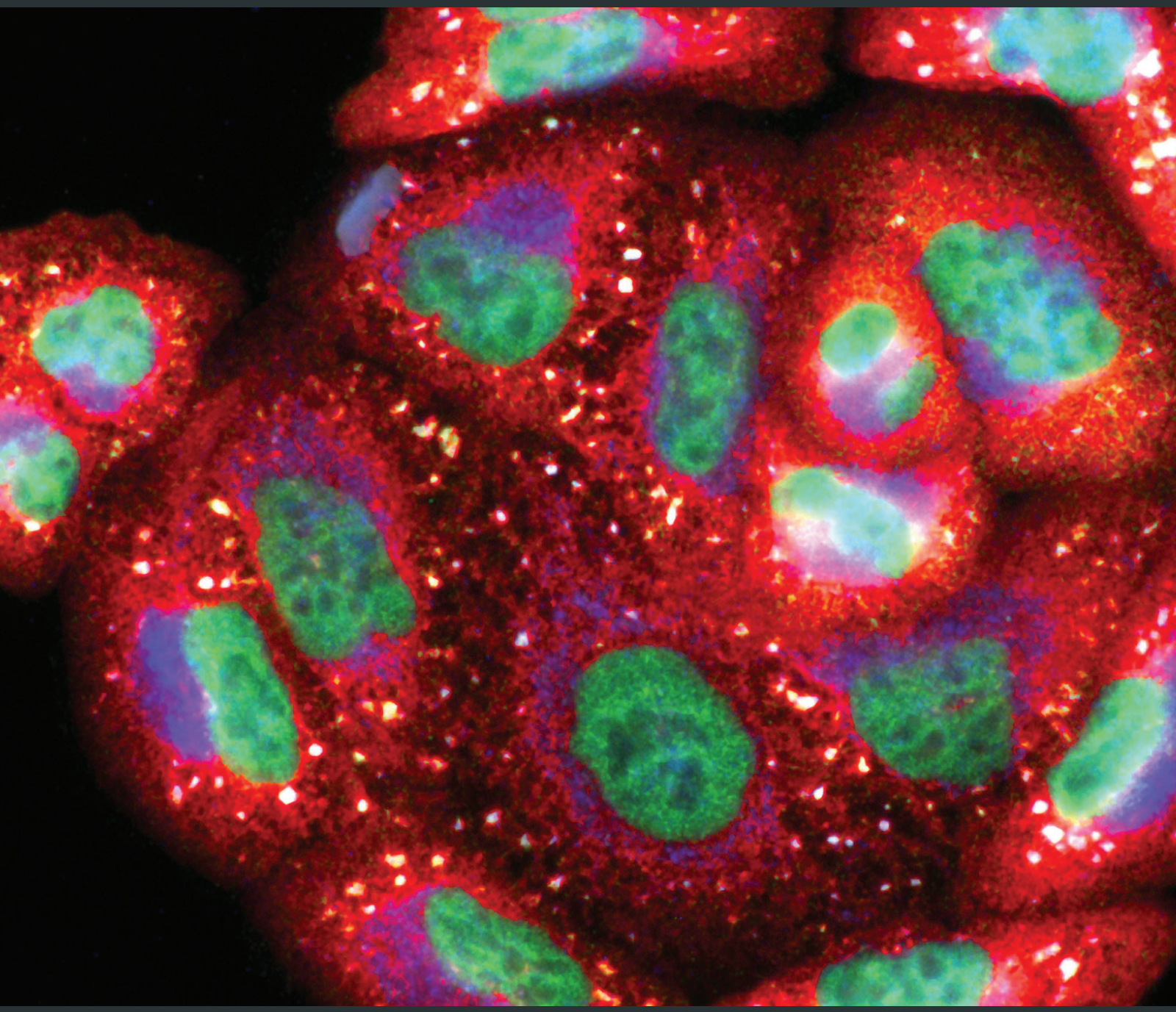
Oxidative Medicine and Cellular Longevity

Antioxidants in Longevity and Medicine 2014

Guest Editors: Narasimham L. Parinandi, Nilanjana Maulik, Mahesh Thirunavukkarasu, and David W. McFadden



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Editorial Board

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Editorial
Antioxidants in Longevity and Medicine 2014

Narasimham L. Parinandi,[1] Nilanjana Maulik,[2] Mahesh Thirunavukkarasu,[2] and David W. McFadden[2]

[1]*Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Medicine, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, 473 W. 12th Avenue, Room 601, Columbus, OH 43210, USA*
[2]*Department of Surgery, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06032, USA*

Correspondence should be addressed to Narasimham L. Parinandi; narasimham.parinandi@osumc.edu

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The reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated from the enzymatic and nonenzymatic sources from superoxide ($O_2^{\bullet -}$) and nitric oxide (NO), respectively, are established to cause oxidative, nitroxidative, nitrative, and nitrosative stresses in the biological systems. Scientific evidence is mounting in favor of the involvement of ROS-induced oxidative stress and RNS stress in diverse pathophysiological states in experimental animal models and humans including the cardiovascular, cerebrovascular, and renal diseases to name a few. Among many proposed and tested theories of aging, the oxidative stress theory of aging underscores the ROS-induced damage as being instrumental in the progressive and unalterable alterations at the molecular, cellular, and organ levels during aging and shortening of life span. The balance between the ROS/RNS and antioxidants is highly critical to maintain the cellular health and any alteration in that balance caused by either the elevation of production of ROS/RNS or decrease of antioxidant status in the cells will lead to diverse pathophysiological states. Antioxidants (enzymatic and nonenzymatic), either endogenous or exogenous, are known to counteract the deleterious actions of ROS/RNS leading to protection against the oxidative stress and RNS stress. Therefore, this special issue focuses on the possibilities of protection against the ROS-induced oxidative stress and RNS stress which could potentially impact aging, life span, and diseases among humans. Several experts in the fields of ROS and RNS biology have contributed original research and review articles highlighting the state-of-the-art cellular and molecular mechanisms and protection of pathophysiology of oxidative stress and RNS stress in diseases, aging, and age-associated pathophysiological states.

The articles published in this special issue are broadly grouped into (1) conditions that elevate oxidative stress, (2) conditions that decrease/attenuate oxidative stress, and (3) aging and oxidative stress.

Articles on Conditions/Factors That Elevate Oxidative Stress. As the deficiency of estrogen in postmenopausal women is shown to have a direct impact on their susceptibility to cardiovascular diseases, A. Pósa et al. conducted a study on the endogenous estrogen-mediated heme oxygenase (HO) regulation in rats under conditions of menopause. In this study, the investigators used an estrogen depletion model and showed that the sensitivity of the animal model to myocardial ischemia was associated with the suppression of HO activity and expression of HO-1/HO-2 as well as an increase in the secretion of proinflammatory cytokines and biomarkers. Also, the authors demonstrated elevated myeloperoxidase activity as well as the depression of the electrocardiogram ST segment in the estrogen-depleted rats which was exacerbated by inhibiting heme oxygenase activity. Obstructive sleep apnea (OSA) is identified as an independent risk factor in cardiovascular diseases through a state of intermittent hypoxia (IH) that induces oxidative stress and inflammation.

The endogenous transition metal-binding protein, metallothionein (MT), is identified as an inducible antioxidant that may offer protection against oxidant-induced damage. Along these lines, S. Zhou et al. conducted the study on the deletion of MT which exacerbated the IH-induced oxidative and inflammatory injury in the aorta. The results of this study suggested that IH could be an important condition/factor that could lead to aortic injury through oxidative stress and inflammation, wherein MT might protect against the IH-induced vascular injury. Goeckerman therapy (GT, skin treatment with a combination of 3% crude coal tar ointment and UV irradiation) is a current form of skin treatment for children suffering from plaque psoriasis, a multifactorial skin disease. L. Borska et al. carried out a prospective cohort study on children with plaque psoriasis receiving the GT and showed adverse (side) effects of the treatment on the subjects following the GT. The authors reported an increase in several oxidative stress markers including the oxidative nucleic acid damage, formation of the benzo(a)pyrene-7,8-diol-9,10-epoxide-DNA (BPDE-DNA) adducts, and chromosomal abnormalities (index of genotoxicity) in lymphocytes of the subjects following the GT. However, the authors acknowledged that elevated levels of oxidative stress and genotoxic biomarkers might not necessarily be associated with the side effects of the GT and additionally the treatment showed significant clinical benefit. L. A. Rabelo et al. provided a comprehensive review on arginase as a critical prooxidant mediator in the binomial endothelial dysfunction atherosclerosis. This review focused on the involvement of arginase in vascular endothelial function and its role in atherosclerosis. Also, the authors discussed adverse actions of arginase in relation to oxidative stress and in various metabolic diseases. The authors specifically described the role of arginase in endothelial function and the production of atherosclerotic lesions with an emphasis on the mechanisms of regulation of the enzyme and potential development of strategies of pharmacological intervention of CVDs.

Articles on Conditions/Factors That Decrease Oxidative Stress. Currently, there is a dearth of evidence/information on the interaction(s) between biomarkers of oxidative stress and chemokines in humans, specifically among adults without overt clinical disease(s). Therefore, Y. Li et al. focused on studying the relationship between antioxidants and markers of lipid peroxidation along with different chemokines in adults. In this study, the authors observed a positive correlation between total antioxidant status and different chemokines. However, the authors were unable to establish association between chemokines and biomarkers of lipid peroxidation. Mitochondrial respiratory chain dysfunction and elevated oxidative stress are markedly encountered in the brain during aging. With the aim to investigate whether natural products would alleviate/mitigate the age-associated cerebral mitochondrial respiratory dysfunction and enhanced oxidative stress, A. B. de Sá-Nakanishi et al. investigated the effects of treating old rats with aqueous extracts of *Agaricus blazei* (medicinal mushroom) on oxidative and functional outcomes of the brain tissue and brain mitochondria of 21-month-old rats. The results of this study revealed that the intragastric administration of mushroom extract offered protection against elevated oxidative stress (ROS production and lipid peroxidation) and increased the antioxidant (nonenzymatic and enzymatic) status in the brain of old rats. Furthermore, administration of the mushroom extract showed enhancement of the activity of different mitochondrial respiratory enzymes and mitochondria-coupled respiration. This study revealed that natural products present in the medicinal mushroom extract offered protection against the age-associated oxidative stress and enhanced mitochondrial activity in the brain of old rats. The natural product, 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside (TSG) obtained from the Chinese herb, *Polygonum multiflorum* (traditionally used as an antiaging plant remedy), was investigated by C. Büchter et al. for its actions to increase the life span and stress resistance of *Caenorhabditis elegans*. This study revealed that TSG increased the antioxidative potential and stress resistance and prolonged life span in the nematode as compared to the actions exhibited by resveratrol. In a review article, S. Zhou et al. extensively discussed the role of nuclear factor-erythroid (NF-E) 2-related factor 2 (Nrf2) in cardiac remodeling and heart failure. In this review the authors described the role of Nrf2 in various antioxidative phenomena, cytoprotection, inducible expression of antioxidant genes, and phase II detoxifying enzymes in the cardiovascular system. Their focus of this review was to disclose the antioxidative capability of Nrf2 as well as its protective actions against cardiac remodeling and heart failure. S. C. Khor et al., in their review, discussed in detail vitamin E in sarcopenia with the current evidences on its role in prevention and treatment of sarcopenia. This review revealed vitamin E as a potential treatment for sarcopenia, a disease of the old age associated with decreased muscle mass and strength. In addition, the authors discussed the protective/beneficial actions of vitamin E in combating oxidative stress and improving muscle health. Moreover, the authors expressed that further studies are warranted to establish the specific mechanism(s) of protective actions of vitamin E in sarcopenia.

Articles on Aging and Oxidative Stress. Currently, evidences are accumulating in support of micro-RNAs (miRNAs) as the posttranscriptional regulators of aging. Aiming at this, S. G. S. Khee et al. studied the expression of senescence-associated miRNAs and their target genes in cellular aging and their modulation by tocotrienol-rich fraction (an isomer of vitamin E) in young and old human diploid fibroblasts. This study suggested that tocotrienol prevented cellular senescence of human diploid fibroblasts through the modulation of certain senescence-associated miRNAs and the expression of target genes. Sirtuins (silent information regulator proteins) are focused to prevent oxidative damage that threatens female fertility. SIRT1 and SIRT3, the main members of the sirtuin family, have been recognized as the sensors and guardians of redox status in oocytes, granulosa cells, and embryos. Taking this into consideration, C. Tatone et al. reviewed in detail the functions of sirtuins in female fertility and their possible role in oxidative stress and aging. This review discussed the role of sirtuins in the prevention of female infertility arising from

oxidative stress. More specifically, the authors addressed the actions of SIRT1 and SIRT3 and their functions as sensors and protectors of oxidative stress at various stages of the reproductive cycle. The authors envisioned future studies that would lead to the use of sirtuins as targets to rectify female infertility. L. Marseglia et al. discussed in detail in their review the oxidative stress-mediated aging during the fetal (pregnancy) and perinatal stages focusing on the onset of common disorders in the newborn as an outcome of the early aging process. The authors proposed the onset of aging prior to birth. This review described the significant role of oxidative stress in the pathogenesis of various pregnancy-related and neonatal disorders.

Attenuating or inhibiting the overwhelming production and deleterious actions of ROS and RNS by treatments with several antioxidants is increasingly becoming sought-after therapeutic strategies to treat age-related diseases and extend the life span wherein the ROS-induced oxidative stress and RNS stress play crucial roles. The goal of this special issue is to disclose the latest understanding of the mechanisms and protection of oxidative stress and RNS stress and to create opportunities to move the field forward that will impact the oxidative stress-mediated diseases and aging in humans. Hence, the articles in this special issue contributed by the experts in the fields of oxidative and RNS stress biology will nurture continued discoveries towards better understanding of the mechanisms and protection of oxidant injury, oxidative stress-mediated diseases, and age-related pathophysiological states.

Narasimham L. Parinandi
Nilanjana Maulik
Mahesh Thirunavukkarasu
David W. McFadden

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Research Article

Endogenous Estrogen-Mediated Heme Oxygenase Regulation in Experimental Menopause

Anikó Pósa,[1] Renáta Szabó,[1] Anett Csonka,[1] Médea Veszelka,[1] Anikó Magyariné Berkó,[1] Zoltán Baráth,[2] Rudolf Ménesi,[1] Imre Pávó,[1] Mariann Gyöngyösi,[3] Ferenc László,[1] Krisztina Kupai,[1] and Csaba Varga[1]

[1]*Department of Physiology, Anatomy and Neuroscience, Faculty of Science and Informatics, University of Szeged, Kozep Fasor 52, Szeged 6726, Hungary*
[2]*Faculty of Dentistry and Department of Orthodontics and Pediatric Dentistry, University of Szeged, Szeged 6720, Hungary*
[3]*Department of Cardiology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria*

Correspondence should be addressed to Anikó Pósa; paniko@bio.u-szeged.hu

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Estrogen deficiency is one of the main causes of age-associated diseases in the cardiovascular system. Female Wistar rats were divided into four experimental groups: pharmacologically ovariectomized, surgically ovariectomized, and 24-month-old intact aging animals were compared with a control group. The activity and expression of heme oxygenases (HO) in the cardiac left ventricle, the concentrations of cardiac interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), the myeloperoxidase (MPO) activity in the cardiac left ventricle, and the effects of heme oxygenase blockade (by 24-hour and 1-hour pretreatment with tin-protoporphyrin IX, SnPP) on the epinephrine and phentolamine-induced electrocardiogram ST segment changes *in vivo* were investigated. The cardiac HO activity and the expression of HO-1 and HO-2 were significantly decreased in the aged rats and after ovariectomy. Estrogen depletion was accompanied by significant increases in the expression of IL-6 and TNF-α. The aged and ovariectomized animals exhibited a significantly elevated MPO activity and a significant ST segment depression. After pretreatment with SnPP augmented ST segment changes were determined. These findings demonstrate that the sensitivity to cardiac ischemia in estrogen depletion models is associated with suppression of the activity and expression of the HO system and increases in the secretion of proinflammatory cytokines and biomarkers.

1. Introduction

Many epidemiological studies have suggested the involvement of free radicals and oxidative stress in aging and certain age-related processes that often accompany the menopause [1–3]. An increased level of production of reactive oxygen species (ROS) is considered to be one of the major causes of age-related morbidity (e.g., coronary artery and general cardiovascular dysfunctions) [4]. Estrogen protects women against cardiovascular diseases and seems to play a major role in sex-related differences in hypertension in experimental models [5]. Antioxidant properties may also be involved since estradiol can reduce the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits and increase the expression of superoxide dismutase [6, 7].

Estradiol protects endothelial cells against damage by oxidants and induces the generation of endothelial derived vasodilators, such as nitric oxide (NO) [8]. Recent data indicate that another system associated with cardioprotection, the heme oxygenase (HO) system, is also affected by estrogen [9]. HO-1 can catalyze the oxidative degradation of heme to yield equimolar amounts of biliverdin, free iron, and carbon monoxide (CO). Biliverdin is metabolized to bilirubin by biliverdin reductase. Among the products of HO-1, bilirubin and biliverdin are the most potent endogenous scavengers of ROS and CO exerts antiapoptotic and anti-inflammatory

effects through the induction of soluble guanyl cyclase. CO additionally suppresses the production of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) [10].

The aim of our work was to investigate the effects of estrogen depletion on the activation and expression of HO in the cardiovascular system and on inflammatory biomarkers, such as the levels of TNF-α and interleukin-6 (IL-6) and the activity of myeloperoxidase (MPO) in experimental menopause.

2. Methods

2.1. Experimental Groups of Animals. All experimental procedures were performed in accordance with the standards of the European Community guidelines on the care and use of laboratory animals and had been approved by the Institutional Ethics Committee.

Female Wistar rats were divided into four groups: 4-month-old sham-operated controls, 4-month-old pharmacologically ovariectomized (POVX) animals, 4-month-old surgically ovariectomized (OVX) animals, and 24-month-old ovary-intact (aged) animals.

Ovariectomy surgery was performed on anesthetized animals via a small ventral abdominal midline incision. The ovaries were clamped bilaterally and removed. The uterine horns were tied and the uterus was left intact. The abdominal wall was then sutured. In the sham procedure, the animals were anesthetized and the abdominal wall was opened but the ovaries were not removed. The ovaries were exteriorized to create a similar degree of stress. Other groups of female rats received treatment with 750 μg/kg triptorelin (Decapeptyl depot, Ferring, Germany) i.m. every 4 weeks in order to achieve pharmacological ovariectomy [11]. After a 6-week resting period to verify the surgically or pharmacologically induced menopause and to ensure that all animals were killed at the same stage of the estrus phase (all the control rats were in the proestrus phase) via Giemsa staining method we checked the estrogen level with estrogen quantitative enzyme-linked immunosorbent assay according to the manufacturer's directions (Quantikine rat Estrogen Elisa kit, R&D Systems Inc.) [12].

2.2. Cardiac Left Ventricle HO Activity. The cardiac left ventricle (LV) tissues were homogenized (Ultra Turrax T25; 13.500 min^{-1}; 2 × 30 s) in ice-cold 10.0 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 32.0 mM sucrose, 1.0 mM dithiotreitol, 0.10 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), 10.0 μg/mL trypsin inhibitor, 10.0 μg/mL leupeptin, and 2.0 μg/mL aprotinin; pH 7.4. The supernatant was collected by centrifugation at 15000 g for 20 min at 4°C. The reaction mixture contained the following compounds in a final volume of 1.50 mL: 2.0 mM glucose-6-phosphate, 0.14 U/mL glucose-6-phosphate dehydrogenase, 15.0 μM hemin, 120.0 μg/mL rat liver cytosol as a source of biliverdin reductase, 2.0 mM $MgCl_2 \times 6H_2O$, 100.0 mM KH_2PO_4, and 150.0 μL of supernatant. To start the reaction 100.0 μL the reduced form of β-nicotinamide adenine dinucleotide phosphate, reduced form (150.0 μM), was added to the samples and they were then incubated in the dark at 37°C for 60 min. The reaction was stopped by placing the samples on ice. The bilirubin formed was calculated from the difference between the optical densities obtained at 464 and 530 nm. Bilirubin solution was used as standard (58.47 μg/mL; 10.0 μM). Protein content was determined by spectrophotometric assay (Bio-Rad Protein Assay).

One unit of HO activity was defined as the amount of bilirubin (nmol) produced per hour per mg protein.

2.3. Cardiac LV HO-1 and HO-2 Expression. The expression of HO-1 and HO-2 enzymes was determined by Western blot analysis. The cardiac LV tissues were homogenized (Ultra Turrax T25; 13.500 min^{-1}; 2 × 30 s) in ice-cold Tris-mannitol buffer (2.0 mM Tris 7–9, 50.0 mM mannitol, 100.0 μM phenyl methyl sulfonyl fluoride, 2.0 μM leupeptin, 0.50 mU/mL aprotinin, and 0.50% Triton X-100). Homogenizates were centrifuged at 12000 g for 20 min at 4°C. Protein content was measured by spectrophotometric assay (Bio-Rad Protein Assay).

Aliquots of 25.0 μg of total cellular protein were denatured by mixing and boiling with 20.0 mM Tris 7–9, 3.0 mM EDTA, 2.0% sodium dodecyl sulfate, 10.0% β-mercaptoethanol, and 20.0% glycerol. The samples were electrophoresed (100 V, 50 mA) on 10.0% polyacrylamide gel and transferred (100 V, 100 mA, 2 h) to a nitrocellulose membrane (Amersham, Pharmacia Biotech., Buckinghamshire, UK). Equal protein loading was determined by staining the blot with 0.10% Ponceau red in 5.0% acetic acid. Two h after blocking with PBS (pH 7.4), 0.25% Tween 20, and 5.0% fat-free dried milk, the membrane was probed for 2 h with mouse anti-HO-1 monoclonal antibody (1/10.000; StressGen Biotechnologies Corp., Victoria, Canada) or anti-HO-2 monoclonal antibody (1/1000; StressGen Biotechnologies Corp., Victoria, Canada) at room temperature, washed 3 times with PBS-Tween 20, and then incubated for 1 h with horseradish peroxidase-conjugated bovine anti-mouse antibody (1/2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature. Membranes (Hybond ECL Nitrocellulose membrane, Amersham, Pharmacia Biotech., Buckinghamshire, UK) were developed by using an enhanced chemiluminescence system (ECL+Plus, Amersham Pharmacia Biotech., Buckinghamshire, UK) and exposed to Hyperfilm (Biomax light-1, Eastman Kodak Comp., Rochester, NY, USA). Films were analyzed by using ImageQuant Software (Amersham Pharmacia Biotech., Buckinghamshire, UK) after scanning with GelAnalyst 3.01 Software (Iconix, Toronto, Canada).

2.4. Measurement of Cardiac LV IL-6 and TNF-α Concentrations. The IL-6 and TNF-α levels were determined by means of quantitative enzyme-linked immunosorbent assays according to the manufacturer's directions (Quantikine rat IL-6 and TNF-alpha Elisa kit, R&D Systems Inc.). Optical density was measured at 450 nm (Benchmark Microplate reader; Bio-Rad). IL-6 and TNF-α were expressed as pg/mg protein.

2.5. Cardiac LV MPO Activity. The cardiac LV tissues were homogenized (Ultra Turrax, T25, 13 500 r.p.m., twice for 30 s) in ice-cold phosphate buffer (50 mM, pH 6.0), freeze-thawed three times, and then centrifuged twice (each time at 15 000 g for 15 min at 4°C). A 12 μL aliquot of the supernatant was next mixed with 280 μL of phosphate buffer (50 mM, pH 6) containing 0.167 mg mL^{-1} of O-dianisidine dihydrochloride and the reaction was started with 10 μL of 0.03% hydrogen peroxide and assayed spectrophotometrically at 490 nm (Benchmark Microplate reader; Bio-Rad, Budapest, Hungary) after 90 s of shaking. MPO activity was expressed as mU/mg protein.

2.6. Experimental Angina Provoked by Epinephrine Plus Phentolamine. The standard limb lead II of the surface electrocardiogram (ECG) was recorded by the HAEMOSYS system (Experimetria Ltd., Budapest, Hungary). The change in the ST segment was measured and used as the index of angina severity. The mean ECG voltage 13 ms after the peak of the S wave was defined as the value of the ST segment, as described previously [13, 14]. The difference in the amplitude of the ST segment after and before the administration of the angina-provoking agents was calculated and expressed as the depression of the ST segment in mV. In the epinephrine plus phentolamine model, a single dose of epinephrine (10.0 μg/kg) and 30 s later the α-adrenoceptor antagonist phentolamine (15.0 mg/kg) were administered into the tail vein of the rat. Each agent was dissolved in 0.20 mL of physiological saline and injected over 2 s. The ECG was recorded simultaneously. To investigate the effects of HO enzyme activity inhibition on ST segment changes tin-protoporphyrin IX (30.0 μmol/kg, s.c., pH 7.4) was administrated 24 h and 1 h before treatment.

2.7. Chemicals. Apart from epinephrine (Tonogen, Richter Gedeon, Hungary), Phentolamine (Regitin, P; Ciba-Geigy, Switzerland), SnPP (Frontier Scientific Europe, United Kingdom), and triptorelin (Decapeptyl depot, Ferring, Germany), all chemicals were from Sigma Aldrich Company.

2.8. Statistical Analysis. Data are reported as means ± S.E.M. of the results from at least 3 independent experiments. Western blots are shown as representative photographs of three independent experiments. Statistical significance was assessed by ANOVA; $P < 0.05$ was taken as significant.

3. Results

3.1. Cardiac LV HO Activity. As shown in Figure 1, the activity of HO was determined by measurement of bilirubin formation in the cardiac LV. We found that HO activity was significantly ($^*P < 0.05$) decreased in the ovariectomized (POVX: 0.68 ± 0.1 nmol bilirubin/h/mg protein, $n = 12$; OVX: 0.73 ± 0.1 nmol bilirubin/h/mg protein, $n = 11$) and aged (0.61 ± 0.13 nmol bilirubin/h/mg protein, $n = 11$) animals compared to the control group (1.59 ± 0.2 nmol bilirubin/h/mg protein, $n = 11$).

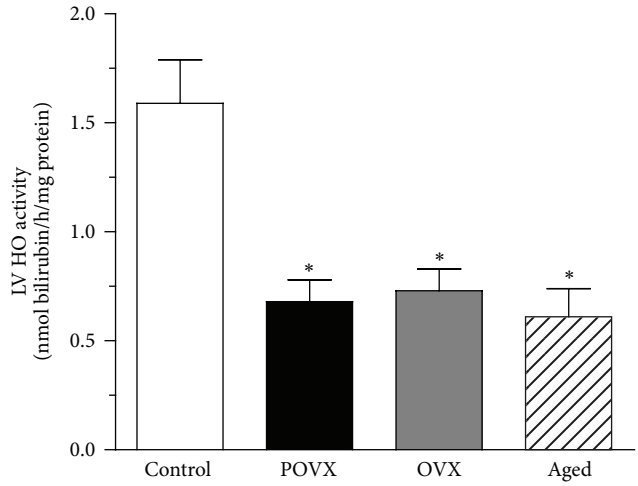

Figure 1: Heme oxygenase activity (HO; expressed as nmol/bilirubin/h/mg protein) in the cardiac left ventricle of control, ovariectomized (pharmacologically POVX and surgically OVX), and aged rats. A significant decrease was observed in the HO activity of the ovariectomized and aged rats as compared with the controls. Data are expressed as means ± S.E.M., $n = 11$-12. Statistical significance: $^*P < 0.05$ relative to the control (sham-operated) group.

3.2. Cardiac LV HO-1 and HO-2 Expression. HO-1 and HO-2 protein were determined by Western blot techniques. Data are shown in Figure 2. In both the POVX and OVX rats, the cardiac expression of HO-1 (POVX: 32.87 ± 3.92%, $n = 10$; OVX: 32.92 ± 3.1%, $n = 10$) and HO-2 (POVX: 36.4 ± 5.3%, $n = 10$; OVX: 35.8 ± 5.22%, $n = 10$) was significantly ($^*P < 0.05$) lower than in the controls (HO-1: 84.33 ± 4.3%, $n = 10$; HO-2: 89.29 ± 2.6%, $n = 10$). Aging was also accompanied by a significantly ($^*P < 0.05$) reduced cardiac expression of HO-1 (32.62 ± 2.89%, $n = 10$) and HO-2 protein (37.95 ± 2.42%, $n = 10$) relative to the control group.

3.3. Cardiac LV IL-6 and TNF-α Concentrations. The levels of the proinflammatory cytokines IL-6 and TNF-α were significantly ($^*P < 0.05$) elevated during aging and after ovariectomy in both the POVX and OVX groups in comparison with the control animals. Additionally, the increase in cardiac LV IL-6 concentration was more marked in the aged group than in the ovariectomized and control animals (IL-6 concentration in the aged group: 89.434 ± 5.817 pg/mg protein, $n = 8$; in the POVX group: 62.503 ± 7.339, $n = 9$; in the OVX group: 61.403 ± 5.512 pg/mg protein, $n = 8$; and in the controls: 41.797 ± 4.673 pg/mg protein, $n = 9$; TNF-α concentration in the aged group: 6.622 ± 0.657 pg/mg protein, $n = 8$; in the POVX group: 5.648 ± 0.598 pg/mg protein, $n = 8$; in the OVX group: 6.015 ± 0.415 pg/mg protein, $n = 6$; and in the controls: 3.520 ± 0.502 pg/mg protein, $n = 7$). The results are presented in Figure 3.

3.4. Cardiac LV MPO Activity. The activity of MPO was significantly ($^*P < 0.05$) higher in the cardiac LV of the POVX (71.0 ± 8.34 mU/mg protein, $n = 7$), OVX (75.0 ± 8.42 mU/mg protein, $n = 8$), and aged (76.0 ± 4.192 mU/mg

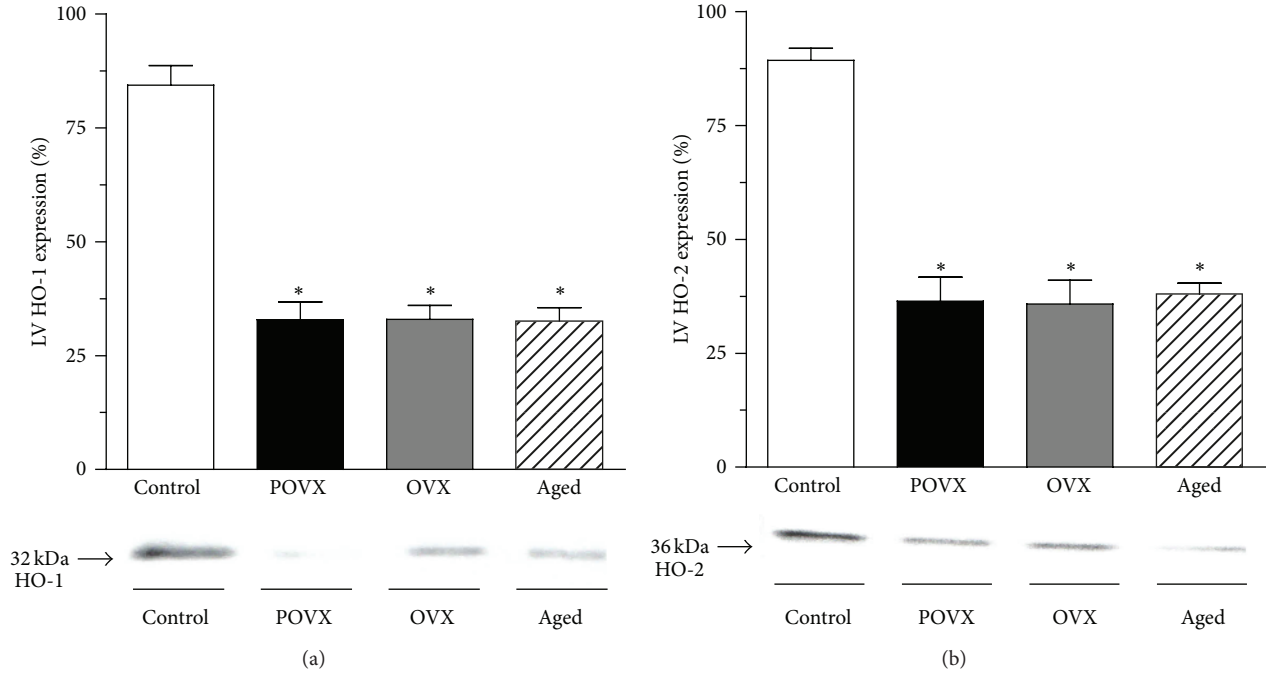

Figure 2: Detection of heme oxygenase expression (HO-1 and HO-2; expressed as %) by Western blot analysis in the cardiac left ventricle of control, ovariectomized (pharmacologically POVX and surgically OVX), and aged rats. (a) Heme oxygenase-1 enzyme (HO-1; expressed as %, 100% being the maximal expression) in the cardiac left ventricle of control, ovariectomized (pharmacologically POVX and surgically OVX), and aged rats with densitometric assessment. There was a significant decrease in HO-1 expression in the ovariectomized and aged groups. Data are expressed as means ± S.E.M., $n = 10$. Statistical significance: $^{*}P < 0.05$ relative to the control (sham-operated) group. (b) Heme oxygenase-2 expression (HO-2; expressed as %, 100% being the maximal expression) in the cardiac left ventricle of control, ovariectomized (pharmacologically POVX and surgically OVX), and aged rats, with densitometric assessment. The HO-2 expression proved to be significantly decreased in the ovariectomized and aged rats. Data are expressed as means ± S.E.M., $n = 10$. Statistical significance: $^{*}P < 0.05$ relative to the control (sham-operated) group.

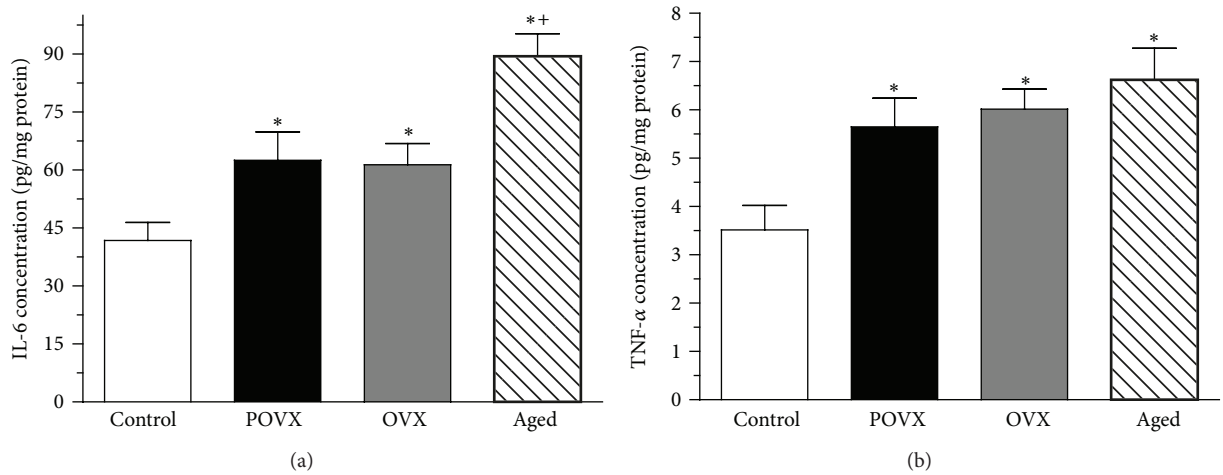

Figure 3: The levels of the proinflammatory cytokines interleukin- (IL-) 6 and tumor necrosis factor-alpha (TNF-α) in control, ovariectomized (pharmacologically POVX and surgically OVX), and aged rats. (a) Cardiac LV IL-6 level (expressed as pg/mg protein) in control, ovariectomized (POVX, OVX), and aged rats. The IL-6 level was significantly increased in the ovariectomized rats as compared with the control. The aged group exhibited a more significant increase than that of the ovariectomized group. Data are shown as means ± S.E.M., $n = 8$–10. Statistical significance: $^{*}P < 0.05$ relative to the control group, and $^{+}P < 0.05$ relative to the ovariectomized animals. (b) Cardiac LV tumor necrosis factor-alpha concentration (TNF-α; expressed as pg/mg protein) in control, ovariectomized (POVX, OVX), and aged rats. The TNF-α concentration was significantly higher in the ovariectomized and aged animals. Data are expressed as means ± S.E.M., $n = 6$–8. Statistical significance: $^{*}P < 0.05$ relative to the control (sham-operated) group.

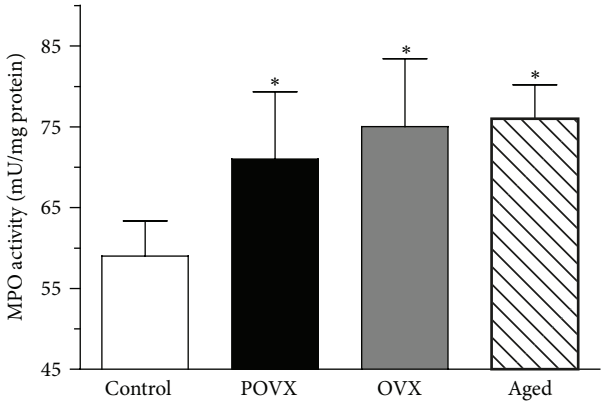

Figure 4: Myeloperoxidase activity (MPO; expressed as mU/mg protein) in the myocardium of control, ovariectomized (pharmacologically POVX, surgically OVX), and aged rats. The MPO enzyme activity was significantly enhanced in the ovariectomized and aged animals. Results are shown as means ± S.E.M., n = 7-8. Statistical significance: $^{*}P < 0.05$ relative to the control (sham-operated) group.

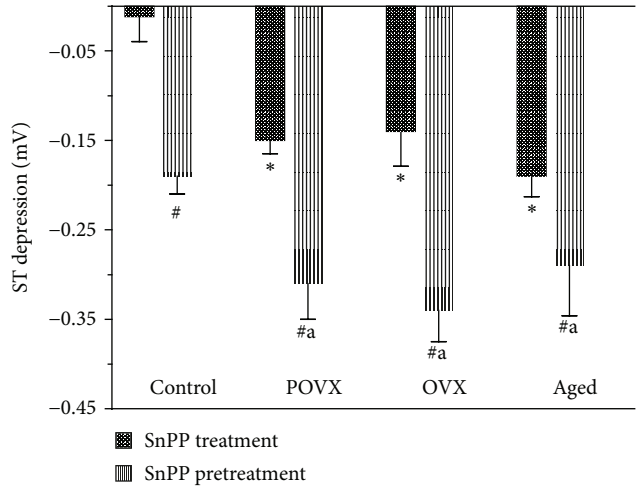

Figure 5: The effects of HO activity inhibition by tin-protoporphyrin-IX (SnPP) on the ST segment (measured in a lead II standard surface ECG, expressed in mV) following intravenous injection of epinephrine (10.0 μg/kg) and 30 s later phentolamine (15.0 mg/kg). The columns show significant decreases in the ST segment of ovariectomized and aged animals without SnPP treatment. SnPP pretreatment (30.0 μmol/kg, s.c., pH 7.4, 24 h and 1 h pretreatment) caused a significant ST depression in the sham-operated control animals. Following ovariectomy (POVX and OVX), or in aged rats, the SnPP treatment induced a more marked significant decrease in ST as compared with the control. Results are shown as means ± S.E.M., n = 11–13. Statistical significance: $^{*}P < 0.05$ relative to the ovary-intact sham-operated control group; $^{\#}P < 0.05$ a significant difference between groups with and without SnPP pretreatment; $^{a}P < 0.05$ a significant difference between the data for the ovariectomized and aged groups after SnPP pretreatment and those for the controls.

protein; $n = 8$) groups as compared to control ones (59.00 ± 4.367 mU/mg protein, $n = 8$). The data are to be seen in Figure 4.

3.5. Effects of Inhibition of HO Activity on Cardiac Ischemia. The administration of phentolamine caused a significant ($^{*}P < 0.05$) ST segment depression 30 s following epinephrine administration in the POVX (−0.15 ± 0.015 mV, $n = 13$), OVX (−0.14 ± 0.0389 mV, $n = 13$), and aged (−0.19 ± 0.023 mV, $n = 13$) rats. In the ovary-intact sham-operated females, the ST segment depression was −0.0116 ± 0.028 mV, $n = 4$. Pretreatment with the HO inhibitor SnPP (30.0 μmol/kg, s.c. pretreatment 24 h and 1 h prior to the measurement) caused an ST depression in the sham-operated animals (−0.19 ± 0.02 mV, $n = 11$), and significantly ($^{\#}P < 0.05$) augmented the ST depression in the POVX (−0.31 ± 0.04 mV, $n = 3$), OVX (−0.34 ± 0.035 mV, $n = 11$), and aged (−0.29 ± 0.056 mV, $n = 11$) groups. The data are depicted in Figure 5.

4. Discussion

We conclude that pharmacological treatment with an aromatase inhibitor triptorelin (POVX) and surgical ovariectomy (OVX) are valuable conditions for effective estrogen studies.

The female cardiovascular system is influenced by changes in the endocrine system. During the menopausal transition, dramatic hormonal changes such as declining levels of estrogen and rising levels of gonadotropins may affect the cardiovascular system. Estrogen deficiency induces an imbalance between enhanced ROS production and inadequate antioxidant activity. The decline in ovarian function accompanying the menopause, OVX, and POVX contributes to the induction of proinflammatory cytokines such as TNF-α and IL-6. Estrogen is a well-known regulator of inflammation. Cardiovascular and immune system abnormalities have been reported in females with estrogen deficiency, exerting a number of known anti-inflammatory effects through a variety of different mechanisms, both genomic and nongenomic. The anti-inflammatory role ranges from generating NO and regulating leukocyte recruitment to reducing oxidative stress and promoting cell survival. These effects contribute to dampening inflammation in the vascular system. Estrogen deficiency has been shown to upregulate TNF-α levels in aged animals with deleterious effects on vascular function. Such effects are largely mediated through increased oxidative stress and can be reversed through exogenous estrogen, a TNF-α inhibitor, or antioxidants [15]. Sex hormones are known modifiers of the inflammatory response to injury, an important aspect of myocardial dysfunction and cardiomyocyte death following ischemia. Hamilton et al. in their experiments showed that the overall effect of OVX on myocardial gene expression was increased expression of genes involved in the inflammatory response. OVX increased IL-6 receptor, TNF-α, complement 8, and SOCS2 and SOCS3 expression [16]. During aging and after ovariectomy, estrogen deficiency is able to stimulate the spontaneous secretion of such proinflammatory cytokines. Along with the effects of cytokines, the MPO

activity is determined as an inflammatory biomarker. Inflammation and oxidative stress are associated with atherosclerosis and cardiovascular disease. Conversely, inflammation also triggers vascular remodeling, aggravates vessel injury, and exacerbates the processes of hypertension and atherosclerosis [17]. MPO plays a significant role in the development of the atherosclerotic lesion and renders the plaques unstable, which is associated with the aging mechanism and cardiovascular disease [18]. Similar results demonstrate the role of HO-1 in HO-1 knockout mice, in which the HO-1 deficiency leads to an increased production of proinflammatory cytokines [9] while HO-1 upregulation successfully slows the processes of hypertension and myocardial infarction [19]. Preclinical and clinical evidence clearly suggest that the progression of atherosclerosis is associated with inflammation. The CO derived from HO has been demonstrated to protect against TNF-α-induced apoptosis [20]. Aging leads to significantly increased levels of the proinflammatory cytokines in the liver of aged female rats as compared with young controls. Hence, the diminished activity of the HO system contributes to increased oxidative stress [21]. Ross and Howlett demonstrated that the ventricular myocytes from young female rats were more resistant to ischemia and reperfusion injury than were cells from males, and then ischemia and reperfusion injury in female myocytes was exacerbated by aging or by OVX. Advancing age abolishes the beneficial effects of the female sex on the cell viability and the contractile function [22]. Our data indicated that aging and estrogen depletion process abolished the beneficial effects of the female sex in the myocardium. This suggests that the cardioprotection of the female sex may decline with age in response to a reduction in circulating estradiol levels. Indeed, we found that the removal of estrogen through OVX or POVX exacerbated the adverse effects of ischemia, which can be augmented via HO activity inhibition. Our results indicate that decreases in the expression of HO-1/HO-2 and in the activity of the HO system are important factors that contribute to the enhanced sensitivity of the OVX or POVX heart to ischemia. Moreover, we found that aging and OVX induced diminished HO activity and expression. Direct vascular effects of estradiol are believed to play a significant role in the cardioprotectivity of estrogens. These findings on OVX and POVX female rats may have important clinical implications. Extensive epidemiological studies of the morbidity and mortality rates of postmenopausal women have revealed a significant increase in cardiovascular mortality as compared with that in women who are still menstruating. After the menopause, women lose the relative cardiovascular protection that they had previously enjoyed over men [23, 24]. Although estrogen has been reported to protect the cardiovascular system, the mechanisms involved remain unclear. Estrogen exerts genomic and nongenomic effects via estrogen receptor-dependent and receptor-independent mechanisms, to confer protective effects on the cardiovascular system. Alterations in plasma concentrations of lipoproteins (decreases in low-density lipoprotein levels and in oxidized low-density lipoprotein formation and increases in high-density lipoprotein levels), hemostatic factors, glucose, insulin, and endothelium-derived factors (decreases in endothelin and increases in NO and prostaglandins) and the inhibition of smooth muscle cell migration and proliferation induced by various mitogens are thought to contribute to the vasoprotective effects of estrogen [25]. Of these biological effects, the antioxidant effects of estrogen may play a critical role in eliciting vasoprotective effects [26]. Endogenous and exogenous estrogens have antioxidant potential both *in vitro* [27] and *ex vivo* [28]. An estrogen deficiency in the menopausal state therefore leads to changes in the homeostatic environment of the body, for example, a gradual increase in oxidative stress. A previous report from our laboratory supports this concept, demonstrating the sexual dimorphism in HO activity and expression. The present study demonstrated similar results in that the HO levels in the hearts of OVX rats were markedly lower than those in the sham-operated controls. HO has potent cytoprotective effects that are likely to be mediated by its products, CO, biliverdin/bilirubin, and free iron, and we were interested in the role of HO in an estrogen-deficient environment. The HO levels proved to be reduced in the aged and ovariectomized rats, which might result from the downregulation of HO in the heart tissues. After OVX, a marked reduction in estrogen synthesis led to decreases in the activity and expression of HO.

We also found that the cardiac LV levels of IL-6 and TNF-α were significantly reduced after ovariectomy and the menopause. An estrogen deficiency may lead to an increased activity of MPO.

However, only a small amount of data is available concerning the effects of HO expression *in vivo*. It emerged from the current study that an HO activity inhibitor caused an ST depression in control female rats and augmented the ST depression in the estrogen-withdrawal model. The data suggest that the HO activity protects the heart at a fertile age.

This study has also demonstrated that the inhibition of HO activity in ovariectomized rats significantly increases the ST depression, implying that HO plays a protective role in the cardiovascular system. Although a number of studies have clearly pointed to the vasculoprotective effects of estrogen in animal models, the targets of estrogen are numerous and estrogen deficiency affects the cardiovascular system at different points. In summary, the decreased level and activity of HO in the hearts of OVX and POVX rats may cause the development of atherosclerosis after the menopause.

Conflict of Interests

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

Authors' Contribution

All authors participated in the design and interpretation of the studies, in the analysis of the data, and in the drafting and final approval of the paper. Anikó Pósa and Renáta Szabó contributed equally to this paper as first authors.

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Review Article

Sirtuin Functions in Female Fertility: Possible Role in Oxidative Stress and Aging

Carla Tatone,[1,2] Giovanna Di Emidio,[1] Maurizio Vitti,[1] Michela Di Carlo,[1] Silvano Santini Jr.,[1] Anna Maria D'Alessandro,[1] Stefano Falone,[1] and Fernanda Amicarelli[3]

[1]*Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio, 67100 L'Aquila, Italy*
[2]*Infertility Service, San Salvatore Hospital, Via Vetoio, 67100 L'Aquila, Italy*
[3]*Institute of Translational Pharmacology (IFT), National Council of Research (CNR), 67100 L'Aquila, Italy*

Correspondence should be addressed to Carla Tatone; carla.tatone@univaq.it

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In search for strategies aimed at preventing oxidative threat to female fertility, a possible role of sirtuins has emerged. Sirtuins (silent information regulator 2 (Sir2) proteins), NAD^+ dependent enzymes with deacetylase and/or mono-ADP-ribosyltransferase activity, are emerging as key antiaging molecules and regulators in many diseases. Recently, a crucial role for SIRT1 and SIRT3, the main components of sirtuin family, as sensors and guardians of the redox state in oocytes, granulosa cells, and early embryos has emerged. In this context, the aim of the present review is to summarize current knowledge from research papers on the role of sirtuins in female fertility with particular emphasis on the impairment of SIRT1 signalling with oocyte aging. On this basis, the authors wish to build up a framework to promote research on the possible role of sirtuins as targets for future strategies for female fertility preservation.

1. Introduction

In humans the first aging phenotype is represented by the decline of female reproductive function. After maximum efficiency in the early 20s, fertility gradually decreases until menopause occurs at a mean age of 50-51 years [1]. This process becomes more dramatic in the late 30s, when, in spite of ovulatory cycles, fertility decline is manifested in increasing rates of infertility, miscarriage, and birth defects [1]. The same condition arises from *in vitro* fertilization (IVF) studies where female age is the most significant factor influencing clinical outcome. Certainly the biological clock regulating female reproductive lifespan is established in order to minimize pregnancy and birth delivery complications in advanced age [2] and to preserve energy for somatic maintenance [3]. Nevertheless, increasing postponement of the first pregnancy in most industrialized societies has been contributing to the wide spread of subfertility related to female aging [1, 4].

Although it is generally accepted that aging is a result of both inborn and environmental factors [5], gradual accumulation of damage by oxidative stress and its interplay with dicarbonyl stress has been considered the major mechanism underlying ovarian aging [6–8]. Beyond aging, oxidative stress may jeopardize ovarian function during endometriosis, metabolic syndromes, that is, diabetes and polycystic ovarian syndrome (PCOS), and anticancer therapies [9].

In search for strategies aimed at preventing oxidative threat to female fertility, a possible role of sirtuins has emerged. Sirtuins (silent information regulator 2 (Sir2) proteins) belong to a well conserved family of NAD^+ dependent enzymes with deacetylase and/or mono-ADP-ribosyltransferase activity that have evolved to respond to a variety of stresses and are emerging as key antiaging molecules and regulators in many diseases [10, 11].

The aim of the present review is to summarize current knowledge on the role of sirtuins in the regulation of ovarian

functions in an effort to identify common threads. On this basis, the authors wish to build up a framework to promote future research on the possible role of sirtuins as targets for female fertility preservation.

2. Ovarian Functions and Aging

Ovarian lifespan is the main determinant of female reproductive function. It provides a reserve of germ cells established prior to or soon after birth. Renewal potential of the follicle pool has been demonstrated in a limited number of species where neo-oogenesis in adult ovaries has been observed [12]. Independently of renewal, during reproductive lifespan follicle reserve gradually decreases. At puberty the gonad is endowed with 300.000 primordial follicles containing oocytes blocked at prophase of the first meiotic division (GV stage) that are periodically recruited into the growing phase and stimulated towards differentiation under the influence of regulators and pituitary hormones, which play the role of survival or death factors [13, 14]. As a result a few follicles reach the large antral follicle stage and complete meiosis until the metaphase II stage (MII) during ovulation to become competent for fertilization [15]. During folliculogenesis the oocyte undergoes a remarkable array of genetic, epigenetic, and cytoplasmic changes aimed at developing full competence for fertilization and production of normal offspring. This process relies on a continuous cross talk between oocytes and granulosa cells that ensure coordination of all the events orchestrated in the ovary under the influence of paracrine and endocrine factors [16]. Maintenance of ovarian reserve and development of oocyte competence at all stages can be hampered by extrinsic and intrinsic factors, which may target ovarian microenvironment during entire reproductive lifespan. Extrinsic factors include environmental insults, that is, xenobiotics and anticancer drugs [17]. Intrinsic factors may enclose ovarian or systemic diseases endometriosis, metabolic syndromes, that is, diabetes and PCOS, and ovarian aging [9].

This process is represented by gradual loss of quantity and quality of ovarian follicles. The size of oocyte/follicle pool declines exponentially with age, with a marked increase in the rate of disappearance from age of 37-38 years onwards. When the menopause is reached, the supply is reduced to a thousand or less follicles, a number insufficient to sustain the cyclic hormonal process necessary for menstruation [18]. Ovarian functional decline with aging is also characterized by reduced ability to produce oocytes competent for fertilization and further development (the so-called aged oocytes) [19]. In addition to several biological and molecular evidences, the central role of oocyte aging is proved by the observation that age-related decline in female fertility can be overcome by oocyte donation from younger women [20]. Aneuploidy, associated in most cases with spindle aberrations, disturbances in chromosome congression, mitochondrial alterations, and changes in gene and protein expression represent the most frequent phenotype of aged oocytes [19, 21].

Evolved from the "free radical theory of aging" [22], the oxidative stress theory of aging suggests that, as unavoidable by-products of metabolism, reactive oxygen species (ROS) are continuously generated within cells, mainly by the electron transport chain (ETC) of mitochondria. If these reactive chemical species are not efficiently scavenged, oxidative damage to biomolecules may occur [23]. More than a decade after the free radical theory of ovarian aging first proposed by Tarin [24], biological and clinical research has provided numerous evidence that increased ROS may contribute to follicular atresia and to oocyte aging in ovaries [25]. Oxidative stress generated by mitochondrial dysfunction is considered the main cause for telomere shortening, chromosomal segregation disorders, maturation and fertilization failures, or oocyte/embryo fragmentation [26, 27]. Looking for the aetiology of oxidative stress, a possible role could be ascribed to impaired follicular vascularization with aging [21, 28]. Factors contributing to perturbation of ovarian microenvironment are *advanced glycation end-products*. These factors may hamper ovarian stroma vessels, follicular growth, assembly of an efficient system of antioxidant enzymatic defence, and development of an efficient perifollicular vascularization [9].

Given their high reactivity and short half-life, measurement of ROS levels in the follicle microenvironment has led to conflicting results about their role in fertility [21]. In this regard, relevant findings are those by Lim and Luderer [29], who revealed significant age-related increases in oxidatively damaged lipids, proteins, and DNA in different ovarian compartments, including granulosa cells and ovarian interstitial tissue, along with alterations of antioxidant enzyme expression. Further evidence of oxidative stress in the ovarian follicle was obtained by research on stress signaling pathways in older granulosa cells [30, 31] and reduced ROS scavenging efficiencies in the follicular environment have been confirmed by observations in cumulus cells [32]. Enzymatic activity and protein level of superoxide dismutase (SOD), the enzyme that reacts with superoxide anion radicals to form oxygen and H_2O_2, were found to decrease with age, and lower levels of SOD activity are associated with unsuccessful IVF outcomes. Although the involvement of oxidative stress in ovarian aging is clear, there are poor evidences of possible benefits from antioxidant treatments in humans suggesting that further actors with a potential role in modulating redox balance in the ovary and its loss with aging need to be investigated [33].

3. Sirtuins

In the last 15 years, the complex process of cellular aging has been tightly linked to the action of sirtuins [34, 35]. Sirtuins are formerly known as class III nicotinamide adenine dinucleotide (NAD^+) dependent histone deacetylases (HDACs), although they may use a variety of substrates that include structural proteins, metabolic enzymes, and histones [11, 36]. All sirtuins remove predominantly acetyl groups from cellular proteins, and this posttranslational chemical modification affects significantly protein localization and function. In this process, the acetyl group from the acetylated substrate is transferred to the ADP-ribose portion of NAD, releasing 2′-O-acetyl-ADP-ribose, nicotinamide, and the deacetylated substrate as products [37]. Since change in NAD^+/NADH ratio controls the activity of sirtuins, all members of this

family may have a crucial role in sensing the energetic status of the cell [38–40].

After the first discovery of the yeast ortholog silent information regulator 2 (Sir2) [41], sirtuins have been identified in prokaryotes and in metazoan, thus suggesting an evolutionary conserved requirement for these proteins in different organisms across phyla [42, 43]. To date, seven members of the sirtuin family have been identified in mammals (SIRT1-7) and each member has peculiar subcellular localization, function, and substrate specificity (for an extensive review, see [44]). Although the initial investigations on mammalian sirtuins focused mainly on SIRT1, as this member appears to be the closest mammalian homolog to yeast Sir2 [45], interest is growing in understanding the function of the related family members. Among SIRT1-7, all except SIRT4 exhibit deacetylase activity, whereas only ADP-ribosyltransferase catalytic activity has been demonstrated for SIRT4 [46]. SIRT1 and SIRT2 have been found in both the nucleus and cytosol; on the other hand, SIRT3, SIRT4, and SIRT5 have been found exclusively in mitochondria, while SIRT6 and SIRT7 have been localized only in the nuclear compartment [11, 46, 47].

Important evidence has been provided regarding the role of sirtuin-activated pathways in the regulation of age-related oxidative stress. Overexpression of Sir2 prevents the aged phenotype reported when *S. cerevisiae* is treated with H_2O_2 [48]. In addition, human fibroblasts treated with sublethal concentrations of hydrogen peroxide show cell cycle arrest, NAD^+ depletion, and decreased sirtuin activity and accelerate cellular senescence [49].

4. SIRT1, a Key Regulator of Energy Metabolism and Oxidative Stress

Strong experimental evidence supports the notion that SIRT1 plays a crucial role in sensing and modulating the cellular redox status thus providing protective effects in cells and tissues exposed to oxidative stressors *in vitro* and *in vivo* [50–52]. SIRT1 is able to directly deacetylate key proteins involved in the cellular stress response, such as forkhead box O (FoxO) transcription factors. Evidence for the interaction between SIRT1 and FoxO was first provided by Brunet et al. [53]. van der Horst et al. [54] showed that mammalian SIRT1 was able to bind FOXO4, thus catalyzing its deacetylation in a NAD dependent manner, while Kobayashi et al. [55] demonstrated that FOXO4 activity was suppressed or enhanced by SIRT1 inhibitor or its activator, respectively. Later, many other authors observed that SIRT1-related cellular protection against oxidative stress can be achieved by upregulating key antioxidant enzymes, such as catalase (CAT), mitochondrial SOD (MnSOD), and peroxiredoxin, through forkhead box O- (FoxO-) dependent mechanisms [56–60].

In *in vitro* study, some authors established the fact that SIRT1 activates proliferator-activated receptor coactivator-1α (PGC-1α), maintaining its deacetylated active form into the nucleus, where it activates genes involved in a variety of biological processes and responses, including antioxidative protection, mitochondrial biogenesis, glucose/fatty acid metabolism, and oxidative phosphorylation (OXPHOS) [61, 62]. Increases in NAD^+ levels due to environmental or endogenous factors may be sensed by SIRT1 that, by deacetylating the transcriptional coactivator PGC-1α at promoter regions, induces the expression of specific genes whose protein products can maintain the bioenergetic state of the cell [63]. SIRT1-mediated control of PGC-1α activity is virtually able to regulate the expression of oxidative stress genes, including glutathione peroxidase (GPx1), CAT, and MnSOD [64]. In muscle, SIRT1 expression is decreased in response to a high fat diet, thus promoting hyperacetylation and inactivation of PGC-1α, which in turn results in lower expression of PGC-1α-targeted genes involved in mitochondrial biogenesis [63].

The nuclear factor κB (NF-κB), which is a major inducer of inflammatory responses, was the first eukaryotic transcription factor described to respond directly to H_2O_2-induced oxidative stress [65]. NF-κB deacetylated and inactivated by SIRT1 exhibits impaired downstream signalling and lowers the cellular ROS load by promoting the resolution of inflammation [66, 67].

Several experiments demonstrated that SIRT1 prevents replicative senescence in mammalian cells [68–70]. Conversely, the selective knockdown of SIRT1 at early passage was found to slow down cell growth, thus accelerating significantly cellular senescence [68]. Xu et al. [71] provided surprising evidence that senescence-promoting effects elicited by miR-22 in cancer cells are in part mediated by SIRT1. Further studies have clearly showed that the activity of SIRT1 can be fine-tuned by its own expression levels that are under the influence of several miRNAs known to suppress Sirt1 mRNA translation or reduce its stability [72–74].

As regards *ex vivo* studies, some authors have described a marked age-dependent decline in SIRT1 activity within rat liver, heart, kidney, lung, muscles, and cerebellum [75–77]. Moreover, our group found that SIRT1 expression decreases as a function of age in hippocampal formation of CD-1 mice [78]. It is also known that the SIRT1 protein levels decline in several murine disease or accelerated aging models [79, 80]. Conversely, both fasting and caloric restriction (CR) seem to induce SIRT1, in association with deacetylation and activation of PGC-1α [81]. Mice fed with a natural SIRT1 activator (e.g., resveratrol) exhibited significantly increased SIRT1 expression, as well as PGC-1α activity, enhanced mitochondrial biogenesis, and higher expression of the slow, oxidative myogenic program [82, 83].

5. Sirtuins and Female Reproductive Functions

5.1. Sirtuins and Reproductive Physiology: Insights from Transgenic Mouse Models. The role of sirtuins in the regulation of fertility has emerged when mice carrying a null allele of Sir2a were generated [84]. In all strains, Sirt1 deficient mice have a small, weak phenotype, with significantly increased postnatal mortality rates. However, as reported by Coussens et al. [85], Sirt1 deficiency is pleiotropic and dependent on cell type and/or stage of development. Sirt1 deficient mice display a number of developmental defects, most noticeably, irregularly shaped eyes and defective cardiac septation. In mouse embryonic fibroblasts, Sirt1 deficiency promotes extension of replicative lifespan and survival following exposure to

genotoxic stress. Female and male Sirt1-null mice are infertile although data on the effect of Sirt1 deficiency on reproductive phenotype are controversial probably because of influence of mouse genetic background [84–86]. Sirt1-null mouse infertility can be readily overcome by assisted reproductive technologies although crossing with wild type is more successful. By crossing female or male wild type and/or knockout problems in gamete interaction have emerged. Indeed Sirt1-null mice exhibit reduced testis development associated with altered expression of 85 genes involved in spermatogenesis and protein sumoylation. In these mice, sperm are immotile and abnormal with increased numbers of small round-shaped heads and elevated DNA damage. Sirt1-null females appear not to cycle efficiently through oestrous and ovulation does not occur although follicles develop normally, perhaps indicating hormonal defects. Finally the reduction of early embryo development may be taken as further evidence for ovarian dysfunction and compromised oocyte developmental potential. Insight from knockout strains has also revealed that Sirt1 genotype is essential for normal embryogenesis: null offspring was smaller than normal at birth and most died during the early postnatal period. The importance of fine regulation of SIRT1 expression in reproductive functions is highlighted by the observation that reproductive maturity was significantly delayed in transgenic mice overexpressing SIRT1 when compared to wild-type controls [87]. In addition to Sirt1, gene-knockout experiments have contributed to elucidation of the role of Sirt3 in the reproductive phenotype [88]. Sirt3 silencing negatively affects mitochondrial activity and basal ATP synthesis although no obvious phenotype is observed in $Sirt3^{-/-}$ mice indicating that the basal metabolic state could stand this reduction. Phenotype might become apparent under certain stress conditions: whereas $Sirt3^{-/-}$ mice were fertile, IVF and *in vitro* cultured $Sirt3^{-/-}$ or Sirt3 siRNA-induced knockdown embryos were susceptible to developmental defects [89].

5.2. Sirtuins in Mammalian Granulosa Cells, Oocytes, and Embryos. The expression of sirtuins has been observed in mammalian ovaries, granulosa cells, oocytes, and embryos (Table 1) [77, 89–91]. In rat ovaries it has been demonstrated that rapamycin and its target, mTOR, may inhibit the transition from primordial to developing follicles and preserve the follicle pool reserve through a fine-tuning of SIRT1 signalling which involves downstream activation of SIRT6 [92, 93]. In the same animal model, Wang et al. [94] observed that caloric restriction has a beneficial effect on ovarian lifespan through suppression of mTOR and activation of SIRT1 signalling. By contrast in ovaries from high fat diet, downregulation of SIRT1 signalling was associated with accelerated ovarian development and follicle loss.

In mammalian granulosa cells (GCs), the oocyte companion cells, which play a main role in follicle differentiation under the influence of oocyte and ovarian paracrine factors, and endocrine molecules, the role of sirtuins has been so far poorly investigated. From studies on SIRT1 and SIRT3, a possible role of this sirtuin in the regulation of proliferation and hormonal metabolism has emerged.

FOXL2 is a transcription factor belonging to forkhead box superfamily, regulating a number of biological processes such as development, differentiation, and proliferation, and is required for the normal development of the GCs [95]. In human granulosa cell lines KGN and COV434, it has been found that SIRT1-FOXL2 axis plays a key role in the maintenance of cell homeostasis [96]. This conclusion has arisen from the observation that SIRT1 inhibition by nicotinamide limits proliferation by promoting G1 arrest and DNA repair through dose-dependent deacetylation of FOXL2. Recent data by Pavlová et al. [97] in porcine GCs have revealed that SIRT1 is shown to stimulate cell proliferation by promoting accumulation of cyclin B1 and Cdc2/p34. In addition the interrelationships among SIRT1, NF-κB/p50, and NF-κB/p65 in transfected cells are relevant for control of proliferation and secretory activity of porcine GCs. In rat GCs overexpressing p50 and p65 and cultured in presence of increasing concentration of FSH, it has been reported that FSH promotes accumulation of both SIRT1 and NF-κB. Therefore the SIRT1/NF-κB system mediates FSH activity on progesterone and IGF-1 release, although it has been observed as a negative feedback control of FSH-induced NF-κB by high amount of SIRT1 [98]. The SIRT1/NF-κB system has also been demonstrated to be strictly connected with p53 and the mTOR; thus granulosa cell fate depends on the balance between inhibitory and stimulatory influences on SIRT1 [99].

Although it is recognized that resveratrol is an indirect and nonspecific activator of SIRT1, it has been employed to show that SIRT1 may play a key role in the activation of steroidogenesis associated with luteinization, the terminal differentiation of rat GCs [98]. Therefore the stimulation of SIRT1 by resveratrol would be potentially beneficial in the treatment of luteal phase deficiency. These observations provide evidence for direct involvement of SIRT1 in upregulation of ovarian hormone secretion. Finally, studies in human GCs and KGN cells show that SIRT1 signalling is involved in the response of ovarian cells to the insulin sensitizer metformin (MetF). MetF is known to increase NAD^+/NADH ratio and SIRT1 abundance and activity in a dose-dependent manner [100, 101]. It has been recently demonstrated that MetF action on SIRT1 influences the expression of visfatin, a cytokine hormone and rate-limiting enzyme in NAD biosynthesis involved in metabolic (obesity, type II diabetes) and immune disorders [102].

Probably by acting as a sensor and regulator of ROS levels and mitochondrial functions, SIRT3 has been recently found to exert a positive role in the folliculogenesis and luteinization processes in GCs. Indeed, SIRT3 targets mitochondrial enzymes, such as glutamate dehydrogenase (GDH), and increases oxidative phosphorylation by deacetylation of enzymes involved in the electron transport chain [103]. In human granulosa and cumulus cells, a decrease has been reported in SIRT3 expression which is dependent on maternal age and ovarian reserve. This reduction has been associated with a lower active deacetylated GDH form, which contributes to the altered metabolism of follicle cells during aging [104]. Finally, SIRT3 depletion resulted in downregulation of steroidogenic enzymes and thus resulted in decreased progesterone secretion in human GC and COV434 cell line [105].

Table 1: Sirtuins and related functions in the ovary.

Sirtuin	Intracellular localization	Ovarian cell type	Species	Function	Mediator	References
SIRT1	Cytoplasmic Nuclear	Ovary	*Rat*	Folliculogenesis	mTOR; FOXO3a; NRF-1; SIRT6	Luo et al. [92]; Zhang et al. [93]; Wang et al. [94]
		Granulosa cells	*Human*	Proliferation; activation of steroidogenesis	Visfatin	Reverchon et al. [102]
			Porcine	Proliferation; secretory activity	p53; NF-κB; MAPK; ERK1-2	Pavlová et al. [97]; Sirotkin et al. [99]
			Rat	Mediation of FSH action; activation of steroidogenesis	StAR	Morita et al. [98]
		KGN		Cell homeostasis; response to metformin; activation of steroidogenesis; proliferation	FOXL2; visfatin	Benayoun et al. [96]; Cantó et al. [100]; Caton et al. [101]; Reverchon et al. [102]
		COV434		Cell homeostasis	FOXL2	Benayoun et al. [96]
		Oocyte	*Mouse*	Chromatin configuration; maturation; oxidative stress response; aging process	FOXO3a; miR-132; SOD2	Kawamura et al. [89]; Manosalva and González [90]; Di Emidio et al. [91]
			Porcine	Maturation		Wang et al. [94]
		Embryo	*Porcine*	Embryo development; regulation of apoptosis		Kwak et al. [112]
SIRT2	Cytoplasmic Nuclear	Oocyte	*Mouse*	Metaphase II spindle assembly and chromosome alignment; aging process	Histone H4K16 and α-tubulin	Kawamura et al. [89]; Zhang et al. [110]
		Embryo	*Porcine*	Embryo development; regulation of apoptosis		Kwak et al. [112]
SIRT3	Mitochondrial	Granulosa cells	*Human*	Follicle metabolism; aging process; folliculogenesis; luteinization; progesterone secretion; oxidative stress response	GDH; SOD1; CAT; 17βHSD1; StAR; P450arom	Pacella-Ince et al. [104]; Fu et al. [105]
		COV434		Folliculogenesis; luteinization; progesterone secretion	SOD1; CAT; 17βHSD1; StAR; P450arom	Fu et al. [105]
		Cumulus cells	*Human*	Follicle metabolism; aging process	GDH	Pacella-Ince et al. [104]
		Oocyte	*Mouse*	Oxidative stress response; maintenance of mitochondrial functionality		Kawamura et al. [89]
		Embryos	*Mouse*	Embryo development; oxidative stress response; maintenance of mitochondrial functionality	p53	Kawamura et al. [89]
			Porcine	Embryo development; regulation of apoptosis; marker of embryo potential		Kwak et al. [112]
SIRT4	Mitochondrial	Oocyte	*Mouse*	Function not investigated		Kawamura et al. [89]
SIRT5	Mitochondrial	Oocyte	*Mouse*	Function not investigated		Kawamura et al. [89]
SIRT6	Nuclear	Ovary	*Rat*	Folliculogenesis	mTOR; FOXO3a; NRF-1; SIRT6	Luo et al. [92]; Zhang et al. [93]; Wang et al. [94]
		Oocyte	*Mouse*	Follicle development		Kawamura et al. [89]; Wang et al. [94]
SIRT7	Nuclear	Oocyte	*Mouse*	Function not investigated		Kawamura et al. [89]

All sirtuin members are expressed in mouse ovulated MII oocytes, and their expression gradually decreases upon fertilization until the blastocyst stage [89].

In mouse GV and MII oocytes, SIRT1 expression has been associated with changes in chromatin configuration [90], oxidative stress response, reproductive aging [91], and postovulatory aging [77].

Since the acetylation status of proteins in oocytes is positively or negatively associated with oocyte aging [106], sirtuin deacetylation activity of histonic and non-histonic targets has recently been investigated in oocytes at different meiotic stages [90].

By changing its intracellular localization, activating GV chromatin rearrangement, and modulating antioxidant enzymatic response, SIRT1 has been shown to orchestrate the adaptive response to oxidative stress in mouse oocytes [91]. Further, SIRT1 is supposed to be relevant to oogenesis rather than fertilization and early embryo development where a relevant role of SIRT3 has arisen [89]. Indeed we observed reduced levels of Sirt1 transcripts in MII oocytes when compared with GV [91]. This is not surprising since degradation of specific transcripts is known to occur during completion of meiosis; thus SIRT1 reduction in MII can be taken as an evidence of the minor role of SIRT1 in postfertilization events [107].

Experimental evidence in mouse oocytes demonstrated the role of redox signalling in the regulation of SIRT1 [91]. Indeed in oocytes arrested at GV stage the gene transcript encoding SIRT1 is induced in response to *in vitro* exposure to H_2O_2. Although the transcriptional regulatory program controlling this process has not been delineated, we can speculate that SIRT1 as a chromatin-regulating factor participates in the changes of transcriptional activity triggered by oxidative stress.

Manipulating SIRT1 activity by means of a specific inhibitor Ex527, SIRT1 downstream pathway appears to rely on the activity of FOXO3A leading to upregulation of MnSod gene and prevention of ROS increase. A novel aspect of the adaptive response to oxidative stress in mammalian oocytes is the finding of an oxidative responsive miRNA. Given the regulatory role of several miRNAs in SIRT1 posttranscriptional regulation [72] and stress response [73], in the mouse oocyte, the indirect correlations between the expression levels of miR-132 and Sirt1 along with the observation that Sirt1 is a validated target of miR-132 strongly support the hypothesis that this microRNA is implicated in Sirt1 mRNA modulation [72]. Finally, the crucial role of SIRT1-FOXO3A-MnSOD pathway under regulation of miR-132 is confirmed by its disruption in aged oocytes showing a lower ability to regulate the Sirt1 gene and miR-132 levels in response to oxidative stress [91]. Inhibition of SIRT1 activity affected meiotic progression and caused a significant increase in ROS levels in concomitance with anomalies of spindle and chromosomal organization in *in vitro* matured oocytes. This suggests that SIRT1 activity protects the oocytes from oxidative damage caused by stress related to *in vitro* culture. Experiments in bovine oocytes have provided evidence that activation of SIRT1 in oocytes may be a potential countermeasure against age-associated events in oocytes derived from aged cows [108]. This conclusion is based on the findings that supplementation of maturation medium with N-acetyl-cysteine (NAC) reduced the levels of ROS in the oocytes independently of age while SIRT1 inhibition increased the ratio of abnormal fertilization [108].

As observed for SIRT1, SIRT2 enzymatic activity seems to be linked to fluctuations in cellular NAD levels [43]. A recent study has established the fact that SIRT2 mediates the acetylation status of tubulin in a NAD dependent manner [109]. In mouse oocytes, depletion of SIRT2 has been associated with increased spindle defects and chromosome disorganization and with impaired microtubule-kinetochore interaction [110]. In addition, in the same paper, the authors observed a lower SIRT2 protein level in oocytes from aged mice, suggesting that decreased SIRT2 may be a contributing factor to oocyte age-dependent deficits [110].

The role of sirtuins as upstream regulators of embryo gene transcription has emerged since 1994 when nicotinamide was found to inhibit mouse embryo development *in vitro* [111]. Nevertheless the interest in the potential role of sirtuins in reproductive physiology has been increasing in the last few years. According to this insight, studies have initially focused on sirtuin involvement in embryo development with particular interest in the role of SIRT3. According to Kawamura et al. [89], sirtuin inhibitors and siRNA-induced knockdown of Sirt3 promote ROS formation and decrease blastocyst formation indicating that SIRT3 acts as a protecting factor against stress conditions during *in vitro* fertilization and embryo culture by ensuring maintenance of mitochondrial functionality. An important role in SIRT3 signalling in mouse embryos is played by p53. Experiments in Sirt3-knockdown embryos have revealed that manipulation of SIRT3 activity results in changes of gene expression and ROS-p53 pathway. These observations in the mouse have been confirmed in porcine embryos where SIRT3 has been found to regulate early embryo development by modulating essential gene expressions in concert with SIRT1 and SIRT3 [112]. Thus targeting SIRT3 might be a potential measure for promoting positive outcome in IVF by improving developmental potential of early embryos.

6. The Role of Sirtuins in Fertility Promoting Strategies Based on Natural Compounds

In addition to extending lifespan in numerous invertebrates, natural and synthetic SIRT1 activating compounds (STACs) exert beneficial effects on age-related diseases (i.e., cancer, inflammation, cardiovascular diseases, and neurodegeneration), when administered by diet. Several classes of plant-derived metabolites such as flavones, stilbenes, chalcones, and anthocyanidins were shown to directly activate SIRT1 *in vitro* [113, 114]. Most of the activators identified were polyphenolic with a structure-activity relationship characterized by planar multiphenyl rings bearing hydroxyl groups [114].

Resveratrol (3,5,4′-trihydroxystilbene) is the most potent *in vitro* natural SIRT1 activator. It was initially identified in 1940 as a phenolic substance in the white hellebore, *Veratrum grandiflorum*, a flowering plant, and later in grape wines [115]. Starting from natural STACs, the search for synthetic SIRT1 activators with greater potency, solubility, and

bioavailability has rapidly advanced in recent years. Initially derivatives of an imidazothiazole scaffold were identified. They were chemically distinct from the polyphenol backbone of resveratrol but had the same KM lowering mechanism as resveratrol and a much lower EC_{50}. Actually a more potent third generation of STACs based on benzimidazole and urea-based scaffolds was screened. However, as far as we know, resveratrol is the only STAC tested on mammalian fertility. A recent paper has provided evidence for the effectiveness of resveratrol in preventing ovarian aging in mice [116]. The authors demonstrated that administration of 7 mg/kg/die of resveratrol for 12 months improved fertility by extending ovarian lifespan, as evidenced by increased number and quality of ovulated oocytes, embryo developmental potential, and litter size. Although SIRT1 activity has not been assessed, increased Sirt1 mRNA levels were considered an indirect evidence for SIRT1 activation by resveratrol. Nevertheless, although it is accepted that STACs act by promoting SIRT1 activity *in vivo*, the mechanisms by which they activate this deacetylase were the subject of intense debate. Direct allosteric activation of SIRT1 through a lowering peptide substrate Km or indirect activation resulting from off-targets effects was proposed [114].

SIRT1 signalling has also been involved in the beneficial effects on fertility attained by dietary supplementation of compounds other than STACs. Indeed, young female mice receiving NAC in drinking water for 2 months presented increased rate of fertilized oocytes and early embryo development in association with higher expression level of Sirt1 and Sirt2 and increased telomerase activity length [116]. In another study, rats treated with 5 mg/kg/die of rapamycin in order to obtain a caloric restriction phenotype throughout mTOR suppression presented enhanced follicle reserve and increased expression of SIRT1 and SIRT6 [93]. On the other hand diet with deleterious effects on fertility results in Sirt1 suppression. This is the case of high fat diet that accelerated rate of follicle loss leading to premature ovarian failure (POF) by activating mTOR and suppressing SIRT1 signalling [94].

7. Conclusions and Future Directions

Our knowledge of sirtuins has grown exponentially over the last few years and has been uncovering the field of fertility for a few years. The majority of the work carried out so far on the role of sirtuins in reproductive functions has focused on SIRT1 and SIRT3, as the main redox regulators. As reported above, SIRT1 as the major nuclear deacetylase plays a pivotal role in the transcriptional response to changes in redox conditions and SIRT3, as the major mitochondrial deacetylase, acts as the *in situ* regulator of proteins which ameliorate damage in mitochondria, the major source of ROS in the cell. Oocytes and embryos are constantly challenged by stresses and privations and require adaptive responses for their survival. In addition to redox perturbations in the intraovarian microenvironment related to aging or diseases with an oxidative basis, reproductive cells have to face stress conditions during their manipulation during assisted reproductive procedure. In this scenario, current findings in mammalian oocytes and embryos fit well with the main roles of SIRT1 and SIRT3 established by previous work in somatic cells. SIRT1 is expressed at mRNA and protein level in ovarian and ovulated oocytes [90, 91] but is likely to play a primary role during oocyte stay in the ovary rather in fertilization event. Many evidences support the role of this sirtuin in the adaptive response to oxidative stress [91] as well as in protecting oocyte against loss of developmental competence with reproductive and postovulatory aging [77, 90]. The SIRT1-FOXO3A axis might be one of the signalling pathways with a main role in this process. Based on the observation of increased ROS levels and disturbed spindle organization under SIRT1 inhibition, a potential role of SIRT1 as a guardian of meiosis might be also suggested. When our attention moves to postfertilization events, maternally derived SIRT3 appears to be a critical protein in the protection of early embryos against stress conditions during *in vitro* fertilization and culture. It is essential in maintaining mitochondrial homeostasis SIRT3 thus preventing the activation of the ROS-p53 pathway responsible for developmental defects. Finally, possible role of SIRT1 and SIRT3 in folliculogenesis and luteinization processes in GCs has emerged, with SIRT1 involved as a sensor of nutritional status and regulator of cell cycle and SIRT3 acting as a guardian of the redox state and steroidogenic metabolism. In addition, the findings open direct or indirect modulation of sirtuin activity by nutritional interventions with beneficial effects on ovarian physiology opening new horizons to their potential clinical implication in fertility care.

Conflict of Interests

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

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Research Article

TSG (2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside) from the Chinese Herb *Polygonum multiflorum* Increases Life Span and Stress Resistance of *Caenorhabditis elegans*

Christian Büchter,[1,2] Liang Zhao,[2] Susannah Havermann,[1,2] Sebastian Honnen,[2] Gerhard Fritz,[2] Peter Proksch,[3] and Wim Wätjen[1,2]

[1] *Institute of Agricultural and Nutritional Sciences, Martin-Luther-University Halle-Wittenberg, Weinbergweg 22, 06120 Halle/Saale, Germany*
[2] *Institute of Toxicology, Heinrich-Heine-University, Moorenstrasse 5, 40225 Düsseldorf, Germany*
[3] *Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Universitätsstraße 1, 40225 Düsseldorf, Germany*

Correspondence should be addressed to Wim Wätjen; wim.waetjen@landw.uni-halle.de

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2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG) was isolated from *Polygonum multiflorum*, a plant which is traditionally used as an anti-ageing drug. We have analysed ageing-related effects of TSG in the model organism *C. elegans* in comparison to resveratrol. TSG exerted a high antioxidative capacity both in a cell-free assay and in the nematode. The antioxidative capacity was even higher compared to resveratrol. Presumably due to its antioxidative effects, treatment with TSG decreased the juglone-mediated induction of the antioxidative enzyme SOD-3; the induction of the GST-4 by juglone was diminished slightly. TSG increased the resistance of *C. elegans* against lethal thermal stress more prominently than resveratrol (50 μM TSG increased mean survival by 22.2%). The level of the ageing pigment lipofuscin was decreased after incubation with the compound. TSG prolongs the mean, median, and maximum adult life span of *C. elegans* by 23.5%, 29.4%, and 7.2%, respectively, comparable to the effects of resveratrol. TSG-mediated extension of life span was not abolished in a DAF-16 loss-of-function mutant strain showing that this ageing-related transcription factor is not involved in the effects of TSG. Our data show that TSG possesses a potent antioxidative capacity, enhances the stress resistance, and increases the life span of the nematode *C. elegans*.

1. Introduction

Ageing is defined as an accumulation of diverse deleterious changes occurring in cells and tissues with advancing age that are responsible for the increased risk of several diseases and finally death [1]. Oxidative stress is believed to play a role in both physiological and pathological ageing processes, for example, age-related neurodegenerative diseases. However, besides the free radical theory of ageing [2], several other theories exist, for example, the hyperfunction theory of ageing [3], the inflammation theory of ageing [4], and the mitochondrial theory of ageing [5], which shows the complexity of the ageing process. A number of herbal medicines have been used traditionally to increase longevity and health. For example, extracts of *Polygonum multiflorum* are used as an anti-ageing treatment in East Asian countries. Besides the traditional use in folk medicine, extracts of *Polygonum multiflorum* have been shown to possess anti-ageing effects in different species: Chan et al. [6] reported that mice fed with *Polygonum multiflorum* extract had less lipofuscin in the hippocampus and lower MDA concentrations in the brain. An extract consisting of *Polygonum multiflorum* reduced the lipofuscin content of liver and brain tissues in both young (1 month old) and adult (11 months old) mice [7]. Li et al. [8] showed neuroprotective effects of an extract on nigrostriatal degeneration in mice. *Polygonum multiflorum* (root) was able to lower Aβ generation by modulating APP processing *in vitro* [9] and to prevent Aβ-induced increase of thiobarbituric acid

reactive substances and cognitive deficits in mice [10]. Steele et al. [11] reported cytoprotective effects of an extract of *Polygonum multiflorum* in astroglia cells. Furthermore, an improvement of cognitive performance in senescence accelerated mice [12] and an attenuation of glutamate-induced neurotoxicity [13] were demonstrated. Since many of the constituents of herbal extracts possess an antioxidative capacity, it is believed that this property may be involved, at least in parts, in the anti-ageing mechanism of the herbal extract. The stilbene glucoside 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside (TSG) is the main bioactive component of *Polygonum multiflorum* [14]; other compounds isolated were physcion, apigenin, hyperoside, rutin, vitexin, beta-amyrin, beta-sitosterol, and daucosterol. TSG is a potent antioxidant: Chen et al. [15] and Ryu et al. [16] investigated the antioxidant activity of compounds isolated from *Polygonum multiflorum* (DPPH-assay) showing TSG as an active constituent. TSG exerts protective effects on experimental colitis through diminishing the level of oxygen and nitrogen free radicals [17]. Wang et al. [18] showed that TSG has protective effects against cerebral ischemia by modulation of JNK, SIRT1, and NF-κB pathways. TSG mediates antagonistic effects on oxidation of lipoprotein, proliferation, and decrease of NO content of coronary arterial smooth muscle cells, which partially explains the antiatherosclerosis mechanism of *Polygonum multiflorum* [19]. Lv et al. [20] reported that TSG efficiently inhibits the formation of advanced glycation end products (AGEs). TSG has been shown to exert positive effects on learning and memory in animal models. This compound was shown to be protective against apoptosis in rat adrenal pheochromocytoma cells by modulating the ROS-NO pathway [21]. Sun et al. [22] showed that TSG protects human neuroblastoma SH-SY5Y cells against MPP^+-induced cell death through improving mitochondrial function, decreasing oxidative stress and inhibiting apoptosis which may be relevant for treatment of Parkinson's disease. Protective effects of TSG against MPP^+-toxicity in PC12 cells were reported to be mediated via modulation of the phosphoinositide-3-kinase/Akt signaling pathway [23] and the JNK pathway [24]. Various studies were performed concerning anti-ageing effects of TSG; however up to now, no direct correlation between ageing and TSG has been published. To investigate the effects of TSG on ageing processes, we used the model organism *Caenorhabditis elegans*. It is known that several polyphenols, for example, quercetin [25], myricetin [26], catechin [27], epigallocatechin gallate [28], or baicalein [29], increase the life span of *C. elegans*. As molecular mechanism for the life span extension, modulation of the insulin/IGF-like signalling pathway or an activation of Sir2 (sir-2.1 in *C. elegans*), a member of the sirtuin family of NAD^+-dependent deacetylases is discussed. Resveratrol acts on a number of target proteins [30]; for example, it was recently shown to activate AMP-kinase and to exert neuroprotective properties independent of Sir2 [31].

Aim of the Study. *Polygonum multiflorum* is widely used as a traditional anti-ageing drug in East Asian countries. The main bioactive component of the extract is 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside (TSG). The aim of this study was to evaluate the effects of TSG on oxidative stress, stress resistance, and life span in the model organism *C. elegans* in comparison to the structural analogue resveratrol and the flavonoid quercetin. For this reason, antioxidative effects were investigated *in vitro* and *in vivo*; effects on stress resistance, lipofuscin accumulation and expression of protective enzymes, and the life span were investigated.

2. Materials and Methods

2.1. Materials. Resveratrol and trolox were purchased from Calbiochem (Merck, Darmstadt, Germany), quercetin was from Extrasynthese (Genay, France), and DMSO was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany).

2.2. Isolation of 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside. The Chinese medicine plant *Polygonum multiflorum* was bought from Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd., Hangzhou, China. Isolation and purification of 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside were performed at the Heinrich-Heine-University, Institute of Pharmaceutical Biology and Biotechnology.

2.3. C. elegans Strains and Maintenance. The strains used in this study were N2 var. Bristol, CF1038 *[daf-16(mu86) I.]*, CF1553 *[muIs84(sod-3p::gfp)]*, and CL2166 *[pAF15(gst-4p::GFP::NLS)]*. All strains were provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Strains were maintained on nematode growth medium (NGM) agar plates at 20°C containing a lawn of *E. coli* var. OP50 (provided by the CGC) as the food source, as described previously [32]. Treatment of *C. elegans* with the substances was performed in 2 mL of liquid NGM containing 1% (w/v) bovine serum albumin, 50 μg/mL streptomycin, and 10^9 OP50-1/mL (provided by the CGC) in 35 mm petri dishes (Greiner Bio-One, Frickenhausen, Germany). Stock solutions (100 mM) were prepared with DMSO. In all assays, the substances were used in a final concentration of 50 or 100 μM; 0.1% (v/v) DMSO was used as the solvent control. Age synchronous animals were obtained by sodium hypochlorite treatment of gravid adults. Briefly, gravid adults were rinsed off from NGM agar plates with liquid NGM, collected in 0.5 mL liquid NGM and mixed with 0.5 mL bleaching solution (50% 5 M NaOH/50% NaClO). Nematodes were then incubated at room temperature for three minutes, occasionally vortexed, pelleted by centrifugation (5000 rpm/4°C/1 min) and the supernatant was discarded. The animal pellet was washed three times with liquid NGM and transferred onto fresh NGM agar plates (containing OP50 lawn) and maintained for three days at 20°C to obtain an age synchronous population of mainly L4 larvae.

2.4. Determination of Antioxidative Capacity In Vitro. To determine the antioxidative capacity of quercetin, resveratrol, and TSG, the TEAC assay was used. This assay is a cell-free method for the measurement of radical scavenging

properties of compounds [33]. The principle of this reaction is a reductive conversion of a stable, blue-green radical by an antioxidant. The solution decolorizes when an antioxidant is added and can be quantified spectrophotometrically. The decolorisation of the radical solution indicates the antioxidative capacity of a compound which is compared to the potency of the reference substance trolox, which is a synthetic vitamin E derivative. The reference- and test-substances were measured in a concentration range from 0 to 25 μM. The radical scavenging activity was measured at 734 nm spectrophotometrically (Lambda 25 UV/VIS Spectrometer, Perkin Elmer, Wellesley, MA, USA) two minutes after starting the reaction.

2.5. Measurement of Intracellular ROS Accumulation In Vivo. The fluorescent probe H_2DCF-DA (2′,7′-dichlorodihydrofluorescein-diacetate) was used to detect the intracellular ROS level in living individual nematodes. H_2DCF-DA is able to freely cross cell membranes; however, after entering the cell, nonfluorescent H_2DCF-DA becomes deacetylated to form the nonfluorescent derivative H_2DCF that is trapped within the cell. Then H_2DCF can quickly be oxidised by intracellular ROS to form fluorescent DCF that can be measured in a fluorescence spectrophotometer (excitation wavelength 485 nm; emission wavelength 535 nm). The fluorescence intensity correlates with the intracellular amount of ROS; no saturation of the DCF fluorescence was observed up to 8 h of persistent thermal stress. The experiment was performed as described elsewhere [34]. Briefly, L4 larvae were incubated in liquid NGM ± the compounds (50/100 μM) or 0.1% DMSO for 48 hours at 20°C. During the incubation period, nematodes were transferred to fresh culture media daily. After 48 hours, all animals were transferred into M9T (M9 buffer containing 0.1% Tween 20) for one hour. Then single nematodes were transferred individually in 1 μL M9T into each well of a 384-well plate (384-well μClear plate, Greiner Bio-One, Frickenhausen, Germany) containing 7 μL M9 buffer. Subsequently, when all animals were transferred, 2 μL H_2DCF-DA (250 μM in PBS) was added into each well to obtain a final concentration of 50 μM H_2DCF-DA. A black backing tape (Perkin Elmer) was applied to the top of the plate to avoid evaporation. ROS accumulation was induced by thermal stress at 37°C and recorded every 15 min for a period of 12 hours in a fluorescence spectrophotometer (Wallac Victor^2 1420 Multilabel-Counter, Perkin Elmer, Wellesley, MA, USA).

2.6. Determination of Lipofuscin Accumulation. Over the lifetime of *C. elegans*, the autofluorescent pigment lipofuscin accumulates in gut granules and serves as an established marker of ageing. Randomly picked L4 larvae were placed in liquid NGM ± the compounds (50/100 μM) as described above and incubated for 72 hours at 20°C, followed by 24 hours of incubation in compound free medium. During the incubation period, nematodes were transferred to fresh culture media daily. Lipofuscin fluorescence of seven-day-old nematodes was detected by fluorescence microscopy (excitation wavelength 360–370 nm; emission wavelength 420–460 nm; Olympus BX43; Olympus, Hamburg, Germany) and analysed densitometrically (ImageJ, National Institutes of Health, Bethesda, MD, USA). The experiment was repeated three times and 20 animals per group and experiment were analysed.

2.7. Induction of Antioxidative Enzymes. Immediately after the bleaching procedure, synchronised eggs of the transgenic strains CF1553 (expressing a *sod-3p::gfp* reporter) or CL2166 (expressing a *gst-4p::gfp::nls* reporter) were incubated in liquid NGM containing 100 μM of quercetin, resveratrol, TSG, or 0.1% DMSO, respectively, as described above and incubated for five days at 20°C. After five days of drug treatment, nematodes were placed in PBST (phosphate buffered saline containing 0.1% Tween 20) for 30 minutes to wash off residual bacteria. Each group was then separated into two groups, exposing one of each group to 150 μM of the juglone for 3 hours. The naphthoquinone juglone is a redox cycler, which results in the generation of reactive oxygen species. A cyclic process consisting of (a) reduction of a compound, followed by (b) autoxidaton of the reaction product leads to a prolonged production of ROS. 10 to 15 animals from each group were placed onto microscope slides, anesthetized in 10 μL of 10 mM levamisole, and covered with cover slips. Epifluorescence images were collected from an Axiolab fluorescence microscope (Zeiss, Göttingen, Germany) using a suitable filter set with a CoolSnap CF Digital Monochrome Camera (Intas, Göttingen, Germany) equipped with the Image Pro-Plus software (version 4.5, MediaCybernetics, Silver Spring, MD, USA). Densitometric analysis of GFP expression of the head and anterior intestinal area of at least 10 animals per group and experiment was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Thermotolerance Assay. Survival of individual nematodes at the lethal temperature of 37°C was monitored with an assay according to Gill et al. [35] and Kampkötter et al. [34]. After treating wild type L4 larvae for 48 hours with the compounds (50/100 μM) or 0.1% DMSO (daily transfer of the animals into fresh culture medium), nematodes were washed in PBST for one hour and then individually transferred in 1 μL PBST into the wells of a 384-well plate (384-well μClear plate, Greiner Bio-One, Frickenhausen, Germany) containing 9 μL PBS. Following the complete transfer of the nematodes, 10 μL of 2 μM SYTOX Green Nucleic Acid Stain (Molecular Probes Inc.; Leiden, The Netherlands) in PBS were added to each well and the plate was sealed using black backing tape (Perkin Elmer) to avoid evaporation. SYTOX Green Nucleic Acid Stain is unable to pass the membranes of intact cells. However, thermal stress causes an impairment of the cellular membrane, thereby enabling the dye to enter the cells. There the dye binds to DNA and exerts a bright fluorescence that can be used as a marker for cellular damage and thus for the viability of individual nematodes [35]. The fluorescence intensity was determined with a fluorescence spectrophotometer (Wallac Victor^2 1420 Multilabel-Counter, Perkin Elmer, Wellesley, MA, USA) and was recorded every 15 min for 12 hours (excitation wavelength 485 nm; emission

wavelength 535 nm). The fluorescence curve of each nematode was calculated and the individual cut-off value was determined by multiplying the average fluorescence of the first four measurements by the factor 3. The time point when the fluorescence exceeded the cut-off value for each well was defined as the point of death of the respective nematode. The factor 3 in the calculation of the cut-off value was previously shown to be adequate [35]. Survival curves and mean survival times were determined from these individual times of death (Kaplan-Meier survival analysis, IBM SPSS 19). Experiments were repeated at least three times.

2.9. Life Span Assays. Life span analyses were performed with N2 (three independent experiments) and CF1038 *[daf-16(mu86) I.]* (two independent experiments). About 30–50 L4 larvae per group and experiment were placed in liquid NGM ± compounds (50/100 μM) as described above and incubated at 25°C. The starting day in liquid culture was considered as day 0 of the life span. Nematodes were transferred daily to new culture dishes during their fertile period to prevent overcrowding and to discriminate the test nematodes from their progeny. After the fertile period, nematodes were transferred to fresh medium every other day. Nematodes were scored as dead when they did not respond to gentle prodding and when they showed no pharyngeal pumping movement. Lost nematodes and animals containing hatched larvae were censored. Kaplan-Meier survival analysis was used to detect statistical differences.

2.10. Statistics. Data are given as mean ± S.E.M of at least 3 independent experiments. Statistical analysis was performed with SPSS 19 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., LaJolla, USA) software. Statistical significance was determined by one-way ANOVA with Bonferroni posttest. Life span analyses were performed using Kaplan-Meier survival analysis; animals that were lost, killed, or showed internal hatching were censored. Differences were considered to be significant at $P < 0.05$.

3. Results and Discussion

3.1. Antioxidative Capacity of TSG. To investigate if TSG may exert antioxidative effects in *C. elegans*, we have analysed the radical scavenging capacity of this compound in the trolox equivalent antioxidative capacity (TEAC) assay. In this cell-free assay, the potency of a substance to reduce and thereby decolourise a green radical is detected. We have compared the antioxidative capacity of TSG with the structural analogue resveratrol and quercetin, a major flavonoid with a well-known antioxidant capacity. TSG possesses a strong antioxidative capacity in this system (Figure 1(b)). Even at the lowest concentration analysed (5 μM), TSG showed a strong antioxidative effect; about 30% of the ABTS radical was reduced. The compound reduced the ABTS radical even more efficiently than resveratrol over the complete concentration range. Up to a concentration of 15 μM, TSG shows a higher antioxidative capacity than trolox, the synthetic vitamin E derivative that was used as a reference substance. Out of the compounds analysed, only the flavonoid quercetin showed stronger antioxidative effects over the complete concentration range. The superior antioxidative capacity of quercetin in the TEAC assay can be explained by differences in the molecular structure of this compound compared to resveratrol and TSG. In contrast to the both stilbene derivatives, the flavonoid possesses redox-active moieties which facilitate the reduction of the ABTS radical. These groups are, for example, the catechol group in ring B in combination with the 3-OH-group in ring C. These groups are able to donate electrons to the ABTS radical forming stable semiquinone radicals and quinoid structures. In the case of the stilbene derivatives, the stabilisation of the oxidized form is not favoured as in case of quercetin.

Next, we investigated the antioxidative effects of TSG in the nematode *C. elegans*. To analyse the antioxidative potential *in vivo*, the DCF assay was used. DCF is a probe that becomes highly fluorescent after oxidation; therefore the DCF fluorescence was taken as a marker for the formation of reactive oxygen species within the organism. We induced the formation of reactive oxygen species in *C. elegans* by application of thermal stress (37°C). As shown in Figure 2, the amount of fluorescent DCF increases over time after the onset of stress conditions. After 180 minutes, the DCF fluorescence of DMSO-treated nematodes was approximately 12.4-fold higher compared to the DMSO value at $t = 0$ (3276 ± 22 rfu $\rightarrow$ 40600 ± 2055 rfu). Treatment of the nematodes for 48 h with 100 μM TSG reduced the stress-induced increase in ROS: after 180 min, the rfu-value was 36378 ± 1926. The experiments performed with resveratrol showed no significant modulation of the DCF fluorescence. However, an incubation with the well-known antioxidant flavonoid quercetin caused a significant reduction of DCF fluorescence at 100 μM.

Antioxidative effects of resveratrol are extensively described in the literature: Jang and Surh [36] described protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells; also in H4IIE rat hepatoma cells antioxidative effects were reported in the DCF assay [37]. In a more recent paper, Vanaja et al. [38] described an improvement of the antioxidative properties of resveratrol after loading into liposomes. The antioxidative properties of resveratrol are further reviewed by, for example, Farghali et al. [39]. Concerning the antioxidative effects of TSG, less information is available: Kim et al. [40] reported protective effects of an extract of *Polygonum multiflorum* against oxidative toxicity in HT22 hippocampal cells without showing active components of the extract. Chen et al. [15] identified antioxidative components of *Polygonum multiflorum* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (similar to TEAC assay). They reported a strong antioxidative capacity by only three compounds: gallic acid, catechin, and TSG. Further antioxidative effects are reported by Zhang et al. [41] demonstrating protective effects of TSG against hydrogen peroxide-induced dysfunction and oxidative stress in osteoblastic MC3T3-E1 cells; Tao et al. [21] report a protective effect of TSG on 6-OHDA-induced apoptosis in PC12 cells and Li et al. [24] demonstrate that TSG attenuates

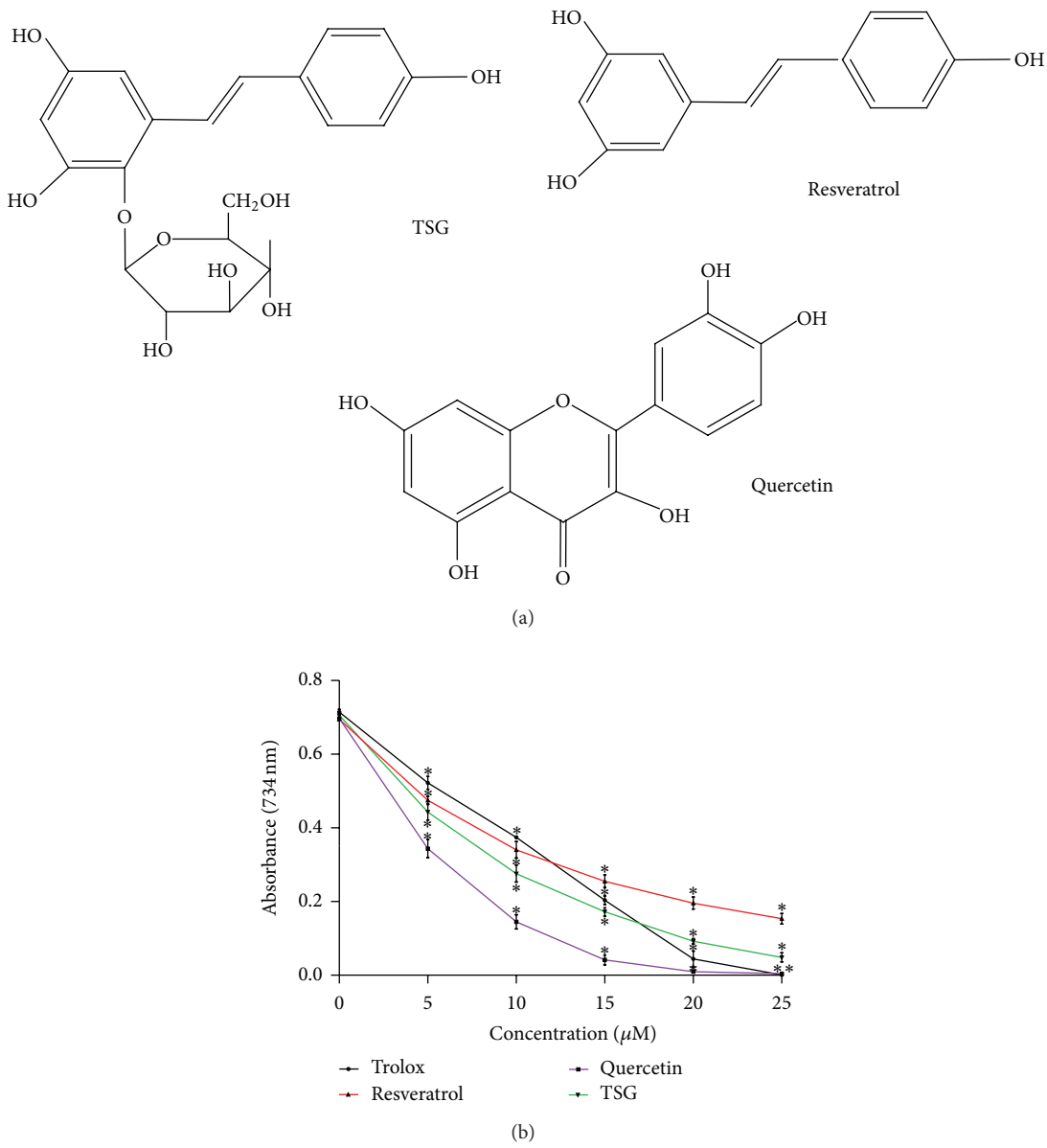

Figure 1: Radical scavenging properties (TEAC assay) of TSG. Chemical structures of resveratrol, quercetin, and TSG are shown in (a); the antioxidative capacity of the substances measured by the TEAC assay is shown in (b). The decolourisation of the radical-solution was detected spectrophotometrically at 734 nm (mean values ± SD, $n = 3$, $^{*}P < 0.05$ versus control value, Student's t-test).

MPP^{+}-induced apoptosis in PC12 cells by inhibiting ROS generation.

3.2. Modulation of Antioxidative Enzyme Expression by TSG. Polyphenols like TSG may protect against oxidative stress either by directly scavenging radicals or by indirectly increasing the stress resistance of the organism, for example, by induction of antioxidative enzymes. We have investigated the effects of TSG on the induction of glutathione-S-transferase-4 (GST-4) and superoxide dismutase-3 (SOD-3), two enzymes that are known to be inducible by oxidative stress.

To analyse the induction of SOD-3::GFP expression in *C. elegans*, we used the transgenic strain CF1553 (*muIs84 [pAD76(sod-3::gfp)]*). Treatment with TSG, resveratrol, or quercetin showed no significant influence on the expression of SOD-3 under basal conditions (Figure 3). Under stress conditions (150 μM of the redox-cycler juglone), SOD-3 expression is induced in *C. elegans*. Compared to the GFP fluorescence of nematodes under basal conditions (657.3 rfu), the fluorescence increased to 970.8 rfu under conditions of oxidative stress. This result shows that the redox-state in the nematode after application of juglone was shifted to the prooxidative state; a relatively high induction of

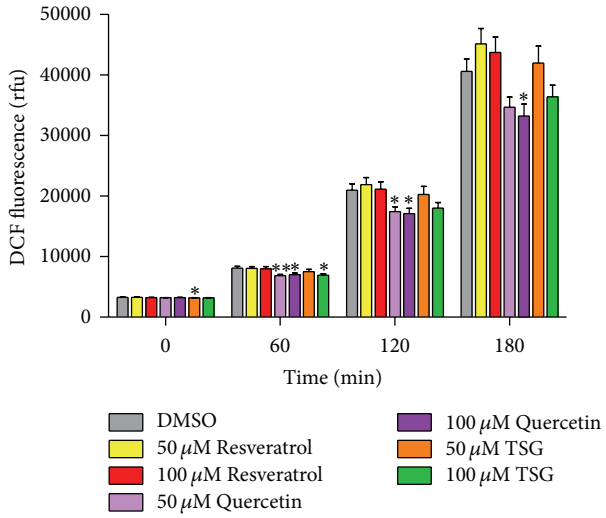

Figure 2: Modulation of ROS accumulation in *C. elegans* by TSG. Modulation of ROS accumulation in wild type nematodes at 37°C after incubation with TSG, quercetin, and resveratrol (50, 100 μM). The fluorescence intensity of DCF (rfu) was used as a marker for intracellular ROS; mean ± SEM, 5–7 independent experiments; at least 80 animals per concentration were analysed; $^{*}P < 0.05$ versus *DMSO value*, one-way ANOVA with Dunnett's post hoc test.

the antioxidative enzyme SOD-3 was needed to compensate this prooxidative condition. Treatment of the nematodes with 100 μM TSG starting 48 h prior to the application of juglone results in a significantly reduced induction of SOD-3 expression (704.1 rfu → 738.7 rfu). This may be interpreted as a reduction of the juglone-induced prooxidative status of the nematode that in consequence results in a lower expression of SOD-3. The effect of resveratrol was comparable to the effect of TSG; the highest modulation was caused by quercetin.

Next, we analysed the effects of TSG on the induction of GST-4, an enzyme that is thought to be involved in the defence against conditions of stress due to the induction via SKN-1. Similar to the SOD-3 reporter gene experiment, we used a transgenic strain (CL2166 *dvIs19 [pAF15(gst-4::gfp::NLS)]*) that expresses GFP under control of the *gst-4* promoter (Figure 4). Comparable to the expression of SOD-3, the expression of GST-4 is strongly increased under stress conditions. A 6.9-fold increase in the GFP fluorescence was detected after application of juglone (150.62 ± 5 rfu → 1043.16 ± 42 rfu). In contrast to the effect observed with the transgenic strain CF1553 (= SOD-3 reporter), TSG and resveratrol only slightly modulated the induction of GST-4; only a tendency can be suggested. Quercetin was the only compound that significantly diminished the induction of GST-4 after application of oxidative stress. The modulation of SOD-3 and GST-4 by the compounds can be explained in two ways. On the one hand, the direct antioxidative potential of the compounds reduces the oxidative stress which is generated by the redox-cycler juglone. The reduction in oxidative stress consequently reduces the amount of antioxidative enzymes which are induced. On the other hand, the reduction of SOD-3 induction may also be due to an indirect antioxidative effect of the compounds by specific activation of the antioxidative response system of the nematode.

Robb and Stuart [42] reported a significantly increased MnSOD expression by resveratrol in mouse C2C12 and primary myoblasts. Khan et al. [43] reported that treatment with 25 μM resveratrol significantly increased SOD activity in PC-3, HepG2, and MCF-7 cells, but not in HEK293T cells. Kavas et al. [44] reported an increase of SOD activity in male Wistar rats by resveratrol (20 mg/kg in drinking water for six weeks). Li et al. [45] reported that resveratrol treatment significantly increased the mRNA expression of GSTA1 in a time-dependent manner. On the other hand, Jiang et al. [46] showed that resveratrol attenuates early diabetic nephropathy by downregulating glutathione S-transferase Mu in diabetic rats. In contrast to the vast information about resveratrol, to our best knowledge, no information about TSG and modulation of antioxidative enzymes is available.

3.3. Modulation of Stress Resistance by TSG. Since TSG reduces the abundance and induction of reactive oxygen species in *C. elegans*, we investigated if the stilbene derivative also mediates a resistance against thermal stress (37°C) which has been shown to be lethal in *C. elegans*. We have analysed the effects of TSG, resveratrol, and quercetin on the tolerance of *C. elegans* against thermal stress using the semiautomated SYTOX Green assay (Figure 5). The mean and median survival time of DMSO-treated nematodes were determined as 4.82 ± 0.14 h and 4.75 ± 0.17 h, respectively. TSG strongly increases the resistance against thermal stress: 50 μM TSG induces a 22% increase in the mean survival time. The mean and median survival time of TSG-treated nematodes were determined as 5.89 ± 0.11 h and 6.00 ± 0.1 h, respectively. In case of stress resistance, TSG was shown to be the most active compound analysed (Table 1). Combined with the experiments shown before, this result clearly indicates that TSG has a high potential to protect *C. elegans* against stress conditions. Chen et al. [47] reported that resveratrol alleviated juglone-induced lethal oxidative stress and significantly prolonged the survival time of *C. elegans* under conditions of acute oxidative damage. However, no information is available about effects of TSG on stress resistance.

3.4. Prolongation of Life Span by TSG. With increasing age, highly oxidised and cross-linked proteins accumulate in the intestine of the nematode. These modified molecules form insoluble, autofluorescent ageing pigments, for example, lipofuscin. We investigated the effect of TSG, resveratrol, and quercetin on this phenomenon (i) to verify the antioxidative effects of the compounds in the nematode and (ii) to estimate effects of these compounds on the ageing process in *C. elegans*.

Treatment with TSG reduces the intestinal lipofuscin accumulation in *C. elegans* by 20 ± 1% compared to the solvent control (DMSO: 952 ± 19 rfu; TSG 817 ± 16 rfu). Resveratrol showed a slightly weaker decrease in lipofuscin fluorescence (846.8 rfu), while quercetin again showed the strongest

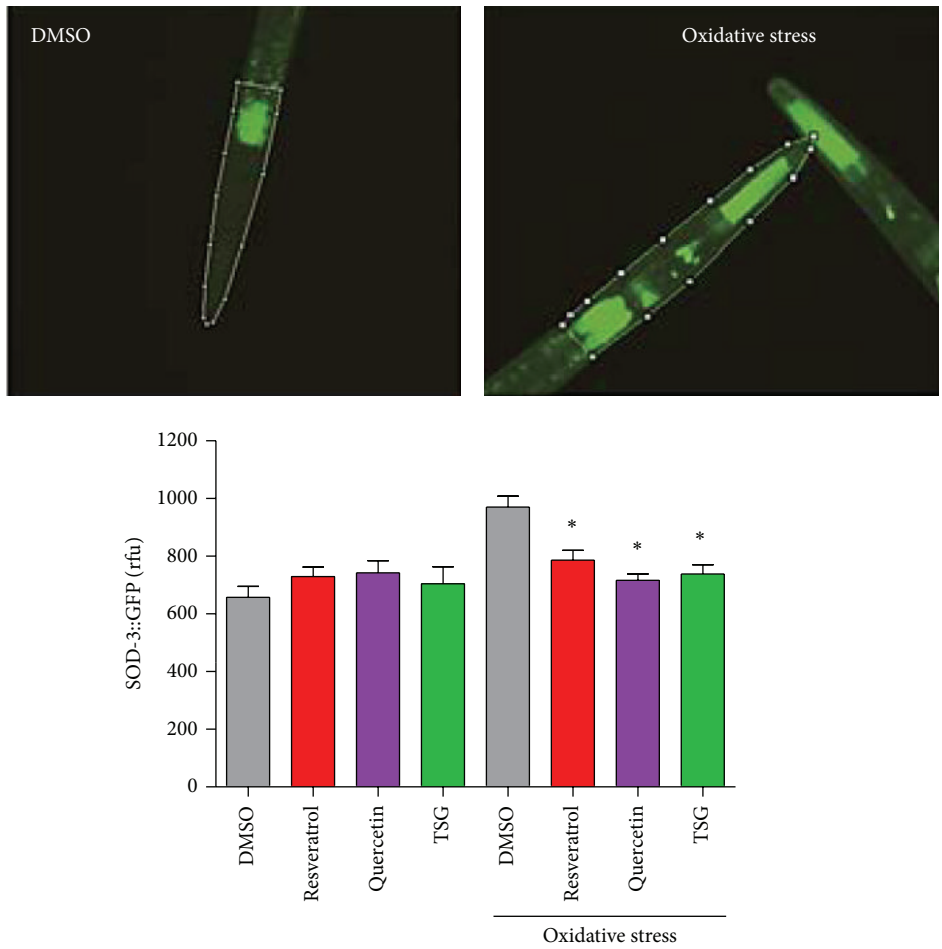

Figure 3: Modulation of SOD-3 expression by TSG. The images show GFP fluorescence of the head and anterior part of the intestine of CF1553 transgenic nematodes pretreated with 100 μM of the compounds without (DMSO) or with oxidative stress (150 μM juglone; 3 h). Data represent mean values ± SEM, $n = 3$, $^{*}P < 0.05$ versus corresponding DMSO value, one-way ANOVA with Dunnett's post hoc test. At least 10 animals per group and experiment were analysed.

reduction in this parameter. Incubation with 100 μM quercetin reduces the lipofuscin fluorescence by 27.85% (686.9 ± 14 rfu). These results show that TSG is capable of decreasing lipofuscin accumulation in *C. elegans* (Figure 6(a)).

Since it is known that a reduction of lipofuscin mostly correlates with an increase in life span of *C. elegans*, we investigated the effect of TSG, resveratrol, and quercetin on the life span of *C. elegans*. All three compounds exerted similar effects (Figure 6(b)). The mean life span of DMSO-treated nematodes was 17.4 ± 0.56 days; in case of TSG, resveratrol, and quercetin, the mean life span was 21.1 ± 0.58 d, 20.7 ± 0.67 d, and 21.3 ± 0.5 d, respectively (Table 2). The median life span of DMSO-treated nematodes was 18.0 ± 0.61 days; in case of TSG, resveratrol, and quercetin, the median life span was 22.0 ± 0.81 d, 22.0 ± 0.71 d, and 23.0 ± 1.01 d, respectively.

From the results of the DCF assay we can conclude that the radical scavenging effects of the compounds cannot be the only explanation for their positive effects on the life span of *C. elegans*. The antioxidative capacity of resveratrol was negligible in comparison to TSG and quercetin, but the effect on the life span of the nematode is comparable to the other two compounds.

To clarify this point, we analysed if the compounds may modulate intracellular pathways to prolong the life span of *C. elegans*. Therefore we used a mutant strain that contains a deletion in the *daf-16* gene leading to a loss-of-function of the corresponding protein. This transcription factor (FoxO homologue in *C. elegans*) has a crucial function in the regulation of ageing. By using a mutant strain defective in this pathway, we analysed if this factor is involved in the life span-prolonging effect of TSG. As we see in Figure 7, the life span extending effect of TSG, quercetin, and resveratrol was not abolished. We conclude that this pathway is not necessary to mediate the effects of TSG, quercetin, and resveratrol.

Resveratrol has been reported to be beneficial in cases of ageing-related cardiovascular and neurodegenerative diseases. However, previous studies on the longevity promoting effect of resveratrol have been partly inconclusive. Upadhyay et al. [48] reported an increase of life span after treatment

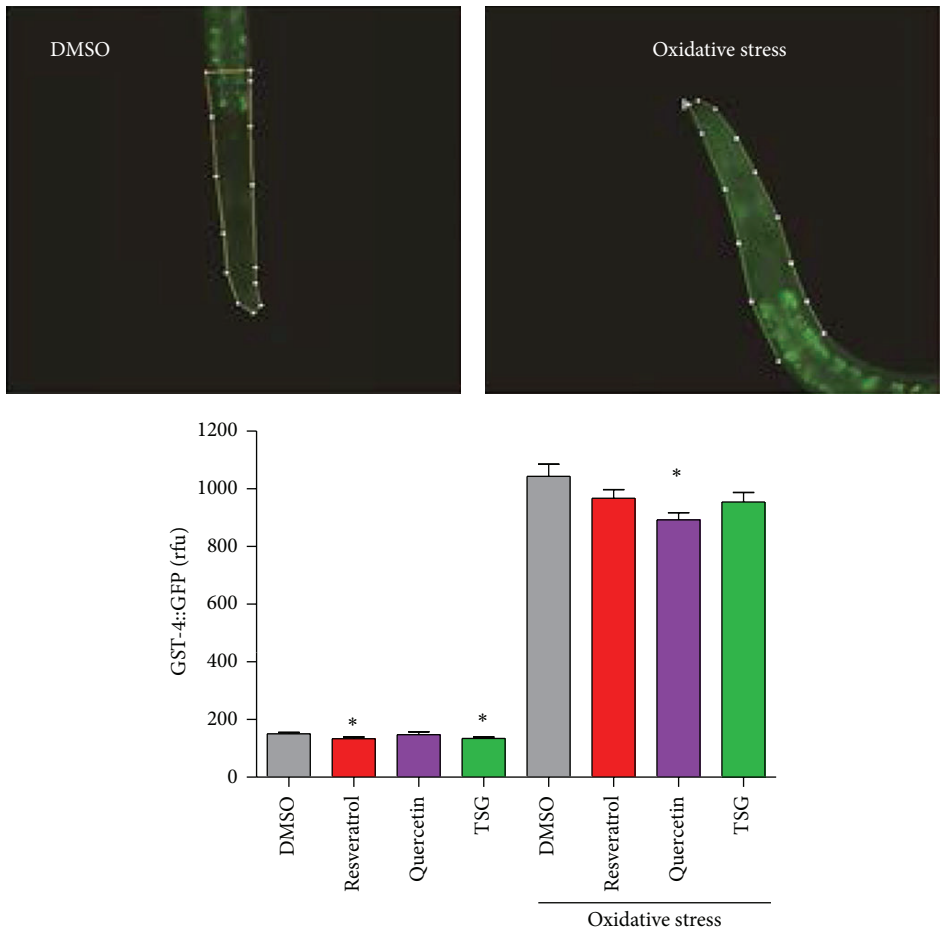

Figure 4: Modulation of GST-4 expression by TSG. The images show GFP fluorescence of the head and the anterior part of the intestine of CL2166 transgenic nematodes pretreated with 100 μM of the compounds followed by an incubation under physiological conditions or under conditions of oxidative stress (150 μM Juglone; 3 h). Data represent mean values ± SEM, $n = 3$, $^{*}P < 0.05$ versus corresponding DMSO value, one-way ANOVA with Dunnett's post hoc test. At least 10 animals per group and experiment were analysed.

with resveratrol (100 μM). Zarse et al. [49] reported that resveratrol significantly extends *C. elegans* life span already at a concentration of 5 μM by 3.6% (mean life span) and 3.4% (maximum life span). On the other hand, Chen et al. [47] observed no extension of the normal life span of *C. elegans* in either liquid or solid growth media containing different concentrations of resveratrol. Bass et al. [50] also analysed effects of resveratrol in *C. elegans* (wild type and sir-2.1 mutant nematodes) but results were variable. Resveratrol treatment results in slight increases in life span in some trials but not others (wild type and sir-2.1 mutant animals). As an explanation for the different effects there may be variations from one study to another concerning the delivery of the compounds to the nematodes. The use of liquid or solid growth media containing different concentrations of resveratrol makes it also difficult to compare results between studies. In our study, we confirm results of Upadhyay et al. [48] showing an extension of life span after incubation with resveratrol. Furthermore, we were the first to show that also application of TSG results in a prolongation of life span comparable to resveratrol. This finding may be a hint for the active component in the *Polygonum multiflorum* extract, which is traditionally used as an anti-ageing drug. It has to be mentioned that no adverse effects of the compounds (TSG, resveratrol, and quercetin) were detectable in the experimental assays up to a concentration of 100 μM. This is important since it was reported that other natural compounds cause toxic effects to the nematode: Mukai et al. [51] reported that a gallate of tannin isolated from the tea plant *Camellia sinensis* L. is toxic to *C. elegans* (LC_{50}: 49 μM).

4. Conclusion

2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG) is the main bioactive component of *Polygonum multiflorum*, a plant which is traditionally used as an anti-ageing agent in many East Asian countries. This compound causes antioxidative effects in *C. elegans*, alleviates the accumulation of lipofuscin, and prolongs the mean life span by 23.5% independently of DAF-16. Furthermore, the stress resistance of the nematode is strongly enhanced by this compound. In addition to direct antioxidative effects of TSG, this compound

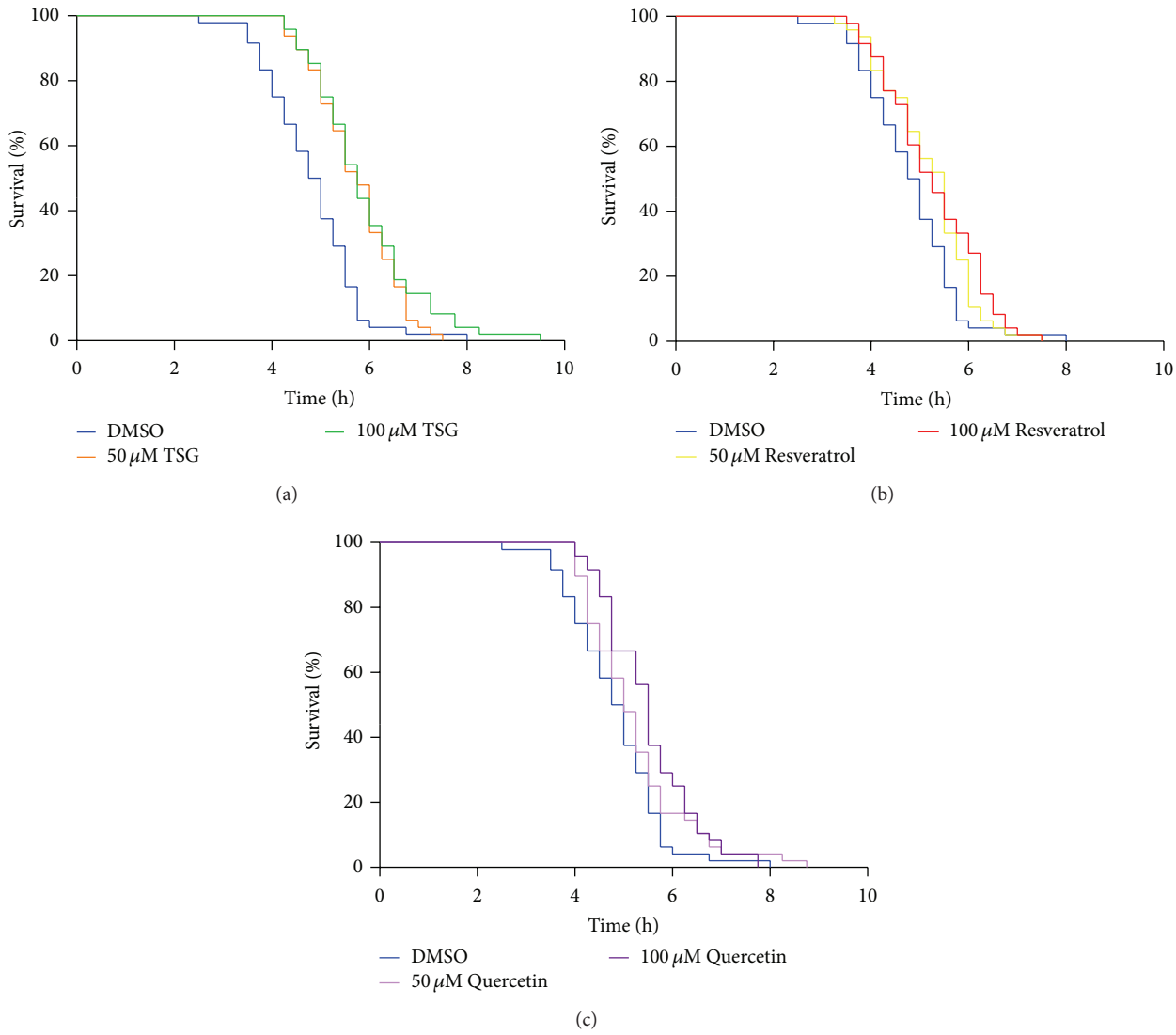

Figure 5: Increased resistance against lethal heat-stress by treatment with TSG. TSG treatment increases the resistance against thermal stress at 50 μM and 100 μM (a). Resveratrol treatment (b) and quercetin treatment effectively increased the survival only at 100 μM. Survival curves represent the data of 4 independent experiments with a total of 48 animals per group (Kaplan-Meier survival analysis); corresponding data are summarised in Table 1.

Table 1: Summary of the heat-stress survival data depicted in Figure 5.

	Adult survival [$h \pm$ SEM] at 37°C			
Treatment	Mean	Median	n	P versus DMSO (log-rank)
DMSO	4.82 ± 0.14	4.75 ± 0.17	48	
Quercetin (50 μM)	5.22 ± 0.15	5.00 ± 0.16	48	0.10
Quercetin (100 μM)	5.50 ± 0.13	5.50 ± 0.09	48	<0.001
Resveratrol (50 μM)	5.20 ± 0.13	5.25 ± 0.16	48	0.05
Resveratrol (100 μM)	5.29 ± 0.14	5.25 ± 0.24	48	<0.05
TSG (50 μM)	5.89 ± 0.11	6.00 ± 0.10	48	<0.001
TSG (100 μM)	5.94 ± 0.17	5.75 ± 0.12	48	<0.001

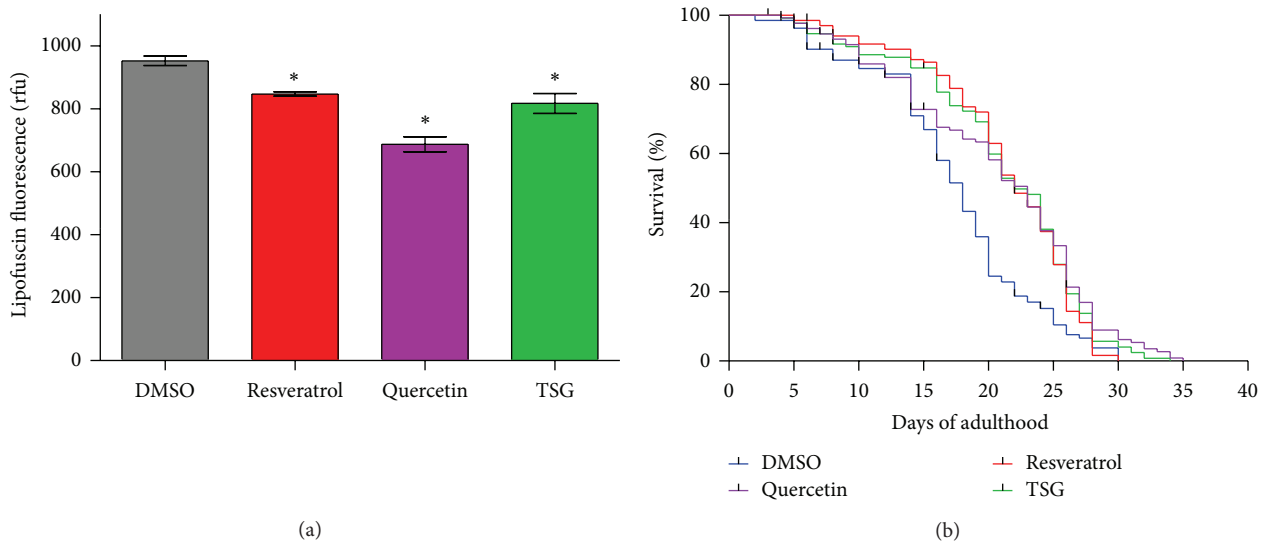

Figure 6: Prolongation of life span by treatment with TSG. (a) Pretreatment with 100 μM of TSG, resveratrol, and quercetin reduced the accumulation of the ageing marker lipofuscin (mean values ± SEM, $n = 3$, $^{*}P < 0.05$ versus control value, Student's t-test). 20 animals per group and experiment were analysed. (b) All compounds (100 μM) induced a prolongevity effect during the complete adult life (Kaplan-Meier survival analysis of three independent experiments with at least 30 animals per group/experiment; survival data are summarised in Table 2).

Table 2: Summary of the life span analyses of wild type nematodes (Figure 6(b)) and *daf-16* loss-of-function mutants (strain CF1038) (Figure 7).

Wild type		Adult life span [d ± SEM]		
Treatment	Mean	Median	n (censored)	P versus DMSO (log-rank)
DMSO	17.39 ± 0.56	18.00 ± 0.61	121 (14)	
Quercetin (100 μM)	20.68 ± 0.67	23.00 ± 1.00	118 (17)	<0.001
Resveratrol (100 μM)	21.31 ± 0.50	22.00 ± 0.71	129 (6)	<0.001
TSG (100 μM)	21.14 ± 0.58	22.00 ± 0.81	128 (7)	<0.001
daf-16(mu86)		Adult life span [d ± SEM]		
Treatment	Mean	Median	n (censored)	P versus DMSO (log-rank)
DMSO	11.05 ± 0.35	11.00 ± 0.37	96 (4)	
Quercetin (100 μM)	12.64 ± 0.43	14.00 ± 0.51	94 (6)	<0.001
Resveratrol (100 μM)	12.01 ± 0.40	11.00 ± 0.38	97 (3)	0.018
TSG (100 μM)	12.21 ± 0.41	12.00 ± 0.63	94 (6)	0.02

also causes indirect antioxidative effects in *C. elegans* via modulation of SOD-3 and GST-4. Our results strongly confirm the potential of TSG to be used as a pharmaceutical anti-ageing drug.

Abbreviations

DCF: 2′,7′-Dichlorofluorescein
DMSO: Dimethylsulfoxid
GST: Glutathione-S-transferase
rfu: Relative fluorescence unit
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TCM: Traditional Chinese medicine
TEAC: Trolox equivalent antioxidative capacity assay
TSG: 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside.

Conflict of Interests

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

Authors' Contribution

Christian Büchter and Liang Zhao contributed equally.

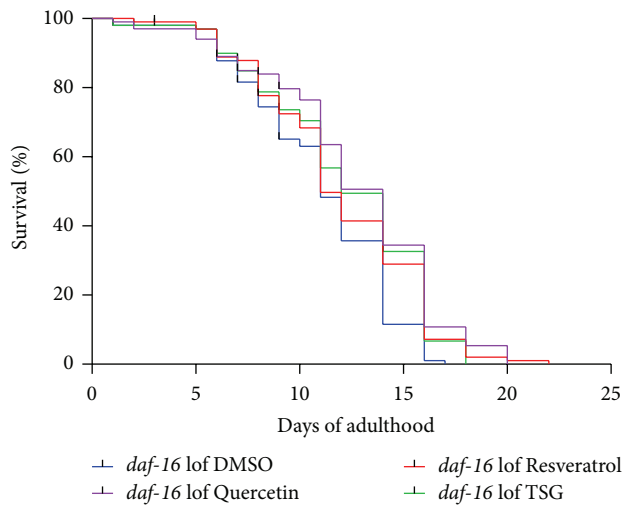

Figure 7: The TSG-mediated prolongation of life span is independent of DAF-16. Treatment with the compounds (100 μM) during the complete adult life induced a prolongevity effect in the *daf-16* loss-of-function mutant strain CF1038 (Kaplan-Meier survival analysis of two independent experiments with at least 30 animals per group/experiment; survival data are summarised in Table 2).

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Review Article

Arginase as a Critical Prooxidant Mediator in the Binomial Endothelial Dysfunction-Atherosclerosis

Luiza A. Rabelo,[1,2,3] Fernanda O. Ferreira,[1,2] Valéria Nunes-Souza,[1,2,3] Lucas José Sá da Fonseca,[1] and Marília O. F. Goulart[4]

[1] *Laboratório de Reatividade Cardiovascular, Setor de Fisiologia e Farmacologia, Instituto de Ciências Biológicas e da Saúde (ICBS), Universidade Federal de Alagoas (UFAL), Avenida Lourival Melo Mota s/n, Cidade Universitária, 57072-900 Maceió, AL, Brazil*
[2] *Instituto Nacional de Ciência e Tecnologia em NanoBiofarmacêutica (N-BIOFAR), Avenida Antônio Carlos s/n, Pampulha, 31270-901 Belo Horizonte, MG, Brazil*
[3] *Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany*
[4] *Instituto de Química e Biotecnologia, Universidade Federal de Alagoas (UFAL), Avenida Lourival Melo Mota s/n, Cidade Universitária, 57072-900 Maceió, AL, Brazil*

Correspondence should be addressed to Luiza A. Rabelo; luizaa.rabelo@gmail.com

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Arginase is a metalloenzyme which hydrolyzes L-arginine to L-ornithine and urea. Since its discovery, in the early 1900s, this enzyme has gained increasing attention, as literature reports have progressively pointed to its critical participation in regulating nitric oxide bioavailability. Indeed, accumulating evidence in the following years would picture arginase as a key player in vascular health. Recent studies have highlighted the arginase regulatory role in the progression of atherosclerosis, the latter an essentially prooxidant state. Apart from the fact that arginase has been proven to impair different metabolic pathways, and also as a consequence of this, the repercussions of the actions of such enzyme go further than first thought. In fact, such metalloenzyme exhibits direct implications in multiple cardiometabolic diseases, among which are hypertension, type 2 diabetes, and hypercholesterolemia. Considering the epidemiological repercussions of these clinical conditions, arginase is currently seen under the spotlights of the search for developing specific inhibitors, in order to mitigate its deleterious effects. That said, the present review focuses on the role of arginase in endothelial function and its participation in the establishment of atherosclerotic lesions, discussing the main regulatory mechanisms of the enzyme, also highlighting the potential development of pharmacological strategies in related cardiovascular diseases.

1. Introduction

Cardiovascular and metabolic diseases have achieved global emphasis and represent one of the main public health problems [1–3]. The augmented burden of such conditions is due to the increase of their risk factors in alarming epidemic proportions, becoming an important cause of morbidity and mortality in occidental countries. In this scenario, atherosclerosis is recognized as a hallmark in the development of key cardiovascular disorders, including myocardial infarction and stroke [3–5].

Thereby, many studies have aimed to elucidate the pathophysiological mechanisms involved in the onset and development of these diseases, highlighting the role of endothelial dysfunction in vascular disorders, which is mainly caused by the reduced bioavailability of nitric oxide ($^{\bullet}$NO; $^{\bullet}$N=O nitrogen monoxide) [6]. When it comes to the $^{\bullet}$NO bioavailability, it is of note to emphasize the role of the semiessential amino acid L-arginine, a common substrate for both nitric oxide synthase (NOS) and arginase enzymes [7].

In a pioneer work, Buga and coworkers showed that the NG-hydroxy-L-arginine, an intermediate compound in the process of $^{\bullet}$NO synthesis from L-arginine, is an endogenous inhibitor of arginase activity [8]. In this context, it becomes evident the important role of these metabolic enzymes, with the necessity to keep the balance for the axis NOS/L-arginine/arginase for maintaining the $^{\bullet}$NO homeostatic levels. The two fundamental mechanisms for reduced levels

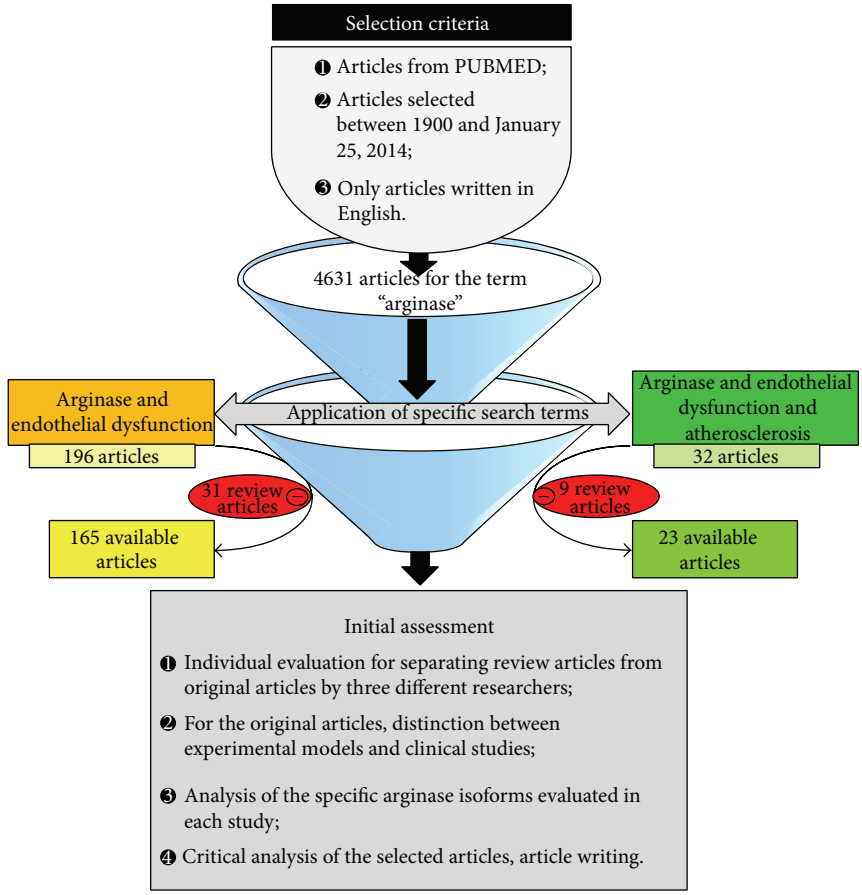

Figure 1: Workflow: steps for articles selection and for preparing the paper.

of bioactive •NO are its reduced synthesis by NOS and its increased oxidative inactivation by reactive oxygen species (ROS) intermediates, ultimately leading to a potential impairment in cardiovascular homeostasis [9].

Mammalian arginase (EC 3.5.3.1), a manganese-metalloenzyme [10], hydrolyzes L-arginine to L-ornithine and urea [11].

By presenting this action on L-arginine, arginase participates in the regulation of the •NO synthesis by using the same enzyme substrate for the three known NOS isoforms: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3) [12]. Thus, increased expression of arginase may imply extensive consumption of L-arginine to be converted into urea and L-ornithine, this way reducing the availability of L-arginine to •NO synthesis by NOS.

Various studies have demonstrated increase in arginase activity in different clinical conditions, such as hypertension [13, 14], type 2 diabetes mellitus [15, 16], hypercholesterolemia [17], aging [18–20], and atherosclerosis [21], proposing a critical contribution of this enzyme in the pathogenesis of cardiovascular diseases. In this direction, the present review focuses on the role of arginase in atherosclerosis and its implications in endothelial function, aiming to contribute to the pathophysiological discussion of the main regulatory mechanisms of the enzyme. Also, the paper highlights the potential development of pharmacological actions in related cardiovascular diseases concerning the arginase activity. For carrying out this study, a research in electronic PubMed database was performed taking as reference the period between the year 1900 and January 25, 2014, using the terms "arginase AND endothelial dysfunction," and "arginase AND endothelial dysfunction AND atherosclerosis." Only articles available in English were considered (Figure 1). In the following lines, the term "arginase" will be used to refer to both isoforms of the enzyme, so that arginase I will be specifically presented by the term "Arg I," and the second one, by "Arg II."

2. From the Discovery of Arginase to Its Role in Endothelial Dysfunction: More Than a Century of History

The seminal events of the history of arginase took place more than a hundred years ago. In 1904, Kossel and Dakin [22] described the discovery of this enzyme in mammalian liver. They observed a decrease of arginine after acid hydrolysis in the liver caused by administration of the ferment named arginase, generating urea and ornithine [11, 22]. Thenceforth, other researches have reported the presence of arginase in various organs. In the first decades of 1900, arginase was

found in the liver of several animal classes such as amphibians, fishes, and turtles by Clementi, who also identified the presence of the enzyme in the kidney of birds.

A few years after Clementi's observations [23–25], the records concerning the spectrum of arginase distribution in different organs of certain mammals and domestic fowls were broadened by Edlbacher and Rothler [26], as both authors conducted a large study which brought to light the identification, in mammals and birds, of arginase in the liver, kidneys and testes. Besides showing that the number of units of arginase normalized by body weight differed between sexes, being elevated in males when compared to females, the authors also reported the presence of arginase in the placenta and thymus of some mammals. Later on, in 1927, Chaudhuri performed a study in 32 birds showing a quantitative estimation of arginase mainly in kidneys and then in testes. He also defended a gender distinction in the arginase distribution due to the presence of this enzyme in male sexual organs and the absence in female ones [27]. In the following years, the differential role of arginase between sexes and the participation of sex steroid hormones in its modulation would also be discussed, pointing to the possible effects of arginase in multiple pathophysiological pathways [28].

In 1930, it was reported the activation of arginase by metal complexes of thiols, such as reduced glutathione and Fe^{+2} and Cu^{+2} ions. However, Purr and Weil [29] suggested that a specific oxidation-reduction potential, and not only the SH group, was related to arginase activation [29]. In 1986, Dizikes and coworkers published the screening of human liver arginase cDNA, which was not completely homologous to the genes found in the human kidney [30]. Such apparent discrepancy was posteriorly ruled out when the sequence found in the kidney was described as the gene of Arg II, cloned in 1996 [31]. Throughout the years, several studies have demonstrated the presence of arginase not only in various organs and species but also in animal models related to vascular disorders [20, 21, 32–38]. Buga and coworkers, for example, demonstrated for the first time the constitutive expression of arginase in rat aortic endothelial cells, also observing that its endogenous inhibition may represent a means for enabling the availability of proper amounts of arginine for $^{\bullet}NO$ production [8]. Furthermore, by comparing young and old Wistar rats, Berkowitz and coworkers first showed the association between arginase activity and the endothelial dysfunction of aging [20]. Following these breakthroughs in the comprehension of arginase contribution to vascular health, Ryoo and coworkers [21] discussed the connections among atherosclerosis, arginase activity, and endothelial dysfunction in atherosclerosis-prone mice, highlighting the therapeutic potential of arginase in atherosclerotic vascular disease [21]. Nevertheless, the pathophysiological mechanisms concerning the arginase participation in different metabolic diseases are not completely understood and the researches in humans still require further advances in order to better define the molecular pathways through which arginase interferes in health and disease.

3. In the Backstage of the Enzyme Function: Expression, Regulation of Arginase, and the Crosstalk in the Signaling Pathways

Arginase is an enzyme that participates in the urea cycle, being described in two isoforms: Arg I and Arg II, both of them catalyzing the same biochemical reaction. Human Arg I is a 322-amino acid protein, sharing 58% sequence with her sister, Arg II [39]. Arg I is known as the hepatic isoform because it is primarily found in the liver [40, 41]. However, it has been described in other tissues such as endothelial cells and vascular smooth muscle cells (VSMC), being also a cytosolic enzyme [42]. On the other hand, in mammalians, Arg II is a mitochondrial enzyme and is distributed in several organs and tissues, including prostate, kidney [39], and blood vessels. Their isoforms are expressed in the vasculature, as well as have many actions that interfere with vascular dynamics and contribute to endothelial dysfunction found in various cardiovascular pathologies [21].

In the course of the enzyme activity, the hydrogen bonding established between the guanidinium group and Glu227 keeps L-arginine in its proper position/location in the active site of the enzyme. Such molecular interaction is critical for enabling L-arginine to be attacked by the metal-associated hydroxide ion at the guanidinium group, leading to the formation of a tetrahedral intermediate. Thus, the hydroxyl group in that intermediate and the developing sp^3 lone electron pair on the NH_2 group are stabilized by the manganese ions [10].

According to the aforementioned statements, in the endothelial layer, the competition between Arg II and eNOS leads to a decrease in the $^{\bullet}NO$ bioavailability, resulting in impaired vasodilation and, consequently, endothelial dysfunction [20, 21]. The consumption of L-arginine also stimulates the production of reactive oxygen species (ROS), greatly contributing to oxidative stress. In addition, arginase induces the synthesis of polyamines and proline, promoting VSMC proliferation and remodeling [43, 44]. It has also been reported that the overexpression of Arg I reduced inflammatory activity in rabbits, via interaction with endothelial nitric oxide synthase (eNOS) [45]. Furthermore, it has been reported that conditions such as hypoxia [46], as well as proinflammatory mediators [47], reactive oxygen and nitrogen species (RONS), glucose, and oxidized low-density lipoprotein (ox-LDL) [40] stimulate arginase expression. Together, factors including stimulation and expression of this enzyme provide a framework for the analysis of the arginase isoforms into the atherogenesis. In endothelial cells, the abovementioned inducing phenomena are particularly of note, once the reduction in L-arginine levels is presented as one of the main mechanisms which may lead to the establishment of endothelial dysfunction [45]. Accordingly, several signaling pathways involve the activation of arginase, such as receptors for ox-LDL [21, 48], inflammatory mediators, and RhoA/ROCK kinases [45]. At first, after the oxidation of LDL, the resulting lipoprotein binds to the lectin-like LDL receptor-1, LOX-1, stimulating the activation of arginase [21]. Furthermore, ox-LDL increases the expression of caveolin I,

a molecule that interacts with eNOS, hindering the formation of $^{\bullet}$NO [48, 49].

With regard to inflammatory mechanisms, macrophages, cells involved in the generation of the atheromatous plaque, induce the expression of arginase via lipopolysaccharide (LPS), interleukin (IL)-4, IL-6, and interferon-gamma (INF-γ) [45]. In addition, in inflammatory conditions, cationic amino acid transporters (CAT), which act as L-arginine transporters, have their function impaired, then reducing the production of $^{\bullet}$NO and consequently promoting the progression of atherosclerosis [50]. Moreover, the route RoA/ROCK corresponds to a cascade of intracellular signaling through which protein kinases, stimulated by various factors, such as proinflammatory cells, ROS, and ox-LDL, induce the activation of arginase [45, 51].

4. Vascular Dysfunction in Atherosclerosis: A Brief Update

The endothelium is a central component for the maintenance of cardiovascular homeostasis. For a long time, it was thought that the vascular endothelium predominantly would act as a surface for blood flow. In a landmark study, Furchgott and Zawadzki [52] changed this concept. These authors first demonstrated the existence of an endothelium-derived relaxing factor which was subsequently identified as $^{\bullet}$NO [53–55]. Further work showed that the endothelium is an active participant in the regulation of cardiovascular homeostasis [56]. The network involved in the endothelial regulation is complex. Under physiological conditions, this cell layer is a sensor of hemodynamic changes and releases both relaxing and contracting factors. The disturbance in this sensitive balance leads to endothelial dysfunction, a common feature in cardiovascular, renal, and metabolic diseases [57], as well as in the atherosclerotic plaque formation [4, 58].

Atherosclerosis represents a multifactorial process and one of the well discussed marks of this structural and functional phenomenon is the role of cholesterol and inflammatory mediators in atheroma formation [3, 5]. Currently, it is defined as a chronic and progressive disease in which the deposition of atherosclerotic plaques occurs on the inner face of great arteries, causing reduction of the vascular lumen and damage to the underlying layers [3, 4].

The initial step in the formation of an atherosclerotic plaque is characterized by damage, either structural or functional, in the inner surface of the arteries, which promotes the increased expression of adhesion molecules in the surface of endothelial cells, thereby stimulating leukocyte adhesion to the intima layer. In the establishment of this process, proinflammatory mediators and shear stress play an important role, favoring this adherence. After physically contacting the endothelial cells, the adhered monocytes may infiltrate the subendothelial space via migration. Concomitantly, an accumulation of LDL particles in that space may also occur. In the presence of oxidative stress, the increased amounts of ROS, among which superoxide anion ($^{\bullet}O_2^-$), lead to the oxidation of such lipoproteins (ox-LDL). The circulating monocytes, now recruited and turned into macrophages in the subendothelial space, progressively phagocyte ox-LDL particles, ultimately being converted in foam cells. In this process, VSMC are stimulated to migrate from the tunica media to the intima, producing a cape of collagen and elastin that covers the plaque. Additionally, it is also observed the formation of a necrotic core due to debris and lipids released from cells that suffered apoptosis [5, 59].

Experimental and clinical evidence show that the major risk factors implicated in the impairment of cardiovascular functions are directly associated with endothelial dysfunction [56, 60, 61]. The seminal experiments of Ludmer and coworkers [58], using angiography, characterized the paradoxical effect of acetylcholine (ACh) in the coronary circulation, as this study showed that, in patients with atherosclerosis, this muscarinic agonist significantly decreased vascular diameter. The authors postulated that the impairment in endothelial function is responsible for vasoconstriction observed after intracoronary administration of ACh [58].

The link between endothelial cells and the elementary changes in the vascular wall which precede the establishment of the atherosclerotic process has passed from drafted speculations to concrete evidences, once some studies have clearly shown the connection between endothelial dysfunction and the early development of atherosclerosis [5, 45]. In order to elucidate the relationship between $^{\bullet}$NO and endothelial dysfunction in atherosclerosis, for example, Dhawan and colleagues [62] demonstrated a decrease in atherosclerotic plaque formation in an experimental model with monkeys fed a high-cholesterol diet after administration of L-arginine, with improved endothelial function by elevation of $^{\bullet}$NO levels and thereby reduced atherogenesis. In this context, several studies have been conducted to characterize the relationship between arginase and the cardiovascular endpoint of progressive endothelial dysfunction, marked by atherosclerosis.

Taking into account that the regulation in $^{\bullet}$NO metabolism represents a mediator for vascular health [63] and that arginase activity in particular conditions may play "the bad guy" by compromising the $^{\bullet}$NO bioavailability [7, 9], it becomes easy to understand the reason why the $^{\bullet}$NO/arginase axis is now recognized as a pivotal regulatory pathway of the vascular system. Indeed, for arginase activity there is only one substrate, L-arginine, but the repercussions of this single action may reach massive proportions, implying pleiotropic harmful outcomes on the endothelial function, once the beneficial effects of $^{\bullet}$NO, among which the prevention of abnormal vasoconstriction, inhibition of platelets aggregation, and reduced expression of adhesion molecules in the surface of endothelial cells [64], could be nullified.

5. Role of Arginase in the Development of Endothelial Dysfunction and Atherosclerosis

Studies have demonstrated an increase in the regulation of arginase in various cardiometabolic diseases such as hypertension [65–67], atherosclerosis [15, 21], ischemia-reperfusion injury [36, 68], diabetes mellitus [15, 57], and ageing [20, 69]. These findings have prompted researchers to discover whether arginase inhibition would then result in improved endothelial function in these conditions.

As previously discussed, a decrease of the •NO bioavailability plays an essential role in the pathogenesis of various cardiovascular events [70]. Interestingly, endothelial dysfunction derived from the •NO reduction causes vascular stiffness even in the absence of atherosclerosis, with arginase presenting itself as a key element in the progression of vascular disorders [71] (Figure 2).

Arginase acts in atherogenesis mostly via reduction of •NO, a free radical responsible for the inhibition of platelet aggregation and leukocyte adhesion to the blood vessel wall, being an important factor in the formation of atherosclerotic plaque [5]. Furthermore, •NO helps in the preservation of endothelial function due its vasodilator effect, so that its deficiency corresponds to another mark that leads to the progression of atherosclerosis [72] (Figure 2). Ryoo and coworkers [21], while studying the role of Arg II in atherosclerosis, found that ox-LDL, frequently observed in this disease, stimulates the release of Arg II, reducing the production of •NO. In such work, the researchers used mice with genetic deletion for Arg II (Arg II$^{-/-}$) fed a high-cholesterol diet, observing that the reduction in arginase activity improved the endothelial function compared to ApoE$^{-/-}$ mice fed the same diet. These findings suggest that the genetic deletion of arginase provides endothelial protection. Another crucial atherosclerotic pathophysiological mechanism consists in the oxidation of LDL particles [40, 48], that contributes to the formation of foam cells, after being captured by phagocytes. This also participates in the formation of ROS via NADPH oxidase [73] and uncoupling of eNOS [40], hindering the •NO production. In addition, it was shown that hypercholesterolemia increases the generation of asymmetrical dimethyl-L-arginine (ADMA), an endogenous inhibitor of eNOS [9, 64], in experimental models with monkey, inhibiting competitively the binding of L-arginine to eNOS [74].

ox-LDL causes a separation of arginase from the microtubule cytoskeleton, then increasing the expression of this enzyme in human aortic endothelial cells (HAECs) [40]. Ryoo and colleagues [48] showed that the isoform in question corresponds to Arg II via LOX-1 receptor. Furthermore, as a consequence of arginase activity in the formation of the atherosclerotic plaque, it is observed a reduction in •NO levels, thereby inducing the oxidation of LDL (which generates a stimulatory form of atherogenesis) and other effects such as increased endothelial permeability, cell proliferation, and leukocyte adhesion on the vasculature, resulting in atherosclerosis [75].

Several studies have reported endothelial dysfunction as a consequence of increased activity and/or expression of arginase in experimental models of hypertension [70], diabetes mellitus [15], aging [20], erectile dysfunction, sickle cell disease, and atherosclerosis [70], the latter corresponding to the focus of this paper. In Arg II null mice, for example, this enzyme activity was substantially reduced in the vascular endothelium, suggesting that Arg II is the main isoform present in blood vessels. Also, the inhibition of Arg II restored endothelial function, led to increased vascular •NO levels and decreased vascular stiffness in ApoE$^{-/-}$ mice [21]. Moreover, even in ApoE$^{-/-}$ mice, the high-cholesterol diet increased arginase activity compared with control mice fed a standard diet. In addition, inhibition of Arg II prevented the •NO decrease induced by high-cholesterol diet [21].

The rise of RONS due to the increased expression of arginase has emerged as one of the factors that induce endothelial dysfunction in animal models of atherosclerosis [41], a fact that has also been observed in patients with coronary artery disease and vascular impairment in type 2 diabetes mellitus [76]. However, it is not completely understood how arginase acts on endothelial dysfunction *in vivo* [15].

An established concept, however, defines that the production of RONS is also stimulated by inflammatory conditions, leading to endothelial injury and consequent development of atherosclerosis [50].

The formation of ROS is reported as a hallmark in the physiopathology of atherosclerosis. In this regard, the oxidative modification hypothesis as a critical step in the development of atherosclerosis deserves to be mentioned. According to this hypothesis, the simple presence of circulating LDL particles is not the only initial influencing factor for the onset of atheromatous plaques. Instead, such particles are required to undergo structural changes so that they can be properly recognized by specific macrophage receptors, being then engulfed and accumulated in phagocytes [77, 78]. In line with these observations, several studies have demonstrated the role of ox-LDL and proinflammatory mediators in the generation of atheromatous plaque [5]. One of the mechanisms arising from these stimuli corresponds to apoptosis of endothelial cells, which contributes to the induction of atherogenesis. Given this context, Suschek and coworkers [50] demonstrated that the expression of IL-1, tumor necrosis factor alfa (TNF-α), and INF-γ in blocked iNOS rat models resulted in cell apoptosis induced by hydrogen peroxide (H_2O_2), whereas under conditions of high levels of •NO, no protection against cell death was observed, concluding that a greater supply of L-arginine helps to reduce the development of atherosclerosis [50].

As previously outlined, endothelial dysfunction and atherosclerosis are directly linked to proinflammatory states in blood vessels. Several cytokines, such as IL-4, IL-6, and TNF stimulate the activity of arginase and impair the expression of eNOS [79], besides increasing the production of ROS (Figure 2). Concerning this issue, Spillman and colleagues [80] investigated the relationship between liver X receptors (LXR), a hormone receptor that participates in the reverse transport of cholesterol and TNF upregulation, demonstrating a decrease in Arg II activity and mRNA expression by LXR agonist, with restoration of •NO bioavailability.

In another study focused on inflammatory factors involved in endothelial dysfunction, Witting and coworkers [81] showed an increase in arginase protein expression and activity in rat aorta exposed to protein serum amyloid A (SAA), an apolipoprotein produced by the liver that is deposited in atherosclerotic plaques and is costimulated by inflammatory mediators such as TNF-α, IL-1, and IL-6. Reinforcing the critical role of arginase in the metabolism of •NO, experiments conducted by Sikka and coworkers [12] showed that, in C57Bl/6 mice exposed to cigarette smoke for 2 weeks, the knockout mice for Arg II showed better endothelial

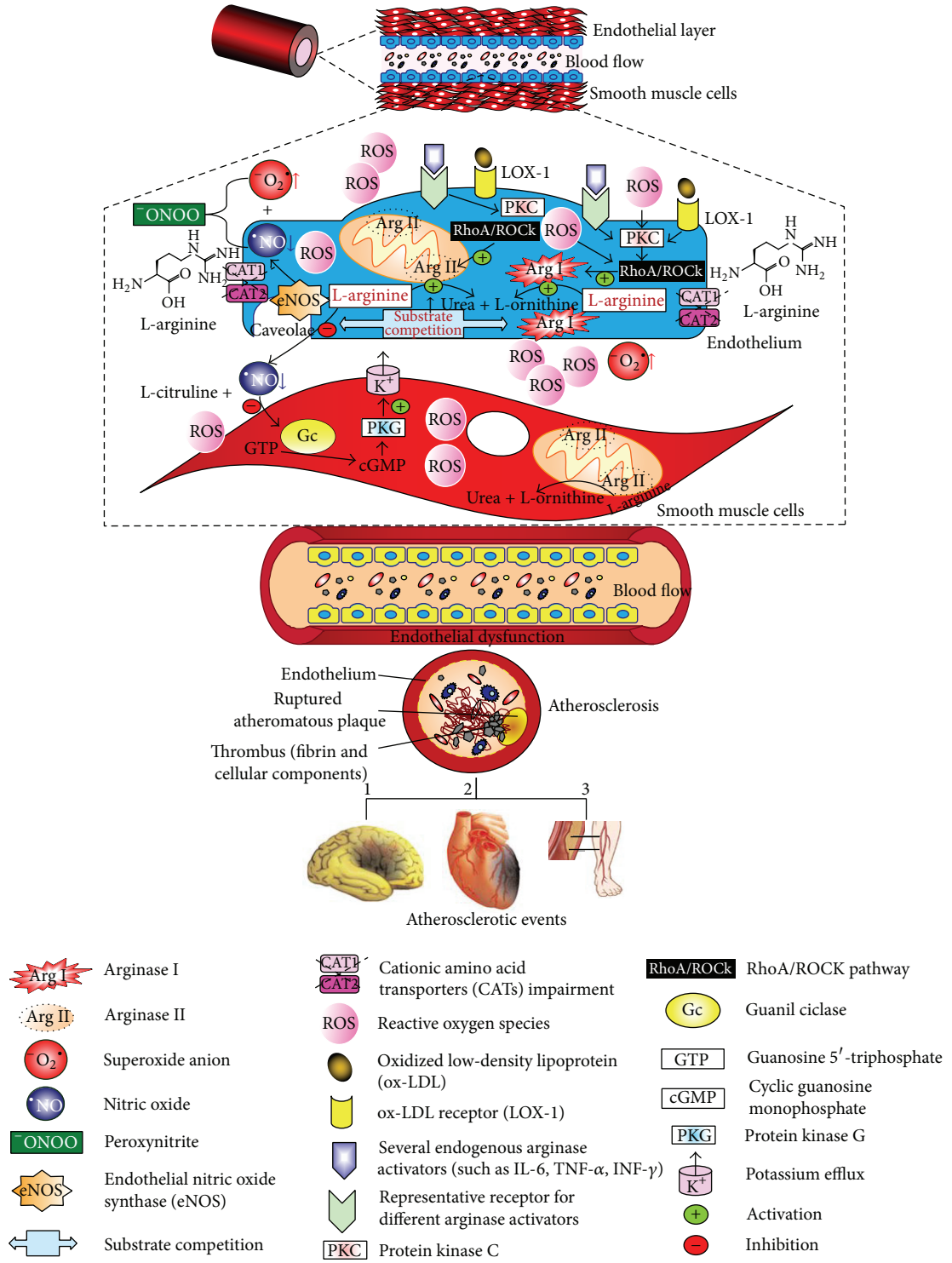

FIGURE 2: Role of arginase isoforms in nitric oxide metabolism in the vasculature. The complex web of interactions among circulating factors, membrane receptors, and intracellular signaling pathways directly interferes with vascular homeostasis. The balance between the activities of the endothelial nitric oxide synthase and the arginase isoforms is critical for maintaining the adequate nitric oxide bioavailability. Once the imbalance is established, either for increased reactive oxygen species production, decreased nitric oxide availability, or both, the phenomenon of endothelial dysfunction (in this figure represented by endothelial cells in yellow) may occur, being the initial event in the establishment and progression of atherosclerosis. As a consequence of such vascular damage, the arterial impairment progresses, increasing the risk of developing different atherosclerotic events, among which are stroke (1), myocardial infarction (2), and peripheral artery disease (3).

function compared to the controls. This observation was attributed to the gene deletion of Arg II and the consequent increase in $^{\bullet}$NO bioavailability [12] (Figure 2).

One of the mechanisms that lead to endothelial dysfunction is characterized by shear stress, reported as a predisposing factor for plaque formation. In a novel study with porcine endothelial cells and carotid artery, Thacher and colleagues [82] demonstrated an increase of Arg II expression after the induction of oscillatory shear stress compared to unidirectional high shear stress, for three days. To corroborate the role of this enzyme with the related alteration in the blood flow, the arginase inhibitor N^{w}-hydroxy-nor-L-arginine (Nor-NOHA) was administered, resulting in a decrease in ROS production [82]. Furthermore, an elevated proliferation of VSMC was also observed. With regard to this effect, Xiong and coworkers [83] studied the role of Arg II in VSMC from human umbilical veins, also demonstrating a proliferative action in these cells when the enzyme was activated. However, the authors observed an induction to senescence and apoptosis in the lack of Arg II function, contributing to the rupture of the plaque burden, due to the resulting weakness of the vascular layers [83] (Figure 2).

With regard to arginase stimulation, the administration of thrombin in human umbilical vein cells was able to elevate this enzyme expression after 18 hours of exposure, with peak in 24 hours [84]. In this study, it was also administered the HMG-CoA inhibitor fluvastatin, which impairs the RhoA/ROCK pathway, leading to reduction of arginase expression by thrombin. In addition, similar effects were observed with other ROCK inhibitors used in the study [84]. Also, while studying the role of Arg II in the macrophage inflammatory responses, Ming and coworkers found a protective profile against insulin resistance, type 2 diabetes, and atherosclerosis in Arg II-deficient mice [38]. In addition, by comparing the vascular function between transgenic C57Bl/6 mice overexpressing human Arg II and nontransgenic controls, Vaisman and colleagues [70] found impaired endothelium-dependent vasodilation induced by ACh in the transgenic group. The authors also observed that the increased expression of Arg II itself, irrespective of changes in lipid concentrations in plasma, was sufficient to feed the development of atherosclerotic lesions, ultimately highlighting the critical role of Arg II in such inflammatory process.

Aside from being described as a potentially deleterious enzyme in the regulation of endothelial function, of great importance are also the observations that arginase may exert beneficial effects in the vasculature. Such capacity of, in specific circumstances, embodying both a protective and harmful role, inserts arginase in an even more complex and intriguing context. In rabbits, for example, while studying genes of atherosclerosis susceptibility, Teupser and coworkers [85] showed that the high expression of Arg I in macrophages contributes to atherosclerosis resistance, possibly by exerting an anti-inflammatory mechanism in the vascular environment [85]. Furthermore, the atheroprotective role of Arg I may be expressed through multiple pathways, such as differential activation of macrophages, modulation of inflammatory response in VSMC, and changes in plaque stability [41].

All original studies assessed in the present paper, concerning the role of arginase isoforms in the binomial endothelial dysfunction-atherosclerosis, are presented in Table 1.

6. The Therapeutic Potential of Arginase: A Short Update

Arginase inhibitors have been developed since the 1990s, to evaluate more accurately the effects of the enzyme activity reduction [86], with the observation of promising results concerning the improvement in endothelial function and associated disorders in animal models of diabetes [34], hypercholesterolemia [87], hypertension [32, 37, 88], and metabolic syndrome [88].

Therefore, the elucidation of mechanisms involved in the transcription and transduction, as well as in activity of arginase represents a promising area for the development of pharmacological approaches in cardiovascular and metabolic diseases [45, 86]. Despite the growing number of studies and the evident role of arginase in the regulation of $^{\bullet}$NO bioavailability [8, 68, 89, 90] and endothelial dysfunction [20, 33, 91], development of atherosclerosis [21, 40], and other cardiometabolic diseases [34, 37, 57, 92], several questions still need to be better understood.

In fact, a major obstacle for a more accurate understanding of the effects of the two different human arginases still rests on the difficulty in creating a specific inhibitor for each isoform, due to the close similarity of chemical structure between them, which contains almost identical metal clusters and active site configuration [86]. In addition, the nonspecificity of the inhibitors becomes a challenge in studies with animals, as well as for future clinical trials, because the unspecific inhibition would not enable to define for sure to which isoform the observed effects could be attributed.

In this scenario, despite the encouraging findings concerning enzyme inhibition, the use of arginase inhibitors still claims for advances, since the pharmacological agents currently available are not isoform-specific. This fact is of particular importance, considering the differential organic distribution, concentrations, and specific actions of arginase isoforms, as stated above.

So far, different studies have already pointed to the potential of arginase inhibition, either directly or indirectly. In rats presenting with metabolic syndrome treated with arginase inhibitors (citrulline, norvaline, and ornithine), for example, it was observed amelioration in blood pressure levels directly by increase in $^{\bullet}$NO bioavailability and indirectly by inhibition of insulin resistance and hypertriglyceridaemia [88]. Also, Holowatz and Kenney demonstrated that essential hypertension in humans was associated with attenuated reflex cutaneous vasodilation and that acute inhibition of arginase improved such reflex [93]. Similarly, oral administration of atorvastatin for three months was capable of restoring cutaneous microvascular function in hypercholesterolaemic patients, with the improvement observed mediated by decreased arginase activity [94]. Taken together, these observations highlight the multiple ways through which arginase may be targeted in order to improve cardiometabolic profiles.

Table 1: Original articles approaching the arginase isoforms in different clinical and experimental studies.

Arginase actions and their responses in the vasculature					
Reference	Type of study	Study design	Type of arginase involved	Actions/results	Vascular damage
[12]	Preclinical	*In vivo* study with *postmortem* analysis in wild type C57Bl/6 and arginase II knockout male mouse (assessment of cigarette smoke effects)	Arg II		
[15]	Clinical	*In vivo* study performed in patients with coronary artery disease and type 2 diabetes mellitus	Arg I and Arg II		
[21]	Preclinical	*In vivo* study with male ApoE^{-}/$^{-}$ and C57Bl/6 mouse and *postmortem* analysis	Arg II		
[70]	Preclinical	*In vivo* model of transgenic C57Bl/6 mouse overexpressing human arginase II, with *postmortem* analysis	Arg II		
[82]	Preclinical	*In vitro* study in cell culture of isolated porcine carotid endothelium	Arg II		
[96]	Clinical	Clinical study with blood samples of patients with type 2 diabetes mellitus	Arg I and Arg II		
[97]	Preclinical	*In vitro* study using isolated mouse aortic endothelium and HUVECs cultures	Arg II		
[6]	Clinical	Human popliteal and tibial vessels from amputation specimens (*ex vivo* model)	Arg II		
[48]	Preclinical	*In vivo* and *in vitro* study using culture of human aortic endothelial cells (HAECs) and C57Bl/6 mouse aortic rings	Arg II		
[50]	Preclinical	*In vitro* study performed on rat aorta endothelial cells (AECs)	Arg I and Arg II	Reduction of $^{\bullet}$NO bioavailability	Endothelial dysfunction
[80]	Preclinical	*In vitro* study performed on HUVEC and Wistar rat aortic rings	Arg II		
[81]	Preclinical	*In vitro* study performed on culture of human aortic endothelial cells and Wistar rat aortic rings	Arg I and Arg II		
[95]	Preclinical	*In vitro* study with treatment in HUVECs and mouse aortic rings	Arg I and Arg II		
[98]	Preclinical	Study performed on HUVECs culture *in vitro* and isolated C57Bl/6 mouse aorta	Arg I and Arg II		
[69]	Preclinical	*In vitro* study using HUVECs culture and *in vivo* treatment in C57Bl/6 mice	Arg I and Arg II		
[12]	Preclinical	*In vivo* study with *postmortem* analysis in C57Bl/6 and arginase II knockout male mice (assessment of cigarette smoke effects)	Arg II		
[21]	Preclinical	*In vivo* study with male ApoE^{-}/$^{-}$ and C57Bl6 mice and *postmortem* analysis	Arg II		
[48]	Preclinical	*In vivo* and *in vitro* study using culture of human aortic endothelial cells (HAECs) and C57Bl/6 mouse aortic rings	Arg II	Uncoupling of eNOS	Increased ROS production
[69]	Preclinical	*In vitro* study using HUVECs culture and *in vivo* treatment in C57BL/6 mice	Arg I and Arg II		
[80]	Preclinical	*In vitro* study performed on HUVEC and Wistar rat aortic rings	Arg II		
[97]	Preclinical	*In vitro* study using isolated mouse aortic endothelium and HUVECs cultures	Arg II		
[95]	Preclinical	Study performed on HUVECs culture *in vitro* and isolated C57BL/6 mouse aorta	Arg I and Arg II		
[98]	Preclinical	Study performed on HUVECs culture *in vitro* and isolated C57BL/6 mouse aorta	Arg I and Arg II		
[69]	Preclinical	*In vitro* study using HUVECs culture and *in vivo* treatment in C57Bl/6 mouse	Arg I and Arg II		
[82]	Preclinical	*In vitro* study in cell culture of isolated porcine carotid endothelium	Arg II		
[83]	Preclinical	*In vitro* (VSMCs from human umbilical vein) and *in vivo* (ApoE^{-}/$^{-}$ and C57Bl/6 mouse) study	Arg II		

Arg I: arginase I; Arg II: arginase II; eNOS: endothelial nitric oxide synthase; HAECs: human aortic endothelial cells; HUVECs: human umbilical vein endothelial cells; $^{\bullet}$NO: nitric oxide; ROS: reactive oxygen species; VSMC: vascular smooth muscle cells.

7. Conclusions and Perspectives

In summary, increases in arginase expression and/or activity undoubtedly repercute in the atherosclerosis physiopathology, mainly by regulating the •NO production via eNOS, so that the upregulation of arginase favors the formation of the atherosclerotic plaque [95].

Thus, further studies are required to explain the degree with which arginase contributes in the intrinsic mechanisms of endothelial dysfunction, principally in the clinical setting. In this regard, clinical research is still limited, with few human studies that assessed the role of arginase in atherosclerosis. Indeed, by allowing the dissection of the molecular and metabolic pathways trodden by the actions of such enzyme, this investigation represents a promising field for developing new targeted therapies for different clinical conditions.

Finally, considering the amount of knowledge that has been accumulated in the last decades, pointing to the well-recognized participation of arginase in the onset and development of endothelial dysfunction and cardiovascular diseases, a myriad of fresh perspectives under the molecular point of view was opened widely right before our eyes. If in 1927, Chaudhuri would say that "*As regards the function of the enzyme, very little is as yet known except that it hydrolyses arginine into ornithine and urea*" [27], the increasing attention given to that newborn and mysterious enzyme in the following years would change that statement dramatically. The pieces of this puzzle were randomly spread on Kossel and Dakin's table, inciting the curiosity of other pioneer researchers in the study of arginase from the first half of the twentieth century. It is our work, from now on, to keep on putting them back together, once recent advances in the study of the triad arginase/endothelial dysfunction/atherosclerosis have shown that these pieces, little by little, have finally began to fit properly with each other. It is not possible to foresee what the future might hold, but it seems to be just a matter of time for us to evidence so many other hidden connections linking the paths of this journey and learn as much as possible from what they can tell us.

Conflict of Interests

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

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Research Article

Positive Relationship between Total Antioxidant Status and Chemokines Observed in Adults

Yanli Li,[1] Richard W. Browne,[2] Matthew R. Bonner,[1] Furong Deng,[3] Lili Tian,[4] and Lina Mu[1]

[1] *Department of Epidemiology and Environmental Health, School of Public Health and Health Professions, State University of New York at Buffalo, Buffalo, NY 14214, USA*
[2] *Department of Biotechnical and Clinical Laboratory Sciences, State University of New York at Buffalo, Buffalo, NY 14214, USA*
[3] *Department of Environmental Health, Peking University Health Science Center, Beijing 100191, China*
[4] *Department of Biostatistics, State University of New York at Buffalo, Buffalo, NY 14214, USA*

Correspondence should be addressed to Lina Mu; linamu@buffalo.edu

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Objective. Human evidence is limited regarding the interaction between oxidative stress biomarkers and chemokines, especially in a population of adults without overt clinical disease. The current study aims to examine the possible relationships of antioxidant and lipid peroxidation markers with several chemokines in adults. *Methods*. We assessed cross-sectional associations of total antioxidant status (TAS) and two lipid peroxidation markers malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) with a suite of serum chemokines, including CXCL-1 (GRO-α), CXCL-8 (IL-8), CXCL-10 (IP-10), CCL-2 (MCP-1), CCL-5 (RANTES), CCL-8 (MCP-2), CCL-11 (Eotaxin-1), and CCL-17 (TARC), among 104 Chinese adults without serious preexisting clinical conditions in Beijing before 2008 Olympics. *Results*. TAS showed significantly positive correlations with MCP-1 ($r = 0.15751$, $P = 0.0014$), MCP-2 ($r = 0.3721$, $P = 0.0001$), Eotaxin-1 ($r = 0.39598$, $P < 0.0001$), and TARC ($r = 0.27149$, $P = 0.0053$). The positive correlations remained unchanged after controlling for age, sex, body mass index, smoking, and alcohol drinking status. No associations were found between any of the chemokines measured in this study and MDA or TBARS. Similar patterns were observed when the analyses were limited to nonsmokers. *Conclusion*. Total antioxidant status is positively associated with several chemokines in this adult population.

1. Introduction

Oxidative stress and inflammation are hypothesized to play important roles in a wide variety of diseases, such as cardiovascular diseases, diabetes, cancer, and autoimmune diseases [1–3]. Oxidative stress occurs when antioxidants fail to provide sufficient protection against the overproduced free radicals, and it might lead to oxidative damage to lipids, proteins, and DNA [4–6]. Chronic inflammation is characterized by continuous inflammatory response and tissue destruction [7–11]. A progressive rise of oxidative stress and increased level of proinflammatory mediators appear to be one of the hallmarks of the aging process [3, 12, 13].

Oxidative stress and inflammation pathways are inseparably interconnected [14]. Oxidants activate transcription factor nuclear factor kappa B (NF-κ B) that further stimulates production of proinflammatory molecules. Conversely, inflammation causes oxidative stress, because production of free radicals is an inherent property of activated immune cells [14, 15]. The interaction between oxidative stress and inflammation might play an important role in the pathogenesis of a number of human diseases [16–19].

Inflammatory chemokines are a large family of structurally related chemoattractant cytokines that play a pivotal role in orchestrating inflammation [20]. The chemokine system has been demonstrated to be redox regulated in experimental studies [21, 22]. Epidemiological studies reported associations between oxidative stress markers and chemokines among several diseased populations, including patients with acute coronary syndromes, systemic lupus

erythematosus and rheumatoid arthritis, preeclampsia, and end-stage renal diseases undergoing hemodialysis [23–26]. However, the potential for the disease process to cause elevated expression of both chemokines and oxidative stress cannot be ruled out. To our knowledge, no human study has been conducted to examine the possible association between chemokines and oxidative stress biomarkers among adults without clinical disease. In the current study, we aim to examine the cross-sectional relationships of serum chemokines with biomarkers of total antioxidant capacity and lipid peroxidation among adults without serious preexisting clinical conditions.

2. Materials and Methods

2.1. Study Population. A total of 104 participants were recruited from Haidian District, Beijing, in July 2008. Participants were restricted to adults aged from 18 to 70 years, with no previous medical history of cancer, serious immunological diseases, chronic respiratory diseases, cardiovascular diseases, or diabetes. The study population has been described in detail previously [27]. Prior to recruitment, IRB approvals were obtained from the State University of New York at Buffalo and Peking University Health Science Center. Written inform consent was obtained from all study participants.

Trained interviewers administered standardized, structured questionnaires to participants querying information about demographics and lifestyle factors. Current height and weight at the time of interview were measured by trained nurses. Blood samples (5 mL) and first morning urine samples were collected.

2.2. Laboratory Analysis of Serum Chemokines and Oxidative Stress Markers. The collected blood samples were immediately transferred to Peking University for processing. Serum and blood clots were separated on the centrifuge and stored at −80°C freezer. All oxidative stress biomarkers were measured at the Department of Biotechnical and Clinical Laboratory Sciences, University at Buffalo. Total antioxidant status rate (TAS) in serum samples was measured using Randox total antioxidant status kit (Randox Laboratories Ltd.) adapted to the COBAS MIRA automated chemistry analyzer (Roche Diagnostic Systems). Biomarkers of oxidative damage to the lipids, including malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS), were measured in urine samples. Total MDA was measured using HPLC according to the procedure described by Giera et al. [28]. TBARS was measured using a colorimetric microplate assay (Oxford Biomedical Research). Both MDA and TBARS were expressed in nmoles/milliliter of MDA equivalents. Urinary creatinine was measured using "Creatinine-a" assay kit (Genzyme Diagnostics) on the ABX PENTRA 400 analyzer (Horiba Instruments Levine, CA, USA). Urinary MDA and TBARS measurements were normalized to the urinary creatinine concentrations to control for variations in urine output. The intra-assay coefficients of variation (CVs) for TAS, MDA, and TBARS were 2.39%, 5.00%, and 3.56%; the interassay coefficients of variation were 7.10%, 9.63%, and 6.30%, respectively.

Chemokines in serum samples were analyzed using the Q-Plex Human Chemokine ELISA-based chemiluminescent assay from Quansys Biosciences. This assay allows the concurrent measurement of chemokines CXCL-1 (GRO-α), CXCL-8 (IL-8), CXCL-10 (IP-10), CCL-2 (MCP-1), CCL-5 (RANTES), CCL-8 (MCP-2), CCL-11 (Eotaxin-1), and CCL-17 (TARC). All study samples were analyzed in triplicate in the same plate. The intra-assay CVs using the triplicate samples were all less than 10% (see Supplementary Material 1 available online at http://dx.doi.org/10.1155/2014/693680). External quality control samples collected in healthy volunteers in the USA were repeatedly measured in all the plates to assess the intra-assay and interassay reproducibility. The intra-assay CVs of the external samples were all less than 15%, except for GRO-α; the interassay CVs were less than 20%, except for GRO-α and IL-8 (Supplementary Material 1). The higher variations for GRO-α and IL-8 assays using the external quality control samples might relate to the significantly lower expression levels in the external quality control samples compared to the samples from the study population (Supplementary Material 2).

2.3. Statistical Analysis. To describe basic characteristics of the study participants, we calculated mean values and standard deviations for continuous variables and frequencies and percentages for categorical variables. We calculated medians and interquartile ranges (IQRs) for all the chemokines.

Simple Pearson correlation coefficients were calculated to examine the possible relationships between levels of serum chemokines and oxidative stress biomarkers. We used a logarithmic transformation to normalize the distributions for IP-10, Eotaxin-1, TARC, TBARS, and MDA to meet the normality assumption of this model. Partial Pearson correlation coefficients were also calculated by controlling for age, sex, BMI, smoking, and alcohol drinking status.

To examine possible nonlinear relationship and minimize distortion of the Pearson correlation coefficient due to outliers, we further categorized chemokines into tertiles and compared the mean levels (95% confidence intervals) of oxidative stress markers in each tertile of chemokines. P values for trend were calculated to assess possible trends of oxidative stress markers across the tertiles of chemokines. We also assessed whether differences in the mean concentrations of oxidative stress markers between the high and low tertiles of chemokines were statistically significant.

All statistical analyses were performed using the SAS 9.3 (SAS Institute, Cary, NC, USA). All statistical tests were two-sided and considered statistically significant at $P < 0.05$.

3. Results

Table 1 shows the characteristics of 104 participants (50 males and 54 females) included in this analysis. Average age of the participants was 48.3 years. About 60.6% of study participants were underweight or normal weight, 28.9% were overweight, and 10.6% were obese. Smokers constituted about 35.6% of the participants and about 32.7% were alcohol drinkers.

Table 1: Basic characteristics of study participants.

Variables	Distribution
	Mean (SD)
Age (years)	48.3 (9.1)
BMI (kg/m^2)	23.7 (3.4)
	N (%)
Sex	
Male	50 (48.1)
Female	54 (51.9)
Age groups	
≤40	16 (15.4)
40–50	36 (34.6)
>50	52 (50.0)
BMI categories	
Underweight and normal (<24 kg/m^2)	63 (60.6)
Overweight (24–28 kg/m^2)	30 (28.9)
Obesity (≥28 kg/m^2)	11 (10.6)
Smoking status	
Smokers	37 (35.6)
Nonsmokers	67 (64.4)
Alcohol drinking	
Drinkers	34 (32.7)
Nondrinkers	70 (67.3)
	Median (IQR)
GRO-α (pg/mL)	47.20 (25.35, 86.98)
IL-8 (pg/mL)	40.01 (19.63, 92.89)
IP-10 (pg/mL)	75.61 (58.40, 103.89)
MCP-1 (pg/mL)	121.75 (90.56, 163.27)
RANTES (ng/mL)	25.78 (18.89, 32.21)
MCP-2 (pg/mL)	38.45 (27.42, 53.90)
Eotaxin-1 (pg/mL)	146.02 (109.26, 192.29)
TARC (pg/mL)	169.02 (108.40, 243.05)

Medians and IQRs of the measured chemokines are also summarized in Table 1.

TAS showed significantly positive correlations with MCP-1 ($r = 0.15751$, $P = 0.0014$), MCP-2 ($r = 0.3721$, $P = 0.0001$), Eotaxin-1 ($r = 0.39598$, $P < 0.0001$), and TARC ($r = 0.27149$, $P = 0.0053$) (Table 2). After controlling for the effects of age, sex, BMI, smoking, and alcohol drinking status, TAS still showed significantly or borderline significantly positive correlations with MCP-1 ($r = 0.19606$, $P = 0.0518$), MCP-2 ($r = 0.30958$, $P = 0.0018$), Eotaxin-1 ($r = 0.27219$, $P = 0.007$), and TARC ($r = 0.21458$, $P = 0.0329$) (Table 2). In the categorized analyses, participants in the higher tertiles of MCP-1, RANTES, MCP-2, Eotaxin-1, and TARC showed significantly or borderline significantly higher levels of TAS (Table 3). Similar patterns were observed when the analyses were limited to nonsmokers (Supplementary Material 3).

TBARS and MDA were not associated with any of the chemokines we measured (Table 2) or in subanalyses restricted to nonsmokers (Supplementary Material 3).

4. Discussion

In this cross-sectional study conducted in adults without serious preexisting conditions, we observed significantly or borderline significantly positive correlations between TAS level and chemokines MCP-1, MCP-2, Eotaxin-1, and TARC. TBARS and MDA showed no associations with any chemokines.

Humans have evolved complex antioxidant strategies against prooxidant conditions. The antioxidant defense system has many components, and a deficiency in any of these components can cause a reduction in the overall antioxidant status of an individual. TAS, a measure of overall antioxidant capacity, describes the dynamic equilibrium between different prooxidants and antioxidants in blood [29].

The observed positive correlations between TAS and several chemokines in this study might reflect their interactions in response to xenobiotic insults. Environmental stimuli, such as smoking and air pollution, are known to induce the production of free radicals [30, 31]. Free radicals have been shown to activate redox-sensitive transcriptional factor NF-κB, which may further stimulate the production of chemokines [14, 15]. In response to overproduction of free radicals, TAS might be elevated as a compensatory response to reestablish "redox homeostasis" [32]. In the current study population, we observed higher TAS concentrations among smokers or during period with higher air pollution levels [33]. Therefore, our finding suggested possible biological interactions between chemokines and excessive formation of free radicals in response to xenobiotic insults. One previous study found that TAS was positively correlated with proinflammatory cytokines IL-1α, IL-6, and TNF-α in bronchoalveolar lavage fluid in lung cancer patients [34]. It implicated that the interactions between chemokines and TAS might further contribute to the pathogenesis of human disease.

Oxidative damages to lipids, proteins, and DNA within cells may occur when the production of free radicals exceeds the antioxidant capacity of the cell [6]. MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells and it is a widely used lipid peroxidation marker. TBARS is one commonly used marker to measure MDA; however, it lacks specificity [35]. In the current study, we did not observe any associations between chemokines and MDA or TBARS. Contrary to the null associations observed in our current study, positive associations were found between chemokines and lipid peroxidation markers among several diseased populations [23, 24, 26]. Several explanations are possible for the conflicting results. First, antioxidant defense systems for the adults in this study might be activated in response to xenobiotic insults, as we observed higher levels of TAS in smokers or when air pollution was higher. Unlike the diseased populations, the activated antioxidant protection among the current population might be sufficient to maintain the "redox homeostasis," which may prevent further oxidative damages to the lipids. Therefore, no correlations between chemokines and lipid peroxidation markers were observed in current population. Second, lack of specificity of the lipid peroxidation assays used in this study might be another explanation for the null findings. TBARS assay does not

Table 2: Simple and partial Pearson correlation coefficients between chemokines and oxidative stress biomarkers.

	TAS		TBARS		MDA	
	Simple correlation coefficients	Partial correlation coefficients[1]	Simple correlation coefficients	Partial correlation coefficients[1]	Simple correlation coefficients	Partial correlation coefficients[1]
GRO-α	−0.01974	0.02379	0.14517	0.17243	0.13731	0.15975
IL-8	−0.13489	−0.14856	−0.09451	−0.10745	−0.00719	0.01839
IP-10	0.05952	0.10126	−0.11125	−0.11266	−0.13101	−0.12015
MCP-1	0.30966**	0.19606	−0.10333	−0.10115	−0.05286	−0.06448
RANTES	0.09351	0.17431	−0.0015	0.04048	−0.02415	0.00592
MCP-2	0.3721***	0.30958**	−0.09373	−0.05383	−0.1378	−0.1185
Eotaxin-1	0.39598***	0.27219**	−0.03503	−0.03171	0.07175	0.07696
TARC	0.27149**	0.21458*	0.03133	0.0541	0.03365	0.04368

Note: 1. Adjusted for age, sex, smoking status, BMI, and alcohol drinking. 2. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Table 3: Concentrations of TAS by tertiles of chemokines.

	TAS level			Crude P for trends	Adjusted P for trends	Crude P for high versus low tertile	Adjusted P for high versus low tertile
	Low tertile	Middle tertile	High tertile				
GRO-α	0.94 (0.81, 1.06)	1.03 (0.92, 1.14)	0.94 (0.85, 1.04)	0.9753	0.9454	0.9633	0.8587
IL-8	0.99 (0.89, 1.09)	1.06 (0.94, 1.17)	0.87 (0.77, 0.97)	0.1070	0.1203	0.1022	0.1105
IP-10	0.92 (0.81, 1.03)	1.02 (0.91, 1.13)	0.98 (0.87, 1.08)	0.4363	0.1661	0.4357	0.1658
MCP-1	0.87 (0.78, 0.97)	0.95 (0.83, 1.06)	1.10 (1.00, 1.20)	**0.0021**	0.1772	**0.0022**	0.1808
RANTES	0.89 (0.78, 1.00)	1.03 (0.94, 1.13)	0.98 (0.86, 1.10)	0.2159	**0.0138**	0.1721	**0.0077**
MCP-2	0.83 (0.73, 0.94)	0.98 (0.89, 1.08)	1.10 (1.00, 1.20)	**0.0003**	**0.0058**	**0.0004**	**0.0061**
Eotaxin-1	0.81 (0.72, 0.89)	0.99 (0.90, 1.08)	1.12 (1.00, 1.24)	**<0.0001**	**0.0037**	**<0.0001**	**0.0039**
TARC	0.90 (0.80, 1.00)	0.93 (0.83, 1.03)	1.08 (0.97, 1.20)	**0.0133**	0.1038	**0.0133**	0.1091

measure MDA exclusively, since it can react to compounds other than MDA. In addition, MDA is also not generated exclusively by breakdown of lipid hydroperoxide [35, 36].

The present study, to our knowledge, is the first epidemiological study that observed positive relationships between chemokines and TAS, a marker of total antioxidant capacity, among individuals without serious preexisting conditions. These findings supported that inflammation and oxidative stress might interact with each other in response to different environmental stimuli and in the pathogenesis of different human diseases. However this study was subjected to certain limitations. First, we only examined a global marker for antioxidant capacity and two lipid peroxidation markers in this study. Future research is warranted to examine the possible correlations between chemokines with individual antioxidants and/or oxidative damage markers to DNA and protein. Second, although TBARS and MDA are the most commonly used measures for lipid peroxidation, the lack of specificity of the two assays might have limited our ability to assess the actual lipid peroxidation status. Third, GRO-α and IL-8 demonstrated large interassay CVs using the external quality control samples; however, this might relate to the significantly lower expression levels of GRO-α and IL-8 in the external quality control samples compared to our study samples. Lastly, we did not control for uric acid, an important contributor to serum total antioxidant capacity, when evaluating the relationship between TAS and chemokines. Uric acid is a product of purine nucleotide metabolism that has antioxidant [37] as well as prooxidant [38] capacity. In plasma, as much as half of the total antioxidant capacity as measured by total radical scavenging assays like the assay employed in this study has been attributed to uric acid which acts as a scavenger of singlet oxygen, peroxyl radicals, and hydroxyl radicals [38]. Paradoxically in prooxidant environments, particularly intracellular, uric acid has been shown to form free radicals in a variety of radical-forming systems [39]. The prooxidant potential of uric acid does support considerable literature on the epidemiology of uric acid as a risk factor for disease including cardiovascular disease, hypertension, and metabolic syndrome among others [40–48]. The antioxidant-prooxidant paradox has been thoroughly reviewed previously [38]. Here we acknowledge the lack of normalization or adjustment of the plasma TAS levels by uric acid as a limitation of the study design.

5. Conclusions

In this cross-sectional study, we observed serum TAS was positively correlated with chemokines MCP-1, MCP-2, Eotaxin-1, and TARC and this implicated possible interaction between chemokines and oxidative stress biomarkers among this group of adults without serious preexisting clinical conditions.

Abbreviations

TAS: Total antioxidant status
MDA: Malondialdehyde
TBARS: Thiobarbituric acid-reactive substances
95% CI: 95% confidence interval
IQR: Interquartile ranges
CV: Coefficients of variation
ICC: Intraclass correlation coefficient
BMI: Body mass index
NF-κ B: Nuclear factor kappa B.

Conflict of Interests

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

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Review Article

Oxidative Stress-Mediated Aging during the Fetal and Perinatal Periods

Lucia Marseglia,[1] Gabriella D'Angelo,[1] Sara Manti,[2] Teresa Arrigo,[2] Ignazio Barberi,[1] Russel J. Reiter,[3] and Eloisa Gitto[1]

[1] *Neonatal and Pediatric Intensive Care Unit, Department of Pediatrics, University of Messina, Via Consolare Valeria 1, 98125 Messina, Italy*
[2] *Unit of Pediatric Genetics and Immunology, Department of Pediatrics, University of Messina, Via Consolare Valeria 1, 98125 Messina, Italy*
[3] *Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX 40729, USA*

Correspondence should be addressed to Lucia Marseglia; lmarseglia@unime.it

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Oxidative stress is worldwide recognized as a fundamental component of the aging, a process that begins before birth. There is a critical balance between free radical generation and antioxidant defenses. Oxidative stress is caused by an imbalance between the production of free radicals and the ability of antioxidant system to detoxify them. Oxidative stress can occur early in pregnancy and continue in the postnatal period; this damage is implicated in the pathophysiology of pregnancy-related disorders, including recurrent pregnancy loss, preeclampsia and preterm premature rupture of membranes. Moreover, diseases of the neonatal period such as bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis, and periventricular leukomalacia are related to free radical damage. The specific contribution of oxidative stress to the pathogenesis and progression of these neonatal diseases is only partially understood. This review summarizes what is known about the role of oxidative stress in pregnancy and in the pathogenesis of common disorders of the newborn, as a component of the early aging process.

1. Introduction

Aging is an inevitable natural phenomenon mainly characterized by increased oxidative stress (OS), elevated inflammatory responses, accelerated cellular senescence, and progressive organ dysfunction that lead to a gradual decline in the physical and mental faculties of individuals. In aging, these homeostatic imbalances significantly alter cellular responses to injury [1]. Identification of factors that regulate aging is limited by the complexity of the process and by its considerable heterogeneity among individuals. At the cellular level, the most prominent event in an aging tissue is cell senescence, a consequence of exposure to intrinsic and extrinsic aging factors, characterized by gradual accumulation of DNA damage and epigenetic changes in DNA structure that modulate correct gene expression leading to altered cell function [2].

Aging is a multifactorial process that is determined by genetic and environmental factors. The genotype determines the variation in lifespan among species or individuals. Many aging disorders, including atherosclerosis, diabetes, and hypertension, result from years of impact of a combination of environmental assaults and genetic susceptibility [3]. Although there has been a great deal of focus on the genes that determine aging, the nongenetic regulation of aging is gaining increased attention. Specifically, aging is associated with profound epigenetic changes, resulting in alterations of gene expression and disturbances in broad genome architecture and the epigenomic landscape [4]. Overall, more than 300 theories have been proposed to explain the aging process [5], but none has yet been generally accepted by gerontologists. However, the free radical theory of aging seems to be the one receiving the widest acceptance as a plausible explanation of the primary cell biological changes that are basis of the aging process [6]. Partially in contrast with the OS related theory of aging evidence has emerged in recent years where reactive

oxygen species (ROS) may actually work as essential and potentially lifespan-promoting, signaling molecules which transduce signals from the mitochondrial compartment to other organelles of the cell [7, 8]. Low-level oxidative damage eventually culminates in increased stress resistance and ultimately in longevity. This reflects an adaptive response commonly defined as hormesis [9] and better named mitochondrial hormesis or mitohormesis, which refers to ROS-related stress emanating from the mitochondria [10, 11].

Regarding the free radical theory of aging, OS, which is enhanced by smoking and bad dietary habits, is recognized as a fundamental underlying component of the aging process. The changes result in hyperactivity of progrowth factors, accumulation of toxic molecular aggregates, and activation of death/survival pathways which culminate into cellular apoptosis or necrosis [1]. OS and proinflammatory processes are strongly related [12, 13]. Upon activation, many immune cells generate free radicals (FR) and, equally, overproduction of partially reduced oxygen species induces an inflammatory response.

Since OS occurs precociously before conception, it can be stated that the aging processes begin before birth [14]. OS clearly occurs early in pregnancy and continues in the postnatal period. This molecular damage contributes to the pathophysiology of many disorders during pregnancy, at childbirth, and during the neonatal period. Pregnancy, in fact, is a state associated with enhanced OS related to high metabolic turnover and elevated tissue oxygen requirements [15]. During intrauterine life, many factors such as hypoxia, inflammation, and infections can induce overproduction of FR [16]. Whenever produced, these toxic species can induce OS and tissue injury. The placenta represents an important source of oxidatively damaged lipid peroxides, because of its high polyunsaturated fatty acid content [17]. Circulating levels of peroxidation markers, such as lipid hydroperoxide and malondialdehyde, are higher in pregnant than in nonpregnant women [18] and these products are elevated in the second trimester and decrease after delivery [19].

OS occurs when the production of toxic FR exceeds the capacity of defensive mechanisms to neutralize them. There is normally a critical balance between FR generation and antioxidant defenses. FR reactions lead to the oxidation of lipids, proteins, and polysaccharides and to DNA fragmentation, base modifications, and strand breaks; as a result, radicals have a wide range of biologically destructive effects [20, 21]. The placenta is the exchange organ between the mother and the developing fetus. Adequate functioning of this organ is clearly essential for a proper progress of gestation and the development of a healthy offspring as final outcome. As with every developing organ, the placenta has an adaptive capacity to cope with variations in maternofetal conditions, with short and long-lasting responses resulting in potential morphostructural and functional changes [22].

Placental function and fetal development are dependent on oxygen availability and limiting ROS generation. In fact, overproduction of ROS causes altered placental remodeling and abnormal fetal development and growth. The mechanisms involved not only impact fetal life, but also may mediate long-lasting effects in the newborn [23, 24]. The programming effects on the fetoplacental unit due to intrauterine stress and the potential mechanisms are currently under intensive research. The proposed main modulating mechanisms are epigenetic, such as DNA methylation and histone deacetylation [25].

Newborns, especially if preterm, are particularly vulnerable to OS because they exhibit accelerated production of FR and limited antioxidant protection, which increases the susceptibility of rapidly growing tissues to damage [26, 27]. These "FR-related diseases" of neonates promote cellular, tissue, and organ damage. Oxygen therapy, often required in the neonatal period, exaggerates OS since oxygen is a proven source of FR. In 1988, Saugstad coined the phrase "oxygen radical disease in neonatology" to highlight the crucial role of OS in a wide range of neonatal disorders [28].

This review summarizes what is known about the role of OS in healthy and complicated pregnancies and in the pathogenesis of common diseases of the newborn, as a component of the early aging process.

2. Oxidative Stress and Pregnancy-Related Disorders

Pregnancy is a state of metabolic challenge to be met by the mother and the developing fetus and, even under normal conditions, it is associated with an elevated levels of OS compared to that what occurs during the nonpregnant state [18, 19]. Due to its high metabolic rate and level of elevated mitochondrial activity, the placenta is a key source of this OS [29, 30]. Intrauterine OS during pregnancy is a physiologic response to fetoplacental energy demands [31]. Placental tissues contain low concentrations and activities of antioxidant enzymes during the first trimester, and so, trophoblastic cells are particularly susceptible to oxygen-mediated damage [32]. During the first trimester the oxygen tension within the intervillous space is about 20 mmHg [33], and the placental tissues display low activities of the principal antioxidant enzymes including catalase, glutathione peroxidase, and Cu/Zn and Mn superoxide dismutase [32]. Concentrations are particularly low in the syncytiotrophoblast, and so this tissue is especially vulnerable to OS. Thus, when the oxygen tension raises threefold in the intervillous space with the onset of maternal arterial flow at the start of the second trimester, a burst of OS is observed in the placenta. This oxidative injury could alter placental remodeling and function, affecting the subsequent course of gestation [34]. The generation of FR is an intrinsic result of aerobic energetics, but the process is well balanced in healthy pregnancy by enzymatic and nonenzymatic antioxidant redox systems [35]. The ability of placental antioxidant defenses to reduce the effects of highly reactive and potentially damaging FR is critical for healthy placental function and optimal growth and development of the fetus. Occasionally, however, the developing fetus may be exposed to high levels of OS due to overproduction of FR and a reduced antioxidant defense capability [33].

Abnormal placentation in the first trimester leads to OS and the resultant endothelial dysfunction plays a key role in the emergence of complications of pregnancy, such as recurrent abortions and preeclampsia [32]. OS, in fact, has emerged

as a likely promoter of several pregnancy-related disorders including preeclampsia, fetal growth restriction (FGR), recurrent pregnancy loss (RPL), preterm birth (PTB), and preterm prelabor rupture of the membranes (pPROM) [36].

Preeclampsia is a serious multisystem syndrome and a major cause of maternal, fetal, and neonatal morbidity and mortality that complicates approximately 5% of all pregnancies and 10% of all first pregnancies. This condition is associated with elevated oxidatively damage molecules in the maternal circulation. Placental OS resulting from the ischemia-reperfusion injury is involved in the pathogenesis of preeclampsia [37]. A significant rise in lipid peroxidation levels in the placenta of preeclamptic women has been demonstrated [38]. Excess iron levels and a decreased unsaturated iron binding capacity are factors associated with OS and contribute in the pathogenesis of preeclampsia/eclampsia. Negi et al. [39] demonstrated a significant elevation in the levels of 8-hydroxydeoxyguanosine, protein carbonyls, nitrite and iron along with reduced levels of catalase, vitamins A, E, and C, and total antioxidant status in the umbilical cord blood of preeclamptic and eclamptic pregnancies. Also, the production of the antioxidant melatonin is significantly depressed in the placenta of preeclamptic women [40]. These parameters might be influential variables for the risk of FR damage in infants born to preeclamptic/eclamptic pregnancies [39].

It has been demonstrated that OS is associated with FGR; in fact, this condition is often complicated by intrauterine hypoxia and impaired blood flow to the fetus [41]. A chronic restriction in uterine blood flow elicits placental and fetal responses in the form of growth adaptation to hypoxia. Intrauterine hypoxia may induce FR generation and, therefore, fetal OS [42, 43].

OS also plays a critical role in the etiopathogenesis of RPL. Safronova et al. [44] found an elevated production of toxic oxygen species in the granulocytes of patients with a history of RPL versus control subjects with normal reproductive function. Increased lipid peroxidation and reduced antioxidant levels in women with a history of abortions were reported by Şimşek et al. [45], supporting the concept that OS plays a central role in the etiopathogenesis of RPL. Inflammatory immune responses also are a key factor in reproductive failures, such as in multiple implantation failures, early pregnancy loss, and RPL, because cellular immune responses, especially those mediated by natural killer and T cells, are often dysregulated in these conditions [46].

OS is a postulated etiology of spontaneous PTB and pPROM, although the precise mechanistic role of FR in these complications is unclear. Animal studies have shown that decidual senescence can lead to PTB by activating p53, a proapoptotic factor, and inflammatory cytokines [47, 48]. Recent biomolecular and histologic data on pPROM and PTB suggest that increased ROS and oxidative damage to lipids and DNA in fetoplacental cells play an important pathophysiological role in these disorders [49, 50].

Argüelles et al. [51] measured OS markers including protein carbonyl groups, lipid peroxides, and total antioxidant capacity and found a good correlation between the oxidative state of the normal mother and the neonate, with a high level of maternal OS corresponding to an even higher OS in newborn umbilical cord blood.

Recently, the use of antioxidant therapies has attracted much attention. Vitamin C, an antioxidant which reacts with superoxide and lipid hydroperoxide radicals, has been studied, but data from large randomized controlled trials do not support the routine supplementation of pregnant women with high doses of the vitamins C or E [52, 53]. The reasons are unclear. First, it is possible that although OS plays a major role in the pathophysiology of preeclampsia, it is not the only causal pathway of the disorder. On the other hand, OS could be relevant to the pathogenesis of preeclampsia in only a subgroup of women, with no appreciable benefit of vitamins C and E for the entire population [54]. In addition, vitamin C and E, although considered antioxidants, can act as conditional prooxidants increasing OS paradoxically. Prooxidative reactions can be greatly enhanced in the presence of transition metal ions [55]. In the presence of redox-cycling oxidizing agents such as Fe, Cu, or Cr, antioxidant protection might take on a prooxidative form because of Fenton-like chemistry [56].

Encouraging results have been reported, however, after the administration of the antioxidant melatonin [57, 58], in both pregnant women and newborns [59]. Melatonin has a great capacity to scavenge radicals and reduce oxidative damage in the placenta [60] by increasing antioxidative enzymes and decreasing lipid peroxidation [61]. It neutralizes many more toxic reactants than vitamins C or E [58, 62] and acts as an indirect antioxidant by stimulating antioxidative enzymes [63]. Moreover, melatonin synthesis occurs in the placenta and the villous trophoblasts contain the classic transmembrane receptors for the indole, MT1, and MT2 [64]. To document this, villous cytotrophoblasts were isolated from human term placentas after vaginal delivery, demonstrating that the villous cytotrophoblasts and the syncytiotrophoblasts from the human placenta also contain the two enzymes, serotonin N-acetyltransferase and N-acetylserotonin methyltransferase, which metabolize serotonin to melatonin [64]. Locally generated melatonin functions in the protection of the placenta from OS employing both receptor-dependent and receptor-independent processes. Furthermore, this hormone reduces the loss of villous cytotrophoblasts by preventing apoptosis of these cells [65]. Also, when human villous trophoblast cells from term placentas of uncomplicated pregnancies were isolated and treated with melatonin, the placental trophoblast was protected against hypoxia-reperfusion-induced OS and apoptosis [66, 67]. Owing to its strong antioxidant capacity and its ability to cross the placenta and blood-brain barrier [68, 69], melatonin is of particular interest as a candidate for antenatal neuroprotectant [70]. In the human and animal models, melatonin treatment before and during transient severe fetal asphyxia reduces oxidative damage to the fetal brain [71], reduces lipid peroxidation [72] and cell death, and stabilizes the blood-brain barrier [73, 74].

The relationship between the oxidative status of the mother and the newborn at the moment of birth has been investigated. During the transition from fetal to neonatal life at birth, the fetus is transferred from an intrauterine hypoxic environment with an oxygen tension (PO_2) of 20–25 mmHg

to an extrauterine normoxic environment with a PO_2 of 100 mmHg. This increase induces an elevated production of ROS as well as reactive nitrogen species (RNS) [75]. Oxygen, vital to survival, is highly damaging to fetal tissues which are known to be poorly equipped to neutralise its toxic derivatives. Although, the resuscitation of newborn infants was traditionally performed with pure oxygen [76, 77], it is currently recognized that OS is elevated when resuscitation is performed with 100% oxygen [78]. The American Heart Association guidelines focused on the optimal management of oxygen during neonatal resuscitation, emphasizing that either insufficient or excessive oxygenation can be harmful to the newborn infant [79].

3. Oxidative Stress and Neonatal Diseases

Increased levels of OS and reduced antioxidant capacities may contribute to the pathogenesis of disorders in the perinatal period and excessive formation of ROS may be related to morbidity. Newborns, especially when born prematurely, are very susceptible to FR-mediated oxidative damage for several reasons: (a) infants at birth are naturally exposed to the hyperoxic challenge due to the transition from the hypoxic intrauterine environment to extrauterine life, and this difference is even more significant for newborns who require supplemental oxygen during resuscitation in the delivery room; (b) infants are more susceptible to infection, especially if born prematurely; (c) they have reduced antioxidant defense processes; (d) they possess high levels of free iron that enhances the Fenton reaction causing the production of the highly toxic hydroxyl radical [80, 81].

The contribution of OS to the pathogenesis and progression of neonatal diseases is only partially understood, and the statement "oxygen radical disease in neonatology" has been proposed to underline the key role of OS in a wide range of neonatal morbidities [28] including hypoxic ischemic encephalopathy (HIE) [20], intraventricular hemorrhage (IVH) [82], periventricular leukomalacia [83], bronchopulmonary dysplasia (BPD), chronic lung disease (CLD) [84], retinopathy of prematurity (ROP) [85], and necrotizing enterocolitis (NEC) [86]. Subsequently, it became clear that FR influences the ductus arteriosus and pulmonary circulation [87, 88]. If the concept of "oxygen radical diseases of neonatology" is valid, it might mean that the abovementioned conditions are not different disease entities but are simply different organ manifestations of the same complex processes of OS and metabolism.

The fetal and neonatal brain are particularly vulnerable to the effects of oxygen and nitrogen-based FR. OS is implicated in the pathogenesis of many neurological diseases such as IVH [89], HIE [90], and epilepsy [91]. The high concentration of unsaturated fatty acids in the neonatal brain predisposes to the propagation of OS. The propensity of the preterm brain to respond to the dangerous effects of OS relates not only to the deficient antioxidant defenses, but also to several prooxidant characteristics; in fact, developing neural tissues have a high metabolic rate supported almost exclusively by oxidative metabolism, which is an important source of FR. The degree of damage depends on the region of the brain that is affected, the severity of the injury, and the stage of development. The morbidity and mortality of infants are strongly affected by their inability to maintain physiologic homeostasis and to counteract the effects of toxic oxygen derivatives. When an inflammatory response is initiated during a perinatal brain injury, it is associated with the induction of a variety of cytokines, including TNF-α, IL-1, and IL-6 [92, 93]. A similar cytokine response has been found to be involved in several neurodegenerative diseases that typically affect humans in old age [94–96].

Elevated OS and/or reduced endogenous antioxidant defenses may also play a role in the pathogenesis of a number of inflammatory pulmonary diseases including respiratory distress syndrome (RDS) in the newborn [97, 98]. Oxygen, which is obviously vital to survival, can be highly damaging to tissues such as neonatal lungs, which are known to be poorly equipped to neutralize oxygen's toxic derivatives. ROS and RNS induce ultrastructural changes in the cytoplasm of pulmonary capillary endothelial cells and cause focal hypertrophy thereby altering metabolic activity. ROS also have been implicated in the molecular damage seen in the bronchoalveolar lavage (BAL) fluid of patients with RDS [99, 100]. In fact, hydrogen peroxide is detected in the expired air of RDS patients, and myeloperoxidase and oxidized-1-antitrypsin have been found in BAL fluid. CLD or BDP is a general term for long-term respiratory problems in premature babies. The underling mechanisms of BPD are not clearly defined, although OS in ventilated infants with immature lungs is crucial component of the injury process that leads to CLD [101]. Barotrauma, volutrauma, and oxygen toxicity during mechanical ventilation are assumed to be important factors in the pathogenesis of CLD as they cause pulmonary damage, resulting in a release of multiple proinflammatory cytokines, including IL-6, IL-8, TNF-α, and the production of extracellular matrix components and growth factors [102, 103]. Balinotti et al. [104] also found that surface area for diffusion in the lung increased during the first 2 years of life and is proportional to alveolar volume. In BPD patients, an impaired alveolar growth, decreased pulmonary diffusion capacity, and susceptibility to develop chronic obstructive pulmonary disease phenotype with aging have been reported. Premature senescence might be a common physiological response to pulmonary OS in children and adults [105].

Yet another neonatal disease in which the OS plays a critical role is ROP, a proliferative condition of the retinal vasculature in premature infants that may cause severe visual loss; it is more common in premature infants exposed to high concentrations of oxygen as it causes generation of FR. Hypoxia as well is also known to be involved in the genesis and progression of ROP [106, 107]. In animal studies, intermittent hypoxia induced a more severe form of ROP than equally distributed hypoxemic episodes [108]. Di Fiore et al. [106] demonstrated that an increase in the prevalence of hypoxemic episodes during the first 8 weeks of life in preterm newborns is correlated to severe ROP requiring laser therapy. The pathologic blood vessel proliferation causing blindness in proliferative retinopathy of premature infants is also the main cause of visual impairment in diabetic retinopathy, one

of the most common complications of diabetes in working age adults [109].

NEC is a gastrointestinal tract disorder of premature neonates that results in inflammation and bacterial invasion of the bowel wall. Many etiologic factors including immaturity, hypoxia/ischemia, hyperosmolar feedings, bacterial colonization, and OS have been identified [110]. Perrone et al. [111] measured nonprotein bound iron and total hydroperoxides in cord blood and showed that the determination of OS biomarkers was useful in identifying babies at high risk for NEC.

4. Conclusions

FR, normally produced in living organisms, are highly reactive molecules containing one or more unpaired electrons. If they are produced in excess, they are important mediators of cell and tissue injury [112], causing or accelerating senescence processes. Physiological pregnancies and even more so complicated pregnancies represent a condition of increased exposure to FR. Furthermore, we emphasize the strong correlation between the oxidative status of the normal mother and the neonate, showing that a high maternal OS level corresponds to even higher OS in the newborn, with accelerated cellular senescence. Infants have limited protective mechanisms against OS, which contributes to maternal and neonatal disorders [37, 80, 113]. The critical perinatal susceptibility to OS indicates that prophylactic use of antioxidants could help to prevent or at least reduce OS-related diseases in pregnancy and in newborns. Recently, the use of the antioxidant melatonin has been proposed to protect fetus and newborn against perinatal OS [59]; this molecule also has a very high safety profile [114] further supporting its use.

In conclusion, the scientific evidence concerning OS in development of pregnancy complication and several diseases of the newborn confirm that aging process starts at the time of conception.

Conflict of Interests

The authors report no conflict of interests.

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Research Article

Oxidative Damage to Nucleic Acids and Benzo(a)pyrene-7,8-diol-9,10-epoxide-DNA Adducts and Chromosomal Aberration in Children with Psoriasis Repeatedly Exposed to Crude Coal Tar Ointment and UV Radiation

Lenka Borska,[1] Ctirad Andrys,[2] Jan Krejsek,[2] Vladimir Palicka,[3] Marcela Chmelarova,[3] Kvetoslava Hamakova,[4] Jan Kremlacek,[1] and Zdenek Fiala[5]

[1] *Institute of Pathological Physiology, Faculty of Medicine in Hradec Kralove, Charles University in Prague, 50038 Hradec Kralove, Czech Republic*
[2] *Institute of Clinical Immunology and Allergology, Faculty of Medicine in Hradec Kralove, Charles University in Prague, Simkova 870, 50038 Hradec Kralove, Czech Republic*
[3] *Institute of Clinical Biochemistry and Diagnosis, Faculty of Medicine in Hradec Kralove, Charles University in Prague, 50038 Hradec Kralove, Czech Republic*
[4] *Clinic of Dermal and Venereal Diseases, University Hospital Hradec Kralove, Novy Hradec Kralove, 500 05 Hradec Kralove, Czech Republic*
[5] *Institute of Hygiene and Preventive Medicine, Charles University in Prague, Faculty of Medicine in Hradec Kralove, 50038 Hradec Kralove, Czech Republic*

Correspondence should be addressed to Lenka Borska; borka@lfhk.cuni.cz

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The paper presents a prospective cohort study. Observed group was formed of children with plaque psoriasis ($n = 19$) treated by Goeckerman therapy (GT). The study describes adverse (side) effects associated with application of GT (combined exposure of 3% crude coal tar ointment and UV radiation). After GT we found significantly increased markers of oxidative stress (8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanosine, and 8-hydroxyguanine), significantly increased levels of benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) DNA adducts (BPDE-DNA), and significantly increased levels of total number of chromosomal aberrations in peripheral lymphocytes. We found significant relationship between (1) time of UV exposure and total number of aberrated cells and (2) daily topical application of 3% crude coal tar ointment (% of body surface) and level of BPDE-DNA adducts. The findings indicated increased hazard of oxidative stress and genotoxic effects related to the treatment. However, it must be noted that the oxidized guanine species and BPDE-DNA adducts also reflect individual variations in metabolic enzyme activity (different extent of bioactivation of benzo[a]pyrene to BPDE) and overall efficiency of DNA/RNA repair system. The study confirmed good effectiveness of the GT (significantly decreased PASI score).

1. Introduction

Oxidative stress, characterized by a high degree of reactive oxygen species (ROS), is known to induce damage to cellular components including strand breaks and base modifications in nucleic acids [1]. Oxidative damage to nucleic acids has been found to be associated with a variety of diseases including cancer and aging, but the precise mechanisms are still remaining to be elucidated [2, 3].

Psoriasis is a multifactorial relapsing and remitting inflammatory skin disease. The prevalence of psoriasis ranged from 1.4% to 3.3% in North America and Europe [4]. The disease represents about 4% of all dermatoses which originally manifested at 16 years of age or younger, often as

plaque psoriasis [5, 6]. Psoriasis can adversely affect quality of life of children [7].

Goeckerman therapy (GT) represents effective treatment of plaque psoriasis in children [8, 9]. GT is based on daily dermal application of pharmaceutical grade crude coal tar (CCT) ointment with subsequent whole body exposure to UV radiation (UVR). Duration of the treatment is usually 2-3 weeks [10]. Retrospective study demonstrated that GT is effective in children and adolescents with moderate to severe psoriasis [9].

The use of GT has recently decreased for several reasons, including suspected mutagenicity/carcinogenicity of CCT [11–13]. The mutagenicity/carcinogenicity of CCT is probably caused by the presence of polycyclic aromatic hydrocarbons (PAHs). Typical representative of mutagenic/carcinogenic PAHs, benzo[a]pyrene (BaP), is metabolized into a highly reactive metabolite benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) that is able to bind to the structure of DNA, RNA, and proteins [14, 15]. The complex of reactive metabolite and DNA structure is known as a DNA adduct. In the case of BPDE it is a BPDE-DNA adduct [16]. An alternative PAHs carcinogenic pathway includes the induction of oxidative stress caused by redox cycling between PAHs dihydrodiols and PAH-o-chinons with subsequent formation of various oxidized forms of nucleobases and nucleosides [17, 18].

Children are more susceptible to the harm effects of environmental exposure and medical treatments than adults as their organism is developing [19, 20]. Recent trends in childhood cancers in the USA and Europe seem to confirm children's increased exposures to genotoxic substances [21].

Elevated levels of biomarkers confirm deleterious effects of carcinogenic PAHs on human health and underline the importance of biomarker analysis as a preventive measure in children. Suitable biomarkers include, among others, PAH-DNA adducts, markers of oxidative stress, and chromosomal aberration [19].

Presented study used biomarkers of oxidative stress and genotoxicity for description of adverse (side) effects associated with application of GT (combined exposure of 3% coal tar ointment and UV radiation) in children. Mentioned effects can damage cells, accelerate aging, induce diseases, and shorten life expectancy.

2. Materials and Methods

2.1. Study Group. The group was formed of children with chronic stable plaque psoriasis, treated by GT at the Clinic of Dermal and Venereal Diseases, University Hospital, Hradec Kralove (Czech Republic). Over the period of two years, we collected data of 19 children. The group consisted of 12 girls and 7 boys (average age of 12 years, age range 5–17 years). Exposure history of the patients was checked by the questionnaire. Regardless of the young age of respondents, we also assessed their smoking habits. Those patients who had significant prior exposure to PAHs and/or artificial UVR were excluded from the monitored group. The study was approved by the Ethics Committee of the University Hospital in Hradec Kralove, Czech Republic. Informed written consent was obtained from parents of each patient.

Table 1: Detailed characteristic of the treatment.

Number of patients	Total duration of GT (days)	Time of UVR exposure (days)	Daily topical application of CCT ointment (% of body surface)
1	22	18	32
2	17	10	19
3	18	13	37
4	22	22	38
5	22	19	31
6	18	15	20
7	14	9	40
8	14	10	28
9	17	17	31
10	14	13	40
11	19	15	28
12	21	21	37
13	22	22	37
14	14	9	30
15	14	10	17
16	17	17	38
17	22	23	28
18	17	17	36
19	18	18	28

2.2. Goeckerman Therapy (GT). Within the therapy, dermatological ointment containing 3% of CCT was administered daily overnight on psoriatic lesions. According to the extent of lesions, 17–40% of the total body surface was covered by CCT ointment. Each morning the residues of CCT ointment were removed from the body (using oil bath) and the patients were whole-body irradiated by UVR.

UVR exposure was dosed according to skin condition and in cases of adverse reactions has been temporarily suspended. For this reason, the total duration of GT (days) may not be equivalent to the time of UVR exposure (days). Irradiation time was individual according to the disease status (1–15 min/day). The density of used radiation was 249.75 μW/cm^2 of UV-B and 131.8 μW/cm^2 of UV-A (controlled by Sola-Scope 2000 spectrometer; Solatell, UK). Duration of the treatment was modified according to the treatment effectiveness (average duration of 18 days; range of 14–22 days). Detailed individual characteristic of GT is summarized in Table 1. The effectiveness of the therapy was calculated from basic characteristics of actual disease status (erythema, desquamation, and skin infiltration) and expressed as the PASI score (Psoriasis Area and Severity Index) [22]. Calculation of the efficiency of the treatment was performed according the formula (100 − [PASI after GT/PASI before GT] × 100).

Table 2: The content of selected PAHs in pharmaceutical grade crude coal tar (CCT).

PAHs	IARC[a]	mg/g	PAHs	IARC[a]	mg/g
Benzo(a)pyrene	1	0.008	Acenaphthene	3	0.104
Dibenz(a,h)anthracene	2A	0	Anthracene	3	2.494
Benz(a)anthracene	2B	0	Benzo(g,h,i)perylene	3	0
Benzo(b)fluoranthene	2B	0	Fluoranthene	3	0.413
Benzo(k)fluoranthene	2B	0	Fluorene	3	0.299
Chrysene	2B	0.028	Phenanthrene	3	2.520
Indeno(1,2,3-c,d)pyrene	2B	0	Pyrene	3	0.241
Naphthalene	2B	3.286	Acenaphthylene	—	0.153

[a] Classification according to WHO (IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, Volumes 1–109, 2014):
Group 1: the agent is carcinogenic to humans;
Group 2A: the agent is probably carcinogenic to humans;
Group 2B: the agent is possibly carcinogenic to humans;
Group 3: the agent is not classifiable as to its carcinogenicity to humans.

2.3. Selected PAHs in Pharmaceutical Grade Crude Coal Tar (CCT). The pooled sample of CCT (0.1973 g) was sonicated (30 min) with 25 mL of n-hexane and subsequently was diluted fifty times. Instrumental analysis of the extract was performed by gas-chromatography-mass detection: GC Agilent HP 6890/MS HP 5975; column HP-5 ms, Agilent, 30 m/0.25 mm/0.25 μm.

2.4. Oxidative Damage of DNA and RNA. Samples of blood were collected from the cubital vein a day before the first treatment and immediately after the last procedure of GT (BD Vacutainer sampling tubes). Blood serum was isolated by centrifugation and stored under −70°C until analysis. Repeated thawing and freezing were avoided. The extent of DNA/RNA oxidative damage was evaluated by EIA Kit (Enzyme Immunoassay, Cayman Chemical Company, Michigan, USA). Oxidative damage of DNA and RNA was presented as the sum of three oxidized guanine species in serum: 8-hydroxy-2′-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. The level of DNA/RNA oxidative damage was expressed in picograms of all guanine species per milliliter of serum with detection limit of 33 pg of species/mL of serum.

2.5. Benzo[a]pyrene-7,8-diol-9,10-epoxide-DNA Adducts (BPDE-DNA Adducts). EDTA-treated peripheral blood samples were collected a day before the first treatment and immediately after the last procedure of GT. DNA isolation from lymphocytes was performed and samples were subsequently diluted to a concentration of 2 μg of DNA in 1 mL. The level of BPDE-DNA adducts was determined using the standard method OxiSelect BPDE-DNA Adduct ELISA Kit (Cell Biolabs, Inc., San Diego, USA). The results were expressed as nanograms of BPDE-DNA adducts per microgram of DNA with detection limit of 3.9 ng BPDE-DNA/μg of DNA.

2.6. Chromosomal Aberration in Peripheral Lymphocytes. Heparin-treated peripheral blood samples were collected a day before the first treatment and immediately after the last procedure of GT and the level of chromosomal aberration in peripheral lymphocytes was evaluated by standardized method [23]. The method is based on microscopic analysis of lymphocytic chromosomes undergoing mitotic metaphase. In each blood sample, 100 of mitotic sets were evaluated. We counted structurally aberrated cells (SAC), numerically aberrated cells (NAC), and total number of aberrated cells (ABC).

2.7. Statistical Analysis. Obtained data were analyzed by using MATLAB rel. 2013b software (MathWorks, Inc., Massachusetts, USA). Because the Lilliefors test of normality had rejected the hypothesis of normal distribution, the data were analyzed by the Wilcoxon signed rank test. The possible associations between monitored biomarkers and important factors of exposure were evaluated by using Spearman Rank Order Correlations.

3. Results

For analysis of selected PAHs, we used a pooled sample of CCT (from five different samples of CCT collected in different time of the study). The analyses were done for 16 selected PAHs reported by the US EPA standards (US Environmental Protection Agency). The results are listed in Table 2. The total content of 16 selected PAHs was 9.546 mg/g CCT and used dermatological ointment contains 3% of CCT. This means that 100 g of 3% dermatological ointment contained altogether 28.6 mg of 16 selected PAHs. As resulted from analysis of CCT (Table 2), one PAH is sorted into the group of proven carcinogens, one PAH in the group of probably carcinogens, and six PAHs into the group of possibly carcinogens. Remaining PAHs are registered by IARC; however they are not classifiable as to their carcinogenicity to humans.

Table 3 summarizes the results of biomonitoring of oxidative stress and genotoxic effects associated with the application of GT in observed group. After GT (3% of CCT) we found significantly increased serum levels of oxidative damage of DNA and RNA ($P < 0.05$), BPDE-DNA adducts ($P < 0.01$), and total number of aberrated cells ($P < 0.001$). Increased levels of biomarkers are clearly visible in

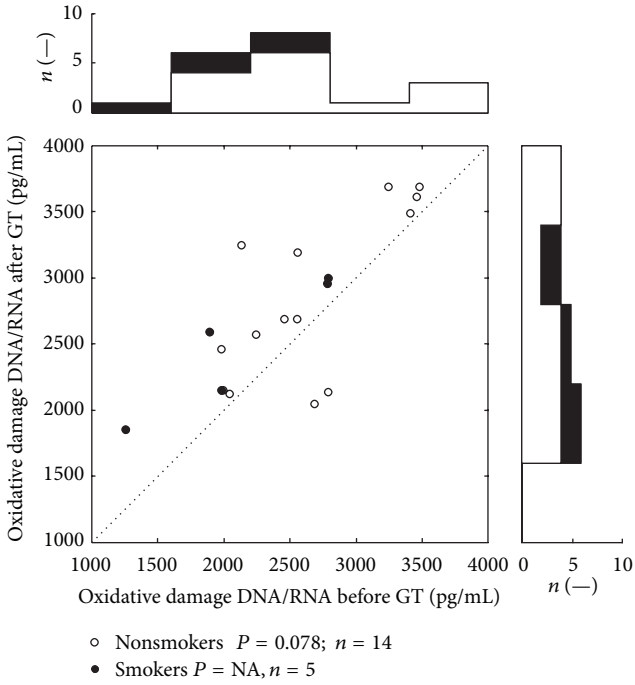

Figure 1: Distribution of values of oxidative damage of DNA and RNA. The oxidative damage of DNA/RNA is presented as the sum of three oxidized guanine species per mL of serum. Scatter plot depicts anti-BPDE-DNA values before and after the GT therapy. All together 19 dots represent 38 measurements; each dot belongs to a one patient. The top histogram shows data distribution before treatment; the right side histogram corresponds to the posttreatment values distribution. The white zones represent nonsmokers data, and the black zones depict smokers' ones. NA: statistically not available.

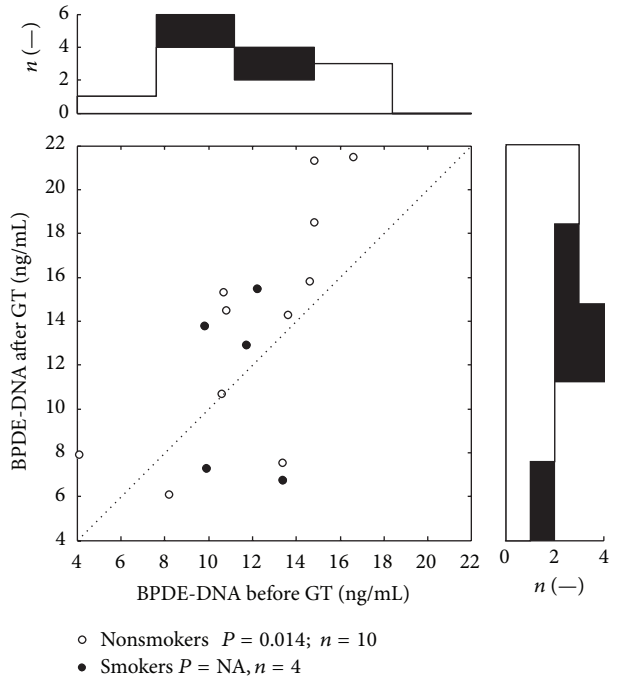

Figure 2: Distribution of values of BPDE-DNA adducts. The levels are expressed as ng of BPDE-DNA adducts per μg of DNA. Scatter plot depicts BPDE-DNA values before and after the GT therapy. All together 14 dots represent 28 measurements; each dot belongs to a one patient. The top histogram shows data distribution before treatment; the right side histogram corresponds to the posttreatment values distribution. The white zones represent nonsmokers data, and the black zones depict smokers' ones. NA: statistically not available.

the attached scatter plots (Figures 1, 2, and 3) which depicts distribution of values of oxidative damage of DNA and RNA, BPDE-DNA adducts, and chromosomal aberration in peripheral lymphocytes (total number of aberrated cells) before and after the GT. Each dot belongs to at least one patient (number of patients belonging to one dot is depicted in histograms). Values above the dotted diagonal line reflect the increase of the oxidative and genotoxic damage after the therapy. Probability of difference between the pre- and the posttreatment medians was evaluated by Wilcoxon signed rank test. The top histogram shows distribution of followed parameters before treatment; the right side histogram corresponds to the posttreatment values distribution. On these histograms the development of changes in the distribution of values can be well observed.

The effectiveness of GT was high and the values of PASI score significantly decreased after GT ($P < 0.001$). Before the therapy we found 20.4/12.8–22.3 (median/lower-upper quartile of PASI) and after the therapy 9.4/5.3–10.2 (median/lower-upper quartile of PASI). Figure 4 depicts the PASI score with a similar layout as Figures 1–3. The values representing positive treatment effect are below the diagonal dotted line. The Wilcoxon's test for smokers for such small group did not have sufficient power to detect differences between pre- and posttreatment values and therefore P is reported as not available (NA).

Table 4 presents the analysis of potential associations between monitored biomarkers (values after GT) and important characteristics of exposure. We found significant relationship between (1) time of UV exposure and total number of aberrated cells ($P < 0.05$), (2) daily topical application of CCT ointment (% of body surface) and BPDE-DNA adducts ($P < 0.05$), and (3) daily topical application of CCT ointment (% of body surface) and PASI score ($P < 0.01$).

Cigarette smoking was reported by 5 patients. Number of smoked cigarettes was overall low and irregular and ranged from one to four cigarettes per "ordinary" day (during hospitalization they almost did not smoke at all). Group of respondents who admitted smoking was not statistically evaluable because of low number of the patients (4-5). For the reasons above, the statistical evaluations in Tables 3 and 4 were performed for the whole group of respondents and the values of potential smokers were marked only in Figures 1–4.

4. Discussion

The carcinogenicity of individual PAHs and PAHs containing mixtures has been studied in experimental animals. Virtually no data exist on the carcinogenicity of individual PAHs in humans, and only a limited amount of data on

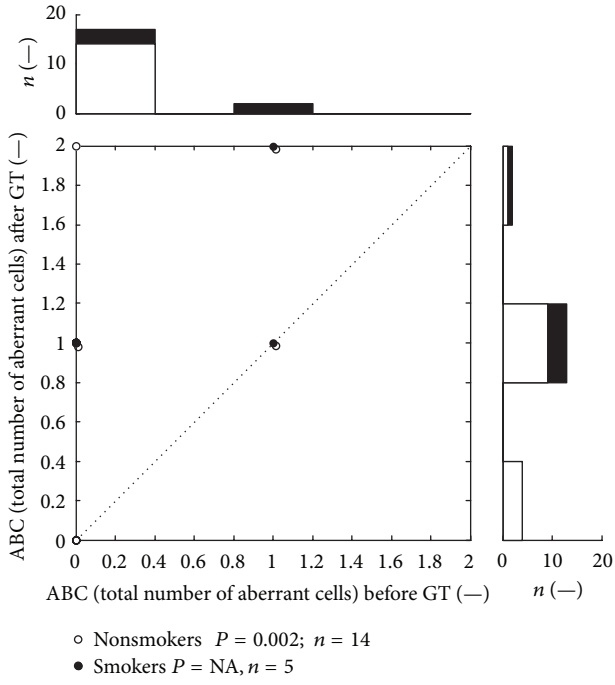

Figure 3: Distribution of values of chromosomal aberration in peripheral lymphocytes (total number of aberrated cells). The levels are expressed as percentage of total number of aberrated cells (ABC). Scatter plot depicts ABC values before and after the GT therapy. All together 5 dots represent 19 subjects and 38 measurements; number of patients belonging to one dot is shown in histograms. The top histogram shows data distribution before treatment; the right side histogram corresponds to the posttreatment values distribution. The white zones represent nonsmokers data, and the black zones depict smokers' ones. NA: statistically not available.

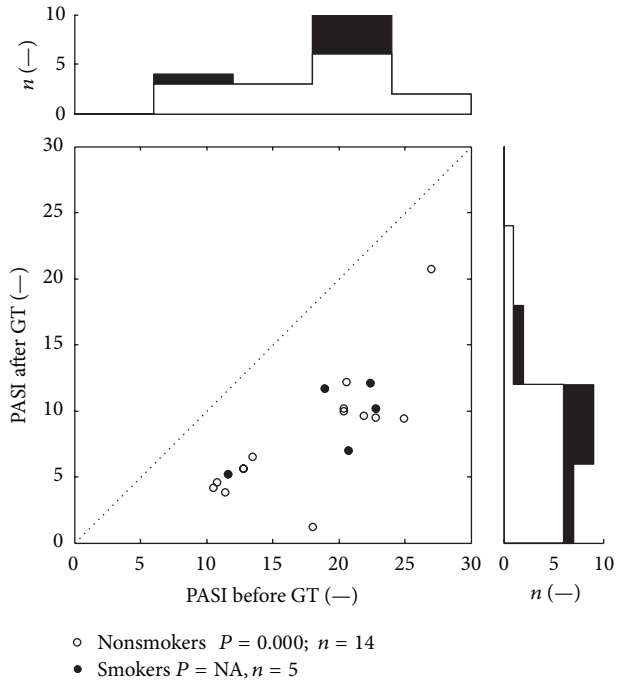

Figure 4: Distribution of PASI score. The levels are dimensionless. Scatter plot depicts PASI score before and after the GT therapy. All together 18 dots represent 38 measurements; each dot belongs at least to a one patient. The top histogram shows data distribution before treatment; the right side histogram corresponds to the posttreatment values distribution. The white zones represent nonsmokers data, and the black zones depict smokers' ones. NA: statistically not available.

the carcinogenicity of PAHs containing mixtures is available for humans. There is evidence that a number of individual PAHs are carcinogenic in experimental animals, while others have been found to be noncarcinogenic. Because of the varying carcinogenic potency of individual PAHs, it is not possible to provide a single weight of evidence carcinogenicity assessment for PAHs as a class. As it was shown in Table 2, the carcinogenicity of each PAHs should be assessed separately [24].

While it is not possible to provide a carcinogenicity assessment for PAHs as a class, it may be possible to evaluate the carcinogenicity of PAHs containing complex mixtures. There is good evidence that some PAHs containing mixtures are carcinogenic to humans (e.g., bitumen, carbon black, coal tars, creosote, and soot). Coal tars are classified according to IARC (International Agency for Research on Cancer) in group 1: the agent is carcinogenic to humans [24].

As can be seen from the results the amount of 100 g of 3% CCT ointment contains 0.024 mg of proved carcinogens and 9.942 mg of possible carcinogens. The average amount of 3% CCT ointment applied to treated skin was approximately 4 mg/cm^2. Extent of psoriatic lesions ranged approximately from 10 to 80% of the body surface and, thus, corresponding total dose of applied CCT ointment ranged from units to tens of grams. The total amount of carcinogenic PAHs applied to the skin of given patient (and related hazard) can be assumed on the base of these data. As an example we can describe the hazards of some patient with a body surface area of about 14.000 cm^2 and with the 50% of skin affected by psoriatic lesions (7.000 cm^2). Then the theoretical total daily dose of 16 selected PAHs will be 8.0 mg and of carcinogenic PAHs 2.7 mg (PAHs from the group of 16). However, although the group of 16 selected PAHs is toxicologically significant and representative it should be noted that the group of selected 16 PAHs represents only a small fraction of the total content of PAHs in the CCT.

It must be noted that the mixture of UV-A and UV-B radiation (part of GT) is also ranked by IARC into Group 1 with definition: ultraviolet radiation, wavelengths 100–400 nm, encompassing UVA, UVB, and UVC. It is evident that both components of the therapy (CCT and UVR) present a relatively high carcinogenic potential. Moreover, in situation of combined exposure to PAHs and UV radiation even their synergistic effects on generation of ROS, lipid peroxidation, and DNA damage can be expected [25, 26].

PAHs may exert their mutagenic, genotoxic, and carcinogenic properties by two major mechanisms. One pathway includes induction of oxidative stress [17, 18, 27]; second pathway includes formation of specific PAHs-DNA adducts [16, 28, 29].

Table 3: Oxidative damage of DNA/RNA, BPDE-DNA adducts, and chromosomal aberrations in children treated by GT.

Oxidative damage of DNA and RNA[a]		
Before GT ($n = 19$)	After GT ($n = 19$)	Significance of differences
2557 (2008–2789)	2687 (2146–3238)	$P < 0.05$
BPDE-DNA adducts[b]		
Before GT ($n = 14$)	After GT ($n = 14$)	Significance of differences
5.65 (4.97–7.3)	7.2 (5.35–7.9)	$P < 0.01$
Chromosomal aberration in peripheral lymphocytes[c]		
Before GT ($n = 19$)	After GT ($n = 19$)	Significance of differences
Structurally aberrated cells		
0.0 (0.0-0.0)	1.0 (0.0–1.0)	$P < 0.01$
Numerically aberrated cells		
0.0 (0.0-0.0)	0.0 (0.0–1.0)	NS
Total number of aberrated cells		
0.0 (0.0-0.0)	1.0 (0.0–1.0)	$P < 0.001$

[a] Oxidative damage of DNA and RNA is presented as the sum of three oxidized guanine species in serum: 8-hydroxy-2′-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. The levels are expressed as pg of the sum of oxidized guanine species per mL of serum. The average concentration is presented as median and lower-upper quartile because of nonnormal data distribution; *P*: Wilcoxon matched-pairs test.
[b] The levels are expressed as ng of BPDE-DNA adducts per μg of DNA and the average concentration is presented as median and lower-upper quartile because of nonnormal data distribution; *P*: Wilcoxon matched-pairs test.
[c] The levels are expressed as percentage of structurally aberrated cells (SAC), numerically aberrated cells (NAC), and total number of aberrated cells (ABC). The average levels of aberration are presented as median and lower-upper quartile because of nonnormal data distribution; *P*: Wilcoxon matched-pairs test; NS: nonsignificant difference.

Table 4: The associations between monitored biomarkers (levels after GT) and important characteristics of exposure (Spearman Rank Order Correlations).

	Total duration of GT (days)	Time of UVR exposure (days)	Daily topical application of CCT ointment (% of body surface)
Oxidative damage of DNA and RNA[a]	NS	NS	NS
BPDE-DNA adducts[b]	NS	NS	$r = 0.62$ $P < 0.05$
Total number of aberrated cells[c]	NS	$r = 0.55$ $P < 0.05$	NS
PASI score[d]	NS	NS	$r = 0.65$ $P < 0.01$

[a] Unit: pg of the sum of three oxidized guanine species per ml of serum.
[b] Unit: ng of BPDE-DNA adducts per μg of DNA.
[c] Unit: percentage of total number of aberrated cells (ABC).
[d] Dimensionless.

Oxidative stress represents a deregulation of the homeostasis between the reactive oxygen species and the mechanisms of detoxification and repair [27]. The currently available epidemiological and laboratory data indicate that oxidative stress plays a central role in carcinogenic potential effect of PAHs [30]. PAHs can be metabolized by the cytochrome P450 enzymes to active semiquinones, which are known free radicals intermediates and can go through redox cycling (between PAHs dihydrodiols and PAH-o-chinons) and generate reactive oxygen species (ROS) [28]. Subsequently, the ROS can then cause oxidative modification of DNA and lipids in the body [30]. Oxidative damage to nucleic acids has been

found to be associated with a variety of diseases, including cancer and aging. Guanine is the base that is the most prone to oxidation. The 8-hydroxy-2′-deoxyguanosine (from DNA) is the form of oxidized guanine that is the most commonly studied critical biomarker of oxidative DNA damage in adults and children exposed to PAHs [31–33].

In recent years, it has become increasingly clear that both DNA and RNA are damaged by oxidation in disease states and that the repair processes that are initiated to correct this damage release multiple oxidized guanine residues, including the ribose free base (8-oxo-guanine or 8-hydroxyguanine), the nucleoside from RNA (8-oxo-guanosine or 8-hydroxyguanosine), and the deoxynucleoside from DNA (8-oxo-deoxyguanosine or 8-hydroxy-2′-deoxyguanosine) [2, 18]. These residues are released into the body fluid, including cerebrospinal fluid, plasma/serum, and urine under the condition of oxidative stress [1, 2].

While 8-hydroxy-2′-deoxyguanosine is the form most researches are familiar with, other published studies have reported that the RNA residue 8-hydroxyguanosine is a better biomarker of age-related oxidant damage [17] and that the base (8-hydroxyguanine) is a better biomarker in some cancer patients [3, 27, 34]. From the reasons described above it is recommended to analyze more than one oxidation (damage) product, since if just one damage product is measured, there is a risk that the specific damage (product) may not be a good marker of the oxidative stress, and thus not give a true picture of the amount of oxidative stress the subject in question is exposed to [35].

It was shown in Table 3 that the level of oxidative damage of DNA and RNA was significantly increased after the therapy ($P < 0.05$). This exposure scenario indicates (in given group of children) increased hazard of oxidative stress related to the treatment. However, it must be noted that although higher level of oxidized guanine residues indicates elevated level of oxidative stress, it can also reflect a high level of efficiency of the processes that work to repair this damage (oxidative stress can be high and the repair processes eliminate its effects). Therefore the interpretation of this bioindicator for the purpose of health risk assessment requires a certain degree of caution.

In the presented study the oxidative damage of DNA and RNA was presented as the sum of three oxidized guanine species in serum: 8-hydroxy-2′-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA (pg/mL of serum). To the best of our knowledge the state of oxidative stress (expressed as different serum oxidized guanine species) in children dermally exposed to CCT and UVR has not been yet reported. Previously published studies were focused mostly on relationship between inhalation exposure to PAHs and different guanine species in urine (not in serum). For example the study of Rossnerova et al. presented inhalation exposure to benzo[a]pyrene and related urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine in children living in highly polluted area. The level of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine of these children ranged from 5.96 to 6.73 nmol/mmol creatinine [32]. Other authors described impact of inhalation exposure of children to PAHs (traffic pollution; polluted versus nonpolluted area) on urinary 8-hydroxy-2′-deoxyguanosine [31]. They did not find significant difference for urinary 8-hydroxy-2′-deoxyguanosine concentration between the groups from polluted and nonpolluted areas (20.87 versus 16.78 μmol/mol creatinine). It can be seen that urinary 8-hydroxy-2′-deoxyguanosine concentration was only slightly higher in the group from polluted area. The authors assume that the potential of coexposure of the children to other pollutants affects 8-hydroxy-2′-deoxyguanosine concentration besides the PAHs. However, as stated previously, it cannot be excluded that the used oxidation product (8-hydroxy-2′-deoxyguanosine) was not the most suitable marker of the oxidative stress and thus did not give a real picture of the amount of oxidative stress [35]. On the other hand a clear link between heavy occupational exposure to PAHs (coke oven workers) and the levels of urinary 8-hydroxydeoxyguanosine was found in the work of Kuang et al. [30]. The levels of urinary 8-hydroxydeoxyguanosine ranged from 81.7 (control) to 115.7 (heavily exposed group) nmol/mmol creatinine.

Benzo(a)pyrene is notable for being the first chemical carcinogen to be discovered. BaP is a five-ring PAH known to be procarcinogen. Its mechanism of carcinogenesis is dependent on a three-step enzymatic metabolism to final mutagen. The steps include generation of benzo(a)pyrene-7,8-epoxide, benzo(a)pyrene-7,8-diol, and finally benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE). Very reactive BPDE binds covalently to proteins, lipids, and DNA (guanine residues) to produce BPDE-DNA adducts. If left unrepaired, DNA adducts may lead to permanent mutations resulting in cell transformation and ultimately to tumor development [15]. Biological monitoring includes indicators of exposure, biological effect, and susceptibility. BPDE-DNA adducts can be indicative of both exposure and genotoxic effect of BaP. The level of BPDE-DNA adducts is determined by the character of BaP metabolism (metabolic enzyme activities) and by the degree of reparation mechanisms.

It was shown in Table 3 that the level of BPDE-DNA adducts was significantly increased after the therapy (5.65 versus 7.2 ng BPDE-DNA adducts/μg DNA; $P < 0.01$) and it indicates increased hazard of genotoxic effects related to the treatment. However, similar to the scenario with oxidative stress, it must be noted that the final level of BPDE-DNA adducts reflects combined effect of intensity of exposure to BaP, metabolic activity (production of BPDE-DNA adducts), and efficiency of DNA/RNA repair mechanisms. The worst scenario should include high level of exposure, high production of BPDE-DNA adducts (high metabolic activity of responsible CYP enzymes), and limited activity of repair system. This indicates (analogously to the biomarkers of oxidative stress) that the interpretation of the level of BPDE-DNA adducts for purposes of health risk assessment requires a certain degree of caution.

The number of epidemiological studies related to BPDE-DNA adducts in children is limited [15, 29, 36]. In addition, these studies are different in used methods, units, and analyzed biological materials. Thus, the comparison of results is a little bit complicated. For example, Pauk et al. compared the levels of total DNA adducts in lymphocytes between different groups of respondents [15]. These groups

included healthy persons, patients with chronic obstructive pulmonary disease (noncancerous lung disease which occurs mainly due to smoking and other long-term respiratory exposure to chemical substances), and patients with lung cancer. The level of total DNA adducts was determined using P^{32}-postlabeling method and ranged between 0.710 and 1.329 A.U. (arbitrary units). The patients with diseases largely associated with smoking (PAHs) showed increased levels of total DNA adducts in comparison to nonsmoking patients. The influence of tobacco smoke (PAHs) on the level of total DNA adducts and B(a)P-like adducts in newborns and mothers was studied by Topinka et al. [36]. PAH-DNA adducts and B(a)P-like adducts were determined using P^{32}-postlabeling method. In subjects unexposed to tobacco smoke, the level of total DNA adducts in lymphocytes ranged from 1.06 (newborns) to 1.13 (mothers) and the level of B(a)P-like adducts ranged from 0.16 (newborns) to 0.19 (mothers) DNA adducts/108 nucleotides. In subjects exposed to tobacco smoke the level of total DNA adducts in lymphocytes ranged from 0.92 (newborns) to 1.18 (mothers) and the level of B(a)P-like adducts ranged from 0.15 (newborns) to 0.22 (mothers) DNA adducts/108 nucleotides.

Genotoxic exposures occurring during childhood may continue for several years, become chronic, and eventually play a relevant role in the etiology of childhood as well as adulthood cancers. Chromosomal aberrations in human peripheral lymphocytes represent well-established biomarker of genotoxicity, probably the only one which has been internationally standardized and validated for children [19, 21, 37].

In our study we found significantly increased levels of ABC ($P < 0.01$) and SAC ($P < 0.05$) after GT and these findings support assumption of elevated genotoxic hazard (Table 3). Before GT we found only two aberrations (ABC) in the total number of 1900 cells (0.10%). One aberration was classified as a structural abnormality (SAC) and one cell as a numerical abnormality (NAC). After GT we found seventeen aberrations (ABC) in the total number of 1900 cells (0.80%). Eleven aberrations were classified as structural abnormalities (SAC) and six as numerical abnormalities (NAC). Declared reference value for the total number of aberrated cells (ABC) of healthy Czech children ranges from 0 to 1.85%. This is a range of values for a group of children ($n = 20$) [23, 38]. From the above it is evident that treatment increased the level of aberrated cells in lymphocytes (genotoxic hazard). Under this condition we can consider that a similar situation of elevated genotoxic hazard was obvious also in other tissues/organs that are well perfused with blood. In conclusion, it must be emphasized that the level of abnormalities which was found after the therapy (0.80%) is still below the upper level of the reference range for healthy Czech children (1.85%; $P < 0.01$). This means that although there was an increase of genotoxic risk, this risk is within the acceptable values.

The results of the present study agree well with the results of our previous work [8]. In that work, we studied a group of children treated with GT using 5% coal tar ointment. After the treatment, there were found significantly increased levels of aberrations (ABC; median 2.0%; lower-upper quartile 2.0-3.0%). As it can be seen, the median value of ABC in the case of foregoing study (5% of CCT) was greater than median value presented in this paper (3% CCT) and exceeded the upper level of the reference range for healthy Czech children (1.85%). The results from both studies suggest possible dose dependency.

Nowadays, it is generally accepted that the high frequency of cytogenetic biomarkers, chromosomal aberrations, can predict an increased risk of genotoxic effect in children after exposure to PAHs. Exposure situations can be described either by simply increasing the levels from initial state (if known) or by comparison with the reference value(s). It is known that cytogenetic biomarkers may vary with gender and age in children (adults). The age extent of children in our group ranged from 5 to 17 years and the group includes boys and girls. It may therefore be the question whether this age range and gender differences can lead to some shifts of reference values of chromosomal aberrations. Merlo et al. analyzed data from 16 published epidemiologic studies performed on pediatric population and from a large sample of Czech children aged 7–16 years. For the whole referent population (age range 0–19 years) the mean frequency of chromosomal aberration was 1.24 (95% CI = 1.05–1.47). Similar baseline levels were found for chromosome breaks frequency in boys and girls: 1.22% and 1.21%, respectively. In conclusion, based on the reviewed studies, baseline levels for chromosomal aberrations were similar in boys and girls and failed to show any increase with age [37].

While it appears that most of genotoxic effects of GT can be attributed to PAHs, it is likely that UVR, as integral part of GT, increases the risk for mutagenicity and carcinogenicity. UVR is known to increase the toxicity of PAHs through photoactivation and photomodification [39]. Skin damage caused by exposure to BaP is further increased by exposure to both UV-A and UV-B radiation. The effects related to UV-A radiation seem to be more significant [40]. It has been shown that BaP, in combination with UV-A radiation, synergistically induced oxidative DNA damage. These facts indicate that UV-A radiation is able to transform BaP into more harmful compounds [39]. Another research demonstrates that BaP in combination with UV-A radiation can substantially increase oxidative damage of DNA via a ROS mechanism (primarily represented as elevated levels of 8-hydroxy-2′-deoxyguanosine) [16, 25, 41]. In the presented work (Table 4) we found significant relationship between time of UV exposure and total number of aberrated cells ($P < 0.05$).

The effectiveness of the therapy was expressed as the PASI score. In the presented study, the PASI score was significantly decreased after GT ($P < 0.001$) and confirmed generally assumed high effectiveness of the GT for children [8, 9]. We found significant relationship between the PASI score and daily topical application of CCT ointment (% of body surface).

5. Conclusion

We found significantly increased markers of oxidative stress (8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanosine,

and 8-hydroxyguanine), significantly increased levels of BPDE-DNA adducts, and significantly increased levels of total number of chromosomal aberration in peripheral lymphocytes of children with psoriasis treated by GT. These findings can indicate increased hazard of oxidative stress and genotoxic effects related to the treatment. However, it must be acknowledged that the oxidized guanine species and BPDE-DNA adducts also reflect the individual variations in metabolic enzyme activity (different extent of bioactivation of BaP to BPDE) as well as the overall efficiency of DNA/RNA repair system. The study confirmed good clinical effect of the GT (significantly decreased PASI score).

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Deletion of Metallothionein Exacerbates Intermittent Hypoxia-Induced Oxidative and Inflammatory Injury in Aorta

Shanshan Zhou,[1,2] Yonggang Wang,[1,2] Yi Tan,[2,3] Xiaohong Cai,[4] Lu Cai,[2,5] Jun Cai,[2] and Yang Zheng[1]

[1] *The Center of Cardiovascular Diseases at the First Hospital of Jilin University, 71 Xinmin Street, Changchun 130021, China*
[2] *Kosair Children's Hospital Research Institute at the Department of Pediatrics, University of Louisville, 570 South Preston Street, Baxter I, Suite 321B, Louisville, KY 40202, USA*
[3] *Chinese-American Research Institute for Diabetic Complication, Wenzhou Medical College, Wenzhou 325035, China*
[4] *Department of Pediatrics, The Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University, Wenzhou 325027, China*
[5] *Departments of Radiation Oncology and Pharmacology and Toxicology, University of Louisville, Louisville, KY 40202, USA*

Correspondence should be addressed to Jun Cai; j0cai002@louisville.edu and Yang Zheng; zhengyang@jlu.edu.cn

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The present study was to explore the effect of metallothionein (MT) on intermittent hypoxia (IH) induced aortic pathogenic changes. Markers of oxidative damages, inflammation, and vascular remodeling were observed by immunohistochemical staining after 3 days and 1, 3, and 8 weeks after IH exposures. Endogenous MT was induced after 3 days of IH but was significantly decreased after 8 weeks of IH. Compared with the wild-type mice, MT knock-out mice exhibited earlier and more severe pathogenic changes of oxidative damages, inflammatory responses, and cellular apoptosis, as indicated by the significant accumulation of collagen, increased levels of connective tissue growth factor, transforming growth factor β1, tumor necrosis factor-alpha, vascular cell adhesion molecule 1,3-nitrotyrosine, and 4-hydroxy-2-nonenal in the aorta. These findings suggested that chronic IH may lead to aortic damages characterized by oxidative stress and inflammation, and MT may play a pivotal role in the above pathogenesis process.

1. Introduction

Obstructive sleep apnea (OSA) has been recognized as a common respiratory disorder with the estimated prevalence of 3–7% in the general populations [1, 2]. OSA is characterized by recurrent episodes of partial or complete collapse of the upper airway during sleep, resulting in repetitive apneas or hypopneas. These obstructive respiratory events can cause episodes of hypoxia and reoxygenation, which are known as nocturnal intermittent hypoxia (IH) [3–5]. Epidemiological studies have revealed independent associations between OSA and coronary [6–9] and cerebral vascular diseases [9–11]. Because chronic IH (CIH) is a prominent feature of OSA [12], there has been a great interest to understand how CIH exposures cause pathological changes of artery [13–17]. Although results of some experimental studies did not support that CIH exposures cause atherosclerosis in mice [13] even after long-term (8.3 months) exposures [18], there is evidence that CIH may induce preatherosclerotic artery damages. Increased carotid intima-media thickness (IMT) has been observed in patients with OSA [19]. There were also studies that revealed independent associations between the hypoxic stress and increased carotid artery IMT [20] in OSA that can be reversed by continuous positive airway pressure therapy (CPAP) [9].

Oxidative stress, endothelial dysfunction, and inflammation have been considered to be key pathophysiologic processes that mediate cardiovascular injury in patients with

OSA [5]. Oxidative stress is defined as an imbalance between the excessive production of reactive oxygen or nitrogen species (ROS or RNS) and the reduced antioxidant capacity. ROS and RNS exerted their cellular harmful effects, which can be counteracted by a variety of specific antioxidants [21]. The plasma concentration of an oxidative marker malondialdehyde (MDA) was found to be higher in patients with OSA than in control subjects [22], suggesting a possible role of oxidative stress in the pathogenesis of OSA. It is also known that inflammation and oxidative stress are reciprocal causes and outcomes [23], and oxidative stress is associated with vascular inflammation. Impaired endothelial function has been accepted as a major pathologic process involved in IH-induced vascular alteration, which mainly may result from IH-induced inflammatory response [24]. Indeed, OSA patients had increased plasma levels of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6, as well as adhesion molecules for leukocyte recruitment, including intercellular adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [25, 26]. Proinflammatory cytokines and oxidative stress lead to endothelial dysfunction [27] and to formation of fatty streaks, the early stage of atherosclerosis [28]. Inflammatory molecules influence each other in a complex cascade while forming an atheroma [29].

Metallothionein (MT) is a family of cysteine-rich, low molecular weight proteins that could bind to both physiological (such as zinc and copper) and xenobiotic heavy metals through the thiol group of its cysteine residues, which represents about 30% of its amino acid content [30]. Experimental data suggested that MT exerts cellular protection effects not only against metal toxicity, but also against a variety of oxidative stimuli [31, 32]. Our group has shown protective effects of MT in animal models of diabetes [32, 33]. Based on the results of these studies, it seems reasonable to assume that endogenous MT in aorta may also be protective against IH-induced endothelial damages. However, it should be noted that the potential molecular mechanisms in response to oxidative stress caused by CIH in aorta are different from those in diabetes. The CIH mainly induces hydrogen peroxide [34], while in models of diabetes or doxorubicin induced injury, superoxides were predominant mediators that interact with NO to form peroxynitrite [32, 35, 36]. Therefore, whether MT plays a pivotal role in CIH induced aortic damage cannot be simply extrapolated and has to be investigated experimentally. Therefore, using a mouse model of IH to mimic hypoxia-reoxygenation events that occur in OSA patients, we investigated aortic oxidative damage and inflammatory responses in a time-dependent manner during this process, particularly focusing on the potential role of MT.

2. Materials and Methods

2.1. Animals. MT-KO and WT 129S1 mice were purchased from Jackson Labs. In MT-KO mice (stock number: 002211), both MT1 and MT2 genes were simultaneously disrupted using a vector that inserted in-frame stop codons into the exons of the two genes. Mutant alleles are transcribed but not translated. Six mice were initially used in each group. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care.

2.2. IH Exposures. The murine model of IH exposures during sleep was used in this study as previously reported [37, 38]. Briefly, adult mice were exposed to an IH profile designed to produce similar nadir hemoglobin oxygen saturations (50~60%) and apnea/hypopnea index (AHI: 21–50 times/hour) as observed in moderate to severe OSA patients. The IH paradigm consisted of alternating cycles as 20.9% O_2/8% O_2 FiO_2 (30 episodes per hour) with 20 seconds at the nadir FiO_2 during the 12 hr light phase. After IH exposures, mice were transferred to room air and sacrificed for tissue collection.

2.3. Aorta Preparation and Histopathological Examination. After anesthesia, thoraxes were opened and the descending thoracic aortas were isolated carefully without rips or cuts. Aortic tissues were fixed in 10% buffered formalin overnight. The fixed tissues were cut into ringed segments (approx. 2-3 mm in length) for being dehydrated in graded alcohol series, cleaned with xylene, embedded in paraffin, and sectioned at 5 μm thickness for pathological and immunohistochemical staining.

Histological evaluation of aorta was performed after H&E staining with Image Pro Plus 6.0 software for measuring the tunica media width size as the thickness of aortic tunica media. For immunohistochemical staining, paraffin sections from aortic tissues were dewaxed and incubated with 1X Target Retrieval Solution (Dako, Carpinteria, CA) in a microwave oven for 15 min at 98°C for antigen retrieval, followed by 3% hydrogen peroxide for 10 min at room temperature and 5% animal serum for 60 min, respectively. These sections were then separately incubated with primary antibodies against connective tissue growth factor (CTGF) at 1 : 100 dilution (BD Biosciences, San Jose, CA), transforming growth factor (TGF-β1) at 1 : 100 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA), tumor necrosis factor-alpha (TNF-α) at 1 : 50 dilution (Abcam, Cambridge, MA), vascular cell adhesion molecule 1 (VCAM-1) at 1 : 100 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 3-nitrotyrosine (3-NT) at 1 : 400 dilution (Millipore, Billerica, CA), 4-hydroxy-2-nonenal (4-HNE) at 1 : 400 dilution (Alpha Diagnostic International, San Antonio, TX), and metallothionein (MT) at 1 : 100 dilution (Dako Inc, Carpinteria, CA) overnight at 4°C. After the sections were washed with PBS, they were incubated with horseradish peroxidase conjugated secondary antibodies (1 : 100–400 dilutions with PBS) for 1 h at room temperature. For color development purposes, immunohistochemical staining sections were treated with peroxidase substrate DAB kit (Vector Laboratories, Inc. Burlingame, CA) and counterstained with hematoxylin to localize the nucleus.

The quantitative analyses of these immunohistochemical staining were achieved from 6 mice of each group. 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 staining density in area of interest to an integrated optical density (IOD), and the ratio of IOD/area 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 Sirius-red staining of collagen, as described in our previous study [39]. Briefly, sections were stained with 0.1% Sirius-red F3BA and 0.25% Fast Green FCF. The stained sections were then assessed for the presence of collagen using a Nikon Eclipse E600 microscopy system.

2.5. Terminal Deoxynucleotidyl-Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Staining. TUNEL staining was performed with formalin-fixed, paraffin-embedded sections using Peroxidase *in situ* Apoptosis Detection Kit S7100 (Millipore, Billerica, MA), according to the manufacturer's instructions. The positively stained apoptotic cells were counted randomly in five microscopic fields at least in each of the three slides from each mouse under light microscopy. The percentage of TUNEL positive cells relative to 100 nuclei was presented.

2.6. Quantitative Real-Time PCR (qRT-PCR). Aortas were frozen with liquid nitrogen and stored 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 (Promega, Madison, WI, USA). Reverse transcription was run in a Master cycler gradient (Eppendorf, Hamburg, Germany) at 42°C for 50 min and 95°C for 5 min with 0.5 μg of total RNA in a final volume of 20 μL that contained 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 dT primer, and nuclease-free water. Primers of CTGF, TGF-β, TNF-α, and VCAM were purchased from Applied Biosystems (Carlsbad, CA, USA). The qPCR was carried out in a 20 μL solution including 10 μL of TaqMan universal PCR master mix, 1 μL of primer, and 9 μL of cDNA with the ABI 7300 Real-Time PCR system. Data were expressed as fold increase compared with levels measured in controls by using the $\Delta\Delta$Ct method and β-actin as a reference gene.

2.7. Statistical Analysis. Data were presented as mean ± standard deviation (SD, $n = 6$). One-way ANOVA was used to detect the differences between groups, followed by repetitive comparing Tukey's test with Origin 7.5 Lab data analysis and graphing software. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. MT-KO Mice Exhibited Earlier and More Severe IH-Induced Aortic Pathological Changes and Fibrosis. At the end of experiment, aortas were examined pathologically by H&E staining, which displayed significantly increased tunica media thickness in wild-type mice after exposure to IH for 8 weeks as compared with the room air controls (Figure 1(a)). Sirius-red staining also revealed an increased collagen accumulation in tunica media of aortas by IH exposures for 8 weeks (Figure 1(b)). Moreover, all these pathological changes were observed as early as 3 weeks and exacerbated at 8 weeks after IH exposures in the aorta of MT-KO mice (Figure 1). To further detect the effect of MT on IH-induced aortic fibrosis, immunohistochemical staining and qRT-PCR for both protein and mRNA levels of profibrotic mediators, CTGF (Figure 2(a)) and TGF-β1 (Figure 2(b)), were measured. Compared to wild-type mice, aortic CTGF and TGF-β1 levels in MT-KO mice were significantly increased after exposures to IH only for 3 weeks, and the differences of CTGF and TGF-β1 levels between the two groups were even more remarkable after 8 weeks of IH (Figures 2(a) and 2(b)).

3.2. MT-KO Mice Exhibited Earlier and More Severe IH-Induced Aortic Inflammation and Oxidative Damage. Previous studies have suggested that exposures to IH could induce systemic inflammation, as shown by increased ICAM-1 expression in mesenteric vessels [40] and tumor necrosis factor (TNF)-α in lymphocytes from OSA patients [41]. In view of both inflammation and oxidative damages being primary risk factors for the vascular endothelium remodeling, the protein and mRNA levels of TNF-α (Figure 3(a)) and VCAM-1 (Figure 3(b)) were examined via immunohistochemical staining and qRT-PCR, which showed that aortic tunica media were significantly increased in wild-type mice after 8 weeks of IH. It was also noticed that expression of TNF-α and VCAM-1 was significantly increased in MT-KO mice even exposed to IH for only 3 weeks, which is significantly earlier than those in wild-type mice, and the differences were even more remarkable after 8 weeks of IH (Figures 3(a) and 3(b)).

Since it is well accepted that inflammation and oxidative stress are reciprocal causes and outcomes [23], we went on to examine the markers of oxidative stress in the aortas of each group. Results of immunohistochemical staining showed a significant increase in oxidative and nitrative damage in the aortic tunica media of wild-type mice after 8 weeks of IH, as shown by the accumulation of 3-NT (Figure 4(a)) and 4-HNE (Figure 4(b)). 3-NT and 4-HNE levels were also significantly increased in aorta of MT-KO mice after exposures to IH for 3 weeks, and the differences of 3-NT and 4-HNE levels were even more remarkable after 8 weeks of IH (Figures 4(a) and 4(b)). eNOS expression was upregulated in response to 3-day IH but significantly decreased at 8 weeks of IH in wild-type mice, and MT-KO mice showed no increased of eNOS expression at early stage of IH exposures but further aggravated decrease in eNOS expression after exposures of IH for 8 weeks (Figure 5(a)). P47phox expression significantly increased in aorta of MT-KO mice after exposures to IH for 3 weeks and further increased after 8 weeks of IH compared to wild-type mice (Figure 5(b)).

3.3. MT-KO Mice Exhibited Earlier and More Severe IH-Induced Aortic Cell Death. To examine the effect of IH on

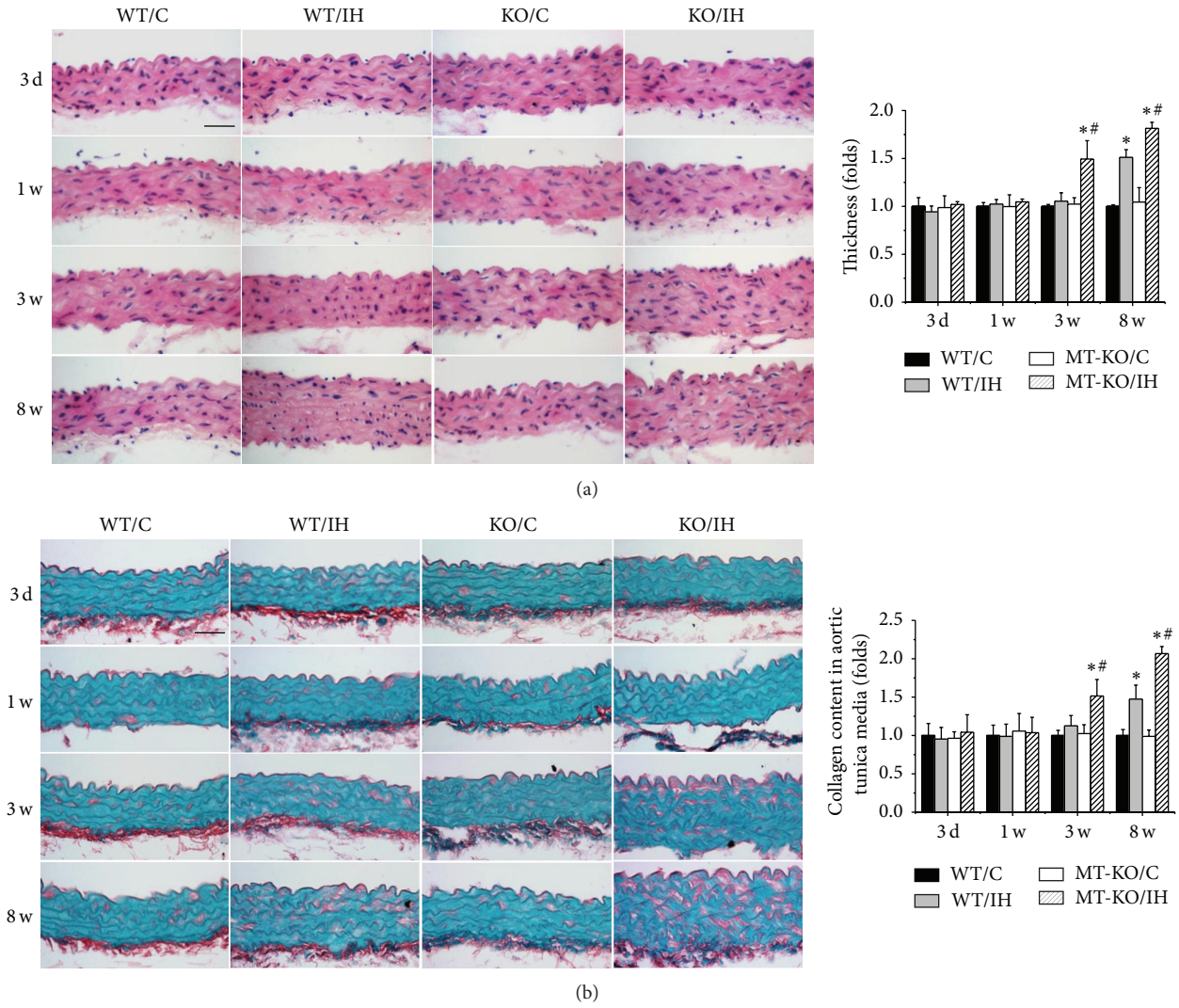

Figure 1: MT-KO mice exhibited earlier and more severe IH-induced aortic pathological changes. The pathogenic changes of aortas were examined by H&E staining (a), and the accumulation of collagen was detected by Sirius-red staining (b), followed by semiquantitative analysis. Data were presented as means ± SDs ($n = 6$); $^{*}P < 0.05$ versus WT/C; $^{\#}P < 0.05$ versus WT/IH. Bar = 50 μM.

aortic cell death and the role of MT in IH-induced cell death, we examined aortic apoptosis in MT-KO and wild-type mice by TUNEL staining (Figure 6). The results showed that cell death in aortas of wild-type mice was significantly increased after 8 weeks of IH. Compared to wild-type mice, cell death in MT-KO mice was exhibited earlier after 3 weeks of IH exposures and the differences of cell death between these two groups were even more remarkable after 8 weeks of IH.

3.4. Aortic MT Level Was Increased at the Early Stage and Decreased at the Late Stage of CIH Exposures. Above findings indicated that, in response to IH exposures, MT gene deletion exacerbated IH-induced aortic fibrosis, inflammation, oxidative damage, and apoptosis. So next we detected the dynamic changes of MT protein level in aorta directly. Intriguingly, in wild-type mice, results of immunohistochemical staining showed that there was an early induction of MT expression at the 3rd day of IH, which subsequently returned to normal and then significantly decreased at the 8th week of IH exposures, as compared to controls (Figure 7). No MT expression was detected in aorta of MT-KO mice at each time point independent of whether the mice were exposed to IH or not.

4. Discussion

The present study was designed to determine whether aortic expression of MT was changed in response to IH exposures and whether MT deletion exacerbated IH-induced aortic pathological changes. We demonstrated that short-term IH induced the protein level of MT in aorta, while long-term IH inhibited the expression of MT. Moreover, MT levels in aorta seemed to be negatively associated with aortic pathologic damages, including aortic remodeling, oxidative stress, and inflammation, suggesting a possible compensative response

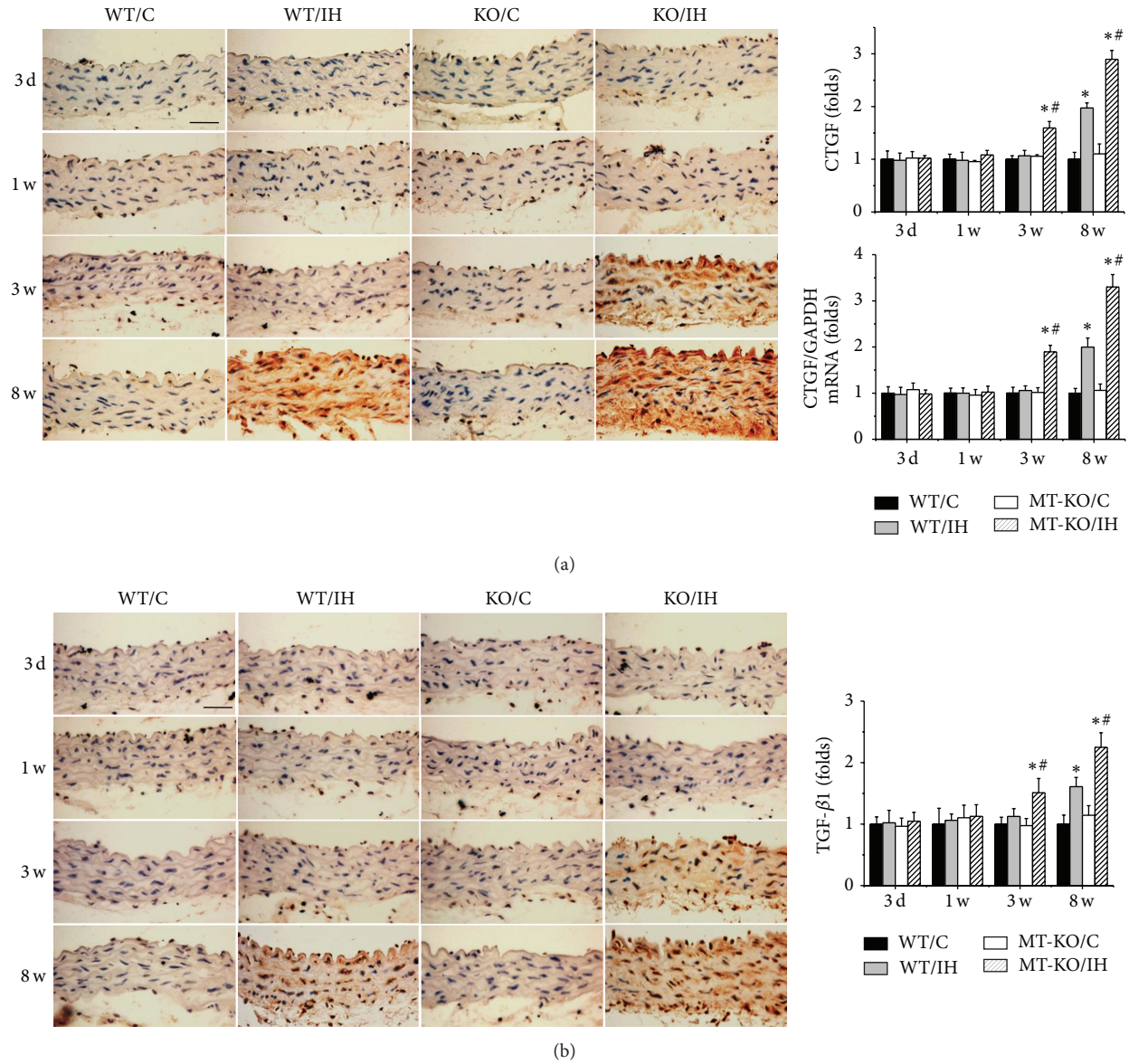

FIGURE 2: MT-KO mice exhibited earlier and more severe IH-induced aortic fibrosis. Aortic fibrosis was examined by immunohistochemical staining and qRT-PCR for the expression of CTGF (a) and immunohistochemical staining for TGF-β1 (b), followed by semiquantitative analysis. Data were presented as means ± SDs ($n = 6$); $^{*}P < 0.05$ versus WT/C; $^{\#}P < 0.05$ versus WT/IH. Bar = 50 μM.

of MT induction. By using global MT-KO mice, we further confirmed that deletion of MT aggravated IH-induced aortic pathological changes.

Chronic inflammation plays an important role in the development of various chronic diseases, including OSA [42, 43]. The effects of chronic inflammation include induction of oxidative stress, apoptotic cell death, and endothelial dysfunction, all of which could contribute to the structural and functional abnormalities of the cells [42, 44]. In the present study, we demonstrated the IH induced aortic inflammation, as shown by increased expression of TNF-α and VACM-1 in the aortic tunica media after 8 weeks of IH, which was accompanied with increased expressions of markers of aortic oxidative stress (3-NT, 4-HNE), cell death, and remodeling (aorta tunica media thickness, collagen accumulation, and the expressions of CTGF and TGF-β1) in IH group. These findings are consistent with the previous concept that inflammation and oxidative stress are reciprocal causes and outcomes [23], both of which are main pathogenic factors for the development of various cardiovascular diseases under stressed conditions. All these pathogenic alterations were exhibited as early as 3 weeks and got more severe after 8 weeks of IH exposures in aorta of the MT-KO mice.

Oxidative stress has been accepted as an imbalance between the excessive production of ROS and the reduced antioxidant capacity, which plays a pathogenic role in the

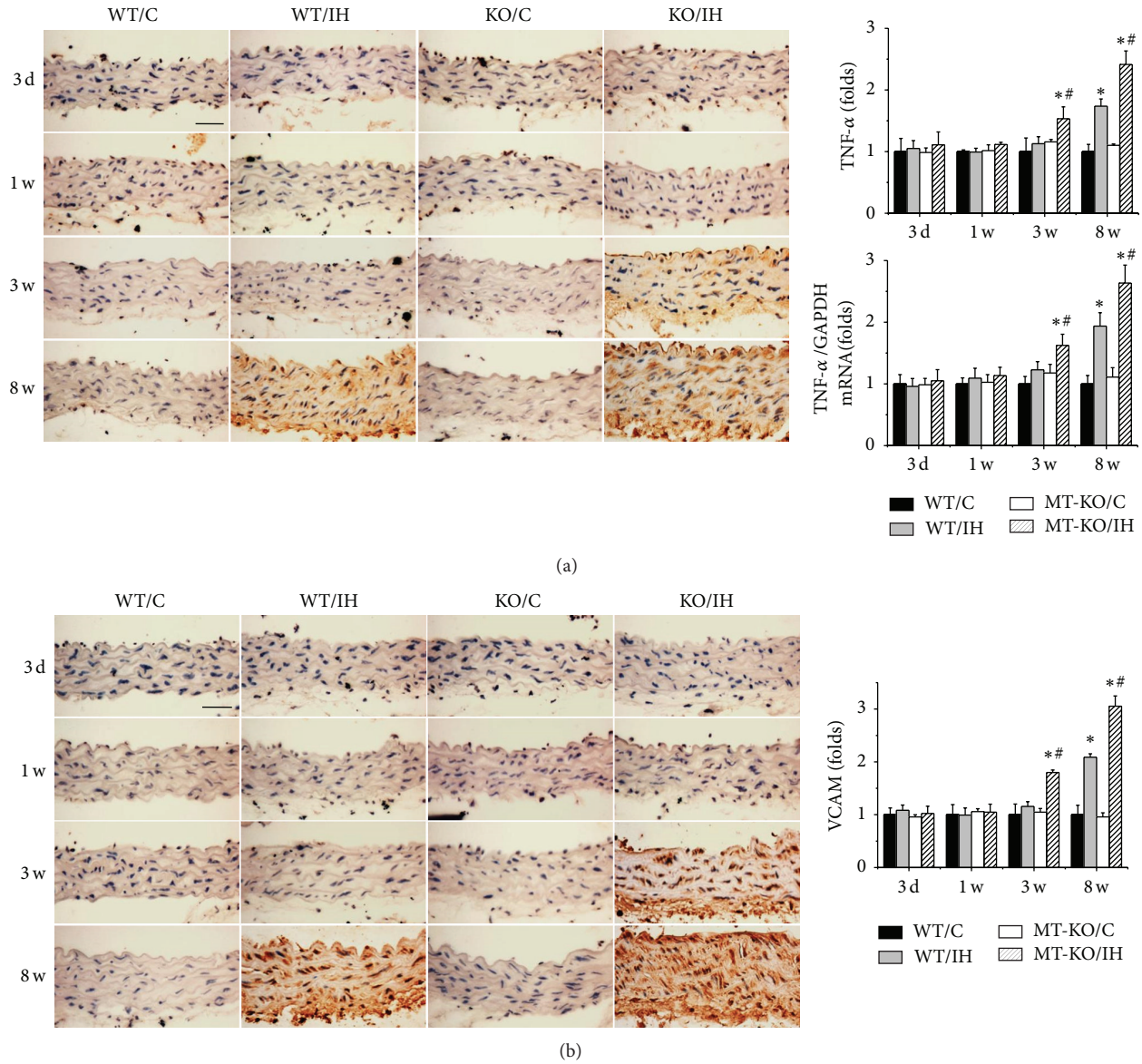

FIGURE 3: MT-KO mice exhibited earlier and more severe IH-induced aortic inflammation. Aortic inflammation was examined by immunohistochemical staining and qRT-PCR for the expression of TNF-α (a) and immunohistochemical staining for VCAM-1 (b), followed by semiquantitative analysis. Data were presented as means ± SDs ($n = 6$); $^{*}P < 0.05$ versus WT/C; $^{\#}P < 0.05$ versus WT/IH. Bar = 50 μM.

IH-induced hypertension in patients with OSA [45]. This study showed that IH-induced aortic oxidative stress damage closely involved the NO pathway. NO plays a key signal molecule in regulating vascular function; it is generated by NO synthases (NOSs), which comprise endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS [46]. Intimal eNOS-derived NO is transported to the vascular smooth muscle cells of the vascular media and regulates vascular tension mainly by relaxing the vessels, thereby maintaining vascular function [47]. In our study, we showed that eNOS expression was upregulated in response to 3-day IH but significantly decreased at 8 weeks of IH. Compared to their WT controls, MT-KO mice showed no increase of eNOS expression at early stage of IH exposures but decreased at 3 weeks of IH and further aggravated after exposures of IH for 8 weeks. NADPH oxidase is the main source of oxidative stress in the cardiovascular system [48]. Generation of ROS by mitochondria or NADPH oxidase (Nox) may contribute to the altered activity of the carotid chemoreceptor and brain injury in sleep apnea [49, 50]. The expression patterns of the multiple Nox subunits differ between cell types, resulting in different contributions to disease development. ApoE null mice that are deficient in p47phox, a regulatory subunit for both Nox1 and Nox2, have a greater reduction in atherosclerotic lesion size than mice deficient in either Nox1 or Nox2 alone [51–53]. In the present study, we reported that MT deletion exacerbated the IH-induced increased Nox subunits p47phox expression. Taken together,

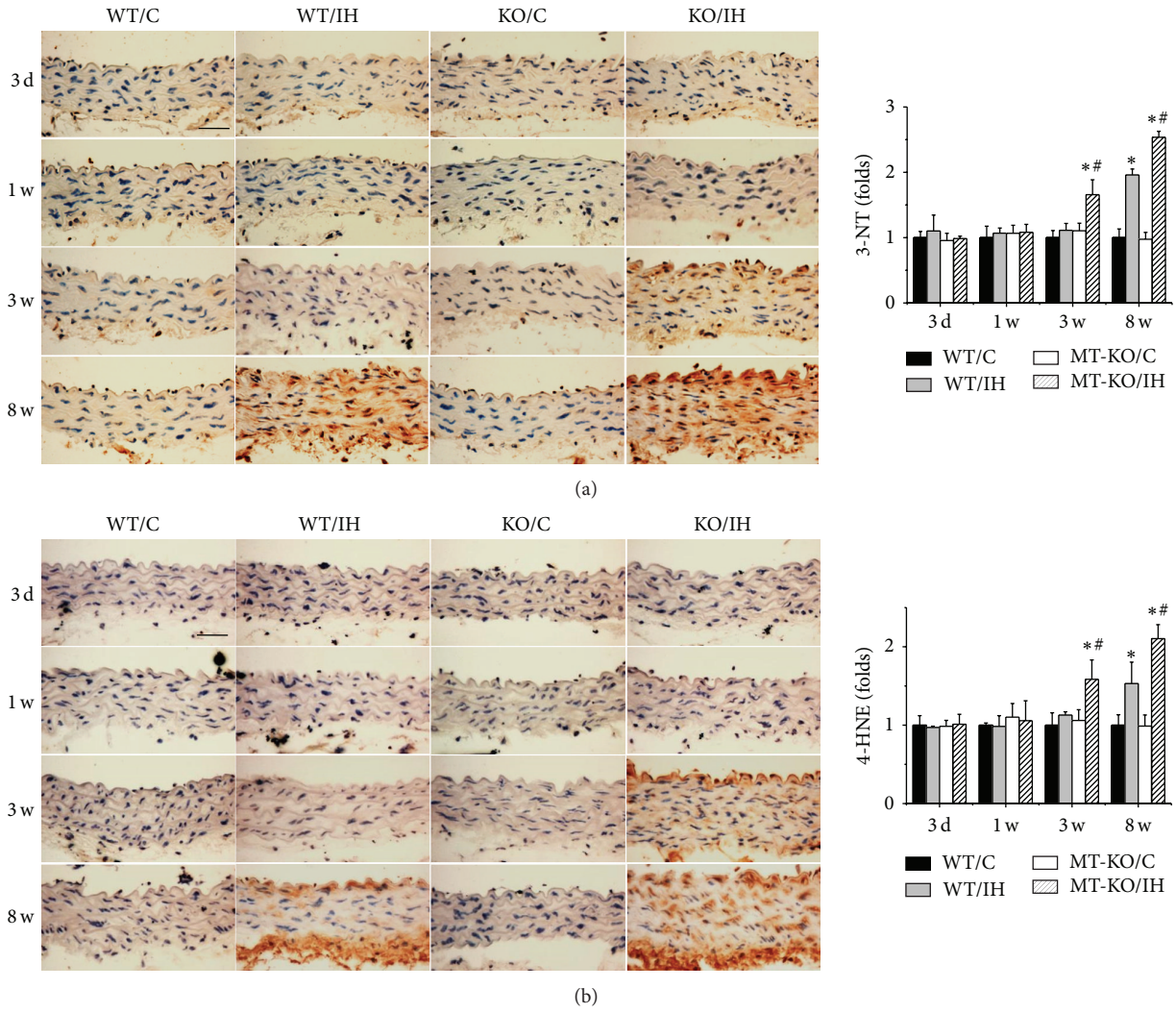

FIGURE 4: MT-KO mice exhibited earlier and more severe IH-induced aortic oxidative damage. Aortic oxidative damage was examined by immunohistochemical staining for the accumulation of 3-NT (a) and 4-HNE (b), followed by semiquantitative analysis. Data were presented as means ± SDs ($n = 6$); $^{*}P < 0.05$ versus WT/C; $^{\#}P < 0.05$ versus WT/IH. Bar = 50 μM.

the early upregulation of eNOS expression was assumed as an important compensatory response to induce NO to protect aorta, while downregulation of eNOS and upregulation of p47phox at the late stage may imply the dyscompensatory response so that there was significant increase in oxidative stress and damage.

MT, a potential antioxidant, was found to be increased at the early stage (i.e., at the end of 3-day IH exposures), slightly downregulated at 3 weeks, and significantly decreased at 8 weeks in aorta of wild-type mice. The early induction of aortic MT expression may be assumed as an important compensatory response, while downregulation of MT at the late stage may suggest a decompensatory response, as reflected by the fact that the oxidative stress and damages were significantly increased in the late stages of IH exposures. This hypothesis was supported by the findings that MT-KO mice are more sensitive to CIH-induced aortic damage, as shown by either increased severity of pathologic markers that were observed in both wild-type and MT-KO mice or induction of these markers early only in MT-KO mice. Thus, our study indicated that MT plays a critical role in IH-induced aortic oxidative damage, inflammation response, and vascular remodeling.

One of the special characteristics of MT, as compared with the other antioxidants, is that it can be endogenously expressed in most of the organs and it is also inducible. MTs exist in multiple organs with several molecular forms, including isoforms of MT1, MT2, MT3, and MT4 [32, 54]. A multigene family with at least 14 closely related and pseudogenes encodes MT proteins, and most MT genes, including the functional MT genes (MT1A, MT1B, and MT2A), lie on human chromosome 16 [55]. An earlier study compared the +838 C/G MT2A polymorphism for 288 patients with atherosclerosis and 218 healthy controls showed increased

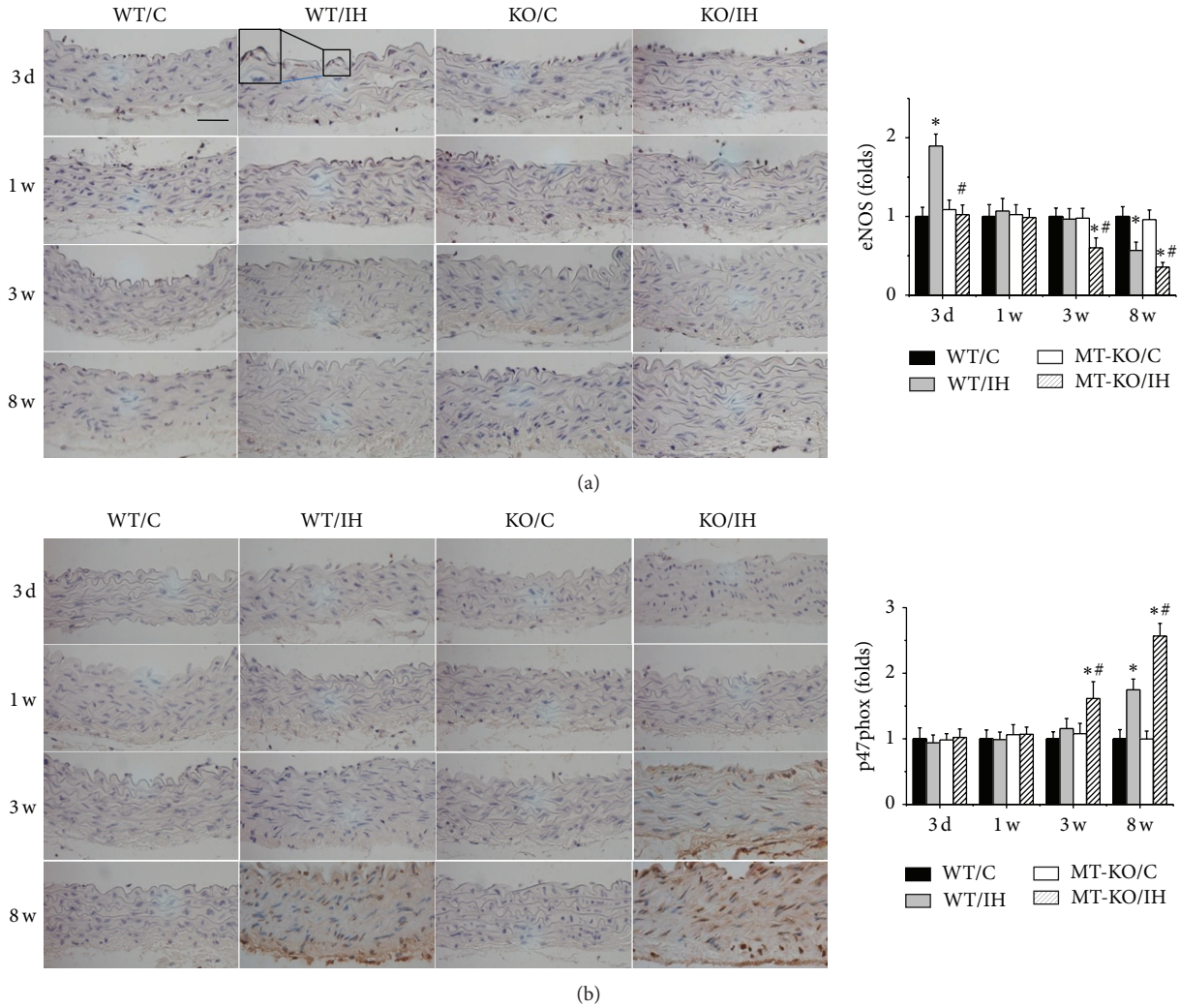

Figure 5: Effects of MT on eNOS and p47phox expression in aorta. Aortic eNOS (a) and p47phox (b) expression were examined by immunohistochemical staining, followed by semiquantitative analysis. Data were presented as means ± SDs ($n = 6$); $^{*}P < 0.05$ versus WT/C; $^{\#}P < 0.05$ versus WT/IH. Bar = 50 μM.

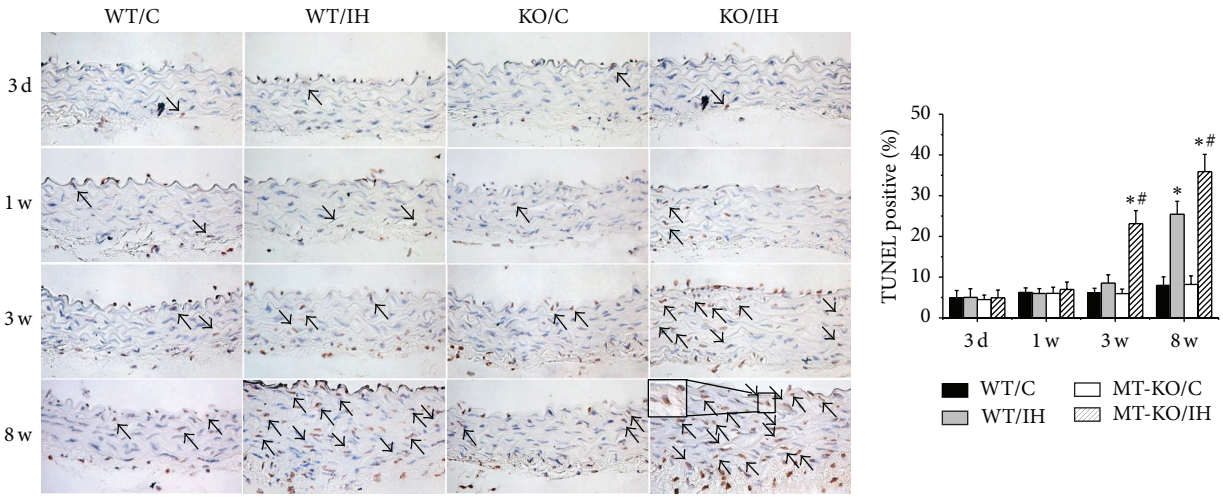

Figure 6: MT-KO mice exhibited earlier and more severe IH-induced aortic apoptosis. The apoptotic cell was examined by TUNEL staining followed by semiquantitative analysis. Data were presented as means ± SDs ($n = 6$). $^{*}P < 0.05$ versus WT/C; $^{\#}P < 0.05$ versus WT/IH. Bar = 50 μM.

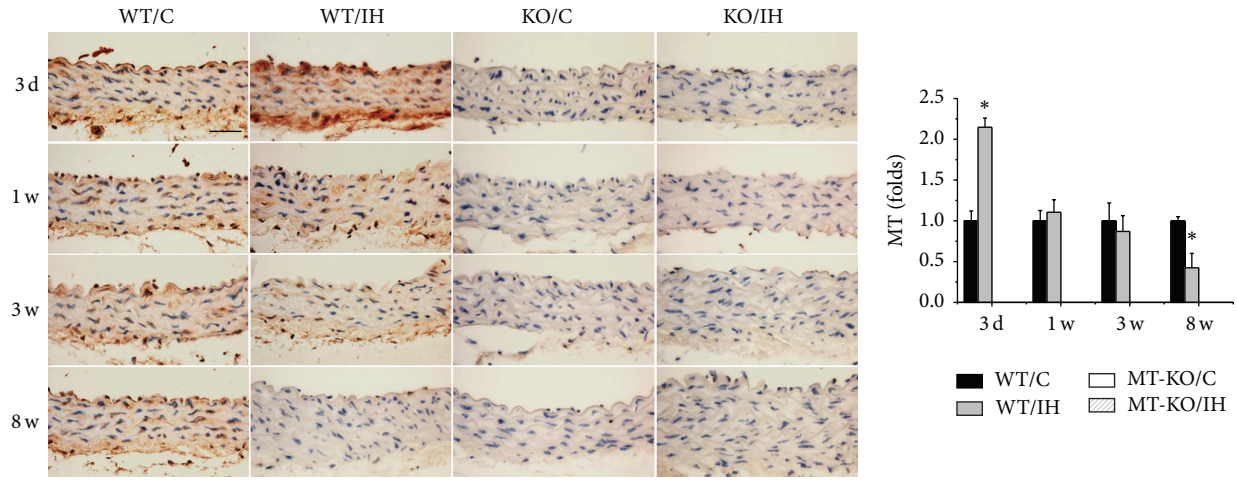

FIGURE 7: Effects of IH on aortic expression of MT. Aortic expression of MT was examined by immunohistochemical staining (a) with semiquantitative analysis (b). Data were presented as means ± SDs ($n = 6$). $^{*}P < 0.05$ versus WT/C; $^{\#}P < 0.05$ versus WT/IH. Bar = 50 μM.

inflammatory cytokines in the patients with atherosclerosis [56]. MT1A gene in rs8052394 SNP is also most likely the predisposition gene locus for diabetes or the changes of serum superoxide dismutase activity [57]. In the present study, we also found deletion of MT aggravated IH-induced aortic pathological changes which may contribute to formation of atherosclerosis by using MT-KO mice in which both MT1 and MT2 genes are deleted. Furthermore, overexpression of MT decreases the sensitivity of pulmonary endothelial cells to oxidant injury [58]. In one of our previous studies, we have also demonstrated that zinc supplementation of diabetic mice induced a significant increase in aortic MT expression, accompanied with significant prevention of diabetes-induced pathogenic changes in the aorta [39]. So induction of MT may be one of the important compensatory components, which protects aorta from chronic IH-induced damages. Therefore, induction of MT may be considered to be clinically applied for patients with OSA to prevent the development of aortic pathological processes induced by CIH exposures.

Conflict of Interests

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

Acknowledgments

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Research Article

Expression of Senescence-Associated microRNAs and Target Genes in Cellular Aging and Modulation by Tocotrienol-Rich Fraction

Sharon Gwee Sian Khee, Yasmin Anum Mohd Yusof, and Suzana Makpol

Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

Correspondence should be addressed to Suzana Makpol; suzanamakpol@yahoo.com

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Emerging evidences highlight the implication of microRNAs as a posttranscriptional regulator in aging. Several senescence-associated microRNAs (SA-miRNAs) are found to be differentially expressed during cellular senescence. However, the role of dietary compounds on SA-miRNAs remains elusive. This study aimed to elucidate the modulatory role of tocotrienol-rich fraction (TRF) on SA-miRNAs (miR-20a, miR-24, miR-34a, miR-106a, and miR-449a) and established target genes of miR-34a (CCND1, CDK4, and SIRT1) during replicative senescence of human diploid fibroblasts (HDFs). Primary cultures of HDFs at young and senescent were incubated with TRF at 0.5 mg/mL. Taqman microRNA assay showed significant upregulation of miR-24 and miR-34a and downregulation of miR-20a and miR-449a in senescent HDFs ($P < 0.05$). TRF reduced miR-34a expression in senescent HDFs and increased miR-20a expression in young HDFs and increased miR-449a expression in both young and senescent HDFs. Our results also demonstrated that ectopic expression of miR-34a reduced the expression of CDK4 significantly ($P < 0.05$). TRF inhibited miR-34a expression thus relieved its inhibition on CDK4 gene expression. No significant change was observed on the expression of CCND1, SIRT1, and miR-34a upstream transcriptional regulator, TP53. In conclusion tocotrienol-rich fraction prevented cellular senescence of human diploid fibroblasts via modulation of SA-miRNAs and target genes expression.

1. Introduction

Tocotrienols, the lesser known isomer of vitamin E, have gained increasing scientific interest in the study of aging and aging-related diseases due to its eminent antioxidant effects and nonantioxidant activity [1]. Palm oil is one of the richest natural sources of tocotrienol. Tocotrienol extracted from palm oil consists mainly of α-tocopherol and a mixture of four tocotrienol isomers (α, β, γ, and δ), referred to as tocotrienol-rich fraction (TRF) [2].

Accumulating evidences demonstrated that tocotrienol modulates several mechanisms associated with aging. In individuals over 50 years old, tocotrienol-rich fraction supplementation decreased DNA damage [3] and reduced the level of advanced glycosylation end products (AGE) and protein carbonyls, which are the oxidative damage indicators during aging [4]. In animal model of aging, tocotrienol extended mean lifespan by reducing protein carbonylation [5]. In replicative cell aging model, tocotrienol-rich fraction reversed cellular aging by preventing cell cycle arrest while restoring telomere length [6].

Human diploid fibroblasts (HDFs) undergo irreversible proliferative arrest, termed as replicative senescence, after around 50 cell divisions when cultured *in vitro*. This makes HDFs a suitable experimental model in the study of cellular aging [7]. Permanent arrest of proliferation accompanied by striking changes in cellular phenotype is the hallmark of cellular senescence. Deposition of senescent cells with age disrupts the normal tissue structure and function, further suggesting a relationship between senescence and aging [8].

Proliferating cells succumbed to cell cycle arrest when cellular macromolecules (DNA, protein, and lipid) are damaged by reactive oxygen species (ROS) constantly generated during physiological metabolism [9]. Besides the free radical

theory of aging, various signal transduction pathways that regulate aging have been proposed, including insulin/IGF-1 signalling, TOR signalling, AMPK, and sirtuins [10].

The role of miRNAs in regulating aging process has been established recently, with the discovery of miRNA, lin-4 that regulates the lifespan in *Caenorhabditis elegans* [11]. Since then, various studies have characterized numerous microRNAs (miRNAs) that are differentially expressed during aging at cell, tissue, and organism levels. Individual miRNAs contribute to accelerate or decelerate aging by targeting components of conserved aging signalling pathways [12]. These small and noncoding RNAs (≈22 nucleotides) regulate gene expression at posttranscriptional level by binding to its target mRNA mainly at the 3′ untranslated region (3′-UTR). The binding may inhibit protein translation or result in mRNA degradation [13].

Several miRNAs (including miR-20a, miR-24, miR-34a, miR-106a, and miR-449a) that funnel proliferating cells to senescence regulate cellular senescence via either or both p53/p21 and p16/pRb pathways [14]. The coordinated action between SA-miRNAs in p53/p21 and p16/pRb pathway with transcription factors (Myc and E2F) in cell cycle regulation contributes to the inhibition of cell proliferation during cellular senescence [15]. The SA-miRNAs control cell transition, mainly through the G_1/S checkpoint during cell cycle progression by targeting the components of cell cycle including cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) [16].

Despite the reported discrepancies between the upregulation and downregulation of miRNAs during aging and cellular senescence, such as miR-34a [17, 18], study of the modulatory effect of dietary compound on miRNAs may aid in the understanding of how SA-miRNAs can be regulated in favour of slowing down aging process or reducing aging phenotypes. Modulation of miRNAs by dietary and pharmacological agents has been reported recently [19]. In view of this, the present study was designed to evaluate the possible modulatory role of tocotrienol-rich fraction on the expression of SA-miRNAs and their target genes, which could potentially be exploited for reversing cellular aging.

The present study aimed to elucidate the molecular mechanism of TRF in reversing cellular aging through cell cycle arrest prevention focussing on the modulation of SA-miRNAs expression and, hence, alteration of their target genes expression which are involved in cell cycle regulation.

2. Materials and Methods

2.1. Sample Collection. This research was conducted with the approval of Ethics Committee of Universiti Kebangsaan Malaysia (Approval Project Code: FF-215-2013). Primary HDFs were derived from circumcised foreskins of 9 to12 year-old boys. Written consents were obtained from parents of all subjects.

2.2. Cell Culture and Serial Passaging. Aseptically collected skin samples were rinsed several times with 75% alcohol and phosphate buffered saline (PBS) containing 1% antibiotic-antimycotic solution (PAA, Austria). After removing the epidermis, the dermis was cut into small pieces and transferred into 0.03% collagenase type I digestive buffer (Worthington Biochemical Corporation, USA). Pure dermis was digested in incubator shaker at 37°C for 6–12 h. The isolated cells were rinsed with PBS before being cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) (PAA, Austria) and 1% antibiotic-antimycotic solution at 37°C in 5% CO_2 humidified incubator. After 5-6 days, the cultured HDFs were trypsinized and culture-expanded into new T25 culture flasks. When the subcultures were 80–90% confluent, serial passaging was done by trypsinization while the number of population doublings (PDs) was monitored until HDFs reached senescence. For subsequent experiments, HDFs used were at passage 6 (young HDFs, PD < 12) and passage 30 (senescent HDFs, PD > 55).

2.3. TRF Preparation and Treatment. Stock solution of TRF was freshly prepared in dark by dissolving 1 g Gold Tri E 50 (Sime Darby Bioganic Sdn. Bhd., Malaysia) in 1 mL 100% ethanol (1 : 1) and kept at −20°C for not more than one month. TRF was activated by incubating 45 μL stock TRF (1 g/1 mL) with 60 μL FBS overnight at 37°C. To prepare TRF at 50 mg/mL, 90 μL DMEM with 10% FBS and 105 μL 100% ethanol were added to the activated TRF, after which 600 μL mixture containing FBS and 100% ethanol (1 : 1) was also added. TRF at 0.5 mg/mL was prepared in culture medium by mixing 5 μL TRF (50 mg/mL) and 495 μL DMEM with 10% FBS. Cells were plated at 2×10^4 in 24-well plate and incubated overnight. Treated groups were incubated with 0.5 mg/mL TRF for 24 h; untreated HDFs were incubated with DMEM containing 10% FBS (PAA, Austria) while transfected untreated HDFs were incubated with DMEM containing 5% FBS (PAA, Austria) without antibiotic. Media for untreated cells were changed parallel to the treated cells and both were harvested on the same day.

2.4. Morphological Analysis and Senescence-Associated Beta-Galactosidase (SA-β-gal) Staining. SA-β-gal staining was performed with a senescent cells staining kit (Sigma, USA) according to the manufacturer's instructions. 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 in the absence of CO_2.

2.5. Primer Design. Forward primers for miRNAs were designed according to the miRNAs sequences listed in miRBase database (http://www.mirbase.org). Table 1 shows the forward primer sequences for validated miRNAs. Primers for human GAPDH, CCND1, CDK4, SIRT1, and TP53 were designed from listed NIH GenBank database using Primer 3 software and blasted with GenBank database sequences for specificity confirmation. The efficiency and specificity of each primer set were confirmed via standard curve (Ct value versus serial dilution of total RNA) and melting profile evaluation. The primers sequences for quantitative gene expression analysis are shown in Table 2.

Table 1: Forward primer sequences for validated miRNAs.

Accession number	miRBase ID	Mature miRNA sequences (5′ → 3′)	Size (bp)
miRBase			
MIMAT0000075	hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	23
MIMAT0000080	hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG	22
MIMAT0000255	hsa-miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU	22
MIMAT0004517	hsa-miR-106a-3p	CUGCAAUGUAAGCACUUCUUAC	22
MIMAT0001541	hsa-miR-449a	UGGCAGUGUAUUGUUAGCUGGU	22
NCBI			
NR_002752	RNU6B	CGCAAGGAUGACACGCAAAUUCGUGAAGCGUUCCAUAUUUUU	42

Table 2: Primers sequences for quantitative gene expression analysis.

Accession number	Gene	Primer	Primer sequences (5′ → 3′)	PCR product size (bp)
NM_002046	GAPDH	Forward	TCCCTGAGCTGAACGGGAAG	217
	GAPDH	Reverse	GGAGGAGTGGGTGTCGCTGT	
NM_053056	CCND1	Forward	AGACCTTCGTTGCCCTCTGT	181
	CCND1	Reverse	CAGTCCGGGTCACACTTGAT	
NM_000075	CDK4	Forward	TGGCCCTCAAGAGTGTGAGA	147
	CDK4	Reverse	ATGTGGCACAGACGTCCATC	
NM_012238	SIRT1	Forward	GCAGATTAGTAGGCGGCTTG	152
	SIRT1	Reverse	TCTGGCATGTCCCACTATCA	
NM_000546	TP53	Forward	GGAAGAGAATCTCCGCAAGAA	177
	TP53	Reverse	AGCTCTCGGAACATCTCGAAG	

2.6. RNA Extraction. Total RNA was extracted from different groups of HDFs using TRI Reagent (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions. Polyacryl Carrier (Molecular Research Center, Cincinnati, USA) was added to each extraction to precipitate the total RNA. Extracted RNA pellet was washed with 75% ethanol and dried prior to dissolving it in RNase-free and DNase-free distilled water. Aliquots of total RNA were stored at −80°C immediately after extraction. The yield and purity of extracted total RNA were determined by Nanodrop (Thermo Scientific, USA).

2.7. Transfection. Young HDFs were reverse transfected with mirVana miR-34a Mimic 1 (Ambion, USA) at a final concentration of 10 nM to overexpress miR-34a in the cells, using Lipofectamine RNAiMAX (Invitrogen, USA). 3×10^4 cells were plated and transfected in DMEM containing 2% FBS (PAA, Austria) without antibiotic for 24 h. mirVana miRNA mimic and Negative Control #1 (Ambion, USA) were used as control. Total RNA was extracted from nontreated and TRF treated transfected cells after treatment for another 24 h.

2.8. Real Time qRT-PCR. For quantitative analysis of miRNAs, reverse transcription (RT) was first performed using Taqman MicroRNA Reverse Transcription kit (Applied Biosystems, USA) according to manufacturer's instructions with total RNA at 10 ng. PCR reactions were then performed according to manufacturer's instructions to quantitate the expression levels of miRNAs (miR-20a, miR-24, miR-34a, miR-106a, and miR-449a) using Taqman Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, USA), and Taqman microRNA assay (Applied Biosystems, USA) for the miRNAs of interest. The PCR amplification was performed in iQ5 Multicolor Real Time PCR (Bio Rad, USA) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The PCR incubation profile was extended to 45 cycles for miR-20a and miR-449a. PCR reactions were performed in triplicate. All miRNAs expressions were normalized to the expression of RNU6B. The relative expression value (REV) of miRNAs was calculated using the $2^{-\Delta Ct}$ method of relative quantification [20] as the equation

$$\text{REV} = 2^{\text{Ct value of RNU6B} - \text{Ct value of miRNA}}. \quad (1)$$

Gene expression levels of CCND1, CDK4, SIRT1, and TP53 were analysed with KAPA SYBR Fast 1-Step qRT-PCR kit and Bio-Rad iCycler (KAPA Biosystems, USA). Each qRT-PCR mixture contained 11.7 μL nuclease free water, 10 μL KAPA SYBR Fast master mix, 0.3 μL RT enzyme, 1 μL 100 μM forward primer, 1 μL 100 μM reverse primer, and 1 μL total RNA at 50–100 ng. Reactions were performed in iQ5 Multicolor Real Time PCR (Bio Rad, USA) at 42°C for 5 min and 95°C for 4 min, followed by 40 cycles of 95°C for 3 s and 60°C for 20 s. qRT-PCR reactions were performed in duplicate. GAPDH was used as a reference gene in gene expression normalization [21]. The relative expression value (REV) of genes of interest was calculated using the $2^{-\Delta Ct}$ method of relative quantification [22] as the equation

$$\text{REV} = 2^{\text{Ct value of GAPDH} - \text{Ct value gene of interest}}. \quad (2)$$

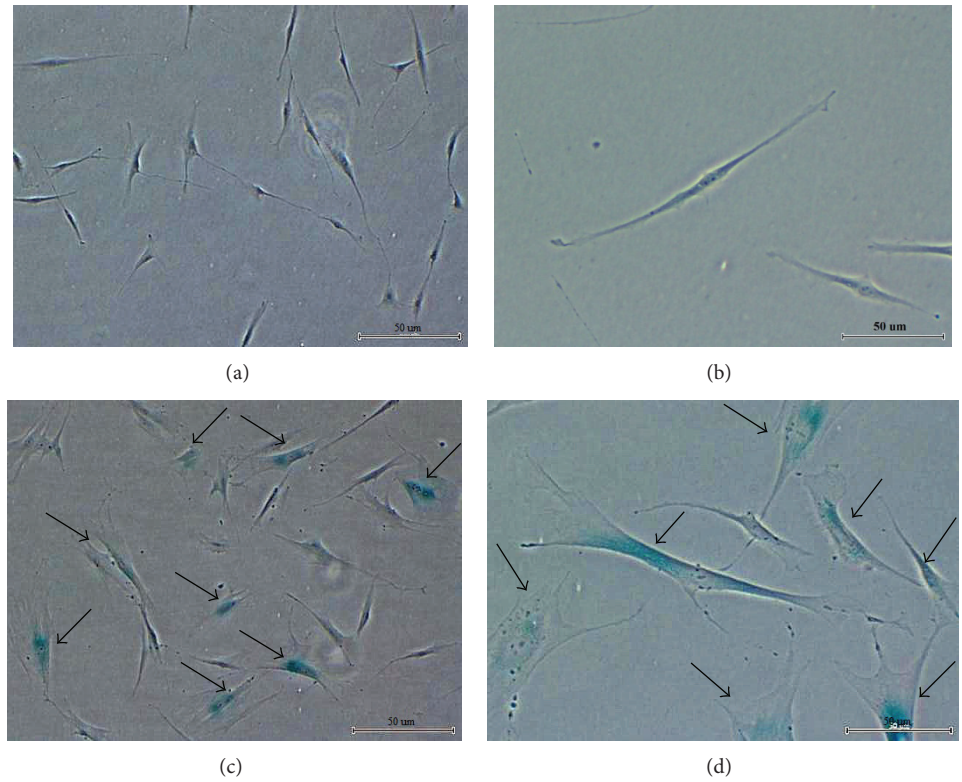

Figure 1: Morphological changes and SA-β-gal staining of young HDFs (magnification 40x) (a), young HDFs (magnification 100x) (b), senescent HDFs (magnification 40x) (c), and senescent HDFs (magnification 100x) (d). Senescent HDFs showed morphological changes during replicative senescence with the loss of original fibroblastic shape, appearance of flattened morphology, and increased in the size of cells and nucleus. Only senescent HDFs showed positive SA-β-gal staining in blue as indicated by arrow.

2.9. Statistical Analysis. Data were presented as mean ± SD. ANOVA was used for multiple comparisons of groups. Mann-Whitney U test was used to assess statistical significance between groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Morphological Analysis and SA-β-Galactosidase Staining. Changes in cell morphology and increase in SA-β-gal activity were characterized as aging phenotypes. Young HDFs displayed the normal spindle shape of a typical fibroblast and were not stained blue in SA-β-gal staining (Figures 1(a) and 1(b)). However, senescent HDFs were mainly characterized by cellular enlargement and flattening with a concomitant increase in the size of nucleus. Positive blue stain of SA-β-gal staining mainly appeared in HDFs at passage 30 suggesting that HDFs at this passage had reached senescence (Figures 1(c) and 1(d)).

3.2. TRF Treatment Modulates the Expression of SA-miRNAs. Changes in miRNAs expressions were observed in HDFs with senescence. The expression of miR-20a and miR-449a was decreased while the expression of miR-24 and miR-34a was increased significantly in senescent HDFs as compared to young HDFs ($P < 0.05$) (Figure 2). No noticeable level of miR-106a was expressed with trials using 10 ng, 20 ng, and 30 ng total RNA (data not shown). TRF treatment increased miR-20a expression in young HDFs, reduced miR-34a expression in senescent HDFs, and increased miR-449a expression in both young and senescent HDFs ($P < 0.05$). No significant effect was observed on the expression of miR-24 with TRF treatment.

3.3. Effect of TRF Treatment on miR-34a Expression in Transfected HDFs. The expression level of miR-34a increased significantly ($P < 0.05$) in young HDFs transfected with miR-34a mimic indicating that transfection process had successfully introduced miR-34a into young HDFs (Figure 3). TRF treatment reduced miR-34a expression significantly in young HDFs transfected with miRNA negative control and senescent HDFs.

3.4. TRF Treatment Modulates the Expression of Target Genes and Upstream Regulator of miR-34a. Ectopic expression of miR-34a reduced the gene expression of CDK4 significantly ($P < 0.05$), while no significant changes were observed on the gene expression of CCND1 (cyclin D1), SIRT1, and TP53 (Figure 4). TRF treatment was found to increase the expression of CDK4 significantly in young HDFs, young HDFs with ectopic expression of miR-34a, and young HDFs transfected with miRNA negative control ($P < 005$). TRF treatment also reduced CCND1 gene expression in all groups

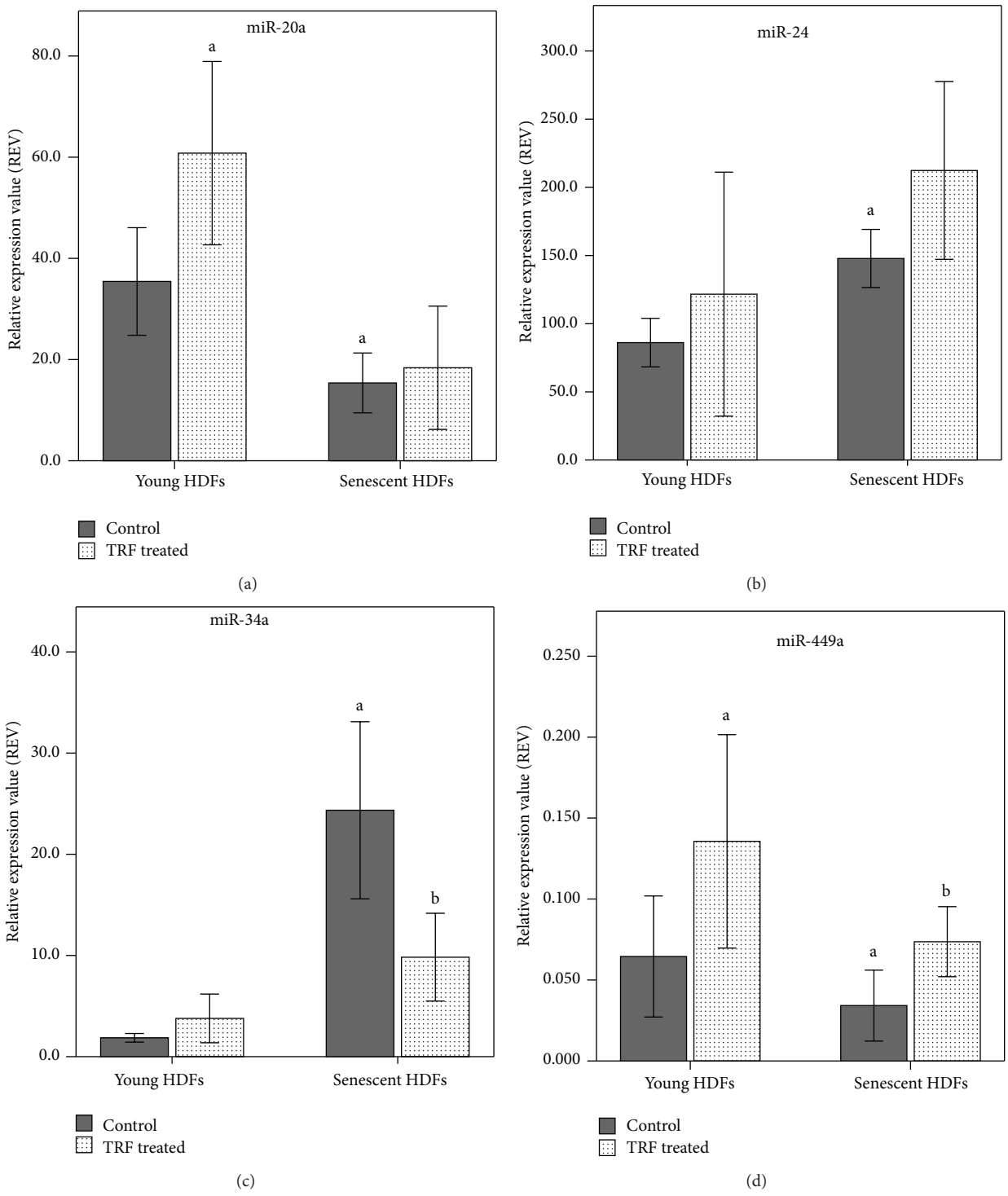

Figure 2: Effect of TRF treatment on the expression levels of miR-20a (a), miR-24 (b), miR-34a (c), and miR-449a (d) in young and senescent HDFs measured by real time qRT-PCR. [a]denotes $P < 0.05$ compared to control young HDFs and [b]$P < 0.05$ compared to control senescent HDFs. Data are presented as relative expression value (REV) normalized to RNU6B expression (mean ± SD, $n = 9$).

of cells and increased the expression of SIRT1 I in young HDFs with ectopic expression of miR-34a and young HDFs transfected with miRNA negative control ($P < 0.05$). The expression of TP53 increased significantly in all groups of cells treated with TRF ($P < 0.05$).

4. Discussion

In this study, cellular morphological changes and increased SA-β-gal activity clearly differentiate senescent HDFs from young HDFs. Elevated level of matrix metalloproteinase 1 and

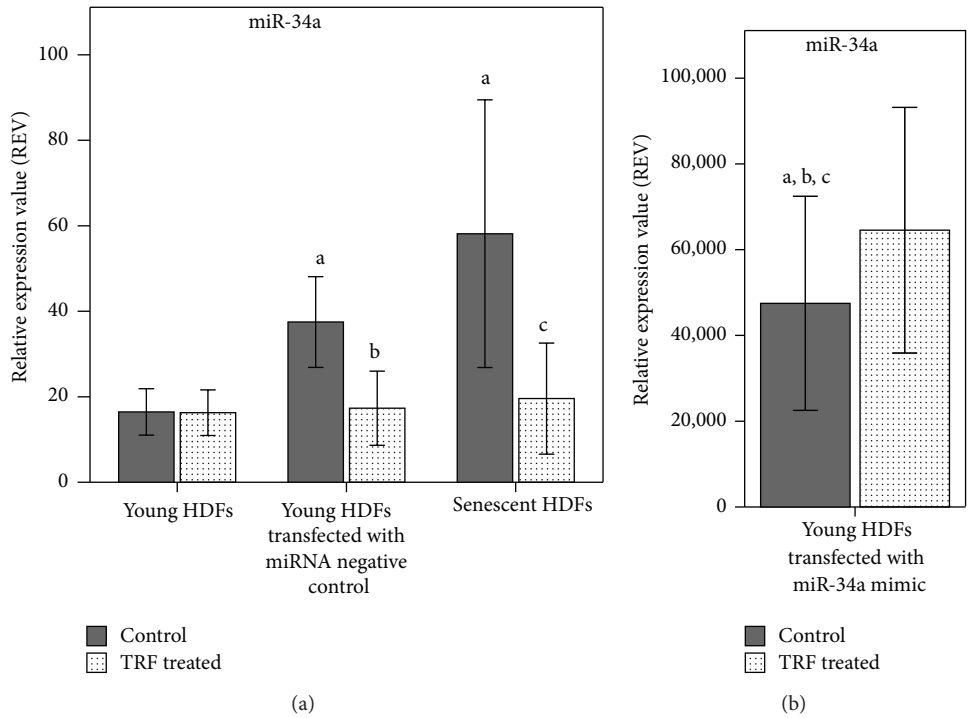

Figure 3: Effect of TRF treatment on the expression level of miR-34a in nontransfected young HDFs, young HDFs transfected with miR-34a mimic, miRNA negative control, and senescent HDFs measured by real time qRT-PCR. Young HDFs were transfected with miR-34a mimic (10 nM) to overexpress miR-34a or miRNA negative control as control for 24 h, followed by TRF treatment for 24 h. [a]denotes $P < 0.05$ compared to control young untransfected HDFs, [b]$P < 0.05$ compared to young HDFs transfected with miRNA negative control, and [c]$P < 0.05$ compared to control senescent HDFs. Data are presented as relative expression value (REV) normalized to RNU6B expression (mean ± SD, $n = 9$).

decreased level of extracellular matrix components such as elastin and collagen I-1a were believed to have contributed to the shift in the senescent fibroblasts phenotypes towards matrix degradation [23]. Furthermore, increase in the size of HDFs during replicative senescence may be attributed to the increase in size of nucleus and nucleoli and increase in number of vacuoles, Golgi, endoplasmic reticulum, cytoplasmic microfilament, and intracellular vesicles mainly lysosomes [24]. Increased lysosomal content in senescent cells was reflected by the increase of SA-β-gal activity [25], widely used as the biomarker to demonstrate the onset of replicative senescence in multiple cell types including human fibroblast cultures [26].

It has been reported that changes in miRNA expression occurred with human aging [18]. Our data on SA-miRNAs expression showed upregulation of miR-24 and miR-34a and downregulation of miR-20a and miR-449a in senescent cells.

Decrease in miR-20a expression in senescent cells observed in this study, which was also reported in previous studies [27, 28], may be attributed to the increase in CCND1 gene expression [29] which was also observed in senescent HDFs. Young HDFs rather than senescent HDFs responded towards TRF treatment, by showing an increase in miR-20a expression. Upregulation of miR-20a in young HDFs after TRF treatment may increase the inhibition effect on $p21^{Cip1}$ [30] and hence increase CDK2 level to form active CDK complexes with cyclin E and cyclin A to promote higher cell proliferation rate in young HDFs [31].

Deep sequencing analysis [17] and loss-of-function analysis [32] supported the upregulation of miR-24 in senescent cells observed in this study despite the contradictory findings that were reported earlier [33, 34]. Increased miR-24 expression in senescent HDFs may inhibit cell proliferation by suppressing cell cycle regulatory genes including E2F2 [32], which then prevent miR-20a promoter activation resulting in decreased miR-20a expression [35]. Interestingly, the expression of one miRNA may affect the other miRNA via its target genes, which is at the same time the transcriptional regulator of the other miRNA. However, TRF treatment did not have any modulatory effect on miR-24 expression in senescent HDFs and also young HDFs.

Increased miR-34a expression in senescent HDFs observed in this study which is in agreement with earlier reported literature [17, 36] may halt cell cycle progression by regulating several components in cell cycle regulation including CCNE2, CDK4 [37], CCND1, and CDK6 [38]. It is intriguing to report that TRF treatment decreased the expression of miR-34a in senescent HDFs. This finding triggered the interest to further study how TRF affects the target genes of miR-34a in reversing cellular aging.

To characterize miR-34a targets, we have identified CDK4, CCND1, and SIRT1 as the target genes of miR-34a, using database of experimentally verified targets of miRNAs (TarBase 6.0) [39] and the bioinformatics miRNA target prediction tools: TargetScan (http://targetscan.org) and microRNA.org (http://www.microRNA.org). CDK4 and

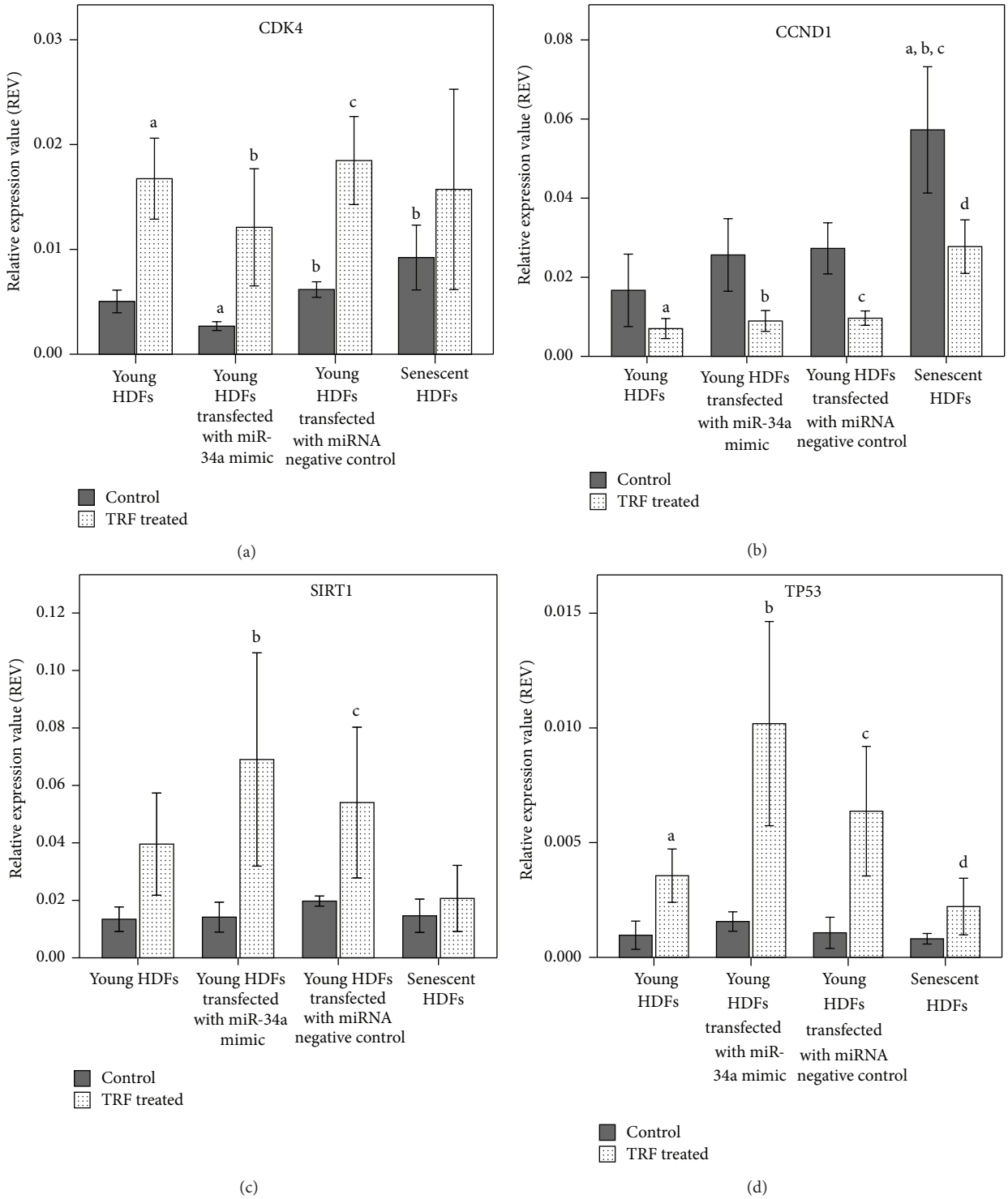

Figure 4: Effect of TRF treatment on the expression level of CDK4 (a), CCND1 (b), SIRT1 (c), and TP53 (d) in nontransfected young HDFs, young HDFs transfected with miR-34a mimic, miRNA negative control, and senescent HDFs measured by real time qRT-PCR. [a]denotes $P < 0.05$ compared to control young untransfected HDFs, [b]$P < 0.05$ compared to control young HDFs transfected with miR-34a mimic, [c]$P < 0.05$ compared to control young HDFs transfected with miRNA negative control, and [d]$P < 0.05$ compared to control senescent HDFs. Data are presented as relative expression value (REV) normalized to GAPDH expression (mean ± SD, $n = 6$).

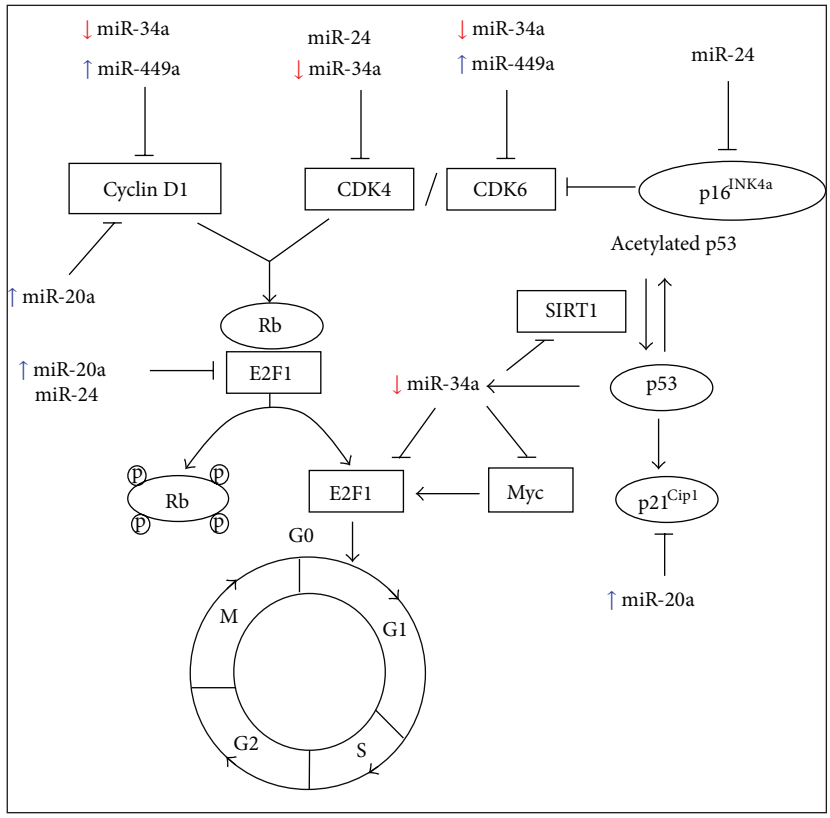

Figure 5: Modulatory effect of tocotrienol-rich fraction on the expression of SA-miRNAs at transcriptional level.

CCND1 are involved in cell cycle regulation [40] while SIRT1 is responsive towards oxidative stress which is prominent during aging [41].

Our findings showed that ectopic delivery of miR-34a in young HDFs significantly increased miR-34a expression level which increased the inhibitory effect of miR-34a on target genes. Transfection of miR-34a mimic into young HDFs resulted in sufficient increase in miR-34a levels to cause a corresponding decrease in the expression of the predicted target, CDK4, whereas the gene expression of CCND1, SIRT1, and TP53 was not affected.

Elevated level of miR-34a in senescent HDFs was not sufficient to repress CDK4 gene expression. However, ectopic expression of miR-34a showed significant inhibition effect on CDK4 gene expression, suggesting that miR-34a level is important in determining its effect on CDK4 gene expression. TRF treatment increased CDK4 gene expression in young nontransfected and transfected HDFs but not senescent HDFs. This interestingly suggested that TRF treatment suppressed miR-34a expression and thus relieved its inhibition on CDK4 gene expression. Increased CDK4 level encourages more cyclin D1/CDK4/CDK6 complexes to be formed, which favours cell cycle progression and cell proliferation. In addition, high level of CDK4 ensures its function will not be diminished completely by $p16^{INK4a}$ [31, 34].

Decreased CCND1 gene expression was reported with ectopic expression of miR-34a with a higher concentration of miR-34a duplex (50 nM) [38]. Increased CCND1 gene expression in senescent HDFs was observed in this study, in accordance with previously reported data [23]. CCND1 mostly formed inactive CDK complex with inactive unphosphorylated CDK2 [42]. TRF treatment decreased CCND1 gene expression directly in young and senescent HDFs regardless of miR-34a modulation. One of the isomer of TRF, γ-tocotrienol, has been reported to decrease CCND1 gene expression [23].

Increased expression of miR-34a did not result in SIRT1 mRNA degradation even though translational inhibition of SIRT1 by miR-34a upregulation has been reported [43]. TRF treatment was found to increase SIRT1 gene expression directly without miR-34a modulation. Increased SIRT1 gene expression by TRF may compensate the reduction of this gene during aging [40] and hence increase the oxidative stress response.

Although previous study demonstrated that miR-34a is the direct transcriptional target of p53 [42], upregulation of miR-34a expression in this study was not accompanied with the increase of TP53 gene expression. However, increased transcriptional activity of p53 in senescent cells without elevated p53 gene and protein expression was reported [44]. Alternatively, upregulation of miR-34a in senescent HDFs may be independent of p53 and modulated by other transcription factor, such as ELK1 [45]. TRF increased TP53 gene

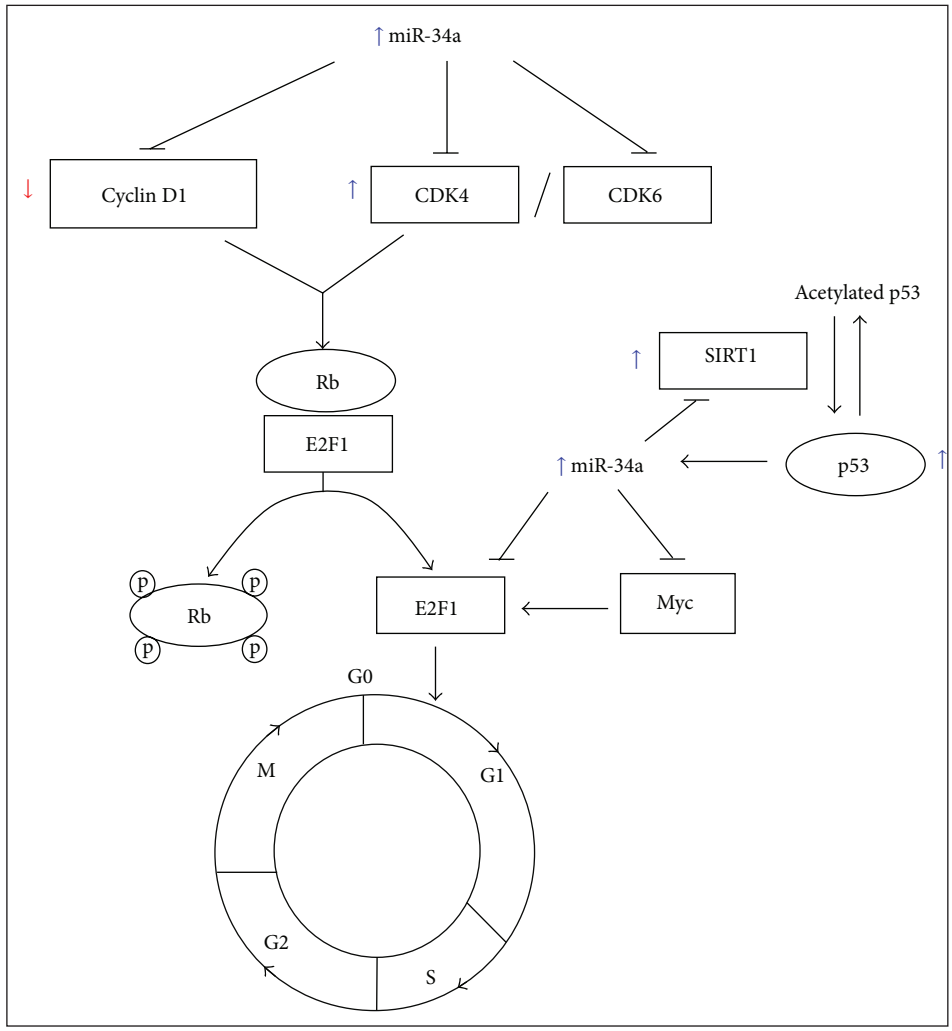

Figure 6: Modulatory effect of tocotrienol-rich fraction on the expression of miR-34a associated genes at transcriptional level when miR-34a is overexpressed.

expression directly in young nontransfected and transfected HDFs and senescent HDFs.

p53-miR-34a-SIRT1 positive feedback loop suggested that p53 induces miR-34a expression which suppresses SIRT1, increasing p53 activity [46]. However, this positive feedback loop was vague when miR-34a expression increases in senescent cells and by ectopic delivery at transcriptional level. TRF highlighted the positive feedback loop by increasing SIRT1 expression to enhance p53 deacetylation when miR-34a is overexpressed.

This study also observed the downregulation of miR-449a in senescent HDFs. Similarly, genome-wide analysis of miRNA expression revealed miR-449a was downregulated with age [18]. In contrary, increased miR-449a expression was reported in deep sequencing analysis [17]. However, miR-449a expression was found to be negatively associated with CCND1 expression [47]. Increased CCND1 observed in senescent HDFs may contribute to the downregulation of miR-449a in senescent HDFs observed in this study. Furthermore, the seed sequences of miR-449a are similar to that of miR-34a (UGGCAGUGU) [48], indicating similar target genes including CCND1 [46], CCNE2 [47], and CDK6 [49]. Increase of miR-34a expression with higher relative expression value (REV) suggested miR-34a may have a more important role than miR-449a during replicative senescence of HDFs.

TRF treatment was found to have increased miR-449a expression in both young and senescent HDFs, indicating that TRF modulated miR-449a expression but not specifically for senescent cells. Increased miR-449a expression in young and senescent cells may be accompanied with the elevated level of miR-449a transcription regulator, E2F1, to promote cell cycle progression [50].

In this study, the proposed mechanism which underlies TRF mediated regulation of miRNAs may be attributed to its radical-scavenging effect [51]. RNase III enzyme Dicer is responsible in the production of mature miRNAs. Its function is inhibited by multiple stresses including reactive oxygen

species [52], which is normally accumulated during aging [12]. TRF is suggested to modulate miRNAs posttranscriptionally by alleviating the effect of stress on Dicer, therefore affecting miRNAs biogenesis and expression levels.

Figure 5 summarized the modulatory effect of TRF on the expression of SA-miRNAs while Figure 6 summarized the modulatory effect of TRF on the expression of miR-34a associated genes when miR-34a is overexpressed. Our results revealed that TRF is a potential anticellular aging agent by modulating the expression of specific SA-miRNAs and its target genes involved in cell cycle regulation during cellular senescence.

5. Conclusion

In the present study, we demonstrated that tocotrienol-rich fraction with antioxidant and nonantioxidant properties altered the expression of SA-miRNAs specifically miR-34a and, therefore, alters the expression of miR-34a target genes involved in cell cycle regulation to promote cell cycle progression in senescent HDFs.

Abbreviations

AGE:	Advanced glycosylation end product
AMPK:	Adenosine monophosphate-activated protein kinase
CCND1:	Cyclin D1
CCNE2:	Cyclin E2
CDKI:	Cyclin-dependent kinase inhibitor
CDK:	Cyclin-dependent kinase
DMEM:	Dulbecco's modified Eagle medium
ELK:	ETS-like gene 1
E2F:	E2 promoter binding factor
FBS:	Foetal bovine serum
GADPH:	Glyceraldehydes 3-phosphate dehydrogenase
HDF:	Human diploid fibroblast
IGF-1:	Inculin-like growth factor 1
miRNA:	MicroRNA
PBS:	Phosphate buffered saline
PD:	Population doubling
pRb:	Retinoblastoma protein
qRT-PCR:	Quantitative reverse transcription-polymerase chain reaction
ROS:	Reactive oxygen species
SA-miRNAs:	Senescence-associated microRNAs
SA-β-gal:	Senescence-associated beta-galactosidase
SIRT1:	Sirtuin 1
TOR:	Target of rapamycin
TP53:	Tumour protein 53
TRF:	Tocotrienol-rich fraction
UTR:	Untranslated region.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Vitamin E in Sarcopenia: Current Evidences on Its Role in Prevention and Treatment

Shy Cian Khor, Norwahidah Abdul Karim, Wan Zurinah Wan Ngah, Yasmin Anum Mohd Yusof, and Suzana Makpol

Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

Correspondence should be addressed to Suzana Makpol; suzanamakpol@yahoo.com

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Sarcopenia is a geriatric syndrome that is characterized by gradual loss of muscle mass and strength with increasing age. Although the underlying mechanism is still unknown, the contribution of increased oxidative stress in advanced age has been recognized as one of the risk factors of sarcopenia. Thus, eliminating reactive oxygen species (ROS) can be a strategy to combat sarcopenia. In this review, we discuss the potential role of vitamin E in the prevention and treatment of sarcopenia. Vitamin E is a lipid soluble vitamin, with potent antioxidant properties and current evidence suggesting a role in the modulation of signaling pathways. Previous studies have shown its possible beneficial effects on aging and age-related diseases. Although there are evidences suggesting an association between vitamin E and muscle health, they are still inconclusive compared to other more extensively studied chronic diseases such as neurodegenerative diseases and cardiovascular diseases. Therefore, we reviewed the role of vitamin E and its potential protective mechanisms on muscle health based on previous and current *in vitro* and *in vivo* studies.

1. Sarcopenia: A Progressive Geriatric Syndrome

The term "sarcopenia" has been used to describe the progressive loss of muscle structure and function which is associated with increasing age. The origin of "sarcopenia" is from Greek's words, "sarx" and "penia," which means "poverty of flesh" [1]. Later, geriatricians found that the definition of sarcopenia is inadequate and suggested that the definition of sarcopenia should include the classification of target groups, guidelines to identify high risk patients, and a purpose definition of sarcopenia [2]. Therefore, a consensus definition of sarcopenia was recommended by International Working Group on Sarcopenia. They define sarcopenia as age-associated loss of skeletal muscle mass and function, which could be due to disused muscle, endocrine shift, chronic diseases, inflammation, or nutrient deficiency. Thus older patients with symptoms of declining physical fitness, strength, or overall health can be diagnosed with sarcopenia. Further analysis on body composition using dual energy X-ray absorptiometer (DXA) is needed if patients are bedridden, unable to rise from a chair independently, or with gait speed less than 1.0 m/s. The diagnosis of sarcopenia is confirmed in an individual with gait speed less than 1 m/s and low muscle mass; for example, appendicular fat lean mass relative to height^2 is less than 7.23 kg/m^2 in men and less than 5.67 kg/m^2 in women. Cachexia is included as part of sarcopenia even though they are two different conditions [2]. The European Working Group on Sarcopenia in Older People (EWGSOP) stated that an older person (age of more than 65 years old) with low muscle mass and either low muscle strength or low physical performance (gait speed $\leq$ 0.8 m/s) can be diagnosed with sarcopenia [3].

Skeletal muscle mass and strength will gradually decrease each year. For example, about 1 to 2% of skeletal muscle mass declines each year after the age of 30 years [4]. It has been reported that the percentage of muscle mass degeneration is higher in men than in women, at a rate of decline of around 12.9% and 5.3%, respectively, for each decade [5]. Besides the decline in muscle mass, loss of muscle strength is also obvious. Longitudinal studies showed that older adults lose their leg strength around 10 to 15% each decade before their age reaches 70 years, and a more dramatic loss was observed in advanced age, in a range of 2 to 4% per year

[5, 6]. Furthermore the declined muscle strength is more severe than the progressive shrink of muscle mass [6, 7].

Both men and women can suffer from sarcopenia but the prevalence is higher in men [8]. The prevalence of sarcopenia reaches 5 to 13% in older people aged between 60 to 70 years old [9] and it increases in individuals with age of more than 80 years. The progressive loss of muscle strength in women, however, is slower than in men as reported in various studies [5, 6]. A certain degree of divergence was observed between different populations in the world. For instance, about 53% of men and 31% of women in Caucasian population were diagnosed with sarcopenia [8], while a lower prevalence was shown in Taiwan population with values of 26% for men and 19% for women over 80 years old who are sarcopenic [10].

Sarcopenia results in disability, hospitalization, and death [2]. About 14% of elderly aged between 65 and 75 years old lose independence on daily works and nearly 45% of them require support in their daily life when their age reaches 85 years [11]. The severe muscle mass loss and low muscle strength which occur in the elderly aged 85 years old will further lead to frailty resulting in failure in handling even the easiest task [8]. It has been reported that sarcopenia is one of the main contributors for frailty in older people [12, 13]. Frailty is a condition of high susceptibility for unfavorable prognosis including falls, hospitalization, loss of independence, and eventually death.

The duration of hospital admission has been shown to be correlated with hand grip strength of the elderly whereby individual with stronger hand grip strength has shorter hospitalization period [14]. Data from Aging and Longevity Study in the Sirente Geographic Area, Italy (ilSIRENTE Study), showed that older individuals with sarcopenia have three times higher risk of falling during the 2-year follow-up period as compared to nonsarcopenic individuals [15]. Other studies reported a higher risk of mortality among sarcopenic individuals in a 7-year follow-up period [16], while both quadriceps and grip strength have been shown to be correlated with mortality [17]. These findings strongly indicated that sarcopenia increases vulnerability and leads to poor prognosis in aged population.

Current world life expectancy has increased as compared to last century. Latest report forecasts individuals who were born in year 2000 may live until their age reaches 100 years old [18]. Consequently, world aging population will increase as a result of longer lifespan and lower birth rate, especially in developed nations which are expected to have a spectacular increment in the year 2050 (United Nations 2011). Therefore solution to tackle the problems arising from an aging population has to be obtained, especially in relation to debilitating age-related diseases [18]. Researchers predict that the number of sarcopenic individuals will increase fourfold in 40-year time to more than 200 million patients suffering from sarcopenia [3]. The alarming number of sarcopenia patients will not only affect the health and life status of the population but will also increase the healthcare costs of the country. In the year 2000, 1.5% of healthcare expenses in the United States (US) were for the management and treatment of sarcopenia which were around 18.5 billion dollars for both genders [19]. This condition imposed an economic burden but is potentially modifiable if the prevalence of sarcopenia is reduced. With the global aging population drift, sarcopenia has become a major health problem that could not be ignored. Therefore efforts to discover novel interventions which can concurrently prevent and delay the onset and progression of sarcopenia are important.

2. Pathogenesis of Sarcopenia

The underlying cause of sarcopenia remains unknown. Although some researchers claimed that environmental factors play the biggest role during advanced age, the influence of genetics has to be considered [20]. Figure 1 illustrates the various possible underlying factors that contribute to the onset of sarcopenia as reported in several studies.

The search for "sarcopenia genes" has drawn the attention of many researchers. Researches have been extensively done to identify candidate genes for sarcopenia in different populations [21]. Amongst the genes that have been identified to play important roles in the pathogenesis of sarcopenia are hormone and receptors genes such as vitamin D receptor (*VDR*) and androgen receptor (*AR*); growth factors and cytokines genes such as ciliary neurotrophic factor (*CNTF*), myostatin (*MSTN*), and insulin-like growth factor 1 (*IGF1*); structural and metabolic genes such as angiotensin I converting enzyme 1 (*ACE*) and alpha actinin 3 (*ACTN3*) [21]. However, these genes are still inconclusive as candidate gene/s that will accelerate the onset of sarcopenia.

Reallocation of fat mass from fat-free mass (FFM) concurrently occurs with decreased muscle power and fitness in advanced age [22, 23]. The muscle fibers in older adults become thinner and shorter, which cause loss of muscle strength [24]. Type II muscle fibers loss has been reported to significantly contribute to the decline in muscle strength during old age [25].

Skeletal muscle aging is very much associated with loss of muscle regenerative capacity [26]. The number of satellite cells that are responsible for muscle repair declines in the elderly aged above 70 years old [27]. However, aged progenitor cells which were exposed to young systemic environment regain their regenerative capacity [28] indicating loss of permissible environment is imperative to cause age-related shift on muscle rejuvenation rather than the number of satellite cells [29]. There is also the possible role of myogenic regulatory factors (MRFs) which affect the severity of sarcopenia. Increased MRFs expression was observed in senescent muscle which is associated with increasing severity of sarcopenia [30]. These changes could be a compensation mechanism of muscle in response to the loss of muscle mass. In contrast other findings reported a decreased MRFs expression at the early stage of senescent cells differentiation and smaller myotubes were formed from senescent myoblasts suggesting early conditions occurring during muscle aging and its potential to develop into sarcopenia [31].

Changes in the nervous system have been considered as one of the causes of failure in muscle power generation during aging [32]. Different expression of neuromuscular junction genes and proteins has been observed in sarcopenic rats,

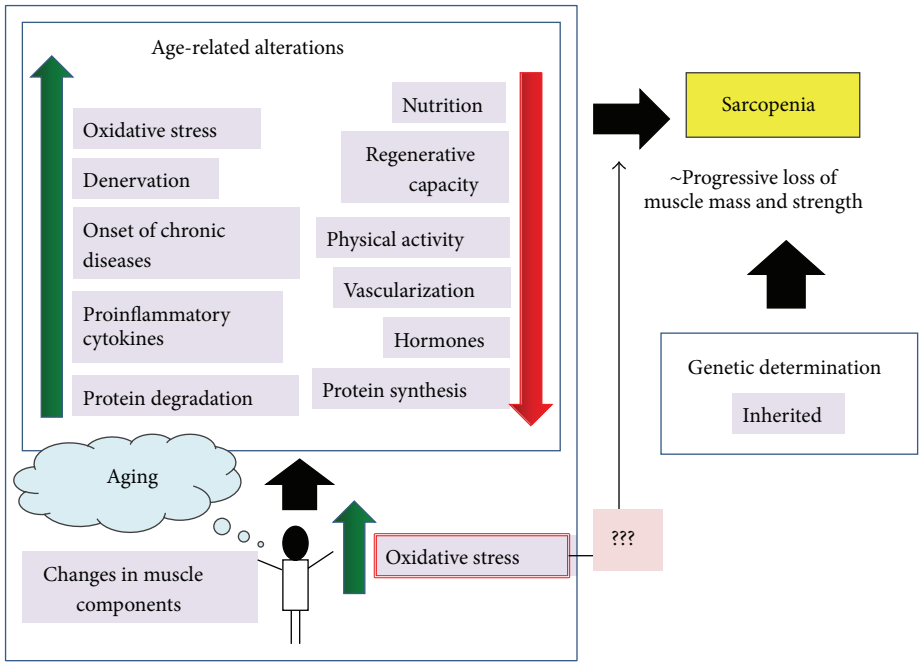

Figure 1: Schematic diagram of the risk factors underlying the progression of sarcopenia. Despite genetic determination, most of the age-related changes are modifiable and can be the target in preventing sarcopenia. Among these factors, increased oxidative stress in aging is more likely to modulate a bunch of signaling cascades that will lead to sarcopenia.

which explains the link between denervation and sarcopenia [33].

Changes of muscle constituent are another important cause of sarcopenia which is interrelated with other factors such as food intake, lifestyle, and chronic diseases including diabetes mellitus and cardiovascular diseases [34]. Other factors may include hormonal changes and the presence of proinflammatory cytokines [35, 36]. High level of proinflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor (TNF) has been reported to reduce muscle mass and strength [36].

Redox and protein imbalance present in advanced age have been reported to cause age-related muscle wasting. Its mechanism is complicated and involves interactions at gene and protein levels. Increased oxidative stress in aging can damage the DNA, protein, and lipid in the muscle. An association between serum protein carbonyls and handgrip strength shown in recent study indicated that oxidative damage affects muscle strength [37]. Sarcopenia may also result from deteriorating anabolic responses in which protein degradation is higher than protein synthesis. At rest, the synthesis rate of myofibril mass is at 0.05% for every hour and this rate increases with nourishment or physical activity in young adults. Similar results, however, were not observed in older adults [38, 39].

3. Role of Oxidative Stress on Human Skeletal Muscle Aging

Skeletal muscle can be a privileged site for oxidative damage as it is the organ with the highest consumption of oxygen in the body. Thus, the correlation between the status of cellular antioxidant defense system and muscle injury can be substantial. Under normal conditions, the resistance of skeletal muscle cells towards oxidative stress is higher than other cell types, such as fibroblasts. Young fibroblast cells that were treated with 150 μM H_2O_2 once or 75 μM H_2O_2 twice in a fortnight developed into stress-induced premature senescence (SIPS) [40]. However, a higher dosage of H_2O_2 (1 mM) was required for premature senescence induction in myoblasts [41]. This may indicate that skeletal muscle has a more established antioxidant defense system which is able to quench a high level of reactive oxygen species (ROS).

Increased ROS or other free radicals in skeletal muscle during exercise were first reported in 1982 [42]. However, ROS production is also increased in the muscle after prolonged immobilization and causes disused muscle atrophy [43]. The contradictory findings observed in these two conditions perhaps could be explained by the different mechanisms involved. For instance, different intensity and duration of ROS production influence the final outcome. A reasonable production of ROS in skeletal muscle in a short period is able to activate cellular defense machinery which provides further protection against oxidative stress. However, different mechanisms are engaged when there is a substantial increase of ROS over a long period of time, resulting in increased protein catabolism and decreased cell survival instead of activation of cellular defense mechanism [44]. Increased free radicals level in aging is congruent with the prooxidative effect of exercise, resulting in a prominent elevation of oxidative stress as expected in aged muscle. Studies have proven that the biomarkers of oxidative stress increased in both

resting and exercise stages in the muscle of older adults [45]. Similarly, in a rodent model, free radicals that are released from isolated mitochondria are higher in older group, either with or without contraction induction [46]. However, reports on the mechanism of antioxidant defense system in the muscle of aged individuals are still inconsistent and not well explained. A few findings indicate decreased enzymatic antioxidant system in the muscle of old individuals as shown by decreased catalase and glutathione transferase activities in the elderly [47, 48], suggesting lower oxidative capacity in aged skeletal muscle. In contrast, increased superoxide dismutase (MnSOD) and catalase activities were observed in old individuals as compared to young [49].

Recent reports have shown increased oxidative damage on protein, lipid, and DNA in human skeletal muscle with increasing age which is congruent with increased lipid peroxidation, protein carbonylation, and DNA damage [45, 47, 49]. The major free radicals that are present in muscle fibers are superoxide anion and nitric oxide which affect the mitochondria, sarcoplasmic reticulum, sarcolemma, cytosol, and transverse tubules [50]. Mitochondria become the main target of oxidative attack, as they act as the primary ROS production factory in the cells. In 1972, the mitochondria theory of aging postulated that increased oxidative stress in aging is responsible for cellular damage [51]. Continuous oxidative attack to the mitochondrial DNA (mtDNA) and other proteins can cause damage and results in mitochondrial dysfunction [52]. Therefore, aging is very much related to age-associated decline in mitochondrial function. Several studies have shown that the degree of mtDNA damage in skeletal muscle is associated with increasing age [49, 53] and changes in mtDNA are linked to the abnormality in aged muscle [53, 54]. Recently, Szczesny and colleagues [55] reported that declined muscle regenerative capacity during sarcopenia is the result of myoblasts vulnerability to oxidative damage. Oxidative insults will disturb the process of mtDNA repair and decrease the viability of myoblasts. Accumulation of incomplete mtDNA repair products in the mitochondria leads to cell death. Since this defect occurs mainly in growing myoblasts, skeletal muscle regeneration capacity is affected and results in sarcopenia [55].

The imbalance between ROS production and antioxidant defense responses modulates the expression of many transcription factors, responsible for shifting protein synthesis to protein degradation and leading to muscle wasting [50, 56]. Accumulation of ROS will activate mitogen-activated protein kinase (MAPK): extracellular signal-regulated kinase (ERK) and p38 [57]. These MAPKs act as the initial contact between cellular surface and nucleus. ROS will induce the signaling cascade of this pathway resulting in a change in skeletal muscle mass and function. MAPK signaling pathway during resting and exercise suggests a degree of divergence between young and old individuals [58]. Older adults have shown higher resting MAPKs, phosphorylating ERK 1/2, p90RSK, Mnk 1, p38, and JNK/SAPK as the muscle is under stress insults, even during the resting state. However, resistance exercise increased phosphorylation in young subjects, but not in old subjects [58]. Principally, ERK is responsible for cell survival, because it can regulate c-Myc, activator protein 1 (AP-1), and B cell lymphoma-2 (Bcl-2) [59], while p53, nuclear factor-κB (NF-κB), and activating transcription factor 2 (ATF2) are the targets for p38. Both NF-κB and AP-1 are important for the activation of cellular adaptive mechanisms. However, these two transcription factors were not activated in the muscle of old mice [60], suggesting the presence of compromised adaptive responses.

NF-κB is involved in both exercise and disuse-induced skeletal muscle adaptive mechanism. In normal condition, NF-κB presents in the cytosol and forms a complex with IκB protein. In the presence of oxidative stress, IκB-α kinase (IKK) will activate and phosphorylate the IκB proteins, unleashing NF-κB, followed by dimerization (with p50 and p65) and nuclear translocation [61]. NF-κB will then activate several genes involved in cell growth, apoptosis, stress responses, and inflammatory processes [62]. NF-κB is also involved in muscle differentiation as indicated by the formation of p65/p50 heterodimer complex which binds to the repressor YinYang1 (YY1) and halts the myogenesis process [63]. Increased NF-κB, p50, and Bcl-3 expression was observed in the muscle of unloaded rats indicating the activation of NF-κB by p50 in disused muscle atrophy [64]. Another study using a transgenic mouse model also reported a similar activation of NF-κB resulting in induction of muscle atrophy [65]. In a muscle-specific transgenic expression of activated IκB kinase β (MIKK) mice model, whereby the IKKβ protein is constitutively active (SS177/181EE mutant), a severe muscle wasting phenotype was observed in both muscles of the limb and trunk, as well as a decline in muscle mass with increasing age [65]. The role of NF-κB in the muscle is further proven by using knockout mouse IKK2^{mko} which is the first NF-κB muscle-specific knockout mice model that has been developed [66]. These mice showed enhanced physical performance and increased resistance to the denervation-induced atrophy [66].

There are contradictory findings reported regarding the effects of exercise where it can either promote or suppress the activation of NF-κB signaling pathway. In a study using rodent, increased NF-κB was reported in the exercised group but decreased in the unexercised group [67]. A concurrent reversible decline of IKK and IκB-α in the cytoplasm was observed, whereby the NF-κB signaling cascade was attenuated with antioxidant treatment [67, 68]. Another study showed that high level of NF-κB during aging can be suppressed by regular exercise [69], while acute fatiguing exercise is able to decrease NF-κB activity in human muscles [70]. These findings indicated that NF-κB plays an important role in the muscle as it can be activated or suppressed which results in either muscle wasting or muscle regeneration.

A significant correlation between ROS, mtDNA damage, and TNF-α [71] was reported. TNF-α stimulates ROS production and may directly cause mitochondrial dysfunction through its receptors [72]. TNF-α is also responsible for NF-κB and MAPKs activation [73]. At the same time, increased free radicals may act as second messenger which induces TNF-α production. It can be postulated that a cycle exists between TNF, mitochondria, and ROS [74]. In myotubes, activation of NF-κB enhanced IL-6 production [75] and

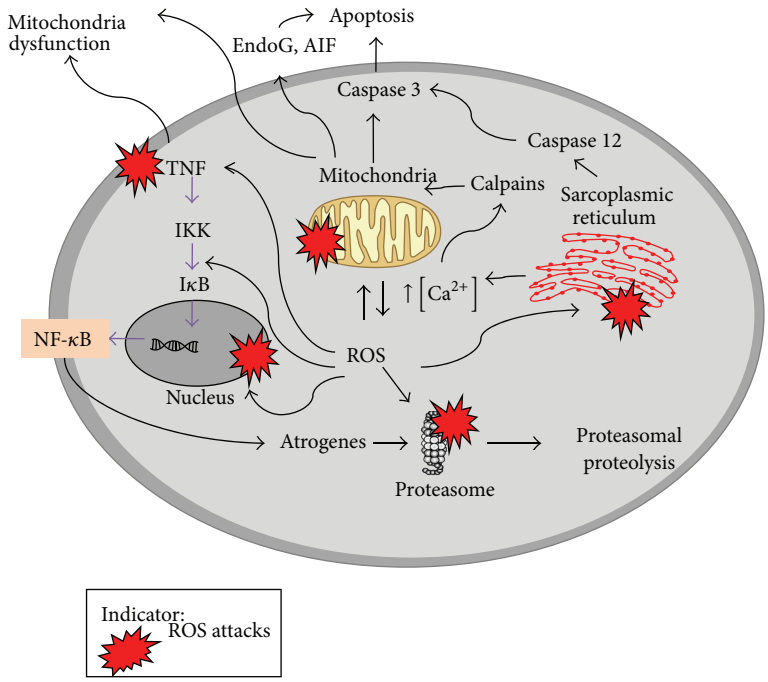

Figure 2: Effects of reactive oxygen species (ROS) on muscle cells. Accumulation of ROS will affect organelles and cell membranes. Alteration on genes and proteins expression leads to muscle wasting during aging. The important mechanisms involved are apoptosis and proteolysis.

resulted in inflammation which is extensive during aging. Since elevated levels of TNF-α and IL-6 are associated with total muscle quality and quantity [36], the role of proinflammatory cytokines on muscle adaptive mechanism in association with redox imbalance may be crucial in the development of sarcopenia.

How does increased activation of skeletal muscle NF-κB in aging lead to muscle atrophy? NF-κB induces muscle breakdown by promoting proteolysis in skeletal muscle. A previous study using a transgenic mice model has shown that NF-κB increased murine ring finger-1 (MuRF-1) by binding to its promoter, supporting the notion of NF-κB regulated ubiquitin-proteasome pathway (UPS) [65]. This pathway can be regulated by ROS and contributes to muscle mass loss [76]. Furthermore, p38 which is affected by ROS was found to increase the expression of E3 ubiquitin ligases (atrogin-1) in myotube [77]. In senescent muscle cells, the expression of E3 ubiquitin ligases, atrogin-1, and MuRF-1 can either increase [78], remain unchanged [79], or decrease [80]. Even though inconsistent results were reported, their roles in sarcopenia are still worth exploring. Researchers suggested that Akt and FOXO may contribute to the decrease of atrogenes expression in the muscle old rats [80]. However, atrogin-1 and MuRF-1 at transcription level may not represent their actual protein concentrations [81]. Findings on the degradation of oxidized protein without ubiquitination in proteasome 20S served as evident that these atrogenes play an important role in accelerating proteolysis in response to oxidative stress [82]. In addition, reactive nitrogen species (RNS) can activate NF-κB and its cascade response on UPS and matrix metalloproteinases (MMPs), as well as degrading muscle-specific proteins in L6 myotubes [83]. Protein catabolism that occurs during oxidative stress via the activation of NF-κB and other proteins leading to muscle wasting is summarized in Figure 2.

Another cellular process prominent in aged skeletal muscle that leads to muscle atrophy and causes elderly to be more susceptible to sarcopenia is apoptosis [84]. Apoptosis occurs following the activation of p53 by ROS, which further induces Bax to promote apoptosis via the activation of procaspase 3 to caspase 3 [85]. Bax translocates into the mitochondria membrane, releasing cytochrome c and activating caspase 3 [86, 87]. Caspase 3 has also been reported to play an important role in disused muscle atrophy [88]. However, a different apoptosis pathway was suggested in a study involving young and old rats as no significant results were obtained with cytochrome c, Bax, and Bcl-2 expression [89]. Increased apoptosis-inducing factor (AIF) and increased caspase 12 in aged muscle were observed in another study indicating that apoptosis during aging is promoted via the activation of mitochondria independent pathway [90]. Another important protein that may contribute to sarcopenia is endonuclease G (EndoG). EndoG is a mitochondria-specific nuclease which is elevated in old skeletal muscle but not in young skeletal muscle [91]. The apoptotic pathway that will be activated in the muscle depends on the type of muscle fiber. It has been demonstrated that high level of TNF-α in aging generates an antiapoptotic response in soleus muscle, but proapoptotic response is generated in vastus lateralis muscle [92]. Thus, further studies are required to elucidate the apoptotic events in aged muscle leading to sarcopenia. Findings from a study on apoptotic events in satellite cells derived from aged adults showed that, compared to young satellite cells, aged satellite cells are more vulnerable to apoptosis at all time points of

Table 1: The potential interventions for sarcopenia.

	Treatments	References
Exercise	PRT	[97]
	aerobic (running)	[98]
Hormone replacement	Testosterone	[102]
	Growth hormone	
Pharmacological intervention	CR mimetics, for example, rapamycin	[104]
	Angiotensin-converting enzyme inhibitors, for example, enalapril	[105]
Diet and nutrition	Protein	[108]
	Vitamin D	[110]
	Antioxidants, for example, vitamins E and C	[114, 115]
Combination	Diet and exercise	[111, 112]

* CR: caloric restriction; PRT: progressive resistance exercise training.

the experiment (4 to 72 hours) with upregulation of *CASP6* and *CASP9* genes, while *FADD* gene is downregulated leading to decreased survival rate of satellite cells which results in decreased muscle regenerative capability [93] and finally muscle wasting.

Activation of MAPK can cause cell death as MAPK regulates calpains, a cysteine protease, and causes cell death through calcium dependent pathway, leading to sarcopenia [94, 95]. An overall increase in calpain activities which is associated with muscle aging was observed in a study involving 3- and 24-month-old rats [94]. This observation suggested the role of calcium (Ca^{2+}) dependent proteolytic pathway in the development of sarcopenia. Apart from the Ca^{2+} channel, ryanodine receptor 1 (RyR1) in sarcoplasmic reticulum may also be involved in the proteolytic pathway since its opening capacity is affected by oxidative stress. This can be explained by an elevated concentration of Ca^{2+} with increasing age in both skeletal muscle and satellite cells [48]. Also the loss of muscle function in the aged group correlated with the permeability of RyR1. This RyR1 channel is more susceptible to oxidation during aging as compared to young rodents [96]. Whilst there is substantial evidence to support a role of ROS in the progression of sarcopenia, the search continues on ways to reverse or prevent this undesirable physiological change during aging.

4. Management of Sarcopenia

Elucidating the underlying mechanisms of sarcopenia may aid in discovering potential interventions which will attenuate the decline in muscle mass and strength in advanced age. The potential interventions of sarcopenia are summarized in Table 1.

Since immobilization or inactivity leads to muscle wasting, exercise or increased physical activity may be an effective approach to improve muscle performance. Exercise intervention such as progressive resistance exercise training (PRT) and low intensity exercise is able to provide positive outcomes on the functions of skeletal muscle of the elderly [97, 98]. By engaging exercise, senior subjects have been shown to improve their balance and decrease their falling rate [99, 100]. Nevertheless, running also exerts neuroprotective effect on exercised muscle as well as modulating the expression of growth-related and muscle breakdown-related genes [98, 101].

A systematic review on the role of testosterone and growth hormone replacement therapies has shown the possibility of replacement therapy in improving muscle endurance in older people [102]. However, concern arises in relation to the safety of these therapies. One of the reported adverse effects is the significant correlation between testosterone replacement therapy and formation of prostate cancer as reported by Baltimore Longitudinal Study on Aging which reported a positive correlation between free testosterone level in the blood with aggressive prostate cancer among older men [103].

Other pharmacological interventions that have been used to combat sarcopenia include treatment with caloric restriction mimetic and angiotensin-converting enzyme inhibitors. Introducing rapamycin as an antisarcopenic agent is also beneficial because low level of mammalian target of rapamycin (mTOR) protein has been reported to cause muscle atrophy in aged Fischer 344 X Brown Norway (F344BN) rats [104]. Also, the usage of angiotensin-converting enzyme inhibitors in preventing age-related physical fitness declining has been documented [105].

Nutrient intervention is another potential strategy to prevent sarcopenia [106]. Older adults normally have lower food intake and are more vulnerable to inadequate nutrient consumption [107]. Therefore, modifying the dietary practice of the elderly based on their body requirement may help in improving their muscle performance. For example, malfunction of anabolic system in the aged group reduced the need for increased protein consumption [108]. Furthermore, the role of vitamin D in the conservation of muscle mass and strength can be imperative. Genetic polymorphisms in the vitamin D receptor (VDR) have been linked to sarcopenia occurrence [109] and supplementation of vitamin D decreases the rate of falls among older adults [110].

While diet and exercise alone can be beneficial in improving physical functions, combining nutrient supplementation and exercise is recommended in determining their synergistic effects on sarcopenia [97]. Combination treatment may involve increased protein intake coupled with exercise. A significant increase in muscle protein synthesis in the

group with high consumption of protein and performing resistance exercise was reported compared to the group with high protein intake alone [111]. This finding is supported by findings from another study which showed an increment in leg muscle mass in a group with physical exercise and nutrient intervention [112].

Another factor that can be considered in preventing sarcopenia is the central cause of aging which is redox imbalance in the muscle cells. Elimination of ROS may provide a rational solution for sarcopenia prevention particularly in aging as it is a condition where the antioxidant capacity decreases. Therefore, researchers have introduced antioxidants as potential intervention for sarcopenia [113]. However, at present, direct evidence that showed the relationship between antioxidant supplementation and muscle aging prevention is scarce, even though a study has shown that vitamins E and C supplementation in aged rodents was able to improve oxidative stress status and muscle function [114]. Another study reported that an antioxidant cocktail which consists of rutin, vitamin E, vitamin A, zinc, and selenium was able to restore the defective leucine stimulation of protein synthesis and function of aged rats, which triggers the application of antioxidants for sarcopenia prevention [115].

5. Vitamin E: A Potential Intervention for Sarcopenia

Vitamin E is a lipid soluble vitamin that exerts antioxidant properties, by scavenging ROS and boosting cellular antioxidative capacity to reduce oxidative damage. It consists of two subgroups which are known as tocopherols and tocotrienols. Both groups have similar fundamental chromanol head but different phytil tail with a saturated phytil tail for tocopherols and unsaturated for tocotrienols. There are four isomers of tocopherol and tocotrienol which are α, β, γ, and δ [116] depending on the number and position of methyl groups. Vitamin E is a potent peroxyl radical scavenger that prevents propagation of free radicals in cell membranes and in plasma lipoproteins. The less reactive tocopheroxyl radical produced will react with vitamin C, thereby recycling the vitamin E to its reduced state [117]. Many researches have shown that vitamin E does not only act as an antioxidant but also act as a signaling molecule. However, its action on signaling pathways is still not well explained [118] and studies are ongoing [119, 120]. The interactions between vitamin E and genes in aging and inflammatory age-related diseases have been documented in many studies, whereby supplementation of vitamin E protected against oxidative stress and inflammation [120]. Even though the role of vitamin E in maintaining muscle health per se is rarely mentioned in the literature, a study has shown the role of vitamin E in repairing dystrophic muscle [121].

Vitamin E can be beneficial for aging prevention and treating infections, atherosclerosis, cardiovascular diseases, cancer, diabetes mellitus, and neurodegenerative diseases [120]. The positive effects of vitamin E in aging have been shown in a randomized, double-blinded, placebo-controlled study. A daily dose of 160 mg Tri E tocotrienol when given to 64 participants aged between 37 and 78 years old for 6 months was found to reduce free radical damage as shown by a decreased percentage of DNA damage, sister chromatid exchange frequency (SCE), and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in aged group (>50 years old) [122]. In the same study, supplementation of Tri E was found to improve health markers especially by decreasing protein damage and improving HDL cholesterol level besides reducing serum advanced glycosylation end products (AGEs) in adults aged above 50 years old [123]. Interestingly, another study reported a correlation between AGEs level and muscle strength among Japanese men. A significant association between increased AGEs and lower muscle strength was observed which requires further clarification [124]. As vitamin E has been found to reduce AGEs, supplementation could lead to delaying or preventing muscle wasting in the elderly.

The association between vitamin E level and sarcopenia has been reported in various studies, as indicated by grip and knee strength. A prospective population-based study of older adults in Italy done between 1998 and 2000 was known as Invecchiare in Chianti (aging in the Chianti area; InCHIANTI) study. In this study, a total of 1156 subjects aged between 65 and 102 years old were recruited using multistage stratified method, whereby 986 participants without daily vitamin supplementation and capable of conducting physical performance and knee extension strength tests were included for analysis. In an unadjusted analysis, vitamin E daily intake level has been shown to positively correlate with knee extension strength and total physical performance score [125]. After the data was adjusted according to plasma concentration and daily intake, a strong correlation was found between γ-tocopherol and muscle strength, as well as a significant correlation between α-tocopherol and both measurements [125]. However, final analysis on the correlation between vitamin E and frailty was carried out on 827 participants in InCHIANTI study after excluding the disabled subjects, subjects with cancer, and subjects with vitamin E supplementation [126]. Since sarcopenia is one of the main contributors to frailty in aged populations, hence the correlation between vitamin E and frailty may indicate the role of vitamin E in sarcopenia. Ble and colleagues found a significantly low circulating vitamin E level in frail individuals as compared to nonfrail individuals, suggesting a higher load of free radicals attack in frail individuals [126].

To date, research findings have shown that a satisfactory supply of dietary vitamin E is essential for maintaining muscle health. Although vitamin E deficiency is seldom reported in human, but, in animal models, severe α-tocopherol deficiency has been found to affect muscle performance [127, 128] which indicates the importance of vitamin E in protecting muscle from oxidative damage during aging. Muscle morphological changes due to vitamin E deficiency have been observed under electron microscope, including mitochondrial degeneration, sarcoplasmic reticulum fragmentation, and accumulation of myelin figures indicating the progression of muscle dystrophy [121]. As a result, various studies have been carried out to further elucidate these histological

observations. Aiming to elucidate the effect of aging and antioxidant deficiency on mitochondrial respiratory chain function in skeletal muscle, a chronic deprivation of vitamin E in rodent model was developed [127]. In this study, rats were fed with a vitamin E-deficient diet for 12, 24, 36, and 48 weeks before their gastrocnemius muscle was obtained for analysis. Data from this study showed accelerated aging in vitamin E-deficient rats as compared to normal rats. In normal rats, increased lipid peroxidation and membrane fluidity of the mitochondria have been observed together with a decrease in cytochrome oxidase and NADH coenzyme Q_1 reductase indicating greater ROS and lipid peroxides production, as well as impaired mitochondria respiratory chain function during aging. Skeletal muscle, however, starts to show structural abnormality after 12 weeks of exposure to vitamin E-deficient diet. The lipid peroxidation level increases, while declined GSH/GSSG ratio was observed, indicating that the vitamin E-deficient rats are experiencing severe oxidative stress insults. The decline in mitochondria respiratory chain function is more obvious in vitamin E-deficient rats suggesting that vitamin E deficiency may accelerate the aging process [127].

With current technologies, researchers are able to identify the changes caused by vitamin E deficiency, especially at the molecular level to facilitate better understanding of the mechanisms involved. In 2006, Nier and colleagues [128] used Fisher 344 rats to determine the effects of vitamin E deficiency at the transcriptome level. Total RNA was isolated from the musculus quadriceps of the hind leg at 5 time points (days 17, 91, 191, 269, and 430 of deficient vitamin E diet). Results showed that more than 56 genes were differentially expressed in response to vitamin E deficiency at 4 consecutive time points (day 91 to day 430). Most of the genes that were differentially expressed encode for proteins responsible for muscle structure, extracellular matrix, growth and development, oxidative stress, inflammation, and protein degradation. These genes were upregulated during vitamin E deprivation, whereby the severity was time dependent. Nier and colleagues [128] suggested that a defense mechanism has been evoked to protect the muscle structure from oxidative stress during vitamin E deficiency. In another model using α-tocopherol transfer protein knockout mice, increased sarcolipin and ubiquitin carboxyl-terminal hydrolase 1 mRNA was observed in ataxia muscle, indicating the effects of vitamin E deprivation on muscle contraction and protein degradation [129]. These findings provide further insight into the molecular mechanisms of vitamin E in the maintenance of skeletal muscle strength and function.

More evidences are emerging to confirm that deficiency of vitamin E leads to muscle atrophy. In other words, supplementation of vitamin E may aid in reversing damaged muscle or at least in maintaining muscle function.

6. Protective Mechanisms of Vitamin E against Muscle Aging

Several *in vivo* and *in vitro* studies using different models of muscle aging have provided data on the protective action

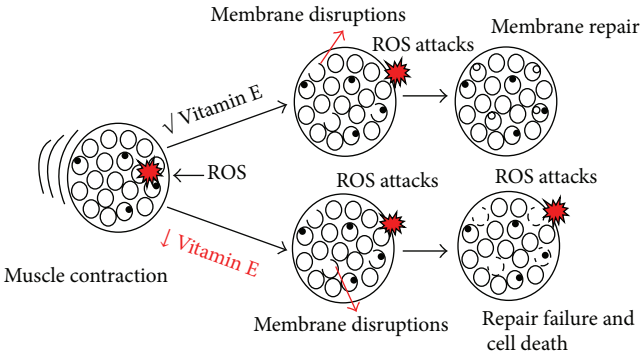

Figure 3: Role of vitamin E on cell membrane repair. During muscle contraction, production of ROS may cause membrane injury which is repaired by vitamin E [131].

of vitamin E against muscle aging. Recently, a study showed that vitamin E (tocotrienol-rich fraction (TRF)) can reverse senescence in a stress-induced presenescence (SIPS) model of myoblasts [130] indicating the potential therapeutic effects of vitamin E on muscle cells, although further studies are required to confirm the mechanism involved. In another study, Howard and colleagues [131] have shown that vitamin E aided the restoration of myoblasts membrane, rather than protecting it from laser-induced membrane disruption. However, this poses questions on actual mechanisms involved.

In the presence of adequate vitamin E levels, skeletal muscle survived, even though there is a massive production of ROS during muscle contraction because vitamin E helps in repairing the myoblasts membrane (Figure 3). In a study using C2C12 myoblasts, Howard and colleagues indicated that the antioxidant property alone is not sufficient to repair the injured myoblast membrane. α-Tocopherol has been reported to be better than other antioxidants as it acts as a stabilizer for the membrane due to its nature lipid soluble properties that allow it to enter the hydrophobic core of plasma membrane. In addition, its chromanol-head group with antioxidant activity can bind to phospholipids head on the membrane surface and scavenges the ROS effectively [131]. As reported earlier, membrane fluidity increases with increasing age, which causes instability of membranes [127]. Therefore, vitamin E acting as a "stabilizer" of the plasma membranes will equally be effective in repairing the damaged membrane during aging as well as in sarcopenic muscle. Besides preventing lipid peroxidation, vitamin E is able to induce Ca^{2+}-triggered fusion events that are involved in membrane repair by stimulating the negative impulsive bend [131].

In human studies, vitamin E has been shown to affect muscle strength of the elderly [126]. Several studies have shown the positive effects of vitamin E in reversing muscle damage during extensive muscle contraction (exercise) in healthy men. Vitamin E supplementation at a dose of 800 IU for 28 days resulted in lowering the expression of oxidative stress markers after a downhill run in both young and older men [132]. In another study, a longer supplementation period (12 weeks of vitamin E supplementation) lowered creatinine

kinase level after exercise in young men, whereas older men showed decreased lipid peroxidation in both resting state and after exercise, indicating that vitamin E promotes adaptation against exercise induced-oxidative stress and reduced muscle damage [133]. In animal models, similar results were obtained. Increased swimming performance and endurance capacity were achieved after vitamin E supplementation [134, 135] besides attenuation of the early onset of muscle weakness in the elderly [134].

Besides being a powerful antioxidant, vitamin E has been shown to act as an anti-inflammatory agent. The role of ROS and inflammatory processes in inducing muscle atrophy has been suggested in several studies. Aoi and colleagues [136] used *in vitro* L6 myotubes and exercise-induced rats for their *in vivo* model demonstrated that myotubes which were exposed to oxidative stress (H_2O_2) showed increased nuclear translocation of NF-κB as well as increased expression of chemokines (cytokine-induced neutrophil chemoattractant-1 (*CINC-1*) and monocyte chemoattractant protein-1 (*MCP-1*)). However, these elevations were prevented by vitamin E treatment. In rat models without vitamin E supplementation, increased neutrophils infiltrated tissues were observed as indicated by elevated neutrophil enzyme myeloperoxidase (MPO), while elevated nuclear translocation component, p65, was demonstrated in gastrocnemius muscle cells. In the vitamin E-supplemented rats, however, significant improvement was seen in MPO, thiobarbituric acid-reactive substances (TBARS), and p65 protein expression after exercise [136] indicating that vitamin E supplementation decreases the activation of cytokines and adhesion molecules. In addition, the expression of a key protein, NF-κB, was attenuated and preventing muscle damage [136]. A more recent report showed that vitamin E reduces inflammation in muscle wasting through NF-κB activation in lipopolysaccharide-(LPS-) induced mice [137] where male *Balb/C* mice were divided into four groups receiving placebo-saline, placebo-LPS, vitamin E-saline, and vitamin E-LPS. Their findings showed that LPS significantly upregulated IL-6 gene and increased protein expression in skeletal muscle. However, vitamin E lowered the expression of IL-6 at both gene and protein levels suggesting that vitamin E may regulate pretranslation level and modulated IL-6 expression as its expression was halted at the mRNA stage. Similar findings were observed for IL-1β, whereby vitamin E attenuated the LPS induced increment of IL-1β at both gene and protein levels. The positive effect of vitamin E supplementation was also observed in the regulation of TNF-α and NF-κB expression [137]. As previously reported, NF-κB was able to stimulate the expression of IL-6 in myotubes [75]. Vitamin E may halt the overall proinflammatory cytokines responses by suppressing NF-κB and thus offering benefits to the population that is suffering from inflammation, such as the elderly [137]. As inflammatory responses affect muscle mass and strength [36], these findings may indicate that muscle weakness in the elderly can be ameliorated by vitamin E through NF-κB suppression.

Mitochondrial dysfunction and apoptosis are very much related to ROS and muscle wasting which are affected by vitamin E supplementation in protecting against skeletal muscle atrophy. Studies have been carried out using male Charles River mice to investigate the effectiveness of vitamin E in maintaining mitochondrial membrane integrity and preventing mitochondrial dysfunction and apoptosis induced by severe high-altitude hypobaric hypoxia in skeletal muscle [138, 139]. These findings demonstrated that vitamin E attenuated the impact of hypoxia on mitochondrial function and apoptotic signaling pathways. Vitamin E prevents mitochondrial alteration, as well as decreasing Bax expression and improving Bcl-2/Bax ratio in skeletal muscle [138, 139]. The apoptotic pathways involving calpains and caspases are also modulated by vitamin E which counteracted muscle wasting. Vitamin E was reported to prevent increases in μ-calpains expression and caspases activity (caspase 3, caspase 9, and caspase 12) in unloading-induced rats which further reduced apoptosis in a stressful condition and thus prevented muscle atrophy [140].

Apart from apoptosis, proteolysis is likely to be involved in the onset of sarcopenia. *In vitro* study using proteolysis-inducing factor (PIF) and angiotensin II- (Ang II-) induced myotubes has shown rapid increment of ROS production and protein degradation in muscle cells. These unfavorable elevations, however, were attenuated by α-tocopherol [141]. Vitamin E inhibited phosphorylation of IκB and suppressed NF-κB activation, which finally attenuated protein degradation. These findings supported the role of ROS, NF-κB, and UPS in the progression of muscle atrophy [76, 141, 142], where NF-κB may be the central transcription factor that is regulated by vitamin E in protecting skeletal muscle against sarcopenia.

Another study demonstrated the ability of vitamin E in counteracting unloading-induced soleus muscle atrophy [140] indicated by decreased severity of muscle atrophy with vitamin E treatment. Vitamin E prevented the decline in type I and type II muscle fiber mass and decreased the level of TBARS, promoted heat shock protein 72 (HSP72) expression, and downregulated proteolysis-related genes. Induction of HSP72 protein by vitamin E induced protective mechanism against muscle wasting and promoted repair of oxidative damaged proteins [140]. These findings also supported the data reported by Russell and colleagues [141], which showed vitamin E prevented proteolysis by decreasing MuRF-1 expression which was elevated during unloaded γ-tocopherols induction [140].

In a study involving a diabetic model, myoblast cells were exposed to high glucose medium for 14 days to induce diabetes [131]. Vitamin E reversed the impaired membrane repair system after diabetes induction as indicated by decreased membrane disruption. These findings are in parallel with the findings from another study which showed that γ-tocopherols prevented glucose oxidase-induced insulin resistance in L6 myotubes [143]. As mentioned previously, diabetes mellitus may be one of the underlying causes of sarcopenia [34]. Thus the promotion of membrane repair and increased insulin sensitivity by vitamin E are important in diabetes mellitus which further prevent the development of myopathy.

The role of another antioxidant vitamin C in muscle repair has been reported in several studies. Vitamin C exerts

its effect by regenerating vitamin E and subsequently maintains the level of vitamin E, which is responsible for muscle protection [131]. A cocktail of antioxidants which consists of vitamin C, vitamin E, and α-lipoic acid improved exercise perfusion after 5 minutes of plantar flexion exercise as well as improving muscle oxidative capacity in the elderly. These findings showed that oxidative stress contributes to age-related decline in skeletal muscle perfusion during physical activity which is improved by antioxidants [144]. In animal studies, vitamins E and C have been shown to exert a protective effect in aged rodents. An improvement in oxidative indices of chronically loaded muscle of aged F344BN rats was observed with vitamins E and C supplementation [114] indicating that combination of vitamins E and C may provide a stable antioxidant supply in aged muscle, subsequently reducing muscle atrophy.

In contrast, other researchers reported that vitamin E alone or in combination with vitamin C did not alter the unfavorable responses in the muscle during exercise [145–147]. In young healthy men, supplementation of vitamin E at a dose of 1200 IU for 30 days did not normalize the elevated creatinine kinase, torque deficit, and the extent of Z-band disruption caused by contraction-induced muscle damage [145]. Eccentric exercise resulted in muscle damage and poor muscle performance and affected blood redox balance and haemolysis despite vitamins E and C supplementation raising a query on the needs of antioxidant supplementation in healthy individuals for protection against muscle damage [147].

In short, several studies have suggested the possible pathways of vitamin E protection against muscle atrophy. Vitamin E is able to combat oxidative stress and stabilized the plasma membrane in protecting the muscle against oxidative insults. Interestingly, the nonoxidative way of vitamin E in preventing inflammation, proteolysis, and apoptosis is also described in various skeletal muscle wasting models.

However, there are contradictory results as well reporting the effects of vitamin E supplementation against muscle atrophy with no or little improvement seen on physical performance indicating inconsistency of findings due to discrepancy in subject recruitment and vitamin E supplementation. A supplementation study showed that 1000 IU of vitamin E given to young and old men who did downhill running did not affect the exercise-induced response in a consistent manner [146]. This finding highlighted the variation in the metabolic system of different age groups. Furthermore, the definition of vitamin E which only includes α-tocopherol in most studies provides unexplored research area especially in skeletal muscle aging for the other 7 isomers of vitamin E especially tocotrienols.

7. Future Prospects of Vitamin E in the Prevention and Treatment of Skeletal Muscle Aging

Most of the studies that have been carried out used α-tocopherols as a representative of vitamin E. However, it did not reflect other possible effects of different isomers of vitamin E in protecting against muscle atrophy and muscle aging. The beneficial effects of tocotrienols beyond tocopherols have been reported in several studies [116]. Hence, the role of tocotrienols in muscle aging prevention should not be ignored.

Comparison between tocopherols and tocotrienols has been carried out on their antioxidant and nonantioxidant properties. Tocotrienols have been reported to be a more potent antioxidant compared to tocopherols due to their structural differences which facilitate cell membrane penetration [148, 149]. Tocotrienols may have higher recycling efficiency and cellular uptake than tocopherols [148, 150]. These features may contribute to tocotrienol superior effectiveness in certain conditions. Corresponding to this, Mazlan and colleagues [151] reported that a lower dose of γ-tocotrienol was able to protect cells from H_2O_2-induced apoptosis, while α-tocopherol requires higher dose to preserve cell viability. Among the evidences which highlighted the importance of tocotrienols in muscle aging prevention is a study that compared the effects of α-tocopherols and tocotrienol-rich fraction (TRF) in forced swimming rats. TRF-treated rats have shown better physical performance and oxidative status than α-tocopherol-treated rats, as indicated by longer swimming period and better antioxidants profile in the muscle [135]. Hence, a certain degree of divergence may be present between the different isomers of vitamin E in their actions on muscle aging. This will further initiate exploration on the molecular mechanism of tocotrienols or TRF in preventing sarcopenia and muscle aging.

The molecular and cellular events modulated by vitamin E in protecting muscle from aging are still unknown. Even though there are studies which reported the possible genes and pathways modulated by vitamin E in various cell types [119], data are still limited, especially studies on skeletal muscle cells. Therefore, more pieces of research should be carried out to explore the role of vitamin E at molecular level in finding novel genes or elucidating the role of NF-κB [141] and HSP72 [140] that has been shown to be affected by vitamin E in the muscle. These proteins may influence the inflammatory and adaptive pathways, which contribute to aging as well as age-related muscle atrophy. With the development of new technologies, molecular research can be very exciting. Recently the role of epigenetics has drawn the focus of many researchers in exploring the molecular mechanism of muscle aging. In skeletal muscle, epigenetics may control or regulate myogenesis. For instance, interaction between Myb-binding protein 1a (Mybbp1a) and miR-546 has been reported to suppress myoblast differentiation [152]. Thus, it is possible that protection of muscle function by vitamin E could be via epigenetics modulation.

8. Conclusion

Sarcopenia is a geriatric syndrome, which is characterised by progressive loss of muscle mass and strength. It may lead to unfavorable consequences, including morbidity and mortality. Understanding the risk factors and its underlying mechanisms is imperative to manage this disease, even

though the theory of oxidative stress in aging is well established. *In vivo* and *in vitro* studies have demonstrated that increased free radicals in aging are a major contributor to sarcopenia development. Thus, the rationale of introducing antioxidants, such as vitamin E, in the prevention and treatment for sarcopenia is justified, even though further studies are required to elucidate its molecular effects.

Vitamin E, either as single isomer or in combination, can be introduced as an antiaging intervention for the muscle. The reduced level of vitamin E in the body has been reported to be associated with increased risk of muscle atrophy. Current evidences indicated that vitamin E can prevent muscle atrophy and promote muscle regeneration, although further investigations are required to confirm molecular mechanisms involved. Other effects of vitamin E, including modulation of signaling pathways, have been proven indirectly in other cell types initiating studies to elucidate molecular effects and antisarcopenic properties.

Most of the studies reported using α-tocopherol as a representative of vitamin E which does not reflect the actual effects of various forms of vitamin E in protecting against muscle atrophy and muscle aging. Hence, the role of tocotrienols in muscle aging prevention warrants further investigation.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

The Role of Nrf2-Mediated Pathway in Cardiac Remodeling and Heart Failure

Shanshan Zhou, Wanqing Sun, Zhiguo Zhang, and Yang Zheng

The Cardiovascular Center, The First Hospital of Jilin University, 71 Xinmin Street, Changchun 130021, China

Correspondence should be addressed to Yang Zheng; zhengyang@jlu.edu.cn

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Heart failure (HF) is frequently the consequence of sustained, abnormal neurohormonal, and mechanical stress and remains a leading cause of death worldwide. The key pathophysiological process leading to HF is cardiac remodeling, a term referring to maladaptation to cardiac stress at the molecular, cellular, tissue, and organ levels. HF and many of the conditions that predispose one to HF are associated with oxidative stress. Increased generation of reactive oxygen species (ROS) in the heart can directly lead to increased necrosis and apoptosis of cardiomyocytes which subsequently induce cardiac remodeling and dysfunction. Nuclear factor-erythroid-2- (NF-E2-) related factor 2 (Nrf2) is a transcription factor that controls the basal and inducible expression of a battery of antioxidant genes and other cytoprotective phase II detoxifying enzymes that are ubiquitously expressed in the cardiovascular system. Emerging evidence has revealed that Nrf2 and its target genes are critical regulators of cardiovascular homeostasis via the suppression of oxidative stress, which is the key player in the development and progression of HF. The purpose of this review is to summarize evidence that activation of Nrf2 enhances endogenous antioxidant defenses and counteracts oxidative stress-associated cardiac remodeling and HF.

1. Introduction

Despite recent advances in treatment, the morbidity, mortality, and economic burden of heart failure (HF) still remain very high. Hypertension, ischemia, diabetes, and some anticancer drugs used in the clinic are common causes of cardiac remodeling and HF. Cardiac remodeling, a term that refers to cardiac maladaptation at the molecular, cellular, tissue, and organ levels, is the key pathophysiological process leading to HF. It has been well established that oxidative stress is a major cause of HF [1–8].

Free radicals and other reactive small molecules have emerged as important regulators of many physiological and pathological processes [9]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) serve as signaling messengers to mediate various biological responses [10, 11], including numerous cardiovascular diseases, such as HF, coronary heart disease, and cardiac arrhythmias [12]. Whether the effects of ROS/RNS are beneficial or harmful depends on the site, type, and amount of ROS/RNS production and the activity of the organism's antioxidant defense system [13]. As a rule, heart and cardiovascular diseases are characterized by ROS overproduction, whereas the formation of major RNS, nitric oxide (a free radical) and peroxynitrite (a diamagnetic molecule), can decrease or increase depending on the nature of the heart injury [12]. ROS include superoxide anion ($\bullet O^{2-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), and hypochlorite ($OCl^{\bullet-}$), which impair cardiac function [14] and increase susceptibility to cardiac arrhythmia [15] through direct toxic effect, resulting in increased necrosis and apoptosis of cardiomyocytes [16]. Critical components of the cellular antioxidative defense mechanisms are the ROS scavengers, phase II detoxification enzymes, and other detoxification proteins which contain antioxidant response elements (AREs) in their promoter regions. A major regulator of the AREs is the highly conserved transcription factor, nuclear factor-erythroid-2- (NF-E2-) related factor 2 (Nrf2). Many of the Nrf2-regulated enzymes are essential in the pathogenesis of cardiovascular diseases [17] and are strongly associated with HF; in addition, they serve as sensitive and specific markers to reflect the ventricular function in HF patients [18]. Nrf2 also can prolong graft survival and

modulate innate and adaptive immune responses after heart transplantation [19]. Emerging evidence has revealed that the Nrf2/ARE signaling pathway plays an important role in preventing oxidative cardiac cell injury *in vitro* [20, 21] as well as protecting the heart from maladaptive remodeling and cardiac dysfunction [7, 22–27].

The main purpose of this review is to discuss the current evidence for the cardioprotective role of Nrf2 and its target genes in the development of cardiac remodeling and HF caused by hypertension, ischemia, diabetes, and anticancer drugs.

2. ROS and Cardiac Remodeling

ROS refer to a group of small reactive molecules that include $O2^{\cdot-}$, H_2O_2, $OH^{\cdot-}$, and $OCl^{\cdot-}$. These molecules react avidly with other molecules such as cellular lipids, proteins, and nucleic acids [28]. The fine balance between ROS generated during normal physiological processes and endogenous antioxidants presented in the body is essential for redox homeostasis. Increased production of ROS or impaired endogenous antioxidant defense of the body that leads to oxidative stress may cause adverse effects due to irreversible modification of macromolecules, membranes, proteins, and DNA [29]. Cardiac remodeling is an adaptive, regulatory process of cardiomyocytes that occurs over time in order to maintain homeostasis against external stress [30]. Progression of cardiac hypertrophy, cardiomyocyte apoptosis, and interstitial collagen deposition into systolic dysfunction has been reported in numerous clinical and animal studies [31–35]. ROS can activate specific pathways leading to adaptive or maladaptive cardiac remodeling processes [28]. In experimental studies, oxidative stress has been identified as one of the molecular mechanisms and the key players involved in the development of cardiac hypertrophy [36]. In addition, antioxidants have been found to prevent the development of cardiac hypertrophy [37]. Various studies have suggested that oxidative stress is also an important regulator of profibrotic processes in the myocardium [34, 35]. NADPH oxidase-dependent ROS production contributes to the development of left ventricular (LV) interstitial fibrosis, reduction of ROS generation, and restoration of the redox balance; thus, it may be important in preventing or treating myocardial fibrosis in HF [38]. Programmed cell death (apoptosis) of cardiomyocytes has been identified as an essential process in the progression to HF [39]. ROS and the resulting oxidative stress also play a pivotal role in this pathological process. Antioxidants and thiol reductants, such as N-acetylcysteine, and overexpression of manganese superoxide dismutase (MnSOD) can block or delay apoptosis [40]. Taken together, ROS play an important role in the development of cardiac remodeling and HF. The findings of the majority of the studies suggest that antioxidants may be possible therapeutic candidates against cardiac remodeling.

3. The Nrf2-ARE Signaling Pathway

Nrf2 is the master regulator of oxidative stress signaling. It is a member of the cap-n-collar (CNC) family of transcription factors, which include Nrf1-3 and Bach1-2 [41–43]. These genes encode transcription factors that belong to the CNC-type of the basic region leucine zipper factor family (CNC-bZIP). CNC-bZIP factors are characterized by a highly conserved 43-amino acid homology region that lies in the immediate N-terminus of the basic DNA-binding domain and is referred to as the "CNC" domain after the *Drosophila* cap-n-collar protein. There is evidence indicating that CNC-bZIP factors function as obligate heterodimers by forming dimers with small Maf proteins (Maf G, Maf K, and Maf F) for DNA binding [44–46].

Under normal conditions, Nrf2 is kept in the cytoplasm by Kelch-like-ECH-associated protein 1 (Keap1) and Cullin 3 [47]. Cullin 3 ubiquitinates its substrate, Nrf2; and Keap1 serves as a substrate adaptor, which facilitates the ubiquitination of Nrf2 by Cullin 3. As a result, Nrf2 has a short half-life that lasts only 20 min under normal conditions [48]. As illustrated in Figure 1, oxidative stress destroys critical cysteine residues in Keap1, disrupting the Keap1-Cul3 ubiquitination system. If Nrf2 is not ubiquitinated, it builds up in the cytoplasm [49] and is translocated into the nucleus. In the nucleus, Nrf2 combines with a small protein called Maf to form a heterodimer, and, by binding to the ARE in the upstream promoter region, it initiates the transcription of a number of antioxidative genes, including heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase (quinone 1) (NQO1), superoxide dismutases (SODs), catalase (CAT), glutathione-S-transferase (GST), γ-glutamylcysteine synthase (γ-GCS), and glutathione peroxidase (GPx) [50, 51] (Figure 1). These are the first line of defense mechanism against ROS-mediated cardiac injury. HO-1 is a rapidly inducible cytoprotective protein that degrades heme to biliverdin, ferrous iron, and carbon monoxide (CO) [52]. HO-1 mitigates cellular injury by exerting antioxidant, antiapoptotic, and anti-inflammatory effects [52, 53]. SOD catalyzes the dismutation of O^{2-} into H_2O_2 and O_2. H_2O_2 is a product of SOD activity and is handled by GPx and CAT. Three SOD isozymes have been identified, including copper/zinc-containing SOD (CuZn-SOD, also SOD1), which is primarily cytosolic in location, Mn-SOD (also SOD2), and extracellular SOD (EC-SOD, also SOD3) [54]. The GPx/glutathione (GSH) system is important in low-level oxidative stress. It scavenges hydroxyl radicals and singlet oxygen directly, detoxifying H_2O_2 and lipid peroxides by the catalytic action of GPx [55]. GPx not only scavenges H_2O_2 but also prevents the formation of other more toxic radicals, such as ·OH [56]. CAT is mainly located in cellular peroxisomes and, to some extent, in the cytosol and catalyzes the reaction of H_2O_2 to water and molecular oxygen [57]. Overexpression of mitochondrial CAT has shown protection against cytotoxic drugs [58].

Through inducing the expression of this battery of genes, Nrf2 is able to augment a wide range of cell defense processes, thereby enhancing the overall capacity of cells to detoxify potentially harmful substances. As such, the Nrf2-Keap1 pathway is generally considered to be a major cellular defense pathway. In addition, recent experimental and clinical studies have shown the important roles that Nrf2 and its downstream

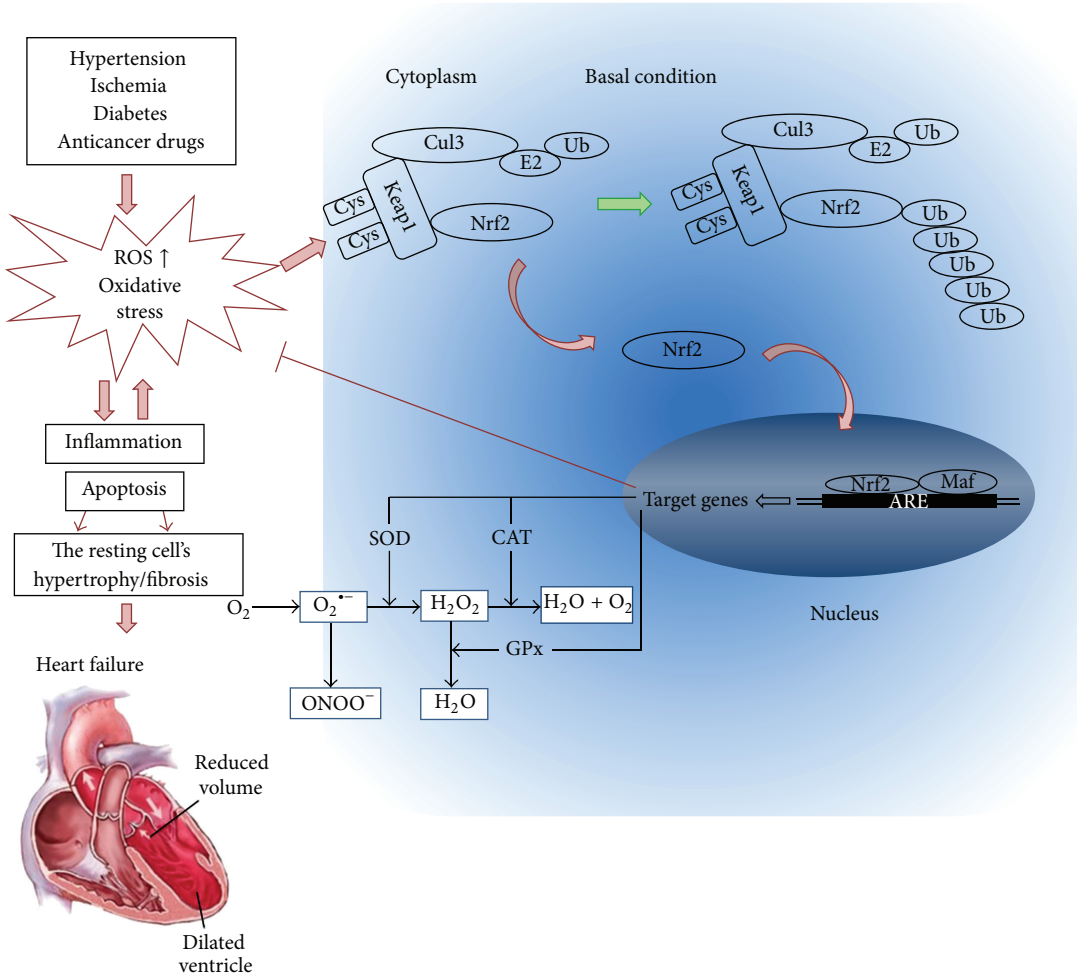


FIGURE 1: Roles of oxidative stress in cardiac remodeling and the potential protection by Nrf2 from oxidative damage. Hypertension, ischemia, diabetes, and anticancer drugs all induce additional generation of reactive oxygen and/or nitrogen species (ROS and/or RNS), leading to oxidative stress. Oxidative stress accelerates inflammation and apoptosis, which in turn causes cardiomyocyte hypertrophy and/or fibroblast proliferation, resulting in the cardiac remodeling (fibrosis). Meanwhile, ROS and/or RNS interact with cysteine residues in Keap1, disrupting the Keap1-Cul3 ubiquitination system. At the early stage of these pathological conditions, the released Nrf2 from Keap1 translocates to nucleus and combines with Maf and ARE to initiate the transcription of a number of antioxidative genes, such as SOD, CAT, and GPx, which are performing a wide range of cell defense processes against this pathological oxidative stress in the heart; however, at the late stage, Nrf2 may be exhausted or downregulated by its abnormal Nrf2 gene expression, leading to the failure to maintain the redox homeostasis by increasing ARE-mediated expression of phase II and antioxidant enzymes. Consequently, the persistently oxidative stress induces cardiac remodeling and finally heart failure.

genes play in the pathogenesis of cardiac remodeling and HF induced by a number of factors.

4. Role of the Nrf2 Pathway in Hypertension-Induced Cardiac Remodeling

Many factors are involved in the pathophysiology of hypertension, such as upregulation of the renin-angiotensin-aldosterone system and activation of the sympathetic nervous system [59]. Common to these processes are the increased bioavailability of ROS due to excess ROS generation, decreased nitric oxide level, and reduced antioxidative capacity in the cardiovascular, renal, and nervous systems [60, 61]. In the cardiovascular system, ROS activate a broad variety of hypertrophy-promoting kinases and transcription factors, and oxidative stress has been identified as one of the key contributing factors in the development of cardiac hypertrophy [29]. In a pathologically hypertrophied myocardium, fibrosis and induction of fetal gene expression promote the development of cardiac dysfunction [62–65]. Activation of Nrf2 and its target genes provides a novel mechanism to protect the heart against pathological cardiac remodeling through suppressing oxidative stress [7, 25, 26, 66].

After transverse aortic constriction (TAC), Nrf2 expression was transiently increased and then declined to the basal level [7]. Compared to wild-type mice, Nrf2-knockout mice after TAC developed pathological cardiac hypertrophy, significant myocardial fibrosis and apoptosis, overt

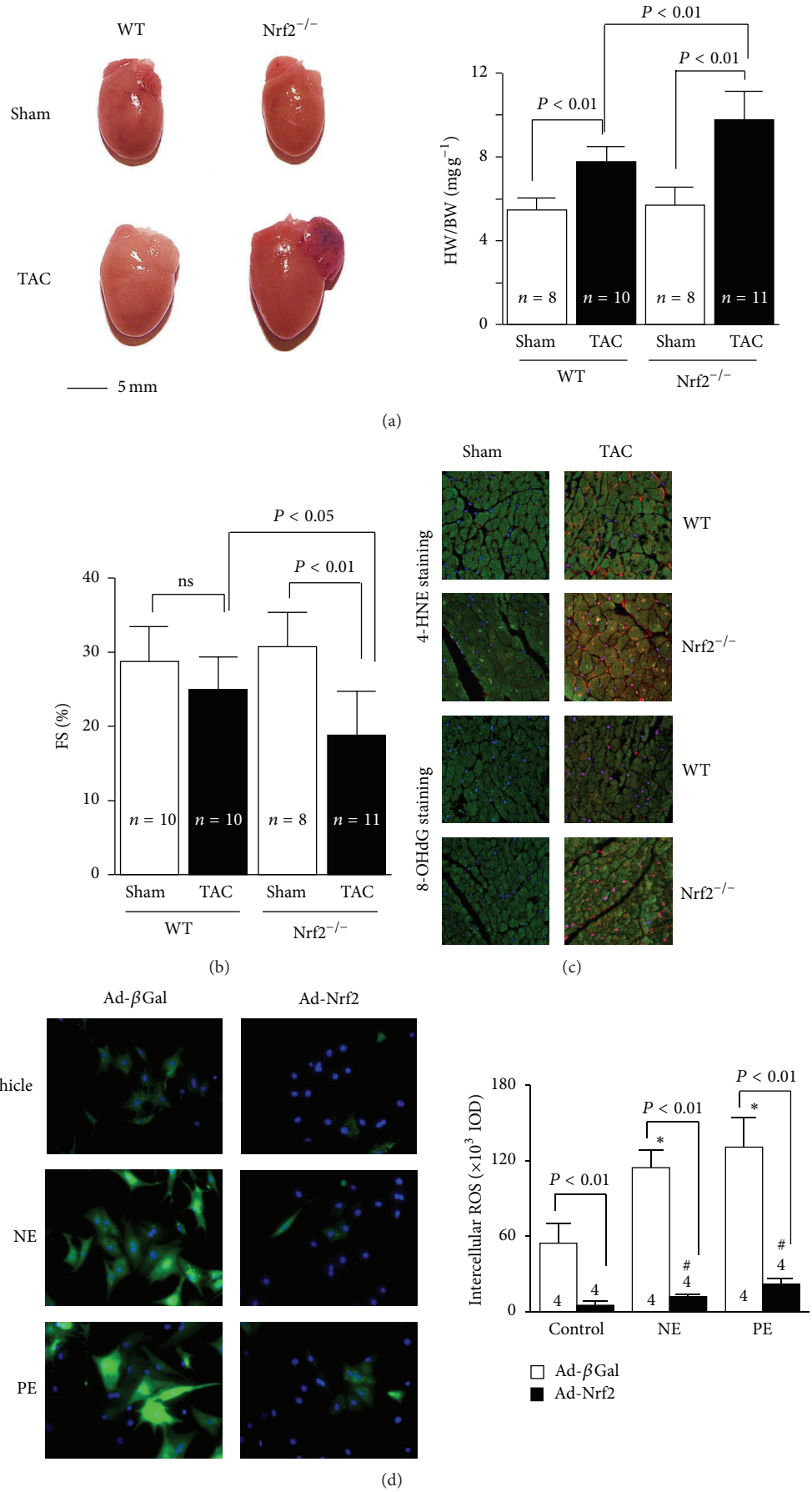
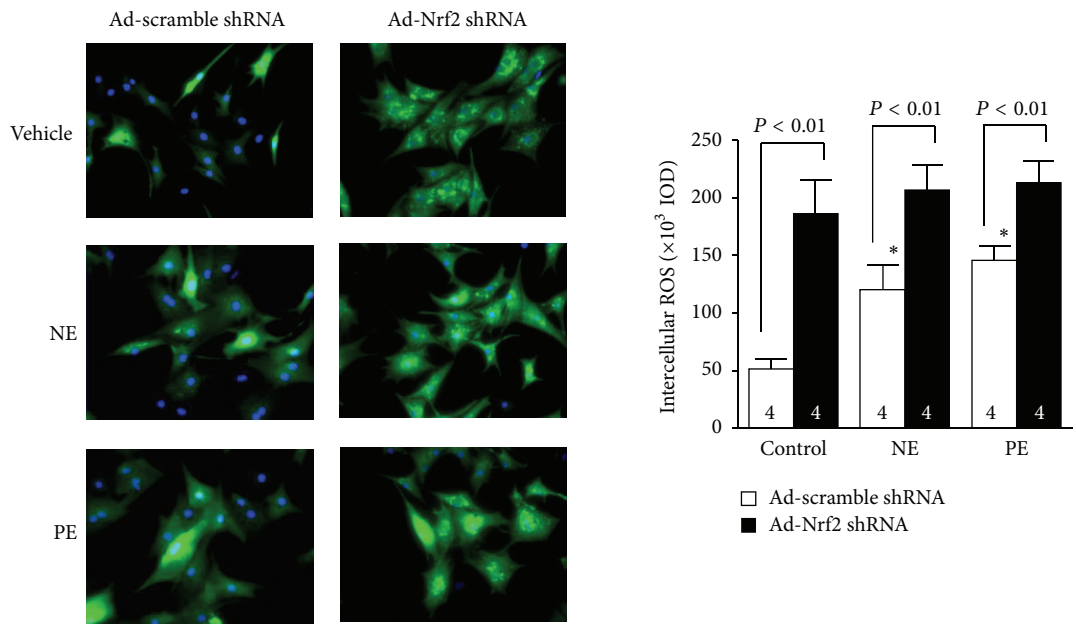


Figure 2: Continued.



(e)

Figure 2: Nrf2 protects against maladaptive cardiac responses to hemodynamic stress. (a) The left panel shows representative pictures of hearts of wild-type (WT) and $Nrf2^{-/-}$ mice after TAC. The right panel shows the heart weight/body weight (HW/BW) ratio. (b) Left ventricle fractional shortening (FS) (%) of WT and $Nrf2^{-/-}$ mice 2 weeks after TAC. (c) Representative confocal microscopic images of LV 4-HNE staining. 4-HNE-positive staining is shown in red. Nuclei are shown in blue (×630). Nrf2 gain- and loss-of-function on hypertrophic factor-induced ROS production in rat neonatal cardiomyocytes. Representative images and quantitative analysis of intracellular ROS production in norepinephrine (NE, 200 μmol/L) or phenylephrine (PE, 100 μmol/L) treated cardiac myocytes that were infected with adenovirus of Nrf2 (d) or rat Nrf2 shRNA (e). $^{*}P < 0.05$ or $^{\#}P < 0.05$ versus control ad-βGal- or ad-Nrf2-infected cells that were treated with vehicles. The combined figures here were collected by the authors from different figures published in the study by Li et al. [7].

HF, and increased mortality, which were associated with elevated myocardial levels of 4-hydroxy-2-nonenal and 8-hydroxydeoxyguanosine and a complete blockade of the myocardial expression of several antioxidant genes. Overexpression of Nrf2 dramatically inhibited hypertrophic factor-induced ROS production and growth in both cardiomyocytes and cardiac fibroblasts, whereas knockdown of Nrf2 exerted the opposite effects in both cells [7]. Thus, Nrf2 is a critical regulator for maintaining structural and functional integrity of the heart that is abnormally stressed, as shown in Figure 2. Activating transcription factor 3 (ATF3) is a cAMP response element-binding protein/ATF family transcription factors member and has been implicated in the cardiovascular and inflammatory systems [67]. Infusion of tert-butylhydroquinone (tBHQ), a selective ATF3 inducer, increased the expression of ATF3 via the Nrf2-related transcriptional factor, inhibited transverse aortic banding-induced cardiac dilatation, and increased LV contractility, thereby rescuing from HF [67].

In addition, HO-1 protects against cardiac hypertrophy [24, 25, 68]. For example, Hu et al. reported that HO-1 attenuated angiotensin II- (Ang II-) induced cardiac hypertrophy both *in vitro* and *in vivo* [25]. Furthermore, Ndisang and Jadhav reported that upregulating the heme oxygenase system suppressed LV hypertrophy in spontaneously hypertensive adult rats and was accompanied with attenuated extracellular matrix remodeling, whereas HO-1 was blocked with chromium mesoporphyrin-exacerbated cardiac fibrosis/hypertrophy [68]. Deletion of Bach1 caused upregulation of cytoprotective HO-1 and also inhibited TAC-induced LV hypertrophy and remodeling [69]. Another group reported that HO-1 gene transfer to cardiomyocytes attenuated Ang II-mediated apoptosis but not hypertrophy [70]. However, Chen et al. reported that, after TAC, more calcineurin protein expression was induced in systemic HO-1 transgenic overexpressing mice than in wild-type mice, and it aggravated pressure overload-induced cardiac hypertrophy [71]. Therefore, the role of HO-1 in pressure overload-induced cardiac remodeling is controversial and further investigation is needed.

Myocardial SOD activity was suppressed in LV remodeling induced by TAC [26, 72]. In addition, Ang II could stimulate collagen production and in the meantime inhibit total SOD, SOD1, and SOD2 activity in rat cardiac fibroblasts [73]. Reduced expression of the manganese-dependent SOD2 resulted in increased cardiac oxidative stress and induced maladaptive cardiac hypertrophy [74]. Moreover, SOD3 deficiency exacerbated TAC-induced myocardial oxidative stress, hypertrophy, fibrosis, and HF [26]. These findings demonstrate that activation of Nrf2 and its downstream genes provides a novel mechanism to protect the heart against pressure overload-induced pathological cardiac hypertrophy, fibrosis, apoptosis, and HF via suppressing oxidative stress.

5. Role of the Nrf2 Pathway in Ischemia-Induced Cardiac Remodeling

Coronary artery disease and ischemic heart disease are prevalent worldwide. Cardiac hypertrophy, apoptosis, and fibrosis after myocardial infarction (MI) have been identified as key detrimental factors in the development of HF. The development of percutaneous coronary intervention and surgical revascularization has brought marked benefits to patients with acute MI. However, ischemia/reperfusion (I/R) injury during revascularization can cause further cardiac injury [75, 76]. Once blood flow is restored, ROS can be produced either by the myocardium itself or by infiltrating inflammatory cells [77]. ROS can subsequently lead to cellular damage through a number of pathways, including direct damage to membranes and proteins or indirect damage through activation of proapoptotic pathways [77]. This damage can further cause cardiac remodeling which leads to progressive heart chamber dilation, ventricular wall thinning, and eventually HF [78]. Nrf2 and its target genes have been shown to play a protective role in cardiac ischemia-associated injury [79, 80].

Some antioxidants protect the heart from ischemia-induced cardiac injury via the Nrf2 pathway. For example, α-lipoic acid and prostaglandin D2 significantly increased Nrf2 nuclear translocation and the expression of its downstream genes reduced lactate dehydrogenase (LDH) and creatine kinase (CK) release, attenuated myocardial infarct size, decreased cardiomyocyte apoptosis, and partially preserved heart function; and this effect was at least partially PI3K/Akt signaling pathway dependent [79, 81]. The phenomenon of ischemic preconditioning (IPC) has been recognized as one of the most potent mechanisms to protect against myocardial ischemic injury, reducing infarct size, attenuating the incidence and severity of reperfusion-induced arrhythmias, and preventing endothelial cell dysfunction [82]. In rat hearts, 30 minutes of left anterior descending coronary artery occlusion resulted in a reduction in the Nrf2 protein level, which was prevented by IPC of the myocardium [83]. Recently, Zhang et al. [23] reported IPC-induced activation of protein kinase C, which then promoted the translocation of Nrf2 and the inductions of antioxidant genes HO-1 and MnSOD and decreased tissue malondialdehyde content compared to control hearts.

Prior studies have reported that HO-1 is upregulated in human failing hearts [84] and in animal models of right ventricular failure [85]. The HO-1 expression in the noninfarct myocardium was increased four weeks after coronary ligation, and cardiomyocyte-specific HO-1 transgenic mice showed improved postinfarction survival and attenuated cardiac hypertrophy, interstitial fibrosis, oxidative stress, and apoptosis [86, 87]. Heterozygous HO-$1^{+/-}$ mice exhibited exaggerated cardiac injury and dysfunction after I/R, patially rescued by antioxidants [88]. In contrast, mice with cardiac-restricted HO-1 overexpression were resistant to I/R injury, with improved contractile recovery and reduced infarct size, inflammatory cell infiltration, oxidative damage, and apoptosis [89]. Similar results were obtained in rat hearts subjected to I/R 8 weeks after human HO-1 gene transfer [90]; the improvement in LV function was maintained for up to 1 year after injury [91]. Kuzuya et al. [92, 93] have shown that the infarct limitation observed in the canine myocardium 24 hours after IPC was accompanied by a significant increase in SOD2 activity. In addition, a recent study demonstrated that SOD3 expression was decreased in MI-induced HF [54]. SOD3-knockout mice had greater increases of nitrotyrosine in the peri-infarct myocardium, and this was associated with a greater reduction of LV ejection fraction, a greater decrease of sarcoplasmic or endoplasmic reticulum $Calcium^{2+}$ ATPase, and a greater increase of atrial natriuretic peptide in the peri-infarct zone compared to wild-type mice at 8 weeks after MI, which means that mice with SOD3 gene deletion developed more severe LV hypertrophy, more fibrosis, and worse cardiac function [54]. Moreover, it has been demonstrated that GPx-knockout mice were more susceptible to myocardial I/R injury and transgenic mice were more resistant to myocardial I/R injury [94–96]. Overexpression of GPx also attenuated post-MI cardiac failure and cardiac remodeling by decreasing myocyte hypertrophy, apoptosis, and interstitial fibrosis in the noninfarct LV [56], which may be related to GPx preserving electron transport chain complex activities [96]. Overexpression of Nrf2 and its downstream genes inhibited ischemia-induced LV remodeling and HF; thus, therapies designed to interfere with oxidative stress might be beneficial to prevent ischemia-induced heart injury.

6. Role of the Nrf2 Pathway in Diabetes-Induced Cardiac Remodeling

Diabetic cardiomyopathy is a very common complication of diabetes and also one of the major causes of mortality in diabetic patients [97]. Evidence suggests that diabetes is associated with a reduced overall antioxidant defense system and increased oxidative stress [98]. Extra production of ROS and RNS causes the development of diabetic complications, including cardiomyopathy; on the other hand, antioxidants can prevent or treat diabetic complications [97, 99, 100]. Therefore, the activation of endogenous antioxidative components has been proposed as an appealing strategy to alleviate diabetic complications [101]. Nrf2 and its target genes may prevent or inhibit this pathological process through their antioxidative stress properties.

Emerging evidence has indicated that high glucose (HG) not only induces ROS and/or RNS production, but also enhances the expression and activation of Nrf2 and its downstream genes, both *in vivo* and *in vitro* [103, 115]. However, Tan et al. have reported a different finding [116]. In tissue sections of left ventricles obtained from autopsied heart specimens of humans with or without diabetes, Nrf2 expression in the nuclei was significantly downregulated compared to control hearts [116]. Tan et al. also demonstrated that Nrf2 protein expression was slightly increased in the hearts of mice with two-month hyperglycemia but significantly decreased in the hearts of mice with five-month hyperglycemia [116], which suggests that Nrf2 was adaptively overexpressed to combat diabetic damages at the early stage of diabetes; at the late

stage, however, cardiac antioxidant function was so severely impaired that it led to a decrease in cardiac Nrf2 expression [117]. Glucose at high concentrations induced ROS production in both primary neonatal and adult cardiomyocytes from the Nrf2 wild-type mouse heart, whereas, in Nrf2-knockout cells, the amount of ROS was significantly greater under basal conditions and high glucose markedly further increased ROS production in concentration- and time-dependent manners. Concomitantly, high glucose induced significantly greater levels of apoptosis at lower concentrations and in a shorter time in Nrf2-knockout cells than in wild-type cells [115].

HO-1 and its reaction products have been shown to have both antioxidative and anti-inflammatory properties [89]. Association of a microsatellite polymorphism in the promoter region of the human HO-1 gene with the risk of coronary artery disease in type 2 diabetic patients has been reported [118]. Patients with type 2 diabetes with longer repeats of the HO-1 gene promoter (with lower HO-1 inducibility) were shown to have more oxidative stress and increased susceptibility to coronary artery disease [118]. In streptozotocin- (STZ-) induced diabetes rodent models, overexpression of HO-1 ameliorated LV dysfunction, myofibril structure disarray, oxidative stress, inflammation, apoptosis, and autophagy [119]. Furthermore, deficiency of HO-1 significantly increased the infarct size in normoglycemic mice and exacerbated myocardial infarction in diabetic mice [120]. In addition to a larger infarct size, mortality was two-fold greater in diabetic HO-1-knockout mice than in wild-type mice after I/R injury, and 55% of the diabetic HO-1-knockout mice survived LV thrombi induced by I/R [120]. Myocardial SOD activity and the GSH level were also significantly increased in the hearts of diabetic rats [114]. Overexpression of SOD2 protected the cardiac morphological changes induced by diabetes and completely normalized the impaired contractility in diabetic cardiomyocytes [121]. Moreover, the plasma type of GPx, GPx-3, was significantly upregulated in diabetic mice compared with control mice [122]. Furthermore, GPx overexpression inhibited the development of LV remodeling and diastolic dysfunction associated with diabetes [27]. These beneficial effects of GPx overexpression are thought to be associated with the attenuation of hypertrophy, apoptosis, and interstitial fibrosis of cardiomyocytes [27]. Thus, Nrf2 and its downstream genes play an essential protective role in the adaptation of diabetic cardiomyopathy. Taken together, these studies may provide a new target for developing therapeutic strategies to treat/prevent diabetic cardiomyopathy.

7. Role of the Nrf2 Pathway in Anticancer Drug-Induced Cardiac Remodeling

There are rising concerns among both cardiologists and oncologists about cardiotoxicity induced by cancer treatment, since it has a significant impact on the management and outcomes of cancer patients. The most typical manifestation of cardiotoxicity is hypokinetic cardiomyopathy leading to HF [123]. Nrf2 and its target genes have been suggested to be useful for protection against cardiotoxicity of anticancer drugs [124, 125].

Anthracycline anticancer drugs (e.g., doxorubicin or daunorubicin) can induce chronic cardiotoxicity and HF, both of which are believed to be caused by oxidative injury and mitochondrial damage [126]. GPx activity was significantly increased in both a daunorubicin-induced rabbit HF model and rat cardiomyocytes exposed to daunorubicin [127]. Incubation of H9c2 rat cardiomyoblasts with doxorubicin resulted in a two-fold increase in Nrf2 protein content and enhanced transcription of several of the Nrf2-regulated downstream genes, including GSTp1 and NQO1 [128]. Chronic arsenic exposure also has been linked to increased risks of vascular diseases. Arsenic exposure affects the activity of Nrf2 [125], and HO-1 has been identified as a response biomarker [129]. H9c2 rat cardiomyocytes exposed to arsenic showed a modest activation of Nrf2 and lower GSH availability [130]. However, the results have been contrastive. Recently, in a daunorubicin-induced rabbit HF model, Jirkovský et al. showed that Nrf2 and its target genes did not show upregulation and that global oxidative stress may not be a factor for the development of anthracycline-induced HF [131]. Therefore, the role of Nrf2 and its target genes in anticancer drug-induced cardiotoxicity is still controversial and more investigations are needed.

8. Potential Clinical Interventions of Cardiac Remodeling by Targeting Nrf2 and Its Target Genes

The exploration of mechanisms underlying the regulation of the Nrf2 pathway has led to the development of agents manipulating Nrf2 to treat HF. In fact, compounds that increase the activity of Nrf2 and its downstream genes are currently being tested for disease prevention [111, 132–134] (Table 1).

8.1. Activation of Nrf2 to Treat Cardiac Remodeling and HF. Many Nrf2 activators are natural products and plant-derived phytochemicals. Some examples of natural Nrf2 activators include sulforaphane, curcumin, resveratrol, and garlic organosulfur compounds [135]. Several synthetic Nrf2 activators have also been developed, such as carbobenzoxy-Leu-Leu-leucinal (MG132), 4-hydroxynonenal (4-HNE), α-lipoic acid, hydrogen sulfate, and 17β-estradiol (E2) [79, 109, 110]. These chemopreventive compounds exert their effects by inducing an Nrf2-mediated defense response as well as activation of phase II detoxification enzymes, antioxidants, and transporters that protect cells from oxidative stress.

8.1.1. Natural Nrf2 Activators. One of the most extensively studied natural products that target the Nrf2-Keap1 signaling pathway is sulforaphane, an isothiocyanate present in cruciferous vegetables such as broccoli [136]. Treatment with sulforaphane decreased infarct size, inhibited an increase in the LV end-diastolic pressure, and improved coronary flow in mice after MI [102]. This protective effect may be partly mediated through HO-1, SOD, and CAT expression [102].

Table 1: Activation of Nrf2 and its downstream genes to treat cardiac remodeling and HF.

	Name	Target disease model	Species	Function	Ways and volume	References
Nrf2 activators	Sulforaphane	Ischemia (I/R, Langendorff)	SD rats	Prevent cardiac apoptosis	0.5 mg/kg/d, 3 days, IP	[102]
		Diabetes (type 1, STZ)	Mice	Prevent cardiac hypertrophy, fibrosis, and apoptosis	0.5 mg/kg/d, 3 months, IP	[103]
	MG132	Hypertension (abdominal aortic banding)	SD rats	Prevent cardiac hypertrophy and fibrosis	0.1 mg/kg/d, 8 or 16 weeks, IP	[104, 105]
		Diabetes (type 1, OVE)	Mice	Prevent cardiac hypertrophy and apoptosis	10 μg/kg/d, 3 months, IP	[106]
	Resveratrol	Ischemia (MI, LAD occlusion)	SD rats	Improve cardiac stem cells' survival, proliferation, and differentiation	2.5 mg/kg/d, 2 weeks, gavage	[107]
	Allicin	Hypertension (Ang II)	SD rats	Prevent cardiac hypertrophy and fibrosis	180 mg/kg/d, 8 weeks, receiving a diet in addition to standard chow	[108]
	α-Lipoic acid	Ischemia (I/R, coronary artery ligation followed by reperfusion)	SD rats	Prevent cardiac apoptosis	15 mg/kg, one time, IV	[79]
	Hydrogen sulfate	Ischemia (I/R, coronary artery ligation followed by reperfusion)	Mice	Prevent cardiac apoptosis	0.1 mg/kg, one time, IV	[109]
	4-HNE	Ischemia (MI, Langendorff)	Mice	Improve cardiac function	4 mg/kg, one time, IV	[110]
HO-1 activators	CoPP	Hypertension (Ang II)	Wistar rats	Prevent cardiac hypertrophy	1 mg/kg, every 2 days, 2 weeks, IP	[25]
		Hypertension plus ischemia (spontaneous hypertensive rats + LAD occlusion)	Wistar rats	Prevent cardiac hypertrophy	4.5 mg/kg, one/week, 6 weeks, IP	[111]
	Hemin	Ischemia (MI, LAD occlusion)	SD rats	Prevent cardiac apoptosis	4 mg/kg, every 2 days, 4 weeks, IP	[112]
SOD activators	EUK-8	Hypertension (transverse aortic banding)	Mice	Prevent cardiac hypertrophy, fibrosis, and apoptosis	25 mg/kg/d, 4 weeks, IP	[113]
	N-Acetylcysteine	Diabetes (STZ)	Wistar rats	Improve cardiac function	1.4-1.5 g/kg/d, 8 weeks, in drinking water	[114]

Curcumin is another well-studied chemopreventive natural product that is capable of activating Nrf2 [132]. Curcumin has been used to attenuate acute doxorubicin-induced cardiomyopathy in rats [133]. In this study, curcumin pretreatment reversed the increase in lipid peroxidation and CAT content, as well as the decrease in GSH content and GPx activity in rat cardiac tissues induced by doxorubicin [133].

By treating rat cardiac stem cells with resveratrol in a rat left anterior descending occlusion model, Gorbunov et al. found that resveratrol significantly improved cardiac function through enhancing Nrf2 expression, as well as significantly increasing the survival and engraftment of implanted cardiac stem cells in the host [134]. Resveratrol can also achieve the same effect by gavage in the same animal model [107].

In an Ang II-induced cardiac remodeling and HF rat model, allicin treatment could prevent the development of cardiac remodeling and the progression of cardiac hypertrophy to cardiac dysfunction, by enhancing the Nrf2 pathway [108]. In addition, dietary phytochemical intake could upregulate the cardiac Nrf2 transcriptome and reduce oxidative damage and HF in hypertensive rats [137]. The protective effect of Nrf2 in myocardial remodeling and HF has been suggested to be mediated through Nox4 [138], which is known to be an important regulator of reduction-oxidation signaling in many cell types including cardiomyocytes [139].

8.1.2. Synthetic Nrf2 Activators. Accumulating evidence has demonstrated that MG132 can protect cells and tissues against oxidative damage, since it can activate the Nrf2-ARE signaling pathway, leading to upregulation of detoxifying and antioxidant genes [140, 141]. Both sulforaphane and MG132 could prevent diabetes-induced high blood pressure and cardiac dysfunction, as well as cardiac hypertrophy, fibrosis, oxidative damage, and inflammation [103, 106]. In pressure-overload-induced rodent HF models, treatment with MG132 significantly attenuated cardiac hypertrophy and cardiac fibrosis as well as improving cardiac function [104, 105]. Mechanistically, MG132 may enhance Nrf2-mediated antioxidative function and inhibit NF-κB-mediated inflammation [142].

4-HNE is an α, β-unsaturated hydroxyalkenal that is produced by lipid peroxidation in cells. α-Lipoic acid is an organosulfur compound derived from octanoic acid. E2 is a sex hormone. All three of these compounds could protect the heart from ischemia-induced cardiac remodeling and HF by activating Nrf2 and its target genes. Treatment with 4-HNE [110], α-lipoic acid [79], or hydrogen sulfate [109] could activate Nrf2 in the heart and increase the intramyocardial GSH content, consequently improving the functional recovery of the LV following I/R in Langendorff-perfused hearts [110] or reducing infarct size, decreasing cardiomyocyte apoptosis *in vivo* [79, 109]. The cardioprotective effect of 4-HNE was not observed for Nrf2-knockout mice [110], indicating that this protection is Nrf2 dependent. In an *in vitro* study, Yu et al. cultured primary cardiomyocytes and established a hypoxia/reoxygenation (H/R) model to simulate myocardial I/R injury [143]. They found that E2 could upregulate Nrf2 expression in nuclear extracts and also increased the expression of HO-1, SOD1, GST, and glutamate-cysteine ligase (GCL) dramatically during H/R injury [143]. These findings indicate that Nrf2 plays a pivotal role in preventing and alleviating cardiac I/R injury-induced oxidative stress.

8.2. Role of Nrf2 Target Genes Activation in Cardiac Remodeling

8.2.1. Role of HO-1 Activation in Cardiac Remodeling. It has been reported that elevation of HO-1 by treatment with cobalt protoporphyrin IX (CoPP IX) or a recombinant adenovirus carrying the human HO-1 gene attenuated cardiac hypertrophy and apoptosis, in both an Ang-II-induced HF model and a spontaneously hypertensive rat model [25, 70], while this pathological process was exacerbated in the presence of tin protoporphyrin, an inhibitor of HO activity [70]. In a spontaneous hypertensive rat model, HO-1 upregulation by CoPP IX administration decreased blood pressure, inhibited inflammation, and improved the ventricular remodeling process and postinfarct cardiac function [111]. In addition, cardiac stem cells (CSCs) were protected by pretreatment with CoPP against apoptosis through activation of the extracellular signal-regulated kinase (ERK)/Nrf2 signaling pathway and cytokine release [144].

Hemin could upregulate HO activity, reduce hypertension, suppress oxidative stress, and attenuate cardiac fibrosis, apoptosis, and oxidative stress. This effect was modulated through enhanced expression of the PI3K p85 regulatory subunit [145]. In an acute I/R with STZ-induced hyperglycemic rat model, intraperitoneal administration of hemin 18 h before I/R increased the levels of HO-1, providing marked protection against myocardial injury [146]. Moreover, zinc protoporphyrin IX (an inhibitor of HO activity) abolished the protective effect by hemin [146]. Furthermore, chronic HO-1 activation by prolonged administration of hemin improved postinfarction survival and exerted protective effects in a rat model of myocardial ischemia, through a potent antioxidant activity [112].

Taurine treatment significantly improved LV systolic and diastolic function in an STZ-induced diabetes rat model, and there were persistent increases in activities of Akt/PKB and SOD, as well as the level of HO-1 protein [147].

Mesenchymal stem cells (MSCs) have been reported to have the potential to release several kinds of cytokines, which could induce angiogenesis [148, 149]. However, almost all transplanted cells seemed to be lost by 6 weeks after transplantation, making it impossible for the limited number of MSCs to achieve a maximum proangiogenesis effect [150]. Due to its multiple catalytic byproducts, HO-1 has been proposed to be involved in a number of cytoprotective effects. HO-1 has been administered to improve the survival environment of MSCs and to achieve maximum functional benefits of MSCs [151]. HO-1 transduced by MSCs can induce angiogenic effects, reduce cardiac remodeling, and improve heart function after acute MI [152, 153]. Intramyocardial delivery of the HO-1 gene using adeno-associated virus-2

(AAV-2) before I/R also prevented cardiomyocyte apoptosis and reduced infarct size and cardiac dysfunction after myocardial I/R in mice [90]. To evaluate the long-term effects of HO-1 gene delivery, Liu et al. have showed that delivery of the HO-1 gene reduced mortality and preserved LV function and chamber dimensions 1 year after acute MI [91]. These results suggest that preemptive HO-1 gene delivery may be useful as a therapeutic strategy to reduce post-MI LV remodeling and HF.

8.2.2. Role of SOD and GSH Activation in Cardiac Remodeling. EUK-8, as a SOD mimetic, improved LV end-systolic dimensions and fractional shortening, prevented myocardial oxidant stress, attenuated necrotic and apoptotic cell death, and attenuated cardiac hypertrophy and fibrosis in mice subjected to pressure overload [113]. *In vitro*, the SOD mimetics tempol and EUK-8 could also reduce collagen production in Ang-II-treated fibroblasts [73]. Moreover, N-acetylcysteine, an antioxidant and GSH precursor, attenuated diabetic myocardial dysfunction via upregulating myocardial GSH and SOD2 activity [114]. Treatment with polyethylene glycol-conjugated SOD ameliorated doxorubicin-induced cardiac dysfunction, and this effect was mediated by inhibition of doxorubicin-induced upregulation of NF-κB signaling, lowering the levels of hexanoyl-lysine (a marker of free radical-induced lipid peroxidation) and suppressing the activation of Akt and Akt-regulated gene expression [124].

Recombinant SOD3 preserved cardiac function following I/R in isolated rat hearts [154–156] and reduced the infarct size when given just prior to coronary reperfusion in pigs [157]. Cardiac-selective expression of SOD3 from the cardiac troponin-T promoter after systemic administration of AAV-9 provided significant protection against both acute MI and LV remodeling [158]. Liu et al. also have demonstrated that encapsulation of CSCs in SOD-loaded alginate hydrogel enhanced CSC survival in the presence of doxorubicin, indicating its potential application as a novel therapy for the treatment of acute and early-onset doxorubicin-induced cardiotoxicity [159].

9. Advantages of Nrf2 Activation in Cardiac Remodeling and HF

Nrf2 dimerizes with members of the small Maf family to bind to antioxidant or electrophile response elements (AREs/EpREs) located in the regulatory regions of cellular defense enzyme genes [50]. The target genes of Nrf2 include many antioxidant genes, such as HO-1, NQO1, SODs, CAT, GST, γ-GCS, and GPx. By inducing the expression of these genes, Nrf2 activates a wide range of cell defense processes, thereby enhancing the overall capacity of cells to detoxify and eliminate harmful substances. As mentioned above, activated Nrf2 and its target genes, such as HO-1, SOD, and GPx, all play important roles in preventing cardiac remodeling. Although it cannot be excluded that some Nrf2 target genes may protect the heart through an Nrf2-independent pathway, Nrf2-induced cell defense processes remain to be the main driving force. Because Nrf2 could induce transcriptional activation of a number of ARE-bearing antioxidants, we speculate that, acting as an upstream gene, activation of Nrf2 may rescue from cardiac remodeling and HF induced by deficiency of an individual downstream gene. So, activation of Nrf2 may have an advantage over its downstream target genes in protecting one from oxidative stress-induced HF, but further investigations are needed before a conclusion can be drawn.

10. Conclusions

Increased generation of ROS in cardiomyocytes leads to increased necrosis and apoptosis, which induce cardiac remodeling and dysfunction. Nrf2 and its target genes are key components to maintain cellular redox homeostasis by attenuating oxidative stress-associated pathological processes. Patients with insufficient Nrf2 levels in their cardiovascular system are more vulnerable to HF development. It is conceivable that Nrf2 orchestrates a group of antioxidative and other cytoprotective genes to provide a protective mechanism against detrimental stress-induced cardiac dysfunction. However, further work is needed to understand the role of Nrf2 in the pathogenesis of cardiac remodeling and HF in more detail before it can be seriously considered as a therapeutic target for HF.

Abbreviations

HF:	Heart failure
ROS:	Reactive oxygen species
RNS:	Reactive nitrogen species
Nrf2:	Nuclear factor-erythroid-2- (NF-E2-) related factor 2
LV:	Left ventricular
AREs:	Antioxidant response elements
CNC:	Cap-n-collar
CNC-bZIP:	CNC type of basic region leucine zipper
Keap1:	Kelch-like-ECH-associated protein 1
HO-1:	Heme oxygenase-1
NQO1:	NAD(P)H dehydrogenase (quinone 1)
SODs:	Superoxide dismutases
CAT:	Catalase
GST:	Glutathione-S-transferases
γ-GCS:	γ-Glutamylcysteine synthase
GPx:	Glutathione peroxidases
TAC:	Transverse aortic constriction
STZ:	Streptozotocin
ATF3:	Activating transcription factor 3
LDH:	Lactate dehydrogenase
GCL:	Glutamate-cysteine ligase
GSH:	Glutathione
MG132:	Carbobenzoxy-Leu-Leu-leucinal
4-HNE:	4-Hydroxynonenal
E2:	17β-Estradiol
H/R:	Hypoxia/reoxygenation
CSCs:	Cardiac stem cells
ERK:	Extracellular signal-regulated kinase
MSCs:	Mesenchymal stem cells
AAV-2:	Adeno-associated virus-2

EpREs: Electrophile response elements
$\bullet O^{2-}$: Superoxide anion
•OH: Hydroxyl radical
H_2O_2: Hydrogen peroxide
$OCl^{\bullet-}$: Hypochlorite
Ang II: Angiotensin II
MI: Myocardial infarction
I/R: Ischemia/reperfusion
HG: High glucose
tBHQ: Tert-Butylhydroquinone
CK: Creatine kinase.

Conflict of Interests

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

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Research Article

Effects of Treating Old Rats with an Aqueous *Agaricus blazei* Extract on Oxidative and Functional Parameters of the Brain Tissue and Brain Mitochondria

Anacharis B. de Sá-Nakanishi, Andréia A. Soares, Andrea Luiza de Oliveira, Jurandir Fernando Comar, Rosane M. Peralta, and Adelar Bracht

Department of Biochemistry, University of Maringá, Avenida Colombo 5790, 87020900 Maringá, PR, Brazil

Correspondence should be addressed to Adelar Bracht; adebracht@uol.com.br

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Dysfunction of the mitochondrial respiratory chain and increased oxidative stress is a striking phenomenon in the brain of aged individuals. For this reason there has been a constant search for drugs and natural products able to prevent or at least to mitigate these problems. In the present study the effects of an aqueous extract of *Agaricus blazei*, a medicinal mushroom, on the oxidative state and on the functionality of mitochondria from the brain of old rats (21 months) were conducted. The extract was administered intragastrically during 21 days at doses of 200 mg/kg. The administration of the *A. blazei* extract was protective to the brain of old rats against oxidative stress by decreasing the lipid peroxidation levels and the reactive oxygen species content and by increasing the nonenzymic and enzymic antioxidant capacities. Administration of the *A. blazei* extract also increased the activity of several mitochondrial respiratory enzymes and, depending on the substrate, the mitochondrial coupled respiration.

1. Introduction

Aging is characterized by a general decline of many physiological functions, with pronounced influence on the cerebral activities [1]. An important theory that explains the diminution of the cerebral activities during aging states that the increased formation of reactive oxygen species (ROS) is an important generator of cellular lesions and disturbances related to old age [2, 3]. The deleterious effects of ROS on biomolecules such as proteins, nucleic acids, and lipid membranes [4] slowly accumulate along the years and have been regarded as an important endogenous factor contributing to aging as well as to the degenerative disturbances associated with the old age [5, 6].

There are numerous routes that lead to the production of ROS, but the mitochondrial energy metabolism is generally recognized as the most important one in most eukaryotic cells [7]. By being the direct intracellular source of ROS the mitochondria are also subject to the direct attack by these molecules [8]. It has been claimed that damage induced by oxidant molecules, including mutations in the mitochondrial DNA, may result in a progressive loss of the cellular capacity of ATP production, cellular degeneration, and eventually cell death [2]. This loss of physiological performance during aging seems to be one of the most important factors involved in the pathogenesis of many disturbances that appear during the old age [9].

The consumption of foods rich in components possessing antioxidant activity has been regarded as a promising measure for the prevention of age related diseases. Mushrooms have been especially recommended due to the fact that they represent a great source of new therapeutic agents [10, 11]. In this sense, the mushroom *Agaricus blazei* Murril, popularly known as *sun mushroom,* has been amply utilized in the form of a medicinal extract for cancer prevention and for treating a variety of conditions such as diabetes, atherosclerosis, hypercholesterolemia, and cardiac diseases [12, 13]. A number of clinical studies have already been conducted, several of them confirming the beneficial effects of *A. blazei*, especially its immunomodulatory activity [14].

Several studies have also shown that *A. blazei* is an important source of potential antioxidant compounds [15, 16], especially phenolics such as gallic acid, syringic acid, and pyrogallol and also polysaccharides [17, 18]. Furthermore, *A. blazei* also contains significant amounts of nucleosides and nucleotides, adenosine, for example, [19], which are important paracrine agents [20, 21] also able to exert neuroprotective actions [22, 23].

Considering, thus, the increased oxidative stress during aging [24–26] and the antioxidant and putative medicinal properties of *A. blazei*, we decided to investigate in detail the influence of an extract of this mushroom on several parameters in the brain tissue of old rats. Besides characterizing the oxidative status of the brain tissue of these rats, cerebral mitochondria were isolated and investigated for their oxidative state and functional properties. The study should answer the question if an aqueous extract of *A. blazei* is or is not able to influence in a positive way the cerebral oxidative state and the mitochondrial functions during aging.

2. Materials and Methods

2.1. Preparation of the Agaricus blazei Aqueous Extract. Basidiomata of *Agaricus blazei* were obtained from a local producer in Maringá, PR, Brazil, in spring 2009. The previously grounded dehydrated basidiomata were submitted to an aqueous extraction as described previously [16] with some minor modifications. The dried basidiomata were milled until obtaining a fine powder. The samples (10 g) were extracted by stirring with 100 mL of water (28°C) at 130 rpm for 3 hours and filtered through Whatman no. 1 paper. The extraction was repeated three times. The filtrates (yield 50%) were lyophilized and stored in freezer until use.

2.2. Animals and Treatment. Male *Wistar* rats kept in laboratory cages received water *ad libitum* and a standard chow diet (Nuvilab). The rats were maintained in automatically timed light and dark cycles of 12 hours. Experiments were done with young adult rats (3 months old, weighing 250 to 300 g) and old rats (21 months old, weighing 450 to 500 g). The *A. blazei* extract was administered intragastrically to a group of old rats at a daily dose of 200 mg/kg during 21 days. These rats are labeled as "*A. blazei*-treated old rats" in the graphs and tables. All control rats received saline (0.9% NaCl) during the same period of 21 days. All experiments were done in accordance with the internationally accepted recommendations in the care and use of animals.

2.3. Preparation of the Brain Homogenate. Rats were starved for 18 hours and then anesthetized by intraperitoneal injection of thiopental (50 mg/kg). The criterion of anesthesia was the lack of body or limb movement in response to a standardized tail clamping stimulus. The brain of each rat was surgically removed with scissors, clamped with liquid nitrogen, and stored at temperatures under −150°C. The tissue suspension (10% w/v in 0.1 M phosphate buffer, pH 7.4) was homogenized by means of a van Potter-Elvehjem homogenizer. Protein contents were determined with the Folin phenol reagent [27] using bovine serum albumin as standard.

2.4. Isolation of Brain Mitochondria. After 18-hour fast the rats were decapitated and their brains removed immediately and cut into pieces with scissors. The fragments were suspended (10% w/v) in a medium containing 0.2 M mannitol, 76 mM sucrose, 10 mM TRIS (pH 7.4), 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 50 mg% fatty acid-free bovine serum albumin. Homogenization of the tissue was done in the same medium using a van Potter-Elvehjem homogenizer. After homogenization the mitochondria were isolated by fractional centrifugation [28, 29]. The isolated mitochondria were suspended in the isolation medium and kept at 0 a 4°C. Protein content was determined with the Folin phenol reagent [27].

2.5. Determination of the Total Antioxidant Capacity (TAC). The total antioxidant capacity of the brain was determined colorimetrically with 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; [30]). Aliquots from the supernatant of a 10000 g centrifugation of the brain homogenate were added to 0.4 M acetate buffer (pH 5.8) plus 150 μL of a cationic ABTS solution (30 mM acetate buffer, pH 3.6, containing 10 mM ABTS and 4 mM H_2O_2). After 5 minutes of incubation in the dark, the absorbance at 734 nm was determined against water. The compound 6-hydroxy-2,5,7,8-tetramethyl-chloraman-2-carboxylic acid (TROLOX) was used as a standard and the results were expressed as μmol TROLOX equivalents per mg protein.

2.6. Determination of Lipid Peroxidation, Reduced Glutathione, and Protein Reduced Thiol Contents. The levels of lipid peroxidation were measured in the brain homogenate and in brain mitochondria by means of the TBARS method (thiobarbituric reactive substances). The concentration of lipoperoxides was determined spectrophotometrically at 532 nm using an extinction coefficient ($\varepsilon_{532\,\mathrm{nm}}$) of $1.56 \times 10^5\,\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$. The results were expressed as nmol malondialdehyde (MDA) per mg protein [31]. The reduced glutathione (GSH) levels of mitochondria and brain homogenate were determined spectrofluorimetrically with o-phthalaldehyde (excitation: 350 nm; emission: 420 nm) [32]. Standards were run in parallel and the glutathione concentration was expressed as nmol per mg protein. The reduced protein thiol groups in the brain homogenate were determined using the compound 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) [33]. The concentration of reduced thiols was calculated using a molar extinction coefficient ($\varepsilon_{412\,\mathrm{nm}}$) of $1.36 \times 10^4\,\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ and was expressed as nmol per mg protein.

2.7. Reactive Oxygen Species (ROS) Determination. The levels of reactive oxygen species were estimated in aliquots from the supernatants of the 10000 g centrifugation of either disrupted mitochondria or brain homogenate using the reaction with 2′,7′-dichlorofluorescein diacetate (DCFH-DA; [34]). The formation of oxidized 2′,7′-dichloro-fluorescein (DCF) was

measured fluorimetrically with excitation at 504 nm and emission at 529 nm. The ROS content was calculated using a standard curve with oxidized dichlorofluorescein (DCF) and the results were expressed as nmol per mg protein. All steps were processed in the dark and a blank containing DCFH-DA was used to correct for autofluorescence.

2.8. Determination of Antioxidant Enzymes. The activity of catalase (CAT) was evaluated by measuring spectrophotometrically the decomposition of H_2O_2 at 240 nm [35]. Aliquots from the supernatants of the 10000 g centrifugation of either disrupted mitochondria or brain homogenate were added to 1 mL of solution containing 50 mM TRIS (pH 8.0), 0.25 mM EDTA, and 30 mM H_2O_2. The drop in absorbance during the first minute of incubation was measured at 25°C. A standard H_2O_2 curve was used to calculate the enzyme activity, which was expressed as μmol min^{-1} mg protein^{-1}.

The activity of the superoxide dismutase (SOD) was assayed by its capacity to inhibit the auto-oxidation of pyrogallol in alkaline medium, which was monitored spectrophotometrically at 420 nm [36]. One unit of SOD is defined as the amount of enzyme promoting 50% inhibition of pyrogallol auto-oxidation. Aliquots from the supernatants of the 10000 g centrifugation of either disrupted mitochondria or brain homogenate were added to 1 mL of a solution containing 0.2 M TRIS (pH 8.2) and 2 mM EDTA. The reaction was started by adding 0.1 mM pyrogallol. The change in absorbance was monitored, the initial rate computed, and the activity expressed as SOD units per mg protein.

The activity of glutathione peroxidase was determined as the decrease in absorbance at 340 nm due to NADPH oxidation dependent on H_2O_2 at 25°C [37]. Aliquots from the supernatants of the 10000 g centrifugation of either disrupted mitochondria or brain homogenate were added to 1.5 mL of a solution containing 40 mM phosphate buffer (pH 7.0), 0.5 mM EDTA, 1.0 mM sodium azide, 1.0 mM reduced glutathione, 1.5 mM NADPH, and 2 units of glutathione reductase. The reaction was initiated by the addition of H_2O_2 (0.2 mM) and monitored during 90 seconds. The initial rates were obtained by extrapolation to zero time and the activity computed as nmol min^{-1} mg protein^{-1} using the molar extinction coefficient of NADPH ($\varepsilon_{340\,nm}$) (6.22×10^3 M^{-1} cm^{-1}).

The activity of glutathione reductase (GR) was determined as the decrease in absorbance at 340 nm due to the NADPH oxidation [38]. Aliquots from the supernatants of the 10000 g centrifugation of either disrupted mitochondria or brain homogenate were added to 1 mL of a solution containing 50 mM phosphate buffer (pH 8.0), 2 mM EDTA, 0.15 mM NADPH, and 0.5 mM oxidized glutathione (GSSG) at 25°C. The initial rates were obtained by extrapolation to zero time and the activity computed as nmol min^{-1} mg protein^{-1} using the molar extinction coefficient of NADPH (6.22×10^3 M^{-1} cm^{-1}).

The activity of glucose 6-phosphate dehydrogenase was measured as the reduction rate of $NADP^+$ in the presence of glucose 6-phosphate [38]. Aliquots from the supernatants of the 10000 g centrifugation of brain homogenate were added to 1.5 mL of a solution containing 0.1 M triethanolamine buffer (pH 7.6), 7 mM $MgCl_2$, and 1 mM $NADP^+$. The reaction at 25°C was initiated by the addition of glucose 6-phosphate (1.0 mM). The increase in absorbance due to NADPH production was monitored during three minutes. The initial rates were obtained by extrapolation to zero time and the activity computed as nmol min^{-1} mg protein^{-1} using the molar extinction coefficient of NADPH (6.22×10^3 M^{-1} cm^{-1}).

2.9. Determination of Mitochondrial Membrane-Bound Enzymatic Activities. The NADH oxidase and succinate oxidase activities as well as the oxidation of ascorbate mediated by TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) were measured polarographically using freeze-thawing disrupted mitochondria in a medium containing 20 mM TRIS (pH 7.4) [39]. The reactions were initiated by the addition of the corresponding substrates, namely, NADH (1 mM), succinate (10 mM), or ascorbate plus TMPD (10 and 1 mM).

The activity of cytochrome c oxidase was determined spectrophotometrically using freeze-thawing disrupted mitochondria [40]. The rate of ferrocytochrome c oxidation was monitored at 550 nm. The results were expressed as nmol min^{-1} mg protein^{-1} using a molar extinction coefficient ($\varepsilon_{550\,nm}$) of 1.9×10^4 M^{-1} cm^{-1}.

The determination of the NADH dehydrogenase in disrupted mitochondria was done spectrophotometrically at 420 nm using ferricyanide as the electron acceptor. The reaction medium contained 20 mM TRIS (pH 7.4), 1.8 μM antimycin, 1 mM NADH, 0.5-0.6 mg mitochondrial protein, and 0.1 mM potassium ferricyanide. The results were expressed as nmol min^{-1} mg protein^{-1} using an extinction coefficient ($\varepsilon_{420\,nm}$) of 1.04×10^3 M^{-1} cm^{-1} [41].

The mitochondrial ATPase activity was quantified by measuring phosphate release from ATP. The reaction medium was 0.2 M sucrose, 10 mM TRIS (pH 7.4), 50 mM KCl 50, 0.2 mM EGTA, and, when appropriate, 100 μM 2,4-dinitrophenol. The reaction was initiated by the addition of 5 mM ATP and interrupted after 20 min incubation at 37°C by the addition of 5% trichloroacetic acid and maintained at 4°C until determination of free phosphate [42].

2.10. Determination of Mitochondrial Dehydrogenases. The α-ketoglutarate dehydrogenase activity was measured in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM thiamine pyrophosphate, 2 mM NAD^+, 1 mM $MgCl_2$, 0.3 mM dithiothreitol, 0.1% Triton X-100 (v/v), 10 mM α-ketoglutarate, and aliquots of freeze-thawing disrupted mitochondria suspensions [43]. The reaction was initiated by the addition of coenzyme A (0.2 mM) and monitored spectrophotometrically as the reduction of NAD^+ at 340 nm ($\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}). The initial rate was expressed as nmol min^{-1} mg protein^{-1}.

For the determination of pyruvate dehydrogenase, the inactive form (phosphorylated) of the multienzyme complex was converted into the active form (dephosphorylated) by incubating freeze-thawing disrupted mitochondria in a medium containing 20 mM TRIS (pH 7.8), 130 mM KCl, 5 mM potassium phosphate, and 10 mM $MgCl_2$. After

5 minutes an aliquot of 50 μL was transferred to the assay medium (1 mL) containing 50 mM TRIS (pH 7.8), 1 mg/mL bovine serum albumin, 50 mM NAD^+, 2.5 mM coenzyme A, 50 mM $MgCl_2$, 5 mM sodium oxalate, 5 mM thiamine pyrophosphate, 100 μM rotenone, 30 mM dithiothreitol 6.5 μM phenazine methosulfate, and 0.6 mM iodonitrotetrazolium chloride. The reaction was started by adding 100 mM pyruvate and the reduction of iodonitrotetrazolium was measured as the increase in absorbance at 500 nm [44]. Activity of the enzyme was expressed as nmol min^{-1} mg $protein^{-1}$ using the molar extinction coefficient of reduced iodonitrotetrazolium (1.24×10^4 M^{-1} cm^{-1} at pH 7.8).

The activity of succinate dehydrogenase was measured in a reaction medium (1 mL) containing 100 mM triethanolamine (pH 8.3), 0.5 mM EDTA, 2 mM KCN, 6.5 μM phenazine methosulfate, 0.6 mM iodonitrotetrazolium, and aliquots from freeze-thawing disrupted mitochondria [45]. The reaction was started by the addition of succinate (10 mM), monitored as the increase in absorbance at 500 nm and expressed as nmol min^{-1} mg $protein^{-1}$ using the molar extinction coefficient of reduced iodonitrotetrazolium (1.93×10^4 M^{-1} cm^{-1} at pH 8.3).

The activity of malate dehydrogenase was measured in a reaction medium (1.5 mL) containing 120 mM phosphate buffer (pH 7.8), 0.25 mM NADH, and aliquots from the supernatant of the 10000 g centrifugation of disrupted mitochondria. The reaction was initiated by the addition of oxaloacetate (0.1 mM) and monitored as the diminution of absorbance at 340 nm [46]. Activity was expressed as nmol min^{-1} mg $protein^{-1}$ using the extinction coefficient of NADH (6.22×10^3 M^{-1} cm^{-1}).

The $NADP^+$-dependent isocitrate dehydrogenase activity was measured in a medium (1 mL) containing 0.1 M TRIS (pH 7.4), 2 mM $MgCl_2$, 2 mM $NADP^+$, and aliquots from the supernatant of the 10000 g centrifugation of disrupted mitochondria [47]. The reaction was initiated by the addition of isocitrate (1.25 mM) and monitored as the increase in absorbance at 340 nm. Activity was expressed as nmol min^{-1} mg $protein^{-1}$ using the extinction coefficient of NADPH (6.22×10^3 M^{-1} cm^{-1}).

The L-glutamate dehydrogenase activity was measured in a medium (1 mL) containing 50 mM triethanolamine (pH 8.0), 0.1 M ammonium sulfate, 95 μM NADH, 2.5 mM EDTA, 1 mM ADP, and aliquots from the supernatant of the 10000 g centrifugation of disrupted mitochondria [48]. The reaction was initiated by the addition of α-ketoglutarate (8.0 mM) and monitored as the increase in absorbance at 340 nm. Activity was expressed as nmol min^{-1} mg $protein^{-1}$ using the extinction coefficient of NADH (6.22×10^3 M^{-1} cm^{-1}).

2.11. Mitochondrial Oxygen Consumption and Oxidative Phosphorylation. Mitochondrial oxygen consumption was measured polarographically using a teflon-shielded platinum electrode [28, 41]. Mitochondria (≈1 mg protein/mL) were incubated in the closed oxygen chamber in a medium (2 mL) containing 0.25 M mannitol, 5 mM sodium phosphate, 10 mM KCl, 0.2 mM EGTA, 50 mg% fatty acid-free bovine serum albumin, 10 mM TRIS-HCl (pH 7.4), and substrates. The latter were succinate (10 mM), α-ketoglutarate (10 mM), or pyruvate + L-malate (10 + 1 mM). ADP (0.125 mM) was added at appropriate times. The rates of oxygen consumption, the respiratory control ratio (RC), and the ADP/O ratios were computed from the slopes of the recorder tracings [49]. Rates were expressed as nmol O_2 min^{-1} mg $protein^{-1}$.

2.12. Mitochondrial Membrane Energization. The mitochondrial membrane energization (transmembrane potential) was estimated fluorimetrically using safranin as a fluorescent probe [49, 50]. Mitochondria (1 mg protein) were incubated in a medium (2 mL) containing 0.25 M mannitol, 5 mM potassium phosphate, 10 mM TRIS (pH 7.4), 0.2 mM EGTA, 50 mg% fatty acid-free bovine serum albumin, and 10 μM safranin. Energization was achieved by the introduction of either 50 μM succinate + 2 μM rotenone or 1 mM ATP. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 10 μM) was added to attain full deenergization. The wavelengths for excitation and emission were 520 and 580 nm, respectively.

2.13. Statistics. All results are presented as mean ± mean standard errors. Evaluation of the statistical significance was done by means of the variance analysis (ANOVA) followed by post hoc Student-Newman-Keuls testing. The 5% level ($P < 0.05$) was adopted as the significance criterion.

3. Results

3.1. Oxidative State of the Brain Tissue and Mitochondria. The total antioxidant capacities of samples from the supernatant of the 10000 g centrifugation of the brain homogenate from young (3 months old) and old rats (21 months) without treatment and treated with the *A. blazei* extract are shown in Figure 1. The ABTS assay revealed a substantial decline of the total antioxidant capacity, the difference between young and old rats amounting to −36%. Figure 1 also shows that the 21-day treatment with the *A. blazei* extract was able to restore the antioxidant capacity of the old rats' brains to a level very close to that of young rats.

The lipid peroxidation levels were measured in both the total brain homogenate and the mitochondrial fraction. The results are shown in Figure 2. Lipid peroxidation was clearly higher in the brain homogenate of old rats (+33%) when compared to that of young rats (Figure 2(a)). A more pronounced difference (+40%) was found in the mitochondrial fraction (Figure 2(b)). The *A. blazei* extract treatment, however, restored almost completely the lipid peroxidation level of the total brain tissue of old rats to that found in young rats. A similar effect of the extract treatment was found for the mitochondrial fraction, though restoration was not complete in this case.

The levels of reactive oxygen species in both total homogenate and mitochondria can be seen in Figure 3. Old rats presented an enormous increase relative to young rats, +106% in the total homogenate (Figure 3(a)) and +119% in the mitochondria (Figure 3(b)). Treatment of old rats with the

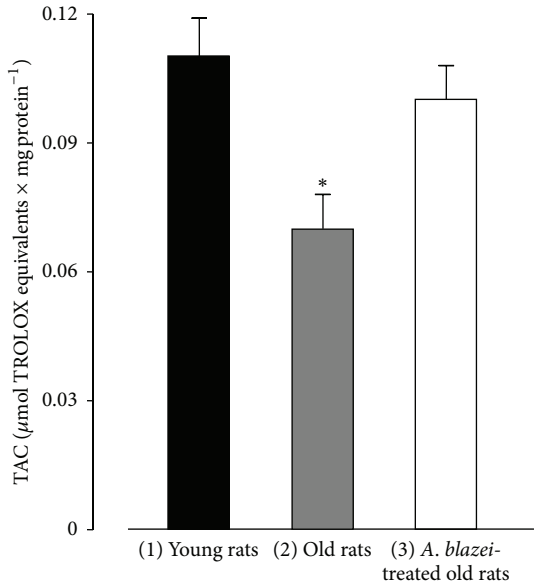

Figure 1: Total antioxidant capacity of the brain homogenates from young, old, and *A. blazei*-treated old rats. The brain homogenate was prepared as described in Section 2. The ABTS assay was used to evaluate the antioxidant capacity of the brain homogenate. The bars represent the mean ± mean standard errors of 5 (young), 8 (old), and 5 (*A. blazei*-treated) rats. The asterisk (∗) indicates $P \leq 0.05$ for the comparison between (1) and (2) and (2) and (3), according to ANOVA followed by Student-Newman-Keuls post hoc testing.

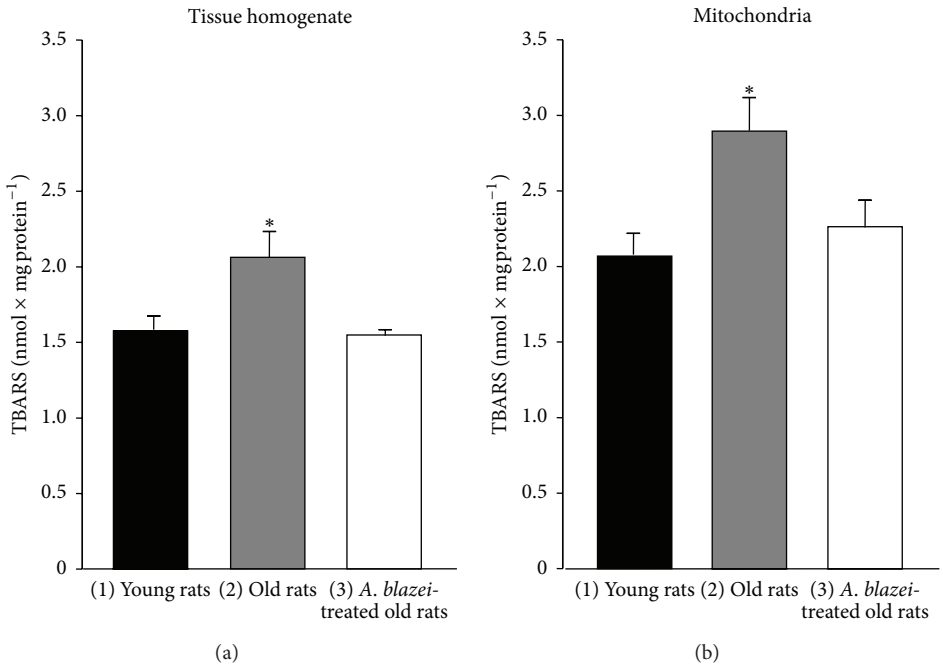

Figure 2: Lipid peroxidation levels of brain homogenate and brain mitochondria from young, old, and *A. blazei*-treated old rats. The brain homogenate and the mitochondria were prepared as described in Section 2. The lipid peroxidation levels were evaluated as the thiobarbituric acid reactive substances (TBARS). The bars represent the mean ± mean standard errors of 5 (young), 5 (old), and 4 (*A. blazei*-treated) rats for the homogenate and of 6 (young), 6 (old), and 7 (*A. blazei*-treated) rats for the mitochondria. The asterisks (∗) indicate $P \leq 0.05$ for the comparison between (1) and (2) and (2) and (3), according to ANOVA followed by Student-Newman-Keuls post hoc testing.

A. blazei extract was partially successful. For the total brain tissue it caused a diminution of 22%. For the mitochondria a diminishing tendency was also found, but lacking statistical significance at the 5% level.

Figure 4 summarizes the results obtained when the GSH levels were measured as well as the protein reduced thiol levels. Aging did not affect the GSH concentration of the total cerebral tissue nor had the *A. blazei* treatment any influence

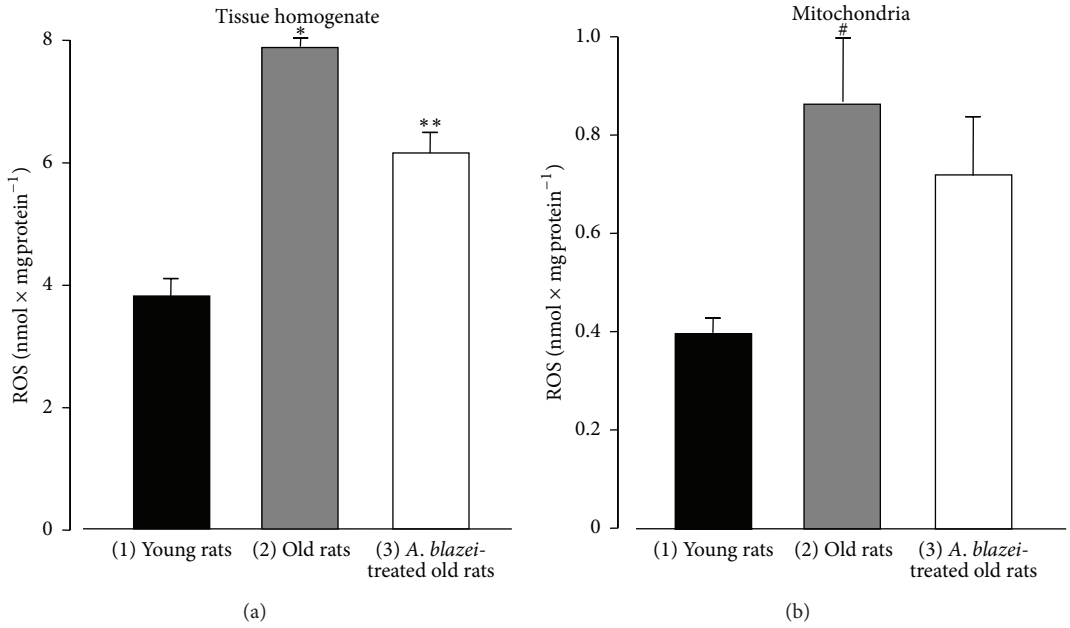

FIGURE 3: Reactive oxygen species (ROS) levels of brain homogenate and brain mitochondria from young, old, and *A. blazei*-treated old rats. The brain homogenate and the mitochondria were prepared as described in Section 2. The ROS levels were evaluated by means of the dichlorofluorescein diacetate method. The bars represent the mean ± mean standard errors of 5 (young), 7 (old), and 6 (*A. blazei*-treated) rats for the homogenate and of 4 (young), 6 (old), and 5 (*A. blazei*-treated) rats for the mitochondria. $^{*}P \leq 0.05$ for the comparison between (1) and (2) and (2) and (3), according to ANOVA followed by Student-Newman-Keuls post hoc testing; $^{**}P \leq 0.05$ for the comparison between (3) and (1); $^{\#}P \leq 0.05$ for the comparison between (2) and (1).

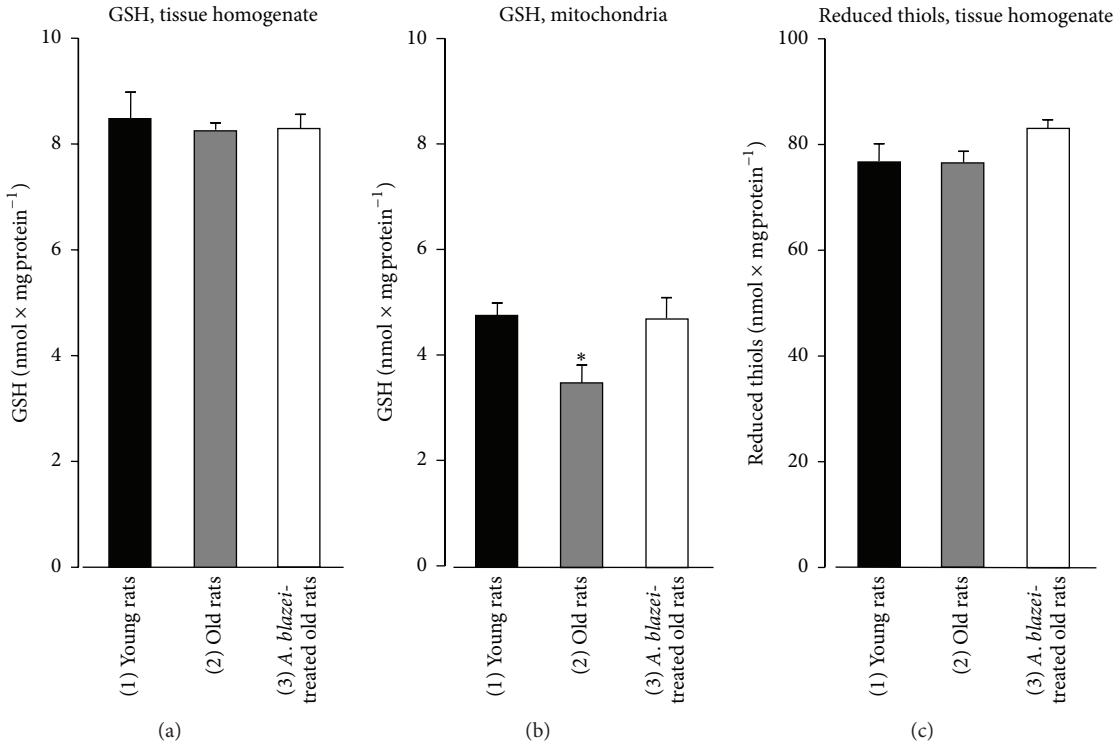

FIGURE 4: Reduced glutathione (GSH) levels and protein thiol groups of brain homogenate and reduced glutathione (GSH) levels of brain mitochondria from young, old, and *A. blazei*-treated old rats. The brain homogenate and the mitochondria were prepared as described in Section 2. The GSH levels were evaluated spectrofluorimetrically by o-phthalaldehyde method. The reduced thiol groups were measured with 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) method. The bars represent the mean ± mean standard errors; in panel (a) (tissue homogenate) the *n* values were 5 (young), 9 (old), and 6 (*A. blazei*-treated); in panel (b) the *n* values were 6 (young), 6 (old), and 6 (*A. blazei*-treated) and in panel (c) *n* values were 5 (young), 6 (old), and 5 (*A. blazei*-treated). $^{*}P \leq 0.05$ for the comparison between (1) and (2) and (2) and (3), according to ANOVA followed by Student-Newman-Keuls post hoc testing.

Table 1: Effects of the *A. blazei* extract treatment on the activity of the antioxidant enzymes in the brain tissue and in brain mitochondria of old rats. All enzymes were evaluated spectrophotometrically as described in Materials and Methods. The values represent the mean ± standard errors of the mean.

Parameters	Experimental groups		
	(1) Young rats	(2) Old rats	(3) *A. blazei*-treated old rats
Brain homogenate (supernatant of 10,000 g centrifugation)			
Catalase (μmol min^{-1} mg protein^{-1})	12.90 ± 0.57 ($n = 5$)	12.89 ± 0.74 ($n = 7$)	17.78 ± 0.86** ($n = 5$)
Superoxide dismutase (units mg protein^{-1})	1.86 ± 0.12 ($n = 5$)	1.70 ± 0.07$^{\#}$ ($n = 8$)	2.04 ± 0.07 ($n = 6$)
Glutathione peroxidase (nmol min^{-1} mg protein^{-1})	20.42 ± 0.60 ($n = 5$)	30.47 ± 3.09* ($n = 6$)	39.29 ± 2.46$^{\#\#}$ ($n = 5$)
Glutathione reductase (nmol min^{-1} mg protein^{-1})	16.09 ± 0.67 ($n = 4$)	14.51 ± 1.05 ($n = 8$)	14.93 ± 0.76 ($n = 6$)
Glucose 6-phosphate dehydrogenase (nmol min^{-1} mg protein^{-1})	34.23 ± 1.63 ($n = 5$)	25.43 ± 0.82* ($n = 8$)	29.29 ± 0.79$^{\#\#}$ ($n = 6$)
Brain mitochondria			
Catalase (μmol min^{-1} mg protein^{-1})	5.72 ± 0.52 ($n = 4$)	3.05 ± 0.12* ($n = 5$)	6.25 ± 1.08 ($n = 5$)
Superoxide dismutase (units mg protein^{-1})	2.14 ± 0.11 ($n = 5$)	1.63 ± 0.10* ($n = 5$)	2.10 ± 0.12 ($n = 5$)
Glutathione peroxidase (nmol min^{-1} mg protein^{-1})	20.51 ± 1.48 ($n = 5$)	20.52 ± 1.32 ($n = 5$)	24.98 ± 2.32 ($n = 5$)

$^{*}P < 0.05$ for (2) versus (1) and (2) versus (3) (ANOVA and Student-Newman-Keuls post hoc testing).
$^{**}P < 0.05$ for (3) versus (2) and (3) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).
$^{\#}P < 0.05$ for (2) versus (3).
$^{\#\#}P < 0.05$ for (3) versus (1).

(Figure 4(a)). In the mitochondria, however, aging reduced the GSH levels by 26% (Figure 4(b)). The *A. blazei* treatment restored the mitochondrial GSH levels of old rats to the levels found in young rats. The reduced thiol levels of the total homogenate (Figure 4(c)) were not affected by aging and *A. blazei* treatment.

3.2. Antioxidant Enzymes of the Brain Tissue and Mitochondria. The activities of five antioxidant enzymes were measured and the results are summarized in Table 1. The catalase activity in the homogenate (supernatant of 10,000 g centrifugation) was not affected by aging, but the *A. blazei* extract treatment induced an increase of 38% in old rats. Singularly, in mitochondria of old rats the catalase activity was considerably smaller than that in young rats (−47%), a condition that was completely abolished by the *A. blazei* treatment.

Superoxide dismutase in the brain homogenate supernatant showed some tendency toward diminution in old when compared to young rats. Treatment with the *A. blazei* extract, on the other hand, produced an increase in the superoxide dismutase activity of old rats (+20%) which was statistically significant. The changes in the superoxide dismutase activity were more pronounced in the mitochondria, where a clear decrease was found upon aging (−24%) and a recovery in consequence of the *A. blazei* extract treatment.

The glutathione peroxidase activities in the homogenate supernatant and mitochondria behaved quite differently upon aging: the activity of this enzyme increased in the total brain homogenate (+49%) but remained the same in the mitochondria. A further increase (+29%) was found in the homogenate upon the *A. blazei* extract treatment, whereas a nonsignificant increasing tendency was apparent in the mitochondria. The combined increases in the brain glutathione peroxidase activity caused by aging and *A. blazei* treatment, thus, amounted to 92%.

Aging was without significant effect on the tissue activity of glutathione reductase, but the glucose 6-phosphate dehydrogenase was substantially diminished in old rats (−26%). The *A. blazei* extract treatment produced a partial recovery.

3.3. Mitochondrial Enzyme Activities. The mitochondrial enzyme activities corresponding to individual proteins or to segments of the respiratory chain that were measured in the present work are listed in Table 2. With respect to their behavior during aging and their response to the *A. blazei* extract treatment they may be divided into three groups. The first group comprises those enzymes that were clearly diminished upon aging: NADH dehydrogenase (−16%), succinate dehydrogenase (−45%), pyruvate dehydrogenase (−27%), and α-ketoglutarate dehydrogenase (−36%). Excepting pyruvate dehydrogenase, the *A. blazei* extract treatment resulted in enzyme levels that were equal or even superior to those found in young rats. For pyruvate dehydrogenase this tendency was much less pronounced. The second group of enzymes in Table 2 comprises only cytochrome c oxidase, whose activity was not affected by aging, but which suffered a very pronounced increase in consequence of the *A. blazei* extract treatment (+90%). And the third group comprises the enzymes that were altered neither by aging nor by the *A. blazei* extract treatment. These enzymes were malate dehydrogenase, glutamate dehydrogenase, and isocitrate dehydrogenase ($NADP^+$-dependent).

Table 3 shows the results of experiments in which three oxidases were measured polarographically using freeze-thawing disrupted brain mitochondria. Aging diminished NADH oxidase by 21%, succinate oxidase by 17%, and the TMPD mediated ascorbate oxidation "TMPD-ascorbate oxidase" by 30%. Treatment of the old rats with the *A. blazei*

Table 2: Effects of the *A. blazei* extract treatment on several enzymatic activities of brain mitochondrial from old rats. All enzymes were evaluated spectrophotometrically as described in Materials and Methods. The values represent the mean ± standard errors of the mean.

Enzymes	Experimental groups		
	(1) Young rats	(2) Old rats	(3) *A. blazei*-treated old rats
	nmol min^{-1} mg $protein^{-1}$		
NADH dehydrogenase	698.90 ± 29.99 ($n = 3$)	588.17 ± 16.51* ($n = 5$)	752.12 ± 36.35 ($n = 6$)
Succinate dehydrogenase	14.22 ± 1.84 ($n = 6$)	7.83 ± 0.94* ($n = 5$)	14.57 ± 0.95 ($n = 6$)
α-Ketoglutarate dehydrogenase	17.24 ± 1.48 ($n = 6$)	10.98 ± 0.54* ($n = 5$)	16.13 ± 0.98 ($n = 6$)
Pyruvate dehydrogenase	23.75 ± 2.37 ($n = 4$)	17.26 ± 0.27# ($n = 5$)	21.03 ± 1.22 ($n = 6$)
Cytochrome c oxidase	287.20 ± 31.30 ($n = 5$)	264.53 ± 16.23 ($n = 5$)	501.32 ± 25.09** ($n = 4$)
Malate dehydrogenase	7540 ± 560 ($n = 6$)	6780 ± 770 ($n = 5$)	6980 ± 270 ($n = 6$)
Isocitrate dehydrogenase	26.68 ± 1.79 ($n = 6$)	25.80 ± 2.89 ($n = 5$)	27.57 ± 1.31 ($n = 6$)
Glutamate dehydrogenase	183.6 ± 11.9 ($n = 6$)	179.5 ± 19.9 ($n = 5$)	179.9 ± 8.4 ($n = 6$)

*$P < 0.05$ for (2) versus (1) and for (2) versus (3) (ANOVA and Student-Newman-Keuls post hoc testing).
#$P < 0.05$ for (2) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).
**$P < 0.05$ for (3) versus (2) and (3) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).

Table 3: Effects of the *A. blazei* extract treatment on the oxidation of NADH, succinate, and TMPD-ascorbate by disrupted brain mitochondria of old rats. The NADH oxidase, succinate oxidase, and oxidation of ascorbate mediated by TMPD were measured polarographically using freeze-thawing disrupted mitochondria as described in Materials and Methods. The values represent the mean ± standard errors of the mean.

Enzymatic activities	Experimental groups		
	(1) Young rats	(2) Old rats	(3) *A. blazei*-treated old rats
	nmol O_2 min^{-1} mg $protein^{-1}$		
NADH oxidase	58.56 ± 2.19 ($n = 6$)	46.12 ± 4.84# ($n = 6$)	46.44 ± 4.77** ($n = 7$)
Succinate oxidase	38.12 ± 1.47 ($n = 6$)	31.50 ± 1.53# ($n = 5$)	32.27 ± 1.32 ($n = 5$)
TMPD-ascorbate oxidation	113.89 ± 5.75 ($n = 4$)	79.40 ± 3.03# ($n = 5$)	98.66 ± 6.25 ($n = 5$)

#$P < 0.05$ for (2) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).
**$P < 0.05$ for (3) versus (1) ANOVA and Student-Newman-Keuls post hoc testing).

Table 4: Effects of the *A. blazei* extract treatment on the ATPase activity of mitochondria from old rats. The ATPase activity was quantified by measured phosphate release from ATP as described in Materials and Methods. The values represent the mean ± standard errors of the mean.

Conditions	Experimental groups		
	(1) Young rats	(2) Old rats	(3) *A. blazei*-treated old rats
	nmol P_i released min^{-1} mg $protein^{-1}$		
Intact mitochondria	85.04 ± 2.11 ($n = 5$)	56.77 ± 2.13# ($n = 5$)	62.29 ± 3.15** ($n = 6$)
Intact mitochondria + 2,4-dinitrophenol	97.28 ± 4.16 ($n = 5$)	75.44 ± 6.55# ($n = 5$)	78.89 ± 4.84** ($n = 6$)
Freeze-thawing disrupted mitochondria	90.49 ± 2.97 ($n = 4$)	58.11 ± 2.85* ($n = 4$)	71.86 ± 4.42** ($n = 5$)

*$P < 0.05$ for (2) versus (1) and for (2) versus (3) (ANOVA and Student-Newman-Keuls post hoc testing).
#$P < 0.05$ for (2) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).
**$P < 0.05$ for (3) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).

extract had a positive effect only on the TMPD mediated ascorbate oxidation (+24%).

The ATPase activity measurements are summarized in Table 4. ATP hydrolysis was measured in three different systems. Aging caused diminutions of 33%, 22%, and 36%, respectively, in the ATPase activities of coupled mitochondria, uncoupled mitochondria, and freeze-thawing disrupted mitochondria. A significant positive effect of the *A. blazei* treatment was found only for the ATPase activity of freeze-thawing disrupted mitochondria (+24%).

3.4. Mitochondrial Respiration and Membrane Energization. The respiratory activity of intact brain mitochondria was measured using three different substrates, namely, succinate, pyruvate + L-malate, and α-ketoglutarate. The classical protocol was used in which the mitochondria were initially incubated in the closed oxygraph chamber without exogenous substrates. After stabilization of the very small respiratory rate, substrates were added and the mitochondria were allowed to respire under these conditions for 2-3 minutes. This respiration is labeled as the −ADP rate of

Table 5: Effects of the *A. blazei* extract treatment on the respiration of brain mitochondria of old rats. The oxygen consumption, ADP/O ratio, and respiration coefficient (RC) were assessed in the mitochondria as described in Materials and Methods. The values represent the mean ± standard errors of the mean.

Substrate	Experimental groups	Rate of oxygen uptake (nmol min^{-1} mg $protein^{-1}$)		ADP/O	RC
		−ADP	+ADP		
Succinate (10 mM)	(1) Young rats	16.11 ± 1.27 ($n = 6$)	51.65 ± 2.06 ($n = 6$)	1.54 ± 0.02 ($n = 6$)	2.67 ± 0.14 ($n = 6$)
	(2) Old rats	14.16 ± 1.477 ($n = 6$)	41.6 ± 2.68* ($n = 6$)	1.22 ± 0.14 ($n = 5$)	2.30 ± 0.32 ($n = 6$)
	(3) *A. blazei*-treated old rats	16.01 ± 0.976 ($n = 7$)	52.20 ± 3.50 ($n = 6$)	1.24 ± 0.16 ($n = 6$)	2.54 ± 0.27 ($n = 6$)
Pyruvate + L-malate (10 mM + 1 mM)	(1) Young rats	9.95 ± 0.26 ($n = 6$)	40.47 ± 2.44 ($n = 6$)	1.78 ± 0.10 ($n = 6$)	1.92 ± 0.10 ($n = 6$)
	(2) Old rats	9.64 ± 0.71 ($n = 5$)	30.71 ± 3.03$^{\#}$ ($n = 5$)	1.66 ± 0.28 ($n = 5$)	1.85 ± 0.16 ($n = 5$)
	(3) *A. blazei*-treated old rats	10.64 ± 0.54 ($n = 7$)	35.48 ± 2.12 ($n = 7$)	1.67 ± 0.04 ($n = 6$)	1.95 ± 0.18 ($n = 7$)
α-Keto-glutarate (10 mM)	(1) Young rats	4.85 ± 0.46 ($n = 6$)	18.50 ± 1.39 ($n = 6$)	1.88 ± 0.08 ($n = 6$)	2.63 ± 0.39 ($n = 6$)
	(2) Old rats	2.99 ± 0.62$^{\#}$ ($n = 3$)	15.53 ± 1.53 ($n = 3$)	2.17 ± 0.21 ($n = 3$)	3.22 ± 0.90 ($n = 3$)
	(3) *A. blazei*-treated old rats	3.51 ± 0.15 ($n = 3$)	16.39 ± 0.81 ($n = 3$)	2.60 ± 0.12** ($n = 3$)	4.43 ± 2.29 ($n = 3$)

$^{*}P < 0.05$ for (2) versus (1) and (2) versus (3) (ANOVA and Student-Newman-Keuls post hoc testing).
$^{\#}P < 0.05$ for (2) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).
$^{**}P < 0.05$ for (3) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).

Table 6: Effects of the *A. blazei* extract treatment on the mitochondrial membrane energization by forward (succinate) and reverse (ATP) electron flow. The mitochondrial membrane energization (transmembrane potential) was estimated fluorimetrically using safranin as a fluorescent probe. Intact mitochondria (1 mg protein) were incubated in a medium as described in Materials and Methods. The values represent the mean ± standard errors of the mean.

Energizing agent	Experimental groups		
	(1) Young rats	(2) Old rats	(3) *A. blazei*-treated old rats
	Fluorescence units		
Succinate	150.7 ± 6.5 ($n = 4$)	187.5 ± 11.7* ($n = 5$)	228.2 ± 8.07$^{\#}$ ($n = 6$)
ATP	81.3 ± 17.4 ($n = 7$)	48.3 ± 3.9 ($n = 4$)	130.5 ± 20.0** ($n = 6$)

$^{*}P < 0.05$ for (2) versus (1) and (2) versus (3) (ANOVA and Student-Newman-Keuls post hoc testing).
$^{\#}P < 0.05$ for (3) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).
$^{**}P < 0.05$ for (3) versus (2) (ANOVA and Student-Newman-Keuls post hoc testing).

oxygen uptake in Table 5. This phase was followed by the addition of a limited amount of ADP. The ADP stimulated respiration is labeled as the +ADP rate of oxygen uptake in Table 5. Table 5 reveals that the −ADP respiration was not significantly affected by aging when the substrates were succinate and pyruvate + L-malate. A diminution of 39% occurred, however, when α-ketoglutarate was the substrate. Coupled respiration (+ADP respiration), on the other hand, was significantly reduced by aging when succinate and pyruvate + L-malate were the substrates, the diminution amounting to 20 and 24%, respectively. The diminution caused by aging in the α-ketoglutarate coupled respiration lacked statistical significance. Treatment of old rats with the *A. blazei* extract was effective only in restoring the ADP-dependent respiration when succinate was the substrate. With pyruvate + L-malate as substrates a tendency toward restoration was apparent, but it lacked statistical significance. The ADP/O ratios were not significantly diminished by aging, nor were the respiratory control ratios affected. With respect to these parameters the only significant action of the *A. blazei* treatment was an increase in the ADP/O ratio when α-ketoglutarate was the substrate.

The membrane energization of brain mitochondria was assayed using safranin as a fluorescent probe [49, 50]. Typical traces are shown in Figure 5 and the mean results of the evaluations are listed in Table 6. The numbers in Table 6 represent the changes in fluorescence that occurred upon the addition of the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) which was done after stabilization of the fluorescence intensity due to succinate or ATP addition (Figure 5). With mitochondria of old rats the addition of succinate caused a more intense decrease in safranin fluorescence (24%) when compared to young rats. This effect was further enhanced by the *A. blazei* extract treatment (22% over nontreated old rats). Energization due to ATP addition, on the other hand, tended to be smaller in mitochondria of old rats when compared to the mitochondria of young rats, but the experimental variation was quite pronounced so that statistical significance could not be demonstrated. The *A. blazei* treatment of old

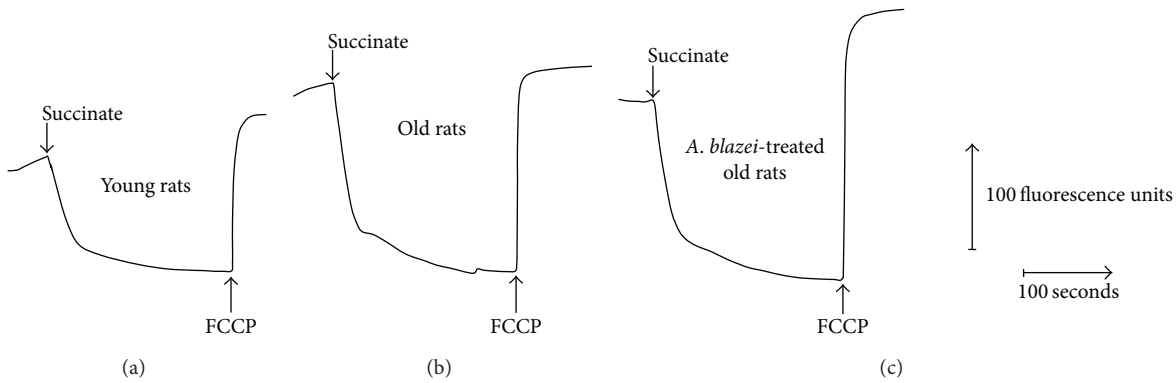

Figure 5: Changes in safranin fluorescence due to succinate in incubations containing brain mitochondria. Mitochondria (1 mg protein) were incubated in a medium containing (2 mL) containing 0.25 M mannitol, 5 mM potassium phosphate, 10 mM TRIS (pH 7.4), 0.2 mM EGTA, 50 mg% fatty acid-free bovine serum albumin, and 10 μM safranin. Succinate and FCCP were added as indicated. The wavelengths for excitation and emission were 520 and 580 nm, respectively.

rats, however, had also an increasing effect on the ATP-induced membrane energization.

4. Discussion

Diminution of mitochondrial enzyme activities and respiration upon aging is a phenomenon that has been observed in several studies including rat brain, liver, and cardiac mitochondria [25, 26, 51–55]. There are also studies with human liver and skeletal muscle mitochondria [56, 57]. In the majority of these studies, however, either enzyme activities or the respiratory activity with various substrates were measured. An exception is a study with human liver mitochondria [56], in which coupled respiration and some oxidases were measured. The simultaneous measurement of these parameters, however, is important for establishing possible rate-limiting relationships. In the present study with brain mitochondria several enzyme activities have been measured in addition to the respiratory activity of intact mitochondria, so that our data offer the opportunity of discussing the way by which these variables are related. Among the enzymes measured in the present work succinate dehydrogenase was the most strongly affected by aging as it was 45% diminished in old compared to young rats. This reduction, however, did not reflect in a similar diminution in the respiratory rates of intact mitochondria. In fact, no diminution was found in the absence of exogenously added ADP, a situation in which the phosphorylation rate limits respiration. When this limitation was abolished by the addition of ADP a 20% diminution of respiration was observed. This diminution was similar to that found in disrupted mitochondria (succinate oxidase), namely, 17%. The diminution of 20% would also correspond to the inhibition of oxidative phosphorylation in mitochondria of old rats, a relatively modest figure if one takes into account both the diminutions of the succinate dehydrogenase activity (−45%) and the ATPase/ATP-synthase activity (−33%), which are presumed to contribute simultaneously to the rate of coupled respiration. In the case of the substrate α-ketoglutarate the discrepancy between enzyme activity and coupled respiration is even more pronounced. Old rats revealed a 36% diminished α-ketoglutarate activity. Coupled respiration with α-ketoglutarate, however, was only minimally affected in mitochondria of old rats to the point that the difference was not even statistically significant. A more close correlation between enzyme inhibition and ADP-dependent respiration was found when pyruvate + L-malate were used as substrates. The pyruvate dehydrogenase was 27% smaller in mitochondria of old rats and the coupled respiration was 24% smaller. Malate dehydrogenase, as a near-equilibrium enzyme (very high activity), is not expected to exert any rate control activity. Clearly, taking the observations as a whole, no prediction about the actual impairment of coupled respiration can be made based on the diminution of enzyme activities because it is difficult to infer a priori the rate-limiting step for a given phenomenon. This occurs probably because there are many factors influencing the actual rate of ADP phosphorylation in intact mitochondria, including the membrane energization which, by the way, was increased in mitochondria of old rats at least when succinate was the substrate. If higher membrane energization, as detected by the safranin probe, represents increased ionic concentration gradients, this could also be meaning an additional drive for oxidative phosphorylation, partly compensating enzyme deficiencies.

The *A. blazei* treatment was quite successful in improving the oxidative state of the brain of old rats. Our observations are consistent with similar effects of the same *A. blazei* extract on the injury caused by paracetamol in both hepatic and brain tissue [18]. The *A. blazei* extract was particularly able to bring back the levels of lipid peroxidation (TBARS) of old rats to those found in young rats. This action can, in principle at least, be attributed to the free-radical scavenging ability of several constituents of *A. blazei*, as, for example, the phenolics [58]. The three phenolics that have been identified in *A. blazei*, gallic acid, syringic acid, and pyrogallol, have also demonstrated to possess pronounced antioxidant activities [17]. They are probably present in the aqueous extract used in the present study if one takes into account their pronounced hydrophilic character.

Important constituents of *A. blazei* that are infrequently mentioned in the specialized literature are adenosine and other nucleosides and nucleotides which are quite abundant in this mushroom [19]. Nucleosides and nucleotides are purinergic agents and purinergic effects of an *A. blazei* extract have been recently demonstrated to occur in the rat liver [19]. Adenosine, but possibly also other activators of A_1 purinergic receptors, confers cytoprotection in the cardiovascular and central nervous systems by activating cell surface adenosine receptors [22, 59]. Activation of these receptors, in turn, is postulated to activate antioxidant enzymes via protein kinase C phosphorylation of the enzymes or of intermediates that promote activation [22]. It is thus possible that the increased activities of antioxidant enzymes caused the *A. blazei* treatment in old rats were partly caused by its contents in adenosine or other purinergic agents.

Antioxidant action can also be expected from the polysaccharides that are present in *A. blazei* [60, 61]. In this respect it must be mentioned that the hepatoprotective action of partially purified fungal polysaccharides (including antioxidant effects) has been recently demonstrated [62, 63]. Further active components of *A. blazei* might also include oligopeptides. In fact an oligopeptide from *A. blazei*, rich in Pro, Lys, and Phe and possessing antioxidant activity, has been recently described [64].

The *A. blazei* treatment was also successful in increasing the activities of several mitochondrial enzymes in old rats especially the succinate dehydrogenase, α-ketoglutarate dehydrogenase, NADH dehydrogenase, and the cytochrome c oxidase. The latter was nearly doubled by the *A. blazei* treatment. These results are close to those obtained in a study in which old rats were treated with *Ganoderma lucidum* extracts using an experimental schedule similar to that of the present work [55]. Furthermore, the *A. blazei* treatment enhanced both the succinate- and ATP-dependent membrane energization of the mitochondrial membrane. In spite of all these effects the influence of the treatment on the respiratory rates of intact mitochondria in the presence and absence of ADP was relatively modest. Only the succinate-driven respiration in the presence of exogenous ADP was significantly increased in such a way as to approach the respiration rates found in brain mitochondria of young rats. No doubt that this event, which is mainly the consequence of the succinate dehydrogenase stimulation by the *A. blazei* treatment, represents a gain in terms of the rat brain energetics. On the other hand, with respect to the other dehydrogenases, the question must be raised about the possible physiological consequences of their stimulations by the *A. blazei* extract treatment. At least for the α-ketoglutarate and pyruvate dehydrogenases their increases may be related to the improvement of the oxidative state of the mitochondria as evidenced by the increased mitochondrial GSH levels found in treated old rats. Enhanced activities of the α-ketoglutarate and pyruvate dehydrogenases are expected to increase the $NADH/NAD^+$ ratio which, in turn, also leads to an increased NADPH nucleotide transhydrogenase activity with a concomitant increase in the $NADPH/NADP^+$ ratio. The consequence will be an increased regeneration of GSH via glutathione reductase and also a more efficient removal of H_2O_2 via glutathione peroxidase [65, 66].

The mechanisms by which the *A. blazei* extract increases the activities of several enzymes linked to energy metabolism in the mitochondria of old rats cannot be inferred with certainty from the data obtained in the present work. However, it is likely that the antioxidant properties of the extract played an important role. This proposition bases on the fact that many alterations in enzymatic activities in the brain of old rats are accompanied by oxidative modifications in proteins such as carbonylations and nitrations [67]. In principle such modifications can be prevented by improving the antioxidant defenses of the cells, a task for which the *A. blazei* extract is well equipped as already discussed above. It is also possible that components of the extract promote the expression of specific proteins. Important candidates for these actions are the purinergic agents of the extract, adenosine and others [19]. Purinergic signalling is very important in the neuronal tissue [21] and it is said that the levels of adenosine increase when there is an imbalance between the rates of energy use and the rates of energy delivery [21].

In conclusion, the experiments of the present work confirm the widespread notion that aging increases oxidative stress in the brain [2, 4, 24, 34] and that it also negatively affects several mitochondrial enzyme activities involved in energy metabolism [24, 51], though not always a precise correlation between coupled respiration and enzyme activities was apparent. And, more important, the results show that treatment with an aqueous extract of *Agaricus blazei* can improve the oxidative state of the brain tissue and can also reverse some of the deleterious effects of the aging process on mitochondrial oxidative enzymes. It remains to be demonstrated if these effects also occur in humans during aging. If confirmed, an extract of this mushroom could be incorporated into the diet as a supplement able to stimulate the body defense against oxidative stress.

Conflict of Interests

The authors state that they have no conflict of interests concerning the present paper.

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