japan) equipped with an X-Cite 120 Fluorescence Illumination System (Lumen Dynamics Group Inc., Mississauga, Ontario). Photographs of hydroxyethidium fluorescence were taken under a rhodamine filter (green excitation 550 nm, red emission 573 nm). Fluorescent intensity, which positively correlates with superoxide generation in tissue, was quantified using MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA, USA).

2.8. Effect of Nitric Oxide Synthase (NOS) Inhibitor in Isolated Hearts. Additional hearts were isolated to determine if sivelestat exerts cardioprotection in the presence of NOS inhibition. Isolated hearts ($n = 4$ per group) were subjected to 25 min of ischemia and 3 min of low flow at 4 mL per min followed by full reperfusion for 120 minutes. During 3 min of low flow, hearts were directly infused with either (a) perfusion buffer; (b) sivelestat (100 μg per mL); (c) the nonspecific NOS blocker N-nitro-L-arginine methyl ester (L-NAME, 100 μM); or (d) L-NAME + sivelestat ($N = 4$ per group). LV function and infarct size were recorded as detailed above.

2.9. Effect of Hypoxia-Reoxygenation in Bovine Aortic Endothelial Cells. To determine the influence of sivelestat on ROS production in endothelial cells, cultured bovine aortic endothelial cells (BAECs) were subjected to hypoxia-reoxygenation in the presence of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) [17]. BAECs were cultured in low-glucose DMEM supplemented with 10% FBS, penicillin/streptomycin, and 0.1% of Endothelial Cell Growth Supplement (Millipore, Billerica, MA, USA). Cells were cultured in a humidified environment of 5% CO_2/21% O_2. When cells achieved 80–85% confluence, they were washed, trypsinized, pelleted, and suspended in low-glucose DMEM without phenol red to achieve a cell count of 5×10^6 cells per mL. To achieve hypoxia, cells were placed in a heated chamber at 37.0°C and flushed with 100% nitrogen for 45 min. Immediately following the rapid addition of DMPO (50 mM final concentration), endothelial cells were flushed with oxygen (95% O_2/5% CO_2, pH 7.4) for one minute at 37.0°C in the presence of (a) no drug, (b) sivelestat (100 μg per mL final concentration), (c) the xanthine oxidase inhibitor oxypurinol (500 μM), or (d) superoxide dismutase (SOD, 1 kU per mL from bovine erythrocytes (Sigma-Aldrich Co., St. Louis, MO, USA). Cells were then transferred to a quartz flatcell inside an EPR-300 X-band (9.7 GHz) spectrometer. Second peak amplitude of the DMPO-OH spectrum was quantified and taken as an indication of the magnitude of superoxide production.

2.10. Data Analysis. Data was expressed as mean ± SEM. Statistical significance between groups was calculated by one-way ANOVA followed by the Tukey range test for multiple comparisons. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Sivelestat Preserved LV Function. Administration of sivelestat during 3 min of low flow following ischemia improved the recovery of developed pressure, dP/dt_{max}, and rate pressure product (RPP) at 60 min of reperfusion as compared to hearts receiving 25 min of ischemia or low flow without sivelestat (Figure 1(a)).

3.2. Sivelestat Administration Preserved Vasoreactivity. The vasoconstrictor acetylcholine was infused for one min at the end of 60 min of reperfusion. Following this, normal buffer was reperfused, and the rebound in coronary flow was taken as a metric of vasoreactivity. Administration of sivelestat significantly improved recovery of coronary flow (CF) following acetylcholine infusion (8.4 ± 1.8%) as compared to hearts receiving 25 min of ischemia (3.4±0.50%) or low flow without sivelestat (−0.20 ± 1.0%) (Figure 1(b)).

3.3. Sivelestat Decreased Myocardial Infarct Size. At the end of 60 min of reperfusion, hearts infused with sivelestat during 3 min of postischemic low flow had significantly lower infarct area (11 ± 2.2%) than hearts given normal perfusion buffer during low flow (54 ± 3.4%) and hearts receiving 25 min of ischemia with no low flow (47 ± 2.1%). Representative images are shown in Figure 2(a).

3.4. Sivelestat Decreased Myocardial Creatine Kinase Levels. Creatine kinase (CK) release was measured in coronary effluent collected during baseline and at 10, 30, and 60 min of reperfusion. CK was significantly reduced at 10 min of reperfusion in hearts treated with sivelestat during 3 min of postischemic low flow as compared to hearts that received 25 min of ischemia with no low flow (Figure 2(b)).

3.5. Inhibition of eNOS with L-NAME Reversed Cardioprotection from Sivelestat. After 2 hours of full reperfusion the percent infarct size was significantly less in hearts treated with sivelestat compared to L-NAME-treated hearts (sivelestat I.S. = 19 ± 3.2% versus L-NAME I.S. = 50 ± 2.7%). This sivelestat-mediated reduction in infarct size was lost in the L-NAME + sivelestat-treated hearts (I.S. = 59 ± 0.97%) (Figure 3(a)). In these same hearts, the percent recovery of rate pressure product (RPP) was significantly greater in sivelestat-treated hearts compared to vehicle-treated hearts (sivelestat RPP = 47 ± 5.3% versus vehicle RPP = 28 ± 4.7%) and to L-NAME + sivelestat-treated hearts (RPP = 26±2.9%) (Figure 3(b)).

3.6. Sivelestat Decreased Superoxide Levels in LV Tissue Sections. The blockade of eNOS by L-NAME reversed the effect of sivelestat by increasing superoxide fluorescence (Figure 4(a)). DHE staining showed that superoxide generation was

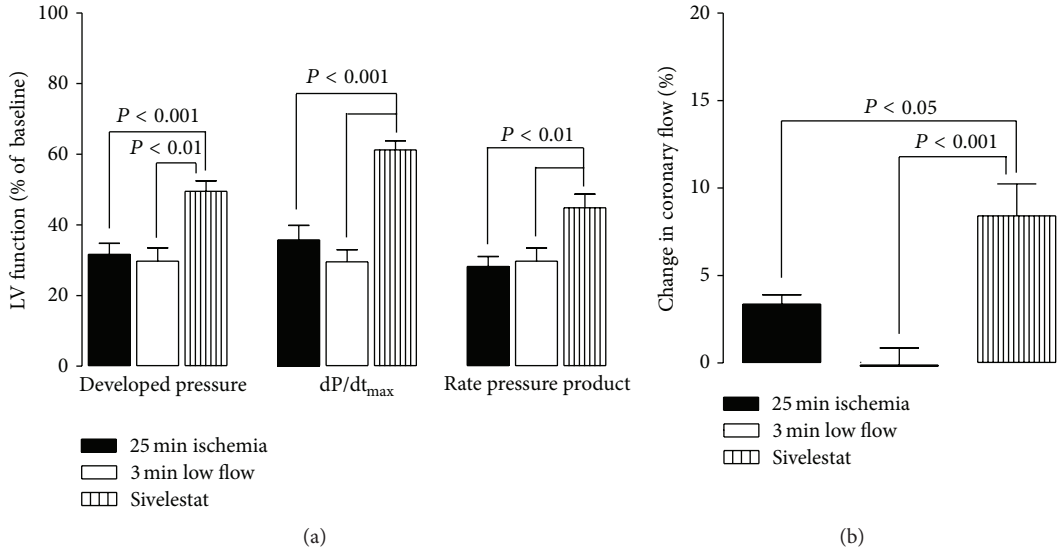

Figure 1: Sivelestat preserves LV and coronary vascular function in ischemia-reperfusion. (a) Effect of sivelestat on LV function and infarct size. Sivelestat significantly improved recovery of developed pressure, dP/dt_{max}, and rate pressure product (RPP) as compared to hearts that did not receive low flow (25 min isc) ($n = 8$ per group). (b) Rebound in coronary flow. Sivelestat significantly preserved vasoreactivity at the end of reperfusion as shown through the rebound in coronary flow after one minute of acetylcholine (1 μM) infusion ($n = 4$/group).

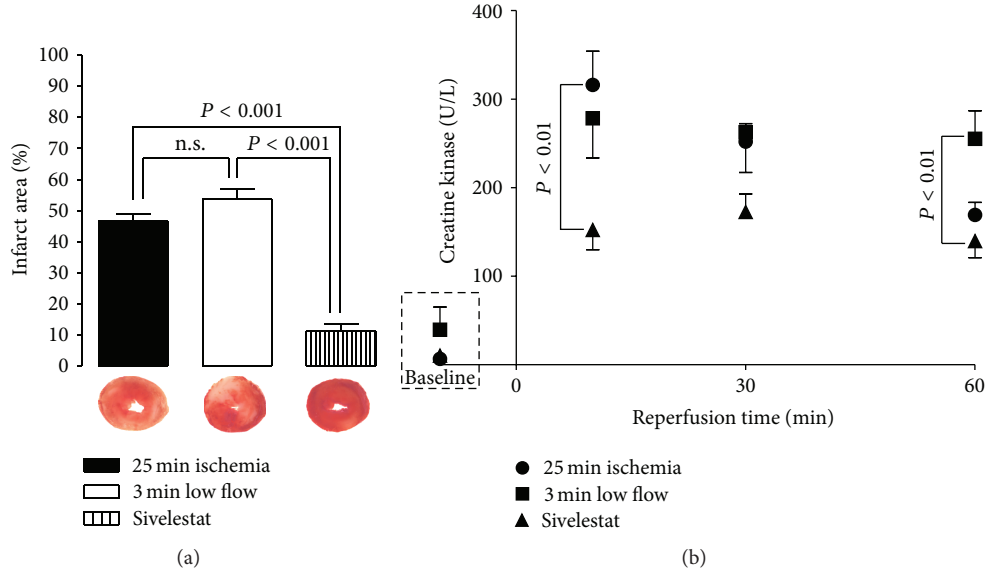

Figure 2: Sivelestat preserves LV tissue in ischemia-reperfusion. (a) Percent infarct area with representative images of left ventricle slices. Sivelestat significantly reduced infarct area as compared to hearts given 25 min ischemia or ischemia and low flow with vehicle only, as observed through TTC staining. Whiter areas indicate regions of tissue infarction, and pink and red areas indicate functional tissue ($n = 4$ per group). n.s.: no significance. (b) Extrusion of intracellular creatine kinase into coronary effluent. Creatine kinase (an intracellular enzyme) release is indicative of cell membrane rupture. Creatine kinase release (U/L) was significantly reduced by treatment with sivelestat at 10 min of full reperfusion, as compared to hearts that did not receive low flow, and at 60 min of reperfusion, as compared to vehicle-treated low flow hearts ($n = 8$/group).

significantly decreased in the sivelestat group (Figure 4(b)). In contrast an increase in superoxide fluorescence was seen in the low flow, L-NAME, and L-NAME + sivelestat treated groups. This data demonstrates the involvement of nitric-oxide-mediated cardioprotection by sivelestat.

3.7. Sivelestat Decreased ROS Formation in BAECs. To measure the production of superoxide by hypoxic-reoxygenated bovine aortic endothelial cells (BAECs), DMPO was added at the moment of reoxygenation as a spin trap for superoxide. The EPR-active molecule DMPO-OH is the molecular

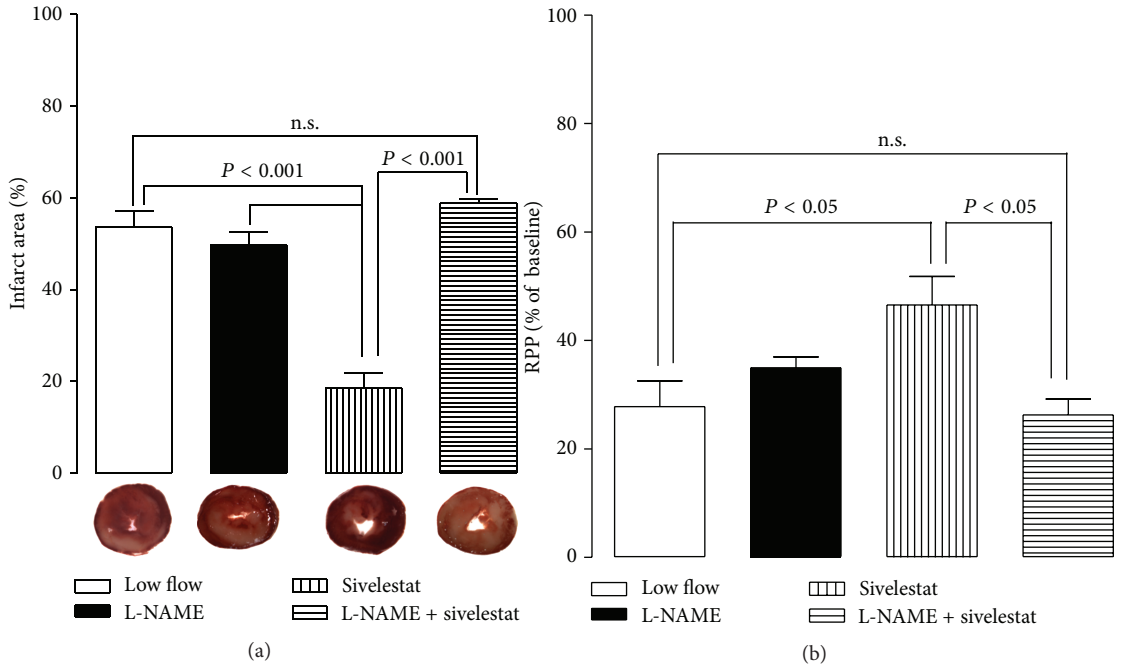

Figure 3: L-NAME reverses the cardioprotective effects of sivelestat in ischemia-reperfusion. (a) Infarct size and (b) recovery of rate pressure product. During low flow, hearts were treated with L-NAME, sivelestat, or both. Though sivelestat preserves RPP and reduces infarct following IR, coinfusion of L-NAME and sivelestat offered no preservation of RPP or infarct reduction as compared to hearts that received vehicle during low flow (n = 4/group). n.s.: nonsignificant.

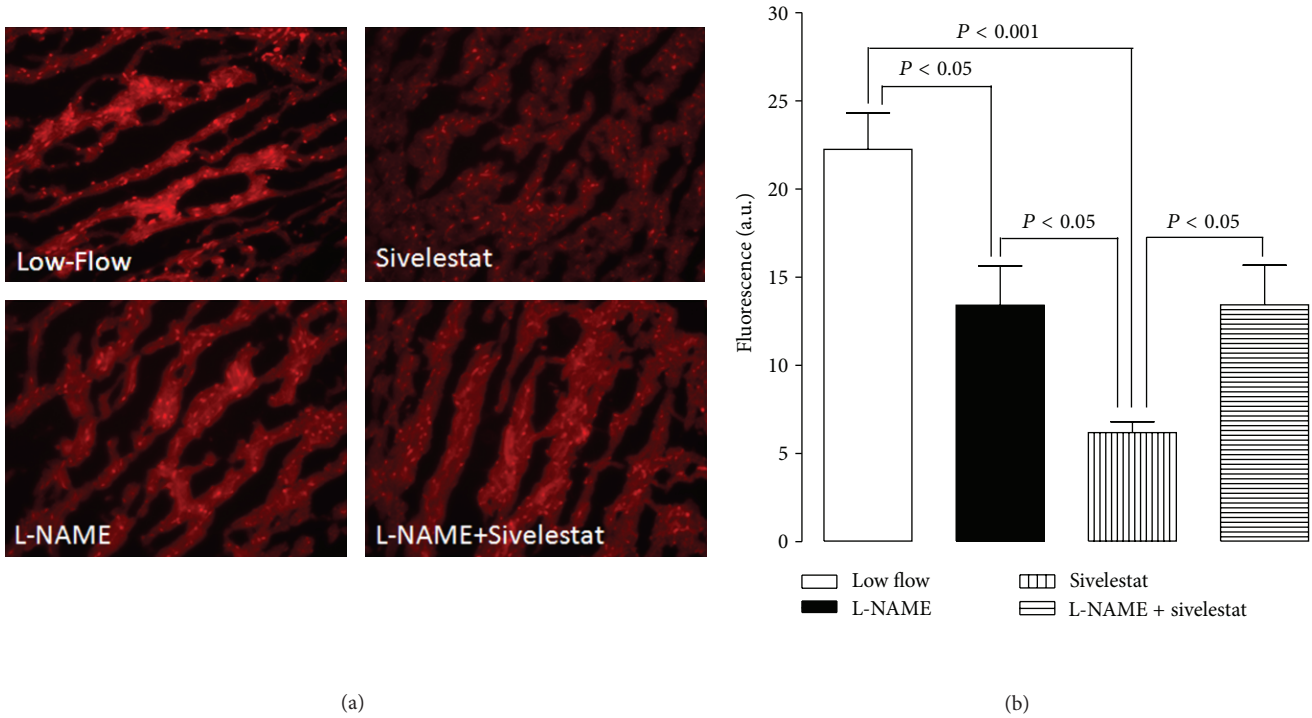

Figure 4: Measurement of ROS in the heart by DHE fluorescence. (a) Representative confocal fluorescence images of cryopreserved LV sections from different groups stained with DHE. (b) Quantification of superoxide generation is indicated by DHE fluorescence, sivelestat decreased tissue ROS during reperfusion, as compared to hearts infused with L-NAME. This decrease was reversed when L-NAME was coinfused with sivelestat (n = 3–5 per group). n.s.: nonsignificant.

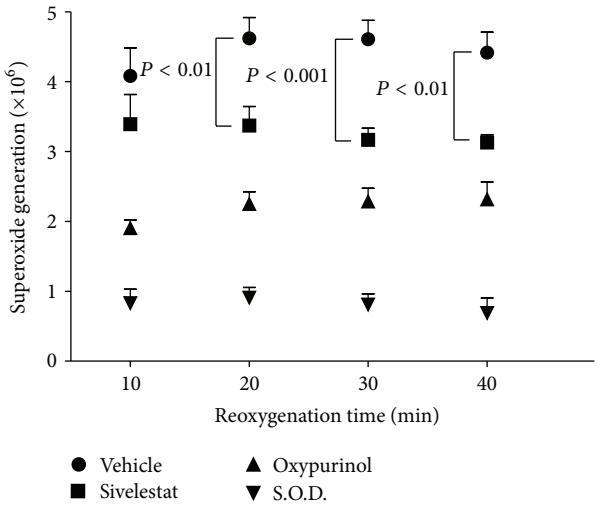

Figure 5: EPR spin-trapping of superoxide with DMPO in hypoxic-reoxygenated bovine aortic endothelial cells. First integral of DMPO-OH, amplitude of the 2nd spectral peak. DMPO-OH is the stable EPR-active adduct of trapped superoxide. All drugs were applied prior to hypoxia. Sivelestat (190 μM) decreased the formation of superoxide (DMPO-OH) in bovine aortic endothelial cells as indicated by spin-trapping with DMPO. Oxypurinol (500 μM) was more effective than sivelestat, and S.O.D. (5 kU/mL) was most effective at decreasing DMPO-OH ($n = 5$ per group).

product of superoxide trapped by DMPO. Sivelestat reduced oxygen radical formation in hypoxic-reoxygenated BAECs exposed to 45 min of hypoxia followed by reoxygenation. As indicated by the 2nd peak height in the 1st integral of the DMPO-OH EPR spectrum, the spin adduct DMPO-OH was significantly reduced in cells treated with 100 μg/mL sivelestat, as compared to untreated endothelial cells, after 20 min, 30 min, and 40 min of reoxygenation. Oxypurinol significantly reduced DMPO-OH formation at every time point as compared to vehicle, but not as effectively as S.O.D. (Figure 5).

4. Discussion

In this study, the combination of postischemic low flow with a novel pharmaceutical agent, sivelestat, for 3 minutes at the onset of reperfusion successfully reduced infarct size, improved contractile function, preserved endothelium-dependent vasodilatation, and reduced creatine kinase release, an indicator of tissue destruction at the cellular level [30]. Sivelestat reduced superoxide production in both the ischemia-reperfused explanted LV tissue and the hypoxia-reoxygenated cultured endothelial cells. However, the functional cardioprotection and reduction in tissue superoxide generation effected by sivelestat was lost with NOS inhibition. Our study is the first to demonstrate the infarct-reducing property of sivelestat in the ischemia-reperfused heart.

Preservation of LV contractile function is in agreement with Kambe et al., who showed a 35% recovery of LV developed pressure versus controls (16%) when sivelestat was infused continuously during the first 10 min of full reperfusion in isolated rat hearts [14]. Also in agreement with Kambe et al., sivelestat significantly preserved endothelium-dependent vasorelaxation following IR. That study showed a 15% increase over controls in the rebound in coronary flow in response to acetylcholine infusion at the end of reperfusion in hearts treated with sivelestat.

The infarct-sparing effects of sivelestat were reversed with simultaneous NOS enzyme inhibition, suggesting that nitric oxide bioavailability is essential during a brief 3-minute period of postischemic low flow. It is likely that sivelestat protects endothelium through dependence on functional NOS enzymes since myocardial protection was abolished by NOS blockade in our study. The idea that preservation of NO bioavailability may lead to myocardial salvage from IR has been widely accepted [31]. Others have shown that sivelestat requires functional NOS enzymes for exertion of protective effects on tissue. Okajima et al. concluded that sivelestat reduced IR-induced liver injury, an effect that was completely inhibited by pretreatment with L-NAME and relied on endothelium-dependent production of NO [32]. Takayama and Uchida showed that sivelestat inhibited substance P-induced contraction of tracheal ring preparations and that this effect was significantly attenuated by either removal of epithelium or blockade of NOS by L-NAME. However, they concluded that sivelestat exerted vasodilatory effects on guinea pig airways by both NO- and epithelium-dependent and NO- and epithelium independent mechanisms [33].

The retention of endothelium-dependent vasoreactivity with sivelestat may be explained by preservation of sensitivity to nitric oxide in the smooth muscle. However, other studies assert that sivelestat mediates vasorelaxation independent of endothelium. Maeda et al. suggested that this mechanism lies in the vasculature, by demonstrating that sivelestat selectively inhibits calcium sensitization to a receptor agonist in porcine vascular smooth muscle strips with or without endothelium, without affecting calcium-induced contraction [34]. Amemori et al. reiterated this work showing that sivelestat induces endothelium-independent vasorelaxation in pre-contracted human gastric arteries [35]. It should be noted that sivelestat also protects the heart in situations where neutrophils are present. Akiyama et al. showed attenuation of myocardial stunning in swine with postischemic infusion of sivelestat [11]. Toyama et al. reported an association of sivelestat infusion with improved fractional area of change in the left ventricle of pediatric patients who underwent cardiovascular surgery with cardiopulmonary bypass, demonstrating that sivelestat is protective in at least one setting of ischemia-reperfusion in humans [12]. However, our results confirm that sivelestat preserves vasodilatation in the coronary endothelium of hearts subjected to IR in an environment without neutrophils.

The role of eNOS in sivelestat-induced cardioprotection must be further explored. In order to produce nitric oxide from L-arginine, eNOS enzymes require tetrahydrobiopterin (BH_4) to couple L-arginine oxidation to NADPH consumption and prevent dissociation of the ferrous-dioxygen complex [36]. When BH_4 is depleted, as in myocardial ischemia, the association of the ferrous-dioxygen complex can decrease. Once perfusion is resumed, eNOS is not able to couple L-arginine oxidation to NADPH consumption and becomes

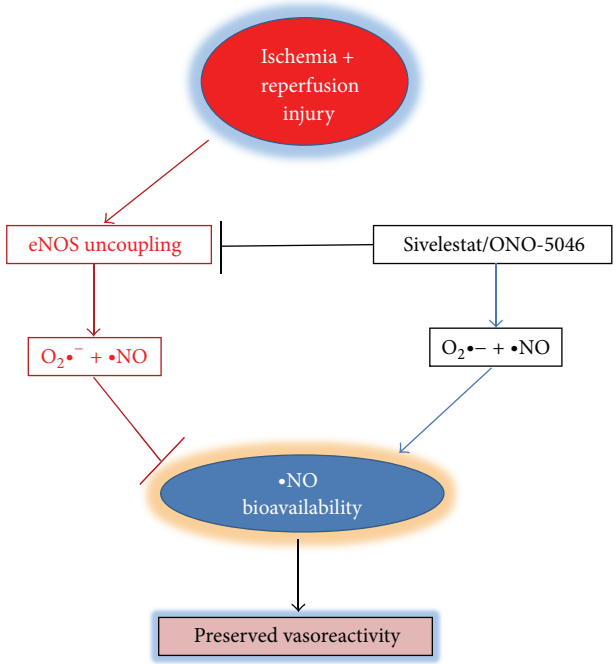

Figure 6: Proposed mechanism of sivelestat cardioprotection. During reperfusion, eNOS enzymes are uncoupled from nitric oxide production and produce superoxide in excess. Sivelestat may prevent eNOS uncoupling, thereby reducing superoxide overproduction and preserving nitric oxide production. Nitric oxide bioavailability preserves vascular function in cardiac ischemia-reperfusion.

a true NADPH oxidase, producing superoxide instead of nitric oxide [37]. It is reasonable to hypothesize that sivelestat preserves the association of the ferrous-di-oxygen complex thus preserving nitric oxide bioavailability. In support of this hypothesis we noted an increase in vascular reactivity to acetylcholine and a reduction of superoxide production in aortic endothelial cells (see Figure 6). However, additional studies are needed to elucidate the exact mechanism(s) of protection exerted by sivelestat. For instance, direct measurements of NO production by coronary endothelium are needed to confirm that sivelestat enhances bioavailability of NO.

In summary, our results demonstrate that sivelestat protects the heart against IR injury by scavenging ROS and thereby decreasing the oxidative damage at reperfusion leading to improved LV function and decreased infarct size. The cardioprotective effect of sivelestat may be attributed to an NOS-mediated mechanism. Sivelestat is effective at salvaging tissue when applied in very brief postischemic low flow conditions. This low flow reperfusion differs from the classic mechanical postconditioning, which consists of stuttered start-stop episodes following ischemia [38].

5. Conclusions

Overall, these results demonstrate that the period of postischemic low flow can be exploited for significant cardioprotection by pharmaceutical intervention. Due to its availability, promising results in animal studies, and current use in humans, sivelestat is a promising translational pharmaceutical for the investigation of myocardial infarction treatment.

Abbreviations

ROS:	Reactive oxygen species
IR:	Ischemia-reperfusion
LV:	Left ventricular
BAEC:	Bovine aortic endothelial cell
CK:	Creatine kinase
RPP:	Rate pressure product
TTC:	Triphenyltetrazolium chloride
DAPI:	4′,6-Diamidino-2-phenylindole
DHE:	Dihydroethidium
DMPO:	5,5-Dimethyl-1-pyrroline N-oxide
ERP:	Electron paramagnetic resonance
$ONOO^-$:	Peroxynitrite
NOS:	Nitric oxide synthase
L-NAME:	L-Nitro-arginine methyl ester
O.D.:	Optical density.

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Review Article

Red Orange: Experimental Models and Epidemiological Evidence of Its Benefits on Human Health

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In recent years, there has been increasing public interest in plant antioxidants, thanks to the potential anticarcinogenic and cardioprotective actions mediated by their biochemical properties. The red (or blood) orange (*Citrus sinensis* (L.) Osbeck) is a pigmented sweet orange variety typical of eastern Sicily (southern Italy), California, and Spain. In this paper, we discuss the main health-related properties of the red orange that include anticancer, anti-inflammatory, and cardiovascular protection activities. Moreover, the effects on health of its main constituents (namely, flavonoids, carotenoids, ascorbic acid, hydroxycinnamic acids, and anthocyanins) are described. The red orange juice demonstrates an important antioxidant activity by modulating many antioxidant enzyme systems that efficiently counteract the oxidative damage which may play an important role in the etiology of numerous diseases, such as atherosclerosis, diabetes, and cancer. The beneficial effects of this fruit may be mediated by the synergic effects of its compounds. Thus, the supply of natural antioxidant compounds through a balanced diet rich in red oranges might provide protection against oxidative damage under differing conditions and could be more effective than, the supplementation of an individual antioxidant.

1. Introduction

In recent years, an increasing interest in plant antioxidants has occurred because of the potential anticarcinogenic and cardioprotective actions mediated by their biochemical properties [1–3]. The antioxidant activity of these compounds may be dependent on the number and arrangement of the hydroxyl groups and the extent of structural conjugation, as well as the presence of electron-donating and electron-withdrawing substituents in the ring structure. Due to the growing interest in these pharmacologically active components in fruits, the demand for studies conducted on specific fruit such as pigmented orange juice is increasing.

Red (or blood) orange (*Citrus sinensis* (L.) Osbeck) is a pigmented sweet orange variety typical of eastern Sicily (southern Italy), California, and Spain. The red orange is noteworthy for its excellent orange flesh color and the consistent appearance of red coloration. The red coloration of red orange is mostly caused by the presence of water-soluble anthocyanin pigments not usually found in other citrus fruits.

In this paper, we discuss the main health-related properties of red orange that include anticancer, anti-inflammatory, and cardiovascular protection activities, and the effects on health of the main constituents of the red orange (namely, flavonoids, carotenoids, ascorbic acid, hydroxycinnamic acids, and anthocyanins) and their antioxidant activity and ability to modulate some key regulatory enzymes.

2. History, Geographical Distribution, and Varieties of Red Oranges

Red oranges may have originated from either China or the southern Mediterranean regions, but their exact origin is not known. It is possible that, in China, northeastern India, and southeastern Asia, *Citrus sinensis* trees were eventually transported along Asian trade routes to Africa, the Mediterranean Sea Basin, and Europe where orangeries were established. A mosaic in a Roman villa built in the first quarter of the 4th century and located about 3 km outside the town of Piazza Armerina, Sicily (southern Italy), demonstrates the presence of lime and lemon in Italy in that period of time. Citrus fruit seems to have been introduced in Sicily by Arab traders during the 7th century and cultivated as ornament until the 16th century. Spaniards introduced orange cultivation in South America in the 1500s and from there also in the United States. The first description of the red orange in Sicily was noted in the 17th century opera *Hesperides* (1646). The author described a particular kind of orange fruit ("*aurantium inducum*"), which is strongly pigmented ("*purpurei coloris medulla*"), imported to the island by a Genoese missionary from the Philippines islands.

The three most common types of red oranges are the *Tarocco*, the *Moro* (both native to Italy), and the *Sanguinello* (native to Spain). Other less common types include *Budd blood orange*, *Maltese*, *Khanpur*, *Washington Sanguine*, *Ruby Blood*, *Sanguina Doble Fina*, *Delfino*, *Red Valencia*, *Burris blood Valencia orange*, *Vaccaro blood orange*, *Sanguine grosse ronde*, *Entre Fina blood orange,* and *Sanguinello a pignu*. While also pigmented, *Cara Cara Navels* and *Vaniglia Sanguignos* have pigmentation based on lycopene, not anthocyanins like blood oranges [4]. The *Tarocco* variety is a medium-sized seedless fruit and is perhaps the sweetest and most flavorful of the three types. It is referred to as "half-blood," because the flesh is not accentuated in red pigmentation as much as with the *Moro* and *Sanguinello* varieties. The *Moro* is the most colorful of the red oranges, referred to as "deep blood orange," with deep red flesh ranges from orange-veined with ruby coloration, to vermilion, to vivid crimson, and nearly to black and a rind that has a bright red blush. This fruit has a distinct, sweet flavor with a hint of raspberry. The *Moro* variety is believed to have originated at the beginning of the 19th century in the citrus-growing area around Lentini (in the Province of Siracusa in Sicily) as a bud mutation of the "*Sanguinello Moscato*." The *Sanguinello* variety, discovered in Spain in 1929, is also present in Sicily as a "full-blood" orange, close in characteristics to the *Moro*. It matures in February, but can remain on trees unharvested until April. Fruit can last until the end of May.

3. The Anti-Inflammatory Capacity of Red Orange Juice

Red orange juice contains elevated quantities of various compounds including polyphenols, flavanones, anthocyanins, hydroxycinnamic acids, and ascorbic acid, and it is supposed to have a high antioxidant capacity depending on all its components (Table 1). After determining the antioxidant profile of several fresh orange juices obtained from five different *Citrus sinensis* (L.) Osbeck varieties (three pigmented varieties: *Moro*, *Sanguinello*, and *Tarocco*, and two blond varieties, *Valencia late* and *Washington navel*), the antioxidant efficiency of orange juices has been attributed, in a significant part at least, to their content of total phenols, whereas ascorbic acid seems to play a minor role [5]. *In vivo* studies conducted on healthy people has shown that red orange juice consumption determines a significant increase in plasma vitamin C, cyanidin-3-glucoside, beta-cryptoxanthin, and zeaxanthin [6]. The effect of red orange juice has been studied also in 19 subjects with increased cardiovascular risk included in a randomized, placebo-controlled, single-blind crossover study and compared with 12 healthy, nonobese control subjects in which consumption of red orange juice ameliorated endothelial functions, improving flow-mediated dilation and reducing inflammation [7].

The antioxidant activity of orange juices is related not only to structural features of phytochemicals but also to their capability to interact with biomembranes [5]. The quality control of cultivation and characteristic freshness of red oranges have demonstrated their active influence on total antioxidant activity and bioactivity of such fruit. The antioxidant capacity of red orange has been explored in two orange-based products: first, pasteurized pure juice with 40 days of shelf life, and, the second, a sterilized beverage containing minimum 12% of concentrated fruit juice [8]. Results obtained revealed that the antioxidant activity was positively related to the content of anthocyanins and the reduction of their content, typical of commercial long-shelf life juices, leading to a remarkable loss of antioxidant power. Similar results were obtained comparing both the phytochemical content (i.e., phenolics, anthocyanins, and ascorbic acid), total antioxidant activity and *in vitro* bioactivity, in terms of the protective effect obtained against oxidative damage at cellular level with organically and nonorganically grown red oranges in cell culture systems [9]. The organic orange extracts showed a higher total antioxidant activity than non-organic orange extracts due to their higher content of total phenolics, total anthocyanins, and ascorbic acid levels than the corresponding nonorganic oranges.

Red orange intake (especially *Moro* juice) has been found to limit body weight gain, enhance insulin sensitivity, and decrease serum triglycerides and total cholesterol in mice [10, 11]. Dietary *Moro* juice markedly improved liver steatosis by inducing the expression of peroxisome proliferator-activated receptor-α and its target gene acylCoA-oxidase, a key enzyme of lipid oxidation. Consistently, *Moro* juice consumption suppressed the expression of liver X receptor-α and its target gene fatty acid synthase, and restored liver glycerol-3-phosphate acyltransferase 1 activity [10]. This action on fat accumulation

Table 1: Main components of red orange fruit.

Food components	Value
Proximates	
Energy, recalculated, kJ	144
Energy, recalculated, kcal	34
Total protein, g	0.7
Animal protein, g	0.0
Vegetable protein, g	0.7
Total fat, g	0.2
Animal fat, g	0.0
Vegetable fat, g	0.2
Cholesterol, mg	0
Available carbohydrates, g	7.8
Starch, g	0.0
Soluble carbohydrates, g	7.8
Dietary total fibre, g	1.6
Alcohol, g	0.0
Water, g	87.2
Minerals and traces elements	
Iron, mg	0.2
Calcium, mg	49
Sodium, mg	3
Potassium, mg	200
Phosphorus, mg	22
Zinc, mg	0.20
Water soluble vitamins	
Vitamin B1, thiamin, mg	0.06
Vitamin B2, riboflavin, mg	0.05
Vitamin C, mg	50
Niacin, mg	0.20
Vitamin B6, mg	0.10
Total folate, μg	31
Retinol eq., μg	71
Retinol, μg	0
β-Carotene eq., μg	426
Vitamin E, α-tocopherol eq., mg	0.24
Vitamin D, μg	0.00
Fatty Acids	
Saturated fatty acids, g	0.03
Monounsaturated fatty acids, g	0.04
Oleic acid, g	0.03
Polyunsaturated fatty acids, g	0.04
Linoleic acid, g	0.03
Linolenic acid, g	0.01

Source: European Institute of Oncology (EIO) database at http://www.ieo.it/bda2008/homepage.aspx.

has been demonstrated to be mediated by the insulin-like effect of anthocyanins cyanidin-3-O-β-glucoside (C3G) and its metabolite protocatechuic acid (PCA) [12]. However, the *Moro* juice antiobesity effect on fat accumulation cannot be explained only by its anthocyanin content and multiple components present in the red orange juice that might act synergistically to inhibit fat accumulation. Likewise, the anti-inflammatory effects of red orange juice do not depend only on a single component [5]. An experimental study has demonstrated that the intake of a single portion of red orange juice provides an early protection of the mononuclear red cell against oxidative DNA damage, whereas, on the contrary, no subsequent effect of a drink supplemented with the same amount of vitamin C was observed [13]. Thus, the protective effect of red orange juice was not explained by utilization of vitamin C alone. Therefore, a variety of phytochemicals contained in red oranges are assumed to be involved.

4. Components of Red Orange

4.1. Flavonoids. Polyphenols are a group of chemical substances found in plants, especially in the genus *Citrus*, characterized by the presence of more than one phenol unit [14]. The most commonly studied polyphenols are the flavonoids, which include several thousand compounds, characterized by a common benzo-γ-pyrone structure [14]. Flavones, flavonols, anthocyanins and, in greater quantities, flavanones are four types of flavonoids present in *Citrus*, in concentration dependent on age of the plant and directly proportional to its mitotic activity [14]. The relatively large number of flavonoids in *Citrus* juices is a result of the many different combinations that are possible between polyhydroxylated aglycones and a limited number of mono- and disaccharides (Table 2). The most abundant flavonoid species that have been so far identified and quantified in *Citrus sinensis*, regardless of variety, is by far hesperidin, followed by narirutin and didymin [15]. These are all flavanone-O-glycosides, which account for most of the flavonoid content in juice, although a higher content has been found in red orange varieties (*Sanguinello*, *Moro*, and *Tarocco*) compared with nonpigmented variants (*Navel*, *Valencia*, and *Ovale*) [16].

The antioxidant properties of flavonoids protect by oxidative stress induced by both reactive oxygen species (ROS) and reactive nitrogen species (RNS) that have been shown to play a role in initiation and progression of CVD and atherosclerosis (Table 3). Several studies suggest that flavonoids act through several mechanisms on the NO-guanylyl cyclase pathway, endothelium derived hyperpolarizing factor(s), and endothelin-1, protecting endothelial cells inducing vasorelaxation [17]. Other evidence suggests that prevention of endothelial dysfunction, blood pressure, and oxidative stress reduction are the main actions of flavonoids [18, 19]. Their mechanism of action seems to be explained by some *in vitro* studies in which flavonoids interacted with various enzyme systems involved in cellular signaling, such as cyclo-oxygenases and lipoxygenases, phosphodiesterases, tyrosine kinases, and phospholipases [20]. These compounds also protect low-density lipoproteins (LDL) against macrophages-induced oxidation by preventing the generation of lipid hydroperoxides and to preserve R-tocopherol, an endogenous antioxidant carried in lipoproteins [21, 22].

Flavonoids have been demonstrated to be able to inhibit the growth of some tumors, such as colon [23], oral cancer [24], human breast cancer cells [25], lung carcinoma [26], and different melanoma cell lines, in which there have been

Table 2: Main polyphenols contained in red orange juice.

Polyphenol group	Single component	Mean content	min	max	SD
Flavonoids					
Anthocyanins	Cyanidin 3-O-glucoside	1.41 mg/100 mL	0.28	4.03	1.32
	Cyanidin 3-O-(6″-malonyl-glucoside)	1.76 mg/100 mL	0.37	3.86	1.30
Flavanones	Hesperidin	43.61 mg/100 mL	18.00	66.50	17.98
	Narirutin	4.80 mg/100 mL	2.90	6.46	1.28
	Didymin	2.43 mg/100 mL	0.89	3.53	1.27
Flavones	Sinensetin	0.26 mg/100 mL	0.26	0.26	0.00
	Nobiletin	0.31 mg/100 mL	0.31	0.31	0.00
	Tangeretin	0.04 mg/100 mL	0.04	0.04	0.00
Flavonols	3-Methoxynobiletin	0.08 mg/100 mL	0.08	0.08	0.00
Phenolic acids					
Hydroxycinnamic acids	Cinnamoyl glucose	1.50 mg/100 mL	0.41	3.74	1.50
	Cinnamic acid	0.02 mg/100 mL	$7.00e-03$	0.06	0.03
	p-Coumaric acid	2.92 mg/100 mL	1.24	4.46	0.84
	Caffeic acid	0.88 mg/100 mL	0.44	1.51	0.34
	Ferulic acid	4.40 mg/100 mL	3.16	6.37	0.75
	Sinapic acid	1.74 mg/100 mL	1.01	3.59	0.63

Source: phenol-explorer at http://www.phenol-explorer.eu/.

Table 3: Name, effect, and mechanisms of action of main components of red orange.

Food components	Effect	Mechanisms of action
Flavonoid	Anti-inflammatory	Modulate apoB secretion and cellular cholesterol; help cholesterol levels by raising HDL and lowering LDL cholesterol
	Antioxidant	Stimulate endothelial NO synthase; normalize lipid peroxidation markers
	Antiaggregation	Inhibit TxA2-mediated responses and dense granule secretion
	Anticarcinogenic	Promote apoptosis in human pre-B NALM-6 cells and colon cancer cells; inhibit HIF-1α and VEGF expression in ovarian cancer and in lung cancer
	Antiproliferative	Inhibit the COX-2 and MMPs in lung, prostate, and hepatocellular carcinoma cells; inhibited the proliferation of MCF-7 human breast cancer cells and testosterone-induced proliferation of LNCaP cells; inhibit lung colonization by melanoma and sarcoma cell line; inhibit formation of new blood vessels in human breast cancer cells
Anthocyanins	Antioxidant	Protect biomembranes from peroxidation by trapping peroxyl radicals in the cytosol; chelate metal ions like Cu2; ability in chelating metal ions like Cu2; form an ascorbic acid metal-anthocyanin complex (copigment)
	Antimutagenicity	Form a cyanidin-DNA copigmentation complex; inhibit the reverse mutation induced by heterocyclic amines in microsomal activation systems
	Growth inhibition	Inhibit the tyrosine kinase activity of the EGFR and the activation of the GAL4-Elk-1 fusion protein
Carotenoids	Antioxidant	React with singlet molecular oxygen and peroxyl radicals
Vitamin C	Blow-flow increase	Enhance generation of NO; reduce nitrite; stabilize atherosclerotic plaques (due to effect on collagen synthesis)
	Antioxidant	Reduce the affinity of LDL-bound apoB protein for transition metal ions; quench aqueous ROS and RNS, decreasing their bioavailability in the plasma; reduce potentially damaging ROS, forming resonance-stabilized and relatively stable ascorbate free radicals; attenuate LDL-oxidation and protection of human vascular smooth muscle cells against apoptosis
Hydroxycinnamic acids	Antioxidant	Effect on phase II detoxification cascade; inhibit of superoxide dismutase and catalase; suppress of PG synthesis and cyclooxygenase-2
	Anticarcinogenic	Prevent the tumor onset and protect the biochemical and molecular abnormalities in mammary, buccal pouch, colon, and skin cancers

EGFR: epidermal growth-factor receptor; HDL: high-density lipoproteins; HIF-1α: hypoxia-inducible factor 1α; LDL: low-density lipoproteins; MMP: matrix metalloproteinase; NO: nitric oxide; PG: prostaglandins; RNS: reactive nitrogen species; ROS: reactive oxygen species.

demonstrated the antiproliferative effects but not the cytotoxic activity [27, 28]. Several flavonoids actions have been explored as possible mechanisms able to explain their anticarcinogenic effects, such as inhibition of matrix metalloproteinase (MMP) secretion, migration, invasion, and adhesion [29–33], as well as inhibition of the angiogenic process by regulating the expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1α (HIF-1α) [34–36], all factors required by cancer cells to acquire metastatic properties. Some citrus flavonoids have been suggested to have potential health benefits due to their proapoptotic activity on several cancer cell lines, thus inhibiting progression of carcinogenesis [37–40].

4.2. Anthocyanins. Anthocyanins are a group of water-soluble plant compounds responsible for the brilliant color of fruits and flowers [41]. They are glycosylated polyhydroxy and polymethoxy derivatives of flavylium salts. The first experimental observation of anthocyanins in red orange was in 1931 by Matlack that demonstrated their presence in citrus fruits, "the red-fleshed variety, the so-called blood orange" [42] confirming a statement noticed even 15 years before [43]. Fruits subjected to thermal stress produce a greater amount of protective substances (i.e., anthocyanins) necessary to guard against unfavorable environmental conditions. The composition of Anthocyanins can be analyzed as a parameter for the assessment of authenticity and quality of foods rich in anthocyanin pigments [44, 45]. Several studies carried out on red oranges have shown that cyanidin-3-glycoside (C3G) was the main component of the fraction [46]. Some differences in anthocyanins content may occur considering different types of red oranges. Indeed, the primary anthocyanins in *Budd* blood orange, a red-colored blood orange typically grown in Florida, USA, were inverted in content, cyanidin-3-(6″-malonylglucoside) (44.8%) followed by cyanidin-3-glucoside (33.6%). Vegetables and fruits, with special regard to pigmented oranges, such as *Moro*, *Sanguinello,* and *Tarocco* varieties, represent a natural source of anthocyanins, especially cyanidins [2]. Each cultivar shows a characteristic seasonal variation of the content of anthocyanins: the cultivar *Moro* contains the highest amount of anthocyanins, with a maximum peak at the first half of April; cultivars *Tarocco* shows the highest value in the first decade of March; cultivar *Sanguinello Nocellare* and *Sanguinello* show the lowest values of anthocyanins with the maximum values in the end of February and in the first half of March, respectively [41].

The antioxidant properties of anthocyanins, and especially of the C3G, depend on their radical scavenging and inhibitory effects on lipid peroxidation [2, 47] by their strong oxygen radical absorbance capacity (ORAC) and nitric oxide (NO) and cyclooxygenase inhibitory activities [48–50] (Table 3). Given the unstable nature of anthocyanins under natural conditions, it was believed that such molecules would not have antioxidant activity in living systems but many studies, instead, have demonstrated the antioxidant activity of C3G also in *in vivo* experiments [51]. The protection against oxidative damage of anthocyanins was observed as a dose-dependent decrease of ROS-mediated tissue damages after different C3G administration in living systems such as isolated Langendorff-perfused rat hearts subjected to ischemia and reperfusion [52], rat liver, kidneys, and brain [53], rabbit erythrocyte membranes, and rat liver microsomes [54]. In rats maintained on vitamin-E-deficient diets for 12 weeks in order to enhance susceptibility to oxidative damage, consumption of dietary anthocyanins significantly improved plasma antioxidant capacity, decreased the vitamin E deficiency, and enhanced hydroperoxides concentrations in liver [47]. These properties seem to be due to the cyanidin structure that allows the compound to be incorporated into the plasma membrane and cytosol of endothelial cells significantly enhancing their resistance to the damaging effects caused by several ROS-generating systems [55]. The antioxidant activity of orally administered C3G in rats was demonstrated in a model for acute oxidative stress in which C3G significantly suppressed the elevations of the liver and serum thiobarbituric-acid-reactive substance concentrations and the serum activities of marker enzymes for liver injury (GOT, GPT, and LDH) caused by hepatic ischemia-reperfusion treatment [56]. It has been suggested only in recent years that orally administered C3G is absorbed into the circulating system as a free form and subsequently metabolized to protocatechuic acid or peonidin 3-glucoside in the blood and tissues [57], and these compounds act as antioxidants in rats [58]. *In vivo* formation of protocatechuic acid following administration of C3G has been demonstrated in three species (humans, rats, and pigs), although protocatechuic acid is not retrieved in blood in 100% of cases due to differences in the experimental models (namely storage conditions, preanalytical treatments of biological samples and extraction procedure) that may affect its fugitive nature [59]. The bioavailability and biotransformation issues have to be always considered when the health efficacy of compounds from oral administration on target organs is considered. Further research should be focused not only on consequent anthocyanins effects but also on their metabolites, rather than their native forms, that reach tissues and may exert biological effects.

A number of biological activities of anthocyanins aimed at preventing cancer have been addressed [60–62]. The antimutagenic activity was demonstrated by a study on colorectal carcinogenesis inducted by 1,2-dimethylhydrazine (DMH) [63] confirming previous reports in which juice or extracts of plants containing large amounts of anthocyanins acted as inhibitors of heterocyclic amine mutagenesis [64, 65]. C3G also prevented genomic DNA damage in human fibroblast [66], hepatoma-derived cell line (Hep G2) [67], colonic adenocarcinoma (CaCo-2) [68], melanoma [69], and vulva carcinoma A431 [70] cell lines. Finally, experiments on protocatechuic acid have been demonstrated promising curative properties in its use against colon cancer [71–73].

4.3. Carotenoids. Fruits and vegetables are a rich source in this phytochemicals and almost 50 carotenoids can be found in the human diet [74]. The carotenoids that have been most studied in this regard are α-carotene, β-carotene, lycopene, lutein, β-cryptoxanthin, and zeaxanthin, almost all contained in red orange in higher quantities than in other sweet oranges (only *Cara Cara orange* demonstrated a superior contents

of carotenoids compared with *Sanguinello*) [75]. Carotenoids have been implicated as important dietary nutrients having many other biological functions such as antioxidant activity, being involved in the scavenging of free radicals. Moreover, carotenoids react with singlet molecular oxygen and peroxyl radicals generated in the process of lipid peroxidation and they have been shown to protect LDL against oxidation (Table 3) [76].

In addition to their antioxidant properties, carotenoids show an array of biological effects including cardioprotective, antimutagenic and anticarcinogenic activities, involving modulation of signal transduction pathways and induction of gap-junctional communication. Dietary intakes of carotenoids have been associated with decreased risk of coronary artery disease [77], CVD [78, 79], atherosclerosis [80]. Lutein plasma changes have been associated with more promising early outcomes and decreased lipid peroxidation in subjects after ischemic stroke [81, 82], reduced risk of ischemic stroke [83]. By contrast, the risk reductions in cardiovascular events subsequent to high carotenoid intakes have appeared only to a small degree [84] or not confirmed in other studies [85, 86]. During the post-intervention follow-up, dietary supplementation with alpha-tocopherol or beta-carotene has produced neither any benefit nor harm [87]. After an average of four years of supplementation, the combination of beta carotene and vitamin A showed no benefits and may even have had an adverse effect on health, with an increased risk of death cause, lung cancer, and CVD [88]. Finally, evidence resulting from a recent randomized controlled trial on specific antioxidant supplementation was insufficient to prove the effectiveness of each of the vitamin supplements in preventing or treating cardiovascular disease [89]. To date, more information is needed to clarify the relation between the intake of single carotenoids, and the risk of heart diseases.

4.4. Ascorbic Acid (Vitamin C). Vitamin C, or ascorbic acid, serves in humans as a co-factor in several important enzyme reactions and is necessary for the synthesis of collagen [90]. Due to the incapacity to synthesize vitamin C, humans require it from natural sources through supplements to the ordinary diet. Lack of vitamin C results in scurvy, a pathological condition characterized by friable vessels, especially in capillary tissues that are most likely to rupture, and also petechial hemorrhages and ecchymosis due to a deficit of collagen synthesis and secretion to form the extracellular matrix or part of the basement membrane [90]. The vitamin C content of red oranges is in the range of 32 to 42 mg per 100 mL, with the highest levels found in the *Sanguinello* varieties, followed by *Cara Cara* navels and *Moro* (the US recommended daily allowance for vitamin C is set at 75 mg for women and 90 mg for men) [46].

At physiological concentrations, vitamin C is a potent-free radical scavenger in plasma, protecting cells against oxidative damage caused by ROS (Table 3) [91]. The antioxidant property of ascorbic acid is attributed to its ability to reduce potentially damaging ROS, forming, instead, resonance-stabilized and relatively stable ascorbate free radicals [92]. These antioxidant capacities lead to numerous effects of vitamin C on vascular bed, such as induction of endothelial-dependent artery dilation and increase of blood flow [93], attenuation of *in vitro* and *in vivo* LDL-oxidation [94–96], and protection of human vascular smooth muscle cells against apoptosis [97, 98]. In light of the several benefits of vitamin C on endothelial cell proliferation, function, and viability, it is plausible that increases in plasma and cell content of such vitamins might help to prevent, delay, or stabilize early endothelial dysfunction associated with atherosclerosis.

Several studies hypothesized that the anti-inflammatory properties of Vitamin C may reduce the incidence of many malignancies in humans due to a number of cytoprotective functions under physiological conditions, including prevention of DNA mutation induced by oxidation by neutralizing potentially mutagenic ROS [99–102]. Indeed, consumption of vitamin-C-rich foods has been found to be inversely related to the level of oxidative DNA damage *in vivo* [103]. However, the inconsistency of the vitamin C cancer correlation and lack of validated mechanistic basis for its therapeutic action underline its potential role as a preventive rather than therapeutic drug.

4.5. Hydroxycinnamic Acids. Hydroxycinnamic acids (hydroxycinnamates) are a class of polyphenols having a C6-C3 skeleton. These compounds are hydroxy derivatives of cinnamic acid. In the category of phytochemicals that can be found in red orange, the most common are caffeic, p-coumaric, ferulic, and sinapic [104]. Free and bound ferulic acid represent the major component in all cases, followed by p-coumaric acid, sinapic acid, and caffeic acid. However, hydroxycinnamic acids have been found to be more abundant in red orange than in blond juices. Ferulic acids and caffeic acid are among the most studied hydroxycinnamic acids (Table 3).

Ferulic acid is an abundant phenolic phytochemical found in plant cell wall components such as arabinoxylans as is found, for instance, in covalent side chains. It is related to *trans*-cinnamic acid. As a component of lignin, ferulic acid is a precursor in the manufacture of other aromatic compounds. It has been demonstrated to be successfully employed as topical protective agents against UV radiation-induced skin damage after *in vitro* and *in vivo* evaluation [105], often in combination with other antioxidants such caffeic acid [106] and vitamin C [107]. Its mechanisms of action may depend on preventing DNA damage and restoring antioxidant status and histopathological changes [108]. The anticarcinogenic properties of ferulic acid have been studied on mammary [109], buccal pouch [110], colon [111], and skin [112] carcinogenesis in experimental models findings showing that oral administration of ferulic acid significantly prevented the onset of tumors and the biochemical and molecular abnormalities. Although the exact chemopreventive mechanism of the ferulic acid is unclear, its antigenotoxic and antioxidant potentials as well as modulatory effect on phase II detoxification cascade could play a possible role [109].

Caffeic acid is an organic compound that is classified as a hydroxycinnamic acid. This acid consists of both phenolic and acrylic functional groups. It is found in all plants because

it is a key intermediate in the biosynthesis of lignin, one of the principal sources of biomass [113]. The caffeic acid phenethyl ester has been found to be a potent free radical scavenger [114] and studied for its antioxidant capacities in several experimental rat models on renal impairment [115–117], retinal oxidative stress [118], myocardial oxidative stress [119], age-related vascular remodeling and cardiac damage [120], and at the same time helping to prevent the metabolic consequences in diabetes mellitus [121, 122], erythrocyte membrane ischemia/reperfusion injury [123], cerebral damage induced by ischemia reperfusion [124], and oxidative stress [125]. The mechanisms by which caffeic acid phenethyl ester exerts its anti-inflammatory action seems to depend on its effect on lipid peroxidation (LPO) and the inhibition of antioxidant enzymes such as superoxide dismutase and catalase [126]. Moreover, caffeic acid phenethyl ester has also been shown to cause dose-dependent suppression of prostaglandins synthesis suppressing the expression of cyclooxygenase-2 in cultured human oral epithelial cells and in an animal model of acute inflammation [127] and in the protection of mice from lethal endotoxin shock. It also inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible NO synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-κB pathways, therefore providing mechanistic insights into the anti-inflammatory and chemopreventive actions of caffeic acid phenethyl ester in macrophages [128].

5. Conclusions

On the basis of these findings, evidence has shown that red oranges demonstrate both potent antioxidant activity and also cytoprotective effects that reflect their substantial role in preventing chronic pathological conditions such as cardiovascular diseases and in many forms of cancers. A synergic action between organic farming and social activities may amplify the advantages and reciprocal benefits in order to obtain a "social and environmental sustainability" and spread consumption of healthy products [129, 130]. The supply of natural antioxidant compounds through a balanced diet rich in red oranges might provide protection against oxidative damage under different conditions and could be more effective than supplementation of an individual antioxidant.

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Review Article

Site-Specific Antioxidative Therapy for Prevention of Atherosclerosis and Cardiovascular Disease

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Oxidative stress has been implicated in pathophysiology of aging and age-associated disease. Antioxidative medicine has become a practice for prevention of atherosclerosis. However, limited success in preventing cardiovascular disease (CVD) in individuals with atherosclerosis using general antioxidants has prompted us to develop a novel antioxidative strategy to prevent atherosclerosis. Reducing visceral adipose tissue by calorie restriction (CR) and regular endurance exercise represents a causative therapy for ameliorating oxidative stress. Some of the recently emerging drugs used for the treatment of CVD may be assigned as site-specific antioxidants. CR and exercise mimetic agents are the choice for individuals who are difficult to continue CR and exercise. Better understanding of molecular and cellular biology of redox signaling will pave the way for more effective antioxidative medicine for prevention of CVD and prolongation of healthy life span.

1. Introduction

Oxidative stress has been implicated in pathophysiology of aging and age-associated disease. Reactive oxygen species (ROS) have been considered as harmful molecules for organisms by destroying DNA and cell structures, thereby deteriorating multiple organ function, leading to aging. However, recently emerging paradigm reinforces a novel role of ROS as a messenger of redox signaling that modulates aging processes [1]. Perturbation of the redox signaling due to oxidative stress deteriorates endothelial function and promotes atherosclerosis. Thus, elucidation of the origin of ROS and the mechanism of ROS generation in endothelial cells are pivotal to develop effective strategies for prevention of atherosclerosis, aging, and cardiovascular disease (CVD).

The major origins of ROS in endothelial cells are mitochondrial electron transfer chain (ETC), NADPH oxidase (Nox), endothelial nitric oxide synthase (eNOS), and xanthine oxidase (XO). Mitochondrial ETC inevitably generates ROS associated with oxidative phosphorylation and energy production. The amount of ROS production by mitochondria increases with age and under certain pathophysiological conditions such as excessive food intake and sedative lifestyle [2, 3]. Besides this intrinsic mechanism of oxidative stress, there are extrinsic mechanisms of oxidative stress that enhances ROS generation by stimulating ROS generating machinery within endothelial cells. Endothelial Nox activity is known to be increased by proinflammatory cytokines [4]. Visceral adipose tissue is a main source of proinflammatory cytokines such as tumor necrosis factor-α and interleukin-6 in individuals with abdominal obesity [5, 6]. Proinflammatory adipocytokines contribute to ROS generation and endothelial dysfunction through upregulation of Nox, leading to insulin resistance or type 2 diabetes (DM), hypertension, and a variety of CVDs. Local activation of renin-angiotensin system (RAS) also contributes to the enhanced expression and activation of Nox [7, 8]. Nox-derived ROS then promotes uncoupling of eNOS and exaggerates oxidative stress and endothelial dysfunction [9–11]. Endothelial dysfunction by any causes including cigarette smoking, stimulation with angiotensin II, or inflammatory cytokines results in activation of xanthine oxidase and further production of ROS [12, 13]. Thus, once ROS generation overwhelms the antioxidative capacity, oxidative stress propagates by creating a self-perpetuating cycle and accelerates endothelial dysfunction and atherosclerosis.

Extensive efforts have been exerted to ameliorate oxidative stress in the cardiovascular system especially to endothelial cells by general antioxidants. However, these antioxidants have conferred only limited success to prevent CVD. On the other hand, a growing body of evidence suggests that the site-specific blockade of ROS production might represent an alternative strategy to prevent atherosclerosis and CVD. The present review will discuss the issue as to why general antioxidants have failed to provide appreciable antiatherosclerotic effects, and how the site-specific antioxidative therapy exerts beneficial effects on the cardiovascular system.

2. Effects of General Antioxidants on Cardiovascular Disease

The use of general antioxidants has become a common practice for prevention of CVD and age-associated disease. However, there are as yet no clinical indications for the routine use of antioxidants for treatment of these diseases. This is because no appreciable benefits have been demonstrated in multiple clinical trials that employed general antioxidants. For example, a large trial of vitamin E and β-carotene failed to show any protective cardiovascular effects when smokers with acute myocardial infarction (MI) were treated for a long term with these agents [14]. Conversely, this clinical study revealed that the risk of fatal CVD increased in the groups that received either β-carotene or the combination of vitamin E and β-carotene. Some explanations have been offered to give an insight into the failure of clinical studies using general antioxidant therapies. It may be argued that more than one antioxidant is required for clinical effectiveness. This idea is based on the assumption that antioxidants exist as a "network," wherein both lipid soluble (like vitamin E) and water soluble (ascorbate, glutathione, and dihydrolipoic acid) molecules work in a network for the removal of oxidative stress in conjunction with the regeneration of oxidant defenses. To date clinical trials have generally not used synergistic combinations of lipid soluble and water soluble antioxidants despite the theoretical advantages and basic science demonstrations of effectiveness of "antioxidant cocktail." Another explanation for the failure of clinical antioxidant studies is that antioxidants can act as prooxidants by retaining high energy electrons when scavenging ROS or reducing iron. It is also possible that antioxidants or by-products may impair mitochondrial oxidative phosphorylation and ATP production. For example, the β-carotene cleavage products have been shown to strongly inhibit state-3 respiration in isolated liver mitochondria [15]. Therefore, it is desirable for antioxidants to scavenge ROS without unfavorable side effects to effectively eliminate oxidative stress.

Astaxanthin, a xanthophyll carotenoid, which is contained in a variety of seafoods such as crabs, crayfish, krill, lobsters, salmon, shrimp, and trout, may represent a novel class of general antioxidant. Unlike other general antioxidants, astaxanthin is not converted into a prooxidant when scavenging ROS. Astaxanthin neutralizes ROS or other oxidants by either accepting or donating electrons, and without being destroyed or becoming a prooxidant in the process, because its linear, polar-nonpolar-polar molecular layout equips it to precisely insert into the membrane and span its entire width. In its position spanning the membrane, astaxanthin can intercept reactive molecular species within the membrane's hydrophobic interior and along its hydrophilic boundaries, thereby providing versatile antioxidant actions.

Diverse benefits of astaxanthin in the clinical arena, with excellent safety and tolerability, have been extensively reviewed [16]. It appears that astaxanthin clinical success extends beyond protection against oxidative stress and inflammation to promise for slowing age-related functional decline. Nevertheless, larger sized randomized controlled trials for subjects with lifestyle-related diseases are required to address a question whether astaxanthin prevents CVD and the related mortality, which are the most important clinical end point.

3. Effects of General Antioxidants on Ischemic Tolerance

The use of general antioxidants may not be an ideal choice for treatment of CVD especially in subjects at high risk of MI or cerebral infarction. Indiscriminate removal of ROS may reduce the tolerance to hypoxic or ischemic stress, which may worsen the prognosis of heart attack. It is known that ROS is necessary for acquisition of tolerance to lethal ischemic insult. The most representative example of this phenomenon is ischemic preconditioning (IPC). IPC was first discovered by Murry and associates [17] who demonstrated that a brief period of repetitive cardiac ischemia/reperfusion exerts a protective effect against subsequent lethal periods of ischemia. It is now evident that IPC has two distinct phases: an early phase, which lasts from a few minutes to 2-3 hours and a late phase, termed late IPC, which develops after 12 hours, peaks between 24 and 48 hours, and lasts for 72–96 hours [18]. Recent advances in preconditioning research at molecular and cellular levels suggest that cardioprotective signal transduction in early IPC proceeds through a self-perpetuating cycle of redox signaling including activation of protein kinase C-ε and phosphatidylinositol-3 kinase which culminates in protection of mitochondria against ROS- and Ca^{2+}-induced opening of mitochondrial permeability transition pore through activation of mitochondrial K_{ATP} channels and inhibition of glycogen synthase kinase-3β [19]. Positive feedback and feedforward amplification of redox signaling induced by activation of mitochondrial K_{ATP} channels plays a crucial role in developing the memory of cardioprotection that converges on mitochondria.

ROS generated during brief ischemia and reperfusion cycles have been consistently implicated in the trigger of IPC [20–22]. In contrast to detrimental effects by massive generation of ROS, sublethal amounts of ROS could serve as a trigger of IPC. Because IPC is implemented by pretreatment with single or multiple brief periods (<10 min) of ischemia and reperfusion prior to more prolonged and potentially lethal period of ischemia, it is conceivable that IPC procedures generate relatively small amounts of ROS compared with a lethal period of ischemia and reperfusion.

Such ROS production could function as a messenger of signaling cascades to protect against lethal oxidative stress induced by a subsequent prolonged period of ischemia and reperfusion by inhibiting robust increase in ROS generation in mitochondria [23]. In addition, redox signaling may be a universal feature of cardiomyocyte response to all forms of stress such as hyperthermia and a mechanism for acquisition of ischemic tolerance [24]. Therefore, antioxidant medicine must be more site specific, being targeted to a specific ROS or cellular compartment, without a deleterious effect on favorable redox-sensitive signaling pathways.

4. Site-Specific Inhibition of NADPH Oxidase

4.1. Blockers of the Renin-Angiotensin System. Nox is a major ROS generating enzyme in the cardiovascular system. It has been demonstrated that Nox-derived ROS plays a physiological role in the regulation of endothelial function and vascular tone and a pathophysiological role in atherosclerosis and angiogenesis. There are seven homologs of Nox (Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2) [25]. Nox2 also known as $gp91^{phox}$ was originally found in phagocytes such as neutrophils and macrophages. Nox2 plays a crucial role in host defense against microorganisms by releasing ROS upon the respiratory burst. The absence of Nox2 gene causes repetitive infections and premature death known as chronic granulomatous disease at an early stage of life [26]. Thus, indiscriminate inhibition of Nox2 abrogates bactericidal capacity of the phagocytes. Nox2 is inducible in cardiovascular systems such as in endothelial cells, vascular smooth muscle cells (SMCs), and cardiomyocytes by stimulation with inflammatory cytokines and angiotensin II [27, 28]. Nox1, which is the first homolog of Nox2, is also activated in endothelial cells under the pathological conditions that lead to atherosclerosis [29]. Conversely, Ray et al. [30] demonstrated that, unlike Nox1 or Nox2, Nox4 is constitutively activated in endothelial cells without stimulation with inflammatory cytokines or angiotensin II and acts as a vasodilator through H_2O_2-induced hyperpolarization of endothelial cells. These findings indicate that the use of nonselective Nox inhibitors is not suitable for ameliorating oxidative stress in the cardiovascular system and provides a basis for employing specific inhibitors that block only inducible Nox.

Some of the recently emerging drugs for CVD possess a property to inhibit inducible Nox as a preferable side effect. Angiotensin converting enzyme inhibitors (ACEs) and angiotensin II type 1 (AT1) receptor blockers (ARBs) are such a class of antihypertensive drugs. A growing body of evidence suggests that enhanced activation of the RAS is a key to promote endothelial dysfunction and hypertension [31, 32].

Nox plays a crucial role in the RAS-mediated development of atherosclerosis. Nox-derived ROS oxidizes LDL that is incorporated into the intima of the blood vessel. Oxidized LDL is then taken up by macrophages via their scavenger receptors CD36 to form foam cells. Macrophage recruitment to the intima is likely to be regulated not only by a multiplicity of adhesion molecules, integrins, and selectins, but also by chemokines such as macrophage chemoattractant protein-1, which is constitutively synthesized and secreted by endothelial cells and SMCs migrated from the media [33, 34]. Transcriptional upregulation of these molecules is enhanced by ROS, which are derived at least in part from Nox in endothelial cells and SMCs, creating a self-amplifying loop in foam cell accumulation and atherosclerotic plaque formation.

Local activation of the RAS in the myocardium induced by hypertension and after myocardial infarction plays a crucial role in pathological remodeling of the heart. The RAS is activated in response to mechanical or hypoxic stress to cardiomyocytes under these conditions. AT1 receptor stimulation induces oxidative stress on cardiomyocytes by activating Nox, which triggers cardiomyocyte hypertrophy by stimulating hypertrophy signaling such as protein kinase C and ERK [35, 36]. AT1 receptors are also known to activate transforming growth factor-β signaling and promote interstitial fibrosis [37]. Cardiomyocyte hypertrophy and interstitial fibrosis are the two important events for transition to heart failure from physiological adaptation against pressure overload to the heart.

There is another reason for the preferential use of ACE inhibitors and ARBs for hypertensive subjects with visceral obesity. Adipocytes in the visceral adipose tissue are a suggested source of components of the RAS, with regulation of their production related to obesity-induced hypertension. Angiotensin II has been demonstrated to promote oxidative stress via overexpression of Nox in adipocytes. It has been demonstrated that blockade of AT1 receptors reduces oxidative stress in adipose tissue and ameliorates production of proinflammatory cytokines responsible for systemic inflammation and oxidative stress [38]. Thus, ACEs and ARBs are capable of inhibiting Nox which is upregulated in the cardiovascular system and the visceral adipose tissue by local activation of the RAS. Importantly, ACEs and ARBs do not affect Nox activity in cells without activation of the RAS.

4.2. Inhibitors of HMG-CoA Reductase. Because atherosclerosis is facilitated by dyslipidemia in conjunction with oxidative stress, numerous studies have investigated relative contribution of dyslipidemia and oxidative stress to atherogenesis. It has been demonstrated that a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor lovastatin significantly reduced total cholesterol, selectively decreased non-HDL-cholesterol, and significantly reduced fatty streak lesion formation in the aortic arch of the diabetic hamsters fed the atherogenic diet [39]. In this study, while vitamin E and probucol were effective in reducing several indices of oxidative stress including plasma lipid peroxides, cholesterol oxidation products, and in vitro LDL oxidation, they had no effect on fatty streak lesion formation. It appears that in this combined model of type 2 DM and hypercholesterolemia, lovastatin prevented progression of fatty streak lesion formation by reducing total cholesterol and non-HDL-cholesterol and inhibiting oxidative stress.

The pleiotropic effects of statins that prevent oxidative stress and atherosclerosis have been extensively investigated. Emerging evidence suggests that these cholesterol-independent effects are predominantly due to their ability to inhibit isoprenoid synthesis, particularly geranylgeranylpyrophosphate and farnesylpyrophosphate, which are important posttranslational lipid attachments of the Rho GTPases and activation of its downstream target, Rho-kinase (ROCK) [40]. Angiotensin II-induced activation of Nox requires Rho GTPase association with Nox [41, 42]. Inhibition of ROCK by statins may also be associated with inhibition of oxidative stress mediated by activation of Nox. It has been shown that rosuvastatin attenuated the angiotensin II-mediated upregulation of Nox subunits associated with downregulation of AT1 receptors and the lectin-like oxidized LDL receptor LOX-1, leading to the reduction of oxidized LDL [43]. In this regard, improvement of endothelial dysfunction, cerebral ischemia and coronary vasospasms by statin inhibition of ROCK is also attributed to upregulation of eNOS, which decreases vascular inflammation and reduces atherosclerotic plaque formation [44].

4.3. Exercise. A number of studies show that increased physical activity reduces oxidative stress. The mechanism underlying reduced oxidative stress by exercise may be related to the decreased expression of Nox in the visceral adipose tissue associated with the prevention of dysregulated production of inflammation-related adipocytokines, suggesting that exercise is a fundamental approach to protect against cardiovascular risk. Szostak and Laurant [45] have proposed that exercise promotes atheroprotection possibly by reducing or preventing oxidative stress and inflammation through at least two distinct mechanisms. Exercise, through laminar shear stress activation, downregulates endothelial AT1 receptor expression, leading to decreases in Nox activity and ROS generation, and preserves endothelial NO bioavailability and its protective antiatherogenic effects. These observations suggest that lifestyle modification can prevent Nox activation and cardiovascular oxidative stress without relying on ARBs, ACEs, or statins.

5. Site-Specific Inhibition of Xanthine Oxidase

A substantial body of experimental and epidemiological evidence suggests that serum uric acid is an important and independent risk factor for cardiovascular and renal disease, especially in patients with heart failure, DM, and hypertension [46]. The level of uric acid in the blood is increased with age, body mass index, and hypertension [47]. Hyperuricemia is caused by excessive intake of dietary purine or ethanol, activation of xanthine oxidase (XO), and decreased renal excretion of uric acid. Of these, XO, which metabolizes xanthine as a substrate, is intimately related to endothelial dysfunction. XO is converted from xanthine dehydrogenase in endothelial cells by oxidative stress or stimulation with inflammatory cytokines or angiotensin II [48].

A large body of evidence supports an important role for XO-mediated ROS generation in atherosclerosis [49–51]. Landmesser et al. [52] demonstrated that angiotensin II substantially increased endothelial XO protein levels and XO-dependent superoxide production in cultured endothelial cells, which was prevented by Nox inhibition. Atherosclerosis is prone to be produced in the bifurcation of blood vessels associated with oscillatory shear stress. It has been demonstrated that conversion of XO from xanthine dehydrogenase and resultant ROS production are increased by oscillatory shear stress in an Nox-dependent manner in endothelial cells [53]. These observations suggest that conversion of XO from xanthine dehydrogenase is dependent on angiotensin II-mediated activation of Nox in endothelial cells, and XO plays a crucial role in angiotensin II-induced development of atherosclerosis. XO-derived ROS is also involved in SMC proliferation and death, which are the important steps for atherosclerosis. Interestingly, a single exposure of cultured rat vascular SMCs to XO/xanthine predominantly resulted in cell proliferation, whereas frequent exposures to high levels of XO/xanthine predominantly resulted in cell death by apoptosis [54]. Thus, inhibition of XO is a promising approach to prevent atherosclerosis.

XO mediates tissue injury during reperfusion typically encountered in the heart after percutaneous coronary interventions for acute MI or open-heart surgery. It has been demonstrated that XO in the heart is activated by tumor necrosis factor (TNF)-α, which is upregulated after ischemia/reperfusion, and administration of anti-TNF-α antibodies at the onset of reperfusion partially restores nitric oxide- (NO-) mediated coronary arteriolar dilation and reduces superoxide production [55, 56]. These studies suggest that anticytokine therapy after reperfusion may be effective in ameliorating reperfusion injury by preventing activation of XO. However, the effect of anti-TNF-α antibodies against ischemia/reperfusion injury remains controversial, presumably by the fact that TNF-α also mediates cardioprotective signal transduction as observed in IPC [57].

Allopurinol and its active metabolite oxypurinol are the representative inhibitors of XO. Inhibition of XO by allopurinol is known to prevent atherosclerosis. It has been demonstrated that allopurinol reduces neointimal hyperplasia in the carotid artery ligation model in spontaneously hypertensive rats [58]. In addition, oral administration of allopurinol to ApoE knockout mice markedly ameliorated oxidized LDL accumulation and calcification in the aorta and aortic root [59]. The antiatherosclerotic effect of allopurinol has also been reported in clinical studies for patients with a variety of cardiovascular risk factors. Long-term and high-dose allopurinol therapy significantly improved endothelial function in diabetic normotensive patients [60]. Allopurinol reversed endothelial dysfunction in smokers [13]. Furthermore, there is evidence that allopurinol improves endothelium-dependent vascular function in patients with chronic heart failure [61].

It has been established that allopurinol protects reperfusion injury of the heart. Manning et al. [62] for the first time demonstrated that reperfusion-induced arrhythmias and infarct size are reduced by allopurinol in rats. Similar

findings were reported by a number of other investigators using allopurinol or oxypurinol in diverse animal models such as rat, rabbit, dog, and pig [46]. The mechanism of allopurinol prevention of myocardial reperfusion injury seems to be provoked by protection of endothelial cells from oxidative stress. Gao et al. [56] demonstrated that administration of allopurinol at the time of reperfusion reduced superoxide production and ameliorated coronary endothelial dysfunction. Taken together, these findings suggest that specific inhibition of XO confers protection against ischemia/reperfusion injury.

6. Site-Specific Inhibition of Nitric Oxide Synthase Uncoupling

NO plays a central role in the regulation of cardiovascular function. NO is one of gaseous signaling molecules, which were previously considered to be toxic. However, the identification of NO as the endothelium-derived relaxing factor combined with the discovery of NO generation by NOS primed an explosion of research in this area in the 1990s [63–65]. It is now evident that NO and cognate reactive nitrogen intermediates are involved in a wide variety of pathophysiological processes in the cardiovascular system where it orchestrates a plethora of cellular activities in cardiomyocytes, endothelial cells, vascular SMCs, and circulating inflammatory cells.

Biosynthesis of NO is dependent on enzymatic activity of NOS. NOS is a homodimeric oxidoreductase containing iron protoporphyrin IX (heme), flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin (BH_4) which is a cofactor essential for the catalytic activity of all three NOS isoforms [66, 67]. The flavin-containing reductase domain and a heme-containing oxygenase domain are connected by a regulatory calmodulin-binding domain. In the case of constitutive NOS, that is, nNOS and eNOS, binding of Ca^{2+}/calmodulin orients the other domains to allow NADPH-derived electrons generated in the reductase domain to flow to the oxygenase domain [68], ultimately resulting in the conversion of L-arginine to NO and L-citrulline. This occurs if BH_4 is bound in the dimer interface, where it interacts with amino acid residues from both monomers to stabilize NOS dimerization and participate in arginine oxidation through the N-hydroxyl-L-arginine intermediate and the subsequent generation of NO. BH_4 depletion because of its oxidation and/or reduced synthesis can result in functional uncoupling of NOS. Uncoupled NOS generates more ROS and less NO, shifting the nitroso-redox balance and having adverse consequences on the cardiovascular system [10]. Thus, modulation of the arginine-NO pathway by BH_4 may be beneficial for prevention of CVD.

BH_4 is highly sensitive to oxidation by ROS and peroxynitrite and is converted to dihydrobiopterin (BH_2). Oxidative stress imposed on endothelial cells causes depletion of BH_4 and eNOS uncoupling. The effect of eNOS uncoupling has been investigated in a wide variety of in vitro models, animal models of CVD, and human subjects with cardiovascular risk factors [10, 69, 70]. Inhibition of oxidative stress to endothelial cells and subsequent occurrence of NOS uncoupling provide an ameliorative effect on endothelial function. Therefore, any interventions against oxidative stress to endothelial cells such as lifestyle modification, especially calorie restriction and endurance exercise, or any pharmacological tools such as the use of ARBs, statins, and xanthine oxidase inhibitors, may represent a promising approach to the prevention of NOS uncoupling.

In many cases, supplementation with BH_4 under pathological conditions with oxidative stress has been shown to reverse eNOS dysfunction. However, true mechanistic relationship between endothelial BH_4 levels and eNOS regulation in vivo by administration of BH_4 remains controversial. High extracellular BH_4 concentrations may result in nonspecific antioxidant effects that indirectly increase NO bioactivity by scavenging ROS rather than by modulating eNOS activity. Furthermore, the effects of supplementation with BH_4 or biopterin analogues on NO bioactivity are unpredictable in vascular disease states in which oxidative stress is increased [71, 72]. Indeed, it remains unclear whether adequate eNOS cofactor function in vivo is related to absolute BH_4 levels in the endothelial cell, or whether the relative balance between reduced BH4 and oxidized BH_2 may be more important [73].

Intracellular BH_4 levels are regulated by the activity of the de novo biosynthetic pathway and the salvage pathway. In the de novo biosynthetic pathway guanosine triphosphate cyclohydrolase (GTPCH)-1 catalyzes GTP to dihydroneopterin triphosphate. BH_4 is generated by further steps catalyzed by 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase. GTPCH-1 appears to be the rate-limiting enzyme in BH_4 biosynthesis; transgenic overexpression of GTPCH-1 is sufficient to augment BH_4 levels in endothelial cells and preserve NO-mediated endothelial function in diabetic mice. In the salvage pathway, BH_4 is synthesized from BH_2 by sepiapterin reductase and dihydrofolate reductase [74]. Exogenous BH_4 is labile in physiological solution. It has been reported that in vivo half-life of BH_4 is 3.3–5.1 hour in the plasma of healthy adult humans [75]. Because not all oxidized BH_4 is converted to BH_2, which is further degraded to dihydroxanthopterin and excreted to urine [76], BH_2 availability for the salvage pathway may be limited under oxidative stress even with BH_4 supplementation. Thus, sepiapterin may serve as an effective substrate for BH_4 via the salvage pathway. Folic acid and vitamin C are also able to restore eNOS functionality, most probably by enhancing BH_4 levels through mechanisms yet to be clarified [76].

The effect of BH_4 on CVD has been investigated in various animal models and in human subjects. BH_4 ameliorated endothelial dysfunction and reversed hypoadiponectinemia as a result of oxidative stress in rats [77]. In addition, intramuscular GTPCH-1 gene transfer using atelocollagen was found to serve as a useful method of long-term systemic delivery of BH_4 and the treatment of endothelial dysfunction in insulin-resistant rats [76, 78]. The therapeutic efficacy of BH_4 has been examined in patients with hypertension, peripheral arterial disease, and coronary artery disease, and these studies consistently demonstrated the beneficial effect of BH_4 on endothelial dysfunction [79]. BH_4 also

improved endothelium-dependent vasodilation in chronic smokers [80]. However, a phase 2 clinical trial sponsored by the US pharmaceutical company BioMarin failed to observe an ameliorative effect of oral administration of BH_4 in patients with poorly controlled hypertension. This finding is not surprising, because chronic exposure to NO causes nitrate tolerance through *S*-nitrosylation and desensitization of soluble guanylyl cyclase, an enzyme that generates cGMP which is largely attributed to the vasodilating effect of NO [81]. Although long-lasting treatment with BH_4 may not be effective in ameliorating hypertension through an NO/cGMP-dependent mechanism, recent studies suggest that an NO-dependent *S*-nitrosylation plays a key role in posttranscriptional modification of the variety of key proteins involved in cardiac contractile function and antiarrhythmias, angiogenesis, and protection against ischemia/reperfusion injury [82–86]. These reports lend support to the hypothesis that supplementation with BH_4 may represent a promising therapeutic strategy for heart failure and ischemic heart disease. Further studies are warranted to address whether BH_4 or its precursors truly exert salutary effects on endothelial dysfunction in a variety of CVD.

7. Strategies to Prevent Mitochondrial Generation of ROS

7.1. Stimulation of Mitochondrial Biogenesis. Mitochondria are the critical component in control of aging. Mitochondrial dysfunction and increased generation of ROS are a prominent feature of aging and various age-related neurodegenerative diseases such as Parkinson's disease and Alzheimer disease [87]. Mitochondrial dysfunction has also been implicated in CVD [88]. The heart has the highest oxygen uptake in the human body, and accordingly it has a large number of mitochondria. This high dependence on mitochondrial metabolism has its costs: when oxygen supply is threatened, high leak of electrons from the ETC leads to oxidative stress and mitochondrial failure. Indeed, it is estimated that, of the oxygen consumed by mammalian cells, 0.2–2% of it is converted to ROS, and most of the ROS have mitochondrial origins [89, 90].

Mitochondrial generation of ROS is increased with age due to a decrease in electron transport function especially at the site of complex I in the ETC [91]. This leads to stagnation of electrons in the ETC and increases leakage of electrons that react with oxygen to generate superoxide. Stagnation of electrons is augmented by increased influx of electrons to the ETC due to excessive food intake or reduced physical activity [92, 93]. The elevation of ATP/ADP ratio by excessive food intake or reduced physical activity also enhances stagnation of electrons and the generation of ROS in the ETC by decreasing the passage of protons through ATP synthase [94].

Improvement of ETC function may be the most effective approach to reduce the production of ROS in mitochondria. An increase in mitochondrial biogenesis has been shown to improve ETC function and reduce ROS production. A growing body of evidence suggests that AMP-activated protein kinase (AMPK) plays a pivotal role in mitochondrial biogenesis [95]. AMPK acts as a cellular energy sensor that is activated by a decrease in ATP/AMP ratio [96]. The decrease in ATP/AMP ratio is most commonly induced by calorie restriction (CR) and endurance exercise. CR and endurance exercise have advantageous effects on blood pressure level in humans and also beneficially influence blood lipid profile and glucose homeostasis in individuals displaying features of metabolic syndrome [97]. Thus, CR and endurance exercise may enhance mitochondrial biogenesis through common signaling pathways. The AMPK-mediated increase in mitochondrial biogenesis occurs through the activation of SIRT1, which can mimic several metabolic aspects of CR by targeting selective nutrient utilization and mitochondrial oxidative function to regulate energy balance [98]. SIRT1 then activates peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α through deacetylation reaction. PGC-1α is an end target of events by which AMPK promotes mitochondrial biogenesis. Valle et al. [99] have demonstrated that endothelial cells that overexpress PGC-1α show reduced accumulation of ROS, increased mitochondrial membrane potential, and reduced apoptotic cell death both in basal and oxidative stress conditions.

The mechanism by which AMPK upregulates PGC-1α appears to be triggered by mitochondrial generation of ROS. Irrcher et al. [100] demonstrated that AMPK activation in the presence of H_2O_2 increased PGC-1α promoter activity with concomitant increases in mRNA expression. This observation suggests that mitochondria-derived ROS is necessary for AMPK and SIRT1 to upregulate PGC-1α that feedbacks to inhibit mitochondrial generation of ROS by increasing mitochondrial biogenesis. Thus, AMPK upregulation of PGC-1α is another example for redox signaling-dependent protection of mitochondria.

eNOS-derived NO and AMPK synergistically increase the activity of PGC-1α. NO induces mitochondrial biogenesis in skeletal muscle cells via upregulation of PGC-1α [101]. Similar effects of NO on PGC-1α and mitochondrial biogenesis were also observed in fibroblasts and adipocytes [102]. Thus, NO-induced maintenance of metabolic function and prevention of oxidative stress is at least in part mediated by AMPK and PGC-1α.

Nuclear respiratory factor (NRF) is another redox-sensitive transcriptional factor that is activated in the presence of ROS derived from mitochondria [103, 104]. Mitochondrial biogenesis depends on the interplay between NRF and PGC-1α [105]. NRF is, therefore, an emerging target to counteract mitochondrial dysfunction and ROS generation. NRF mediates the biogenomic coordination between nuclear and mitochondrial genomes by directly regulating the expression of several nuclear-encoded ETC proteins through nuclear mitochondrial interactions with PGC-1α [106]. Taken together, these findings suggest complex interplay between AMPK, SIRT1, PGC-1α, and NRF under the redox regulation upon CR and endurance exercise.

Because the long-term adoption of a CR lifestyle is not realistic in a significant proportion of the human population, the search for substances that can reproduce the beneficial physiologic responses of CR without a requisite calorie intake reduction, termed CR mimetic agents, have gained

momentum. Of these, CR mimetics that activate NRF and PGC-1α, thereby promoting mitochondrial biogenesis and ameliorating oxidative stress, may represent a promising pharmacological tool to prevent atherosclerosis and CVD.

Epidemiological studies suggest that the consumption of wine, particularly of red wine, reduces the incidence of mortality and morbidity from coronary heart disease. This has given rise to what is now popularly termed the "French paradox" [107]. The cardioprotective effect of red wine has been attributed to antioxidants present in the polyphenol fraction. Grapes contain a variety of antioxidants, including resveratrol, catechin, epicatechin, and proanthocyanidins. Of these, recent data provide interesting insights into the effect of resveratrol on the life span of simple eukaryotes such as yeast and flies by activating the longevity genes such as AMPK, SIRT1, PGC-1α, and NRF and has been suggested as a CR mimetic [108–111].

Emerging evidence indicates that resveratrol increases mitochondrial biogenesis and reduces oxidative stress in a wide variety of age-associated disease models. It has been demonstrated that resveratrol induces mitochondrial biogenesis and ameliorates angiotensin II-induced cardiac remodeling through the activation of SIRT1, PGC-1α, and NRF [112]. Csiszar et al. [113] demonstrated that resveratrol induced activation of PGC-1α and NRF, increased mitochondrial mass and mitochondrial DNA content, and upregulated protein expression of the ETC constituents in endothelial cells. Mitochondrial biogenesis induced by resveratrol treatment also has important impact on the liver and brain functions and seems to have significant consequences in the skeletal muscle, because resveratrol-treated mice exhibit an approximately twofold increase in endurance exercise [114]. Future studies are warranted to investigate whether resveratrol or specific activators of AMPK, SIRT1, PGC-1α, or NRF each alone or in combination can ameliorate age-associated diseases and prolong healthy life span in human subjects.

7.2. Upregulation of Uncoupling Proteins. Uncoupling proteins (UCPs) are mitochondrial transporters present in the inner membrane of mitochondria. The term "uncoupling protein" was originally used for UCP1, which is uniquely present in mitochondria of brown adipocytes, the thermogenic cells that maintain body temperature [115]. In these cells, UCP1 acts as a proton carrier activated by free fatty acids and creates a shunt between complexes of the respiratory chain and ATP synthase. Activation of UCP1 enhances mitochondrial respiration, and the uncoupling process results in a futile cycle and dissipation of oxidation energy as heat. In comparison to the established uncoupling and thermogenic activities of UCP1, UCP2 and UCP3 appear to be involved in the limitation of ROS levels in cells rather than in physiological uncoupling and thermogenesis [116, 117]. UCP2 and UCP3 dissipate electrochemical gradient produced by complex I, III, and IV in the mitochondrial ETC to pump protons outside the inner membrane. Such partial uncoupling of respiration prevents an exaggerated increase in ATP level that would lead to stagnation of electrons and increases their leakage from the ETC to produce superoxide. Consistent with this notion is the fact that low concentrations of chemical uncoupling agents attenuate mitochondrial ROS production [118]. Thus, UCP3 exerts an antioxidant function selectively at times when proton motive force is high, such as in resting muscle (low ATP demand), or when activity of the ETC enzyme complexes is inhibited, such as during hypoxia [119].

The mechanism by which UCPs are activated appears to involve redox signaling through mild uncoupling and membrane depolarization of mitochondria that feedbacks to prevent robust ROS generation. It has been demonstrated that superoxide activates UCP3 in rat skeletal muscle mitochondria [120]. Brand and associates [121] also reported activation of UCP3 by 4-hydroxynonenal, a by-product of lipid peroxidation. Furthermore, UCP2 and UCP3 are acutely activated by ROS generated by mitochondria, which then directly modulate the glutathionylation status of the UCP to decrease ROS emission [122]. This observation indicates that GSH/GSSG ratio within mitochondria may determine the activity of UCPs and lend further support to the hypothesis that redox signaling through mild oxidative stress enhances intrinsic antioxidative capacity of mitochondria.

The physiological signals that modulate UCP3 gene expression include fasting [123, 124], acute exercise [125, 126], and increased fatty acid intake [127, 128]. Peroxisome proliferator-activated receptor (PPAR-γ) agonist thiazolidinedione (TZD) or PPAR-α agonist WY-14643 increased UCP gene expression in the brown adipose tissue [129]. PPAR-α induction of UCP2 protected against elevated ROS during drug-induced hepatotoxicity [130]. UCP3 expression has been shown to be increased by PPAR-α agonist WY-14643 in the rodent hearts [131, 132]. It has been demonstrated that activation of PPAR-γ upregulated mitochondrial UCP2 expression, which decreased overproduction of ROS, improved mitochondrial ETC function at the site of complex I, and inhibited the mitochondrial apoptotic cascade leading to neuronal cell death in the hippocampus following status epilepticus [133].

PPAR signaling pathways are known to exert anti-inflammatory effects and attenuate atherosclerosis formation. The TZDs are the agonist for PPAR-γ and promote insulin sensitization and improve dyslipidemia in patients with type 2 DM, although it is unknown how PPAR agonists-induced upregulation of UCPs contributes to their beneficial effects on CVD. The PPAR-γ agonist rosiglitazone exerted a significant vascular protective effect in hypercholesterolemic rabbits, most likely by attenuation of oxidative and nitrosative stresses [134]. However, recent studies have shown that chronic rosiglitazone administration is associated with an increased risk of heart failure, acute MI, and death as a result of cardiovascular complications [135, 136]. Currently, exact mechanisms by which rosiglitazone exerted an adverse cardiovascular effect remain unclear.

Although CR is the fundamental approach to upregulate PPARs [137], a variety of recently emerging drugs, in addition to TZDs, which are used for the management of life style-related diseases are known to act as PPAR agonists. Fibrates are the representative PPAR-α agonist that stimulates the oxidation of free fatty acids in the liver, diverting them away

Table 1: Inhibitors of ROS sources.

ROS sources	Inhibitors	Mechanisms
NADPH oxidase (Nox)	Calorie restriction exercise	(i) Reduction of visceral adipose tissue-derived inflammatory cytokines (ii) Downregulation of AT1R
	ARBs	AT1R-induced activation of Nox
	Statins	Inhibition of ROCK
Xanthine oxidase (XO)	Calorie restriction exercise	(i) Inhibition of Nox-mediated EC damage (ii) Inhibition of Nox-mediated EC damage
	ARBs	Inhibition of Nox-mediated EC damage
	Allopurinol oxypurinol	Direct inhibition of XO
Nitric oxide synthase (NOS)	Calorie restriction exercise	Inhibition of oxidative stress and NOS uncoupling
	ARBs Statins Allopurinol and oxypurinol	Inhibition of oxidative stress and NOS uncoupling
	BH4 Sepiapterin	Direct inhibition of NOS uncoupling
Mitochondrial electron transfer chain (ETC)	Calorie restriction exercise	(i) Inhibition of electron leakage from ETC (ii) Stimulation of mitochondrial biogenesis (iii) Induction of UPs by activating PPARs
	Resveratrol	Stimulation of mitochondrial biogenesis
	TZD	Activation of PPAR-γ and induction of UP2 and UP3
	WY-14643	Activation of PPAR-α and induction of UP2 and UP3
	Fibrates	Activation of PPARs and induction of UP2 and UP3
	ARBs	Activation of PPAR-γ and induction of UP2 and UP3
	Statins	Activation of PPAR-γ and induction of UP2 and UP3

AT1R: angiotensin II type 1 receptor, ARBs: angiotensin II type 1 receptor blockers, ROCK: Rho-kinase, ECs: endothelial cells, CMCs: cardiomyocytes, UP: uncoupling protein, PPAR: peroxisome proliferator-activated receptor, TZD: thiazolidinedione.

from triglyceride synthesis and thus reducing the hepatic synthesis of triglyceride-rich lipoproteins [138]. Fibrates can also activate PPAR-γ by increasing the adiponectin level [139]. The fact that the cardioprotective properties of fibrates may be largely independent of their effects on plasma lipid levels, especially in subjects with features of the metabolic syndrome [140], suggests that the increase in adiponectin may contribute to the protective effect of fibrates against CVD. Recently, some sort of ARBs such as telmisartan and to a lesser extent irbesartan partially activate PPAR-γ and effectively treat insulin resistance and dyslipidemia without the toxicity sometimes associated with full PPAR-γ agonists [141, 142]. Statins can also enhance PPAR-γ activation, which may at least in part be involved in their antioxidant or anti-inflammatory potential [139].

8. Conclusion

Oxidative stress to endothelial cells plays a central role in the development of atherosclerosis and the occurrence of CVD. CR and endurance regular exercise are the fundamental approach to prevent oxidative stress to endothelial cells and prolong healthy life span. In addition to the improvement of lifestyle, recently emerging drugs that are effective in treating CVD have a property to eliminate ROS with a site-specific manner without interrupting favorable redox signaling, thereby ameliorating oxidative stress to endothelial cells. CR and exercise mimetics will also be fascinating pharmacological tools that specifically mitigate oxidative stress to endothelial cells. The sources of ROS and their inhibitors as well as a strategy of the site-specific antioxidative therapy to prevent CVD are presented in Table 1 and Figure 1.

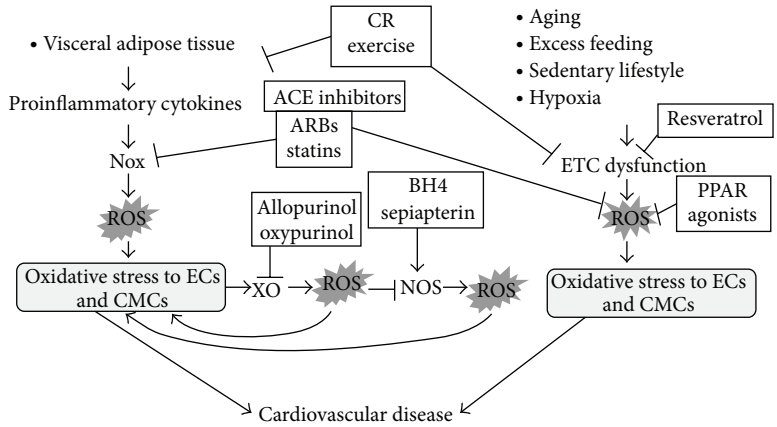

Figure 1: Strategy of the site-specific antioxidative therapy to prevent cardiovascular disease. The visceral adipose tissues are the primary source of proinflammatory cytokines that impose oxidative stress on endothelial cells (ECs) and cardiomyocytes (CMCs) through activation of NADPH oxidase (Nox) and generation of reactive oxygen species (ROS). Oxidative stress to ECs and CMCs then activates xanthine oxidase (XO) that potentiates the generation of ROS and increases oxidative stress to ECs and CMCs. Oxidative stress to ECs and CMCs causes uncoupling of nitric oxide synthase (NOS) through oxidation and depletion of BH_4 that further increases oxidative stress to ECs and CMCs, creating a self-perpetuating cycle for oxidative stress in ECs and CMCs, leading to the development of cardiovascular disease. Calorie restriction (CR) or regular endurance exercise reduces the mass of the visceral adipose tissue, thereby decreasing the production of proinflammatory cytokines responsible for oxidative stress to ECs and CMCs. Angiotensin converting enzyme (ACE) inhibitors, angiotensin type 1 receptor blockers (ARBs), or statins prevents activation of Nox and mitigates oxidative stress to ECs and CMCs. Allopurinol or oxypurinol blocks XO, while tetrahydrobiopterin (BH_4) or sepiapterin prevents uncoupling of NOS, thereby attenuating oxidative stress to ECs and CMCs. Another important source of ROS within ECs and CMCs is the electron transfer chain (ETC) in mitochondria. Aging, excess feeding, sedentary lifestyle, or hypoxia increases the leakage of electrons from the ETC and generation of ROS that imposes oxidative stress on ECs and CMCs. CR and exercise are a fundamental approach to prevent oxidative stress to ECs and CMCs by reducing the leakage of electrons from the ETC and ROS production. Resveratrol mimics the action of CR and exercise, thereby ameliorating oxidative stress to ECs and CMCs. Peroxisome proliferator-activated receptor (PPAR) α or γ agonists induce activation of uncoupling proteins in mitochondria, thereby reducing the leakage of electrons from the ETC and ROS production. ARBs and statins act as the partial agonists of PPARs that may be related to their preferable cardiovascular effects.

Further studies are warranted to establish a more efficacious approach to eliminate oxidative stress to endothelial cells and prevent atherosclerosis.

Conflict of Interests

There is no conflict of interests to disclose.

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Review Article

A Review of Pterostilbene Antioxidant Activity and Disease Modification

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Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a natural dietary compound and the primary antioxidant component of blueberries. It has increased bioavailability in comparison to other stilbene compounds, which may enhance its dietary benefit and possibly contribute to a valuable clinical effect. Multiple studies have demonstrated the antioxidant activity of pterostilbene in both *in vitro* and *in vivo* models illustrating both preventative and therapeutic benefits. The antioxidant activity of pterostilbene has been implicated in anticarcinogenesis, modulation of neurological disease, anti-inflammation, attenuation of vascular disease, and amelioration of diabetes. In this review, we explore the antioxidant properties of pterostilbene and its relationship to common disease pathways and give a summary of the clinical potential of pterostilbene in the prevention and treatment of various medical conditions.

1. Introduction

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a naturally derived compound found primarily in blueberries and Pterocarpus marsupium (PM) heartwood [1, 2]. The amount of daily pterostilbene consumption varies according to dietary fruit intake, and it has been estimated that pterostilbene content per blueberry varies from 99 ng to 520 ng/gram depending on the type of berry ingested [3, 4]. Substantial evidence suggests that pterostilbene may have numerous preventive and therapeutic properties in a vast range of human diseases that include neurological, cardiovascular, metabolic, and hematologic disorders. Further benefits of pterostilbene have been reported in preclinical trials, in which pterostilbene was shown to be a potent anticancer agent in several malignancies [5]. Pterostilbene is structurally similar to resveratrol, a compound found in red wine that has comparable antioxidant, anti-inflammatory, and anticarcinogenic properties; however, pterostilbene exhibits increased bioavailability due to the presence of two methoxy groups which cause it to exhibit increased lipophilic and oral absorption (Figure 1) [6–10]. In animal studies, pterostilbene was shown to have 80% bioavailability compared to 20% for resveratrol making it potentially advantageous as a therapeutic agent [6].

The multiple benefits of pterostilbene in the treatment and prevention of human disease have been attributed to its antioxidant, anti-inflammatory, and anticarcinogenic properties leading to improved function of normal cells and inhibition of malignant cells [11, 12]. Treatments with blueberry extract and PM in similar disease models have yielded paralleled results possibly due to the antioxidant activity and underlying mechanisms of pterostilbene. The evidence presented in this review shows that pterostilbene reduces oxidative stress (OS) and production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), which are implicated in the initiation and pathogenesis of several disease processes [13]. In addition, various cell lines treated with pterostilbene have shown increased expression of the antioxidants catalase, total glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD). In this review, the clinical potential and antioxidant capabilities of pterostilbene in several disease systems will be explored and summarized.

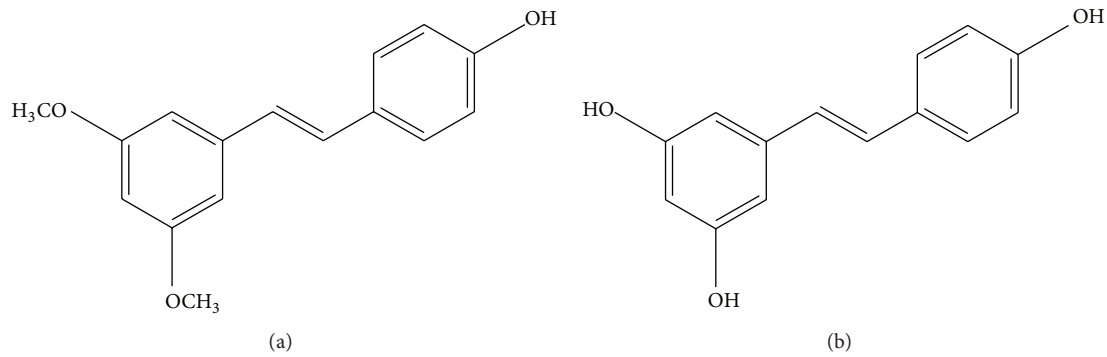

Figure 1: Pterostilbene (a) and Resveratrol (b). Pterostilbene contains two methoxy groups compared to Resveratrol which increases oral absorption and bioavailability.

2. Antioxidant Properties of Pterostilbene

2.1. Breast. Increasing rates of obesity and poor nutrition are major contributors to breast cancer occurrence in women [14]. Several studies have shown that blueberry extract and pterostilbene inhibit breast cancer *in vitro* and *in vivo*. Pterostilbene treatment of breast cancer cells has additionally been shown to alter cellular oxidative activity that may play an important role in pterostilbene-mediated cell death.

Wu and colleagues conducted experiments to examine the effects of blueberry consumption on outcomes of early development of mammary epithelial cells in pregnant and lactating rats [15]. The authors hypothesized that blueberry consumption in pregnant rats would lead to transcriptional modification and mammary gland differentiation during the developmental period. Administering blueberry powder to pregnant and lactating rats and assessing the postnatal effects of blueberry exposure on mammary architecture and differentiation were conducted as experiments. The results of the study demonstrated that rats fed the blueberry diet exhibited higher mammary branching, increased nuclear immunoreactivity of tumor suppressor phosphatase and tensin homolog deleted in chromosome 10 (PTEN), and decreased mitotic rates. Additional experiments were performed to evaluate the effect of blueberry exposure upon *in vitro* expression of PTEN and antioxidant activity in nontumorigenic breast cells, and results revealed that cells treated with blueberry serum showed higher PTEN but similar antioxidant capacity to control cells. The findings show that breast cells are early targets of blueberry-derived mediators, which possess the ability to alter developmental mammogenesis.

Several studies have found that blueberry exposure inhibited breast cancer *in vitro* and *in vivo* [16–18]. The results of experiments conducted by Boivin and colleagues found that blueberry juice from high-bush, low-bush, and velvetleaf blueberries exerted an antiproliferative effect against the breast cancer cell line MDA-MB-231 [17]. Likewise, Adams and colleagues found that high-bush blueberry extract decreased proliferation of triple-receptor negative breast cancer cell lines, HCC38, HCC1937, and MDA-MB-231 but did not affect proliferation of the nontumorigenic cell line, MCF-10A [18]. In the same study, treatment with blueberry extract significantly decreased human-growth-factor (HGF-) induced activation of the PI3 K/AkT/NK-κB pathway, which is implicated in breast carcinogenesis. Blueberry treatment also inhibited the metastatic potential of breast cancer cells *in vitro* by inhibiting HGF-induced cell migration and matrix metalloproteinase-(MMP-) 2 and MMP-9 activity.

In experiments utilizing a breast cancer xenograft model, treatment with blueberry extract produced smaller tumors with decreased expression of Ki-67, a marker of cell proliferation, and increased expression of caspase-3, an apoptosis marker. Blueberry fed mice also exhibited decreased activation of AkT and NK-κB signaling when compared to controls [18]. The cumulative findings suggest that blueberries contain a specific chemical component capable of modifying carcinogenic pathways in breast cancer cells.

Similarly, pterostilbene has been shown to exert anticancer effects in breast cancer through alteration of multiple cancer pathways both *in vitro* and *in vivo*. The carcinogenic pathways inhibited by pterostilbene treatment are similar to pathways altered by blueberry juice making it plausible that pterostilbene is the compound responsible for the anticarcinogenic effects of blueberry treatment in breast cancer. Moreover, pterostilbene's anticancer effects have been associated with its antioxidant-inducing capacity. Alosi and colleagues found that pterostilbene induced apoptosis and inhibited proliferation of MDA-MB-231 and MCF-7 breast cancer cells in a concentration and time-dependent manner [19]. In the same study, the antioxidant activity of pterostilbene was measured using hydroethidine (HE) to detect O_2^- production. The authors hypothesized that pterostilbene when applied to cancer cells would increase O_2^- production which would facilitate mitochondrial membrane depolarization triggering intrinsic mitochondriallyderived apoptosis. The results of the study show a concentration-dependent increase in O_2^- production, increased mitochondrial depolarization, and associated caspase release indicating apoptosis. Mannal et al. reported that treatment with pterostilbene increased caspase release and O_2^- production in breast cancer cells, consistent with the findings of Alosi [20].

Studies performed by Chakraborty and colleagues found that pterostilbene reduced cell proliferation and induced apoptosis through the induction of caspase-3, Bax, and p53

in breast cancer cells [21]. It was also demonstrated by Chakraborty et al. that pterostilbene treatment increased GPx antioxidant activity and the production of H_2O_2 and singlet oxygen indicating a mechanism of ROS-induced apoptosis [21, 22]. To evaluate the hypothesis of ROS-induced apoptosis, pterostilbene-treated breast cancer cells were treated with catalase, H_2O_2 scavenger, and cell survival ratios were compared to pterostilbene-treated controls. The results of the study show that catalase treatment inhibited pterostilbene-induced cell death in MCF-7 cells confirming that H_2O_2 is involved in pterostilbene cytotoxicity [22]. The findings of Alosi, Mannal, and Chakraborty imply that pterostilbene alters cellular oxidation to facilitate mechanisms of apoptosis in breast cancer.

The relationship between oxidation and apoptosis was further examined by Moon and colleagues who evaluated the effects of pterostilbene upon expression of Bcl-2-associated X protein (BAX), Cytochrome C, second mitochondria-derived activator of caspases (Smac/Diablo), and manganese superoxide dismutase (MnSOD), which are markers of intrinsic apoptosis [23]. The results of the study found that pterostilbene treatment produced dose-dependent increases in BAX, cytochrome C, and Smac/Diablo expression implying that pterostilbene induces intrinsic mitochondrially derived apoptosis. Pterostilbene was also shown to significantly increase MnSOD antioxidant activity in breast cancer cells, an antiproliferative mechanism that has been studied extensively [24–26]. The interconnected mechanism of increased MnSOD expression and breast cancer inhibition is not fully understood; however, it is likely that pterostilbene's ability to increase antioxidant activity is related to its function as an apoptotic and antiproliferative agent.

Furthermore, pterostilbene treatment produced a synergistic inhibitory effect when combined with the chemotherapy drug Tamoxifen, demonstrating clinical potential in the treatment of breast cancer [20]. Further research is necessary to investigate the clinical potential of pterostilbene's antioxidant activity in human cases of breast cancer.

2.2. Cardiovascular. Cardiovascular disease is currently the number one cause of mortality in the Unites States, and its high prevalence is attributed to multiple risk factors such as smoking, sedentary lifestyle and low intake of fruits and vegetables [27]. On a cellular level, vascular pathology results from dysfunction of the endothelium due to repeated exposure to mechanical stress and leukocyte generated ROS [28]. Increased mechanical stress of the endothelium predisposes to vascular injury and thrombogenicity, which is then exacerbated by increased levels of OS. The endogenous antioxidant activity of the vascular system is purported to exert a basal protective effect against pathogenesis by reducing OS; however, the antioxidant capacity of the endothelium may become exhausted from increased exposure to ROS creating an imbalance between OS and antioxidant activity [28]. Routine consumption of dietary antioxidants may therefore be protective against ROS-induced vascular injury in high-risk individuals [29].

Several studies have shown that blueberries, and pterostilbene alike, exhibit protective effects against cardiovascular disease possibly due to induction of antioxidant enzymes. In a study performed by Ahmet and colleagues, rats that were fed a three-month blueberry enriched diet had 22% smaller average myocardial infarction (MI) size measured 24 hours after ligation of the left descending coronary artery compared to rats fed a control diet [30]. The authors also determined that rats fed a blueberry diet had higher ejection fractions two weeks after MI compared to controls. A possible explanation for the differences in cardiac outcomes may be due to the antioxidant effects of blueberry-derived compounds leading to cardioprotection of ischemic cardiomyocytes. The theory is supported by findings that show that cardiomyocytes from blueberry fed rats exhibit a 24% increase in the ROS-induced threshold for mitochondrial permeability transition, thereby decreasing the likelihood of apoptosis in ischemic cells [30].

Further studies show that blueberry supplementation attenuated atherosclerosis by upregulating expression of the antioxidant enzymes SOD1, SOD2, GSR, and thioredoxin reductase- (TR-) 1 in ApoE deficient mouse models of atherosclerosis [31]. Blueberry supplementation was also shown to reduce H_2O_2-induced intracellular ROS production in human microvascular endothelial cells (HMVECs) [32].

The antioxidant activity of pterostilbene may play an important role in the cardioprotective effects observed in blueberry supplementation studies. Pterostilbene has demonstrated numerous protective benefits against atherosclerosis through regulation of vascular smooth muscle cells (VSMCs) and vascular endothelial cells (VECs). In experimental studies, VSMCs treated with pterostilbene exhibited reduction in platelet-derived-growth-factor-(PDGF-) induced proliferation and Akt, a serine-threonine kinase [33]. Pterostilbene also produced downregulation of the cell-cycle mediators, cyclin-dependent kinase (CDK)-2, CDK-4, cyclin E, cyclin D1, retinoblastoma (Rb), and proliferative cell nuclear antigen (PCNA), all of which promote unchecked VSMC proliferation resulting in atherosclerosis [33]. The results may be clinically relevant because abnormal proliferation of VSMCs is a significant component of the pathogenesis of atherosclerosis and a major contributor to the development of vascular stenosis. The antiproliferative effects of pterostilbene in VSMCs may therefore confer a defense mechanism against atherosclerosis and subsequent complications of stenosis.

In separate studies conducted by Zhang et al., pterostilbene was shown to inhibit apoptosis and induce autophagy in VECs counteracting the proatherosclerosis effect of oxidized low-density lipoprotein (oxLDL) [34, 35]. The cytoprotective mechanism of autophagy involves removal of abnormal proteins that results from oxLDL accumulation [36]. Zhang and colleagues have demonstrated that pterostilbene treatment induces autophagy in oxLDL-stimulated VECs through activation of AMP-activated protein kinase (AMPK), intracellular calcium (Ca^{2+}), and mammalian target of rapamycin (mTOR) signaling [34]. Apoptosis plays a central role in the pathogenesis of atherosclerosis through induction of plaque instability that occurs when oxLDL induces apoptosis in VECs through activation of the lectin-like oxLDL receptor –1 (LOX-1).

Activation of LOX-1 then triggers a cascade of pro-apoptosis events such as induction of p53, cytochrome C, and

caspase activation. It has been postulated that increased exposure to OS facilitates apoptosis in VECs, which propagates the atherosclerosis process [37]. Treatment with pterostilbene was shown to inhibit oxLDL-induced apoptosis, suppress oxLDL-induced OS, and reduce expression of the pro-apoptosis proteins Bax and p53. Pterostilbene treatment also inhibited NF-κB activation, an instrumental mediator of OS in VECs [34]. Furthermore, pterostilbene treatment suppressed oxLDL-induced expression of MMP, caspase-3/9, and attenuation of LOX-1 signaling [34]. Previous studies have shown that OS leads to LOX-1 activation that further stimulates production of OS creating a positive feedback loop that is pathogenic to vascular cells [38].

Pterostilbene's modulation of LOX-1, NF-κB, and the antioxidant enzymes SOD and catalase indicate strong antioxidant and antiatherosclerosis effects that may be clinically significant. Although, the application of dietary pterostilbene in the prevention of cardiovascular disease is currently undetermined, a study performed by McAnulty and colleagues provided evidence that consumption of 250 g blueberry diets for three weeks attenuates angiotensin converting enzyme (ACE) activity and reduce lipid hydroperoxidase (LH), a marker of OS in chronic smokers [39]. The risk of vascular disease is high in chronic smokers, and reduction of OS by blueberry consumption may decrease the risk and/or severity of atherosclerotic disease.

Future directions may include large-scale clinical trials to determine the impact of blueberry-derived pterostilbene in the prevention or therapy of atherosclerosis in smokers and other high-risk groups.

2.3. Gastroenterology

2.3.1. Esophagus. Esophageal cancer has a poor prognosis with low rates of disease survival [40]. Oxidative damage from smoking, alcohol, and gastroesophageal reflux disease (GERD) increases the risk of esophageal cancer, which some suggest may be mitigated through the use of antioxidant agents [40, 41]. In a study conducted by Stoner and colleagues, the authors evaluated the hypothesis that a blueberry diet induces anticarcinogenic, anti-inflammatory, and antioxidant effects in N-nitroso-methyl-benzylamine (NMBA) treated rats [41]. NMBA is an established carcinogen that induces tumorigenesis in the rat esophagus in a multistep fashion that is analogous to the esophageal carcinogenesis in humans. The authors found that rats treated with NMBA for five weeks, followed by a blueberry diet for a total of 35 weeks, had increased antioxidant activity, reduced tumorigenesis, and decreased expression of interleukin- (IL-) 5 and growth-related oncogene (GRO/KC), two markers of inflammation.

Currently, the relationships between anticarcinogenesis, anti-inflammation, and increased antioxidant activity in the pathogenesis of esophageal malignancy have not been fully elucidated.

It has been postulated that high antioxidant capacity of blueberries facilitates mechanisms of cell death in malignant cells; however, further studies are needed to assess whether the anticancer effects of blueberry treatment in esophageal cancer are related to the antioxidant effects of pterostilbene, which also induces anticancer effects in several digestive malignancies.

2.3.2. Stomach. The risk of gastric cancer is associated with genetics and dietary factors such as high consumption of smoked, salted, and nitrated foods combined with low intake of fruits and vegetables [42]. The anticancer mechanisms of fruit consumption in gastric cancer are complex; however, two studies have found that both blueberry juice and pterostilbene inhibit carcinogenesis of gastric cells. Boivin and colleagues demonstrated that juice from velvet leaf blueberry, low-bush blueberry, and high-bush blueberry, all inhibited cell proliferation of gastric adenocarcinoma cells [17]. Velvet leaf blueberry juice exerted the most significant antiproliferative effect, and high-bush blueberry juice exhibited the least significant effect. However, there was no significant correlation between antiproliferation rates and antioxidant capacity of blueberry juices leading to the conclusion that blueberries may have variable effects upon several carcinogenic mechanisms independent of antioxidant potential. Similarly, in a study performed by Pan and colleagues, pterostilbene treatment inhibited cell proliferation in a dose-dependent manner and induced apoptosis by increasing cytochrome C, Bad, Bax, and caspases 1, 2, 3, 8, and 9 in gastric adenocarcinoma cells *in vitro* [43].

Utilization of blueberry juice and pterostilbene to decrease gastric cancer risk and mitigate progression of malignant tumors may be a feasible option in the future. Additional research to examine the effects of blueberry juice and pterostilbene on gastric cancer should include clinical trials to assess the antioxidant and anticancer effects of blueberry-derived pterostilbene in human subjects.

2.3.3. Colon. The etiology of colon cancer is complex and involves dynamic dietary and genetic and inflammatory components [44]. Epidemiological studies have conclusively shown that chronic inflammation predisposes to high rates of colon cancer, and it has been established that diets low in fiber, fruits, and vegetables increase colon cancer risks [44]. The role of OS in the pathogenesis of colon cancer is not fully understood; however, it has been theorized that inflammation and OS interact in a positive feedback loop leading to aberrant cellular signaling and carcinogenesis. Such theories suggest that dietary antioxidants play a key role in the mitigation of colonic inflammation and colon cancer. In multiple studies, pterostilbene exhibited antioxidant properties and significantly inhibited colon cancer [45]. The anticancer effects of pterostilbene are comparable to the findings of Seeram et al. and Boivin et al. who demonstrated the inhibitory effects of blueberry compounds on colon cancer *in vitro* [16, 17].

Suh et al. and Chiou et al. found that pterostilbene treatment downregulated the inflammatory enzymes nitric oxide synthetase (iNOS) and cyclooxygenase-2 (COX-2), which stimulate production of proinflammatory cytokines and induce proliferation of colon cancer cells [46, 47]. Both

enzymes are upregulated by OS and implicated in the progression of colonic tumorigenesis. The findings indicate that iNOS and COX-2 may be direct and indirect targets of pterostilbene's antioxidant activity in inflammation-mediated colon cancer. Chiou and colleagues also found that pterostilbene decreased expression of aldose reductase (AR), an OS protein, and increased expression of the antioxidant enzymes, GR and hemeoxygenase-1 (HO-1), through NF-E2-related factor 2(Nrf2) upregulation [47]. The transcription factor Nrf2 plays a critical role in regulation of mucosal inflammation and Nrf2 deficient mice have been shown to express increased mucosal inflammation, and OS. The results suggest that pterostilbene exerts its antioxidant effects through multiple interrelated mechanisms.

In addition to anti-inflammation and antioxidation, Chiou et al. and Suh et al. found that pterostilbene treatment inhibited colon cancer proliferation, which is consistent with the research of Remsberg et al. and Priego et al. [12, 48]. Pterostilbene treatment increased expression of the antioxidants, catalase, GPx, GR, and TR-1 by less than 2-fold and downregulated Bcl-2, a proto-oncogene implicated in colon cancer proliferation [48]. The effects of pterostilbene were most significant upon antioxidant SOD2 expression, producing a 5.7-fold increase in enzyme activity [48]. Some authors have proposed that chemotherapeutic regimens target SOD2 as an additional mechanism for tumor suppression making pterostilbene a potential chemotherapeutic agent [49]. Priego and colleagues also found that pterostilbene treatment when combined with quercetin, radiotherapy, and the chemotherapy regimen FOLFOX (oxaliplatin, leucovorin, and 5-fluorouracil) produced tumor regression in rats [48]. The overall evidence suggests that pterostilbene possesses potent anti-inflammatory, antioxidant, and anticarcinogenic properties ideal for the eradication of cancerous colon cells.

The antioxidant properties of pterostilbene may help to explain how blueberry consumption contributes to reduced risk of colon cancer in humans. Pterostilbene's modulation of antioxidant activity may also facilitate anti-inflammatory and anticarcinogenic mechanisms that confer clinical benefits in inflammatory bowel disease and colorectal malignancies. Additional studies are warranted to investigate the preventive and therapeutic effects of pterostilbene in diseases of the colon.

2.4. Hematology. Hemolytic disorders include a broad spectrum of hereditary and acquired conditions that range from mild to severe clinical outcomes [50]. Hemolytic anemias, irrespective of etiology, are exacerbated by exposure to ROS, which produces both internal and external damage to red blood cells (RBCs), accelerating the process of hemolysis. Studies have shown that ROS-induced hemolysis is a modifiable event that can be alleviated with antioxidant treatment [51]. Specifically, treatments with blueberry extract and pterostilbene have been shown to protect RBCs against ROS-induced hemolysis indicating a possible therapeutic effect in the treatment of hemolytic anemia.

Youdim and colleagues conducted experiments both *in vitro* and *in vivo* to assess the antioxidant capacity of blueberry-derived polyphenolic components in vulnerable RBCs [51]. The results of experiments performed *in vitro* show that low-bush blueberry treatment reduced rates of ROS formation at 6 and 24 hours after H_2O_2 treatment in a time- and concentration-dependent manner. *In vivo*, oral blueberry supplementation produced significant inhibition of H_2O_2-induced ROS formation at 6 and 24 hours similar to the results obtained *in vitro*. Serum analysis of blueberry fed rats revealed detectable levels of anthocyanins present at 1- and 6-hour intervals but not at 24 hours, indicating a short-term protective effect. The findings of the study suggest that oral blueberry supplementation protects RBCs against ROS formation after exposure to H_2O_2.

The relationship between antioxidant activity and ROS-induced RBC damage was further explored by Mikstacka and colleagues that studied the antioxidant effects of pterostilbene in RBCs that were treated with 2,2-azobis 2-amidinopropane dihydrochloride (AAPH), a known free radical generator that causes OS in RBCs leading to hemolysis [52]. The authors found that pterostilbene treatment inhibited AAPH-induced hemolysis and AAPH-induced depletion of the antioxidant enzyme GSH. Moreover, pterostilbene treatment was found to inhibit H_2O_2-induced lipid peroxidation, an initiator of OS that produces autoxidation in RBCs [53].

It has been postulated that blueberries and its component pterostilbene protect RBCs against OS by scavenging H_2O_2, altering the harmful effects of ROS and increasing antioxidant activity. The short-term benefits of pterostilbene are observable in RBCs up to 24 hours but long-term effects have not been studied. Currently, it is undetermined whether oral supplementation with blueberries or pterostilbene is able to prevent hemolytic episodes in humans. Future research is needed to elucidate the antioxidant enhancing mechanisms of pterostilbene and prevention of hemolysis in clinical trials.

2.5. Hepatopancreaticobiliary

2.5.1. Liver. Chronic liver disease (CLD) is an end-stage process that results from conditions like Wilson's disease, hemochromatosis, and primary biliary cirrhosis, in addition to infection, alcoholism, and nonalcoholic steatohepatitis (NASH) [54]. The pathogenesis of CLD is complex but follows a consistent model of progression from acute hepatic cellular injury to apoptosis, necrosis, inflammation, and irreversible fibrosis that can culminate in cancer [54]. In experimental studies, OS is a common mediator in the progression of hepatic injury to sustained inflammation and fibrosis regardless of disease etiology. For example, studies have shown that models of CLD due to viral hepatitis, NASH, alcoholism, and excess deposition of copper or iron, all share common pathways of increased OS combined with reduced antioxidant capacity [54]. An imbalance of oxidation, measured by increased ROS, H_2O_2, and O_2^-, can alter critical transcriptional and translational cell signaling leading to increased proliferation and eventually fibrosis of hepatic cells. The combined effect of increased OS and reduced antioxidant capacity is deleterious because it potentiates and amplifies

structural damage in a time-dependent manner leading to permanent cellular and parenchymal hepatic impairment.

Wang and colleagues administered blueberry juice to rats with CCl_4-induced hepatic fibrosis and found an increase in the expression of the transcription factor, NF-E2-related factor 2 (Nrf-2), and its downstream target, the antioxidant enzyme NADPH quinone oxidoreductase (Nqo1), which are central to hepatic stellate cell cytoprotection [55]. Blueberry juice was shown to increase levels of the antioxidant enzymes SOD and GST and decrease malondialdehyde (MDA) levels in CCl_4 treated mice. Levels of hyaluronic acid (HA) and alanine aminotransferase (ALT), two markers of acute hepatocyte injury, were also significantly decreased in blueberry treated rats. The study findings are consistent with results from a previous study, which found that blueberry juice increased expression of Nrf-2, Nqo1, and HO-1 [56]. Furthermore, rats treated with blueberries had increased frequency of CD3+ and CD4+ T-lymphocytes suggesting that blueberries have an immunomodulatory effect *in vivo* [56].

A study conducted by Osman and colleagues reported that pretreatment with blueberry powder led to decreased blood levels of bilirubin and ALT but not aspartate aminotransferase (AST) in rat models of D-galactosamine/lipopolysaccharide-induced hepatic injury [57]. Blueberry treated rats also exhibited decreased levels of proinflammatory cytokines tumor necrosis factor- (TNF-) α and IL-1β and increased levels of GSH in liver tissue, indicating combined anti-inflammatory and antioxidative effects. Additional key findings included decreased lipid peroxidation measured by MDA levels and decreased myeloperoxidase (MPO), a marker of neutrophil-derived inflammation [57]. Treatment with blueberries also inhibited proliferation of hepatic cancer cells which was demonstrated by Schmidt et al. [58]. The cumulative evidence suggests that blueberry supplementation regulates hepatic cell dysfunction through alteration of various anti-oxidative, ant-inflammatory, and antiproliferative mechanisms.

Protection against hepatic cellular dysfunction has also been demonstrated by pterostilbene, which has been shown to thwart cellular dysfunction by inhibiting H_2O_2-induced inhibition of gap junctional intercellular communication (GJIC), a key facilitator of hepatic tumorigenesis [59]. Inhibition of GJIC results from activation of the extracellular receptor kinase (ERK) 1/2 and p38 pathways and phosphorylation of the gap junctional protein connexin 43 (Cx43), resulting in aberrant gap cell communication. Kim and colleagues found that pretreatment with pterostilbene at doses of 0.5, 1.0, and 5.0 μM for 24 hours negated the inhibitory effects of H_2O_2 through dephosphorylation of Cx43 with subsequent restoration of normal GJIC [59]. Pterostilbene's antioxidant effect was found to correlate with repression of an established carcinogenic pathway, making it a potentially advantageous agent for hepatic tumor suppression.

Experiments conducted by Hasiah and colleagues found that antioxidant effects of pterostilbene were present in both cancerous and noncancerous hepatic cells [60]. Treatment with 6.25 to 100 μM pterostilbene increased endogenous antioxidant activity in cancerous HepG2 hepatoma and normal Chang liver cells; however, the effect was higher in HepG2 cells. Pterostilbene also decreased cell viability of HepG2 cells that is consistent with its properties as an anticancer agent. The findings suggest that pterostilbene's antioxidant activity is beneficial to normal cells but antagonistic to the growth of cancerous cells.

Pan and colleagues demonstrated antimetastasis effects of pterostilbene using a12-O-tetradecanoylphorbol 13-acetate-(TPA-) induced metastasis model *in vitro* and *in vivo* [61]. The results of the study found that pterostilbene treatment significantly inhibited TPA-induced vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and MMP activity *in vitro* and *in vivo,* without producing significant toxicity in rodents. The research findings demonstrate pterostilbene's potential as an antimetastasis agent, and future studies may assess whether the anti-metastatic properties of pterostilbene are applicable to human cases of hepatoma as well.

Overall, blueberries and pterostilbene exert anti-inflammatory, antioxidant, and anticarcinogenic effects in models of CLD and liver cancer. The compound may afford clinical protection in a broad range of benign and malignant liver conditions through amelioration of OS and related hepatocyte pathology. Further research should focus upon the medicinal impact of pterostilbene in the management of CLD.

2.5.2. Pancreas. The etiology and pathogenesis of pancreatic cancer is multifactorial and involves various genetic and environmental components. It has been postulated that pancreatic cancer results from an accumulation of multiple genetic mutations making it a highly chemoresistant disease with low rates of survival [62]. Despite extensive scientific efforts, an efficacious strategy for prevention and cure of pancreatic cancer remains elusive.

Several studies have shown that pterostilbene inhibits pancreatic cancer *in vitro* and *in vivo* through mechanisms of mitochondrially derived apoptosis, modification of transcription factors, and inhibition of proliferation [63, 64]. Specifically, pterostilbene-induced apoptosis in the pancreatic cell lines MIA PaCa-2 and PANC-1 has been attributed to mitochondrial membrane depolarization, release of Cytochrome C, and Smac/DIABLO with subsequent activation of caspase 3/7 [63, 64]. A pancreatic cancer genomic analysis of pterostilbene revealed downregulation of multiple apoptosis-related genes including MnSOD, DNA-damage-inducible transcript 3 (DDIT-3), and growth differentiation factor 15 (GDF-15), also known as macrophage inhibitory cytokine 1 (MIC-1) [64]. Further experiments demonstrated that pterostilbene induced upregulation of MnSOD at the genomic level which translated into downstream increased enzymatic activity [64]. Pterostilbene's ability to increase antioxidant activity by altering expression and enzymatic activity of MnSOD contributes to its credence as an anticancer agent because numerous studies show that pancreatic cancer cells have decreased expression of MnSOD when compared to normal cells and overexpression of MnSOD correlates with decreased pancreatic tumor volume [65–68]. In experiments conducted by Kostin et al., pterostilbene demonstrated synergistic inhibition of pancreatic cancer *in vitro* when combined with

the green tea antioxidant epigallocatechin-3-gallate (EGCG) supporting previous evidence of an antioxidant effect [69]. In addition to inhibiting pancreatic cancer, recent research found that pterostilbene ameliorated inflammation and acinar damage in pancreatitis *in vitro* [70]. The collective findings indicate pterostilbene's clinical relevance in the treatment of pancreatic disease. Further studies are warranted to examine the mechanisms involved in pterostilbene-induced antioxidant activity and inhibition of pancreatitis and pancreatic cancer in clinical trials.

2.6. Metabolic. Diabetes mellitus (DM) is a disease that consists of ineffective insulin regulation leading to derangements in carbohydrate, protein, and fat metabolism [71]. The disease is a component of the "metabolic syndrome," a clinical spectrum of abnormal lipid and glucose metabolism. Over recent decades, the incidence of DM has increased worldwide due to sedentary lifestyle and the rising epidemic of obesity [72]. Lifestyle modification is one strategy employed to treat DM and associated complications; however, failure to respond to lifestyle modification is an indication for medical treatment [71]. Unfortunately, treatment with medical agents may have significant side effects, and multiple adjustments may become necessary to achieve positive clinical results. Therefore, the pursuit of new medical agents with minimal side effects remains an enviable option for the successful treatment of DM.

The heartwood of the plant Pterocarpus marsupium (PM) has been shown to exhibit antiglycemic properties in multiple studies. In a study performed by Grover et al., rats were fed high-fructose diets to induce hyperglycemia and insulin resistance and then treated with PM orally for thirty days [73]. The authors hypothesized that PM treatment would counteract the metabolic side effects of a high-fructose diet by mitigating hyperglycemia, hyperinsulinemia and hypertriglycemia. Results of the study show that rats fed high-fructose diets combined with PM treatment had lower levels of hyperinsulinemia, hypertriglycemia, and complete prevention of hyperglycemia. It has been hypothesized that the antiglycemic properties possessed by PM are attributed to pterostilbene. Experiments performed by Manickam and colleagues assessed the antiglycemic effects of pterostilbene isolated from PM in a streptozocin- (STZ-) induced rat model of hyperglycemia and found that oral dosing of 20 mg/kg pterostilbene significantly decreased plasma glucose levels by 42% and body weight by 20% [74].

Further studies were conducted by Pari and Satheesh evaluating the antiglycemic effects of pterostilbene in combination with its antioxidant effect in rodent models of STZ-induced DM [75, 76]. The findings of the studies show that treatment with oral 40 mg/kg of pterostilbene for 6 weeks produced a significant decrease in plasma glucose levels by 56.54% and an increase in plasma insulin levels. It was also discovered that pterostilbene treatment reduced glycosylated hemoglobin (HbA1c), a marker of chronic hyperglycemia, and decreased expression of the gluconeogenic enzymes glucose-6-phosphatase and fructose-1, 6-biphosphatase. In addition, pterostilbene increased expression of the glycolytic enzyme hexokinase. The authors concluded that the effects of pterostilbene were comparable to the experimental effects of 500 mg/kg oral metformin in the STZ-induced DM model.

One proposed mechanism for the antidiabetic effects exerted by pterostilbene is reduction of OS, which plays a critical role in aberrant glucose regulation. Satheesh and Pari hypothesized that pterostilbene treatment in diabetic rats would increase antioxidant activity and lessen the impact of OS on kidney and liver cells [76]. The experimental design measured OS using thiobarbituric acid reactive substance (TBARS) and hydroperoxide (HP) levels in diabetic rats treated with 40 mg/kg oral pterostilbene in comparison to diabetic rats treated with metformin. The authors also evaluated the effect of pterostilbene upon the activity of antioxidant enzymes catalase, SOD, GPx, and GST. Results of the experiments show that DM control rats exhibited marked increases in TBARS and HP in liver and kidney tissue that was subsequently inhibited by pterostilbene treatment [76]. In DM rats, pterostilbene decreased TBARS by 61.5% and 33.3% in liver and kidney tissue, respectively. HP expression in liver and kidney was also significantly decreased by pterostilbene treatment by 27.7% and 28.3%, respectively.

The authors found that activity of the antioxidant enzymes GSH, GST, SOD, GPx, and catalase were decreased in liver and kidney tissue of DM controls; however, pterostilbene treated DM rats had significant increases in activity of all five antioxidant enzymes [76]. Moreover, histopathological examination of the livers of pterostilbene treated DM rats did not show inflammation compared to the DM controls, which exhibited significant portal triad inflammation. Examination of diabetic rat kidneys revealed glomeruli mesangial capillary proliferation with tubular epithelial damage that was significantly reduced in DM rats treated with pterostilbene. Comparable antioxidant and histopathological results were observed in DM rats treated with metformin suggesting that pterostilbene may harbor clinically significant metabolic properties.

The reported antioxidant and antihyperglycemic activities of pterostilbene may confer a protective effect against complications in poorly controlled DM patients by preventing hyperglycemia and associated liver and kidney damage. The exact relationship between antioxidant activity and glucose regulation induced by pterostilbene treatment has not been elucidated; however, it is postulated that pterostilbene increases antioxidant activity leading to improved glucose metabolism. Increased antioxidant activity produced by pterostilbene may improve tissue resilience against hyperglycemia-generated ROS and prevent end-organ damage.

The human applicability of pterostilbene's antidiabetic effects is still unknown. Nemes-Nagy et al. investigated the effect of blueberry extract on antioxidant activity in DM children and found that treatment with blueberry concentrate for two months significantly increased erythrocyte SOD and GPx activity and decreased levels of HbA1c [77]. It is possible that such results are attributable to the antioxidant activity of pterostilbene; however, additional studies are needed to identify the blueberry-derived mediator and investigate a plausible association with pterostilbene.

In addition to mitigating hyperglycemia, pterostilbene *in vitro* and *in vivo* has shown benefits in models of lipid metabolism. In 3T3-L1 preadipocytes, pterostilbene treatment decreased cell population growth, fat droplet formation, and triacylglycerol accumulation [78]. Pterostilbene also altered gene expression of peroxisome proliferator-activated receptor- (PPAR-) γ, CCAAT/enhancer binding protein- (C/EBP-) α, resistin, and fatty acid synthase (FAS), possibly modifying early stage adipocyte differentiation and decreasing the risk of atherosclerosis [79]. Furthermore, pterostilbene demonstrated antiobesity properties by upregulating adiponectin and downregulating leptin, indicating an antilipogenic effect [79]. Expression of adiponectin negatively correlates with body mass index (BMI), glucose, insulin, and triacylglycerol levels in comparison to leptin, which positively correlates with adipocyte size, lipid content, and BMI. Rimando and colleagues found that hypercholesterolemic hamsters fed 20 ppm oral pterostilbene demonstrated a 29% decrease in plasma low density lipoprotein (LDL) cholesterol, 7% increase in high density lipoprotein (HDL) cholesterol, and 14% decrease in plasma glucose levels compared to controls [79]. The authors also found that pterostilbene *in vitro* increased PPAR-γ activation in rat liver cells that was significantly higher than the amount of PPAR-γ activation produced by the lipid-lowering agent clofibrate. Such findings are significant because derangements of glucose metabolism often accompany hyperlipidemia in diabetics and those diagnosed with metabolic syndrome. Ultimately, the glucose and lipid-lowering effects of the dietary compound pterostilbene may contribute to its clinical potential for prevention or treatment of diabetes. Further research is necessary to establish pterostilbene's risk-reducing and therapeutic effects in DM individuals.

2.7. Neurology. The aging process in humans is associated with acquired deficiencies in cognition and motor function. The process is oftentimes innocuous; however, in certain neurological conditions such as Alzheimer's Disease (AD), the effects of aging are pathological and accelerated leading to rapid and permanent neurological decline [80]. Increased OS due to progressive declines in antioxidant activity is a proposed mechanism of age-related neurological deterioration in older adults [81, 82]. Several studies show that consumption of berries rich in antioxidants may effectively thwart neurological deterioration associated with aging [83, 84].

In an experiment conducted by Joseph et al., dopamine (DA) treatment induced OS in fibroblast cells transfected with the striatal muscarinic receptors (MAChr) subtypes M1 and M3 AChR by increasing activation of pCREB and pPKCγ [85]. The MAChr subtypes M1 and M3 AChR exhibit increased sensitivity to OS and are implicated in neurodegeneration making them reliable markers of OS-induced dysfunction. The authors found that treatment with blueberry extract decreased dopamine- (DA-) induced upregulation of the oxidative mediators, CREB and pPKCγ, indicating a significant antioxidant effect [85].

Bickford and colleagues examined the effect of blueberry supplementation upon antioxidant activity in aged rats along with corresponding neurological pathways and behavioral outcomes [86]. In the cerebellar noradrenergic system, reduced β-adrenergic function is associated with motor learning deficits in animal models. For example, cerebellar Purkinje cells in aged rats show a 30% response to β-adrenergic (GABA) potentiation, compared to 70% in young rats. Furthermore, such changes correlate with impaired performance of motor learning and coordination. Bickford and colleagues found evidence that blueberry-fed aged rats had significant improvements in GABA potentiation and increased GSH compared to aged controls. In addition, blueberry-fed aged rats performed rod-running motor tasks at a faster pace compared to controls. The reported findings show that blueberries contain a compound that is capable of increasing GSH antioxidant activity and cerebellar Purkinje cell GABA potentiation resulting in enhanced psychomotor performance in aged rats. Comparable findings were obtained by Malin and colleagues who demonstrated that aged rats maintained on a 1- or 2-month blueberry diet showed significantly higher object memory recognition compared to control rats [87]. The cognitive benefits were seen after termination of the blueberry intervention diet where the 2-month blueberry diet had a longer benefit compared to the 1-month diet suggesting a time-dependent neuroprotective benefit.

Pathologic examination of the cerebellum, cortex, and hippocampal regions of blueberry fed rats that revealed significant expression of blueberry-derived polyphenolic compounds in regions important for learning and memory assessed the impact of blueberry supplementation on brain tissue [88]. The findings suggest that blueberry-derived compounds exert neuroprotective effects by crossing the blood brain barrier and altering central nervous system signals. The study results found that accumulation of polyphenolic compounds in the cortex correlated with Morris water maze (MWM) performance, which indicates a possible risk-reducing relationship between blueberry-derived polyphenolic compounds and memory and spatial learning abilities. Furthermore, in experiments performed by Casadesus et al., supplementation with blueberry extract was shown to enhance hippocampal plasticity and increase levels of insulin-like growth factor (IGF-) 1, IGF-2, and ERK resulting in improved spatial memory [89]. The findings are considerable because age-related memory decline has been attributed to hippocampal deficiencies that are mediated by IGF-1, IGF-2, and ERK pathways.

The beneficial effects of blueberries in the modulation of neurological function may also be applicable to clinical conditions such as stroke and AD. In a study conducted by Sweeney et al., which examined the role of dietary blueberry extract on ischemic brain damage outcomes in rat stroke models, rats that were treated with 14.3% blueberry extract for six weeks had 17% loss of hippocampal neurons compared to 40% in control rats [90]. In experiments using the APP/PS1AD mouse model, it was determined that blueberry supplementation reversed deleterious effects of aging due to observations that blueberry fed APP/PS1 mice did not exhibit deficits in maze performance or have high amyloid beta burden compared to controls [84].

The neuroprotective effects of a blueberry-enriched diet are numerous, and several studies have sought to identify and explain the blueberry-derived compound responsible for the multiple modulatory effects of blueberry supplementation in animal models. To determine whether pterostilbene was involved in neuroprotective outcomes, Joseph and colleagues treated aged rats with low (0.004%) and high (0.016%) dose pterostilbene and evaluated endpoints of cognitive and motor functions [83]. The study results show that pterostilbene fed aged rats performed better on cognitive and motor tasks compared to controls in a dose-dependent manner. Specifically, aged rats treated with pterostilbene had higher level MWM performance, which was similarly shown in a blueberry supplementation study conducted by Andres-Lacueva and colleagues [88]. The study findings suggest that pterostilbene may be involved in modulation of neural plasticity and associated cognitive and motor functions.

Furthermore, Joseph et al. found that low and high dose pterostilbene fed rats had serum levels of 3.951 ± 0.439 ng/mL and 25.576 ± 5.411 ng/mL pterostilbene, respectively [83]. Subsequent pathological examination of hippocampal samples found detectable levels of pterostilbene in high dose fed rats but did not reveal detectable levels in low dose fed animals. Hippocampal levels of pterostilbene correlated with working memory performance that suggests that improvements in neurological function may be directly related to pterostilbene consumption.

In a study performed by Chang and colleagues, the antioxidant potential of pterostilbene was examined in the accelerated aging mouse model SAMP8 to determine a possible relationship between the antioxidant capacity of pterostilbene and neurological markers of disease [91]. SAMP8 mice exhibit increased OS, hyperphosphorylation of tau, and cognitive decline, which were ameliorated in mice that were fed 120 mg/kg pterostilbene for eight weeks. Pterostilbene fed SAMP8 mice also showed improved performance in radial arm water maze trials and significant changes in MnSOD, PPAR-α, phosphorylated JnK, and phosphorylated tau, all of which play an important role in the pathology of AD.

Pterostilbene-induced upregulation of MnSOD in SAMP8 mice indicates a modifiable defense mechanism against the harmful effects of ROS associated with neurological decline. Pterostilbene was also shown to increase levels of PPAR-α, an upstream inducer of MnSOD, and decrease levels of phosphorylated JnK and tau, both of which are associated with OS signaling dysfunction [91]. Pterostilbene-induced upregulation of PPAR-α may have potential clinical benefits since PPAR agonists have been shown to confer central nervous system protection and be therapeutic after stroke [96].

Overall, the antioxidant capacity of pterostilbene has significant effects upon neurological function that may translate into clinical benefits in human subjects. The free radical theory of aging claims that ROSs are involved in the pathogenesis of age-related neurological decline. Moreover, several studies suggest that AD results from decreased activity in major antioxidant defense systems and subsequent increased vulnerability to OS [80, 81]. Blueberry-induced increases in GSH and pterostilbene's ability to increase PPAR-α and MnSOD may abrogate the deleterious effects associated with aging and lead to improved cognition and motor function in older adults and those diagnosed with AD. Additional research is needed to evaluate clinical outcomes associated with pterostilbene treatment in AD and other severe forms of dementia.

2.8. Prostate. Epidemiological trials have shown an association between poor diets and increased risk of prostate cancer [97]. Consumption of dietary antioxidants is thought to reduce prostate cancer risk in some men by reducing inflammation and OS [97]. Specifically, blueberry juice was shown to inhibit proliferation and regulate cell cycle dysfunction in prostate cancer cells [16, 17, 58]. Schmidt and colleagues found that blueberry anthocyanins inhibited cell growth of prostate cancer by 11% and inhibited adhesion of *Escherichia coli*, the bacteria primarily associated with urinary tract infections [58]. Matchett and colleagues discovered that blueberry treatment decreased activity of metastasis mediators MMP-2 and MMP-9 through alteration of protein kinase C (PKC) and mitogen-activated protein (MAP) kinase pathways and increased endogenous tissue inhibitors of metalloproteinases (TIMPs) [92, 93].

It has been postulated that the anticarcinogenic effect of blueberries in prostate cancer is predominantly a result of the anticancer mechanisms of pterostilbene. Studies show that pterostilbene treatment inhibits prostate cancer proliferation and reduces metastatic potential. In p53 wildtype prostate cancer cells, pterostilbene prevented cell cycle progression at the G1 phase by inducing p53 expression and upregulating p21 expression maintaining tight control of proliferation; however, in p53 negative PC3 cells, pterostilbene induced apoptosis [94]. Such findings may help to explain the beneficial effects of pterostilbene in normal cells in contrast to the cytotoxic effects observed in cancerous cells.

Pterostilbene treatment also modified the antioxidant activity of prostate cancer cells suggesting a possible relationship between mechanisms of oxidation and apoptosis. Chakraborty and colleagues found that pterostilbene modified Bcl-2, Bax, and caspase 3, markers of mitochondrial apoptosis, and increased expression of the antioxidant enzymes GPx, GR, and GSH by 1.4-, 1.6-, and 2.1-fold in prostate cancer cells [21]. The same study also determined that pterostilbene increased levels of ROS by 5-fold, which is thought to play a role in the facilitation of mitochondrial depolarization leading to intrinsic apoptosis.

The findings demonstrate the antioxidant properties of pterostilbene in human prostate cancer cells through upregulation of the enzymes GPx, GR, and GSH. The paradoxical increase in ROS production in pterostilbene treated cells may occur through alteration of specific carcinogenic mutations present in prostate cancer that lead to programmed cell death. The findings indicate that pterostilbene is capable of inducing apoptosis through ROS-mediated mechanism in prostate cancer cells, despite upregulation of basal antioxidant activity.

In a study by Wang and colleagues, pterostilbene treatment inhibited cell viability, induced cell cycle arrest at the G1/S phase, and upregulated cyclin-dependent kinase inhibitors, CDNK1A and CDNK1, in prostate cancer [95]. Pterostilbene also decreased prostate-specific antigen (PSA),

Table 1: Antioxidant and disease modification mechanisms of pterostilbene.

Cell type	Mechanism	References
Breast		
Blueberry	↑Mammary branching, ↑PTEN, ↓mitotic rate	Wu et al. [15]
	↓Proliferation	Seeram et al. [16], Boivin et al. [17]
	↓Proliferation, ↓PI3K/AkT/NK-κB, ↓MMP, ↓Ki-67, ↑caspase 3	Adams et al. [18]
	↓Cell viability	Remsberg et al. [12]
Pterostilbene	↓Cell viability, ↑apoptosis, ↑mitochondrial depolarization, ↑superoxide anion, ↑caspase 3/7	Alosi et al. [19]
	↑Caspase 3/7, ↑S phase, ↑superoxide anion, ↓cell viability, ↑apoptosis Synergistic inhibition with Tamoxifen	Mannal et al. [20]
	↑Apoptosis, ↑caspase 3, ↑GPx, ↑Bax, ↑p53, ↓Bcl-2, ↓Akt, ↓MMP ↑Autophagy, ↓mitotic and metastatic potential	Chakraborty et al. [21, 22]
	↑Bax, ↑cytochrome C, ↑Smac/Diablo, ↑MnSOD	Moon et al. [23]
Cardiovascular		
Blueberry	↑Mitochondrial depolarization threshold, ↓myocardial infarction size ↑Ejection fraction	Ahmet et al. [30]
	↑SOD1, ↑SOD2, ↑GSR, ↑TR-1, ↓atherosclerosis lesion	Wu et al. [31]
Pterostilbene	↓ROS in HMVECs	Youdim et al. [32]
	↓Proliferation, ↓Akt, ↓CDK, ↓cyclin, ↓Rb, ↓PCNA in VSMCs	Park et al. [33]
	↓oxLDL-induced apoptosis, ↓oxidative stress, ↓MMP, ↓Caspase 3/9 ↑Autophagy, ↓LOX-1 signaling ↓NF-κB, ↓Bax and p53 in VECs	Zhang et al. [34, 35]
	↓ACE activity, ↓LH in smokers	McAnulty et al. [39]
Esophagus		
Blueberry	↑Antioxidant activity, ↓IL-5, ↓GRO/KC	Stoner et al. [41]
Stomach		
Blueberry	↓Proliferation	Boivin et al. [17]
Pterostilbene	↓Proliferation, ↑apoptosis, ↑cytochrome C, ↑caspases 1, 2, 3, 8 and 9 ↓Bcl-XL, ↑Bad, ↑Bax	Pan at el. [43]
Colon		
Blueberry	↓Proliferation	Seeram et al. [16], Boivin et al. [17]
Pterostilbene	↓Cell viability	Remsberg et al. [12]
	↓Aberrant crypt foci, ↓iNOS, ↓COX-2, ↑MUC-2	Suh et al. [46]
	↓Aberrant crypt foci, ↓lymphoid nodules, ↓NF-κB ↓iNOS, ↓COX-2, ↑HO-1, ↑GR, ↓Aldose reductase	Chiou et al. [47]
	↓Tumor volume, ↓NF-κB, ↓Bcl-2, ↑Bax, ↑Bak, ↑Bad ↑Bid, ↑SOD2 ↑catalase, ↑GPx, ↑GR, ↑TR-1, synergistic inhibition with FOLFOX	Priego et al. [48]
Hematology		
Blueberry	↓ROS	Youdim et al. [51]
Pterostilbene	↓AAPH-induced hemolysis, ↓GSH depletion, ↓lipid peroxidation	Mikstacka et al. [52]
Liver		
Blueberry	↑ Nqo1, ↑SOD, ↑GST, ↑Nrf-2, ↓MDA, ↓HA, ↓ALT ↑Nrf-2, ↑Nqo1, ↑HO-1, ↑T-lymphocytes	Wang et al. [55, 56]
	↓ALT, ↓bilirubin, ↑GSH, ↓TNF-α, ↓IL-1β, ↓lipid peroxidation	Osman et al. [57]
	↓Proliferation of hepatic cancer cells	Schmidt et al. [58]
Pterostilbene	Protect gap junctional intercellular communication ↑Dephosphorylation of Cx43	Kim et al. [59]
	↓Proliferation of hepatic cancer cells	Remsberg et al. [12]
	↓Cell viability, ↑antioxidant activity	Hasiah et al. [60]
	↓Micrometastasis↓PI3K, ↓Akt, ↓NF-κB, ↓MMP-9, ↓VEGF, ↓EGF ↓Lung metastasis	Pan et al. [61]

Table 1: Continued.

Cell type	Mechanism	References
Pancreas		
Pterostilbene	↓Cell viability, ↑apoptosis, ↑caspase 3/7, G0/G and S phase arrest	Mannal et al. [63]
	↓Tumor volume, ↑apoptosis gene expression, ↑cytochrome C, ↑MnSOD ↑Smac/DIABLO, ↓JAK/STAT3, ↓lipase secretion, ↓IL-1β, ↓IL-6	McCormack et al. [64, 70]
	Synergistic inhibition of cancer proliferation with antioxidant EGCG	Kostin et al. [69]
Metabolic		
Diabetes		
Blueberry	↑SOD, ↑GPx, ↓HbA1c	Nemes-Nagy et al. [77]
Pterocarpus marsupium	↓Hyperinsulinemia, ↓hypertriglyemia, ↓hyperglycemia	Grover et al. [73]
Pterostilbene	↓Plasma glucose, ↓rat body weight	Manickam et al. [74]
	↑Plasma insulin, ↓plasma glucose, ↓HbA1c, ↓glucose-6-phosphatase ↓Fructose-1, 6-biphosphatase, ↑hexokinase, comparable to metformin	Pari and Satheesh [75]
	↓Oxidative stress, ↑GSH, ↑GST, ↑SOD, ↑GPx, ↑catalase ↓Portal triad inflammation, ↓renal damage	Satheesh and Pari [76]
Hyperlipidemia		
Pterostilbene	↓Preadipocyte growth, ↓fat droplet formation, ↓triacylglycerol	Hsu et al. [78]
	↑Adiponectin, ↓leptin, ↓LDL, ↑HDL, ↓PPAR-γ, ↓C/EBP-α, ↓resistin ↓FAS, comparable to clofibrate	Rimando et al. [79]
Neurology		
Blueberry	↑Maze performance, ↓amyloid beta burden, ↓CREB, ↓pPKCγ	Joseph et al. [84, 85]
	↑GABA potentiation, ↑GSH	Bickford et al. [86]
	↑Object memory recognition	Malin et al. [87]
	↑Maze performance	Andres-Lacueva et al. [88]
	↑Hippocampal plasticity, ↓IGF-1, ↓IGF-2, ↓ERK	Casadesus et al. [89]
Pterostilbene	↓Ischemic brain damage	Sweeney et al. [90]
	↑Maze performance, ↑cognitive performance	Joseph et al. [83]
	↑MnSOD, ↑PPAR-α, ↓phosphorylated JnK and tau	Chang et al. [91]
Prostate		
Blueberry	↓Cancer cell growth	Schmidt et al. [58]
	↓MMP, ↑TIMP	Matchett et al. [92, 93]
Pterostilbene	↓Cell viability	Remsberg et al. [12]
	↑Apoptosis, ↑caspase 3, ↑GPx, ↑GR, ↑Bax, ↑p53, ↓Bcl-2, ↓Akt ↓MMP, ↑GSH	Chakraborty et al. [21]
	↑Apoptosis, ↑p53, ↑p21, G1 phase arrest	Lin et al. [94]
	↑Apoptosis, G1/S phase arrest, ↑CDNK1A and CDNK1B, ↓PSA	Wang et al. [95]

a human marker of prostate malignancy, indicating potential use as a chemotherapeutic agent [95]. Currently, the preventive and chemotherapeutic potential of pterostilbene in human prostate cancer has not been established; however, the evidence suggests that pterostilbene may have alternate effects on prostate cells based upon genetic composition of each cell, becoming beneficial in the regulation of normal prostate cells and producing inhibition in cancerous cells. Further studies are warranted to investigate the relationship between the antioxidant effects of pterostilbene and clinical outcomes in prostate cancer.

3. Discussion

The antioxidant activity of pterostilbene is an essential component of the compound's interrelated mechanisms of disease inhibition, and the studies presented in this review show that the mechanisms of pterostilbene are comparable to mechanisms exhibited by blueberry treatment in similar disease models (Table 1). The overlap is significant because blueberries are a widely consumed fruit comprised of various concentrations of pterostilbene with proven high antioxidant capacity [3, 4, 98]. Although it is postulated that the pterostilbene component of blueberries exerts clinical benefits, the direct correlation between pterostilbene's therapeutic effects and blueberry consumption remains undetermined.

The results presented in this review exemplify pterostilbene's complicated effect upon antioxidant activity and critical pathways of pathogenesis in multiple organ systems. The benefits of pterostilbene are vast and include neuroprotection, inhibition of malignancy, attenuation of atherosclerosis, protection against hemolysis and liver disease, and metabolic regulation of DM and hyperlipidemia. In breast, esophageal,

stomach, colon, liver, pancreatic, and prostate cancer studies, pterostilbene exhibits profound anticancer mechanisms which include reduction of proliferation rates, induction of apoptosis, alteration of the cell cycle, and inhibition of metastasis [5]. The relationship between pterostilbene and oxidation in cancer cell death has not been fully elucidated; however, it has been discovered that generation of ROS plays a significant role in the apoptotic mechanism in pterostilbene treated breast and prostate cancer cells [19–22]. In contrast, treatment with pterostilbene increased antioxidant activity in esophageal, pancreatic, and colon cancer models but still exerted effective anticarcinogenic effects [41, 47, 48, 64]. The differences in pterostilbene's oxidative influences among cancer cell types may possibly be attributed to the distinctive daily functions of digestion which occur in the esophagus, pancreas, and colon but are absent in the breast and prostate.

Furthermore, numerous studies show that pterostilbene mechanisms vary in each disease system and are tailored toward the correction of aberrant cellular pathways and progressive dysfunction. In disease models of aging, vascular disease, diabetes, and hemolysis, pterostilbene decreases oxidative stress most likely as a protective measure against the progressive cellular damage and dysfunction associated with disease-related deterioration [34, 52, 75, 76, 91]. Interestingly, pterostilbene treatment may upregulate or downregulate specific pathways based upon the nature of the disease process taking place. For example, pterostilbene is efficacious as an anticancer agent because it induces apoptosis in cancer cells; however, the compound has the opposite effect in the vascular system where it inhibits apoptosis in VECs thereby decreasing the risk of plaque instability [5, 34]. Furthermore, in models of hyperlipidemia, pterostilbene increased expression of PPAR-γ, a target for lipid lowering agents, but exerted the opposite effect in AD models where it increased PPAR-α, a key modulator of neural antioxidant activity [79, 91].

Pterostilbene was also shown to exhibit comparable and synergistic effects when compared to medications used in the treatment of human disease, specifically clofibrate, metformin, Tamoxifen, and the chemotherapy regimen FOLFOX indicating that pterostilbene's therapeutic effects may be applicable if administered to human subjects [20, 48, 75, 79]. Additional possible human benefits of pterostilbene include reduction of the clinical markers HbA1C in diabetes and PSA in prostate cancer which was demonstrated by Pari and Satheesh and Wang et al., respectively [75, 95]. However, it is unknown if the beneficial effects of pterostilbene demonstrated *in vitro* and *in vivo* occur in humans as well.

In a recent randomized double-blind placebo-controlled trial, Riche and colleagues report that 100 mg to 250 mg daily of pterostilbene in adults with hyperlipidemia did not produce significant adverse drug events [99]. In addition, treatment with 450 mg daily *Pterocarpus marsupium* extract in healthy volunteers did not produce signs of toxicity and resulted in detectable pterostilbene serum levels up to two weeks after administration [100]. The reported findings show that pterostilbene is safe for administration to humans and further contributes to our understanding of the clinical effects of pterostilbene. Further research should include study designs aimed to delineate pterostilbene's contribution to the antioxidant effects of blueberries in diverse preclinical and clinical disease models. Additional directions should focus upon the creation of human population studies and clinical trials to evaluate the safety and efficacy of pterostilbene in the prevention and treatment of disease.

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Research Article

Therapeutic Effect of MG132 on the Aortic Oxidative Damage and Inflammatory Response in OVE26 Type 1 Diabetic Mice

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The present study tested whether MG132 increases vascular nuclear factor E2-related factor-2 (Nrf2) expression and transcription to provide a therapeutic effect on diabetes-induced pathogenic changes in the aorta. To this end, three-month-old OVE26 diabetic and age-matched control mice were intraperitoneally injected with MG-132, 10 μg/kg daily for 3 months. OVE26 transgenic type 1 diabetic mice develop hyperglycemia at 2-3 weeks of age and exhibit albuminuria at 3months of age with mild increases in TNF-α expression and 3-NT accumulation in the aorta. Diabetes-induced significant increases in the wall thickness and structural derangement of aorta were found in OVE26 mice with significant increases in aortic oxidative and nitrosative damage, inflammation, and remodeling at 6 months of diabetes, but not at 3 months of diabetes. However, these pathological changes seen at the 6 months of diabetes were abolished in OVE26 mice treated with MG-132 for 3 months that were also associated with a significant increase in Nrf2 expression in the aorta as well as transcription of downstream genes. These results suggest that chronic treatment with low-dose MG132 can afford an effective therapy for diabetes-induced pathogenic changes in the aorta, which is associated with the increased Nrf2 expression and transcription.

1. Introduction

Diabetes mellitus is a chronic disease that affects many people worldwide now. There are two major types of diabetes mellitus: type 1 (insulin dependent) diabetes and type 2 (noninsulin dependent) diabetes. Systemic complications are major cause of the mortality in these patients with either type 1 or type 2 diabetes. These complications are divided into two groups: microvascular complications, which include retinopathy, nephropathy, and neuropathy; and macrovascular complications, which include coronary artery disease, atherosclerosis, and peripheral vascular disease.

Increasing evidence indicates that increased production of reactive oxygen or nitrogen species (ROS or RNS) and/or impaired endogenous protective mechanisms is the major risk factor responsible for the development and progression of vascular complications in diabetic patients, although several other mechanisms were also proposed [1, 2]. Exogenous supplementation of a single or a few antioxidants in clinics often fails to efficiently prevent or treat various complications for diabetic patients; therefore upregulation of multiple, endogenous antioxidants may be a better approach for the prevention of diabetic cardiovascular complications.

Nuclear factor E2-related factor-2 (Nrf2), as a transcription factor in regulation of various antioxidative and cytoprotective responses to oxidative stress, has been shown to play an important role in cellular prevention against oxidative stress and damage *in vitro* and *in vivo* [3, 4]. Actin-tethered protein Keap1 is a cytosolic repressor that binds to and retains Nrf2 in the cytoplasm, which promotes Nrf2 proteasomal degradation so as to prevent Nrf2 activation of its transcription [3, 4]. Via blocking Nrf2 proteasomal degradation, therefore, proteasome inhibitors are able to retain Nrf2 in cytoplasm and/or to translocate into nuclei. Therefore, proteasomal inhibitors have become an attractive approach to activate Nrf2-mediated anti-oxidative pathway in the prevention of various oxidative stress-initiated diseases [5]. Among proteasome inhibitors, MG132 is specific, potent, reversible, and cell permeable and plays a key role in blocking the degradation of ubiquitin-conjugated proteins in mammalian cells by the 26S complex without affecting its ATPase or isopeptidase activities [6].

Reportedly, up-regulation of Nrf2 by its activators, including MG132, has been found to potentially be a preventive approach for diabetic complications [5, 7–9], including diabetes-induced vascular injuries [10–13]. However, there was no report yet whether, upregulating Nrf2 can also efficiently cure or slow the progression of established diabetic complications.

Therefore, the present study was to examine the therapeutic effect of chronic treatment with low-dose MG132 on the pathogenic changes of aortas in the transgenic (OVE26) diabetic mouse model. The treatment was started when OVE26 mice exhibited significant increase in albuminuria at 3 months of age. The expression of Nrf2 and its downstream antioxidant genes were examined. We found that the progression of aortic pathogenic change was almost completely stopped by 3-month treatment with MG132, which was associated with a significant upregulated expression of Nrf2 and its downstream antioxidant genes.

2. Methods

2.1. Animals. OVE26 type 1 diabetic mice with FVB background were generated and provided for this study by Epstein et al. [14, 15]. Mice were housed in the University of Louisville Research Resources Center at 22°C with a 12-h light/dark cycle and provided with free access to standard rodent chow and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care.

These OVE26 mice normally develop severe hyperglycemia 2-3 weeks after birth and develop macroproteinuria significantly at 3 months of age [14, 15]. Sixteen 3-month-old female OVE26 mice were randomly divided into two groups: diabetes (DM, $n = 10$) and diabetes treated with MG132 (DM/MG132, $n = 6$). Sixteen age- and sex-matched wild-type (FVB) mice were also randomly divided into two groups: nondiabetic control (control, $n = 10$) and MG132 control (MG132, $n = 6$). MG132 (Sigma-Aldich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.0025 μg/mL. For MG132 and DM/MG132 mice, MG132 was given intraperitoneally at 10 μg/kg daily for 3 months, while control and DM group mice were administered with equal amounts of MG132 vehicle. The dose of MG132 was selected based on a recent study [16], which is significantly lower than that used in other studies to efficiently protect the heart from oxidative damage [17, 18]. Four mice from both control and DM group were sacrificed at 3 months of age, and the rest (6 mice in each group) were sacrificed at the end of 3-month treatment with MG132 (i.e., at 6 months of age).

2.2. Noninvasive Blood Pressure. Blood pressure (BP) was measured by tail-cuff manometry using a CODATM non-invasive BP monitoring system (Kent Scientific, Torrington, CT, USA). Mice were restrained in a plastic tube restrainer. Occlusion and volume-pressure recording (VPR) cuffs were placed over the tail. Mice were allowed to adapt to the restrainer for 5 min prior to starting BP measurement. After a 5 min adaptation period, BP was measured for 10 acclimation cycles followed by 20 measurement cycles. Mice were warmed by heating pads during the acclimation cycles to ensure sufficient blood flow to the tail. Animals were monitored closely throughout the measurement protocol and removed from restraint as soon as possible upon completing the measurement protocol [19]. After three days of training for the BP measurement, formal measurements were performed and BP data were collected.

2.3. Aorta Preparation and Histopathological Examination. After anesthesia, thorax was opened, and descending thoracic aortas were isolated carefully and cleaned of the surrounding fat and connective tissue. Aortic tissues were fixed in 10% buffered formalin and then cut into ring segments (2-3 mm in length) for being dehydrated in graded alcohol series, cleared with xylene, embedded in paraffin, and sectioned at 5 μm thickness in order to perform pathological and immunohistochemical or immunofluorescent examination, as described in previous study [20]. Histological evaluation of aorta was performed by H&E staining with Image Pro Plus 6.0 software for measuring the means of the tunica media width size as the thickness of aortic tunica media.

For immunohistochemical or immunofluorescent staining, paraffin sections were dewaxed, followed by incubation with 1X Target Retrieval Solution (Dako, Carpinteria, CA, USA) in a microwave oven for 15 min at 98°C for antigen retrieval, with 3% hydrogen peroxide for 15 min at room temperature, and then with 5% animal serum for 30 min, respectively. These sections were incubated with primary antibodies, including those against connective tissue growth factor (CTGF) and transforming growth factor (TGF)-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 : 100 dilution, 3-nitrotyrosine (3-NT, Millipore, Billerica, CA, USA) at 1 : 400 dilution, 4-hydroxy-2-nonenal (4-HNE, Alpha Diagnostic International, San Antonio, TX, USA) at 1 : 400 dilution, plasminogen activator inhibitor-1 (PAI-1, BD Bioscience, San Jose, CA, USA) at 1 : 100 dilution, tumor necrosis factor-alpha (TNF-α, Abcam, Cambridge,

MA, USA) at 1 : 100 dilution, Cu-Zn superoxide dismutase-1 (SOD-1) at 1 : 400 dilution, and Nrf2 (both from Santa Cruz Biotechnology) at 1 : 100 dilution, respectively, overnight at 4°C. After sections were washed with PBS, they were incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 300–400 dilutions with PBS) or Cy3-coupled goat antirabbit IgG secondary antibody for 2 h in room temperature. For the color development of immunohistochemical staining, sections were treated with peroxidase substrate DAB kit (Vector Laboratories, Inc., Burlingame, CA, USA) and counterstained with hematoxylin. For immunofluorescent staining, sections were stained with DAPI at 1 : 1000 dilution to localize the nucleus. Three sections at interval of 10 sections from each aorta (per mouse) were selected and at least five high-power fields were randomly captured per section. Image Pro Plus 6.0 software was used to transfer the interesting area staining density to an integrated optical density (IOD) that was divided by the area size of interest to reflect the staining level of the area of interest, and the ratio of IOD/area size in experimental group was presented as a fold relative to that of control.

2.4. Sirius-Red Staining for Collagen. Aortic fibrosis was detected by examining collagen accumulation with Sirius-red staining. Briefly, 5 μm thickness sections were stained with 0.1% Sirius-red F3BA and 0.25% Fast Green FCF and then assessed for the proportion of collagen with a Nikon Eclipse E600 microscopy system [21].

2.5. Real-Time RT-PCR (qPCR). Aortas were snap frozen in liquid nitrogen and kept at −80°C. Total RNA was extracted using the TRIzol reagent (Invitrogen). RNA concentrations and purities were quantified using a Nanodrop ND-1000 spectrophotometer. First-strand complimentary DNA (cDNA) was synthesized from total RNA, according to manufacturer's protocol from the RNA PCR kit (Promega, Madison, WI, USA). Reverse transcription system was performed using 1 μg of total RNA in 12.5 μL of the solution containing 4 μL 25 mM $MgCl_2$, 4 μL AMV reverse transcriptase 5X buffer, 2 μL dNTP, 0.5 μL RNase inhibitor, 1 μL of AMV reverse transcriptase, 1 μL of oligo dT primer, and nuclease-free water that was added to make a final volume of 20 μL. Reaction system was run at 42°C for 50 min and 95°C for 5 min, as described before [21].

Real-time RT-PCR (quantitative PCR, qPCR) was carried out with the ABI 7300 real-time PCR system in a 20 μL reaction buffer, composed of 10 μL of TaqMan Universal PCR Master Mix, 1 μL of primer, and 9 μL of cDNA. Primers of NADPH quinine oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), SOD1, and β-actin were purchased from Applied Biosystems (Carlsbad, CA, USA). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the target gene.

2.6. Statistical Analysis. Data collected from several animals ($n = 4$ for study at 3 months of age; $n = 6$ for study at 6 months of age) were presented as means ± SD. We used Image Pro Plus 6.0 software with an integrated optical density divided area method to identify the positive staining area of interest. Comparisons were performed by one-way ANOVA for the different groups, followed by post hoc pairwise repetitive comparisons using Tukey's test or Student's t-test with Origin 7.5 Lab data analysis and graphing software. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Pathogenic Changes in the Aorta of OVE26 Diabetic Mice at 3-Month-Old. OVE26 mice develop diabetes before 3-week old and exhibit a significant increase in albuminuria at 3 months of age, as an index of renal dysfunction [15]. To define whether there was any pathological change in aortas of 3-month-old OVE26 diabetic mice, microscopic examination of aortas with H&E staining for the general morphology (Figure 1(a)) revealed that there was no significantly morphological abnormality, except for a slight derangement of endothelial and smooth muscle cells, in the aorta of OVE26 diabetic (DM) mice at 3 months of age. Immunohistochemical staining for CTGF as an important fibrotic mediator showed no significant difference between control and DM mice (Figure 1(b)).

However, there was an increase in TNF-α expression in the aorta of DM mice compared to control mice (Figures 1(c) and 1(e)) and also an increase in 3-NT accumulation, as an index of protein nitration, in the aorta of DM mice compared to control mice (Figures 1(d) and 1(f)).

3.2. Therapeutic Effects of MG132 on the Aortic Fibrotic Response. Previous results suggest a significant increase of aortic inflammation and oxidative stress and damage in 3-month-old DM mice compared to age-matched control mice, suggesting an induction of aortic early pathogenesis. To explore the therapeutic effect of MG132 on diabetes-induced progression of aortic pathological changes, we treated 3-month-old DM and age-matched control mice with low-dose MG132 for 3 months.

Figure 2(a) showed no effect of chronic treatment with MG132 on systemic and diastolic blood pressure, although both were significantly higher in DM group than in control. H&E staining of the aortas showed that part of intima was thickened and uplifted; endothelial cells in the surface of intima were swelled and internal elastic membrane was thickened; tunica media is thickened. However, these changes observed in DM group were not observed in DM/MG132 group (Figure 2(b)). By Sirius-red staining, the collagen accumulation in the aorta was also only observed in DM group, but not in DM/MG132 group (Figure 2(c)).

Immunohistochemical staining showed that although there was no significant increase of the CTGF expression in the aorta of DM mice at 3 months of age compared to the age-matched control mice (Figure 1(b)), there was a significantly increased CTGF expression in the aorta of DM at 6 months of age compared to age-matched control mice (Figure 3(a)). Diabetes-increased CTGF expression was not seen in the aorta of DM/MG132 mice (Figure 3(a)). Similarly, aortic expression of another important profibrotic mediator,

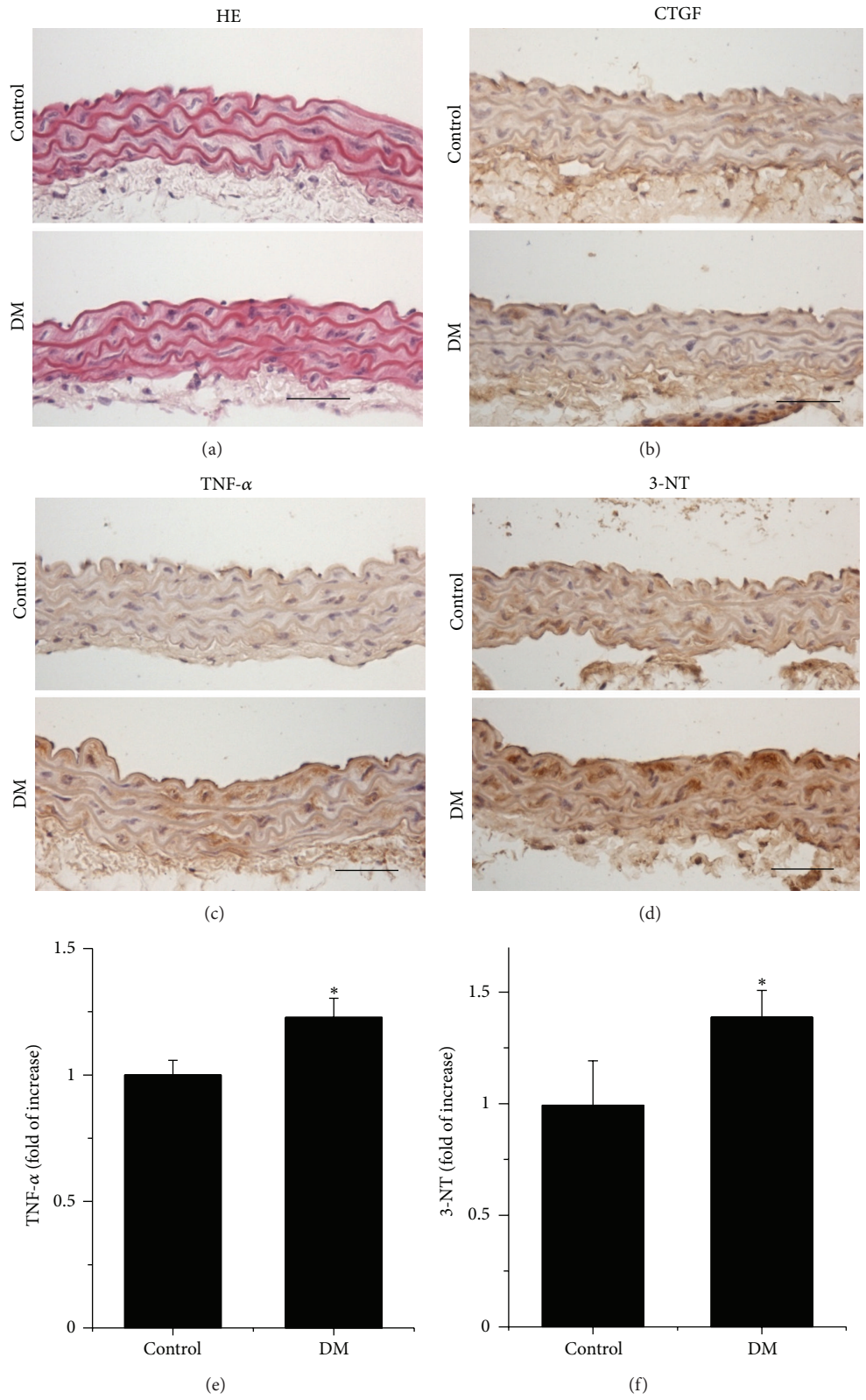

Figure 1: Diabetes-induced aortic pathological changes, inflammation, oxidative stress, fibrosis at 3 months by H&E staining (a), immunohistochemical staining for the expression of CTGF (b), TNF-α (c)-(e) as index of inflammation, and 3-NT (d)-(f) as an index of oxidative damage. Data were presented as means ± SD ($n = 4$). $^{*}P < 0.05$ versus control. Bar = 50 μM.

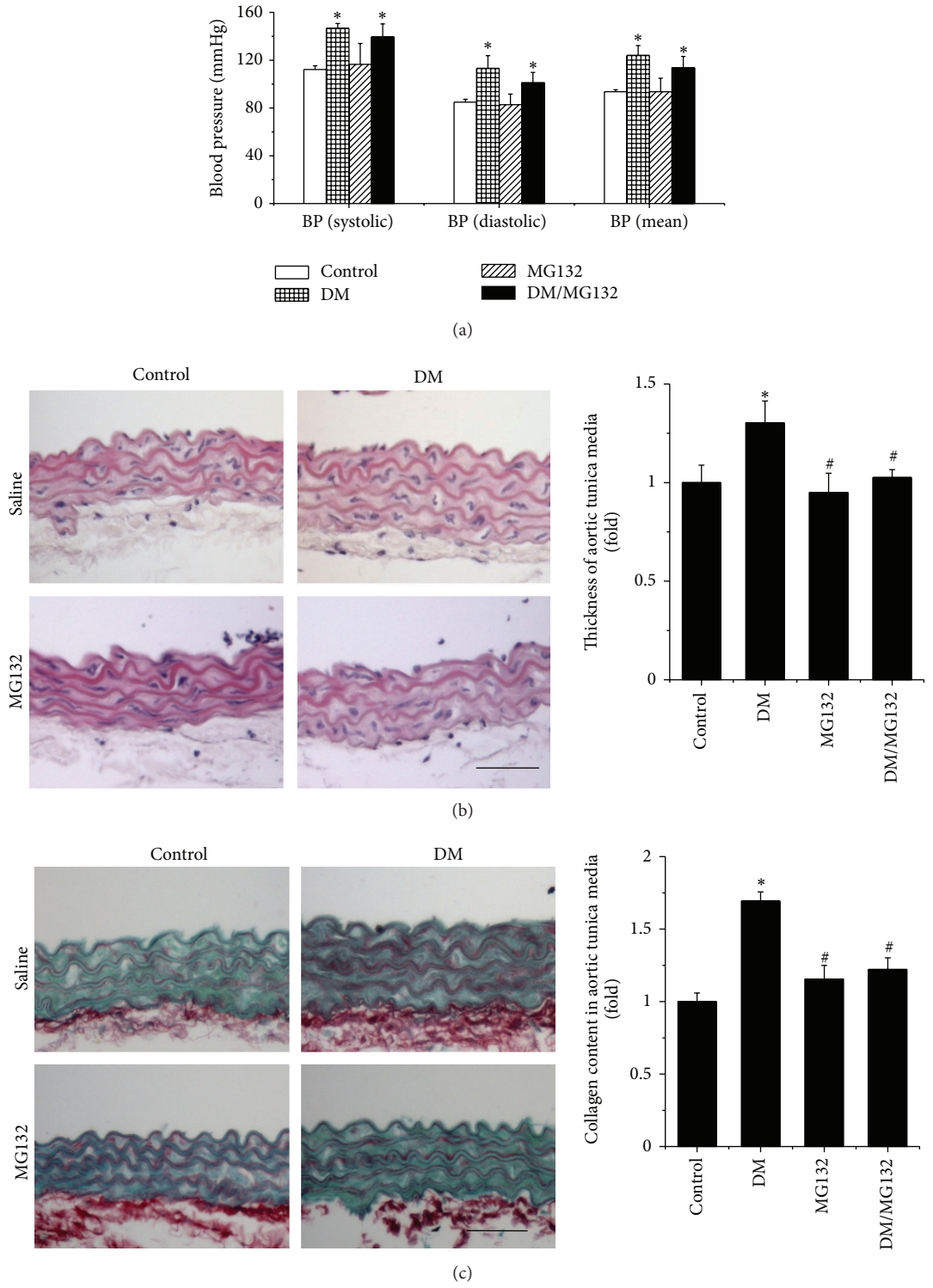

FIGURE 2: Therapeutic effect of MG132 on diabetes-induced blood pressure changes (a) and aortic pathological changes, examined by H&E staining (b), and Sirius-red staining for collagen accumulation (c) with semi-quantitative analysis. Data were presented as means ± SD ($n = 6$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus DM. Bar = 50 μM.

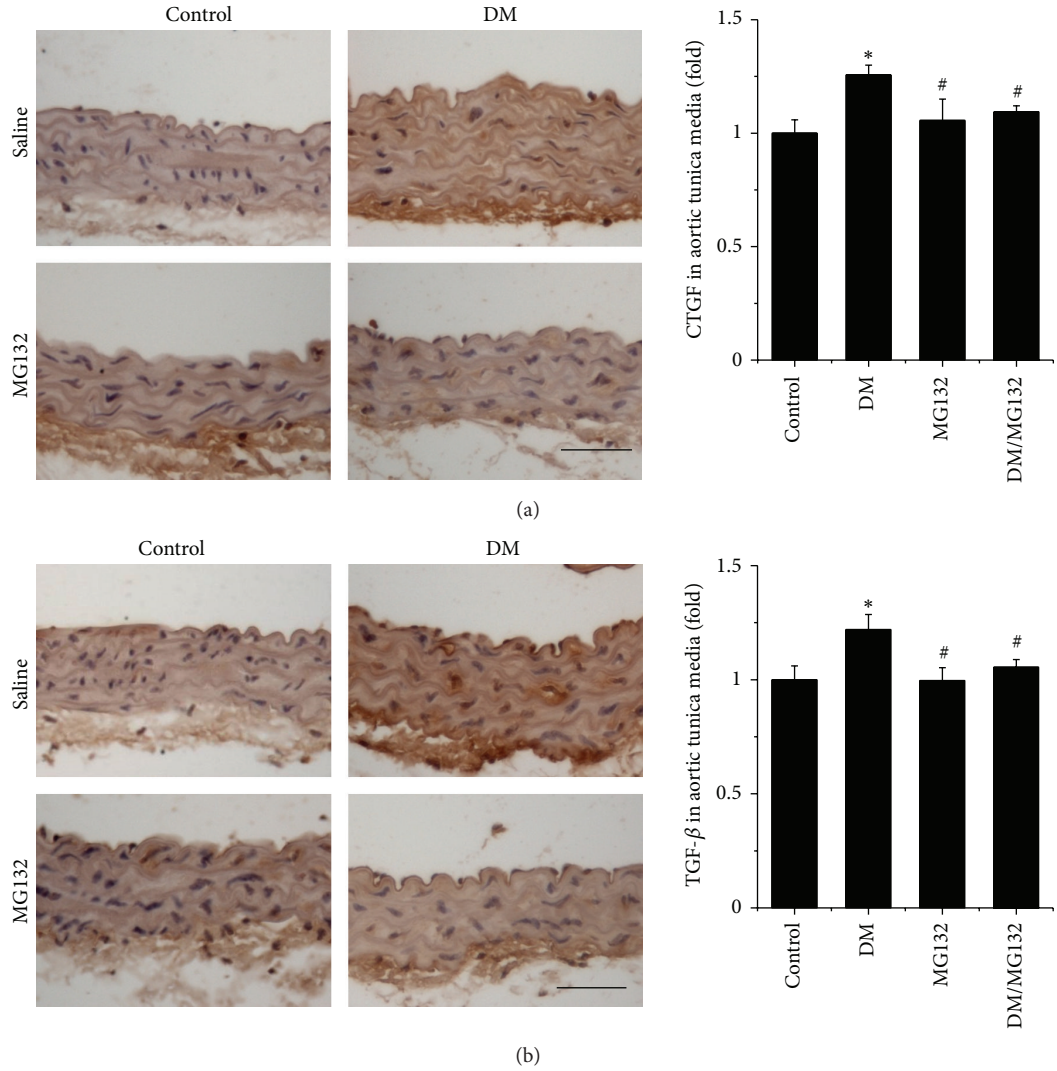

Figure 3: Therapeutic effect of MG132 on diabetes-induced aortic fibrosis by immunohistochemical staining for the expression of CTGF (a) and TGF-β1 (b) with semi-quantitative analysis. Data were presented as means ± SD ($n = 6$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus DM. Bar = 50 μM.

TGF-β1, was also observed only in the DM group and not in the DM/MG132 group (Figure 3(b)).

3.3. Therapeutic Effects of MG132 on Aortic Inflammation and Oxidative Damage. Immunohistochemical staining showed a significant increase in the aortic expression of inflammatory markers, TNF-α (Figure 4(a)) and PAI-1 (Figure 4(b)), in the aortic tunica media of DM mice at 6 months of age, which was significantly progressed compared to that of DM at 3 months of age (Figure 1(e)). However, there was no significant increase in either TNF-α (Figure 4(a)) or PAI-1 (Figure 4(b)) in the aorta of DM/MG132 mice.

Immunohistochemical staining also showed an increased accumulation of oxidative and nitrative damage: 3-NT (Figure 5(a)) and 4-HNE (Figure 5(b)), in aortic tunica media of DM mice, which were significantly attenuated by MG132 treatment.

3.4. Upregulation of Nrf2 Expression and Its Downstream Antioxidant Gene Expression by MG132 in the Aorta. Since previous pathological changes may be all attributed to the increased oxidative stress, the next study is to examine the expression and transcription of Nrf2. Figure 6(a) showed a significant increase in Nrf2 expression in the aorta of DM and MG132 mice, examined by immunofluorescent staining. There was a further increase of the Nrf2 expression in the aorta of DM/MG132 mice (Figures 6(a) and 6(b)). Furthermore, Figure 6(a) also shows an increased accumulation of Nrf2 in nuclei, suggesting the potential increase in its transcriptional function.

Analysis by qPCR revealed a significant increase in the expression of NQO1 (Figure 6(c)), HO-1 (Figure 6(d)), and SOD1 (Figure 6(e)) at mRNA level in the aorta of DM, MG132, and DM/MG132 mice with the highest expression of these endpoints in the DM/MG132 group. By

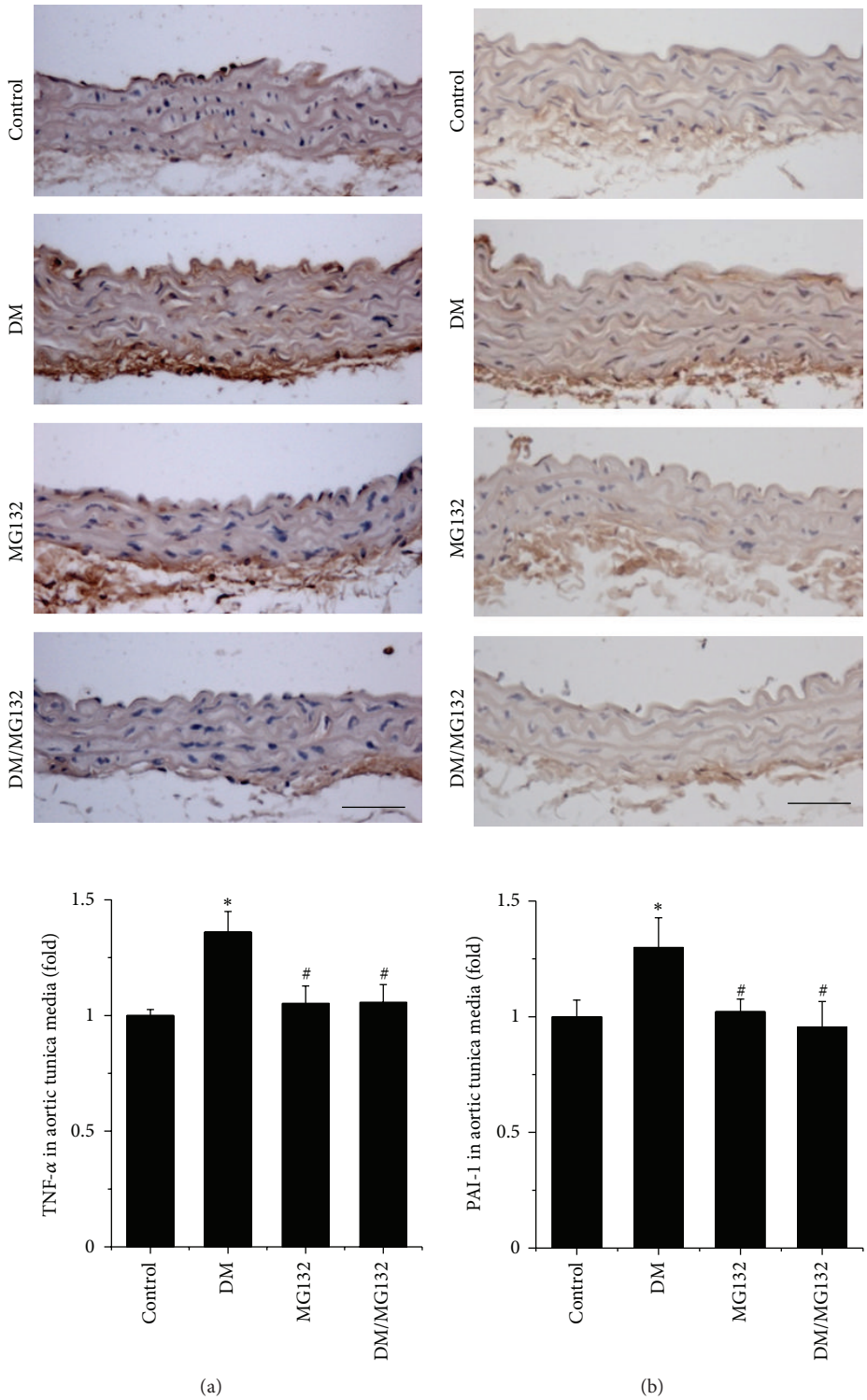

Figure 4: Therapeutic effect of MG132 on diabetes-induced aortic inflammation, examined by immunohistochemical staining for the expressions of TNF-α (a) and PAI-1 (b) followed by semi-quantitative analysis. Data were presented as means ± SD ($n = 6$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus DM. Bar = 50 μM.

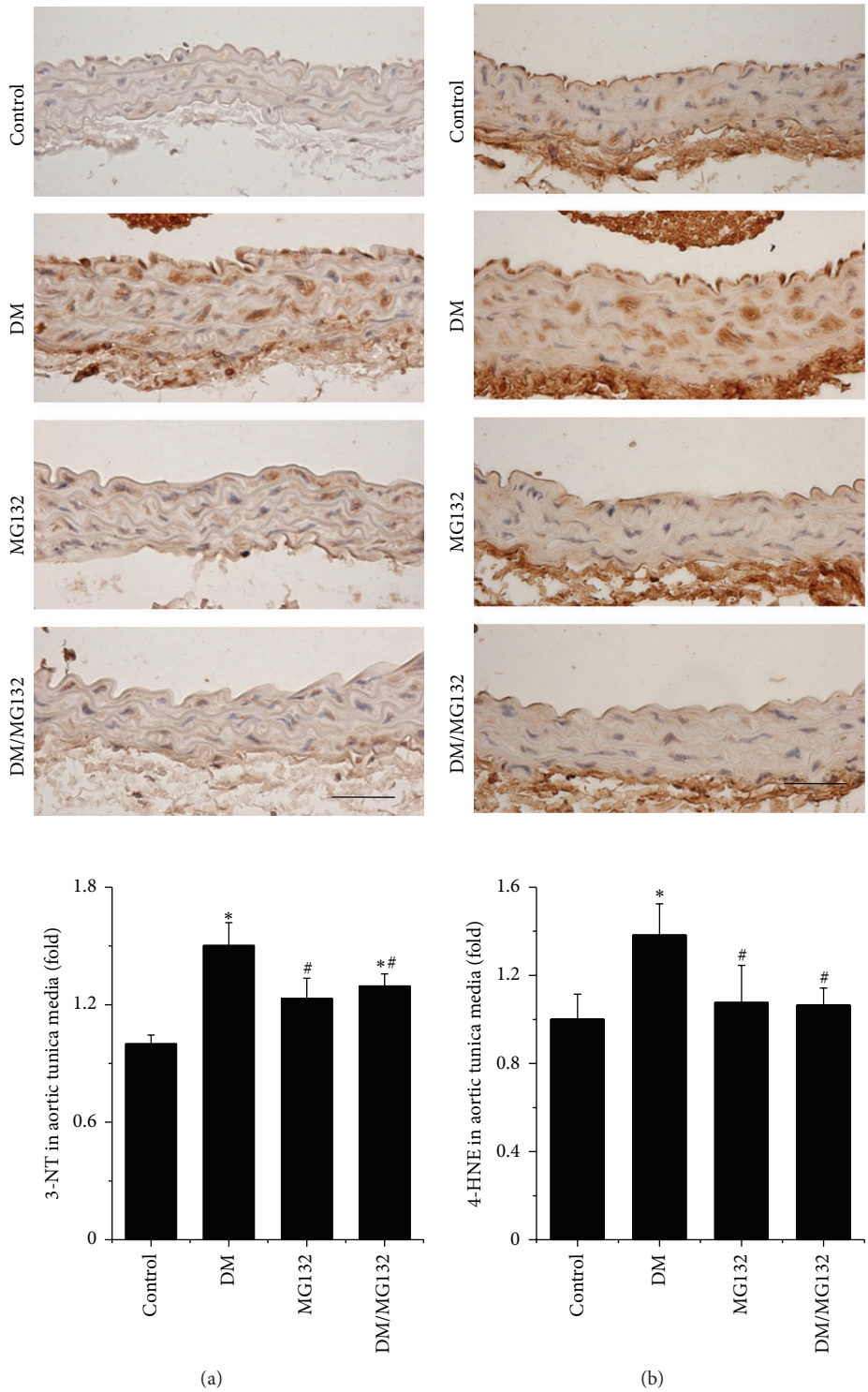

Figure 5: Therapeutic effect of MG132 on diabetes-induced aortic oxidative damage, examined by immunohistochemical staining for the accumulation of 3-NT (a) and 4-HNE (b) with semi-quantitative analysis. Data were presented as means ± SD ($n = 6$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus DM. Bar = 50 μM.

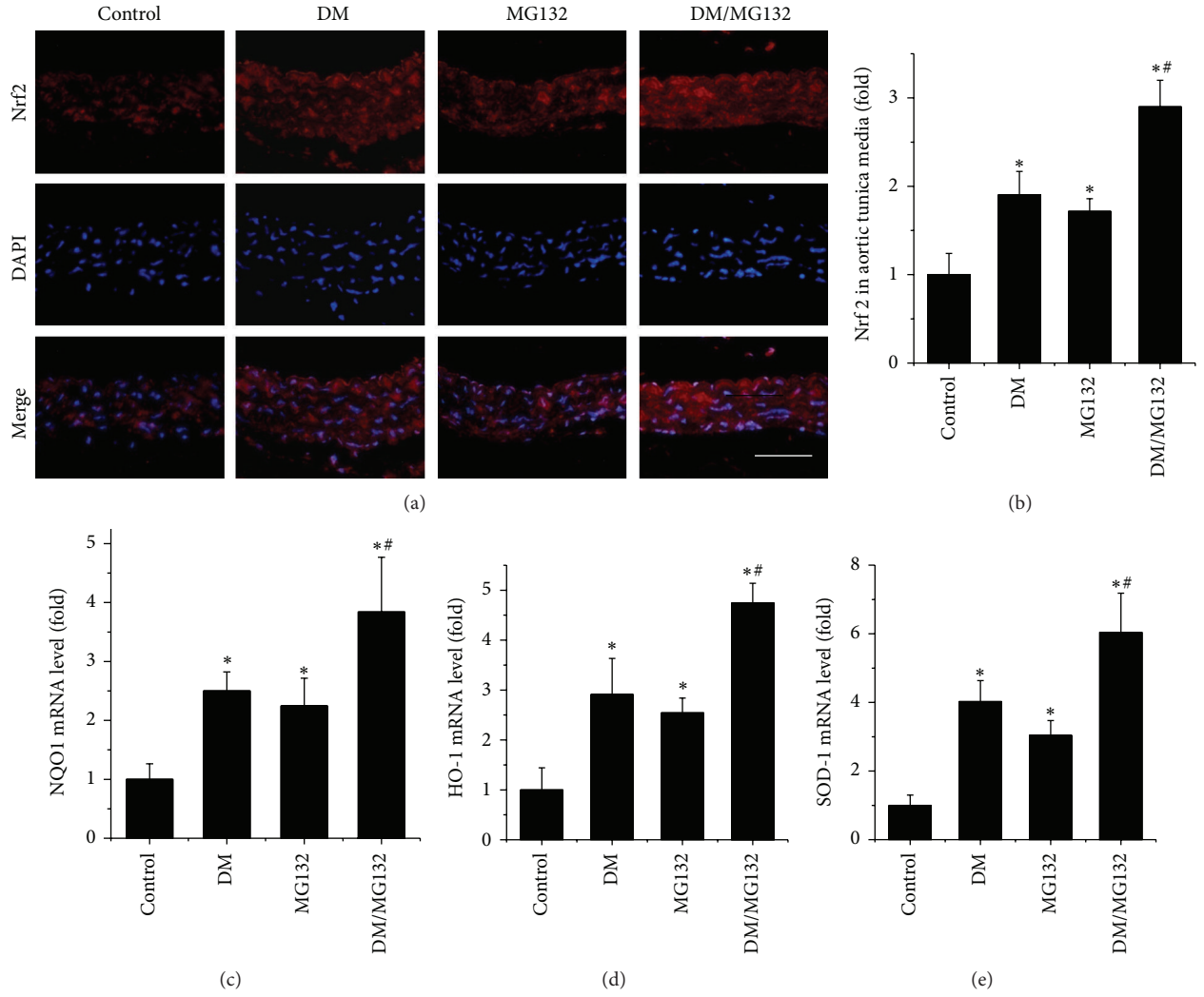

Figure 6: Effects of MG132 on aortic expression of Nrf2 and its downstream genes, examined by immunohistochemical staining for the expression of Nrf2 (red) (a) in aortic tunica media with semi-quantitative analysis (b). Real-time PCR was used to measure the expression of Nrf2 downstream genes at mRNA levels: NQO1 (c), HO-1 (d), and SOD-1 (e). Data were presented as means ± SD ($n = 6$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus DM. Bar = 50 μM.

immunohistochemical staining of SOD1, the upregulated mRNA expression was confirmed with the increased expression of its protein level (Figures 7(a) and 7(b)).

4. Discussion

The preventive effect of Nrf2 in aortic pathogeneses of several disease conditions has been appreciated; however, whether up-regulation of vascular Nrf2 can afford a therapeutic effect on oxidative stress-induced pathogenic changes in vascular system, particularly in the aorta, has not been addressed.

There were two pilot clinical trials that used the proteasomal inhibitor bardoxolone methyl (also known as CDDOMe) as a known Nrf2 activator and demonstrated certain therapeutic effects in the patients with chronic kidney diseases (CKD) and type 2 DM [22, 23]. Since in clinical study the expression of Nrf2 in the kidney of these patients was not measured, it was impossible to attribute the renal therapeutic effect of bardoxolone to Nrf2 up-regulation in these studies. In addition, there was no information regarding aortic pathological change in these studies. It is impossible to imagine whether bardoxolone induces aortic Nrf2 expression and whether aortic pathogenic changes in these diabetic patients were affected or not by bardoxolone treatment. To address such issues, animal studies have to be used.

In the study on diabetes-induced complications, streptozotocin- (STZ-) induced diabetic animals are most frequently used. However, STZ-induced diabetic animals are not perfect models for the study of diabetic complications [24] because STZ may have direct toxic effects on multiple organs [25]. OVE26 mice develop type 1 diabetes because of specific damage to β cells [14]. OVE26 diabetic mice have been previously used in the study of diabetes-induced cardiac and renal complications by Epstein's group [15, 26, 27]. Compared

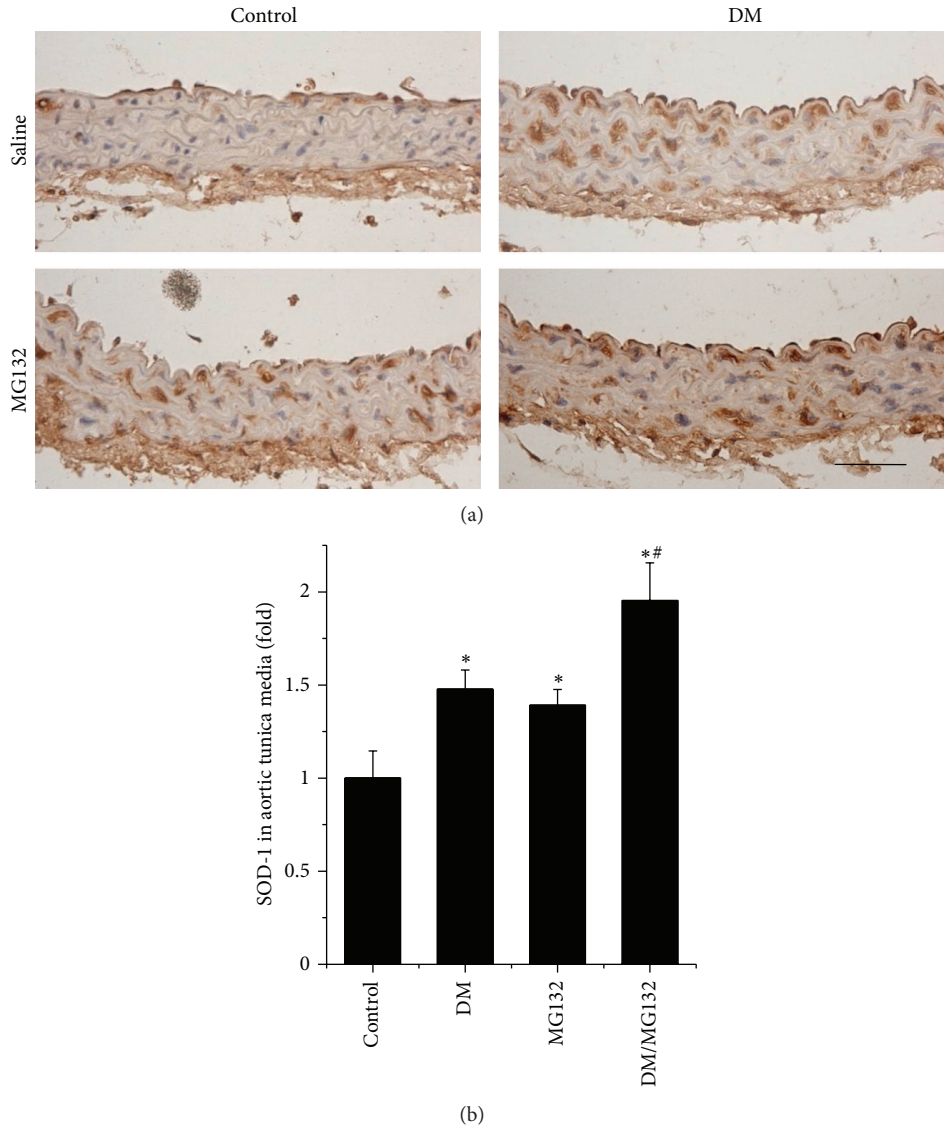

Figure 7: Effect of MG132 on SOD-1 expression at protein level, examined by immunohistochemical staining (a), followed by semi-quantitative analysis (b). Data were presented as means ± SD ($n = 6$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus DM. Bar = 50 μM.

to STZ-induced diabetic mice, OVE26 mice exhibit more characteristics of human diabetic nephropathy, showing the time-dependent proteinuria [15]. One of the strengths of the present study thus is the utilization of the transgenic OVE26 diabetic mouse model.

We demonstrated here, for the first time, that there was an age-dependent development of vascular oxidative damage, inflammation, and remodeling in OVE26 type 1 diabetic mouse model, which is consistent with the observation from STZ-induced-type diabetic model [28]. More importantly we also showed that chronic treatment with low-dose MG132 can afford an effective therapy with an almost complete prevention for the progression of pathology in the aorta when the treatment was started on 3-month-old OVE26 mice that have exhibited renal dysfunction [15] and increased aortic TNF-α expression and 3-NT accumulation (Figure 1). The effectively therapeutic effects include the almost complete abolishment of the aortic oxidative damage, inflammation, and remodeling (Figures 2–5). We also found significant increases in Nrf2 expression and its downstream antioxidant genes, NQO1, HO-1, and SOD1, in MG132-treated control and diabetic mice (Figures 6 and 7). Therefore, we proposed that the therapeutic effect of MG132 on diabetes-induced pathological changes in the aorta may be associated with the upregulated expression of Nrf2 and its downstream antioxidant genes.

However, there may be a concern that diabetes also slightly increased the expression of Nrf2 and its downstream antioxidant genes. Why is increased pathology still observed in the aorta of diabetic mice between 3 and 6 months of age? We speculate that the increase in the expression of Nrf2 and its downstream antioxidants in the aortas of diabetic mice is an adaptive response to diabetes. Although this adaptive response is unable to provide a complete protection, it should

still protect certain levels of pathogenic damage induced by diabetes; otherwise these pathogenic changes would be more severe and appear earlier. To support our assumption, an earlier study has reported that when Nrf2 gene knockout (Nrf2-KO) mice and their wild-type mice were fed a high-fat diet (HFD), HFD induced significant increases in mRNA expression of Nrf2 downstream genes in wild-type mice, but not in Nrf2-KO mice, compared with respective standard diet-fed control mice. Meanwhile, HFD-induced increases in vascular ROS levels and endothelial dysfunction were significantly more severe in Nrf2-KO than in wild-type mice. Their results suggest that adaptive activation of the endogenous Nrf2 pathway could provide certain endothelial protections under diabetic conditions, but not sufficient to completely prevent the progression of aortic pathological changes and dysfunction [11]. However, when up-regulated levels of Nrf2 and its downstream antioxidant genes in MG132-treated diabetic mice are high enough to efficiently reduce diabetes-induced oxidative damage, inflammation, and remodeling, the aortic pathogenesis might be significantly or even completely prevented, as we observed here.

Although we assume previously that Nrf2 up-regulation in the aorta in response to MG132 may be the major mechanism responsible for the aortic protection against diabetes-induced pathogenic changes, we do not exclude the possibility that MG132 may also activate other mechanisms that may also play some roles in the aortic protection against diabetes-induced pathogenic changes. It was reported that hyperglycemic elevation of NF-κB-mediated renal and aortic inflammatory response in early diabetes may be related to the enhanced 26S proteasome activity, since these alterations were abolished by MG132 administration [12]. In addition, MG132 was also found to reduce oxidative stress-induced damage, which probably is related to the suppression of NF-κB activation of NAD(P)H oxidase expression in coronary arterioles in type 2 diabetic mice [29]. Besides NF-κB pathway, MG132 can also play a key role in anti-oxidative system by suppressing MAPK signaling pathway [30–32].

In summary, we have demonstrated here for the first time that chronic treatment with low-dose MG132 can almost completely reverse and/or prevent the progression of diabetes-induced aortic oxidative damage, inflammation, and remodeling in the transgenic OVE26 type 1 diabetic mouse model when it was given to the diabetic mice at 3 months of age, that is, at the time diabetic mice first exhibit renal dysfunction (albuminuria) and mild aortic inflammation and oxidative damage. Mechanisms responsible for the therapeutic effect of MG132 may include up-regulation of Nrf2 expression and function to afford potent antioxidant effect. Although the detailed mechanism requires additional exploration, the present study provides an interesting piece of evidence for the potential application of MG132 for diabetic patient to prevent their cardiovascular complications.

Authors' Contribution

X. Miao, W. Cui, W. Sun, Y. Xin, B. Wang, and Y. Tan researched data. X. Miao, Y. Tan, L. Cai, Y. Fu, L. Miao, G. Su, and Y. Wang reviewed the paper. Y. Tan, L. Cai, G. Su, and Y. Wang contributed initial discussion of and overseeing the project. Y. Wang and L. Cai wrote, edited, and reviewed the paper.

Conflict of Interests

The authors declare no conflict of interest.

Acknowledgments

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Research Article

Gamma-Tocotrienol Modulated Gene Expression in Senescent Human Diploid Fibroblasts as Revealed by Microarray Analysis

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The effect of γ-tocotrienol, a vitamin E isomer, in modulating gene expression in cellular aging of human diploid fibroblasts was studied. Senescent cells at passage 30 were incubated with 70 μM of γ-tocotrienol for 24 h. Gene expression patterns were evaluated using Sentrix HumanRef-8 Expression BeadChip from Illumina, analysed using GeneSpring GX10 software, and validated using quantitative RT-PCR. A total of 100 genes were differentially expressed ($P < 0.001$) by at least 1.5 fold in response to γ-tocotrienol treatment. Amongst the genes were *IRAK3, SelS, HSPA5, HERPUD1, DNAJB9, SEPR1, C18orf55, ARF4, RINT1, NXT1, CADPS2, COG6*, and *GLRX5*. Significant gene list was further analysed by Gene Set Enrichment Analysis (GSEA), and the Normalized Enrichment Score (NES) showed that biological processes such as inflammation, protein transport, apoptosis, and cell redox homeostasis were modulated in senescent fibroblasts treated with γ-tocotrienol. These findings revealed that γ-tocotrienol may prevent cellular aging of human diploid fibroblasts by modulating gene expression.

1. Introduction

Aging is a phenomenon associated with gradual decline in biological functions. Loss of capability to divide besides loss of cellular functions in both mitotic and postmitotic cells is the characteristic of cellular aging [1]. Cellular changes that occur in the cell of an organism have direct impact on the functions of organs, systems and eventually involve the whole organism. The genetic theory of aging is proposed based on the observation that several genes affect longevity [2]. The aging process is regulated by specific genes in many organisms including yeast, *C. elegans*, fruit flies, and mice. In human diploid fibroblasts, several genes including inflammatory genes, cell cycle regulatory genes, cytoskeletal genes, and metabolic genes were differentially expressed [3] during replicative senescence and modifiable by dietary components such as antioxidants [4].

Human aging can be studied *in vitro*, specifically by using normal human diploid fibroblasts (HDFs) which undergo a limited number of cellular divisions in culture and progressively reached a state of irreversible growth arrest, a process termed as replicative senescence [1]. Senescent fibroblast cells are resistant to mitogen-induced proliferation, expressed senescence-associated β-galactosidase (SA β-gal), exhibited enlarged and flattened morphology, and showed altered gene expression [5]. Cultured human fibroblasts cells displayed age-dependent transcriptomic differences. A variety of genes involved in cell cycle regulation, immune response and inflammation, cytoskeleton, stress response, and metabolism are known to be altered during cellular senescence [6].

About 40 micronutrients consist of vitamins, essential minerals, and other compounds are required in small amount for normal metabolism and have been reported as essential components in the diet. Deficiency of macro- and micronutrients in aging is related to global impairments of immune functions, metabolic harmony, and antioxidant defense with subsequent appearance of age-related diseases [7]. The antioxidant vitamin E, usually alpha-tocopherol,

scavenges reactive oxygen species (ROS) thus preventing oxidative damage associated with many degenerative diseases and has been suggested to act as a signaling molecule which modulates signal transduction and gene expression [8].

Vitamin E is a lipophilic vitamin, synthesized by plants, found particularly in plant seeds and oils. There are eight naturally occurring forms of vitamin E which are α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol [9]. Structurally, tocotrienols are different from tocopherols by the presence of three trans double bonds in their hydrocarbon tail [10].

The prenyl side chain of tocotrienols has been postulated to be responsible for the differential membrane distribution and metabolism of tocotrienols. α-tocotrienol possessed higher antioxidant activity against lipid peroxidation than α-tocopherol due to a more uniform distribution in the lipid bilayer membrane providing a more efficient interaction of the chromanol ring with lipid radicals [11]. Tocotrienol also showed novel hypocholesterolemic activity [12] in addition to more recent reports suggesting that it has neuroprotective, antioxidant, anticancer and cholesterol lowering properties that often differ from the properties of tocopherols [10]. Furthermore, tocotrienol was able to delay cellular aging by preventing oxidative damage-induced telomere shortening in aged human fibroblast cells [13]. Also tocotrienol-rich fraction (TRF) has been shown to have antiaging properties by promoting cell cycle progression in senescent human fibroblast cells [5]. Recent research has focused on other biological functions of vitamin E that are unrelated to its antioxidant properties which include its roles in cellular signaling, gene expression, immune response, and apoptosis [9]. Therefore, this study was aimed to determine the effect of γ-tocotrienol in modulating gene expression in cellular aging of human diploid fibroblast cells.

2. Materials and Methods

2.1. Primary Culture of Human Diploid Fibroblast Cells and Treatment with γ-Tocotrienol. This research has been approved by the Universiti Kebangsaan Malaysia Ethics Committee (Approval Project Code: FF-104-2007). Informed written consent was obtained from the parents of all subjects. Primary human dermal fibroblasts were derived from the foreskins (removed during circumcision) of three 9- to 12-year-old boys. The samples were aseptically collected and washed several times with 75% alcohol and phosphate buffered saline (PBS) containing 1% antibiotic-antimycotic (PAA, Austria). After removing the epidermis, the pure dermis was cut into small pieces and transferred into falcon tubes containing 0.03% collagenase type I solution (Worthington Biochemical Corporation, USA). Pure dermis was digested in an incubator shaker at 37°C for 6–12 h. Then, cells were rinsed with PBS before being cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS) (PAA, Austria) and 1% antibiotic-antimycotic at 37°C in 5% CO_2 humidified incubator. After 5-6 days, the cultured HDFs were harvested by trypsinization and culture-expanded in new T25 culture flasks (Nunc, Denmark) with expansion degree of 1 : 4. When the subcultures reached 80%–90% confluency, serial passaging was done by trypsinization, and the number of population doublings (PDs) was monitored until HDFs reached senescence. For subsequent experiments, cells were used at either passage 4 (young cells, PD < 12) and passage 30 (senescent cells, PD > 55).

In the subsequent experiments, treated young HDFs were incubated with 50 μM palm γ-tocotrienol (Malaysian Palm Oil Board), while senescent HDFs were incubated with 70 μM γ-tocotrienol for 24 h. Untreated cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS) (PAA, Austria). The media for the untreated cells were changed in parallel to the treated cells. Both untreated and treated cells were harvested on the same day [14].

2.2. Determination of Senescent Biomarker SA β-Gal Activity. The senescent biomarker of *in vitro* cell aging for HDFs (SA β-gal activity) was determined by senescent cells staining kit (Sigma, USA) according to the manufacturer's instruction. Blue staining was visible after 4 h of incubation with β-galactosidase staining solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) at 37°C. The percentage of blue cells observed in 100 cells under a light microscope was calculated.

2.3. Total RNA Extraction. Total RNA from HDFs in different treatment groups was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instruction. Polyacryl carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA. Extracted total RNA pellet was then washed with 75% ethanol and dried before being dissolved in RNase and DNase-free distilled water. Total RNA was stored at −80°C immediately after extraction. Concentration and purity of the extracted RNA were determined by Agilent 2100 bioanalyzer (Agilent Technologies, USA). RNA with RNA integrity number (RIN) ranging from 7 to 10 and absorbance ratio of A_{260} to A_{280} ranging from 1.5 to 2.1 was utilized for cDNA synthesis.

2.4. Microarray Analysis. Briefly, 250 ng of total RNA from each sample was labeled by using TotalPrep RNA Amplification Kit (Ambion, Austin, USA) for cDNA synthesis. *In vitro* transcription was performed to synthesise the cRNA. Single stranded cRNA was labeled by incorporating biotin-16-UTP, and cRNA was generated by incubation in the hybridization oven for 14 h at 37°C. After 14 h incubation, 750 ng of biotin-labeled cRNA was hybridized (18 h at 58°C) to Illumina's Sentrix HumanRef-8_v3 Expression BeadChips (Illumina, San Diego, USA). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 (Amersham Biosciences, USA) and quantitated using Illumina's BeadStation S455 scanner (Illumina, San Diego, USA). Data was analysed using GeneSpring GX 10 software (Agilent Technologies, USA), and two-way analysis of variance (2-way ANOVA) was applied with the false discovery rate (FDR) for the selection of differentially expressed genes at significance level of $P <$

Table 1: Primers list for qRT-PCR.

Gene	Accession number	Primer sequences (5′-3′)	PCR product size (bp)
HSPA5	NM_005347	F: ggt gaa aga ccc ctg aca aa R: gtc agg cga ttc tgg tca tt	199
HERPUD1	NM_001010990	F: gag cct gct ggt tct aat cg R: gaa agc tga agc cac cca ta	193
ARF4	NM_001660	F: ggg atg ttg gtg gtc aag at R: agc agc act gca tct ctc aa	168

0.001 for baseline (data not shown) and γ-tocotrienol-treated HDFs. Significant gene list was further filtered for differences of more than 1.5-fold between γ-tocotrienol-treated HDFs and untreated control senescent HDFs. Significant genes with expression greater than 1.5-fold were selected for Gene Set Enrichment Analysis (GSEA) by using pathway studio software (Ariadne, USA) with $P < 0.05$.

2.5. Validation of Microarray Data Using Quantitative Real-Time RT-PCR. Genes for validation were chosen from pathway analysis. Quantitative real-time RT-PCR reaction was carried out to evaluate the expression of *ARF4, HSPA5,* and *HERPUD1* genes using 1 μL total RNA as template, 1 μL of forward and reverse primers for genes of interest, and iScript One-Step RT-PCR reagent with SYBR Green (Bio-Rad, USA). Primer sequences for *ARF4*, *HSPA5,* and *HERPUD1* are shown in Table 1. All reactions were run in duplicate with reaction profile as follows: cDNA synthesis for 30 min at 50°C; predenaturation for 2 min at 94°C; PCR amplification for 38 cycles with 10 sec at 94°C and 30 sec at 61°C using Bio-Rad iCycler (Bio-Rad, USA).

3. Results

3.1. Quality Control Assessment of the Samples. Principal Component Analysis (PCA) was used to check the quality of the microarray data. This allows viewing of separation between groups of replicates. Untreated control senescent and γ-tocotrienol-treated senescent HDFs were well separated and clustered into two distinct groups. Similar separation, however, was not observed for untreated control young and γ-tocotrienol-treated young HDFs (Figure 1).

3.2. Hierarchical Clustering of Significantly Expressed Genes. Cluster analysis was performed to organize genes into cluster based on their similarities of expression. Horizontal line represents a single gene, and each column represents a single sample. Red color indicated the upregulated genes, whereas green color indicated the downregulated genes. Statistical analysis of two-way analysis of variance (2-way ANOVA) revealed that a total of 253 genes were significantly regulated in senescent HDFs as compared to young cells (Figure 2(a)). One hundred genes were significantly regulated in tocotrienol-treated senescent HDFs compared to untreated control senescent HDFs (Figure 2(b)). Clustering analysis was able to distinguish gene expression between young and senescent HDFs as well as between untreated

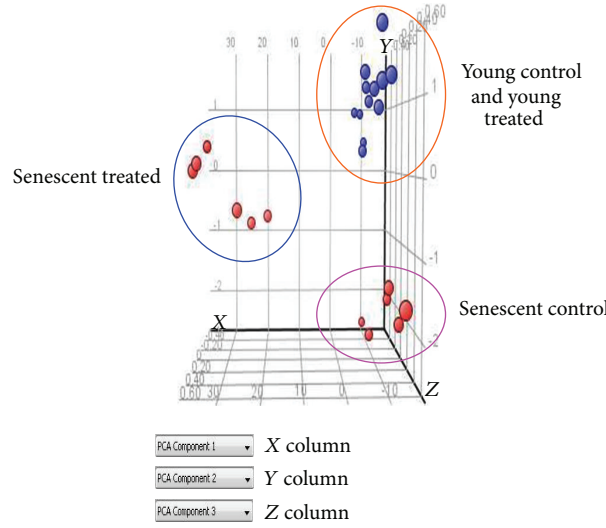

Figure 1: Three-dimensional Principal Component Analysis (PCA) plot was derived from biological replicates of HDFs ($n = 6$). The three dimensional PCA plot showed clustering of the different treatment groups: untreated control senescent HDFs, γ-tocotrienol-treated senescent HDFs, and untreated control young HDFs and γ-tocotrienol-treated young HDFs.

control senescent and tocotrienol-treated senescent HDFs as shown by Hierarchical clustering.

3.3. Analysis of Differentially Expressed Genes in γ-Tocotrienol-Treated HDFs. A total of 100 genes were differentially expressed in γ-tocotrienol-treated senescent HDFs as compared to untreated control senescent HDFs (Table 2). Gene Set Enrichment Analysis (GSEA) revealed the selected significant biological processes involved in response to γ-tocotrienol treatment in senescent HDFs compared to untreated control senescent HDFs (Table 3). Positive value of Normalized Enrichment Score (NES) indicated the upregulated process. The biological processes that were modulated by γ-tocotrienol treatment included the negative regulation of tumor necrosis factor production (inflammation), negative regulation of interleukin-6 production (inflammation), negative regulation of caspases activity (apoptosis), response to stress, transport protein, and cell redox homeostasis. Pathway analysis was carried out for selected genes (*ARF4, HSPA5,* and *HERPUD1*) (Figure 3), and validation on the microarray data was carried out by real time RT-PCR. Results showed an up-regulation of the selected genes in γ-tocotrienol-treated

Table 2: Differentially expressed genes list in γ-tocotrienol-treated senescent HDFs.

Gene Ontology/accession number	Gene symbol	Fold change	Regulation
Protein transport			
NM_020751.1	COG6	2.9372108	Upregulated
NM_022374.1	ARL6IP2	2.4375103	Upregulated
NM_017954.8	CADPS2	2.4749646	Upregulated
NM_004794.2	RAB33A	4.2599683	Upregulated
NM_001660.2	ARF4	3.6646543	Upregulated
NM_021930.3	RINT-1	1.6876434	Upregulated
NM_001031677.2	RAB24	2.3763354	Upregulated
NM_013248.2	NXT1	3.5031867	Upregulated
NM_014177.1	C18orf55	1.5897815	Upregulated
NM_017986.2	GPR172B	2.2754004	Upregulated
Ion transport			
NM_000725.2	CACNB3	2.0575228	Downregulated
NM_002245.2	KCNK1	4.019881	Upregulated
NM_022055.1	KCNK12	2.6714919	Upregulated
NM_012463.2	ATP6V0A2	2.7435005	Upregulated
NM_005175.2	ATP5G1	2.527163	Upregulated
NM_000785.3	CYP27B1	7.801915	Upregulated
Negative regulation of interleukin-6 production			
NM_203472.1	SELS	2.317949	Upregulated
NM_007199.1	IRAK3	2.630288	Upregulated
Immune response			
NM_002162.2	ICAM3	3.910169	Upregulated
Cytokine mediated signal transduction			
NM_203453.2	PPAPDC2	1.8218696	Downregulated
Apoptosis			
NM_018530.1	GSDML	2.4119947	Upregulated
NM_018130.2	SHQ1	1.8244729	Upregulated
NM_018456.4	EAF2	7.062471	Upregulated
NM_018145.1	FAM82C	1.6341798	Upregulated
NM_024310.2	PLEKHF1	2.9315765	Upregulated
NM_001012398.1	AKTIP	2.6247795	Upregulated
Response to stress			
NM_005347.2	HSPA5	5.951358	Upregulated
NM_021237.3	SELK	3.3043954	Upregulated
NM_014445.2	SERP1	3.263752	Upregulated
Cell redox homeostasis			
NM_016417.2	GLRX5	1.7182248	Upregulated
Cell cycle			
NM_006545.4	TUSC4	5.8936715	Upregulated
NM_016948.1	PARD6A	2.2347567	Upregulated
Regulation of transcription			
NM_005444.1	RQCD1	2.0665886	Upregulated
NM_004634.2	BRPF1	1.8511329	Upregulated
NM_003408.1	ZFP37	2.5321555	Upregulated
NM_015394.4	ZNF10	2.5174437	Upregulated

Table 2: Continued.

Gene Ontology/accession number	Gene symbol	Fold change	Regulation
NM_001080485.1	ZNF275	1.8915362	Upregulated
NM_005088.2	CXYorf3	2.2247052	Upregulated
NM_032758.3	PHF5A	2.0002973	Upregulated
NM_022366.1	TFB2M	2.2978535	Upregulated
NM_145805.1	ISL2	2.0560906	Upregulated
Protein binding			
NM_021934.3	C12orf44	2.943061	Upregulated
NM_181291.1	WDR20	1.9003692	Upregulated
NM_006207.1	PDGFRL	3.129943	Upregulated
Protein folding			
NM_021800.2	DNAJC12	4.182389	Upregulated
Endoplasmic reticulum unfolded protein response			
NM_001010990.1	HERPUD1	6.5408597	Upregulated
Regulation of RhoGTPase activity			
NM_153213.2	ARHGEF19	2.899712	Upregulated
Regulation of rab GTPase activity			
NM_022771.3	TBC1D15	2.361947	Upregulated
NM_020705.1	TBC1D24	3.0267975	Upregulated
Cell-cell signalling			
NM_001005914.1	SEMA3B	1.764685	Upregulated
NM_032331.2	MGC2408	2.6944642	Upregulated
NM_001407.1	CELSR3	3.9751458	Upregulated
Translation			
NM_181463.1	MRPL55	3.1179419	Upregulated
NM_003136.2	SRP54	2.6215558	Upregulated
Electron transport chain			
NM_025147.2	COQ10B	1.7724766	Upregulated
NM_007022.3	CYB561D2	3.542795	Upregulated
Signal transduction			
NM_133173.2	APBB3	2.0743015	Upregulated
NM_016115.3	ASB3	2.5060174	Upregulated
Cell proliferation			
NM_206825.1	GNL3	4.2791877	Upregulated
DNA replication			
NM_017443.3	POLE3	3.112311	Upregulated
Oxidoreductase			
NM_017758.2	ALKBH5	3.526197	Upregulated
Microtubule sitoplamic organization			
NM_014171.3	CRIPT	2.496879	Upregulated
Growth			
NM_031479.3	INHBE	24.436758	Upregulated
Biosynthesis process			
NM_014305.1	TGDS	2.4665043	Upregulated
NM_133443.1	GPT2	6.0515475	Upregulated
NM_005768.5	OACT5	2.454743	Downregulated

Table 2: Continued.

Gene Ontology/accession number	Gene symbol	Fold change	Regulation
Modification-dependent protein catabolic process			
NM_203301.1	FBXO33	1.5563589	Upregulated
NM_015984.1	UCHL5	2.2333875	Upregulated
Hydrolase activity			
NM_203453.2	PPAPDC2	1.8218696	Downregulated
Proteolysis			
NM_032549.1	IMMP2L	1.596754	Downregulated

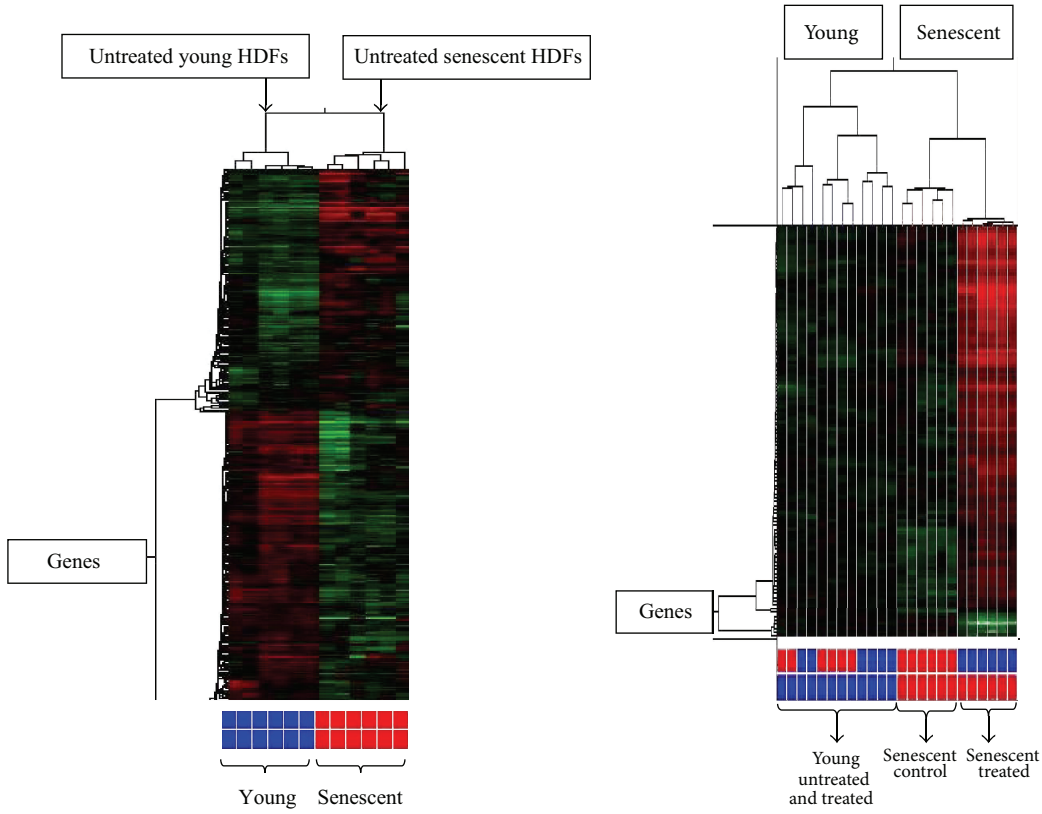

Figure 2: Hierarchical cluster analysis of genes showed the differential expression of genes in young and senescent HDFs (a) and senescent HDFs in response to γ-tocotrienol treatment (b) ($P < 0.001$). Samples were clustered under their conditions.

senescent HDFs as compared to untreated control senescent HDFs (Figure 4). Comparison between microarray and RT-PCR data showed that there was a consistent expression pattern of *ARF4, HSPA5,* and *HERPUD1* genes which are involved in protein transport and negative regulation of apoptosis (Table 4).

4. Discussion

Nonantioxidant activities of vitamin E particularly α-tocopherol has been increasingly reported. For instance, α-tocopherol and α-tocopheryl phosphate were reported to be involved as mediators of lipid metabolism by modulating signal transduction and gene expression [8]. Our findings from the present study showed that another form of vitamin E, γ-tocotrienol, was able to modulate gene expression in human diploid fibroblasts. In response to γ-tocotrienol treatment, a total of 100 genes were differentially expressed in senescent HDFs which included *IRAK3, SelS, HSPA5, HERPUD1, DNAJB9, SEPR1, C18orf55, ARF4, RINT1, NXT1, CADPS2, COG6,* and *GLRX5*. Gene Set Enrichment Analysis (GSEA) revealed that *IRAK3* was involved in inflammation process specifically in the negative regulation of tumor necrosis factor production and negative regulation of interleukin-6 production. *IRAK3* encodes for one of the interleukin

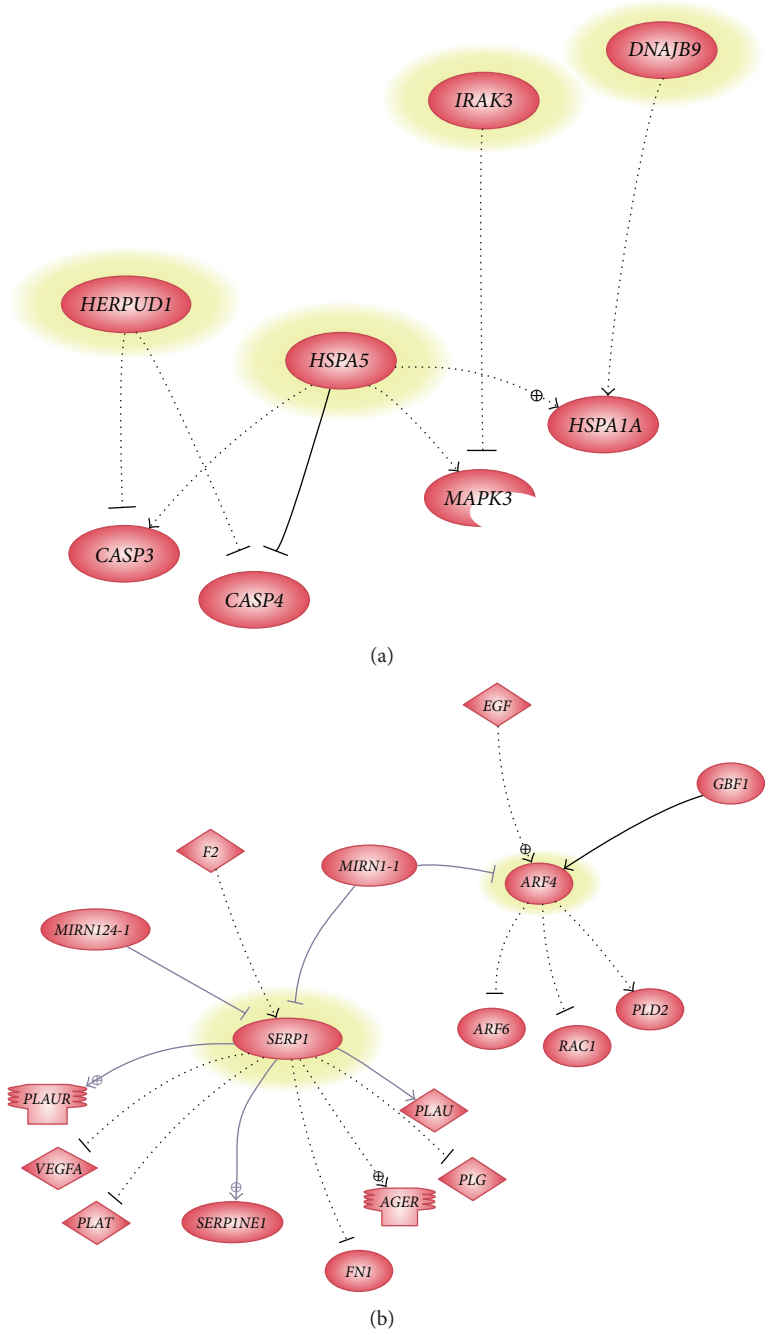

Figure 3: Network of common target for the molecular relationship of selected differentially expressed genes in γ-tocotrienol-treated senescent HDFs. The highlighted genes were the regulator for the downstream genes.

receptor-associated kinase (IRAK) family and has a role in both positive and negative regulation of signal transduction. This gene is also known as *IRAK-M,* and previously, the expression of human *IRAK-M* was limited to monocytes and macrophages [15], However in the present study, its expression was significantly increased in γ-tocotrienol-treated senescent fibroblast cells.

The aging process is attributed to the presence of low chronic inflammation resulting in a stressed condition. Genes related to inflammation are relevant after taking into account that the innate immunity is more involved during inflammation. A chronic inflammatory status called inflame aging appears to be the major component of common age-related diseases including cardiovascular diseases and infections [16]. Among the inflammatory agents that have been identified were interleukin-6, interleukin-1β, cyclooxygenase 2 and tumor necrosis factor (TNF). Up-regulation of proinflammatory mediators was observed during aging due to an age-related redox imbalance that activates several proinflammatory signaling pathways. Dysregulation of these

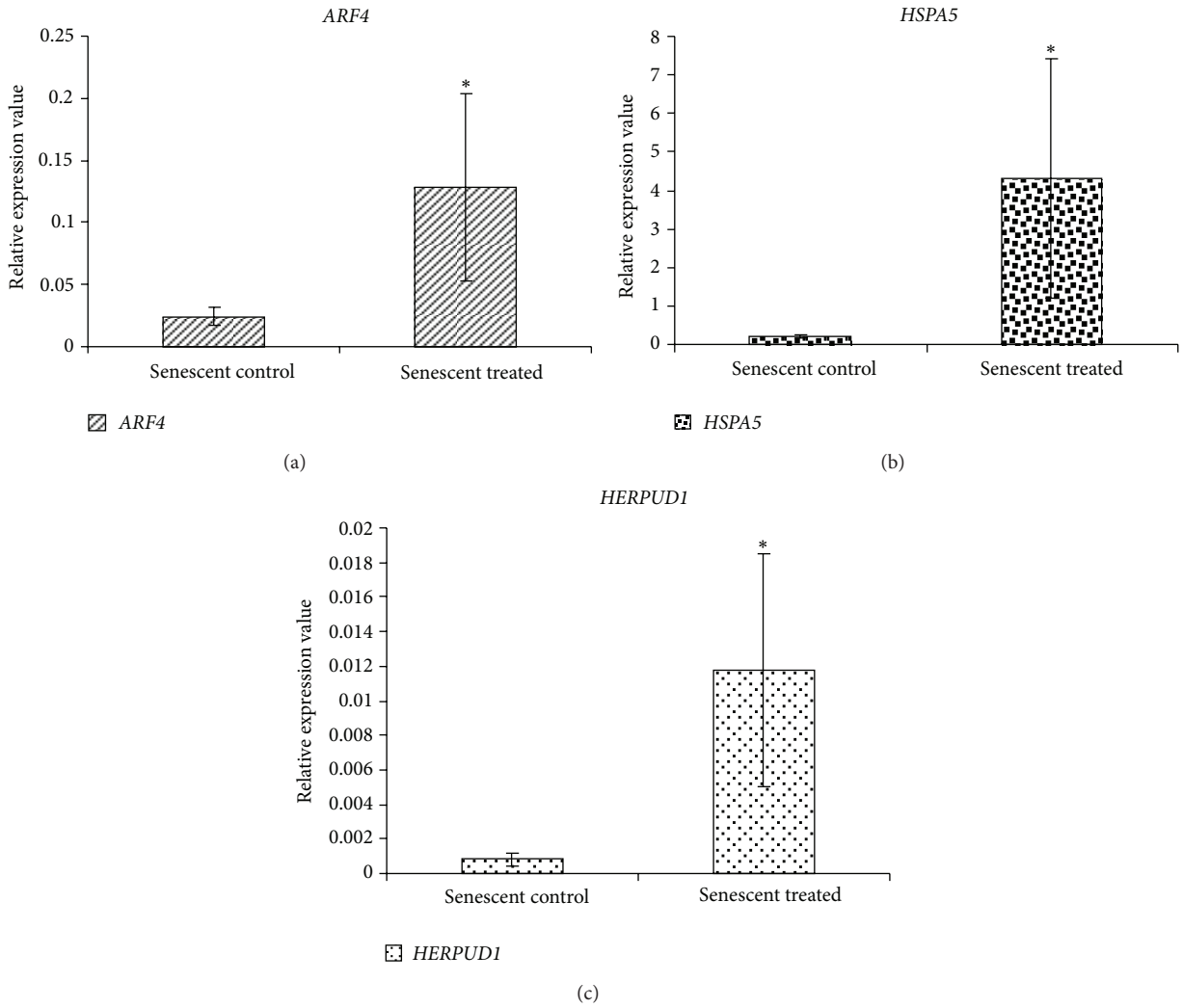

Figure 4: Based on pathway analysis, three genes were selected for validation by qRT-PCR technique. All data were normalized to the housekeeping gene, *GAPDH*. The expression patterns obtained through qRT-PCR were consistent with the microarray results. *ARF4* (a), *HSPA5* (b), and *HERPUD1* (c) were significantly upregulated in γ-tocotrienol-treated senescent HDFs compared to untreated control senescent HDFs ($P < 0.05$).

cytokines promotes inflammation and tissue damage. This indicated that aging is accompanied by chronic low-grade inflammation state showed by 2-to 4-fold increase in serum levels of inflammatory mediators such as C-reactive protein, tumor necrosis factor (TNF) and cyclooxygenase 2 (COX2) [17]. Up-regulation of *IRAK3* in this study may indicate protection against cellular aging and age-related diseases.

In the present study we also found that selenoprotein *S* (*SelS*) was significantly upregulated in γ-tocotrienol-treated senescent fibroblast cells. Selenoprotein S is involved in negative regulation of tumor necrosis factor production, negative regulation of interleukin-6 production, and cell redox homeostasis. It encodes for an endoplasmic reticulum (ER) transmembrane protein and is present in a variety of tissues such as liver, skeletal muscle, and adipose tissue [18]. Selenoproteins contain the twenty-first amino acid, selenocysteine, reported to be involved in cellular defenses against oxidative damage. Furthermore, these proteins are involved in important metabolic and developmental pathways in response to environmental challenges [19]. Many of the selenoproteins are involved in protection against oxidative stress or in maintaining cellular redox balance. SelS is considered as an important component of retrotranslocation channel in endoplasmic reticulum-associated protein degradation (ERAD). This ER membrane protein functions in stress responses to prevent the deleterious consequences of accumulation of misfolded proteins which has been linked to immune and inflammatory processes. Previous findings have suggested that SelS may regulate cytokine production in macrophages, and a regulatory loop between cytokines and SelS has been proposed to play a key role in controlling the inflammatory response [20]. Negative regulation of TNF and interleukin-6 production by *IRAK3* and *SelS* in γ-tocotrienol-treated senescent HDFs observed in this study may indicate inhibition of chronic inflammatory processes that normally accompanies cellular aging. Thus this may suggest one of

Table 3: Selected significant biological processes that were modulated in senescent HDFs after γ-tocotrienol treatment for 24 h.

Biological process	Gene Set Enrichment Analysis (GSEA)		
	Normalized Enrichment Score	Gene symbol	Description
Negative regulation of tumor necrosis factor production	1.54164		
		IRAK3	Interleukin-1 receptor-associated kinase 3
		Sels	selenoprotein S
Negative regulation of interleukin-6 production	1.65684		
		IRAK3	Interleukin-1 receptor-associated kinase 3
		Sels	selenoprotein S
Negative regulation of caspase activity	1.40798		
		HSPA5	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)
		HERPUD1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
Response to stress	1.61171		
		HSPA5	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)
		HERPUD1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
		DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9
		SERP1	Stress-associated endoplasmic reticulum protein 1
Protein transport	1.68316		
		C18orf55	Chromosome 18 open reading frame 55
		ARF4	ADP-ribosylation factor 4
		RINT1	RAD50 interactor 1
		SERP1	Stress-associated endoplasmic reticulum protein 1
		NXT1	NTF2-like export factor 1
		CADPS2	Ca^{2+}—dependent activator protein for secretion 2
		COG6	Component of oligomeric golgi complex 6
Cell redox homeostasis	1.56752		
		GLRX5	Glutaredoxin 5 homolog
		Sels	Selenoprotein S

the mechanisms involved for γ-tocotrienol in slowing down cellular aging of HDFs.

γ-Tocotrienol also modulated the expression of genes that are involved in protein transport. Our results showed that ADP-ribosylation factor 4 (*ARF4*) was significantly upregulated in γ-tocotrienol-treated senescent HDFs. ADP-ribosylation factors have been implicated in several important cellular processes, including membrane trafficking and activation of phospholipase D [21]. They are members of the Ras super family of small guanine nucleotide-binding proteins and were initially identified as proteins that stimulate the ADP-ribosyl transferase activity of cholera toxin *in vitro*. ARFs are ubiquitously expressed from yeast to mammals and function primarily in the regulation of membrane trafficking. Based on their deduced amino acid sequence, protein size, and phylogenetic analysis, these proteins can be divided into three groups which are class I (ARF1, ARF2, and ARF3), class II (ARF4, ARF5), and class III (ARF6). Previous report

Table 4: Comparison between microarray and qRT-PCR data.

Gene	Fold change (RT PCR)	Fold change (microarray)	Regulation
ARF4	5.275	3.66465	Upregulated
HERPUD1	14.26	6.54086	Upregulated
HSPA5	20.99	5.95136	Upregulated

has demonstrated that overexpression of ARF4 in U373MG cells suppresses N-(4-hydroxyphenyl) retinamide (4-HPR)-induced cell death. The findings from yeast-based functional screening in *S. cerevisiae* which facilitates the identification of antiapoptotic mammalian genes showed that ARF4 may function as a novel suppressor of apoptosis [22].

Heat shock proteins (Hsps) have been studied for many years, and there are evidences that demonstrated the role of Hsp up-regulation in tissues and cell protection in a wide variety of stress conditions. Heat shock proteins (HSPs) belong to a class of highly conserved proteins that act physiologically as molecular chaperones to stabilize existing proteins against aggregation and mediate the folding of newly translated proteins in the cytosol and other organelles [23]. Additionally, they have been shown to demonstrate antiapoptotic effects against a wide range of both physical and chemical apoptotic stimuli [24]. Similar finding was found in our study, whereby the *HSPA5* (heat shock 70 kDa protein 5) also known as BiP or GRP78 (glucose-regulated protein, 78 kDa) which is involved in negative regulation of caspase activity in response to stress was significantly increased in γ-tocotrienol-treated senescent HDFs. Besides, treatment with γ-tocotrienol in senescent HDFs caused up-regulation of *HERPUD1* which is also involved in negative regulation of caspase activity in response to stress. HERP (homocysteine-induced ER protein) which was recently identified and characterized as an ER membrane protein was upregulated in response to ER stress. Induction of HERP was reported to be involved in the protection of cells against ER stress. HERP stabilizes ER Ca^{2+} homeostasis and mitochondrial functions in neuronal cells during ER stress [25]. Thus the findings from the present study showed that treatment with γ-tocotrienol in senescent HDFs may delay cellular aging of HDFs by modulating cellular stress responses and regulating the apoptosis pathway.

Oxidative stress is known to be involved in a number of pathological conditions, including neurodegeneration, cardiovascular disease, and stroke, and even plays a role in natural aging. Oxidative stress occurs when the levels of oxidants are higher than the levels of antioxidants, thus overwhelming the system. The imbalance between prooxidants and antioxidants leads to an accumulation of oxidative damage with age in a variety of macromolecules resulting in a progressive loss of functional cellular processes, leading to the aging phenotype [26]. A progressive rise of oxidative stress due to the altered redox homeostasis appears to be one of the hallmarks of the aging process. Changes in the pattern of gene expression through ROS-sensitive transcription factors give rise to both aging and inflammation phenotypes. Chronic oxidative stress and inflammatory reactions lead to many age-associated diseases such as atherosclerosis and arthritis [27].

Cells have developed both nonenzymatic and enzymatic defense mechanisms to counteract the deleterious effects of oxidative stress by either detoxifying reactive oxygen species (ROS) or repairing ROS-induced damage. Nonenzymatic examples of antioxidant include vitamin C, vitamin E, ubiquinone, flavonoids and glutathione (GSH), and examples of enzymatic scavengers include catalase, glutathione peroxidase, thioredoxin, Cu/Zn superoxide dismutase (Cu/Zn SOD), Mn/superoxide dismutase (MnSOD), and glutaredoxins [28]. Interestingly, our finding showed that γ-tocotrienol intervention in senescent HDFs caused increased expression of glutaredoxin 5 homolog (*GLRX5*) gene which is involved in cell redox homeostasis. Glutaredoxins are glutathione-dependent oxidoreductases that help in maintaining cellular redox homeostasis in the cell. The glutaredoxin system consists of GSH, NADPH, and GSH reductase. The mammalian system contains three known members of the glutaredoxin family Grx1, Grx2, and Grx5. The recently discovered glutaredoxin 5, a monothiol glutaredoxin, is hypothetically localized to the mitochondria. The loss of Grx5 in yeast leads to constitutive oxidative damage, sensitization to ROS, iron accumulation and inactivation of iron-sulfur (Fe-S) cluster containing enzymes [29]. Our findings are in agreement with a recent study that showed overexpression of glutaredoxin 5 caused decreased in DNA fragmentation and protect cells against H_2O_2 induced apoptosis and ROS formation [30].

In summary, the findings from this study elucidated the nonantioxidant properties of a vitamin E isomer, γ-tocotrienol in delaying cellular aging indicated by regulation of protective biological processes through the modulation of gene expression in human diploid fibroblasts.

5. Conclusion

γ-Tocotrienol may delay or protect against cellular aging of human diploid fibroblasts by modulating genes expression that are involved in biological processes related to oxidative stress such as inflammation, protein transport, apoptosis, and cell redox homeostasis.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Effects of Caloric Restriction on Cardiac Oxidative Stress and Mitochondrial Bioenergetics: Potential Role of Cardiac Sirtuins

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The biology of aging has not been fully clarified, but the free radical theory of aging is one of the strongest aging theories proposed to date. The free radical theory has been expanded to the oxidative stress theory, in which mitochondria play a central role in the development of the aging process because of their critical roles in bioenergetics, oxidant production, and regulation of cell death. A decline in cardiac mitochondrial function associated with the accumulation of oxidative damage might be responsible, at least in part, for the decline in cardiac performance with age. In contrast, lifelong caloric restriction can attenuate functional decline with age, delay the onset of morbidity, and extend lifespan in various species. The effect of caloric restriction appears to be related to a reduction in cellular damage induced by reactive oxygen species. There is increasing evidence that sirtuins play an essential role in the reduction of mitochondrial oxidative stress during caloric restriction. We speculate that cardiac sirtuins attenuate the accumulation of oxidative damage associated with age by modifying specific mitochondrial proteins posttranscriptionally. Therefore, the distinct role of each sirtuin in the heart subjected to caloric restriction should be clarified to translate sirtuin biology into clinical practice.

1. Introduction

Aging is characterized by a progressive deterioration in physiological functions and metabolic processes, leading to an increase in morbidity and mortality. Although many theories have been proposed to explain the aging process, neither of them appears to be fully satisfactory. Table 1 presents a summary of the major aging theories to date [1, 2]. The free radical theory of aging originally stated that free radicals generated endogenously cause oxidative modification of cellular components; the accumulation of oxidative damage with aging results in cellular dysfunction and eventually cell death [3–5]. The free radical theory has been expanded in light of the fact that reactive oxygen species (ROS) are constantly produced in cells under normal condition and cells have a higher antioxidant capacity per se [3, 5–7]. The oxidative stress theory of aging proposes that the formation of ROS including nonradical hydrogen peroxide and reactive nitrogen species, peroxynitrite, is the major generator of cellular damage and senescence. The age-related increase in oxidative damage to DNA, lipids, and proteins has been well documented [3, 5–7]. The mitochondrion is the main source and target of ROS. Mitochondria have been a central focus of the aging theory because of their critical role in bioenergetics, oxidant production, and regulation of cell death [7–12]. According to the hypothesized role of mitochondria in the aging process, organs that exhibit high rate of oxygen consumption throughout an individual's lifetime, such as the heart, brain, and kidney, may be especially prone to oxidative damage [8]. However, ROS are not just unwanted byproducts of oxidative phosphorylation in the respiratory chain; they are also highly regulated signal molecules involved in the cellular stress response.

Numerous experimental interventions designed to regulate the aging process have been attempted heretofore. To date, an established intervention that has been consistently shown to slow the rate of aging and to increase both mean and maximal lifespan in various species is lifelong caloric restriction (CR) [11, 13, 14]. The beneficial effects of lifelong CR may derive, at least in part, from a reduction of oxidative

Table 1: Major biological theories of aging.

(A) The programmed theory	
(1) Programmed theory Telomere shortening theory	Aging is the result of a sequential switching of certain genes. Telomere plays a role in the genomic instability with aging.
(2) Neuroendocrine theory	Biological clocks act through the neurohumoral system to control the pace of aging.
(3) Immunological theory	The immune system is programmed to decline, which leads to an increased vulnerability to acute and chronic inflammation, resulting in aging and death.
(B) The damage or error theory (Nonprogrammed theory)	
(1) Wear and tear theory	Cells and tissues have vital parts that wear out, that leads to aging.
(2) Rate of living theory Metabolic theory	The greater a rate of basal oxygen metabolism, the shorter its lifespan.
(3) Cross-linking theory Glycation theory	The accumulation of modified constituents, such as cross-linked and glycated proteins, damages cells and tissues, resulting in aging.
(4) Free radical theory Oxidative stress theory Mitochondrial theory	Free radicals and reactive oxygen species (ROS) cause cellular damage and the accumulation of oxidative damage leads to aging. Mitochondria are a main source of ROS and also a target of ROS.
(5) Somatic DNA damage theory	DNA damages occur continuously in living cells. Most of these damages are repaired, whereas some accumulate, resulting in cellular dysfunction and aging. In particular, damages to mitochondrial DNA lead to mitochondrial dysfunction.

damage in organs and tissues [8, 11–15]. In contrast, although overexpression of antioxidant enzymes and antioxidant supplement diets have had some degree of success in attenuating age-associated physiological dysfunction and extending mean lifespan, they have not extended maximal lifespan [16–19]. Clinical investigations demonstrate that antioxidant treatment has either no effect or detrimental effects on health beneficial outcomes in cancer, diabetes, cardiovascular disease, and overall mortality [20, 21]. These results suggest that modest ROS production promotes longevity by inducing the innate adaptive response against oxidative stress; this mechanism may be essential for the development of overall stress resistance and lifespan extension [19, 22]. A favorable response to a low dose of poison is called hormesis. By extension, the beneficial response of mitochondria-derived ROS is named mitohormesis [22]. Since there are contradictory reports on the impact of exogenous antioxidant treatment on mitochondrial biogenesis and endogenous antioxidant defense, experiments that are more definitive are needed to address this issue.

In this paper, we discuss how cardiac mitochondrial dysfunction and oxidative stress contribute to cardiac aging and how CR regulates them.

2. Mitochondrial Theory of Aging

ROS are produced cellularly by enzymatic and nonenzymatic sources. Any electron-transferring protein and/or enzymatic system produce ROS as byproducts of electron transfer reactions. Although ROS are produced from NADPH oxidase, cyclooxygenases, peroxisomes, xanthine oxidase, cytochrome *P-450*, and others, mitochondria appear to produce the majority of oxidants [7, 8, 10–12, 23]. During essential oxygen-dependent ATP production in the electron transport chain (ETC), ROS is generated as a product of electron leakage from complex I and complex III where oxygen is reduced to form the superoxide radical (Figure 1) [23]. The generation of ROS in mitochondria is reported to account for ~1-2% of total oxygen consumption under reducing conditions [24]. However, the intramitochondrial concentrations of superoxide are maintained at very low steady-state levels by mitochondrial superoxide dismutase (SOD), which is present at very high concentrations [25].

In addition to being a main source of ROS, mitochondria are a target for oxidative damage. ROS derived from mitochondrial respiration attack mitochondrial constituents. In particular, the accumulation of somatic mutations in mitochondrial DNA (mtDNA) by oxidative stress is believed to play a key role in physiological decline associated with aging [6–8, 10–12, 26]. MtDNA is located at the mitochondrial matrix where ROS are actively generated [7, 26]. Furthermore, mtDNA lacks protective histones and has relatively low DNA repair capacity [7, 26]. ROS-induced mtDNA damage causes mtDNA mutations if mtDNA damage is not promptly repaired [16, 27]. There is a positive relationship between the increase in oxidative damage to mtDNA and the age-associated increase in mtDNA deletions and point mutations [2]. Because mtDNA encodes 13 of mitochondrial proteins in the ETC complexes [28, 29], mtDNA mutations alter the coupling of electron transport and ATP production. Finally, mtDNA mutations increase electron leakage from the ETC complexes and further damage mtDNA, as well as other important organelles [9]. Therefore, the mitochondrion is believed to be the key organelle in the cellular aging process.

3. Age-Associated Alterations in Cardiac Mitochondrial Function

The heart exhibits a highly aerobic metabolism due to the abundance of large mitochondria, which produce the huge amount of ATP for continuous contraction. Therefore,

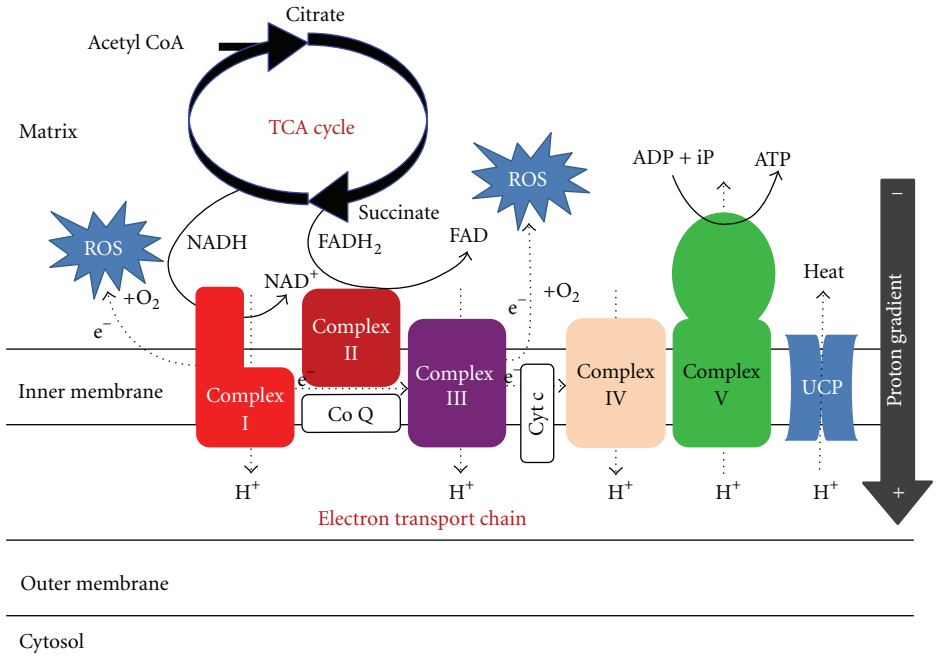

Figure 1: The electron transport chain (ETC) responsible for ATP and ROS production in mitochondria. ROS: reactive oxygen species, TCA: tricarboxylic acid, NADH & NAD^+: nicotinamide adenine dinucleotide reduced form & oxidized form, FADH2 & FAD; flavin adenine dinucleotide reduced form and oxidized form, ADP: adenosine diphosphate, ATP: adenosine triphosphate, Co Q: coenzyme Q, Cyt c: cytochrome c, UCP: uncoupling protein.

the relationship between aging and mitochondrial bioenergetics in cardiomyocytes has been an important research area for many years. However, the interpretation of age-associated alterations in cardiac mitochondrial function has been complicated by several factors. The heart contains two structurally similar but biochemically distinct mitochondrial populations [8, 30, 31]. Subsarcolemmal mitochondria are located beneath the plasma membrane, whereas interfibrillar mitochondria are arranged in parallel with myofibrils [8, 30, 31]. Although evidence demonstrates that 2 populations of mitochondria differ in morphology and function, the isolation procedure to evaluate age-associated alterations in cardiac mitochondrial function yields either subsarcolemmal mitochondria alone or a mixed population of both mitochondria in most reports [31–34]. In addition, cardiomyocytes with dysfunctional mitochondria might be likely to drop out via apoptosis, necrosis, and/or autophagy thus further complicating the detection of bioenergetic changes in cardiac mitochondria with aging because only relatively healthy mitochondria are obtained after the isolation procedure [8]. Compared with those observed in cardiac diseases, alterations in cardiac mitochondria observed in aging might occur heterogeneously in the whole heart and at very low level among mitochondria. Therefore, the early feature of cardiac mitochondrial alterations might be masked by individual differences.

Whether mitochondrial oxidative phosphorylation is gradually impaired with aging remains controversial [33, 35–38]. Some studies have demonstrated a decline in oxygen consumption with age [36, 38], but others have reported no change [35, 37]. Fannin et al. reported that interfibrillar mitochondria but not subsarcolemmal mitochondria obtained from aged rat hearts (24 and 28 months old) exhibited less protein production and oxidative phosphorylation rates, compared with those from adult rat heart (6 months old) [32]. This finding might explain the inconsistency in age-associated alterations in oxidative phosphorylation: the ratio of interfibrillar mitochondria to total mitochondria preparations would be expected to vary.

Whether the activity of each ETC complex decreases with aging is also undetermined [39–46]. Among the five ETC complexes, complex I appears to be susceptible to age-associated decline in activity in the mammalian heart [39, 40, 42, 44–46], although some studies demonstrated no significant change in complex I activity with age [41, 43]. Complex I is believed to be a main source of ROS derived from mitochondrial ETC and to be responsible for the increase in mitochondrial ROS production with aging [12, 23, 42, 44, 47]. Because 7 of 13 mtDNA-encoded polypeptides in the ETC are found in complex I [28, 29], this complex is likely to be most commonly affected by aging, if the mitochondrial theory of aging proves to be true [42]. In addition, the activities of complexes III and IV, which also contain mtDNA-encoded proteins, have been reported to decrease with aging [32, 34, 39–41, 45]. Complex III is another source of ROS production in the mitochondrial ETC [12, 23, 34]. In contrast, most investigations have shown that the activity of complex II appears to be unaffected or rather enhanced by aging [40, 41, 43, 45, 46]. Since mtDNA does not encode any of the polypeptides in complex II [48], this finding further

supports the mitochondrial theory of aging. In addition, protein levels in complexes III, IV, and V were reported to decrease in hearts obtained from old monkeys [38]. Gómez et al. demonstrated that formation of supercomplexes consisting of complexes I, III, and IV decreased in the aged rat heart [49]. Some investigators reported that a decrease in mitochondrial cardiolipin, which is located in the inner mitochondrial membrane and bound to cytochrome c, is closely associated with the decrease in the activity of complex III [50, 51]. However, others have reported that aging does not alter mitochondrial cardiolipin content or composition in subsarcolemmal or interfibrillar mitochondria isolated from rat hearts [44]. Complex V activity is also reported to decline with aging in the heart [52–54]; oxidative modification of β-polypeptides in the F1 complex of complex V may be responsible, at least in part, for this phenomenon [54]. Other types of protein modification, such as 3-nitrotyrosine, have also been found in each ETC complex [55]. Recently, an age-related decline in complexes I and V activity that correlated with increased oxidative modification has been reported in the aged mouse heart, although there was no change in the protein expression levels of them [56]. They may contribute to the decline in mitochondrial function associated with aging.

The mitochondrial proteome has been comprehensively analyzed to clarify the effect of aging and CR on mitochondrial proteins. Chang et al. demonstrated that the effect of aging on the mitochondrial proteome in the heart appears to be slighter than that in the liver, and CR has a minor effect on these changes [57]. These results strongly suggest that posttranslational modifications of mitochondrial proteins are more important than transcriptional changes in the development of age-associated alterations in mitochondrial function and the effect of CR [58].

In conclusion, mounting evidence supports an age-associated decline in cardiac mitochondrial function, especially in the activity of complexes I, III, and IV. However, future studies are required to determine the exact mechanism by which aging impairs cardiac mitochondrial function and to further characterize differential effects of age on the subsarcolemmal and interfibrillar mitochondria populations in the heart.

4. Mitochondrial Oxidative Damage in the Aged Heart

As a major source of ROS production, mitochondria themselves are susceptible to oxidative damage. In fact, the accumulation of oxidative damage in mitochondria is observed in various organs of aged animals [5, 10, 11, 13, 59, 60]. ROS produced by mitochondria damages mitochondrial and nuclear DNA, lipids, and proteins. Oxidized DNA may mutate, lipid peroxidation can attenuate integrity of cellular and intracellular membrane, and oxidized proteins lose their enzymatic activity [16, 60, 61]. These events negatively affect mitochondrial and cellular function and contribute to the decline in physiological function with age. Although oxidative damage increases with age in nuclear DNA and mtDNA [27], Barja and Herrero demonstrated that levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage, were 4-fold higher in mtDNA compared with levels in nuclear DNA in hearts obtained from 8 mammalian species with various lifespans [59]. They found an inverse correlation between 8-oxodG levels in mtDNA and maximal lifespan among the different species. Since the rate of repair for 8-oxodG is believed to be similar between nuclear DNA and mtDNA [62], the higher levels of 8-oxodG in mtDNA are likely caused, at least in part, by the chronic exposure of mtDNA to ROS due to its location in mitochondria. The repair activity for damaged mtDNA increases in the aged heart, indirectly supporting the idea that the rate of mtDNA damage increases with age in the heart [63].

Protein and lipid constituents in mitochondria are also susceptible to oxidative modifications. Increasing evidence demonstrates that polyunsaturated fatty acids contained in membrane lipids are vulnerable to peroxidation by ROS, and lipid peroxidation has been shown to increase in cardiac mitochondria with aging [33, 35, 64, 65]. Lipid peroxidation is a major contributor to the age-associated loss of membrane fluidity; two aldehyde lipid peroxidation products, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), are primarily responsible for this phenomenon [61]. MDA and HNE rapidly react with proteins and exhibit various cytotoxic effects. In particular, HNE is highly reactive with other molecules and exerts numerous effects including inhibition of protein and DNA synthase, enzymatic inactivation, and subsequent cellular dysfunction [66–68]. HNE-modified proteins become resistant to proteolytic degradation and act as noncompetitive inhibitors of the proteasome [67]. In mitochondria, HNE is mainly detoxified by aldehyde dehydrogenase (ALDH) under physiological conditions [66]. Transgenic mice carrying an Aldh2 gene with a single nucleotide polymorphism (Aldh2*2), which impairs ALDH activity, exhibit a senescent phenotype at an early stage of life [69]. However, whether the enzymatic activity and/or the expression levels of ALDH are affected in the aged heart has not been evaluated. Measurement of protein carbonyl content is a commonly used method for assessing protein oxidation. Protein carbonyls can be formed via several mechanisms including site-specific metal-catalyzed oxidation of lysine, arginine, proline, and threonine residues; glycation reactions; and interaction of amino acid side chains with lipid peroxidation products such as MDA and HNE [60, 70]. The accumulation of oxidized proteins may play a role in the loss of physiological function with age because oxidized proteins lose catalytic activity and are prone to forming large and potentially cytotoxic protein aggregates [5, 60, 70]. Several studies have shown an age-associated increase in protein carbonyls in cardiac mitochondria and concluded that this increase may contribute, at least in part, to the decline in mitochondrial function with aging [33, 35, 39, 71].

5. The Effect of CR on Cardiac Oxidative Stress

CR is the only experimental intervention that has consistently shown to slow the rate of aging and increase both mean and maximal lifespan in various species [11, 13–15]. The exact

Table 2: Seven members of sirtuin family.

Sirtuin	Class	Cellular localization	Enzymatic activity	Target molecules
SIRT1	I	Nucleus, Cytosol, (Mitochondria)	Deacetylase	Histone H3, H4, p53, nuclear factor κB (NF κB), peroxisome proliferator-activated receptor-γ co-activator 1 α (PGC1α), forkhead box O transcriptional factors (FoxO) 1 & 3, Notch, hypoxia-inducible factor 1α (HIF1α), liver X receptor (LXR), farnesoid X receptor (FXR), sterol-response element-binding protein 1c (SREBP1c), p300, endothelial nitric oxide synthase (eNOS), peroxisome proliferator-activated receptor (PPAR) γ, CREB-regulated transcription co-activator 2 (CRTC2), and so forth
SIRT2	I	Cytosol, (Nucleus)	Deacetylase	α-tubulin, phosphoenolpyruvate carboxykinase (PEPCK), FoxO1, partitioning defective 3 homologue (PAR3)
SIRT3	I	Mitochondria	Deacetylase	Long-chain acyl CoA dehydrogenase (LCAD), 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2), glutamate dehydrogenase (GDH), NADH dehydrogenase ubiquinone 1 subcomplex 9 (NDUFA9), superoxide dismutase 2 (SOD2), isocitrate dehydrogenase 2 (IDH2), cyclophilin D (CypD), acetyl-CoA synthetase 2 (AceCS2), LKB1, and so forth
SIRT4	II	Mitochondria	ADP-ribosyltransferase	GDH
SIRT5	III	Mitochondria	Deacetylase Demalonylase Desuccinylase	Carbamoyl phosphate synthetase 1 (CPS1)
SIRT6	IV	Nucleus	Deacetylase ADP-ribosyltransferase	Histone H3K9, H3K56
SIRT7	IV	Nucleolus	Unknown	Histone H3K18, p53, RNA polymerase I

mechanisms by which CR extends lifespan have not fully been evaluated, but mounting evidence demonstrates that a reduction in oxidative stress contributes, at least in part, to the antiaging effects of CR [11, 13–15, 19, 71, 72]. CR attenuates the age-associated increase in mitochondrial ROS production, lipid peroxidation, protein oxidation, and oxidative damage of mtDNA in various organs.

Increasing evidence demonstrates that CR has pleiotropic effects on the cardiovascular system [15, 73]. Several studies have reported that CR significantly decreases oxidative damage in the aged heart [47, 71, 74–76]. The levels of 8-oxodG were lower in cardiac mitochondria obtained from CR rats than in those from ad libitum-fed controls [47, 76]. Numerous reports indicate that CR reduces oxidative damage to proteins and lipids in cardiac mitochondria. However, the exact mechanisms involved remain to be determined. In other words, how CR attenuates cardiac oxidative stress has not been established.

Recently, sirtuin 3 (SIRT3) located in mitochondria was shown to play an essential role in enhancing antioxidant defense during CR in the liver and the brain [77, 78]. SIRT3 is a member of the sirtuin family which comprises seven proteins (SIRT1-SIRT7), whose tissue specificity, subcellular localization, enzymatic activity, and target proteins vary (Table 2) [79–81]. Sirtuins have received significant attention since the discovery that a yeast sirtuin, *silent information regulator (Sir) 2,* extends yeast lifespan. Sir2 was identified as an NAD^+-dependent histone deacetylase. Sirtuins deacetylate histones and a wide range of transcriptional regulators and intracellular molecules, thereby controlling their activity. Although it remains controversial whether sirtuins mediate lifespan extension afforded by CR, sirtuins do regulate various aspects of the CR response, namely, glucose homeostasis, insulin secretion, fat metabolism, stress resistance, and physical activity. However, direct evidence that sirtuins play a key role in the reduction of oxidative stress in the cardiovascular system was lacking.

Several studies have reported that CR decreases mitochondrial ROS production in the heart, but failed to clarify the mechanism underlying this observation [47, 82–84]. Figure 2 presents possible mechanisms by which CR attenuates cardiac oxidative damage, which are verified in the latter half of this paper.

6. Antioxidant Defense in the CR Heart

CR might modify mitochondrial ROS production via enhanced mitochondrial biogenesis associated with amplified antioxidant mechanisms. Nisoli et al. demonstrated that 3 months of CR enhanced mitochondrial biogenesis in the murine heart and in skeletal muscle [85]. They concluded that activation of SIRT1 contributes to an increase in mitochondrial biogenesis in the CR heart by upregulating gene involved in mitochondrial biogenesis, including nuclear respiratory factor 1 and peroxisome proliferator-activated receptor γ coactivator 1. Activation of SIRT1 might enhance the expression of manganese SOD (MnSOD) by activating Forkhead box protein O1 as observed in cardiomyopathy hamsters treated with resveratrol [86]. However, we did not find any increase in the mitochondrial DNA and protein content

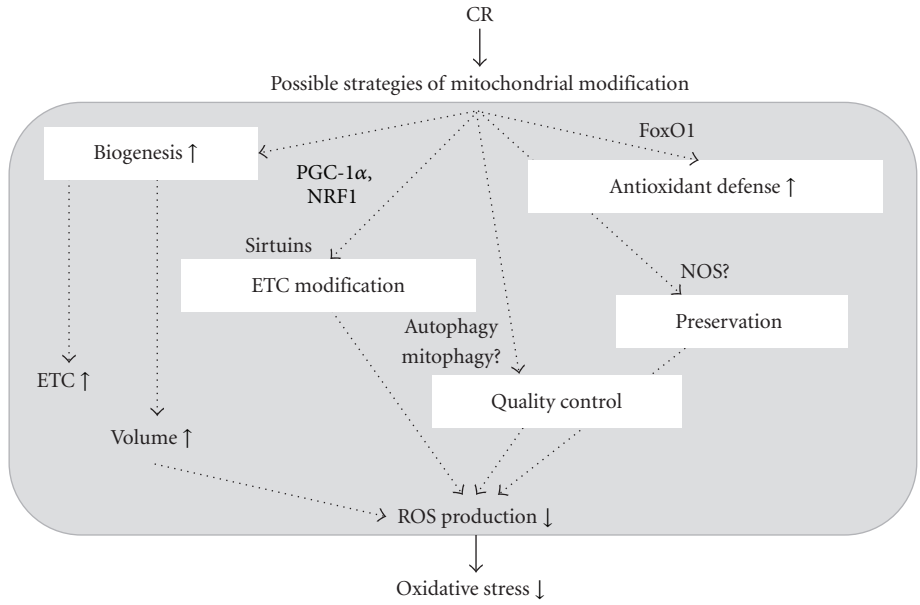

Figure 2: Possible mechanisms by which CR attenuates oxidative stress. CR: caloric restriction, PGC-1α: peroxisome proliferator-activated receptor γ coactivator 1α, NRF1: nuclear respiratory factor 1, FoxO1: forkhead transcriptional factor 1, NOS: nitric oxide synthase.

or in the expression of MnSOD in middle-aged rat hearts treated with 6 months of CR, indicating that CR did not enhance mitochondrial biogenesis associated with amplified antioxidant mechanisms [84]. In this regard, Colom et al. failed to find any increase in the expression levels of MnSOD in 18-month-old rat hearts treated with 3 months of CR [82]. More recently, SIRT3 was reported to enhance the enzymatic activity of MnSOD by direct deacetylation [77]. However, it has not been determined yet whether MnSOD is the target of SIRT3 in cardiomyocytes. Judge et al. demonstrated that the activity of MnSOD was significantly reduced in subsarcolemmal mitochondria from young rat hearts treated with CR for 8 weeks, associated with a decrease in H_2O_2 production [83]. Therefore, the simplest explanation is that CR attenuates mitochondrial oxidative damage by suppressing mitochondrial ROS production, rather than by enhancing antioxidant defense.

7. Mitochondrial Function in the CR Heart

Results on the effect of CR on basal mitochondrial function in the heart are inconsistent [47, 74, 75, 82, 84, 87]. We have evaluated the enzymatic activity of the ETC, baseline mitochondrial respiration, and mitochondrial H_2O_2 production in the hearts of middle-aged (12-month-old) rats treated with 6 months of CR and those of middle-aged rats fed AL [84]. The only difference was that CR attenuated maximal H_2O_2 production in mitochondria; this was assessed by adding rotenone (complex I inhibitor) in the presence of pyruvate/malate.

Several studies demonstrated that prolonged CR improved mitochondrial function at baseline in aged hearts, but other studies failed to find any changes in mitochondria [58]. The discrepancy among studies seems to depend on the strain of rats used, the age of rats analyzed, and the duration of CR treatment. Niemann et al. demonstrated that 6 months of CR improved mitochondrial respiration and enhanced the enzymatic activity of complex I in 30-month-old rat hearts but not in 12-month-old rat hearts [75]. Our results were consistent with their findings in middle-aged rat hearts, suggesting that CR has little effect on basal mitochondrial function in the middle-aged heart, probably because mitochondrial function is maintained at a high level [84].

We speculated that the difference in mitochondrial function between AL and CR might be more remarkable under stressful conditions, such as ischemia/reperfusion. As expected, CR preserved state 3 respiration and increased the respiratory control index in the presence of pyruvate/malate in the ischemic-reperfused heart [84]. These findings suggest that the mitochondria in the CR heart are well coupled during the ischemia/reperfusion sequence. Mitochondria obtained from ischemic-reperfused CR hearts produced less H_2O_2 in the presence of pyruvate/malate [84], suggesting that mitochondria in the CR heart produce less ROS during early reperfusion. The preservation of mitochondrial respiration with attenuated H_2O_2 production in the CR heart subjected to ischemia/reperfusion strongly suggests that mitochondria in the CR heart are more resistant to ischemia/reperfusion. Suppressing mitochondrial ROS production under various stresses could attenuate the long-term accumulation of oxidative damage and might retard cardiac senescence in the CR heart [58].

8. Possible Mechanism of Attenuated Oxidative Damage in the CR Heart

Increasing evidence indicates that several enzymes target mitochondria under stress. Thus, it is likely that mitochondrial proteins are modified posttranscriptionally and

regulated to allow the cell to adapt to various stresses. Among many forms of protein modification, we investigated the acetylation/deacetylation of mitochondrial proteins in order to clarify the effects of CR. Three members of the sirtuin family, SIRT3, SIRT4, and SIRT5, localize specifically in mitochondria and play an important role in regulating mitochondrial metabolism and function by modifying target mitochondrial proteins in various organs under metabolic stress [79–81]. In addition, we previously demonstrated that cardioprotection afforded by prolonged CR is associated with an increase in SIRT1 in the nuclear fraction [88].

We found that the levels of proteins acetylated at lysine residues increased with age in the mitochondrial fraction; furthermore, these changes were partially attenuated with CR [84]. There was no increase in the expression of SIRT3, 4, or 5 in mitochondrial fractions obtained from the CR heart. However, CR significantly increased NAD^+-dependent deacetylase activity in total heart homogenate and the mitochondrial fraction [84]. These results suggest that CR enhances the activity of sirtuins and that activated sirtuins might be responsible for the reverse effect of CR on mitochondrial protein acetylation associated with aging.

Among the mitochondrial proteins deacetylated during CR, we focused on the following 2 mitochondrial proteins involved in the ETC: NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1) and cytochrome bc1 complex Rieske subunit [84]. NDUFS1 is the largest subunit of complex I and is a component of the iron-sulfur fragment of the enzyme. It faces the mitochondrial matrix, receives electrons from NADH, and passes them to the downstream iron-sulfur protein clusters. The Rieske subunit is also a component of the iron-sulfur fragment of cytochrome bc1 complex and transfers electrons to cytochrome c. Although the exact mechanism has not been clarified, we speculate that deacetylation of these proteins might stabilize electron transfer through the ETC and reduce electron leakage during ischemia/reperfusion sequence. As described previously, many mitochondrial proteins in the ETC are targets for oxidative modifications under various stresses. In contrast, it is plausible that some types of posttranscriptional modification play a protective role against oxidative modification under stress [58].

9. Reduction in Mitochondrial Burden during CR

Among various substrates that the heart can utilize, free fatty acids are believed to be the major substrate for the adult heart under physiological conditions. In contrast, utilization of glucose in the heart predominantly increases under pathophysiological conditions such as ischemia/reperfusion and pressure overload. The efficiency of ATP production per one molecule of substrate is better when fatty acids are utilized, but mitochondria require high levels of oxygen for β-oxidation during fatty acid metabolism. Finally, both glucose-derived and fatty acid-derived acetyl CoA enter the TCA cycle. Therefore, the question arises whether the preference of energy substrate is altered in the CR heart.

Sung et al. evaluated the effect of short-term CR (for 5 weeks) on myocardial metabolism [89]. They found that there were no differences in rates of palmitate oxidation and glycolysis during aerobic perfusion between the CR and the AL hearts. However, glucose oxidation was increased by 175% in the CR heart, compared to the AL heart. Consistent with this finding, glucose-derived acetyl CoA production in the TCA cycle was increased in the CR heart. A high rate of glucose oxidation in the CR heart might contribute to improve myocardial energetics under stressful conditions. However, there is no data regarding the effect of long-term CR on myocardial metabolism and this issue remains to be clarified.

Experimental studies demonstrate that animals subjected to CR exhibit a decrease in systemic blood pressure and heart rate, compared with controls fed AL [90, 91]. A clinical observation in which individuals who had been on CR diet for an average of 6 years were compared with age-matched healthy individuals on typical American diet indicated a change in blood pressure similar to that observed in animal experiments [92]. Thus, the decrease in cardiovascular burden during CR might contribute, at least in part, to the antiaging effect of CR on cardiovascular senescence. Recent studies demonstrated that SIRT1 and endothelial nitric oxide synthase (eNOS) colocalize in endothelial cells and that SIRT1 deacetylates eNOS, stimulating eNOS activity, and increasing NO production in endothelial cells [15, 93, 94]. Thus, it is plausible that CR activates SIRT1 and improves NO bioavailability in the cardiovascular system, resulting in a decrease in blood pressure during CR [95]. Reduction of mitochondrial work in the heart by pharmacological intervention has potential for mimicking the effect of long-term CR. In this regard, either pharmacological inhibition of renin-angiotensin system throughout the lifespan or genetic disruption of the angiotensin type 1 receptor gene, which led to a decrease in blood pressure, was reported to promote longevity and retard cardiovascular senescence in rodents [96, 97].

10. Implications for Human Aging and Age-Associated Cardiovascular Diseases

Although it is well accepted that oxidative stress is involved in the aging process and the antiaging effect of CR is closely related to a reduction in ROS-induced cellular damage, there is very little data that demonstrates these relationships in humans or lager animals. Therefore, the translatability to human aging for short-lived small species is always a debating issue. A clear distinction should be drawn between the observation that CR appears to work in humans and a recommendation that individuals embark on its practice. A lot of issues have remained to be understood about the effect of CR on humans.

An increased baseline level of oxidative damage to DNA is reported to be associated with age [98] and several age-related diseases including cardiovascular diseases [99]. Higher levels of protein carbonyls are observed with increased age in healthy human subjects [100]. In contrast, a recent investigation from comprehensive assessment of the long-term

effect of reducing intake of energy (CALERIE) demonstrated that DNA damage was reduced from baseline after 6 months in individuals assigned to CR, but not in controls [101]. Although the effects of CR on lifespan in nonhuman primates were inconsistent [102, 103], preferable outcomes on age-associated oxidative damage by CR could be observed in nonhuman primates [104]. These results suggest that the oxidative stress theory of aging would apply to humans, at least in part.

Clearly, the use of CR mimetics is much easier to incorporate into clinical practice than lifelong CR. The role of sirtuins on lifespan extension by CR remains controversial, but there is the fact that sirtuins regulate various aspects of the CR response. In addition, SIRT1 plays an important role in cardiac adaptive response to various stresses such as ischemia/reperfusion [105] and activation of SIRT1 confers antioxidative and anti-inflammatory effects in the vasculature, resulting in attenuated vascular senescence [106]. Thus, activators of sirtuins are potentially useful for managing age-associated cardiovascular diseases. Our finding further suggests targeted modification of specific mitochondrial proteins by relevant sirtuin-activating compounds is a promising approach for controlling cardiovascular senescence. We found that low-dose resveratrol mimics the effect of CR on deacetylation of specific mitochondrial proteins belonging to the ETC [84]. Resveratrol is reported to be a potentially cardioprotective compound, but not a specific sirtuin activator [107, 108]. Thus, future studies should focus on discovering other sirtuin-activating compounds that deacetylate specific mitochondrial proteins with high specificity and efficiency. The distinct role of sirtuin member should be clarified to understand the mechanism by which CR modifies mitochondrial bioenergetics and cellular oxidative stress in the cardiovascular system. At the present time, the easy use of sirtuin-activating compounds in clinical settings should be avoided.

Conflict of Interests

The author declares that he has no conflict of interests.

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Research Article

Nordihydroguaiaretic Acid Attenuates the Oxidative Stress-Induced Decrease of CD33 Expression in Human Monocytes

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Nordihydroguaiaretic acid (NDGA) is a natural lignan with recognized antioxidant and beneficial properties that is isolated from *Larrea tridentata*. In this study, we evaluated the effect of NDGA on the downregulation of oxidant stress-induced CD33 in human monocytes (MNs). Oxidative stress was induced by iodoacetate (IAA) or hydrogen peroxide (H_2O_2) and was evaluated using reactive oxygen species (ROS) production, and cell viability. NDGA attenuates toxicity, ROS production and the oxidative stress-induced decrease of CD33 expression secondary to IAA or H_2O_2 in human MNs. It was also shown that NDGA (20 μM) attenuates cell death in the THP-1 cell line that is caused by treatment with either IAA or H_2O_2. These results suggest that NDGA has a protective effect on CD33 expression, which is associated with its antioxidant activity in human MNs.

1. Introduction

Nordihydroguaiaretic acid (NDGA) is a natural lignan that is primarily isolated and commercially produced from the desert creosote bush, *Larrea tridentata*, which has long been used in traditional medicine for the treatment of several illnesses including diabetes and inflammation [1]. It is estimated that NDGA comprises approximately 5% to 10% of the dry weight of the leaves, and this corresponds to 80% of all the phenolic compounds in the resin [2]. Cell culture and animal model studies have demonstrated that NDGA has biological properties, including anticarcinogenic, antidiabetic, antiviral, antioxidant, and anti-inflammatory activities [3].

The beneficial effects of NDGA have been essentially attributed to its antioxidant properties. NDGA is an effective *in vitro* scavenger of peroxynitrite, singlet oxygen, hydroxyl radical ($^{\bullet}OH$), and hypochlorous acid [4, 5]. It has been shown that NDGA is capable of protecting rats that are exposed to oxidative stress induced by ozone, potassium dichromate, and cisplatin [4, 6, 7]. In addition NDGA also protects primary rat neuronal cultures against damage that is generated by hydrogen peroxide (H_2O_2) and iodoacetate (IAA) [8–10].

Additionally, it has been found that NDGA induces transcription factor Nrf2 and expression of heme oxygenase-1 (HO-1) in different kinds of line cells [9, 11]. In fact, Nrf2 factor controls the expression of more than 100 genes of cytoprotective proteins including antioxidant enzymes such as HO-1 [11].

On the other hand, it is well established that oxidative stress is implicated in pathologies such as cancer, diabetes, and inflammation. Oxidative stress is an imbalance in the redox state that is generated by exacerbated ROS production or diminished protective systems, such as enzymes or scavenger molecules [12]. In fact, the increased production of

reactive oxygen species (ROS) causes cell damage and even cell death, and antioxidants may help to prevent or alleviate diseases in which oxidative stress is involved.

Glutathione is the most abundant nonprotein sulfhydryl compound and the major intracellular redox buffer in almost all cells. This molecule constitutes the first line of the cellular defense mechanism against oxidative injury [13]. There are evidences that the intracellular redox status regulates various aspects of cellular function and that glutathione (GSH) is important in immune modulation [14, 15]. Recently, it has been described in mice that the pretreatment of NDGA before the treatment with the tumor promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA) mitigated cutaneous lipid peroxidation and inhibited H_2O_2 production. NDGA also was able to restore GSH level and activities of antioxidant enzymes and even to attenuate inflammation [16].

H_2O_2 and IAA are toxic compounds that are utilized commonly to induce oxidative stress in cell models [8–10]. IAA is an alkylating agent that irreversibly inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [17]. IAA reduces adenosine triphosphate (ATP) levels and cell survival in a dose-dependent manner [18]. It has been shown that IAA-induced toxicity is related to ROS production, at least in the hippocampal and cerebellar granule neurons of rats [19].

As an ROS, H_2O_2 is less reactive; however, it can easily penetrate cell membranes and react with transition metal ions to produce $^{\bullet}OH$. This intermediary metabolite reacts rapidly and indiscriminately with biomolecules of all classes, including nucleic acids, free nucleotides, proteins, lipids, and carbohydrates. ROS induce oxidative damage, which may cause DNA mutations, protein inactivation, and cell death [12].

In contrast, monocytes (MNs) play a central role in inflammation and host defense against microorganisms. However, in oxidative stress-related diseases, such as diabetes or atherosclerosis, MNs are permanently activated and produce high levels of ROS and the proinflammatory cytokines IL-6, IL-1, and tumor necrosis factor alpha (TNF-α). This increased ROS production may lead to severe disorders, such as chronic inflammation and even cell death [20].

In this context, it has been suggested that changing glutathione redox status, which is the balance between intracellular reduced (GSH) and oxidized (GSSG) glutathione, in antigen presenting cells (APCs) regulates the helper T-cell type 1 (Th1)/Th2 balance due to the production of IL-12 in mice [21].

In addition, after an oxidative challenge, macrophages, mesangial cells, and monocytes increase the amount of available arachidonic acid, by means of the activation of the phospholipases A (PLA) and C (PLC) [22–24]. The arachidonic acid is the substrate for the synthesis of eicosanoids: prostaglandins (PG), leukotrienes, and thromboxanes, which are involved in the inflammatory responses through the production of IL-8 and TNF-α. The metabolic conversion of arachidonic acid into its byproducts requires the catalytic activity of cyclooxygenase (COX) or lipoxygenase (LOX), and it is known that oxidant molecules can induce the synthesis of the COX through a transcriptional mediated mechanism, involving the IκBα degradation and NFκB nuclear translocation. Therefore the oxidative stress has a dual role in the eicosanoid production; at one hand it is the signal to increase the substrate availability, and on the other hand it activates the biosynthetic pathway [25].

Moreover, diabetic patients with hyperglycemia present oxidative stress and constant inflammation. This is due to diverse mechanisms that are associated with excessive ROS production, such as the irreversible production of advanced glycation end products (AGEs). AGEs stimulate the production of inflammatory cytokines in monocytes and macrophages [26]. Additionally, hyperglycemia may stimulate the production of inflammatory cytokines, such as IL-6, IL-1, and tumor necrosis factor alpha (TNF-α), by increasing the levels of peroxides and free radicals and inducing inflammation [27]. Recently, the decreased expression of CD33 has been described in the macrophages of diabetic patients with hyperglycemia. This disorder contributes to the spontaneous secretion of TNF-α, and this alteration may promote additional inflammation during the early stages of diabetes mellitus type II [28].

CD33 is a myeloid cell-specific type I transmembrane glycoprotein that is constitutively expressed on the surfaces of both myeloid progenitors and mature monocytes. This molecule is a receptor that belongs to the family of sialic acid-binding immunoglobulin Ig-like lectins (SIGLECS) [29].

In this study, we evaluate the potential protective effect of NDGA on IAA- and H_2O_2-induced toxicity in the THP-1 cell line and in human MNs. We also demonstrate that NDGA attenuates the oxidant stress-induced CD33 expression downregulation in human MNs.

2. Materials and Methods

2.1. MNs and THP-1 Cells. Peripheral blood mononuclear cells were obtained from heparinized venous whole blood by gradient centrifugation over a Ficoll-sodium diatrizoate solution (Lymphoprep, Nycomed Pharma, Oslo, Norway) using standard procedures [30]. The layer containing the peripheral blood mononuclear cells (PBMCs) was harvested, and the MNs were enriched by plastic adherence for 1 h at 37°C. The human peripheral blood samples were obtained from the blood bank at the Instituto Nacional de Enfermedades Respiratorias (INER) under approbation of the Institutional Ethical Review Board of INER.

The human MNs were cultured in RPMI 1640 medium (Cambrex, Walkersville, MD, USA) supplemented with 50 μg/mL gentamicin sulfate, 2.0 mmol/L L-glutamine, and 10% heat-inactivated pooled human serum at 37°C in a 5% CO_2 atmosphere (MN medium).

The human acute monocytic leukemia cell line, THP-1, was purchased from the American Type Culture Collection (TIB202, ATCC, Rockville, MD, USA). THP-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD, USA), 0.05 μM β-mercaptoethanol, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (THP medium).

2.2. Effect of NDGA on Cell Viability. The MNs and THP-1 cells were seeded at 3×10^5 cells/well in 48-well plates in culture medium. The NDGA (5–50 μM) was then added to the cells and incubated for 120 h. After incubation, the cell viability was quantified by MTT reduction.

2.3. Effect of Iodoacetate or H_2O_2 on Cell Viability. The human MNs and THP-1 cells (3×10^5 cells/well) were cultured in 48-well plates. First, the cells were treated with 0–100 μM IAA or 0–20 mM H_2O_2 for 2 h to induce oxidative stress. Successively oxidants were replaced by fresh medium, and the incubation was continued until 24 h. Cell viability was monitored by MTT reduction or trypan blue exclusion. In addition cell morphology was observed in bright field micrographs on 40x with phase contrast microscopy (Nikon Co.).

2.4. Protective Effect of NDGA on the Cytotoxicity-Induced by IAA or H_2O_2 in MN and THP-1 on Cell Viability. MNs and THP-1 cells (3×10^5 cells/well) were cultured in 48-well plates (MN or THP medium, resp.). The cells were pretreated with or without 20 μM NDGA by 12 h, and then cells were exposed to (0–100 μM) IAA or (0–20 mM) H_2O_2 for 2 h to induce oxidative stress and refreshed after removal of the toxic compounds. MTT reduction or trypan blue exclusion was determined 24 h after onset of IAA or H_2O_2 exposure.

2.5. Cell Viability Detection by MTT. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) reduction assay [31]. Briefly, MNs (3×10^5 cells/well) were seeded into 48-well plates; after the indicated treatments and time periods, 50 μL MTT stock solution (5.0 mg/mL) was added to the cells and incubated for 2 h at 37°C in a 5% CO_2 atmosphere. The plates were then centrifuged at 1,500 g for 15 min at room temperature (RT), and the medium was carefully removed by aspiration. Subsequently, an acid isopropanol solution (800 μL) was added to the wells, and the plates were shaken at 80 rpm for 5 min. Finally, the absorbance was measured at 570 nm in a microplate reader (Labsystems Multiskan). The number of viable cells was expressed as the index of MTT reduction. The control cells (without treatment) were assigned a maximum value of 1, and the indices of the cells incubated with the different treatments were obtained with respect to the control cells.

2.6. Cytotoxicity Assay by Trypan Blue Exclusion. Cell viability also was monitored by trypan blue negative cells. Concisely, an aliquot on the cell suspension was diluted 1 : 1 (v/v) with 0.4% trypan blue, and the cells were counted with a hemocytometer [32]. The results represent the percentage with respect to control of trypan blue negative cells (without treatment). This assay was done three times for independent experiments.

2.7. Determination of Reactive Oxygen Species (ROS) Production by Flow Cytometry

2.7.1. IAA Treatment. MNs (5×10^5 cells/well) were cultured in 24-well plates. First, the cells were pretreated with or without 20 μM NDGA for 12 h and immediately exposed to nonlethal concentration of IAA (2.5, 5.0 and 7.5 μM) by 48 h. Then, ROS production was determined.

2.7.2. H_2O_2 Treatment. MNs (5×10^5cells/well) were pretreated with or without 20 μM NDGA for 12 h and then exposed to H_2O_2 (0.5, 1.0 and 2.0 mM) by 12 h. Then, ROS production was determined.

ROS detection was measured with the fluorescent marker 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-DCFDA; Invitrogen, Carlsbad, CA), which is an acetylated form of fluorescein, and was used as an ROS indicator [33].

After treatments, the MNs were washed twice with phosphate-buffered saline solution (PBS) and immediately loaded with 10 μM carboxy-H_2DCFDA for 30 min at 37°C in the dark for ROS production. Following this incubation, the cells were centrifuged at 600 g for 5 min. The cells were washed twice with PBS and then fixed with 1% paraformaldehyde and stored at 4°C until acquisition with a FACSCalibur flow cytometer (BD, San Jose, CA, USA). The number of events acquired was 10,000.

2.8. Determination of CD33 Expression by Flow Cytometry

2.8.1. IAA Treatment. MNs (5×10^5cells/well) were cultured in 24-well plates. First, the cells were pretreated with or without 20 μM NDGA for 12 h and immediately exposed to no lethal concentration of IAA (2.5, 5.0 and 7.5 μM) by 72 h. Then, CD33 expression was determined.

2.8.2. H_2O_2 Treatment. MNs (5×10^5cells/well) were pretreated with vehicle or 20 μM NDGA for 12 h and then, exposed to H_2O_2 (0.5, 1.0 and 2.0 mM) by 24 h. Then, CD33 expression was determined.

To measure CD33 in cell surface of MNs, the cells were washed with PBS and immediately loaded with saturating amounts of phycoerythrin- (PE-) labeled mAbs against CD33 and incubated for 15 min at RT in the dark. The cells were fixed with 1% paraformaldehyde and stored at 4°C until acquisition. The number of events acquired was 10,000.

The results of ROS level and CD33 expression were analyzed with CellQuest software (BD Biosciences) and were expressed as the index of the mean fluorescence intensity (MFI) compared to the control without treatment.

2.9. Glutathione/Glutathione Disulfide Ratio Detection. THP-1 cells (3×10^6 cells/well) were cultured in 6-well plates. First, the cells were pretreated with vehicle or 20 μM NDGA for 12 h and immediately exposed to IAA (2.5, 5.0 and 7.5 μM) or H_2O_2 (0.5, 1.0 and 2.0 mM) by additional 24 h. Then, glutathione was measured.

GSH and GSSG levels were measured in cell extracts using the GSH reductase enzyme method [34]. This assay is based on the reaction of GSH with 5, 5′-dithio-bis (2 nitrobenzoic acid) (DNTB) to form 5-thio-2-nitrobenzoic acid (TNB), detectable at $\lambda = 412$ nm. The test is specific to GSH on the basis of the specificity of the GSH reductase enzyme to GSH: the rate of accumulation of TNB is proportional

to the concentration of GSH in the sample. For this assay, the cells extract was diluted 1 : 1 with KPE buffer prior to addition of freshly prepared DTNB and GSH reductase solutions. Following addition of β-NADPH, the absorbance was measured immediately and at 30 s intervals for 1.5 min. The rate of change in absorbance was compared to that of GSH standards. The measurement of GSSG in each sample was identical to that used for the measurement of GSH, but with a previous treatment of each sample with 2-VP, which reacts out with GSH.

2.10. Statistics. The data were analyzed with Prism 5 software (GraphPad, San Diego, CA) using a two-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test or with a one-way ANOVA followed by the Dunnett's test, as appropriate. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of NDGA, IAA and H_2O_2 in Human Monocytes on Cell Viability. First, we evaluated the effect of NDGA on cell viability and determined that NDGA did not decrease the cell viability of MNs at concentrations ranging from 5 to 30 μM (Figure 1(a)); however, the viability of THP-1 cells was slightly decreased at NDGA concentrations of 25 and 30 μM after 120 h incubation. Therefore, the concentration of 20 μM was chosen to evaluate the potential antioxidant effect of NDGA against the damage induced by IAA and H_2O_2 on MNs and THP-1 cells.

We also evaluated the effects of different concentrations of IAA and H_2O_2 on cell viability. The cells were incubated with these oxidants just by 2 h; then the compounds were removed and fresh medium with 20 μM NDGA was added. The cells were incubated until 24 h and viability quantified. As expected, IAA caused a dose-dependent decrease in the viability of the MNs and THP-1 cells. The MN viability decreased progressively and was significant at a concentration of 10 μM IAA. The THP-1 cell viability decreased significantly at 50 to 100 μM (Figure 1(b)). At the same way, H_2O_2 (2.5–20 mM) caused cell death in a concentration-dependent manner. MN cell death increased progressively from 5.0 to 20 mM H_2O_2. THP-1 cell death was substantial and increased significantly from 2.5 to 20 mM H_2O_2 (Figure 1(c)).

We also verified THP-1 cell morphology in bright field micrographs. These cells were round and bright throughout the field when treated with vehicle or 20 μM NDGA (Figure 3). Nevertheless, IAA (25–100 μM) and H_2O_2 (5–20 mM) induced morphological alterations, such as loss of shape round. Both oxidants were able to generate damage on the cells which were dependent on concentrations (Figures 3(a) and 3(b) left side).

We then assessed the protective effect of NDGA on MNs and THP-1 cells cultured in the presence of IAA and H_2O_2 (Figure 2). Our results demonstrate that cell death was decreased in the MNs at all concentrations of IAA (Figure 2(a)), and the NDGA avoided the death of the THP-1 cells by 31% and 41% at 50 μM and 100 μM IAA, respectively (Figure 2(c)). In addition, pretreatment with NDGA decreased cell damage by H_2O_2, and cell death was significantly diminished over a range of 5–20 mM H_2O_2 (Figure 2(b)). For the THP-1 cells, the protective effect of NDGA was significant at 10 mM H_2O_2 (Figure 2(d)). These results also were comparable with percentages of trypan blue negative cells (Figures 2(e) and 2(f)).

Likewise these results were corroborated observing cell morphology in bright field micrographs (Figure 3). Cells incubated with 20 μM NDGA diminished significantly damage in cell morphology when cells were treated with IAA (25 and 50 μM) or H_2O_2 (5 and 10 mM). But the cells pretreated with NDGA and highest concentration of IAA (100 μM) or H_2O_2 (15 and 20 mM) still showed cell damage (Figures 3(a) and 3(b) and right side).

3.2. Determination of ROS Production Induced by IAA and H_2O_2 in Human Monocytes. In this study, we showed that low IAA concentration caused a significant increase of ROS during at 48 h incubation (Figures 4(a) and 4(c)). The relative ROS production was increased for all of the IAA concentrations tested (2.5 to 7.5 μM). Similarly, H_2O_2 induced a significant increase in ROS production (Figures 4(b) and 4(d)) at concentrations of 1.0 and 2.0 mM after at 12 h incubation.

3.3. Glutathione Level in THP-1 Cells Induced by NDGA, IAA, and H_2O_2. The effect of NDGA and toxic compounds (IAA or H_2O_2) on GSH levels was monitored by redox status in THP-1 cells (Figure 5). First, It was found that IAA or H_2O_2 induced reduction of [GSH] + [GSSG] and [GSH] concentrations in a concentration-dependent manner (Figures 5(a) and 5(b)) suggesting that both toxics caused an oxidative stress to the cells. In contrast, pretreatment of cells with 20 μM NDGA (5 μM IAA/NDGA or 1 mM H_2O_2/NDGA) abrogated the reduction of [GSH] + [GSSG] and [GSH] concentrations induced by oxidants. In addition, we also observed that NDGA alone caused slight increase of [GSH] + [GSSG] and [GSH] levels (Figures 5(c) and 5(d)).

3.4. CD33 Expression by IAA and H_2O_2 in Human Monocytes. We showed that treatment with either IAA or H_2O_2 significantly decreased CD33 expression levels in MNs. In IAA-treated cells (2.5, 5.0, and 7.5 μM), CD33 expression levels decreased significantly compared to the control; this decrease in CD33 expression on the cell surface occurred in a concentration-dependent manner (Figures 6(a) and 6(c)). In MNs incubated with 1 and 2 mM H_2O_2, a significant reduction in CD33 expression was observed (Figures 6(b) and 6(d)).

3.5. NDGA Effect on ROS Production and CD33 Expression in Human Monocytes. The protective effect of NDGA was evaluated with regard to H_2O_2- or IAA-induced ROS production and CD33 expression in MNs (Figure 7). NDGA was added to the culture prior to the oxidant exposure (5 μM IAA and 1 mM H_2O_2), and it was maintained in the culture medium during the incubation. The ROS and CD33 expression levels were then measured by flow cytometry.

NDGA prevented oxidative stress because the MNs cultured with NDGA/IAA (Figure 7(a)) or NDGA/H_2O_2

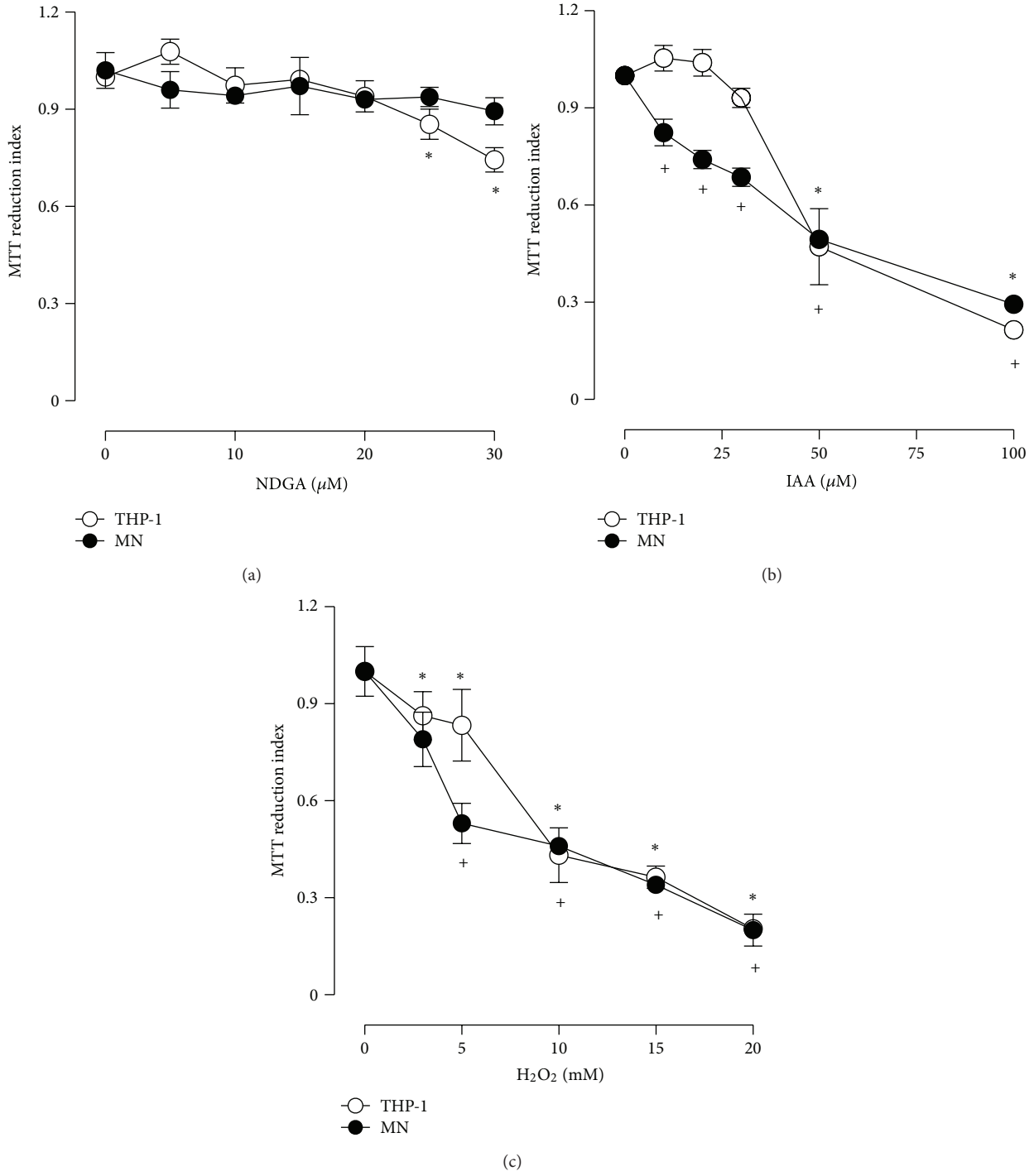

Figure 1: Effect of (a) NDGA, (b) IAA, and (c) H_2O_2 on the viability of human MN and THP-1 cells. (a) NDGA (0–30 μM) was added for 120 h before the measurement of viability. In addition, cells were exposed to (b) IAA (0–100 μM) or (c) H_2O_2 (0–20 mM) for 2 h. At the end of the exposition time, the media containing IAA and H_2O_2 were replaced with fresh medium. Viability was assessed at the end of 24 h of incubation. The number of viable cells is expressed as index of MTT reduction. Data are expressed as mean ± SEM; $n = 5$. $^*P < 0.05$ versus MN (without treatment) and $^+P < 0.05$ versus THP-1 (without treatment). MN: human monocytes; THP1: human acute monocytic leukemia cell line.

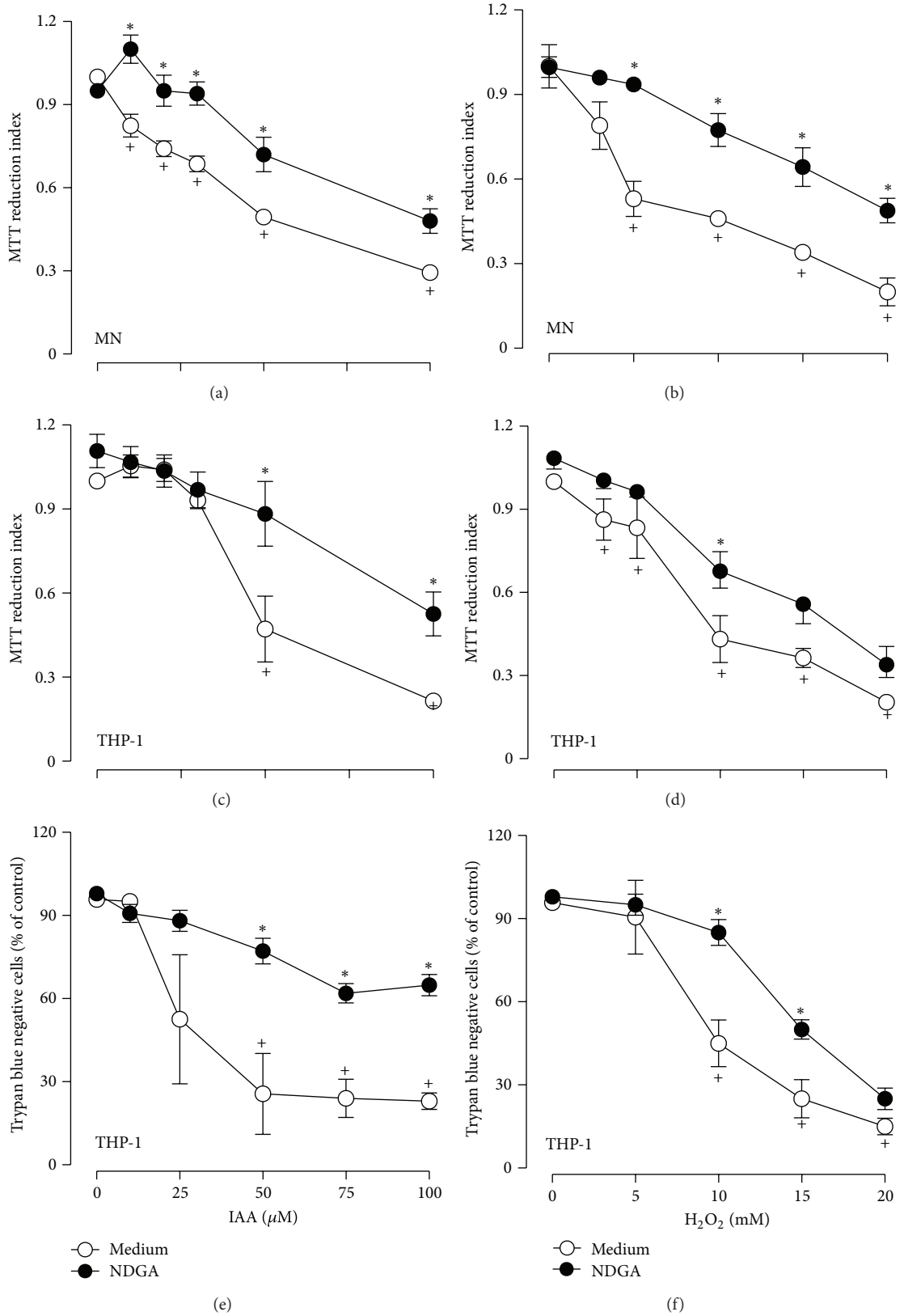

Figure 2: Protective effect of NDGA on the cytotoxicity-induced by ((a), (c), and (e)) IAA and ((b), (d), and (f)) H_2O_2 in human cells. ((a), (b)) MN and ((c), (d), (e), (f)) THP-1 cells were pretreated in absence or presence of 20 μM NDGA by 12 h. Cell cultures were exposed to IAA or H_2O_2 for 2 h and refreshed after removal of the toxic compounds. MTT reduction or trypan blue exclusion was determined 24 h after the onset of IAA or H_2O_2 exposure. The number of viable cells is expressed as ((a), (b), (c), (d)) index of MTT reduction or ((e), (f)) percentage of trypan blue negative cells. Data are expressed as mean ± SEM; $n = 5$. $^{+}P < 0.05$ versus control (without IAA or H_2O_2) and $^{*}P < 0.05$ versus control (without NDGA).

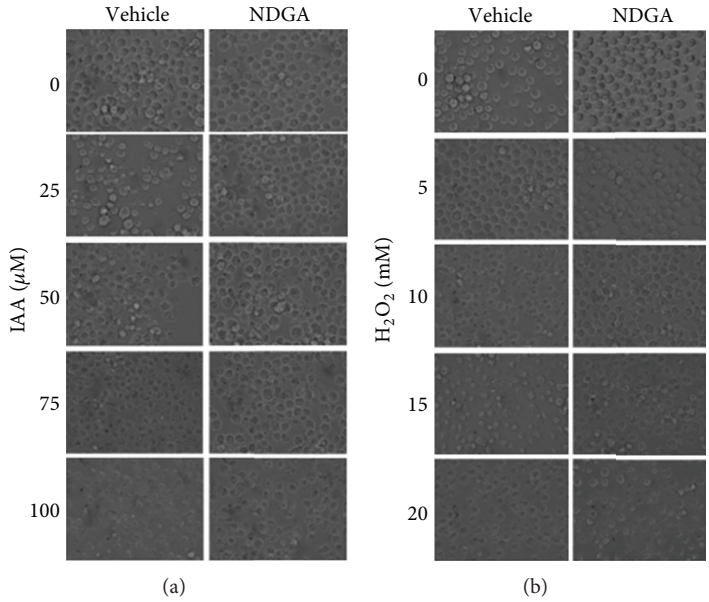

Figure 3: Representative phase contrast micrographs showing the effect of NDGA on the damage induced by different concentrations of (a) IAA or (b) H_2O_2 in THP-1 cells. Cells were treated with vehicle (left side) or 20 μM NDGA (right side) by 12 h; after this the cells were exposed to IAA or H_2O_2 for 2 h. After this time these compounds were withdrawn and fresh medium with NDGA was added. Representative images were obtained 24 h after the onset of IAA or H_2O_2 exposure.

(Figure 7(b)) showed less ROS production. In addition, the decrease of CD33 expression was attenuated by NDGA in the cells exposed to IAA (Figure 7(c)) and H_2O_2 (Figure 7(d)). Notably, the cells exposed to NDGA had a similar CD33 protein expression level compared to the untreated control cells.

4. Discussion

It is well established that NDGA has biological properties, such as anticarcinogenic [35–38], antidiabetic, antiviral, antioxidant, and anti-inflammatory activities in human cell cultures and animal models [3, 39]. Furthermore, NDGA has beneficial health properties, including the growth inhibition of human cancers *in vivo* [40, 41], the degradation of preformed Alzheimer's beta-amyloid fibrils *in vitro* [42], and the protection of cultured rat hippocampal neurons against the toxicity of the amyloid beta-peptide [43].

The purpose of this study was to evaluate the potential protective effects of NDGA on human MNs cultured under oxidative stress conditions. MNs are an essential host defense against microorganisms. MNs use mechanisms that consist of ingesting bacterial material through phagocytosis and killing infectious agents by producing ROS to protect the host [44]. Furthermore, ROS production performs other important physiological functions. For example, ROS participate in signal transduction and gene expression [45, 46]. MNs maintain intracellular redox homeostasis by balancing the production of ROS with their removal through cellular antioxidant defense systems.

However, excessive ROS production can be lethal for the MNs because ROS can attack biomolecules, which causes changes in the structure and function of these molecules. MNs are well known to play a crucial role in the development of ROS-induced pathologies because they can produce non-negligible amounts of ROS. Because of the negative long-term side effects of ROS production by monocytes, modulating ROS generation and maintaining the redox state of the cell at the required physiological level are considered a main therapeutic target [25, 47]. NDGA has a protective effect due to its antioxidant capacity and has garnered increasing interest because it has been reported to contribute to the prevention or delay of oxidative stress-induced damage [4]. In this study, NDGA toxicity was first evaluated in human MNs. Our results demonstrated that, at concentrations of NDGA ranging from 5 to 25 μM, NDGA is not cytotoxic to either THP-1 cells or human MNs over 120 h of treatment. This finding was comparable to animal cells, indicating that NDGA is nontoxic at low doses [4, 9, 10].

The potential protective effect of NDGA was then evaluated in two toxicity models using IAA and H_2O_2 to induce toxicity. Under these conditions, the oxidants caused cell death in a concentration-dependent manner in the THP-1 cells and human MNs. It was previously reported that these oxidants caused damage in primary cultures of rat neurons [9, 10].

IAA injury has been related to ROS production in primary cultures of rat neurons [8, 19]. It also has been demonstrated that in cultured hippocampal neurons, IAA reduces ATP levels and cell survival [18]. Our results suggest that IAA toxicity is related to the exacerbated ROS production and subsequent cell death of MNs (Figures 1(b) and 3(a)).

In contrast, H_2O_2 is a source of $^{\bullet}OH$ in the presence of transition metal ions. This oxygen metabolite reacts rapidly

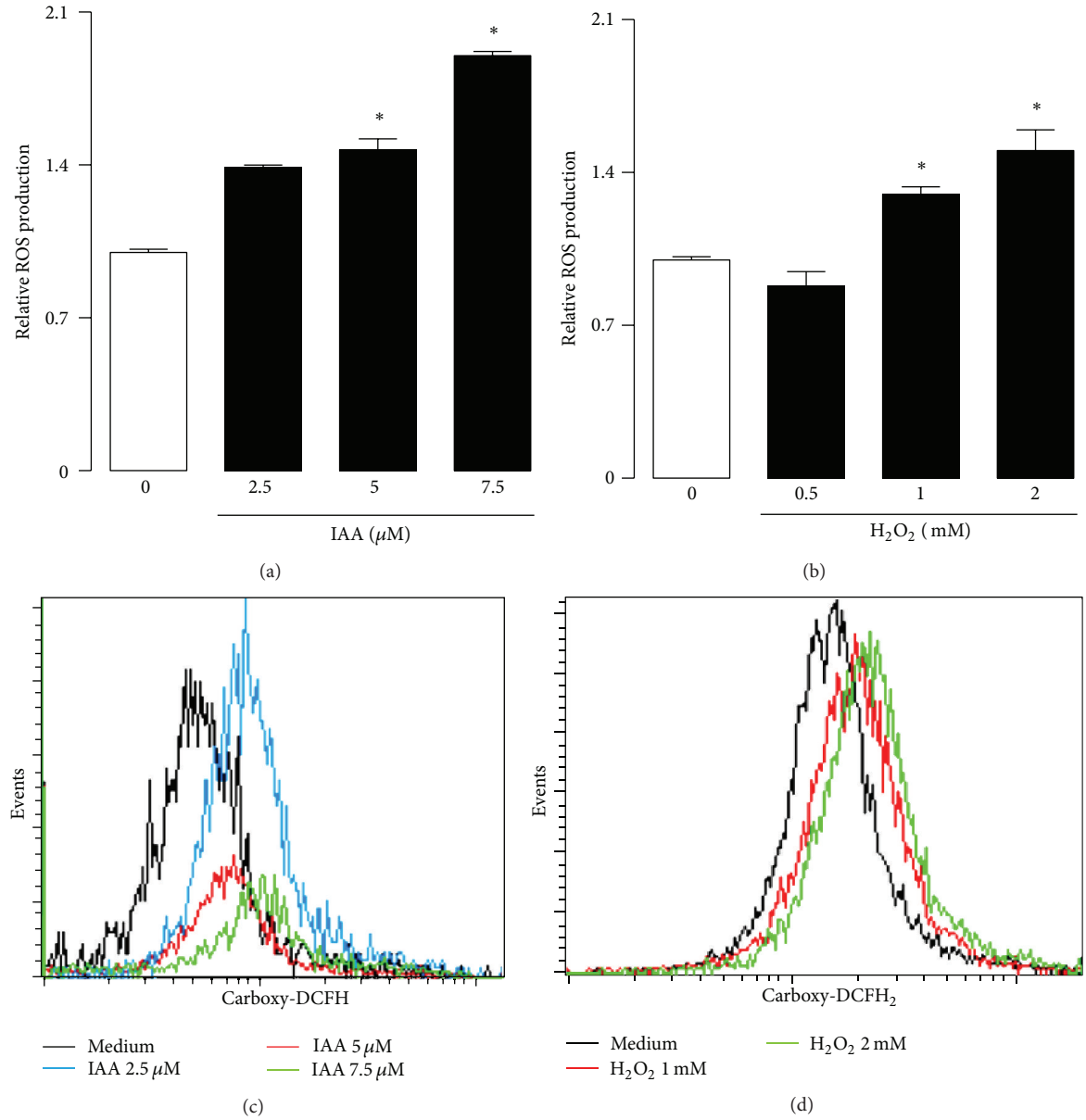

Figure 4: Effect of ((a), (c)) IAA or ((b), (d)) H_2O_2 on ROS generation in MN. Cells were treated 48 h with IAA by 12 with H_2O_2. Then, ROS production was determined by flow cytometry. ROS level was expressed as index with respect to control without treatment. Data are expressed as mean ± SEM; $n = 5$. $^*P < 0.05$ versus control (without IAA or H_2O_2).

and broadly with all biomolecules. As expected, H_2O_2 caused increased ROS production and cell death [12]. In addition, it was recently described that H_2O_2 promotes the opening of the mitochondrial permeability transition pore (PTP), resulting in membrane depolarization, uncoupling of oxidative phosphorylation, and potential cell death in porcine LLC-PK1 cells [11].

In addition, we demonstrated that NDGA protected MNs and THP-1 cells against H_2O_2. We observed that treatment with NDGA prior to the toxic challenges induced by IAA and H_2O_2 significantly diminished the toxicities of these compounds. In this context, it has been shown that the protective effect of NDGA is predominantly due to its antioxidant capacity. In fact, the direct ROS scavenging capacity and induction of antioxidant enzymes via the Nrf2 pathway may be involved in the mechanism by which NDGA exerts its protective effect [4, 9–11]. Recently, it was described that NDGA can prevent the mitochondrial damage that is induced by oxidative stress in renal epithelial LLC-PK1 cell cultures [11] and in an animal model of renal damage [48].

We also explored the effect of NDGA on the oxidant stress-induced downregulation of CD33 expression in human MNs. Our results indicated that NDGA is a potent antioxidant that can prevent low levels of oxidative stress and can

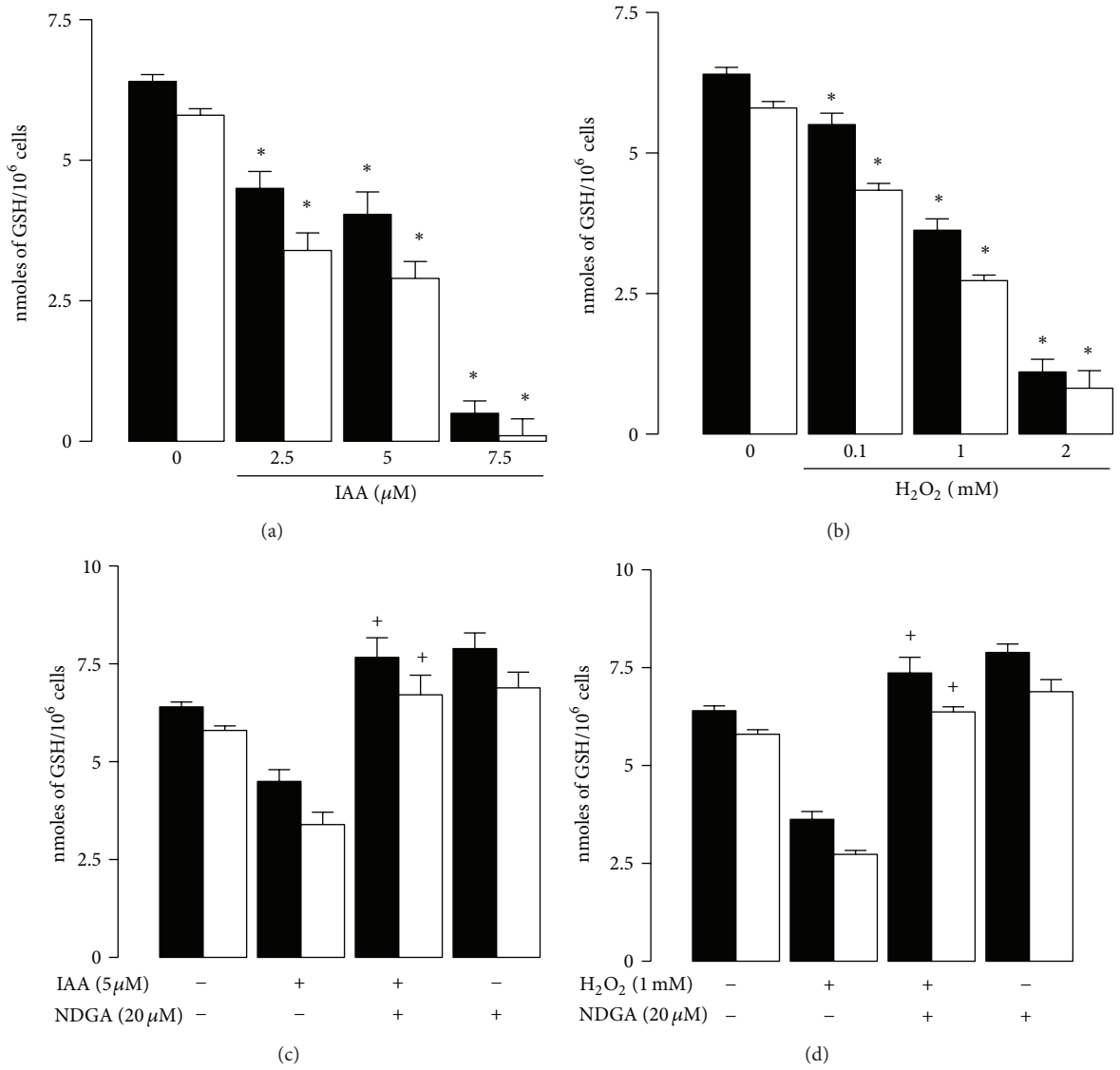

Figure 5: Effect of IAA or H_2O_2 on GSH + GSSG and GSH levels in THP-1 cells. Cells were treated 24 h with (a) IAA or (b) H_2O_2. After this time these compounds were withdrawn and fresh medium was added. GSSG and GSH levels were measured 24 h after the addition of toxic compounds. In addition the effect of NDGA on GSSG+GSH and GSH levels in (c) IAA or (d) H_2O_2 treated cells was also studied. Cells were treated with vehicle or 20 μM NDGA by 12 h; after this the cells were exposed to 5 μM IAA or 1 mM H_2O_2 for 24 h. GSSG and GSH levels were measured 24 h after the addition of toxic compounds. GSH levels were quantified by spectrophotometric/microplate reader assay. Data are expressed as mean ± SEM; $n = 3$. $^*P < 0.05$ versus control (without IAA or H_2O_2) and $^+P < 0.05$ versus the respective toxic compound.

also prevent the decrease in CD33 expression in cells treated with IAA and H_2O_2.

The mechanisms involved in the expression of CD33 have not been fully elucidated. But have been described two mechanisms of repressive control [49, 50]. First, CD33 activity is decreased by SOCS3, which is a member of the suppressor of cytokine signaling (SOCS) protein family. The binding of SOCS3 to the phosphorylated immunoreceptor tyrosine-based inhibitory motifs (ITIM) of CD33 induces the proteosomal degradation of both molecules and the reduction of CD33 expression on surface monocytes and blockades the increased secretion of IL-1β, IL-8, and TNF-α [49, 51]. The second mechanism described in myeloma cells was induced by IL-6. This cytokine upregulates the expression of helix-loop-helix leucine zipper transcription factor (MYC) via transcriptional regulator of the immune response 3 (STAT3) phosphorylation. MYC binds directly to the promoter region of the CCAAT enhancer binding protein α (C/EBPA gene), which downregulates C/EBPA and thus CD33 gene expression is decreased [50]. Finally, Gonzalez et al. (2012) showed that hyperglycemia diminished both mRNA and CD33 expression in surface cell. But, when the human monocytes were treated with α-tocopherol, this negative modulation was prevented. In fact, it is well known that SOCS3 is modulated by oxidative stress in response to hyperglycemia [52, 53] and the TNF-α production is

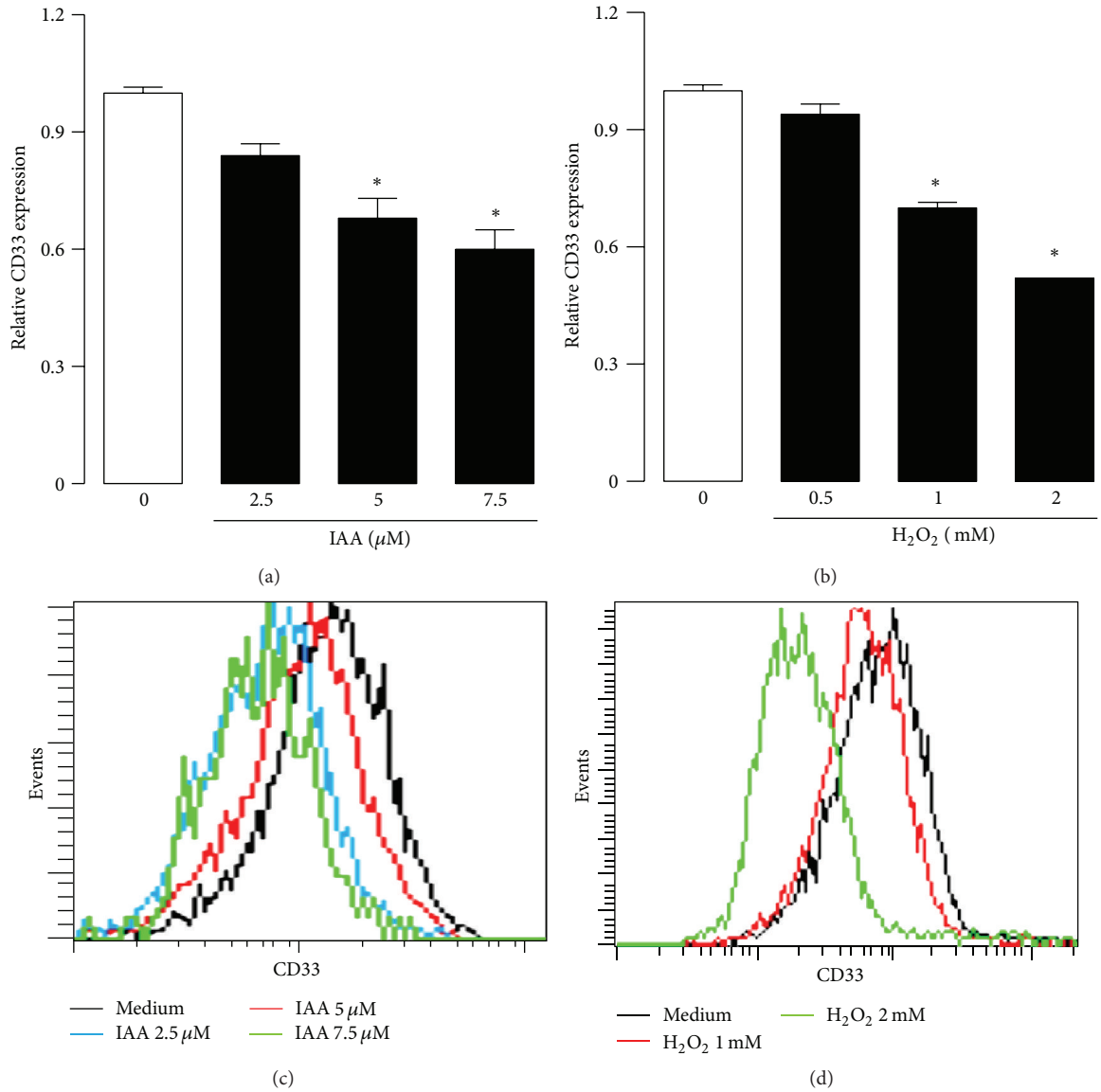

Figure 6: Effect of ((a), (c)) IAA or ((b), (d)) H_2O_2 on CD33 expression in MN. Cells were treated by 72 h with IAA or by 48 h with H_2O_2. Then, CD33 protein presence was determined by flow cytometry. The protein level was expressed as index with respect to control without treatment. Data are expressed as mean ± SEM; $n = 5$. $^*P < 0.05$ versus control (without IAA or H_2O_2).

induced by H_2O_2 via oxidative stress-related signal pathways [54]. Because our previous study found that the antioxidant α-tocopherol prevents TNF-α production and CD33 downregulation, therefore it is possible that ROS induction could be participating in these processes.

Furthermore, MYC is induced by oxidative stress generated by sodium arsenate in the cell line MCG-7 [55]. Even C/EBPA is susceptible to negative regulation by oxidative stress induced by H_2O_2 in 3T3-L preadipocyte cells [56] and by ethanol in HepG2 cells [57]. In addition, we observed a significant decrease of CD33 protein in surface cells in oxidative condition induced by IAA and H_2O_2. This result supports the idea that oxidative stress could alter the transcription of CD33 by modulating the transcription factors such as STAT, MYC, and C/EBPA.

Preliminary studies in our research group demonstrated that oxidative stress induced by hyperglycemia decreases CD33 expression in human monocytes, but the pretreatment with the antioxidant α-tocopherol prevents ROS production and alteration in the CD33 expression. NDGA and α-tocopherol prevent ROS generation. α-Tocopherol inhibits superoxide anion production by impairment of the NADPH oxidase assembly and inhibits p47phox translocation to the membrane [58]. Moreover, NDGA is a selective inhibitor of 12-lipoxygenase (12-LOX), which produces ROS during arachidonic acid metabolism. The mechanism by which ROS induced CD33 downregulation could be through the induction of inflammation, because IAA or H_2O_2 induces the inflammatory cytokine TNF-α, and NDGA is a powerful antioxidant compound that affects a wide variety of cellular

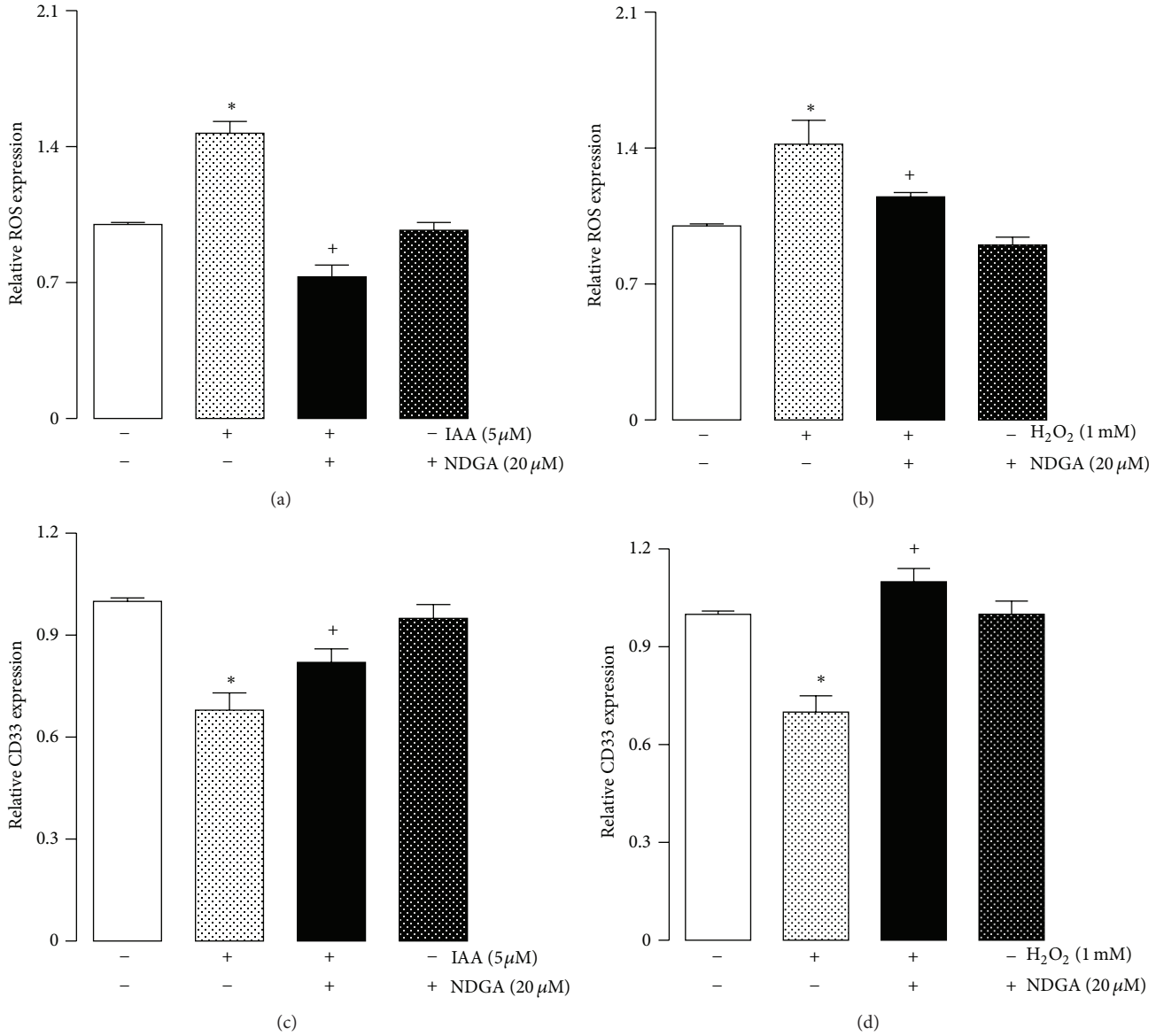

Figure 7: Effect of NDGA on ((a), (c)) IAA or ((b), (d)) H_2O_2 induced increase of ((a), (b)) ROS levels and ((c), (d)) decrease of CD33 expression in MN. Cells were pretreated with vehicle or 20 μM NDGA for 12 h before the addition of 5 μM IAA or 1 mM H_2O_2 and incubated for 72 and 48 h, respectively. ROS levels detection and CD33 expression were determined by flow cytometry and expressed as index compared to control without treatment. Data are expressed as mean ± SEM; $n = 5$. $^{+}P < 0.05$ versus control (without IAA or H_2O_2) and $^{*}P < 0.05$ versus control (without NDGA).

processes including TNF-α. In this work, NDGA prevented the decrease in the expression of CD33 secondary to oxidative stress induced by H_2O_2 or IAA. These data suggest that alterations in CD33 secondary to oxidative conditions may be counteracted by exogenous antioxidants of different structure such as α-tocopherol or NDGA. In addition, low concentration of NDGA may contribute to decreased oxidative stress by either scavenging ROS [4] and/or by the induction of Nrf2-dependent antioxidant enzymes [9, 11] and avoiding inflammation as inhibitor or COX-2 and LOX [39].

In conclusion, this study presents novel findings supporting the ameliorative effect of NDGA on the oxidant condition of human MNs. NDGA could prevent cell death under severe oxidative stress conditions. There was a slight increase of ROS production induced by H_2O_2 and IAA and a significant decrease of CD33 expression on MN surfaces. However, NDGA prevented these negative effects. These results suggest that changes in the redox state induced by hyperglycemia, IAA, or H_2O_2 generate an important signal that causes CD33 modulation in a negative manner, and this state might contribute to MN activation.

Acknowledgments

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