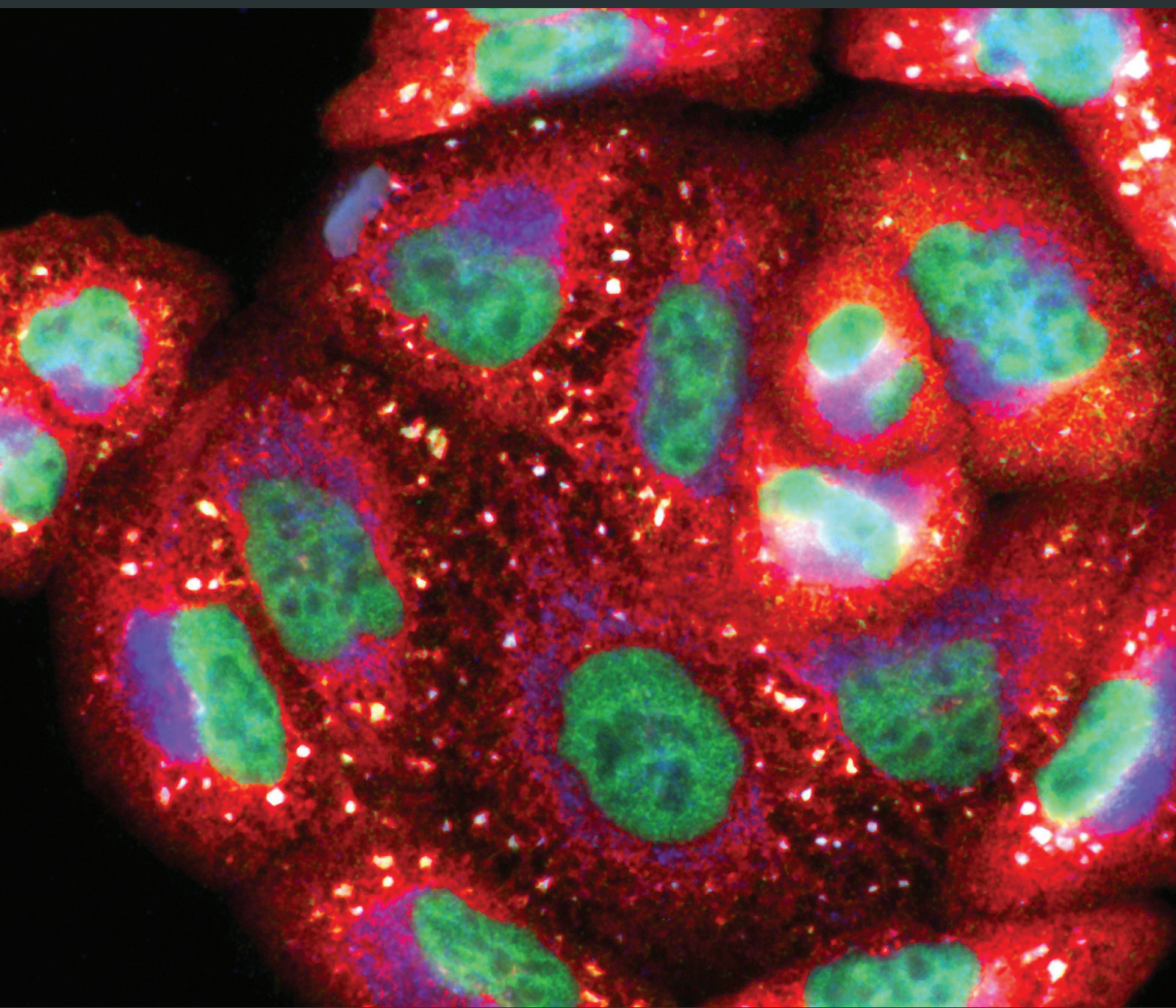
Oxidative Medicine and Cellular Longevity

Antioxidants and Prooxidants: Effects on Health and Aging

Lead Guest Editor: Márcio Carocho
Guest Editors: Isabel C. F. R. Ferreira, Patricia Morales, and Marina Sokovic



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Editorial Board

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Editorial

Antioxidants and Prooxidants: Effects on Health and Aging

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Reactive species are compounds related to two types of molecules, the reactive oxygen species and the reactive nitrogen species. These compounds are produced in the normal metabolism of cells and may take part in the pathological process named oxidative stress, which is promoted when the balance between free radicals and antioxidants tends to favour the former. As a result, the excess of free radicals within the human body leads to oxidative stress, resulting in the development or potentiation of many types of diseases, namely, chronic disorders, cardiovascular problems, diabetes, cancer, and rheumatoid arthritis [1].

A few decades ago, the belief regarding free radicals and oxidative stress was that they were completely undesired and dangerous for the human body and needed to be removed from our bodies immediately after their formation, either through endogenous or exogenous (diet) antioxidants. Today, this demonizing view has mellowed down and it is known that oxidative stress is part of the innate human immune system, and that free radicals are also related to defence mechanisms, but also used as cell mediators, pathway signalling, cell differentiation, proliferation and migration inducers, and release or inactivate the endothelium-derived relaxing factor, among other processes [1, 2].

The aim of this special edition is to shed some light on the effects of antioxidants and prooxidants on diseases and their overall impact on aging, following the free radical theory of aging. The initial focus of aging was centred on the free radical damage to macromolecules; however, it has become clear that hydroperoxides like H_2O_2 play important roles in physiologic signalling. Thus, the dichotomy resides on the fact that alterations in redox signalling are observed in aging, but, on the other hand, deviation from redux homeostasis can result in disease, making the study of redox signalling a hot topic. Furthermore, the free radical theory of aging has been threatened after recent discoveries on the importance of hydroperoxides and other radicals in the human body. Many scientists claim that it is should not be considered a single theory, but a part of a wider general theory of aging [3, 4]. Despite the free radical theory of aging being questioned, the link between free radicals, oxidative stress, and many diseases is scientifically proven. Examples of these diseases are neurodegenerative ones, which, with increasing life expectancy will also increase in the coming decades. New and unusual methods are being tested for these types of diseases, namely, by fine-tuning antioxidant concentrations, exhaustive study of antioxidant mechanisms and interactions, modulation of oxidative stress, and other therapeutic approaches [5–7].

This special edition, comprised of 9 original research articles and 4 reviews on various aspects, namely, pro and antioxidant functions of the peroxisome-mitochondria connection and its impact on aging and disease, the impact of oxidative stress on the regeneration of pancreatic beta cells, the impact of metallothionein in brain disorders, and finally an extensive overview of the role of flavonoids as putative inducers of transcription factors Nrf2, FoxO, and PPARγ. In terms of research manuscripts, four of these describe the

role of specific plant compounds in the human body or in test subjects (mice and rats), namely, the cardioprotective effects of salvianic acid A and raloxifene in mice with elevated homocysteine and estrogen-deficient rats, respectively. The effects of berberine as preconditioning agent to reduce premature senescence of cells were also researched, as well as the effect of resveratrol on rats' cognitive performance with aging. Plant extracts were also described as having cardioprotective effects and reducing oxidative stress, while the *in vitro* antioxidant and antifibrotic profile of other herbal formulations was tested. Dietary supplements, commonly reported as being effective against many neurodegenerative diseases and having antioxidant activity, were also tested in terms of quality control by liquid chromatography with the aid of a mass spectrometer. Finally, using new agar diffusion methods, the risk of tumors was evaluated through the measurement of urinary and serumal antioxidants.

The editors hope that the published articles of this special edition may be of interest to all readers, and overall, be of aid to further improve the research on free radicals, antioxidants and prooxidants, aging, and related diseases.

Acknowledgments

We would like to thank the editorial staff for the help during the whole extension of the preparation of the special edition, but also to the kind reviewers that anonymously contributed to improving the manuscripts. A word of appreciation to all authors that chose to submit their works to this special edition. We hope readers enjoy the latest developments that are published in this special edition.

Márcio Carocho
Isabel C. F. R. Ferreira
Patricia Morales
Marina Soković

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Research Article

Cardioprotective and Metabolomic Profiling of Selected Medicinal Plants against Oxidative Stress

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In this research work, the antioxidant and metabolomic profiling of seven selected medicinally important herbs including *Rauvolfia serpentina*, *Terminalia arjuna*, *Coriandrum sativum*, *Elettaria cardamom*, *Piper nigrum*, *Allium sativum*, and *Crataegus oxyacantha* was performed. The *in vivo* cardioprotective potential of these medicinal plants was evaluated against surgically induced oxidative stress through left anterior descending coronary artery ligation (LADCA) in dogs. The antioxidant profiling of these plants was done through DPPH and DNA protection assay. The *C. oxyacantha* and *T. arjuna* showed maximum antioxidant potential, while the *E. cardamom* showed poor antioxidative strength even at its high concentration. Different concentrations of extracts of the said plants exhibited the protection of plasmid DNA against H_2O_2 damage as compared to the plasmid DNA merely treated with H_2O_2. The metabolomic profiling through LC-MS analysis of these antioxidants revealed the presence of active secondary metabolites responsible for their antioxidant potential. During *in vivo* analysis, blood samples of all treatment groups were drawn at different time intervals to analyze the cardiac and hemodynamic parameters. The results depicted that the group pretreated with HC4 significantly sustained the level of CK-MB, SGOT, and LDH as well as hemodynamic parameters near to normal. The histopathological examination also confirmed the cardioprotective potential of HC4. Thus, the HC4 being safe and inexpensive cardioprotective herbal combination could be considered as an alternate of synthetic drugs.

1. Introduction

Oxidation is a natural phenomenon that leads to the formation of free radicals known as reactive oxygen species (ROS) [1]. Some of the ROS are very important in cell metabolism including intercellular signaling, phagocytosis, and energy production [2]. However, overproduction of ROS during biological processes resulted in extensive pathological alterations like DNA damage and various degenerative disorders. Humans are constantly exposed to natural DNA-damaging agents such as UV light, dietary agents, and endogenously formed free radicals. Damaged DNA accumulates in the brain, muscle, liver, kidney, and in long-lived stem cell, which causes aging, decline in gene expression, and loss of functional capacity [3].

Antioxidants are compounds that slow down or delay the oxidation process by obstructing the initiation of a series of oxidizing reactions [4]. Owing to the presence of antioxidants, medicinal plants have a shielding effect against various diseases, thus emerging as substantial therapeutic agents. Medicinal plants are a time-honored medicine used since the ancient era for treatment of various ailments in human beings [5]. Herbal medicines, in addition to their traditional values, also hold great public and medical interest worldwide as sources of novel lead compounds for drug development. Hence, the medicinal plants will be natural protective strategy and would be freely available with low cost as compared to synthetic drugs [6].

Pakistan is bestowed with a wide range of plant species with unique biodiversity in different climatic zones [7]. These

medicinal plants have been used in scientific research for various cardiovascular disorder in human beings [8, 9]. Currently available synthetic cardioprotective drugs exhibit a number of side effects and are out of reach for poor communities. Cardioprotective effects of some medicinal plants, which are safe and inexpensive, have already been explored [10–13]. Therefore, the green products having cardioprotective and antioxidative potential have attracted many researchers towards metabolomic profiling and phytotherapy. The antioxidative strength of medicinal plants is because of the secondary metabolites present in it [11].

An LC-MS-based metabolomic study has become a powerful analytical tool for assessment of various secondary metabolites in herbal medicine [14]. These secondary metabolites have been found to possess a broad range of therapeutic properties, including antioxidant, cardioprotective, and antihypertensive potential [15]. A thorough integration of information from metabolomics is expected to provide solid evidence-based scientific rationales for the development of modern phytomedicines [16]. Therefore, in this research, the antioxidant potential, metabolomic profiling, and *in vivo* cardioprotective evaluation of *Rauvolfia serpentina*, *Terminalia arjuna*, *Coriandrum sativum*, *Elettaria cardamom*, *Piper nigrum*, *Allium sativum*, and *Crataegus oxyacantha* was done to get the potent role of these natural antioxidants in health. All these medicinal plants were selected as these plants have already been reported to possess cardiotonic, antioxidant, and antilipidemic potential [4, 17]. Moreover, the previous literature and the knowledge of CAM practitioners also endorsed the cardioprotective effect of these selected parts of the plants.

2. Materials and Methods

2.1. Preparation of Herbal Extract. Different parts of the medicinal plant like the roots of *R. serpentina*, bark of *T. arjuna*, seeds of *C. sativum* and *E. cardamom*, leaves of *P. nigrum*, and fruit of *A. sativum*, and *C. oxyacantha* were collected from the Botanical Garden of the University of Agriculture, Faisalabad, Pakistan and from the local herbal market. All the selected parts of the plants were identified by the plant taxonomist in the Department of Botany, University of Agriculture, Faisalabad, Pakistan. These parts of the plants were washed and pulverized to get fine powder. The powdered plants (5 g of each) were macerated in methanol (50 mL). The macerate was kept in an orbital shaker for four days. The supernatant was decanted and the residue was remacerated with methanol. The pooled supernatants were combined and filtered through Whatman's filter paper number 1. The rotary evaporator was used to concentrate the filtrate, and subsequently the filtrate was lyophilized [17].

2.2. Antioxidant Assay

2.2.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Free Radical Scavenging Assay. The antioxidant potential was determined by using 1,1-diphenyl-2-picrylhydrazyl as a free radical. The methanolic solution of DPPH (0.1 mM) and plant extract of different concentrations (20, 40, 60, 80, and 100 μg/mL) were mixed in equal volume. The mixtures was left for 30 minutes in the dark, and the absorbance was noted at 517 nm. Ascorbic acid was used as a standard. The percentage DPPH inhibition of plant extract was calculated as follows:

$$\text{DPPH inhibition}(\%) = \left[\frac{1 - A_1}{A_0}\right] \times 100, \quad (1)$$

where A_1 is the absorbance of the sample, and A_0 is the absorbance of control [4, 18].

2.2.2. DNA Protection Assay. The DNA protection assay of extracts of different concentrations (100, 500, and 1000 μg/mL) of selected plants was performed by using the pBR322 plasmid DNA. pBR 322 DNA plasmid (0.5 μL) was diluted by using 50 mM sodium phosphate buffer (pH 7.4). After dilution, pBR 322 DNA (3 μL) was treated with 5 μL of selected concentrations of plant extracts. 4 μL of 30% H_2O_2 was added to it, and sodium phosphate buffer (pH 7.4) was used to make the volume up to 15 μL. The difference in migration between the oxidized and native DNA was observed on 1% agarose by horizontal DNA gel electrophoresis using a wide mini system (Techview, Singapore). 1% agarose was prepared by dissolving 1 g agarose in 100 mL of 1X × TAE buffer and placed in a microwave oven for two minutes. It was cooled and poured in a casting plate. After solidification, the gel was kept in the sodium phosphate buffer and samples were loaded in the wells one by one. The gel was stained with ethidium bromide and documented by Gene NuGenius unit Syngene model (Cambridge, UK). DNA protection was observed from DNA band corresponding to that of native in the presence and absence of various concentrations (100, 500, and 1000 μL) of each plant's extract [19].

2.3. Metabolomic Profiling. Metabolomic profiling of all the selected medicinal plants was performed through liquid chromatography-mass spectrometry (LC-MS) analysis.

2.4. Liquid Chromatography-Mass Spectrometry (LC-MS). The selected medicinal plants were analyzed by using liquid chromatography combined with electrospray ionization mass spectrometry (LC-ESI-MS). The plant extracts were filtered through a 0.45 μm syringe filter before analysis. Separation was performed on Surveyor plus HPLC System equipped with Surveyor auto (Thermo Scientific, San Jose, CA, USA). The pump was equipped with a Luna Reverse Phase C-18 analytical column (4.6 × 150 mm, 3.0 μm particle size) (Phenomenex, USA). Solvent elution consisted of LC-MS grade methanol and acidified water (0.5% formic acid *v*/*v*) as the mobile phase A and B, respectively. Solvent elution consisted of gradient system which runs at a flow rate of 0.3 mL/min. The gradient elution was programmed as follows: from 5 min in 15% A to 20 min in 25% B and maintained it till the end of the analysis. A 20 minute re-equilibration time was used after each analysis. The column was maintained at 25°C and the injection volume was 5.0 μL. The effluent from the HPLC column was directed to an electrospray ionization mass spectrometer (LTQ XL™ linear ion trap, Thermo Scientific, River Oaks Parkway, USA).

Table 1: Different herbal combinations given to treatment group.

Groups	*R. serpentina*	*E. cardamom*	*P. nigrum*	*A. sativum*	*T. arjuna*	*C. oxyacantha*	*C. sativum*
Herbal ratio							
HC1	1	0.5	1	0.5	—	—	0.5
HC2	1	0.5	1	0.25	—	1	0.5
HC3	1	1	0.5	—	1	—	0.5
HC4	0.5	—	—	0.5	1	0.5	1

Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from 260 to 2000 m/z. The optimum values of the ESI-MS parameters were spray voltage +4.0 kV, sheath gas and auxiliary gas were 45, and 5 units/min, respectively, capillary temperature 320°C, capillary voltage −20 V, and tube lens −66.51 V.s The accurate mass spectra data of the molecular ions was processed through the Xcalibur software (Thermo Fisher Scientific Inc, Waltham, MA, USA) [20].

2.5. Selection of Animals. The eighteen male stray dogs of 1-2 years were selected as experimental animals and acclimatized for one week under laboratory conditions (27°C in 12 hr dark/light cycle). All the animals were housed in the Animal House, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad. They were fed with standard feed. The experiment was performed by taking the approval from the institutional ethics review board in the presence of a licensed veterinarian.

2.6. Herbal Combination Therapy. The four different herbal combinations of selected plant extracts were formed as given in Table 1. These herbal combinations were evaluated for their synergistic cardioprotective potential. The dogs were divided into three groups. The first group of dogs was the control group, to which normal diet was fed for 23 days. The second group was the positive control group, in which the dogs were treated with normal diet for 22 days, and after that, the ligation of the left anterior descending coronary artery (LADCA) was performed on the 23rd day. The third group was the treatment group which was further divided into four subgroups. Each subgroup was pretreated with its respective herbal combination (Table 1) for 22 days. On day 23, all the dogs of the treatment group underwent LADCA ligation. After completion of the surgical procedure, the blood samples were taken at various time intervals (0 to 48 hr) to analyze the cardiac markers (CK-MB, SGOT, and LDH). At the end of the experiment, the dogs were anesthetized by Sodium Pentothal and the hearts were excised for histopathological studies.

2.7. Surgical Induction of Myocardial Infarction. The dogs were anesthetized with Sodium Pentothal (20 mg/kg). Atropine was administered subcutaneously at a dose of 0.1 mg/kg once before the surgery to keep the heart rate elevated during the surgical procedure and to reduce the bronchotracheal secretions. The body temperature was monitored and maintained at 37°C. The animals were ventilated with room air from a positive pressure by using compressed air at the rate of 90 stroke/min and tidal volume of 10 mL/kg. The left jugular vein was cannulated with polyethylene tube for administration of supplemental anesthetic and saline (0.9%) infusion. The neck was opened and left thoracotomy was performed to open the thoracic cavity. Anatomy of the left anterior descending coronary artery (LADCA) was examined visually and then ligated 4-5 mm from its origin and the end of this ligature was passed through polyethylene tube to form a snare. The thoracic cavity was covered with saline-soaked gauze to prevent the heart from drying. After completion of the surgical procedure, the heart was returned to its normal position in the thoracic cavity [21, 22].

2.8. Estimation of Hemodynamic Variables. The mean arterial pressure (MAP) and heart rate of dogs in all the groups were calculated. The left thoracic cavity was opened by an incision at the fifth intercostal space and the heart was exposed. A sterile metal cannula was introduced in the cavity of the left ventricle from the posterior apical region of the heart for measuring left ventricular dynamics at preset time throughout the surgical procedure [23].

2.9. Biochemical Analysis. The blood sampling was performed at different time intervals (0, 12, 24, and 48 hr) during the experimental period. The cardiac biomarkers including creatine kinase-MB (CK-MB), serum glutamic-oxaloacetic transaminase (SGOT), and lactate dehydrogenase (LDH) were analyzed by using "BioMed kits" having patch numbers MBS705376, BGO094144, and LDHK0103016, respectively. All the kits were purchased by "UH Analytics Pakistan."

2.10. Statistical Analysis. The data was statistically analyzed by using two-way ANOVA and Turkey's multiple comparison tests with the help of GraphPad Prism version 7.00, supplied by developer GraphPad software, Inc. [24]. The results have been presented as Mean ± SD.

3. Results

3.1. Antioxidant Assay

3.1.1. DPPH Free Radical Scavenging Activity. The DPPH free radical scavenging activity (in terms of % age inhibition) of *R. serpentina*, *T. arjuna*, *C. sativum*, *P. nigrum*, *E. cardamom*, *A. sativum*, and *C. oxyacantha* at various concentrations (20, 40, 60, 80, and 100 μg/mL) was examined (Figure 1). The *T. arjuna* and *A. sativum* showed higher antioxidant potential even at least concentration of 20 μg/mL as compared to the same concentrations of other selected plants. On the other hand, the *E. cardamom* presented relatively low antioxidant

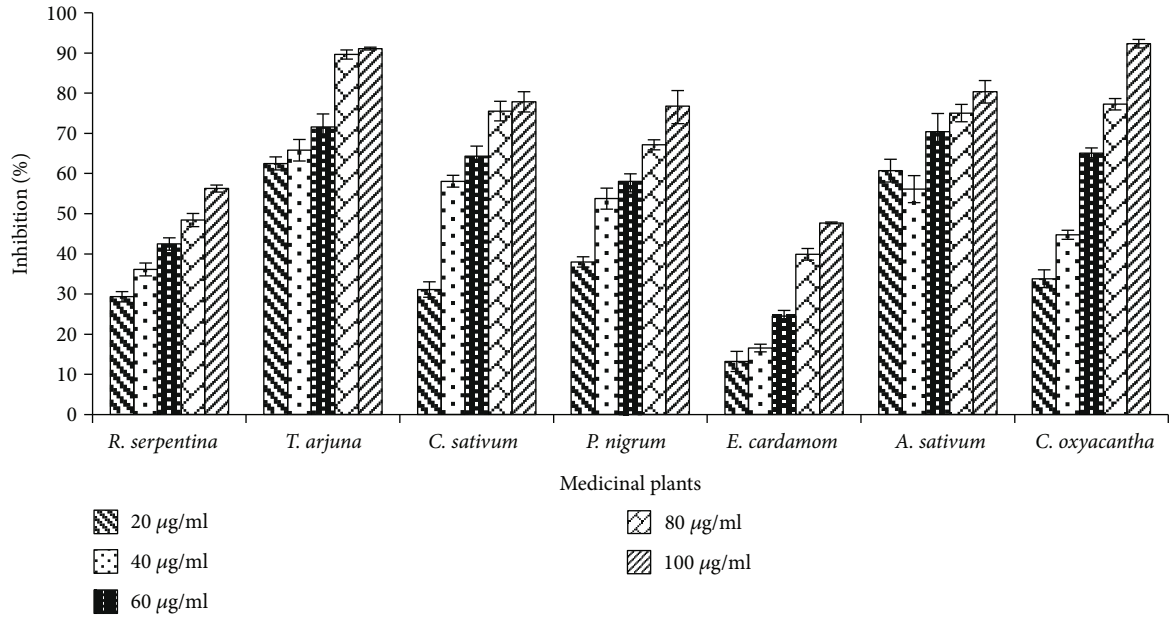


Figure 1: Graphical presentation of antioxidant potential of selected medicinal plants through DPPH radical scavenging activity.

potential even at its higher concentration of 100 μg/mL. In case of *C. oxyacantha*, the concentration of 20 and 40 μg/mL showed low antioxidative strength but it rapidly increased with further increase in concentration from 60 to 100 μg/mL. All the said medicinal plants depicted the dose-dependent response for free radical scavenging potential, that is, the activity of plant extracts in terms of % age inhibition increased with respect to concentrations (Figure 1). The selected medicinal plants could be beneficial to mankind by virtue of their effective antioxidant activity which may able to impart therapeutic role against various diseases.

3.2. DNA Protection Assay. The effect of varying concentrations of medicinal plants on DNA damage along with positive controls (30% H_2O_2, 2 mM $FeSO_4$) has been presented in Figures 2 and 3. The free radicals produced in response to O_2 and $FeSO_4$ caused the strand cleavage of pBR322 plasmid DNA and resulted in DNA band streaking (Figure 2). All the plants exhibited protection of plasmid DNA against H_2O_2 damage as compared to the plasmid DNA merely treated with H_2O_2. The DNA protective potential of all concentrations of said medicinal plants was in concentration-dependent manners which revealed that higher concentrations of extracts are more protective against H_2O_2-induced damage. The least concentration (100 μg/mL) of *P. nigrum* showed the band streaking (lane 7 of Figure 2) while the concentration of 500 and 1000 μg/mL of *P. nigrum* exhibited a good protection of pBR322 plasmid DNA as presented in the corresponding lanes 8 and 9. However, in case of *C. oxyacantha*, the concentrations of 100 μg/mL and 500 μg/mL (lanes 4 and 5 of Figure 2) showed noticeable DNA protection. Minor strand breaks were observed with low concentration (100 μg/mL) of *A. sativum* (Figure 2, lane 13), while its higher concentration (500 and 1000 μg/mL) showed promising protection against DNA damage (Figure 2, lanes 14 and 15).

3.3. Metabolomic Profiling. Metabolomics approaches using LC-MS-based techniques are a useful technique in evaluating the secondary metabolites of medicinal plants. LC-MS-based metabolomics is a powerful new tool for mechanistic studies of drug metabolism.

3.4. Terminalia Arjuna. LC-MS analysis of *T. arjuna* was performed to evaluate the phytoconstituents including phenolics, flavonoids, and alkaloids. The full mass spectrum obtained by LC-MS analysis was presented in Figure 4. The mass spectrum depicted the high peaks at 413.42, 511.50, 321.33, 589.33, and 685.58. The CIDMS-MS-ESI fragments ion of 685.58 peak resulted in three abundant peaks at 667.50, 523.33, and 457.25. The peak of 667.50 indicated the presence of termiarjunoside 1,3,9,22-tetraol-12-en-28-oicacid-3-D-glucopyranoside. The presence of termiarjunoside I from the bark of *T. arjuna* was also reported in a study by Ali et al. 2006. The fast atom bombardment mass spectroscopy (FABMS) of *T. arjuna* also displayed a molecular ion peak at m/z = 666 $[M]^+$ indicating the presence of termiarjunoside I, with a molecular formula of $C_{36}H_{58}O_{11}$, which was also supported by ^{13}C and distortionless enhancement by polarization transfer (DEPT) NMR spectra. The mass spectrum revealed the highest peak at 301.08, 317.25, and 169.08 which indicate the presence of quercetin, myricetin, and gallic acid in *T. arjuna*. The presence of gallic acid was further confirmed by MS-MS using CID (30.00). The peak at 125.08 is the consequence of the removal of COO^- from gallic acid. The MS-MS of the peak 317.25 by CID (21.00) showed the highest peaks at 302.08, 241.08, and 179.06. However, the peaks at 193 and 289 may indicate the presence of ferulic acid and catechin, respectively.

3.5. Crataegus oxyacantha. The LC-MS analysis of *C. oxyacantha* was executed to assess the phytoconstituents. The mass spectrum of *C. oxyacantha* showed the peak at 593.17

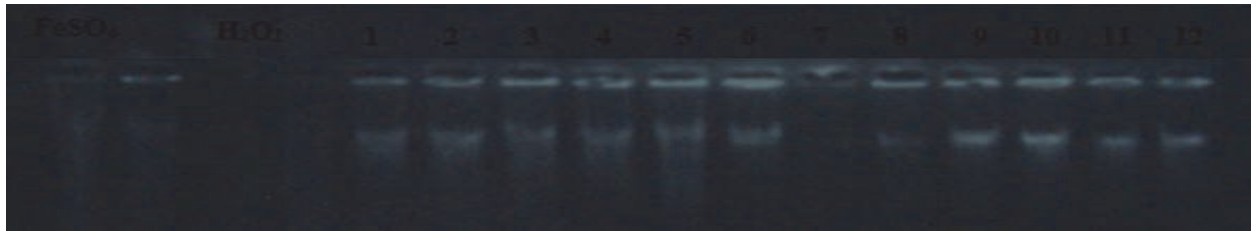


Figure 2: Agarose gel electrophoresis pattern of pBR322 plasmid DNA treated with 30 mM H_2O_2 in the presence and absence of different plants extract. [lane 1: pBR322 DNA + 30 mM H_2O_2 + P1 (100 μg/mL), lane 2: pBR322 DNA + 30 mM H_2O_2 + P1 (500 μ/mL), lane 3: pBR322 DNA + 30 mM H_2O_2 + P1 (1000 μg/mL), lane 4: pBR322 DNA + 30 mM H_2O_2 + P2 (100 μg/mL), lane 5: pBR322 DNA + 30 mM H_2O_2 + P2 (500 μg/mL), lane 6: pBR322 DNA + 30 mM H_2O_2 + P2 (1000 μg/mL), lane 7: pBR322 DNA + 30 mM H_2O_2 + P3 (100 μg/mL), lane 8: pBR322 DNA + 30 mM H_2O_2 + P3 (500 μg/mL), lane 9: pBR322 DNA+ 30 mM H_2O_2 + P3 (1000 μg/mL), lane 10: pBR322 DNA + 30 mM H_2O_2 + P4 (100 μg/mL), lane 11: pBR322 DNA + 30 mM H_2O_2 + P4 (500 μg/mL), lane 12: pBR322 DNA + 30 mM H_2O_2 + P4 (1000 μg/mL)].

Figure 3: Lane13: pBR322 DNA + 30 mM H_2O_2 + P5 (100 μg/mL), lane 14: pBR322 DNA + 30 mM H_2O_2 + P5 (500 μg/mL), lane 15: pBR322 DNA + 30 mM H_2O_2 + P5 (1000 μg/mL), lane 16: pBR322 DNA + 30 mM H_2O_2 + P6 (100 μg/mL), lane 17: pBR322 DNA + 30 mM H_2O_2 + P6 (500 μg/mL), lane 18: pBR322 DNA +30 mM H_2O_2 + P6 (1000 μg/mL), lane 19: pBR322 DNA + 30 mM H_2O_2 + P7 (100 μg/mL), lane 20: pBR322 DNA + 30 mM H_2O_2 + P7 (500 μg/mL), lane 21: pBR322 DNA + 30 mM H_2O_2 + P7 (1000 μg/mL). P1 = *T. arjuna*; P2 = *C. oxyacantha*; P3 = *P. nigrum*; P4 = *R. serpentina*; P5 = *A. sativum*; P6 = *C. sativum*; P7 = *E. cardamom.*

which indicated the presence of bioactive compounds proanthocynidine with positive mode of ESI (Figure 5). The MS-MS of peak 593 gave the highest peaks at 429.25, 457.17, 411.25, and 401.17, where the peak at 457.17 might indicate the presence of ursolic acid. The LC-MS-ESI also revealed the presence of cratagolic acid at peak of 417 (m/z). The CID MS-MS of the peak 381 of *C. oxyacantha* showed the peak at 301.17 may give the idea of the presence of quercetin.

3.6. Rauwolfia serpentina. The LC-MS analysis of roots extract of *R. serpentina* was performed. The full mass spectrums along with the highest peaks at 327.25 and 355.33 indicated the presence of ajmaline and yohimbine, respectively (Figure 6). The MS-MS with CID of 25.00 at peak 327 produced different fragment ion peaks. Among these peaks, the peak at 353.25 m/z may indicate the presence of ajmailacine. The mass spectrum of *R. serpentina* also depicted the presence of serpentine at the peak 349.52.

3.7. Allium sativum. A. sativum was subjected to LC-MS analysis to evaluate the presence of phytoconstituents that might be responsible for cardiovascular diseases, dyslipidemia, and hypertension. The LC-MS analysis of *A. sativum* depicted the highest peaks at 896.92, 917.75, and 782.58 (Figure 7). The MS-MS of 896.92 with CID (25.00) gave the peak at 319.25, which indicated the presence of myricetin. The mass spectrum also showed the presence of apigenin at peak 269.08 with negative mode of ESI.

3.8. Coriandrum sativum. LC-MS analysis of the seed extract of *C. sativum* was performed to evaluate the active phytoconstituents including phenolics, flavonoids, and alkaloids. The full mass spectrum indicated the existence of caffeic acid at peak 179.08 and isorhamnetin-3-O-glucoside at 478.17 m/z. The mass spectrum of *C. sativum* also showed apigenin-6-*C*-glucoside at peak 593.25 m/z with negative mode of electrospray ionization (Figure 8).

3.9. Elettaria cardamom. The mass spectrum obtained by LC-MS analysis of *E. cardamom* represented the high peaks at 195.17133.06 and 333.33. The peak at 195.17 indicated the presence of terpinyl acetate. The mass spectrum also depicted the presence of sebinen at 137.08 peak (Figure 9).

3.10. Piper nigrum. The methanolic extract of *P. nigrum* is subjected to LC-MS analysis to determine its bioactive compounds that impart crucial role in cardioprotection. The pippercide, an active ingredient of *P. nigrum*, showed its peak at 219.08 (Figure 10).

3.11. In Vivo Analysis

3.11.1. Effect of Herbal Combinations on Cardiac Markers. To investigate whether the combinations of herbal extracts under investigation would offer any added advantage over individual herbal treatment, the effects of HCs were compared with normal and the surgically induced MI group. The potential of herbal combinations was evaluated by analysing the cardiac markers including CK-MB, SGOT, and LDH.

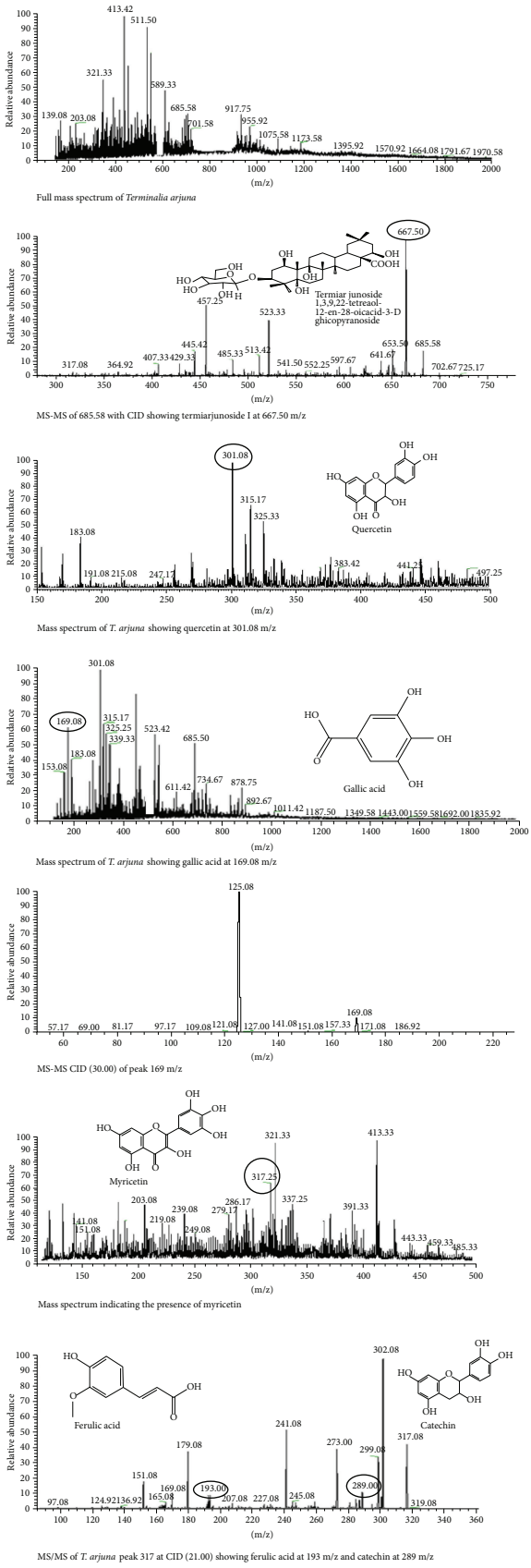


Figure 4: LC-MS analysis of *T. arjuna*.

The effect of different herbal combinations on CK-MB level against surgically induced MI has been presented in Figure 11(a). The normal control group showed the normal CK-MB level (173 ± 3.51 IU/L) throughout the experimental period. There was a considerable increase in the level of CK-MB in the positive control group after 12 hr of left anterior descending coronary artery ligation while the level of enzyme was further raised up to 294.3 ± 1.53 IU/L after 24 hr. The first herbal combination (HC1) did not significantly ($p > 0.05$) restored the CK-MB level after 12 and 24 hr of ligation as compared to the normal control group. In comparison of HC1, the group pretreated with HC2 showed better maintenance of CK-MB level after 12 and 24 hr of ligation. A decrease in CK-MB level was observed in group pretreated with HC4 after 12 hr of ligating left anterior descending coronary artery. After 24 hr of ligation, this group showed considerable decline in the level of CK-MB that was very close to the control group. The prior administration of HC4 depicted the better maintenance of the serum CK-MB as compared to other herbal combinations.

The effect of different herbal combinations on the level of SGOT has been presented in Figure 11(b). In the normal control group, the SGOT level was 43 ± 2 and 46 ± 1.05 IU/L with time intervals of 12 and 24 hr, respectively. The SGOT level was 115 ± 1.527 IU/L and 123 ± 1.154 IU/L after the corresponding time intervals of 12 and 24 hr of LADCA ligation in the positive control group. The HC1 showed the SGOT level with a value of 94 ± 1.53 IU/L after 12 hr and 74 ± 1 IU/L after 24 hr of ligation. The pretreatment of HC2 significantly ($p > 0.05$) maintained at the level of SGOT after 24 hr of ligation in LADCA as compared to the positive control group. There was no considerable variation in the outcomes of HC1 and HC3 preventive treatment. However, the pretreatment of HC4 showed maximum potential against myocardial infarction as it upholds the SGOT level 73 ± 1 IU/L after 12 hr and 53 ± 1.53 IU/L after 24 hr of LADCA ligation.

The preventive treatment of herbal combinations against surgically induced MI on the level of LDH has been presented graphically in Figure 11(c). The serum analysis of the normal control group revealed 223 ± 1.15 to 235 IU/L of LDH from 0 to 48 hr, respectively. The LDH level in the positive control group was considerably higher as compared to the normal control group. The group of dogs pretreated with HC1 showed 382.33 ± 1.53 IU/L of LDH after 12 hr and 283 ± 1.15 IU/L after 24 hr of ligation. In dogs treated with HC2, the LDH level was 291.67 ± 1.15 IU/L and 264 ± 2.08 IU/L at corresponding time intervals of 12 and 24 hr after LADCA ligation. While the pretreatment of HC3 showed 343 ± 1.53 IU/L level of LDH after 12 hr and maintained at the level of 250 ± 1 IU/L after 24 hr of ligation. The preventive treatment of HC4 revealed significant maintenance of LDH level after 12 hr of ligation (Figure 11(c)).

The HC4 showed the prominent cardioprotective potential by maintaining the cardio-specific markers near the normal against surgically induced myocardial infarction after 24 hr of LADCA ligation. Although the precise mechanism of the cardioprotective potential of HCs in surgically

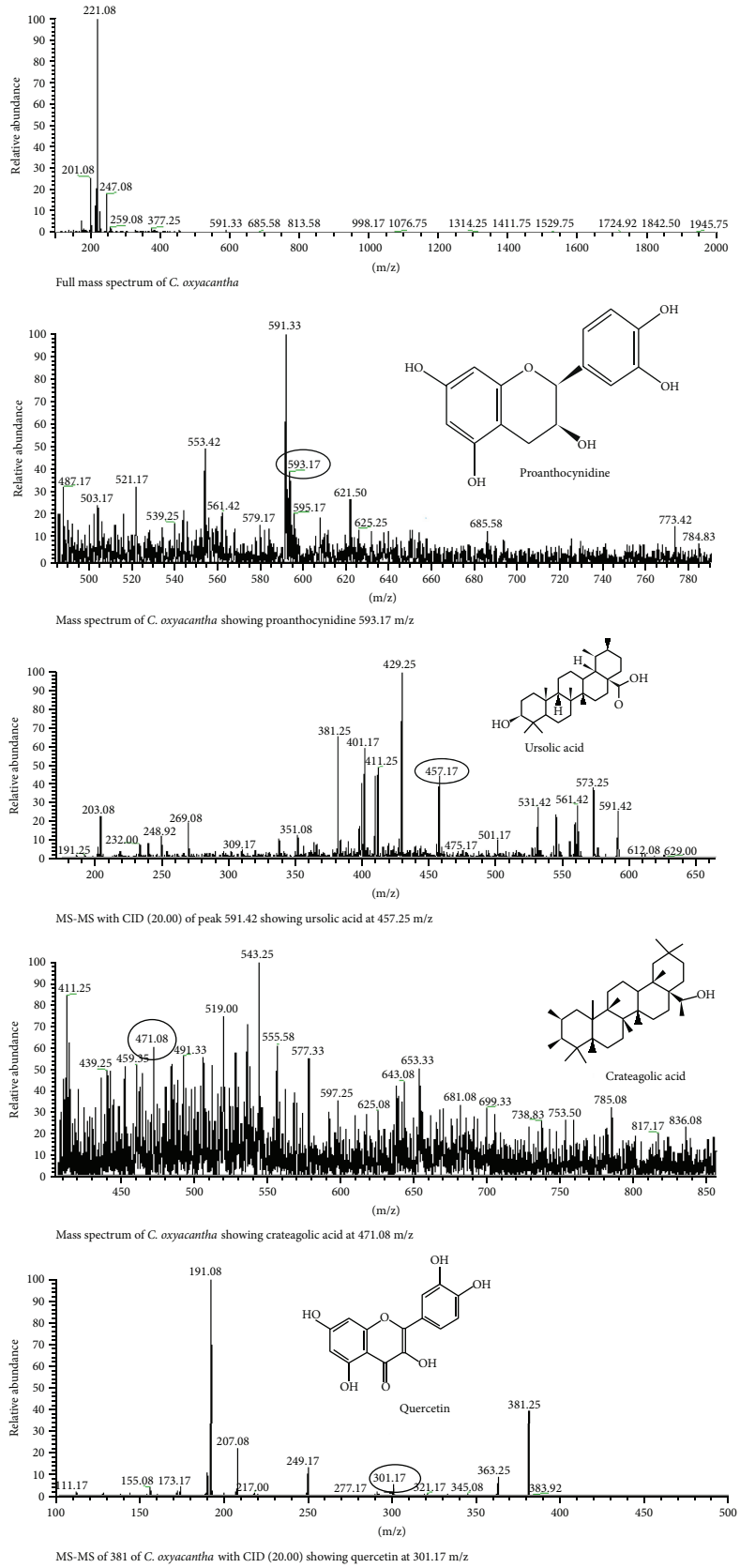
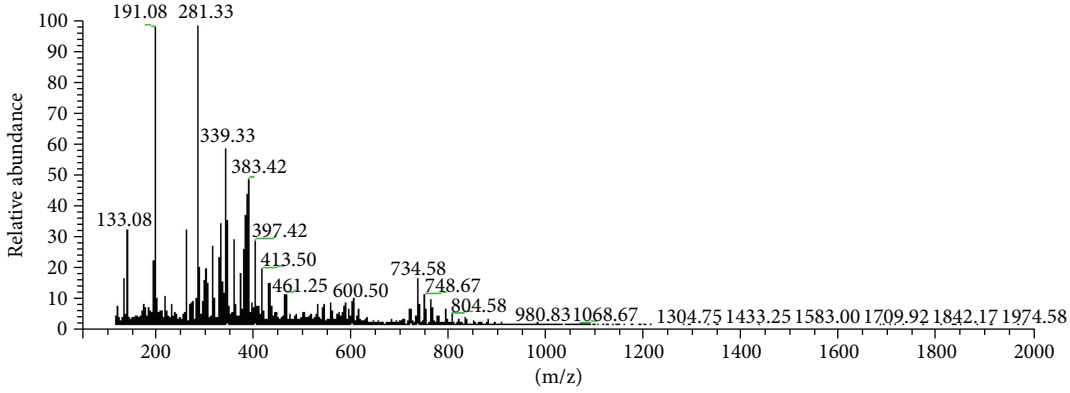
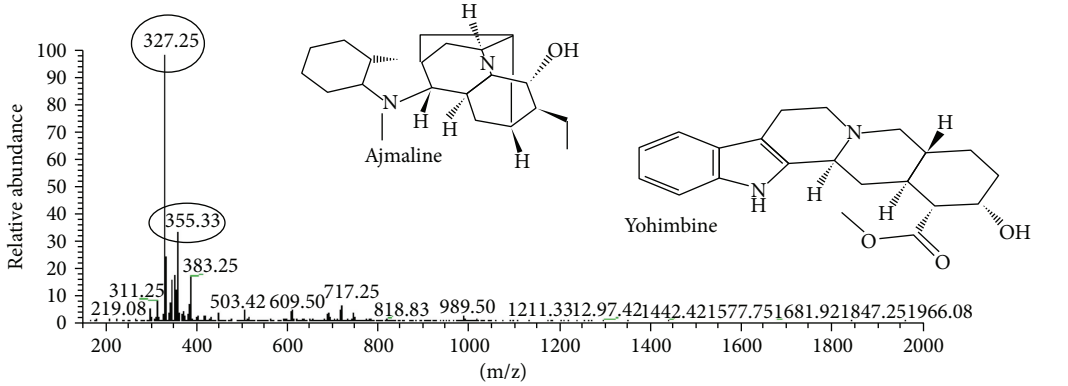


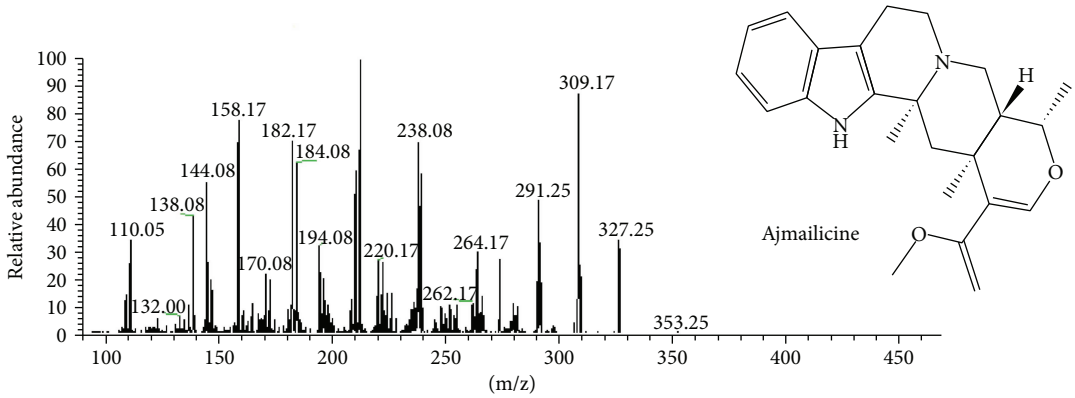
Figure 5: LC-MS analysis of *C. oxyacantha*.



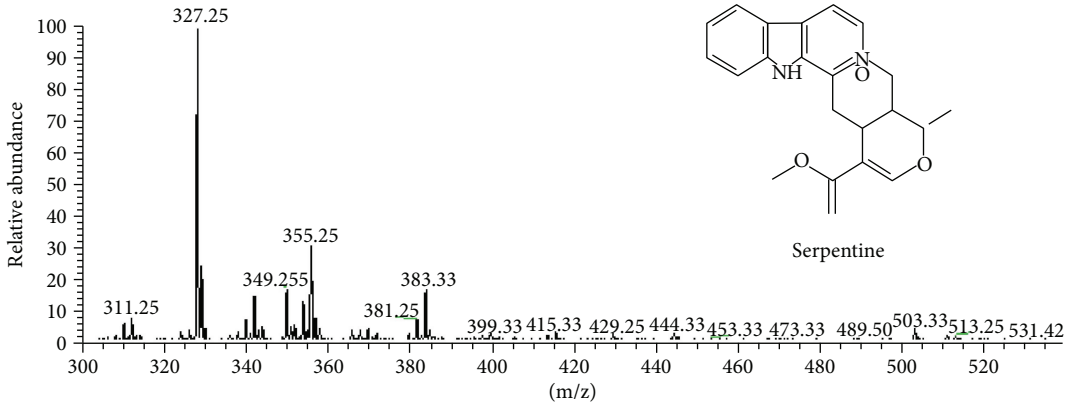
Full mass spectrum of *R. serpentina*



Mass spectrum of *R. serpentina* showing yohimbine at 355.33 and ajmaline at 327.25 m/z

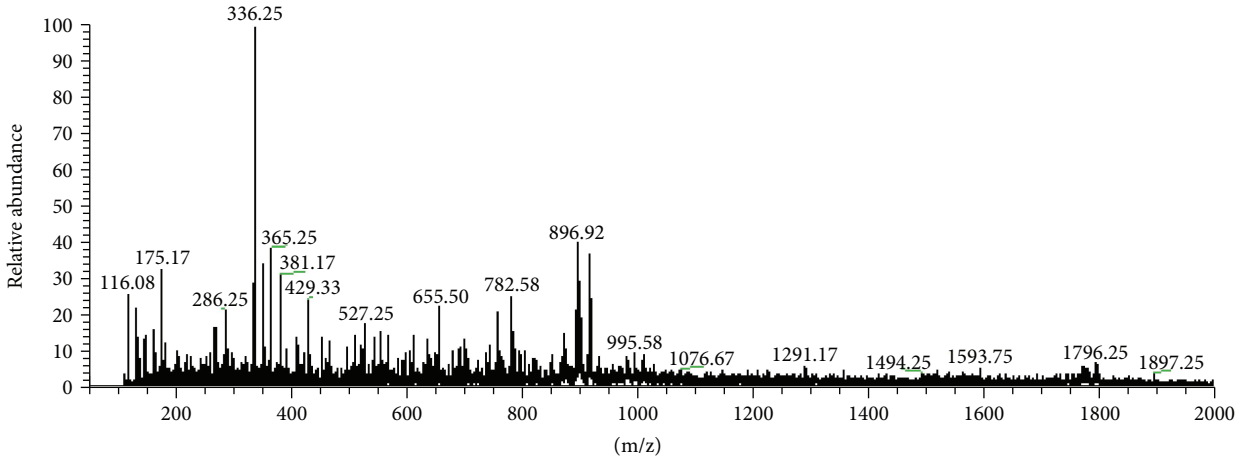


MS2 of peak 327 with CID (25.00) showing ajmailicine at 353.25 m/z

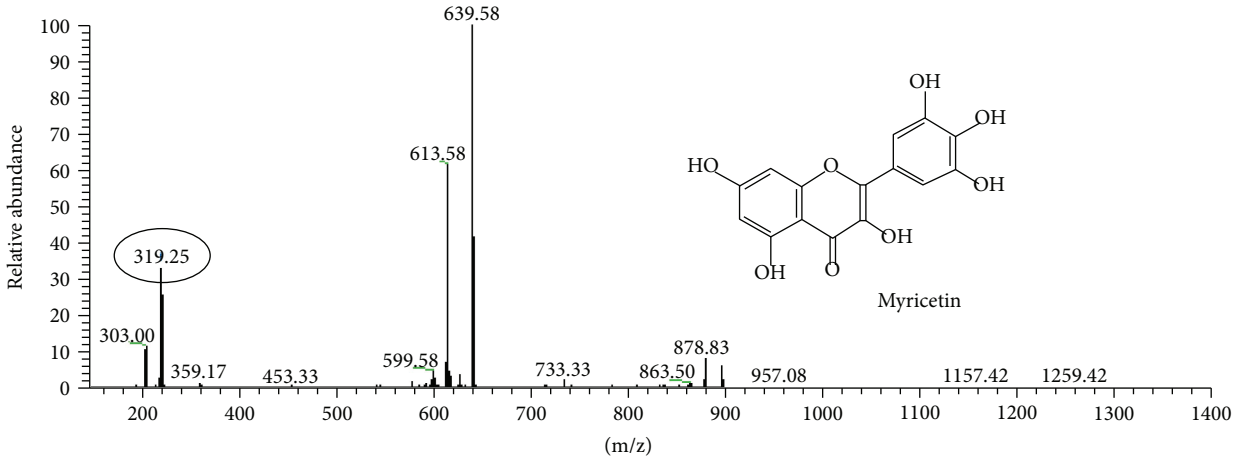


Mass spectrum of *R. serpentina* showing serpentine at 349.25 m/z

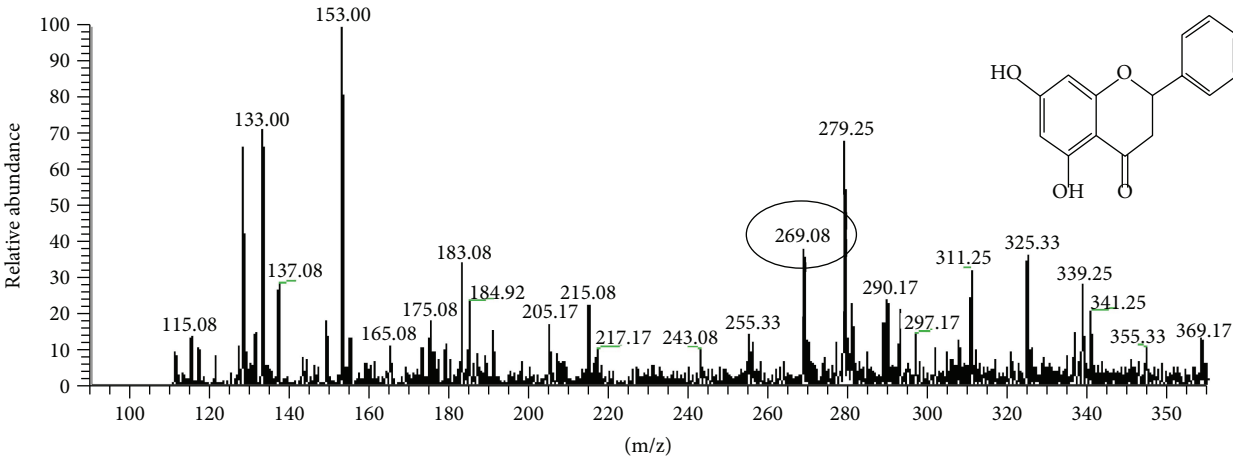
Figure 6: LC-MS analysis of *R. serpentina*.



Full mass spectrum of *A. sativum*



MS^2 CID 25.00 of 896 of *A. sativum* showing myricetin at 319.25 m/z



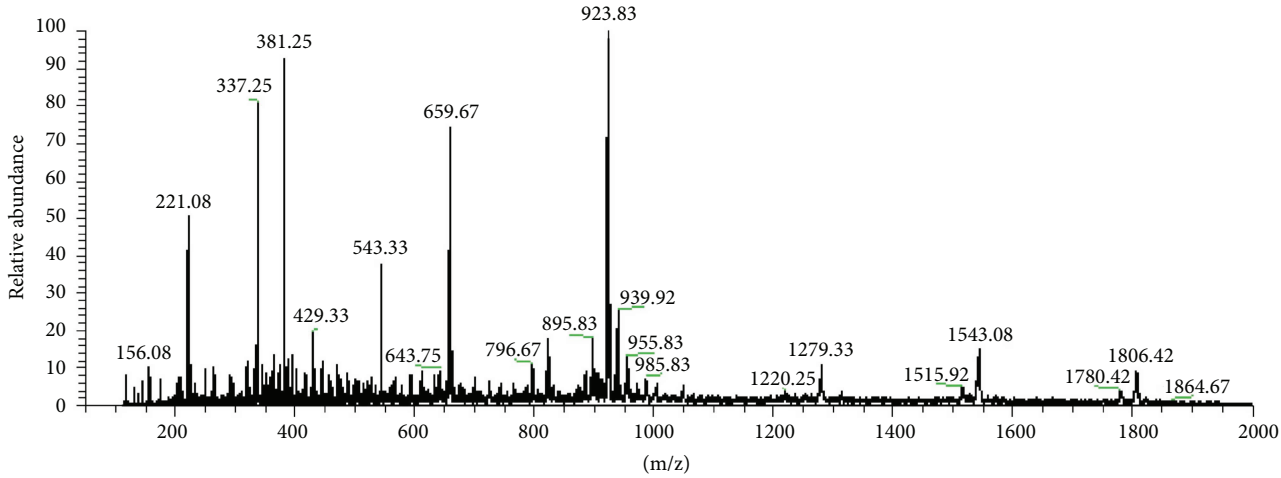
Mass spectrum of *A. sativum* apigenin at 327.25 m/z

Figure 7: LC-MS analysis of *A. sativum.*

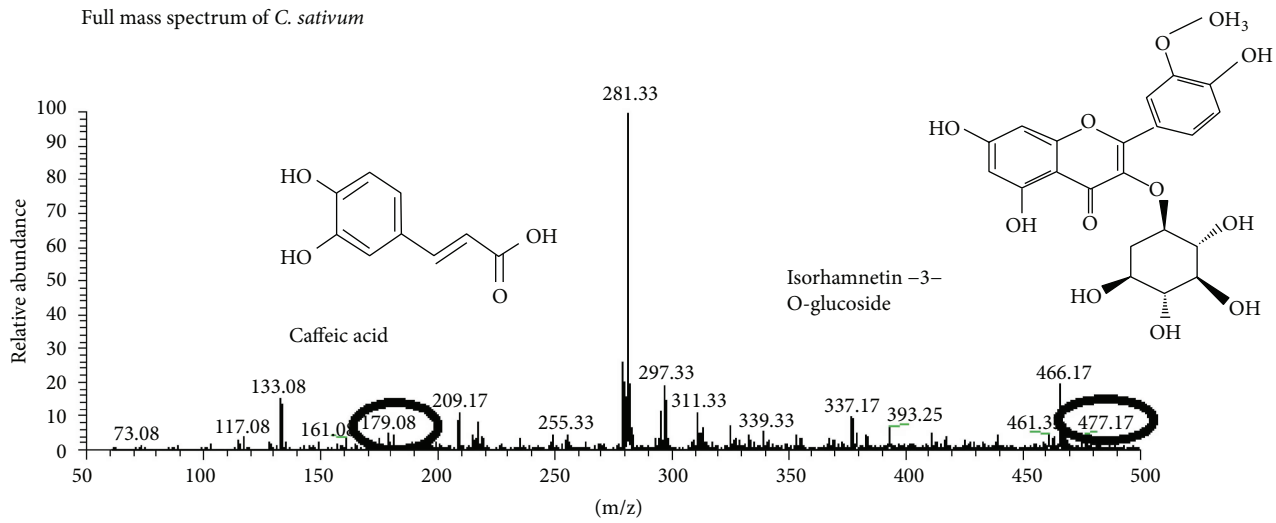
induced myocardial injury is not fully understood, it may be attributed to its favorable myocardial adaptogenic properties. Furthermore, this herbal combination might have the potential for the management of patients at risk of myocardial infarction.

3.12. Effect of Herbal Combinations on Hemodynamic Variable

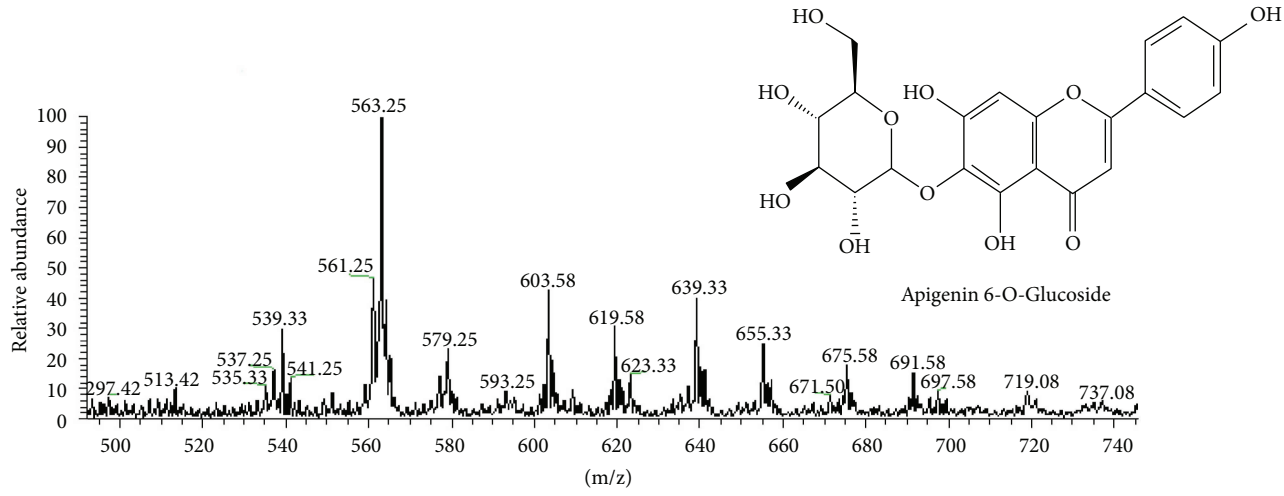
3.12.1. The Mean Arterial Pressure. Measurement of the hemodynamic variables was also incorporated into the experimental design for better understanding and more precise



Full mass spectrum of *C. sativum*



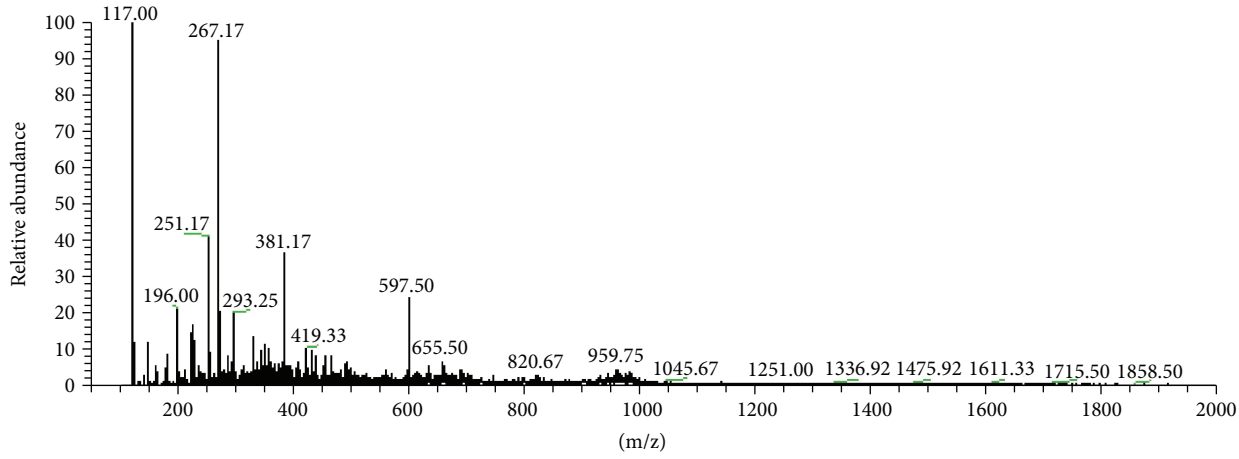
Mass spectrum of *C. sativum* showing caffeic acid at 179.08 m/z and isorhamnetin-3-O-glucoside at 477.17 m/z



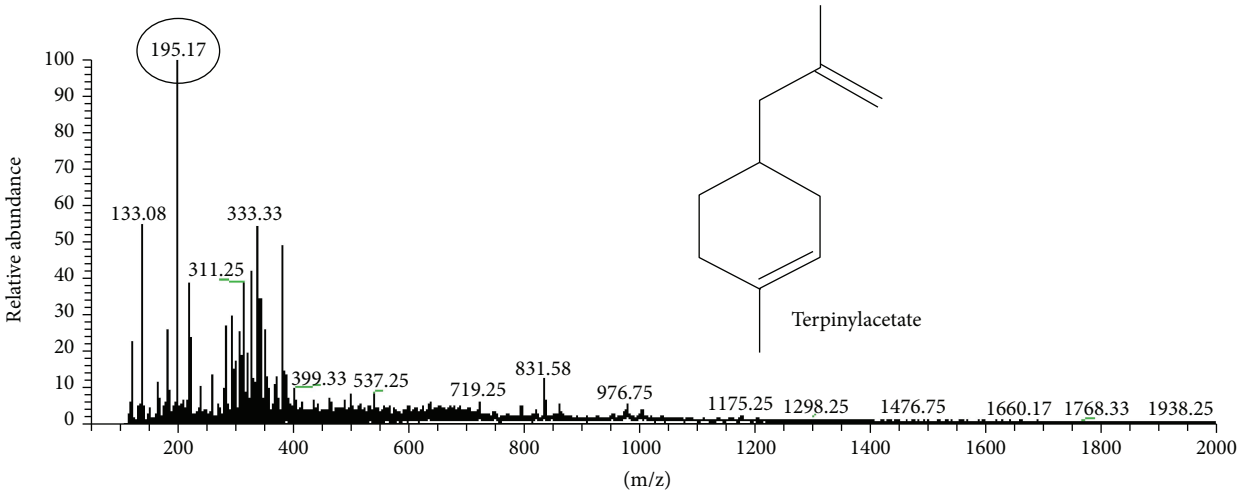
Mass spectrum of *Coriandrum sativum* showing apigenin-6-*C*-glucoside at 593.25 m/z

Figure 8: LC-MS analysis of *C. sativum*.

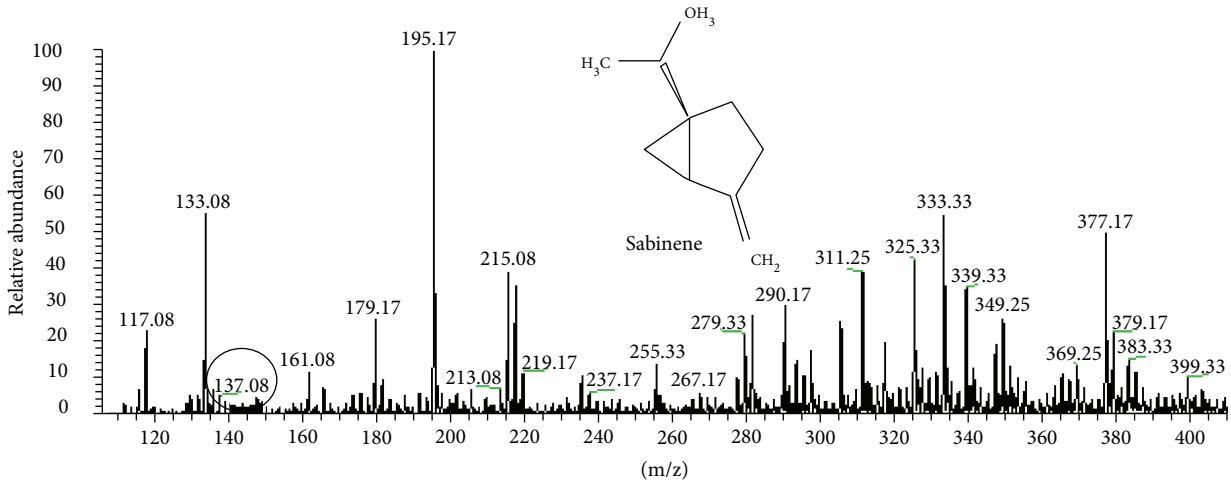
information of the correlation between biochemical and functional changes in the myocardium subjected to surgically induced damage. The normal control group depicted the 85 ± 6.81 mean arterial pressure (MAP) mmHg while the positive control group showed the decline in MAP (33 ± 4.35 mmHg) after occlusion in LADCA (Figure 12). The pretreatment of HC1 tried to sustain the level of MAP up to 52 ± 5.13 mmHg. However, the group treated with HC2 and



Full mass spectrum of *E. cardamom*



Mass spectrum of *E. cardamom* showing terpinylacetate at 195.17 m/z

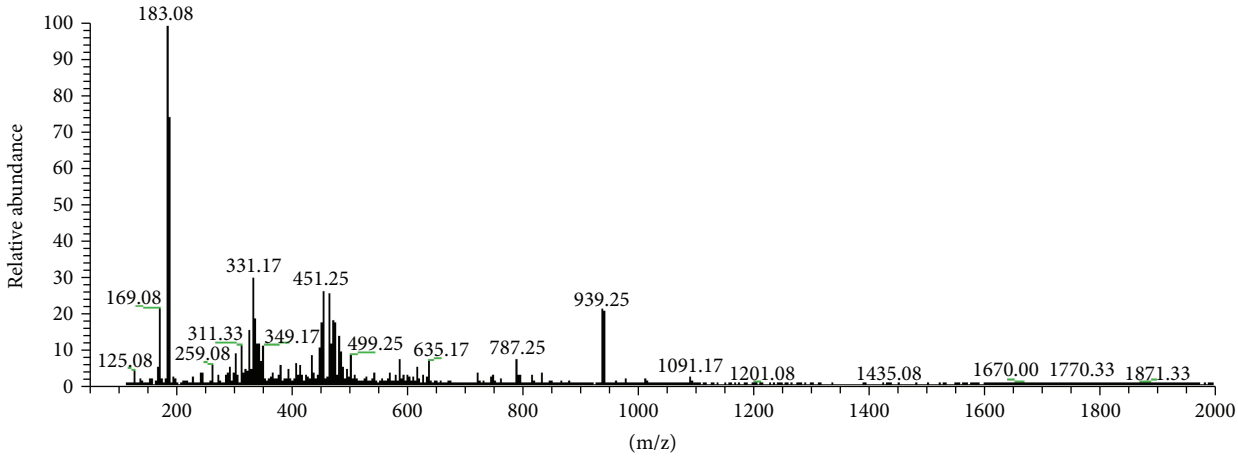


Mass spectrum of *E. cardamom* showing sabinene at 137.08 m/z

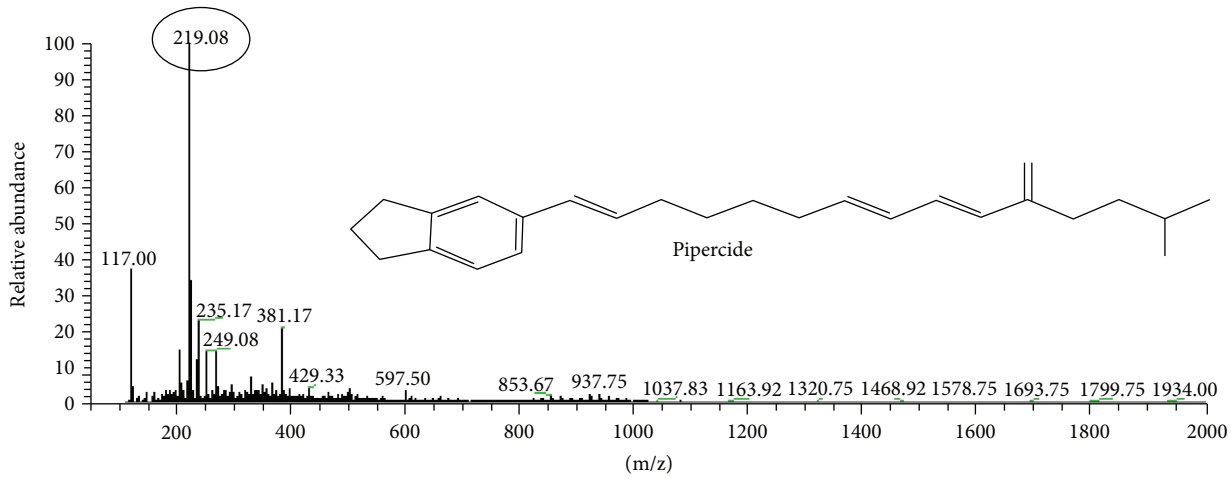
FIGURE 9: LC-MS analysis of *E. cardamom*.

HC4 substantially maintained the MAP 76 ± 4.04 mmHg and 77 ± 5.13 mmHg, respectively, as compared to other groups.

Similarly, in the positive control group, there was an abrupt increase in heart rate (HR) beats/min (277 ± 8.02) as compared to the normal control group (186 ± 4.04 beats/min). On the other hand, the pretreatment of surgically induced MI groups with herbal combinations revealed significant ($p > 0.05$) maintenance of HR as compared to the



Full mass spectrum of *P. nigrum*



Mass spectrum of *P. nigrum* showing pipercide at 219.08 m/z

Figure 10: LC-MS analysis of *P. nigrum*.

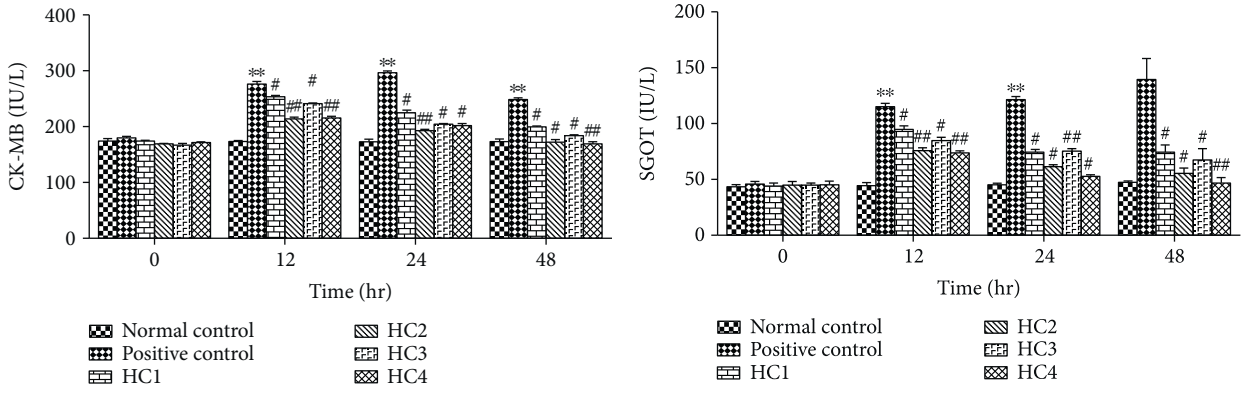
positive control group. Among all the treatment groups, the group pretreated with HC2 and HC4 showed significant ($p > 0.05$) restoration of HR.

3.12.2. Effect of Herbal Combinations on Ventricular Function. A significant decline in left ventricular end-diastolic pressure (LVEDP) (9 ± 3.05) marked the onset of myocardial infarction in surgically induced MI group which remained decreased throughout the experimental period in comparison to the normal control group (32 ± 5.51) (Figure 12). The pretreatment with HC4 and HC2 significantly ($p > 0.05$) maintained the LVEDP level as compared to the surgically induced ischemic group. The HC1 and HC3 also tried to sustain the LVEDP with corresponding values 12 ± 4.04 and 08 ± 1.53.

The positive control group showed the significant decrease in left ventricular systolic pressure (LVSP) as compared to the normal control group. The LADCA ligation resulted in significant cardiac dysfunction evidenced by reduced MAP and increased HR. The left ventricular contractile function was also altered. The pretreatment of HC4 showed the marked restoration as compared to other groups as it maintained the level of LVSP near to the normal control group. It is materialized that the HC4 is more potent in preventing the hemodynamic deteriorations observed in the positive control group.

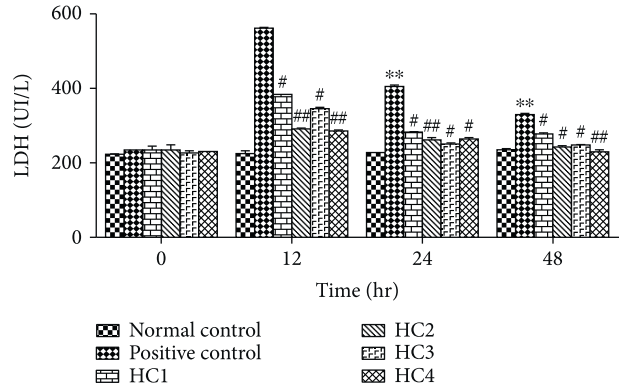
3.13. Histopathological Examination. The histopathological findings of myocardial tissue in the normal control group illustrated clear integrity of the myocardial cell membrane. The myofibrillar structure was normal with no inflammatory cell infiltration. The nuclei were also normal without any pyknotic changes (Figure 13(a)). The histopathological examination of the surgically induced MI group showed extensive myofibrillar degeneration related to infiltration and disruption of cardiac myofibers. There was marked necrosis in the ventricular region. Pyknotic changes in nuclei were also observed (Figure 13(b)).

The treatment of HC1 prior to ligation showed myofibrilation (Figure 13(c)) while the pretreatment with HC2 demonstrated marked improvement in surgically induced alterations, but there was cellular infiltration at few places. The nuclei were also normal (Figure 13(d)). The group treated with HC3 did not protect the cardiac dysfunctions as compared to the other groups. Myocardial fibrillation as



(a) Effect of herbal combinations of plant extracts on the level of CK-MB (IU/L)

(b) Effect of herbal combinations of plant extracts on the level of SGOT (IU/L)



(c) Effect of herbal combinations of plant extracts on the level of LDH (IU/L)

Figure 11: (a–c) Effect of herbal combinations of plant extracts on cardiac markers in the serum of all experimental groups. ∗∗ indicates significance ($p < 0.0001$) compared to the normal control, # indicates significance ($p < 0.001$) compared to the positive control, and ## indicates significance ($p < 0.0001$) compared to the positive control (ANOVA, Turkey's multiple comparison test). Values are presented as the mean ± SEM ($n = 3$).

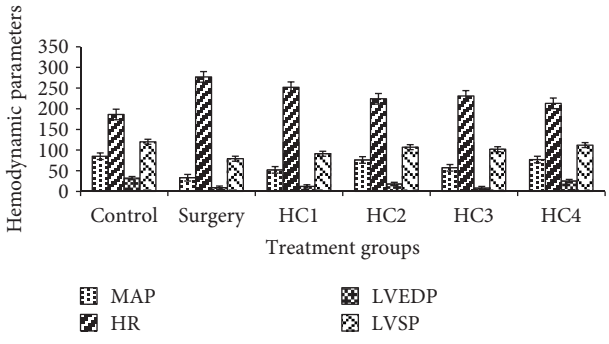


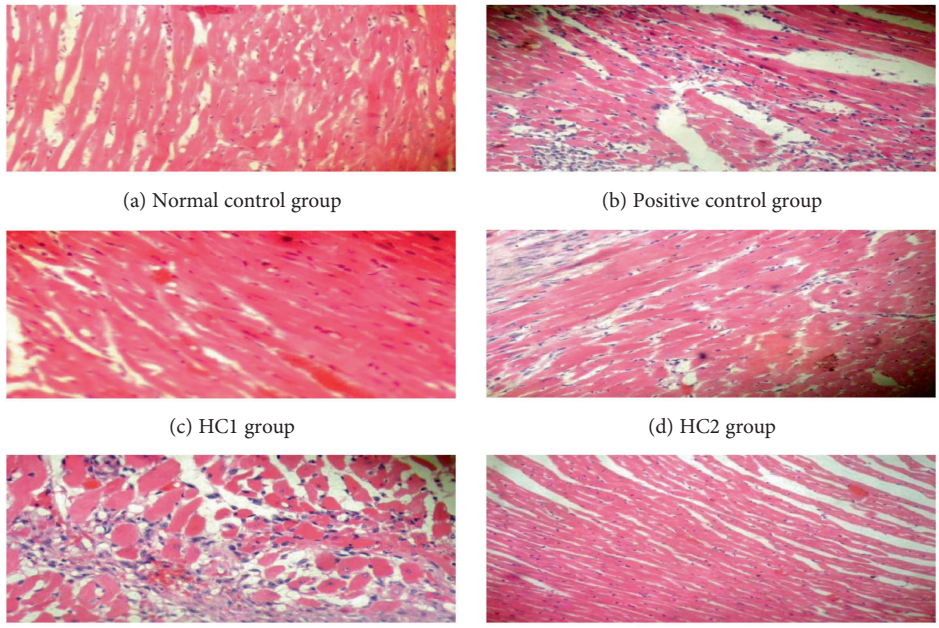
Figure 12: Hemodynamic parameters of various groups treated with different herbal combinations.

well as some pyknotic changes in the nuclei were also seen in the group treated with HC3 (Figure 13(e)). The histopathological examination of the group treated with HC4 showed that there was no myofibrilation, and the cardiac parenchyma was also normal. This confirmed the potential of herbal combination (HC4) over oxidative stress related to cardiac ailment (Figure 13(f)).

4. Discussion

The evidence-based study about metabolomes of medicinal plants is an emerging approach to develop a new group of phytotherapeutics [16]. The therapeutic potential of plant secondary metabolites has augmented an interest in pharmaceutical research for the development of novel therapeutic agents. The antioxidant profiling of the said medicinal plants was explored through DPPH and DNA protection assay. The antioxidative potential of these medicinal plants was found to be dose-dependent. This dose-dependent response of various medicinal plants for antioxidative potential has already been reported by many researchers [18, 25, 26]. The increased antioxidant potential with high dose of medicinal plants may be due to positive correlation with high quantity of powerful chain-breaking antioxidants like phenolics and other phytoconstituents [27]. Different mechanisms like scavenging of free radicals, chelation of metal ions, and inhibition of enzymes may be responsible for good therapeutic antioxidant potential of medicinal plants [28].

In HPLC, the extremely narrow peaks are generated; thus, the high-speed data handling performance demands a blend of MS segment [29]. LC-MS has such features that



(a) Normal control group (b) Positive control group

(c) HC1 group (d) HC2 group

(e) HC3 group (f) HC4 group

Figure 13: (a–f) The histopathological representation of cardiac tissue of all treatment groups.

make it applicable for metabolomic profiling of a wide range of low to high polarity metabolites, including nonvolatile compounds. It also covers a broad range of metabolites, since it operates ionization in negative and positive modes [30]. Hence the LC-MS-based metabolomics is a powerful tool in order to evaluate the important active secondary metabolites which play a vital role to prevent oxidative stress by scavenging free radicals.

The LC-MS analysis of *T. arjuna* revealed the presence of some important phytoconstituents like termiarjunoside I, quercetin, ferulic acid, and gallic acid which were responsible for its antioxidative strength. The HPLC analysis of the *T. arjuna* bark by Jahan et al. [17] also exhibited the existence of polyphenols and phenolic acids including ferulic acid, gallic acid, caffeic acid, and catechin. The fast atom bombardment mass spectroscopy (FABMS) and distortionless enhancement by polarization transfer (DEPT) NMR spectra of *T. arjuna* also displayed a molecular ion peak at $m/z = 666$ $[M]^+$ indicating the presence of termiarjunoside I, with a molecular formula of $C_{36}H_{58}O_{11}$ (Ali et al. 2006). The quercetin and gallic acids are strong antioxidants which play a crucial role in a number of biological and pharmacological activities and also protect DNA damage [31]. The ferulic acid present in *T. arjuna* is not only a good antioxidant in various biological systems but also has the potential to protect the DNA against H_2O_2-induced damage [32].

The metabolomic profiling of *C. oxyacantha* depicted the presence of procynidine, crateagolic acid, ursolic acid, and quercetin. These major phytoconstituents are mainly responsible in curing various diseases like myocardial infarction, coronary heart diseases, hypertension, and diabetes-related complications owing to their antioxidant potential [33]. The presence of ursolic acid in *C. oxyacantha* has also been reported to have angiotensin-converting enzyme-inhibiting and cardioprotective potential (Lacaille et al. 2001). *R. serpentina* has been a popular field of research for decades, and several researchers have explored its excellent phytochemical properties [34, 35]. Various secondary metabolites such as yohimbine, ajmaline, serpentine, and ajmalicine present in the roots of *R. serpentina* contribute for its antioxidant potential [36]. Ajmaline is a sodium channel blocker that illustrated the instant therapeutic potential when given intravenously. It has also been claimed to stimulate respiration and intestinal movements. Serpentine is useful to prevent the oxidative stress-induced DNA damage, hypertension, cardiovascular, and neurological diseases [37]. R. serpentina is a hopeful herbal option in the pharmaceutical world due to the existence of considerable bioactive compounds in the roots [38]. The LC-MS analysis of *A. sativum* indicated the existence of myricetin and apigenin. The myricetin due to its specific chemical structure counteracts oxidative stress generated as a result of reactive oxygen species [39, 40]. The hydroxylated apigenin is found to inhibit tumor cell proliferation and angiogenesis. Caffeic acid is a potent antioxidant and has several therapeutic properties including antioxidants, anti-inflammatory, and anticarcinogenic. It has been reported that caffeic acid inhibits both lipoxygenase activity and suppresses lipid peroxidation thus completely blocks the production of ROS [41]. Cardamom fruit is used against vesicular calculi, dyspepsia, debility, halitosis, and gastrointestinal disorders [42]. Phytochemical investigation of cardamom has revealed highly bioactive components. High-phenolic compounds, in extracts of all plants, could be considered as the key reason behind the antioxidant potential of the said medicinal plants [43, 44].

During *in vivo* trial, the increased cardiac markers in the positive control group are due to the ligation of the coronary artery. The ligation imparts an additional workload on the remaining viable myocytes that may be unbearable, resulting in pathological alterations [11]. Alterations in integrity, fluidity, and permeability of the myocardial membrane due to ligation have been believed to be a reason for the leakage of cardiac markers [21]. The treatment with HCs might salvage viable myocytes, which are at risk of injury, thus preventing cell loss induced by necrosis [45]. The HC4 showed the prominent cardioprotective potential by maintaining the cardio-specific markers near the normal against surgically induced myocardial infarction. The better maintenance of the cardiac markers with HC4 as compared to other herbal combinations might be due to the presence of synergism of some specific phytoconstituents like crateagolic acid, termiarjinoside-I, ajmaline, and serpentine and antioxidants like quercetin, gallic acid, ferulic acid, and myricetin in it. This may render the myocytes less leaky by preventing myocardial membrane destruction [46]. A considerable fall in MAP and increased HR in the surgically induced MI group indicated hemodynamic impairment and ventricular dysfunction due to increased generation of ROS [22]. A fall in MAP due to coronary occlusion is expected to increase HR and myocardial contractility by activating the baroreceptor reflex, which may subsequently result in reflex vasoconstriction and thus worsening the imbalance, between myocardial oxygen demand and supply [47]. The increase in blood flow through the subendocardial region of the left ventricular muscle is the major consequence of the reduction in LVEDP in the surgically induced infarction group [48]. The therapeutic efficacy of HC4 might be due to the improvement in both inotropic and lusitropic function of the heart and considerable maintenance of antioxidant defense capacity of the myocardium [48].

5. Conclusion

The HC4 (*T. arjuna*, *R. serpentina*, *E. cardamom*, and *C. oxyacantha*) considerably ameliorated cardiotoxicity by keeping the levels of biochemical parameters near to normal. The antioxidants property and phytoconstituents of medicinal plants present in this herbal combination might be responsible for its cardioprotective potential. On the basis of this evidence-based study, it can be concluded that the HC4 can be safely used as an alternative product for the management of cardiovascular diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Review Article

Nrf2, a Potential Therapeutic Target against Oxidative Stress in Corneal Diseases

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Corneal diseases are one of the major causes of blindness worldwide. Conservative medical agents, which may prevent sight-threatening corneal disease progression, are urgently desired. Numerous evidences have revealed the involvement of oxidative stress in various corneal diseases, such as corneal wound healing and Fuchs endothelial corneal dystrophy (FECD). Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/Kelch-like erythroid-cell-derived protein with CNC homology- (ECH-) associated protein 1 (Keap1)/antioxidant response element (ARE) signaling is well known as one of the main antioxidative defense systems. To the best of our knowledge, this is the first review to elucidate the different expression profiles of Nrf2 signaling as well as the underlying mechanisms in corneal diseases, implicating that Nrf2 may serve as a potentially promising therapeutic target for corneal diseases.

1. Introduction

The cornea is the transparent front part of the eye and contributes estimated two-thirds of the optical power. Human cornea is mainly composed of corneal epithelium (the outer layer), stroma (the middle layer), and endothelium (the inner layer). Normal morphologies and functions of these cells maintain the transparency of the cornea. The ocular surface mucosa (mainly the cornea) is the first layer of the eye that is exposed to environmental stress. The cornea is susceptible to be damaged by varieties of external stresses due to its constantly direct exposure to harmful factors, such as physical or chemical injuries, UV radiation, and air pollutants (gases, vapors, or cigarette smoke). Oxidative stress is characterized by the generation of reactive oxygen species (ROS), which contains superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^\bullet OH$), and is considered to be involved in these external stresses [1]. Under normal condition, ROS production and cleavage is counterbalanced.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2 or NFE2L2), encoded by the gene of NFE2L2, is a vital nuclear transcription factor for the systemic antioxidant defense system. In basal conditions, Nrf2 binds to Kelch-like erythroid-cell-derived protein with CNC homology- (ECH-) associated protein 1 (Keap1) as a complex and is restricted to the cytoplasm where it undergoes ubiquitination and proteasomal degradation. Under stressed condition, Nrf2 separates from Keap1 (a primary Nrf2 inhibitor) and is translocated into the nucleus, where it binds to the phase 2 of antioxidant response element (ARE) in the DNA promoter region and initiates the transcription of ARE controlled antioxidative enzymes, such as superoxide dismutase (SOD), catalase,

glutathione S-transferase (GSTP) [2], NAD(P)H: quinone oxidoreductase 1 (NQO1) [3], heme oxygenase-1 (HO-1) [4], thioredoxin reductase (TrxR), glutathione reductase (GR), and glutathione-S-transferase (GST) [5, 6], which detoxify ROS through GSH regulation [7]. Nrf2 is known as the molecular switch turning on/off the Nrf2 signaling (also known as Nrf2-Keap1 or Nrf2-Keap1-ARE signaling) and serves as a foremost component of ROS signaling pathway that can be activated by oxidative stress inducers, such as sulforaphane (SFN) [8] and hyperoside [9], and inhibited by oxidative stress factors such as homocysteine (Hcy) [10], O_2 fluctuation [11], and hypoglycemia under hypoxia [12].

Normally, there is a balance between the systemic generation of oxidants and biological antioxidants' capacity to remove the oxidants or to repair the oxidative stress-induced damage. Overproduction of ROS or dysfunction of antioxidative enzymes can result in oxidative stress and lead to cellular damages (e.g., lipid peroxidation of cell membranes and oxidative damage to DNA and proteins). Oxidative stress is known as a vital pathogenesis underlying aging and many human ocular diseases, such as corneal diseases (injuries, keratoconus, Fuchs' endothelial dystrophy (FECD), etc.), dry eyes, cataracts, glaucoma, age-related macular degeneration, and other oxidative-related diseases in the eyes [1]. The protective role of Nrf2 against oxidative stress in ocular surface diseases has been clarified in the following researches: sidestream cigarette smoke (SCS) exposure-induced dry eyes [13] and pterygium [14, 15].

To the best of our knowledge, this is the first review to elucidate the specific role and the underlying mechanism of Nrf2-mediated antioxidative defense in corneal diseases, including wound healing, Fuchs' endothelial dystrophy, and corneal regenerative projects.

2. Nrf2 in Corneal Diseases

2.1. Nrf2 in Corneal Wound Healing. Located in the foremost layer of the cornea, corneal epithelial cells are easily injured by external factors such as physical injuries, chemical injuries, and oxidative stress (UV radiation). Delayed corneal wound healing is often found in the cornea of diabetic patients. It was revealed that the topical application of a high dose of carnosol (1 mM), a well-established Nrf2 activator, accelerated the corneal wound healing in diabetic rats with the corneal epithelium mechanically removed [16]. A Nrf2-Keap1-dependent protective role against oxidative stress in wound healing was also clarified in a heptanol-induced corneal epithelial wound model. The results found that Nrf2 was activated both in the preinjured and postinjured corneal epithelium of wild-type (WT) mice, indicating the involvement of Nrf2 throughout the corneal healing process [17]. Corneal epithelial cells' migration and wound healing were significantly delayed in Nrf2 knockout (KO) mice than those of WT mice, accompanied by the detection of Nrf2 activation and then translocation to the nuclei via immunostaining. However, the corneal cell proliferation was not affected in both KO and WT mice, demonstrated by the immunostaining for Ki-67 (a proliferative marker) [17]. Nrf2 knockdown was performed by siRNA in a corneal epithelial cell line (C/TERT) in vitro to further elucidate the role of Nrf2 during the corneal wound healing, and the results revealed that Nrf2 siRNA significantly delayed C/TERT cell migration (but not proliferation), which is accompanied by the decreased transcriptions of Nrf2-dependent antioxidative genes (HO-1 and NQO1). On the contrary, Keap1 siRNA dramatically accelerated C/TERT cell migration with increased HO-1 and NQO1 expressions [17]. All these studies support the concept that Nrf2 plays a protective role in corneal epithelial wound healing, mainly by accelerating cell migration via the initiation of Nrf2-mediated antioxidative defense system. And the exact mechanisms of Nrf2 regulating corneal epithelial cell migration need to be clarified; Nrf2 would serve as a promising target for the treatment of corneal wound healing.

Corneal keratocytes are activated and transformed into myofibroblasts and fibroblasts upon injury, which is an important biological event during corneal wound repair with scar formation [18]. Ethyl pyruvate (EP), a pyruvate ester which augments pyruvate levels, possesses the ability to ameliorate the cellular oxidative stress [19]. The Nrf2-mediated antioxidant response was enhanced by EP in keratocytes and myofibroblasts and induced phenotypic changes of inactive corneal stromal keratocytes into contractile myofibroblasts cultured in vitro, indicating the therapeutic potential of EP in corneal wound healing [20]. Trichostatin A (TSA), a nonselective inhibitor of histone deacetylase (HDAC), was revealed to possess the capacity for fibrosis prevention [21]. It was reported that treatment with TSA showed a solid protective effect against the oxidative stress induced by transforming growth factor-β (TGF-β) and a strong inhibition of myofibroblast differentiation in TGF-β-stimulated human immortalized corneal fibroblasts cultured in vitro. In addition, TSA decreases ROS and H_2O_2 accumulation, persuades Nrf2 nuclear translocation, and upregulates the transcriptions of Nrf2-ARE-controlled antioxidant enzymes (such as GSH). In opposite, Nrf2 siRNA prevented the inhibitory effect of TSA on TGF-β-induced myofibroblast differentiation. These convincing results implicated that Nrf2 was involved in myofibroblast differentiation, and TSA may serve as a promising medical alternative preventing corneal wound scar formation, via inhibiting Nrf2-ARE-regulated myofibroblast differentiation [22].

2.2. 4-Hydroxynonenal and Nrf2 in Corneal Epithelial Disease. 4-Hydroxynonenal (4-HNE), a major endogenous product of lipid peroxidation and a key marker of oxidative stress, is considered to play oxidant roles in corneal diseases, such as Fuchs endothelial corneal dystrophy (FECD) and keratoconus [23]. 4-HNE was found to inhibit the cell viability by increasing the level of 3-nitrotyrosine (3-NT, a marker of oxidative stress) and NADPH oxidase 4 (NOX4, a vital enzyme of ROS generation) in cultured human corneal epithelial (HCE) cells via Western blot and immunofluorescent staining [24]. 4-HNE also increased the cytoplasmic expression and nuclear translocation of Nrf2 as well as the transcription of Nrf2-dependent effectors: GSTP and NQO1 in cultured HCE cells [24], indicating that 4-HNE can induce oxidative stress in the corneal epithelium through the

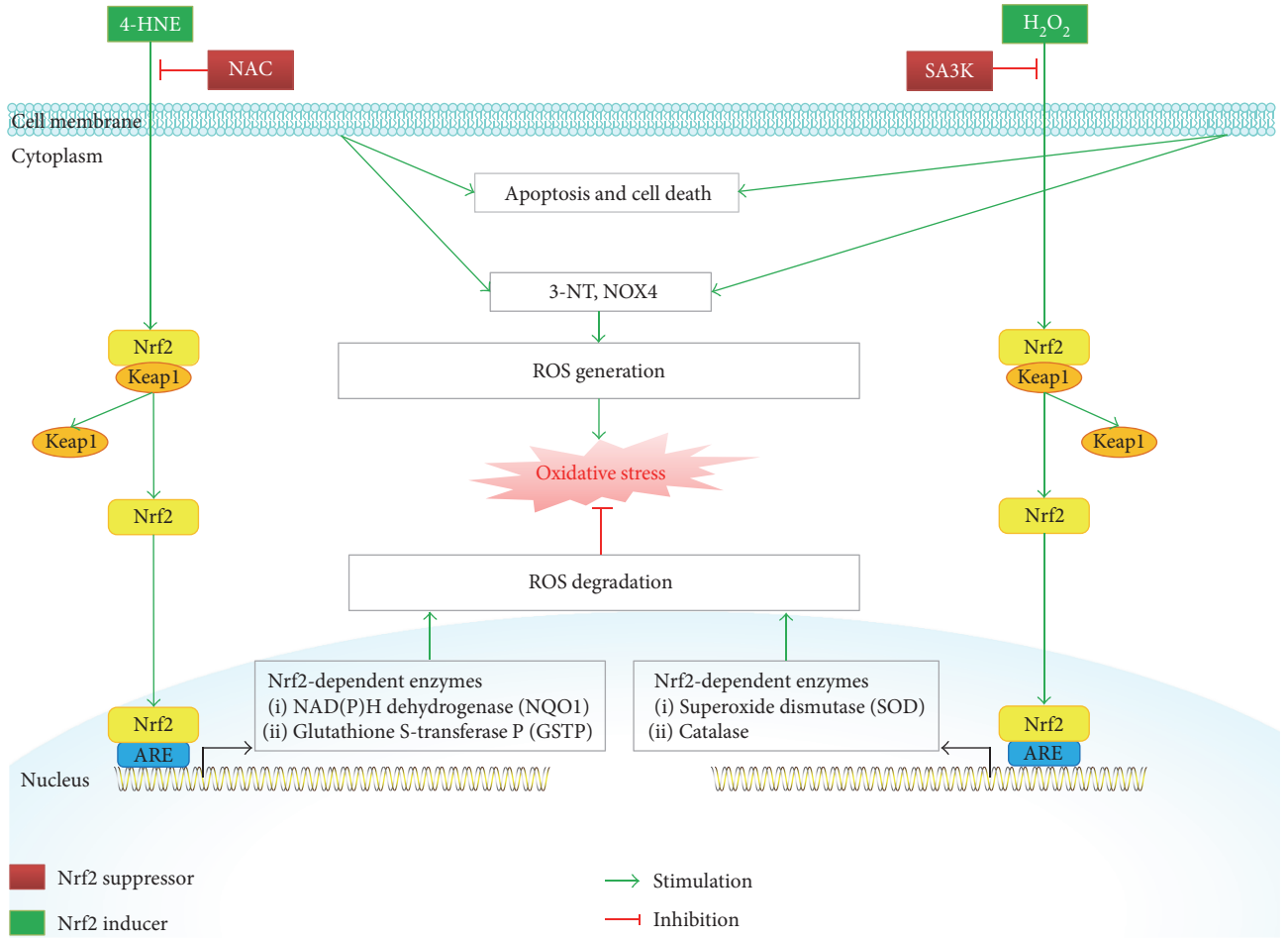

Figure 1: Schematic diagram of Nrf2 signaling and regulation in the corneal epithelial cells. 4-HNE or H_2O_2 induces excessive ROS generation (by upregulation of 3-NT, NOX4 protein expression), leading to oxidative stress in the cultured HCE cells and resulting in cell apoptosis and death of corneal epithelial cells. 4-HNE activates Nrf2/ARE-controlled antioxidant enzyme (NQO1 and GSTP) transcription, which facilitates ROS degradation. On the other hand, H_2O_2 decreases Nrf2/ARE-controlled SOD and catalase transcription, leading to ROS degradation suppression. NAC serves as an antioxidant by antagonizing 4-HNE overexpression and reversing the cell viability of HCE cells. SA3K blocks H_2O_2-induced ROS, 3-NT, and NOX4 overexpression and upregulates ROS degradation by activating Keap1-Nrf2-ARE pathway.

activation of Nrf2 and its effectors. N-Acetylcysteine (NAC), a classic ROS scavenger, antagonized the 4-HNE-induced oxidant effects in the cultured HCE cells, evidenced by the reversed cell viability of HCE cells, and reduced the 3-NT, NOX4, and Nrf2 protein expression induced by 4-HNE (Figure 1) [24]. These results elucidated the relationships among the lipid peroxidation, oxidative stress, and antioxidative defense in the corneal epithelium, providing a potential therapeutic direction for oxidative-related eye diseases. Further intensive studies are still needed to explore the full mechanisms of 4-HNE-induced oxidative stress in corneal epithelium, such as the binding site and other targeting factors.

2.3. H_2O_2 and Nrf2 in Corneal Epithelial Disease. H_2O_2 is a main ROS product and known as an oxidative stressor in experimental researches. Various factors, such as Keap1-Nrf2 pathway and NOX4 (an isoform of NADPH oxidase), are considered to be involved in the complex process of oxidative stress. SERPINA3K (SA3K) belongs to the serine proteinase inhibitor family and possesses antioxidant effect. It prevented against H_2O_2-triggered apoptosis and ROS overproduction as well as repressed GSTP and NQO1 in cultured HCE cells [2]. Meanwhile, it accelerated ROS degradation by upregulating the activity of antioxidant ROS degradation enzymes, such as catalase and superoxide dismutase. In agreement with in vitro study, SA3K is also demonstrated to protect rat corneal epithelium in vivo against oxidative stress by inhibiting ROS generation and suppressing the Keap1-Nrf2 pathway and its downstream factor NOX4 (Figure 1) [2]. Taken together, SA3K protects against H_2O_2-induced oxidative stress in corneal epithelium by restoring the balance between ROS generation and degradation, as well as regulating the Keap1-Nrf2 signaling pathway, indicating that SA3K is a promising antioxidant factor that may serve as a potential therapeutic agent for the oxidative stress-related corneal diseases. Further and in-depth studies based on the transgenic animal models (e.g., transgenic

Tg-SOD mice), which may elucidate the comprehensive mechanisms of SA3K on the Keap1-Nrf2 system, such as SA3K's binding site, the specific pathway (e.g., Wnt pathway) involved in between SA3K and Keap1-Nrf2-ARE system, are needed.

2.4. Role of Nrf2 in Corneal Regeneration via Stem Cells. Under normal circumstance, the injured corneal epithelium is regenerated by the corneal limbal stem cells. The physiological regenerative function of stem cells is lost in corneal limbal stem cell deficiency (LSCD), leading to corneal opacity and vision impairment. Transplantation of stem cells is applied to treat LSCD, using tissue-engineered epithelial cell sheets, such as human oral mucosal epithelial cell (hOEC) sheets [25, 26] and human induced pluripotent (iPS) stem cells [27]. These stem cell-based regenerative cell sheet techniques facilitate the regeneration of the patient's own damaged stem cells and showed positive effects in treating LSCD in clinical trials [28]. A healthy status of stem cells in tissues is the foremost factor in regenerative medicine, and feasible preservation techniques, which may improve the preservation for the cell sheet, are required due to the merging stem cell-based application and transplantation.

Ebselen, an organic selenium-containing redox compound and a well-known Nrf2 activator, has exhibited great potentials as a promising medium for the preservation of tissue-engineered cell sheets and the stem/progenitor cells under hypothermia during preservation. The expression of two tight junction-relevant proteins (ZO-16 and MUC165), which maintain the barrier function of the corneal epithelium, were enhanced, and the reduction of p63 (an epithelial stem cell marker) was prevented by Ebselen in the hOEC sheet under hypothermic stress [25]. Ebselen also maintained the high ATP levels, normal morphology, viability, and function of the hOEC sheet during hypothermic preservation by reducing ROS generation, inducing the Nrf2 activation, decreasing the lactate dehydrogenase (LDH) releasement, and increasing the GSH/oxidized glutathione (GSSG) ratio. Ebselen-initiated Nrf2 activation exerts the antioxidant, as well as the antiapoptotic, effect which protects the cells against oxidative stress-triggered damage caused by the hypothermia during preservation. Though not evidenced during preservation, Nrf2 translocation induced by ebselen in the hOEC sheets was evidenced after 3–6 hours of reculturing at 37°C. These results indicate that ebselen possesses two different biological roles: one is the direct effect of removing hypothermic-induced ROS generation during the preservation period and the other is the indirect effect via Nrf2 translocation during the reculturing period [25]. Moreover, ebselen maintained the normal morphology of limbal epithelial layer and showed meaningfully higher colony-forming efficiency, when the human corneal limbal tissue was preserved in ebselen, when compared to that of control in which a great number of corneal epithelium was lost [25]. Therefore, ebselen was demonstrated to be an effective hypothermic preservation medium for tissue-engineered cell sheets, and it is believed to promote the ocular regenerative medicine, such as LSCD management and cornea transplantation.

It was also reported that murine corneal epithelial progenitor cell line (TKE2) is more resistant to H_2O_2-induced oxidative stress with enhanced atrophy than cultured mature murine corneal epithelial cells (MCE). It was regulated by decreasing ROS production, reducing oxidative enzymes, such as NADPH oxidase 4 (NOX4), and increasing dual specificity phosphatase 6 (DUSP6). TKE2 also activated Nrf2 signaling and upregulated the expression of antioxidative enzymes (SOD and GSTP), indicating that Nrf2 is involved in maintaining the different hemostasis of corneal stem cells and exhibits strong antioxidant capacity against oxidative stress by regulating ROS generation and elimination [25].

2.5. Nrf2-Regulated Antioxidant Defense and Corneal Endothelial Dystrophy. FECD is a blinding disease and a primary reason of endogenous corneal endothelial degeneration. It is a gradually progressing disease with the accumulation of extracellular excrescences (guttae) [29]. Corneal transplantation is the only method to restore lost vision in patients with FECD. Jurkunas et al. reported that the imbalance of oxidant-antioxidant is indispensable in the long-lasting deteriorating progression of corneal endothelium (CE) observed in FECD [30]. Nrf2, a vital nuclear transcriptional inducer, which binds to the ARE in the DNA promoter and initiates antioxidant defense, is downregulated in FECD endothelium. Higher levels of oxidative DNA damage and apoptosis of CE were also detected in FECD endothelium in contrast to controls [30]. 8-Hydroxy-2-deoxyguanosine (oxidative DNA damage marker) localized to the mitochondria, demonstrating that the genome of the mitochondria is the main target of oxidative stress in FECD. This study revealed that oxidative stress plays a vital role in FECD pathogenesis.

2.5.1. Ultraviolet A and Nrf2-Regulated Antioxidant Defense in FECD. Ultraviolet A (UV-A) with the wavelengths of 320 to 400 nm is the main source of solar radiation, which plays an important role in ROS production, and therefore may be related to the etiology of FECD. The cornea is radiated daily by solar ultraviolet (UV) rays, which can lead to DNA damage and induce oxidative stress [31]. Jurkunas et al. reported that lower fluences of UV-A activated the antioxidant defense regulated by Nrf2 and higher fluences initiated p53 and caspase-3, denoting that near-environmental fluences of UV-A may have influence on normal human corneal endothelial cells (CECs) (Figure 2). Other studies have also indicated the key role of Nrf2-mediated antioxidant defense as well as p53 in the regulation of oxidative stress-induced apoptosis in FECD [30, 32, 33]. Those studies can introduce an in vitro oxidative stress model for exploring CEC degeneration, particularly, in FECD pathogenesis.

2.5.2. DJ-1 and Nrf2-Regulated Antioxidant Defense in FECD. DJ-1, encoded by the PARK7 gene, is a multifunctional protein and is universally expressed in most human cells and tissues. Under the influence of oxidative stress, DJ-1 plays an important role in antioxidant defense by regulating several antioxidant gene expressions [34], as a ROS scavenger [35],

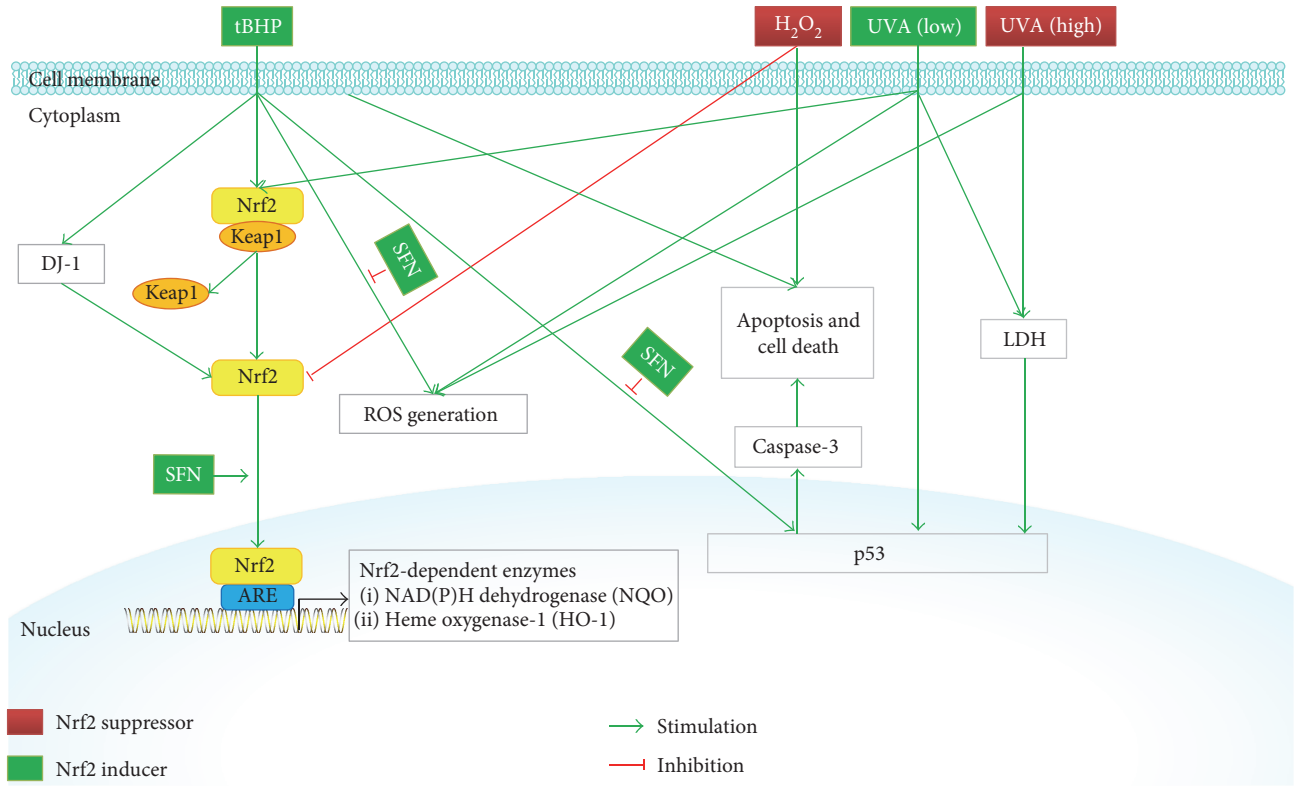

Figure 2: Schematic diagram of Nrf2 signaling and regulation in the corneal endothelial cells. TBHP and H_2O_2, both high and low fluences of UVA, can induce excessive ROS generation in corneal endothelial cells, leading to apoptosis and cell death. SFN can inhibit tBHP-induced ROS generation, apoptosis, and cell death. Meantime, it can increase DJ-1 protein expression and Nrf2 translocation and induce Nrf2/ARE-dependent antioxidant enzyme (HO-1 and NQO1) transcriptions.

and suppresses proapoptotic factors, which guard against oxidative stress- and UV-stimulated cell apoptosis [36]. It is suggested that DJ-1 prevents the Keap1/Nrf2 linkage [37, 38] or nuclear export of Nrf2 [39] to stabilize the function of Nrf2. It is reported that DJ-1 reduced drastically in FECD CECs, while Keap1 (Nrf2 protein repressor) increased under oxidative stress [33]. Nrf2 nuclear localization was detected in normal CECs, whereas the translocation of Nrf2 from cytoplasm to nuclei was not observed in FECD. Decreased levels of DJ-1 in FECD at baseline and under the stressed state were in relation with weakened Nrf2 nuclear translocation and improved cell susceptibility to apoptosis. The DJ-1/Nrf2 axis could be a promising target to delay the degeneration of CECs in FECD [33]. Nevertheless, these opinions are opposed by a report that DJ-1 is not involved in triggering the Nrf2-ARE signal pathway [40]. Recently, Liu et al. further proved that downregulation of DJ-1 resulted in decreased Nrf2 gene expression as well as its target genes HO-1 and NQO1 (Figure 2), which inhibits translocation of Nrf2, leading to attenuate the expression of antioxidant gene and oxidative damage [41]. The decrease of DJ-1 level results in enhanced CEC susceptibility to UV-A light via inducing p53-dependent apoptosis [41]. Therefore, focusing on the DJ-1/Nrf2 axis may offer a prospective treatment of corneal endothelial disorders through improving antioxidant defense.

2.5.3. SFN and Nrf2-Regulated Antioxidant Defense in FECD. SFN is an Nrf2 level enhancer, which is found in green cruciferous vegetables like broccoli [42]. Antioxidative stress effects of SFN have been widely studied [43, 44]. SFN and tBHP have been found to initiate modification of Keap1/Nrf2 proteins, leading to the activation of Nrf2 [33, 45]. Recently, Ziaei et al. had reported that SFN increased the activation of Nrf2 in FECD specimens and reduced p53 staining under oxidative stress. Pretreatment with SFN improved cell viability via diminishing the production of intracellular ROS in FECD. Increased level of Nrf2 resulted in unregulated synthesis of HO-1, DJ-1, and oxidoreductase [46]. Nrf2-ARE pathway may be a vital molecular mechanism involved in degenerative cell loss detected in FECD. SFN can drastically increase ARE-dependent antioxidants and reduce apoptosis induced by oxidative stress in FECD (Figure 2).

2.5.4. SLC4A11 and Nrf2-Regulated Antioxidant Defense in FECD. SLC4A11 is an anion transporter and a dimer located in the plasma membrane. It encodes a 100 kDa protein containing 14 domains [47]. Lately, it has been exposed to take part in Na^+-coupled OH_2 transport in CECs of bovine [48]. SLC4A11 is greatly expressed in human corneal epithelial as well as endothelial cells [49]. Dysfunctional SLC4A11 is supposed to be a contributing factor of corneal endothelial cell death [50]. CHED2 is an autosomal recessive disorder, which is featured by decreasing in corneal endothelial cell

Table 1: Selected studies on the relationship between Nrf2 inducer/suppressor and corneal diseases.

Inducer	Suppressor	Type	Models	Results	Reference
4-HNE	NAC	In vitro	Cultured HCE cells	NAC antagonized the 4-HNE-induced oxidant effects in the cultured HCE cells by the reversed cell viability of HCE cells and reduced the 3-NT, NOX4, and Nrf2 protein expression induced by 4-HNE.	[24]
H_2O_2	SA3K	In vitro	Cultured HCE cells	SA3K reversed H_2O_2-induced cell apoptosis. SA3K upregulated H_2O_2-induced downregulation of SOD2 and catalase gene expression. SA3K blocked H_2O_2-induced ROS and NOX4 overexpression and activated Keap1-Nrf2 pathway by suppressing Keap1-Nrf2-ARE pathway.	[2]
		In vivo	Rat corneal epithelium	SA3K ameliorated H_2O_2-induced corneal epithelium death and decreased the H_2O_2-induced ROS, 3-NT, NOX4, and Nrf2 overexpression.	
	H_2O_2	In vitro	Cultured TKE2 and MCE	TKE2 cells have different homeostasis and strong antioxidant properties compared to MCE by decreasing ROS production and NOX4 and increasing DUSP6, Nrf2, SOD, and GSTP.	[25]
Ebselen		In vitro	hOEC sheet, human corneal limbal tissue	Ebselen maintained the high ATP levels, normal morphology, viability, and function of the hOEC sheets by reducing ROS generation, inducing the Nrf2 activation, decreasing the lactate dehydrogenase (LDH) releasement, and increasing the glutathione (GSH)/oxidized glutathione (GSSG) ratio. Ebselen maintained the normal morphology of limbal epithelial layer and showed meaningfully higher colony-forming efficiency.	[25]
Carnosol		In vivo	Diabetic rat corneal epithelium injury model	Carnosol accelerated the corneal epithelial wound healing.	[16]
EP		In vitro	Cultured keratocytes and myofibroblasts	EP enhanced the Nrf2-mediated antioxidant response and induced phenotypic changes of quiescent corneal stromal keratocytes into contractile myofibroblasts.	[20]
TSA	TGF-β	In vitro	Cultured corneal fibroblasts (HTK)	TSA inhibited of TGF-β-stimulated myofibroblast differentiation in HTK cell line, by decreasing ROS and H_2O_2 accumulation, inducing Nrf2 nuclear translocation and upregulated the transcriptions of Nrf2-ARE-controlled antioxidant enzymes (such as GSH).	[22]
Lower fluences of UV-A		In vitro	CECs	Lower fluences of UV-A activated the antioxidant defense regulated by Nrf2 and higher fluences initiated p53 and caspase-3. UV-A may be related to the etiology of FECD.	[57]
		In vitro	FECD endothelium	Nrf2 is downregulated in FECD endothelium; higher levels of oxidative DNA damage and apoptosis of CE were also detected in FECD endothelium in contrast with normal controls.	[30]
tBHP		In vitro	HCECi and FECDi; FECD corneal buttons	Declined levels of DJ-1 in FECD at baseline and under the condition of oxidative stress were in relation with weakened Nrf2 nuclear translocation and improved cell susceptibility to apoptosis.	[33]
UV-A		In vitro	CECs	Downregulation of DJ-1 resulted in decreased Nrf2 gene expression as well as its target genes HO-1 and NQO1, which inhibits translocation of Nrf2, leading to attenuate the expression of antioxidant gene and increase oxidative damage. The decrease of DJ-1 level results in enhanced CECs susceptibility to UV-A light via inducing p53-dependent apoptosis.	[41]
SFN		In vitro	HCECi and FECDi	SFN increased the activation of Nrf2 in FECD specimens under the condition of oxidative stress. Pretreatment with SFN improved cell viability via diminishing the production of intracellular ROS in FECD.	[46]
	SLC4A11 mutations	In vitro	HEK 293 cells	Mutations in the *SLC4A11* gene can induce ROS generation and mitochondrial dysfunction because of oxidative stress. HO-1, NQO, and NRF2 expression declined drastically, and a higher apoptosis rate was found in cells with mutant proteins under oxidative stress.	[4]

4-HNE: 4-hydroxynonenal; 3-NT: 3-nitrotyrosine; NOX4: NADPH oxidase 4; HCE: human corneal epithelial cells; GSTP: glutathione S-transferase P; NQO1: NAD(P)H dehydrogenase (quinone 1); NAC: N-acetylcysteine; H_2O_2: hydrogen peroxide; SA3K: SERPINA3K; TKE2: murine corneal epithelial progenitor cell line; MCE: mature murine corneal epithelial cells; NOX4: NADPH oxidase 4; DUSP6: dual specificity phosphatase 6; SOD: superoxide dismutase; GSTP: glutathione S-transferase P; EP: ethyl pyruvate; TSA: trichostatin A; TGF-β: transforming growth factor-β; CECs: corneal endothelial cells; UV-A: ultraviolet A; FECD: Fuchs endothelial corneal dystrophy; Nrf2: nuclear factor erythroid 2-related factor-2; FECDi: immortalized FECD human corneal endothelial cell lines; HCECi: immortalized normal human corneal endothelial cell lines; tBHP: tertbutyl hydroperoxide; SFN: sulforaphane; HEK 293: human embryonic kidney.

density [51]. Both CHED2 and FECD result in gradual cloudiness of the cornea and progressive vision loss. Some studies have reported that they have been connected with several mutations in the SLC4A11 gene [52–56], which can induce ROS generation and mitochondrial dysfunction due to oxidative stress. It is detected that 2 major Nrf2 transcriptional targets, HO-1 and NQO, as well as Nrf2 expression, declined drastically, and higher apoptosis rate was found in cells with mutant proteins in overexpressed ROS environment [4]. Further studies are needed to elucidate the specific role of SLC4A11 played in corneal endothelial dysfunction, which may facilitate the management of the corneal endothelial cell degeneration.

3. Conclusions and Outlook

Accumulating evidences denote that oxidative stress is one of the major mechanisms involved in the corneal diseases, which increases the injury in the corneal epithelial and endothelial cells via oxidation of proteins, DNA damage, apoptosis, cell death, and so forth. The Nrf2/Keap1/ARE signaling pathway is related to cell defense mechanisms against oxidative stresses. Therefore, the initiation of the Nrf2-ARE signaling pathway has been estimated as an important target for the design and synthesis of new agents for corneal diseases. Selected studies on the relationship between Nrf2 inducer/suppressor and corneal diseases reviewed in this article have shown notable effects on protecting or deteriorating corneal epithelial or endothelial cells against oxidative stress, decreasing the aberrant proteins and preventing or causing the corneal diseases (Table 1). All these encouraging effects have been linked with the antioxidant and Nrf2-inducing effects of the compounds studied (Figures 1 and 2). In summary, the Nrf2/Keap1/ARE signaling pathway is a promising therapeutic target against oxidative stress for corneal diseases.

Disclosure

Xiu-Fen Liu and Dan-Dan Zhou are co-first authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Effect of Chronic Administration of Resveratrol on Cognitive Performance during Aging Process in Rats

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The increase in the elderly population has generated concern to meet health demands. The research efforts to elucidate the mechanisms of damage associated with aging have also been significantly increased, especially in order to avoid the reduction of the cognitive abilities in geriatric patients, resulting from the damage generated mainly at the level of the hippocampus during old age. At present, many studies describe resveratrol as an antiaging component. There are reports that it can activate the Sirt1 gene related to antiaging, emulating the effects obtained by caloric restriction in rodents. The aim of the study was to evaluate the effect of chronic administration of resveratrol (10 mg/kg) on cognitive performance in behavioral tests after 8 months of treatment and on the preservation of cerebral integrity in the cytoarchitecture of regions CA1 and CA2. Results showed that the cytoarchitecture of the CA1 and CA2 regions in the hippocampus retained their integrity over time in rats treated with resveratrol, and the behavioral test performed revealed that chronic resveratrol administration for 8 months showed improvements in cognitive performance. The results indicate that resveratrol may exhibit therapeutic potential for age-related conditions.

1. Introduction

One of the most important aspects in the adult stage is the deterioration of functional, emotional, and cognitive capacities. These changes limit the carrying out of the habitual activities necessary for the life of the people with the consequent diminution of their independence and the constant need for help. The aging process has been considered as an inevitable physiological change that occurs in living organisms and that progress over time [1, 2].

There is a growing interest in searching for neuroprotective agents from natural products since they contain compounds with high antioxidant power [3]; in this regard, resveratrol has received considerable attention during the last decade [4]. Resveratrol is a stilbenoid constituted by the union of a phenolic ring and a phytoalexin, naturally produced by 72 different plant species, especially vines, pines, and legumes [5]. The presence of these linked rings confers the antioxidant activity because it has been shown that these structures have the ability to be a scavenger of hydroxyl radicals. The most popular form of consumption is in red wine and nuts [6].

It has been shown that resveratrol can be present in cis/transisoforms, among which only the transisoform is biologically active. It is important to mention that transresveratrol can also be produced biotechnologically as a nutritional supplement from *Polygonum cuspidatum* (*Polygonum*, St. Mary's wort, pejiguera herb, pestle, and partridge paw) [7].

It has been demonstrated that a *Polygonum cuspidatum* extract containing 20% transresveratrol had comprehensive suppressive effects on inflammatory and oxidative stress, decreasing TNF-α levels, interleukin, intranuclear NFκB binding, c-jun-N-terminal kinase 1 (JNK 1), and phosphotyrosine phosphatase-1B (PTP-B), as well as reactive oxygen species (ROS) generation in mononuclear cells [8]. Using the model senescence-accelerated mouse (SAM), it was found that resveratrol extracted from Hu Zhang increased the SOD and GPx activities, while decreasing malondialdehyde (MDA) level in SAM *in vivo*. Resveratrol could improve neuromuscular coordination and sensorimotor ability in tightrope test. It could also enhance the learning and memory capacity in the Morris water maze test in SAM [9].

The bioavailability of resveratrol depends on the food matrix where it is incorporated. In humans, when resveratrol is administered orally, a large number of secondary metabolites are detected in plasma and urine, primarily glucuronides. In addition, the bioavailability of resveratrol depends on the vehicle in which it is immersed, that is, oral doses of grape juice report sulfate in plasma and urine while wine administration has increased evidence of glucuronide [10].

In a lot of bioavailability studies, it has been proposed that resveratrol can prevent some types of cancer [11] and confers neutroprotective properties [12], as well as protective effects in cholestatic liver injury [13]. Resveratrol prolongs the useful life of species such as yeasts, worms, and flies [14] by the activation of the silent regulator of information 2 (SIR2), which belongs to the family of sirtuins and has been frequently related to the increase of longevity in some species. Although the pharmacological actions of resveratrol have been linked to antioxidant activity, the possible link between activation of sirtuins and redox regulation by resveratrol is not yet clear. It should be noted that previously the activity of sirtuins had been reported in organisms subject to caloric restriction, so resveratrol is considered a mimetic of the effects of this nutritional practice [15].

The effects of aging on the brain and cognition are very extensive and have multiple etiologies. As we age, there are changes in macroscopic morphology, increases in blood pressure and, with it, the possibility of stroke. In addition, the brain contracts in volume, particularly in the frontal cortex. This change is determinant because it compromises cognitive functions and has been associated with dementia processes [16]. The mechanism by which the brain volume decreases is not yet clear; however, it has been proposed that it is due to the decrease of gray matter because of neuronal death, white matter loss, and changes in dendritic spines [17]. The consequences that are relevant to this loss of brain function are the cognitive changes associated with all types of memory (semantic, episodic, procedural, and working memory) that lead to behavioral disorders, depression, and dementia processes [16].

In this work, we studied the role of chronic administration of resveratrol in oxidative process, the cytoarchitecture of the hippocampus, and the cognitive processes in different periods of rats' life.

2. Materials and Methods

2.1. Experimental Animals. 3-month-old male Wistar rats were obtained from the Bioterio Claude Bernard of the Benemérita Universidad Autónoma de Puebla. The animals were housed in a controlled temperature and humidity environment conditions with light-dark cycles of 12–12 h, with free access to water and food. All treatment methods used in this study were performed according to the guide for the care and use of laboratory animals NOM-062-ZOO-1999. Every effort was made to minimize the suffering of animals.

2.2. Resveratrol Dose Determination. To establish the dose of resveratrol, 30 rats were divided into six groups, according to their administration, divided into the following categories: group administered with vehicle (10% saline and 10% ethanol) and 4 other groups each administered with resveratrol in 2.5, 5, 10, 20, and 50 mg/kg/day, respectively (resveratrol was diluted in physiological solution and 10% ethanol). A stainless steel cannula was used for the oral administration of different doses. Once administrated, rats were returned to their home cage. After daily administration for two months, the rats of diverse groups were sacrificed and decapitated to obtain the brain. Posteriorly, the hippocampus was obtained for quantification of nitrite production, malondialdehyde (MDA), and MDA + 4-hydroxinonenal (4-HDA).

The total protein was quantified by the Sedmak and Grossberg method (1997) using bovine serum albumin (BSA) as standard. Hippocampus was homogenized in PBS 1X solution, in a relation 1 : 4, and the supernatant was pulled apart by a spinning process at 12500 rpm for a period of 30 min at 4°C. Proteins were quantified in 2 μL of supernatant plus 500 μL of Coomassie Brilliant Blue G reactant 0.06% and finally brought to 1 mL with distilled water. The result of the reaction was read in a spectrophotometer (SpectrumVis SP1105) at 620 nm. Protein concentration was determined by interpolation of the optic density of the samples in a BSA standard curve (1 to 10 μg), which was determined parallel in each trial.

The nitric oxide was analyzed through nitrite ion (NO_2^-) content in tissue supernatant by the Griess method (Chao y cols., 1992). The Griess reactant was a compound with equal volumes of N-(1-naphthyl)ethylenediamine dihydrochloride 0.1% dissolved in distilled water and sulfanilamide 1.32% (dissolved in acetic acid 60%). The colorimetric reaction was induced by the addition of 100 μL of Griess reactant to 100 μL of supernatant and was brought to 1 mL with distilled water. After 5 minutes of centrifugation at 500 rpm, the reaction proceeds were read in a spectrophotometer (SpectrumVis SP1105) at 540 nm. The NO2 concentration was determined by the interpolation of the optic density of the samples in a NaNO2 standard curve (0.5 to 10 μL), which was parallel determined in each trial.

The determination of MDA and 4-HDA was counted in the samples to analyze the generation of lipid peroxidation products as oxidative stress markers, using the N-methyl-2-phenyl-indole as chromogenic reactant (10.3 mM). 650 μL of solution which consists of N-methyl-2-phenyl-indole dissolved in a mixture of acetonitrile : methanol (3 : 1) was added

to 100 μL of supernatant; the solution was vigorously shaken and after that 150 μL of HCL or methanosulfonic acid 35% was added. The reaction was incubated at 45°C for 45 minutes or one hour, respectively, then it was allowed to cool for 5 minutes and finally was centrifuged at 3000 rpm for 15 minutes. Later, the reaction proceed was read in a spectrophotometer (SpectrumVis SP1105) at 586 nm. The MDA and 4-HAD concentration was determined by the interpolation of the optic density of the samples in a 1,1,3,3-tetramethoxypropane standard curve (0.5 to 10 μL), which was determined parallel in each trial.

The dose of 10 mg/kg was chosen for a better antioxidant activity.

2.3. Antioxidant Effect of Resveratrol. Four groups of rats were formed: control, vehicle (10% ethanol), vitamin E (2 mg/kg/day), and resveratrol (10 mg/kg/day). The rats were administrated for different periods (2, 4, 6, and 8 months, thus correspond to 5, 7, 9, and 11 months age, resp.). Resveratrol was LEMI & JO Resveratrol® extracted from the root of *Polygonum cuspidatum*; the number of rats in each period was $n = 16$, except for the 8 months administrated, where the number was $n = 32$. It is important to mention that vitamin E was selected to compare the antioxidant effect of resveratrol, because this vitamin is the principal antioxidant against oxidative damage in plasma and erythrocytes [5, 6].

2.3.1. Behavioral Tests. At the end of the corresponding period in each group, the rats were sacrificed, except for the group administered for 8 months, in which before its sacrifice, behavioral tests of the type NOR (novel objects recognition) were realized [18]. The NOR test is suitable for this type of experimental model since it presents advantages over other tests that compromise the integrity of experimental animals, besides that it does not involve positive or negative reinforcements. It is based on spontaneous behavior when accessing a novelty and generating an apparent "unconditioned preference."

Two previous analyses were performed to obtain the recognition index: the object recognition time was measured and the number of contacts (direct interaction of the animal with the object) was counted. The data from these analyses correspond to the recognition index, so that the NOR test is accurately condensed with this indicator. In order to analyze the data obtained, the recognition index with a value of >0.5 corresponds to a process of consolidation of learning, and on the contrary, an index < 0.5 indicates a lack of interest in recognizing the novel objects.

2.3.2. Effect on the Hippocampus Cytoarchitecture. 64 animals were perfused with 1X PBS solution through the left ventricle, cutting the descending aorta artery, and the brain was dissected and preserved in 4% formalin. 30 μm coronal sections in the vibratome were obtained, and for histological studies, the Nissl modified staining was performed [19]. These dyes can bind not only to the DNA content of the cell nuclei but also to the RNA, that is highly concentrated in rough endoplasmic reticulum and ribosomes (Nissl substance) in the cytoplasm. Through the Nissl staining, cell ordering and changes in the cytoarchitecture of the hippocampal CA1 and CA2 structures in the brain tissue sections were observed.

2.4. Statistical Analysis. For all the experiments, the data obtained were expressed as a mean ± standard error of the media (SEM). Statistical analysis was developed through ANOVA and the Dunnett posttest.

3. Results and Discussion

The present work shows the antioxidative effect of resveratrol in rats at different ages. We determined that 10 mg/kg produces a decrement in oxidative stress of the rats from two months of administration to eight; also, we observed that resveratrol produced an increment in the cognitive process and a major conservation of cytoarchitecture of the CA1 and CA2 of the hippocampus in rats. These animals and the NOR test were employed because they are general models in biomedical research. It has been shown that rodents (mice and rats) are very useful for to obtain adequate and uniform results.

In the first place, the dose of resveratrol for subsequent experiments was determined. Figure 1(a) shows that the vehicle group had a concentration of nitrites of 0.6 ± 0.0152 μM/mg of total protein; resveratrol administration during 2 months produced a decrement of $39 \pm 1.6\%$, $48 \pm 0.15\%$, $53 \pm 0.39\%$, and $62 \pm 0.54\%$ in the groups administrated with 2.5, 5, 10, and 20 mg/kg of resveratrol, respectively. To determine lipoperoxidation, MDA + 4-HDA (Figure 1(b)) was measured; a decrement of $60 \pm 0.06\%$, $66 \pm 0.17\%$, $76 \pm 0.19\%$, and $81 \pm 0.38\%$ in the groups 2.5, 5, 10, and 20 mg/kg of resveratrol was found. On the other hand, individual value of NMDA (Figure 1(c)) showed a similar decrease in the different groups ($32 \pm 2\%$, $36 \pm 2.3\%$, $48 \pm 0.54\%$, and $49 \pm 2.8\%$, resp.). Based on these results, it was determined that the appropriate dose that produced antioxidative effect was 10 mg/kg. For analysis, one-way ANOVA and Dunnett's posttest were used. Oral administration of resveratrol promoted a significant decrease in MDA+ 4-HDA levels in the hippocampus. This finding is consistent with previously reported studies in which the appropriate dose was found to be 12.5 mg/kg in subacute administration [20] performed intraperitoneally. In contrast to this trial, the oral administration was the administration of choice and the appropriate dose was 10 mg/kg in weight. It is important to emphasize the importance of this dose, since the administration of higher doses (20 and 50 mg/kg) did not promote a significant increase of the antioxidant activity according to this indicator.

The novel object recognition (NOR) test was applied to the 8-month groups to evaluate the possible protective effect of resveratrol on the hippocampus, and the conservation of cognitive abilities and results are shown in Figure 2. According to the behavioral performance evaluation, the recognition index (parameter relating the number of contacts and the time invested in carrying it out) in the control group was 0.6 ± 0.052 in short- and long-term memory 0.061 ± 0.028. As for the group administered with vehicle, an index of

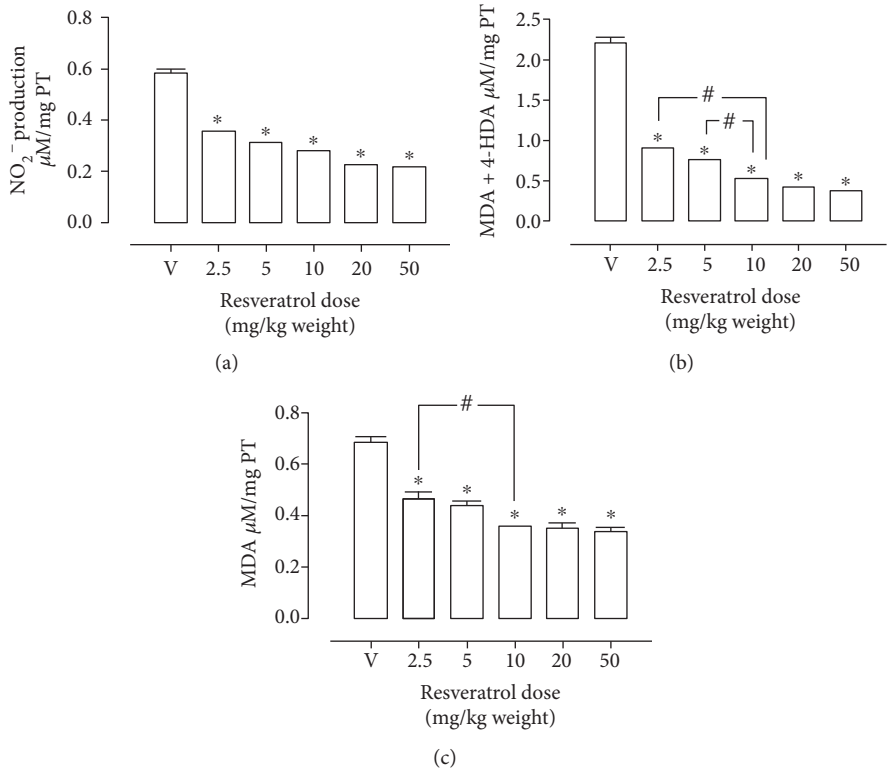

Figure 1: Resveratrol antioxidant effect on the hippocampus: (a) Nitrite levels. Analyzed through Griess method, the nitrite decrease observed is dose-dependent. (b) MDA + 4-HAD levels. It can be observed that resveratrol concentration increment to 10 mg/kg gives the best antioxidant activity. (c) MDA level. The optimum decrease is presented in doses of 10 mg/kg. The lipid peroxidation index was determined by the Gérard-Monnier. The data are means ± standard error of the mean SEM for $n = 4$ rats. *Significantly different from the vehicle group $p < 0.001$ (one-way ANOVA). #Significantly different from the group 10 mg/kg/weight $p < 0.05$ (ANOVA, Tukey's method).

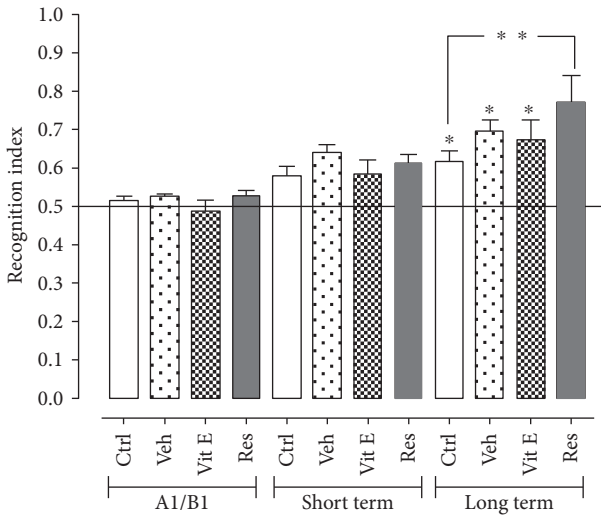

Figure 2: Effect of resveratrol on short-term and long-term memory using the novel object recognition (NOR) test. There are statistically significant differences between the long-term memory of the control compared to the administered group with resveratrol. The data express the mean ± SEM for $n = 8$ rats. * indicates $p < 0.05$ with respect to exposure A1/B1 of its corresponding group. ** indicates $p < 0.05$ with respect to the control group in long-term memory (ANOVA and Dunnett's test).

0.67 ± 0.02 and 0.69 ± 0.027 in the short- and long-term memory, respectively, was reported. The index in the group administered with vitamin E in short- and long-term memory was 0.58 ± 0.037 and 0.67 ± 0.052. Finally, in the group administered with resveratrol, it presented an index of 0.61 ± 0.037 in the short-term memory and 0.77 ± 0.115 in the long-term memory, which represented a 20% increase in comparison with the recognition index observed in the control group. There were no statistically significant differences in other groups.

For the NOR trial, all groups present in this evaluation presented the consolidation of the learning process, including the control group. Additionally, it is observed that the group administered with vehicle has an index similar to that shown by the controls, which allows to rule out that there is any additional effect on the evaluated animals when administering 10% ethanol. On short-term memory, there were no statistically significant differences with their controls or between groups, which reflects that the animals did not recognize a great extension of the novel objects that were exposed to them and their exploration was not significant. As for the group administered with resveratrol, a significant increase in the index of recognition in the long-term memory was found. This important change may be due to the fact that long-term memory is a type of declarative memory whose development involves the cortical area and the

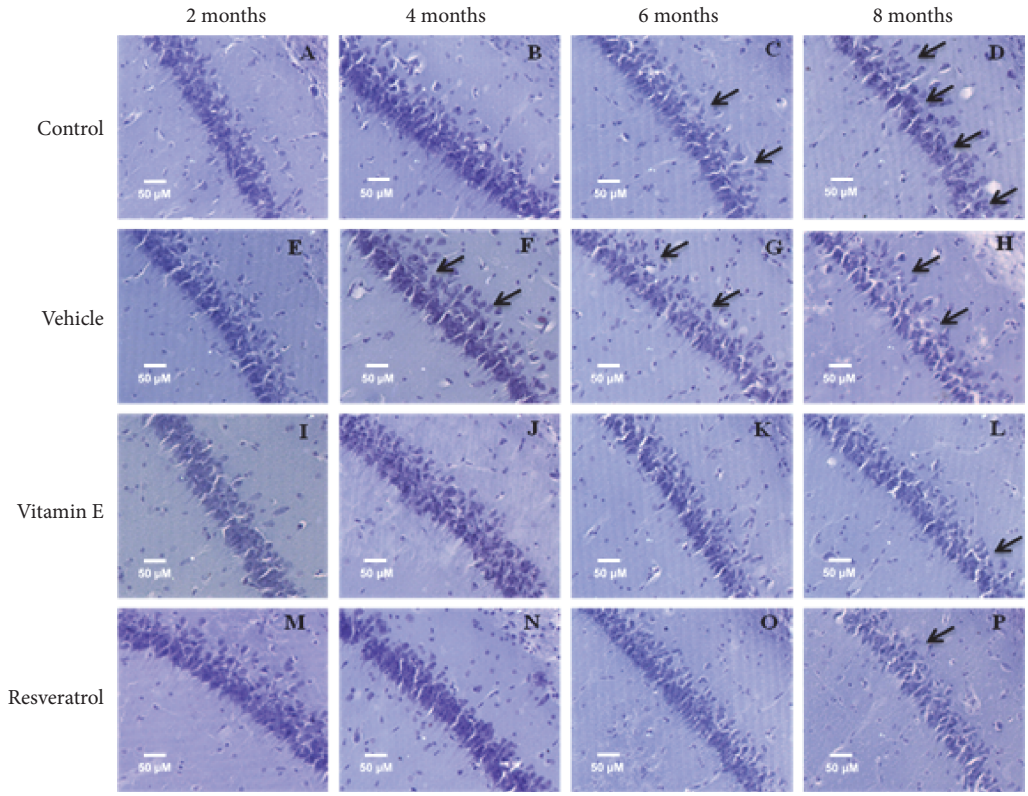

Figure 3: Effect of resveratrol on the hippocampus cytoarchitecture in the CA1 region in 2, 4, 6, and 8-month treatment rats (20×). (a) Control group of 2 months. (b) Control group of 4 months. (c) Control group of 6 months. (d) Control group of 8 months. The CA1 region of the hippocampus and the arrangement of the cells are observed; the cytoarchitecture begins to be modified with respect to the increase of the age. (e) Vehicle group of 2 months. (f) Vehicle group of 4 months. (g) Vehicle group of 6 months. (h) Vehicle group of 8 months. In these groups, greater dispersion of the cells is observed. (i) Group administered with vitamin E for 2 months. (j) Vitamin E group of 4 months. (k) Vitamin E group of 6 months. (l) Vitamin E group of 8 months. During the different periods, it is observed that the CA1 region is conserved; however, the space that is observed between the cells is remarkable. (m) Group administered with resveratrol for 2 months. (n) Group administered with resveratrol for 4 months. (o) Resveratrol group of 6 months. (p) Resveratrol group of 8 months. The structure of CA1 is shown with an order in the arrangement of the cells and the axonal projections are observed aligned, the integrity of the structure is conserved largely and the scattered cells are scarce, and even at 8 months, the resveratrol group is observed with less cellular disorganization. The arrows indicate areas where cells are scattered in the CA1 region.

parahippocampal gyrus, where associations and conjunctions among stimuli are carried out, that is, the neurochemistry of the hippocampus is essential for recognition memory [Stanley et al., 2012], so it indicates that long-term memory is being favored by the protective effect of resveratrol on this region. It has been reported that this neuroprotective effect is due to the preservation of hippocampal integrity, and the treatment selectively protects neurons in the CA1 and CA2 regions [21].

CA1 (Figure 3) and CA2 (Figure 4) regions of the hippocampus were visualized, which are closely related to memory and learning processes. The changes in the cytoarchitecture refer especially to the cellular organization, where the arrangement and order of the axonal projections are observed. In the hippocampal CA1 region, we observed a gradual change in the cytoarchitecture due to the time of administration and the type of treatment. In the intact specimens (Figures 3(a), 3(b), 3(c), and 3(d)), a subtle modification of the cellular confluence is seen from 2 to 8 months of treatment. In addition, it shows a greater dispersion in the cells and even a decrease in the number of cells. On the other hand, in the groups administered with vehicle (Figures 3(e), 3(f), 3(g), and 3(h)), the changes are evidenced due to the time. The cellular disorganization is appreciable from the beginning of the administration and the changes are drastic when the group of 2 months is compared with the last period of treatment. In contrast to these observations, the groups administered with antioxidants (Figures 3(i), 3(j), 3(k), 3(l), 3(m), 3(n), 3(o), and 3(p)), that is, with vitamin E and resveratrol, show that the integrity of the structure is better preserved, especially when administering resveratrol (Figures 3(m), 3(n), 3(o), and 3(p)), where the axonal projections are observed to be aligned and ordered, and conserve a greater number of cells in comparison with the other groups.

The analysis and visualization of the CA2 region of the hippocampus revealed subtle changes that were reproduced throughout the treatment time. The 2-month control group (Figure 4(a)) is observed with a greater confluence of cells, which through the months, is diminished until there is an evident dispersion in the arrangement of the cells in the last

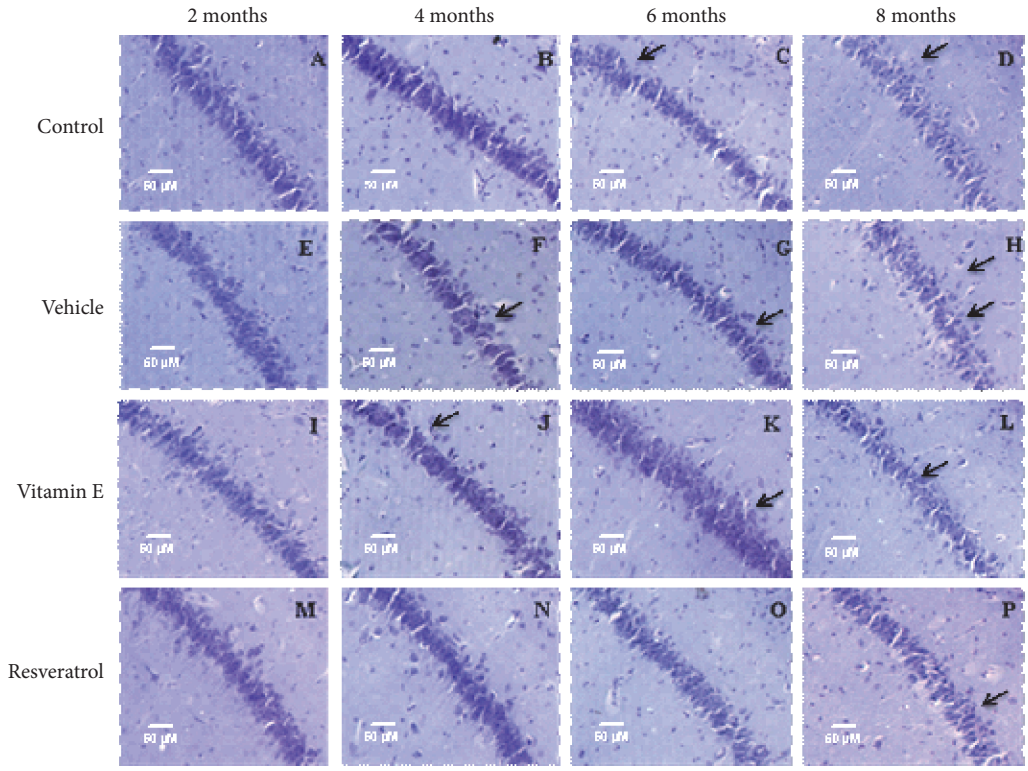

Figure 4: Effect of resveratrol on the hippocampus cytoarchitecture in the CA2 region in rats of 2, 4, 6, and 8 months of treatment (20×). (a) Control group of 2 months. (b) Control group of 4 months. (c) Control group of 6 months. (d) Control group of 8 months. The CA2 region of the hippocampus is observed, and it is interesting to note the arrangement of the cells and the axonal projections; this region does not result in severe alterations as observed, but the gradual changes lead to the disorganization that as observed in the CA1 region is present in different brain regions. (e) Vehicle group of 2 months. (f) Vehicle group of 4 months. (g) Vehicle group of 6 months. (h) Vehicle group of 8 months. Cellular cytoarchitecture is compromised over time, and changes due to cellular disorganization are more noticeable. (i) Group administered with vitamin E for 2 months. (j) Vitamin E group of 4 months. (k) Vitamin E group of 6 months. (l) Vitamin E group of 8 months. The organization of cells is conserved and changes with respect to time are minimal. (m) Group administered with resveratrol for 2 months. (n) Group administered with resveratrol for 4 months. (o) Resveratrol group of 6 months. (p) Resveratrol group of 8 months. The CA2 region of the hippocampus retains its integrity until after 6 months of treatment and then presents characteristic changes of aging. The arrows indicate areas where there are scattered cells or changes in the organization of cells.

group of 8 months (Figure 4(d)). The cellular disorganization that can be distinguished in the group administered with vehicle is evident in relation to the chronology of the treatment, where in addition a reduction in the number of cells in this region becomes appreciable, and the dense layer of cells is diminished, being observed spaces between cells. In the groups administered with vitamin E (Figures 4(i), 4(j), 4(k), and 4(l)), the CA2 region is observed with better conservation and cellular order and is appreciable that it is considerably similar to the control group; however, after 8 months of treatment, it is poorly defined and with a decrease in cell density (Figure 4(l)). In the groups treated with resveratrol (Figures 4(m), 4(n), 4(o), and 4(p)), the integrity of the CA2 region is conserved until after 6 months of treatment (Figure 4(o)), and after 8 months, the cytoarchitecture was modified (Figure 4(p)), since an undefined region is observed and with very dispersed cells in its conformation.

In addition to these observations, it was considered to compare brain mass between the different treated groups, because a decrease in brain mass is related to the process of cerebral atrophy, which is another characteristic of the aging process. The differences in the brain mass were analyzed (Figure 5), and these differences were reported between the brain weights of animals administered with resveratrol compared to those treated with vehicle for six months. Although no statistical differences were found in other groups, it is important to note that in all groups administered with a vehicle, there is a trend of brain mass decreasing and, also, a lesser extent in the control groups. This evidence might suggest that if experimental animals were brought to a greater age, significant changes would be reflected in the difference in brain mass. It has been reported that major changes are evident in rats during adulthood, specifically in older adults. In correspondence to the lifetime and the weight of the rat, the animals that were intervened in this experimentation were in the young adult to the middle-aged adult phase of life. To reach the aging stage in the rat model, age ranges from 15 to 24 months according to the correlation made for this model organism [22, 23]; therefore, the most severe changes are significant and forceful until reaching this age.

With this evidence, it can be suggested that the efficiency of the antioxidant activity of resveratrol in rats was

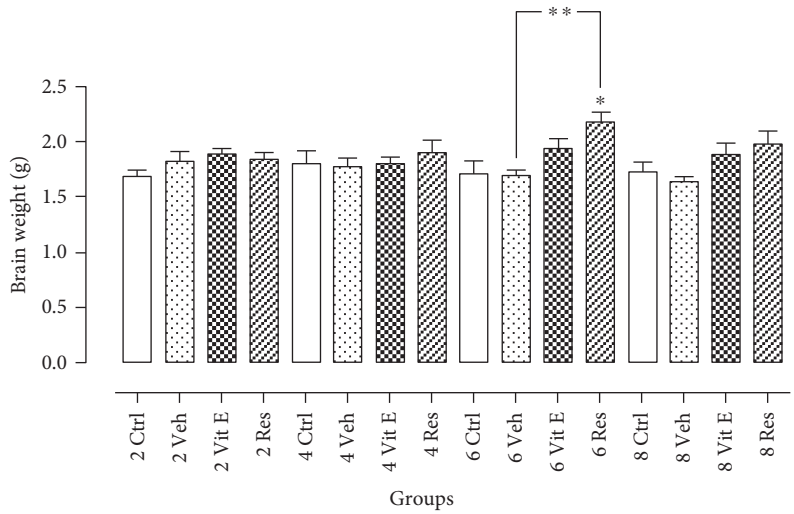

Figure 5: Effect of resveratrol on brain atrophy. Reduced brain size is a hallmark of aging. Comparing each of the groups, the resveratrol group has a tendency to avoid the decline in brain mass. Statistically significant differences were observed between the brain weights of animals treated with resveratrol for six months compared to control group and those administered with vehicle. The data express the mean ± SEM for $n = 4$ rats, with a $p < 0.05$. $*$ indicates $p < 0.05$ with respect to the 6-month control group. $**$ indicates $p < 0.05$ with respect to the 6-month vehicle group (ANOVA and Dunnett's test).

transcendental practically throughout the period between 2 and 8 months of administration, the cerebral integrity and the cognitive functions seem to be conserved when chronic resveratrol is consumed, the oral administration of resveratrol for 8 months allowed a development in cognitive performance, particularly on long-term memory, and cellular disorganization in the CA1 and CA2 region of the hippocampus was attenuated with chronic resveratrol administration.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Metallothionein in Brain Disorders

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Metallothioneins are a family of proteins which are able to bind metals intracellularly, so their main function is to regulate the cellular metabolism of essential metals. There are 4 major isoforms of MTs (I–IV), three of which have been localized in the central nervous system. MT-I and MT-II have been localized in the spinal cord and brain, mainly in astrocytes, whereas MT-III has been found mainly in neurons. MT-I and MT-II have been considered polyvalent proteins whose main function is to maintain cellular homeostasis of essential metals such as zinc and copper, but other functions have also been considered: detoxification of heavy metals, regulation of gene expression, processes of inflammation, and protection against free radicals generated by oxidative stress. On the other hand, the MT-III has been related in events of pathogenesis of neurodegenerative diseases such as Parkinson and Alzheimer. Likewise, the participation of MTs in other neurological disorders has also been reported. This review shows recent evidence about the role of MT in the central nervous system and its possible role in neurodegenerative diseases as well as in brain disorders.

1. Introduction

In 1957, the metallothionein was first identified by Margoshes and Vallee [1] in the cortex of horse kidney. This protein was later characterized biochemically by Kagi and Valle [2] in 1960, since then it has been widely studied in several aspects. This family of proteins is characterized by elevated heavy metal and cysteine content, so it was named metallothionein (MT). Different functions have been described for this protein, among them are storage and transport of essential metals such as copper and zinc, angiogenesis processes, protection against reactive oxygen species (ROS) and DNA damage, gene regulation, inflammatory processes, and cell survival among others [3, 4]; also the MTs have been implicated in processes of neuroprotection and neuroregeneration. The scientific interest in the functional role of MT has been increasing as more physiological functions have been described, and MT is now considered to be protein protecting against oxygen radicals and oxidative damage to the central nervous system; although MT was discovered over 60 years ago, its physiological functions are still unclear in the brain disorders. In this brief review, we focus mainly on emphasizing the physiological role of MT in brain disorders.

2. Classification and Biochemical Properties of MT

MT is a family of proteins characterized by their low molecular weight, they have a single peptide chain containing 61–68 amino acids, 20 of them are cysteines distributed in two domains α and β-clusters [5, 6], and they are bound in total 7 ions of divalent metals. The single polypeptide chain of MT has the structure cys-x-cys, cys-xy-cys, and cys-cys where the x and y represent noncysteine amino acids; the stoichiometric form of the protein shows 7 ions for each 20 cysteines (Figure 1) forming metal-thiolate complexes, enabling the MT to bind to 7–10 g of metal/mol MT atoms [7–10]. The binding of MT to metals is through the thiol group (SH) found in cysteine residues; the metal-free protein named as apo-metallothionein or thionein has a predominantly disordered structure, which makes it highly vulnerable

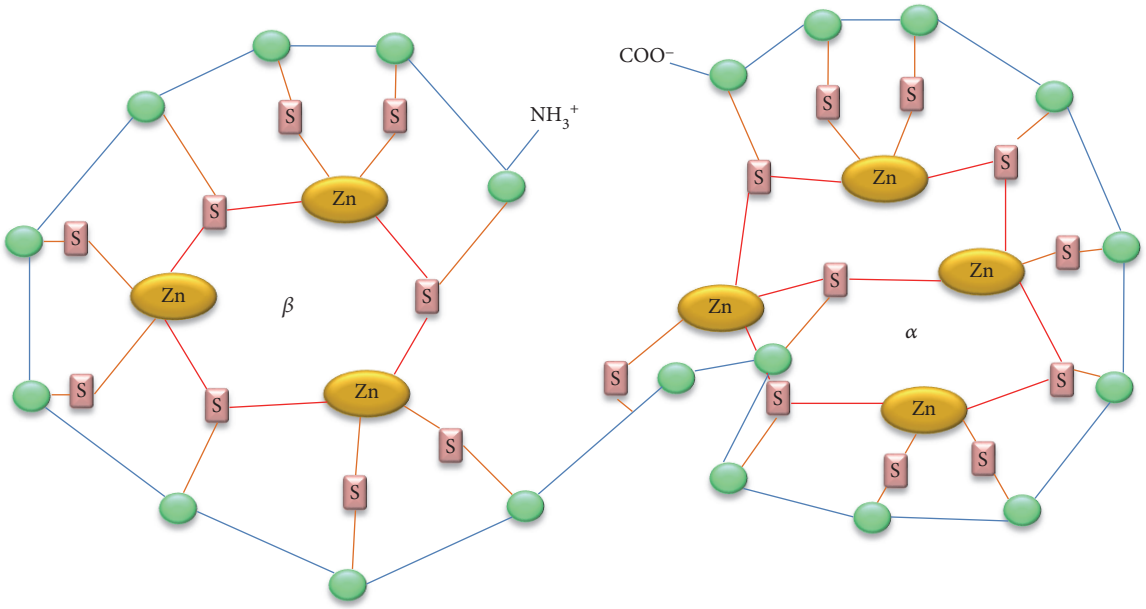

Figure 1: Schematic representation of the MT structure. The metal-thiolate complexes (with C-terminal domain of the α- and N-terminal chain in the β chain); the green ovals represent the 20 cysteine residues bound to the sulfur compound (S) which binds divalent to zinc, three bonds to zinc of the β chain and four to the α chain by adding seven divalent bonds.

to proteolytic processes [11]. Normally, MT has a high affinity for divalent essential metals such as Cu and Zn, although it also has shown a high affinity for toxic metals like cadmium and mercury giving rise to metal-thiolate clusters [12]. Up to now, 4 isoforms of the protein are recognized, which are structurally homologous and enough conserved during evolution [13].

The existence of four MT genes in a region of 50 kb on chromosome 8 has been reported in mice, whereas that in humans, the MT genes have been found on chromosome 16q13 which are encoded by a group of closely related multigene; the presence of seven MT-I functional genes: A, B, C, D, E, F, G, H, and X have been described, which represent microheterogeneity of the MT-I protein as well as a single gene encoding the other isoform of MT-II (MT-IIA), MT-III, and MT-IV [6, 14]. The isoforms of MT-I and MT-II are predominantly expressed in various animal tissue; MT-III and MT-IV are isoforms that are expressed in more specific tissues.

3. Expression and Regulation of MT Genes

Expression of the MT-I and MT-II genes is achieved through two important elements present in the regulatory region of the gene: the TATA box (the core promoter element) and several cis-acting response elements, which are metal-responsive elements (MRE) and antioxidant-responsive elements (ARE). Both elements are mediated by the metal response element binding transcription factor (MTF-1), glucocorticoid responsive elements (GRE), signal transducer, and activator of transcription (STAT); the AREs can be downregulated positively or negatively for transcriptional factors such as Sp1, TPA, USF, Ap1, and Ap2 [9, 15, 16]. The expression of MT gene is significantly regulated by the Zn finger transcription factor MTF-1, which is a metalloregulatory protein, that binds to the proximal MER promoters, through their Zn fingers; the DNA binding activity is reversibly activated in response to changes in free intracellular Zn concentrations (Figure 2) [14, 16]. Similarly, previous studies have reported that linkers between the six different fingers participate actively in the modulation of MTF-1 translocation from the cytoplasm to the nucleus and its binding to the promoter of the MT-I gene; it should be noted that Zn is the only recognized metal that activates MTF-1; however, it has also been seen that this factor can be activated by oxidative stress [11, 16, 17]. It is commonly accepted that the expression of MT-I and MT-II is highly inducible in response to a wide variety of stimuli including metals, hormones, oxidative stress, and inflammatory mediators (such as cytokines); the synthesis of this protein is activated through the action of cis-acting DNA sequences in response to stimuli [9, 12, 14].

4. Physiology of MTs

Several papers have supported the cellular functions of MTs, which mainly include homeostasis and transport of essential metals (Zn and Cu), detoxification of toxic metals, apoptosis, modulation of intracellular redox balance, anti-inflammatory processes, free radical scavenging, and protection of neurons against neuronal lesions [18–20]. As mentioned above, MT was initially described as a protein responsible for the accumulation of cadmium in the kidneys of horses [15]. It is now known that this protein is mainly involved in the detoxification of heavy metals such as cadmium and mercury; these metals bind to the protein by sequestering them and decreasing the acute effects of these toxic metals [21]. The induction in the expression of MT by heavy metals and

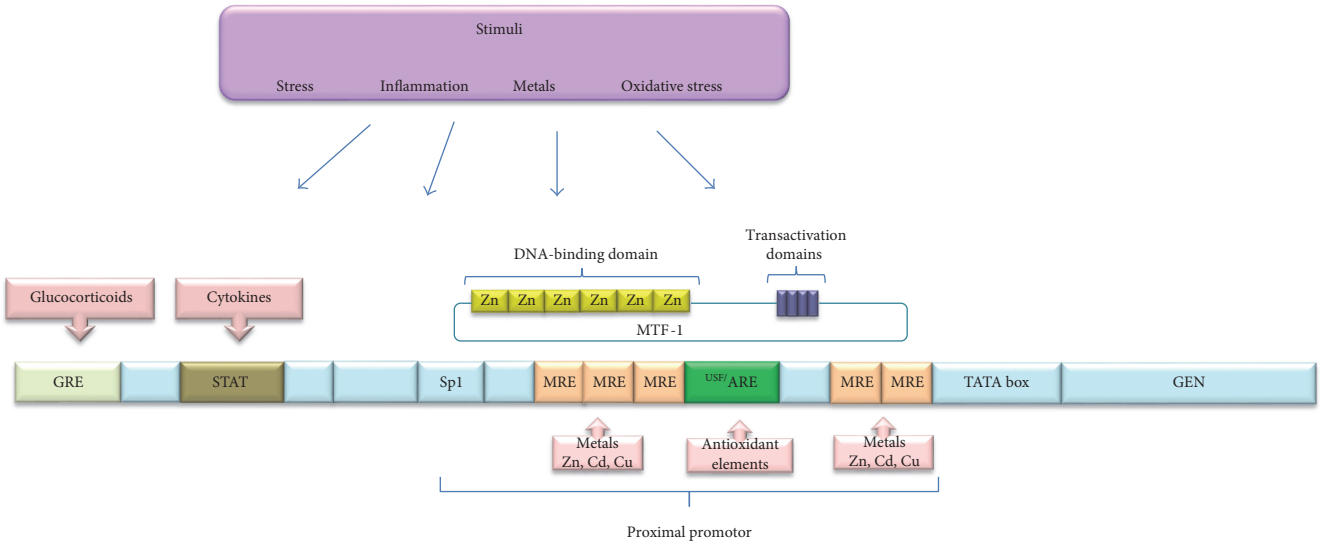

Figure 2: Schematic representation of MT-I and MT–II gen. The cis-acting response elements: glucocorticoids responsive elements (GREs), metal responsive elements (MREs), and antioxidant responsive elements (AREs). Signal transducter and activator of transcription (STAT) Sp1-binding site (Sp1), upstream stimulatory factor (USF), and metalloregulatory transcription factor with functional domains (MTF-1).

subsequent accumulation of metal in the cell has been used as a biomarker in the field of environmental toxicology (Figure 3) [12].

MT has as its main physiological function to maintain the homeostasis and transport of essential metals such as Zn and Cu. Zn is a physiologically important metal that provides structural and catalytic functions to a variety of proteins. The Apo-MT form is a Zn acceptor (which binds with high affinity), increasing intracellular Zn concentrations; when these Zn levels are insufficient to stabilize the protein, the MT is rapidly proteolyzed, so the Zn is released by the degradation of MT, causing the intracellular Zn to remain at a balanced concentration [14]. Traditionally, MT has been considered as an intracellular protein, is localized in cytoplasm, and by translocation can also be found in the nucleus; however, more recent reports suggest that MT can be localized in a variety of extracellular spaces [22].

MT is a vital protein in the cellular defense antioxidant system, and its protective role against ROS damage in biological systems has been widely reported. Different studies have shown that the thiolate ligands in cysteine residues confer the redox activity of MT; these residues can be oxidized by cellular oxidants, and during this process, Zn is released, causing a decrease in lipid peroxidation levels [23]. It is known that, when there is an increase in oxidative stress levels, MT is able to scavenge a variety of ROS including hydroxyl radicals and superoxide anion, hydrogen peroxide, radicals of reactive nitrogen species, and nitric oxide radicals [4, 6, 24, 25]. Compared with other antioxidants such as super oxide dismutase, catalase, and glutathione peroxidase, MT may be considered a more effective antioxidant [26, 27].

Also, there are reports suggesting an anti-inflammatory role of MT, which includes protection of the nervous system [28], in lung lesions, and acute hepatic injury [29]; it is proposed that this effect may be due to activation or inhibition of the expression of pro- and anti-inflammatory interleukins depending on the physiological conditions present [27, 30].

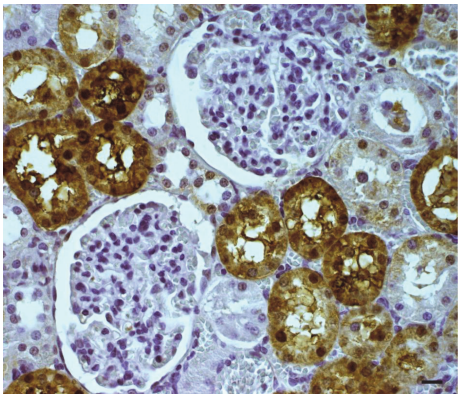

Figure 3: Representative images of immunohistochemical study of the presence of MT in rat kidney. The MT immunoreactivity (brown color) in the cells of the proximal and distal convoluted tubules in kidney. The image shows a glomerular corpuscle with the presence of Bowman's capsule surrounded by numerous contoured tubules. 1000x magnification.

5. MT in the Central Nervous System

The brain is highly susceptible to oxidative stress due to the high levels of oxygen consumption, so that the role of MT as a protein that protects against these processes is very important; as mentioned above, maintaining intracellular Zn homeostasis is one of the main functions of this protein and the brain contains high levels of this metal hence showing the importance of MT in the central nervous system.

MT-I and MT-II are regulated in a coordinated manner; their biosynthesis can be induced by a diverse variety of stimuli such as stress conditions, glucocorticoid levels, cytokines, ROS, and metal ions, which have been reported to occur through various regions of the central nervous system (the cortex, brainstem, spinal cord, thalamus, hippocampus, basal forebrain, neocortex, cranial nerve nuclei, olfactory bulb, and cerebellum) [22, 31]. MTs are proteins predominantly expressed in the cellular cytoplasm, mitochondria and lysosomes, within astrocytes, cells, meningeal cells, ependymal cells, choroid, arachnoid, and pia mater [32, 33]. It has recently been reported that MT-I and MT-II are also distributed intra- and extracellularly, so neurons can incorporate these proteins into their cytoplasm through a family of low-density lipoprotein receptors (megalin) [34–36]. Although the cells of microglia, oligodendrocytes, and neurons commonly do not show MT-I or MT-II expression, it has been observed that in response to brain damage, the protein can be expressed [4, 30].

MT-III was isolated and reported for the first time in 1991 by Uchida et al. [37] in patients with Alzheimer's disease and was identified as a growth-inhibitory factor (GIF) or neuronal growth-inhibitory factor. Subsequent *in vitro* and *in vivo* studies revealed that MT-III isolated from human and bovine brains is predominantly expressed in neurons and contains 4 divalent metal ions of Cu and 3 or 4 divalent Zn metals, differing from the divalent Zn metals which are normally found in the MT-I and MT-II [12]. Another difference with respect to MT-I and MT-II is that MT-III does not appear to be regulated by metals or glucocorticoids, so it is not considered inducible and its neuroanatomical distribution is well related to areas with high concentrations of Zn [8]. In normal brain, expression of MT-III is more restricted; it has been reported that mRNA is related to neuronal cells, whereas MT-III upregulation can be observed in glial cells after brain damage [38]. Although there are contradictory reports about the expression of MT-III, it is generally accepted that the cellular localization of MT-III is mainly in neurons especially those with high concentrations of Zn, suggesting a specific function; none of the other MT isoforms have a growth-inhibitory activity suggesting that this is a specific functional characteristic of MT-III [30, 39–42].

The neuroinflammation process independently contributes to neuronal dysfunction as well as the process of cell death and is an important factor in the development of neurodegenerative diseases since the increase in the formation of ROS and RNS can generate an increase risk; when brain tissue is damaged due to an acute or chronic injury, there is an increase in the response of neuroinflammatory and oxidative stress causing the activation of the immune system with the presence of reactive astrocytes, cerebral parenchyma, and macrophage and microglia cells [27, 43]. MT regulates the expression of inflammatory factors mainly cytokines IL-6, IL-3, and TNF-α and interferons which are present in astrocytes [44, 45]; IL-6 is one of the important cytokines upregulated during brain damage, as it increases phagocytosis and the release of inflammatory mediators which in turn induce the site of MT as neuroprotection proteins [43, 44].

Oxidative stress, a condition that occurs due to imbalance between the oxidant and antioxidant status, increases with age and can therefore be considered as a major causative factor in neurodegenerative diseases [46]. Several studies have shown sufficient evidence that oxidative damage plays a vital role in the pathophysiology of neurodegenerative diseases (Alzheimer's disease and Parkinson's disease) and other brain disorders such as cerebral ischemia and epilepsy [47–51]. Increased oxidative stress in brain disorders is often associated with antioxidant defense mechanisms, including MT-I and MT-II; the MT is able to reduce oxidative damage by attenuating the damage caused by oxidative stress, suggesting that the functions of this protein family are more complex and varied than were initially considered [52].

6. Alzheimer's Disease

Alzheimer's disease (AD) is the most common cause of dementia, an age-related neurodegenerative disorder, characterized clinically by a progressive deterioration of higher mental functions and cognitive functions, resulting in severe dementia [53]. The neuropathology is characterized by the accumulation of two types of fibrous material in the brain: the Aβ-amyloid extracellular peptide deposited in senile plaques and intraneuronal neurofibrillary tangles (NFT) composed mainly of abnormal and hyperphosphorylated forms of the microtubule-bound tau protein, in addition of the presence of a large number of reactive astrocytes, signs of neuroinflammation, metal dyshomeostasis, and oxidative stress [53–56]. The formation of NTFs and the resulting degeneration first appear in the hippocampus, especially pyramidal neurons in CA1 and layer II of the entorhinal cortex and the neocortex [57]. Several hypotheses have been proposed to explain the pathophysiology of AD, including the amyloid cascade with accumulation of Aβ, the metal ion hypothesis that proposes that the underlying cause of AD is the homeostasis of the deteriorated metal, in particular Zn, Cu, and Fe which leads to amyloid imbalance, oxidative stress leading to mitochondrial dysfunction, metallic ion dyshomeostasis, inflammation, improved apoptosis, and imbalance Aβ [56, 58, 59].

It is commonly accepted that ROS play an important role in the pathogenesis of AD disease with low brain regeneration rate and insufficient antioxidant potential [60–62]; the brain favors oxidative stress, due microglia activation to neuronal lesions which generate excess superoxide radicals [56, 63]. Different reports have suggested that oxidative stress promotes the production of Aβ-amyloid and induces alteration of antioxidant enzymes by increasing levels of oxidative stress generating an increase in Aβ deposition [49, 64, 65]. The Aβ-amyloid peptide present in the astrocytes constituting the senile plaques observed in AD disease is considered a source of free radicals; evidence has shown that Aβ-amyloid enters the mitochondria, and induces the generation of free radicals that promote cross-linking of proteins, through the formation of amyloid-fibril, and Aβ itself can form free radical peptides which can lead to oxidative damage in *in vivo* and *in vitro* neurons [54, 66]. Likewise, experimental studies found that Aβ

affects mitochondrial DNA and proteins, which leads to deficiencies in the electronic transport chain (ETC) and, finally, mitochondrial dysfunction [67], thus contributing to the neurodegeneration.

Reactive astrocytes present in patients with AD show significant high levels of MT expression suggesting that MT levels can serve as a marker of AD [68]. The overinduction of MT-I and MT-II in AD stimulated primarily by the presence of free radicals, metal ions, and cytokines may be a defense cellular response against inflammatory signals supporting a neuroprotective effect of MT [3, 56]. Likewise, experimental studies on transgenic animals also have shown that astrocytes and microglia/macrophages surrounding amyloid plaques have high levels of expression of MT-I and MT-II in regions of the cortex and hippocampus [48, 69]. On the other hand, Valko et al. [47] demonstrated that predominantly upregulated MT-I in reactive astrocytes has a neuroprotective effect induced by dual mechanisms in AD, direct attenuation of neurotoxicity of Aβ and indirectly due to MT-I inhibition of Aβ-induced microglial activation and subsequent neurotoxicity.

Another neuropathological feature of AD is the alteration of the Zn metabolism and the accumulation of the metal inside the amyloid plaques. *In vitro* studies have shown that Zn is capable of inducing amyloid Aβ formation, while Cu and Fe only produce partial aggregation [41, 70]. Deibel et al. [71] reported a significant decrease in Cu and significant increases in Zn and Fe levels in the hippocampus and amygdala which are areas that show severe histopathological alterations in patients with AD; due to its role in the homeostatic maintenance of Zn and Cu, the MT is considered an important factor within the pathophysiological mechanisms of the disease. It has also been reported in *in vitro* studies that MT-2A has been able to prevent aggregations of Aβ40 and Aβ42 which are countermediated by preventing neurotoxicity [31].

Expression of MT-III has been observed in different neurological diseases; however, the molecular mechanisms of MT-III downregulation in neurodegenerative disease are not yet known, and unfortunately there are several reports with conflicting results of MT-III function in AD. MT-III is expressed in abundance in normal but largely reduced in AD brains; in *in vitro* studies, an inhibition in neurite formation and survival of cortical neurons has been reported [37, 72, 73]. MT-III and alterations in Zn homeostasis have been correlated with neuronal loss or duration of disease implying increased susceptibility to oxidative stress and metal-induced neurotoxicity [41]. MT-III can bind more metal ions, its role as Zn buffer and sensor/signaling, and also has a periodic response to neuronal insult, while other isoforms are uniformly protective [56]. In contrast to MT-I, the expression of MT-III mRNA was not significantly altered in any of the studied models of AD, probably because MT-III is insensitive to inflammation [48, 69]. MT-III has anti-Aβ activity and antagonizes the neurotoxic effects of Aβ peptides primarily by abolishing the formation of toxic aggregates of Aβ peptides [74]. Decreased MT-III expression in patients with AD suggests a possible decreased protective effect in the AD brain [73].

7. Parkinson's Disease

Parkinson's disease (PD) is the most common neurodegenerative disease of the elderly and is a neurological syndrome manifested by the combination of tremor, stiffness, bradykinesia, and loss of postural reflexes; other symptoms known as nonmotor symptoms but associated with the disease are the autonomic, sexual, and sleepy disease which is capable of causing cognitive, psychiatric, autonomic, and sensory disturbances [75, 76]. PD occurs predominantly in the 6th decade of life, and men have a 1.5 to 2 times more likely of contracting disease than women [77]. The neuropathology of this disease is characterized by a gradual and selective loss of dopaminergic neurons in all basal ganglia, causing both dopamine and its metabolites to decrease in the caudate nucleus, putamen, *globus pallidus*, and compact pars of the substantia *nigra* [78].

An important neuropathological feature within the disease is the presence of Lewi bodies in the brain tissue that are intraneuronal filamentous inclusions containing phosphorylated neurofilaments called α-synuclein protein [79]. The molecular mechanisms described in PD include damage to striatal neurons by exogenous and endogenous toxin such as free radical, due to deficiencies in mitochondrial function, exitotoxicity, inflammation, and cellular apoptosis, and all these processes eventually result in neurodegeneration. Low levels of antioxidants and high levels of free iron make black matter highly vulnerable to ROS attack, so that oxidative stress has been recognized as an important component of this disease [78]; therefore, it has been proposed that MT may play a role in protecting neurons from oxidative stress damage.

Studies in patients with Parkinson's disease by molecular analysis and immunocytochemical techniques demonstrated an increase in the expression of megalin (neuronal metallothionein receptor) and MT in reactive astrocytes of the substantia *nigra*, supporting the neuroprotective role of MT in these cells [80]. Experimental studies have shown that in advanced Parkinson's disease, dopamine and its metabolites are depleted and damaged dopaminergic neurons can release excess cytosolic dopamine out of the synaptic vesicle, inducing neurotoxicity through the generation of reactive oxygen and dopamine species quinones. In studies performed on hemiparkinsonian models of M-I and MT-II knockout mice, dopamine-doped cells treated with dopamine and L-DOPA injections showed that intrinsic MT protects against neurotoxicity induced by dopamine quinone [81]. On the other hand, studies in 6-hydrodopamine- (6-OHDA-) induced hemiparkinsonian rat models showed that treatment with Levodopa significantly increased MT-III mRNA expression in the *striatum* on the uninjured side, but did not show any significant effect on the injured side with 6-OHDA; these results suggest that regulation in MT-III mRNA expression may be related to progressive degeneration in parkinsonism [82].

Several studies carried out by the Ebadi group have shown in the brain of mice from transgenic and knockout PD models that induction of MT is involved in the synthesis of coenzyme Q10, through the activation of lipoamide

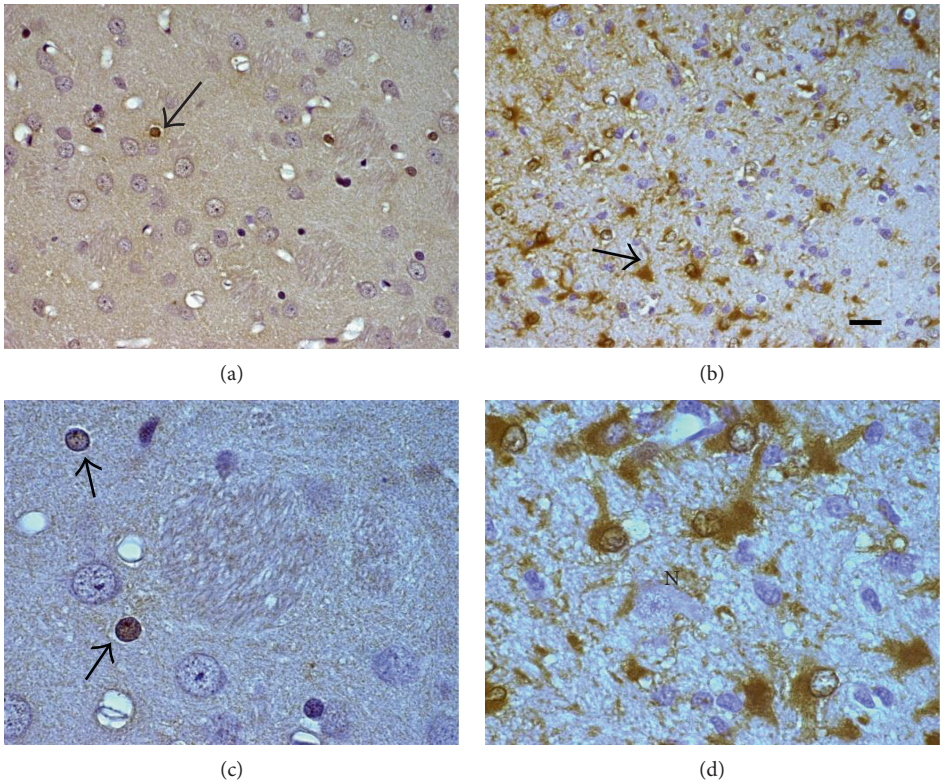

Figure 4: Representative images of immunohistochemical study of MT in the brain (striatum) after MCAO. Control rats (a, c) and MCAO (b, d). (a, c) Neurons, astrocytes, and fibers with normal appearance. (b, d) Interstitial edema, altered neurons (N), and reactive astrocytes are observed after MCAO. Strong immunoreactivity to MT (brown color) was consistently detected in reactive astrocytes (↑) in treated animals. Magnification (a, b) 400x and (c, d) 1000x.

dehydrogenase, enhancing the antioxidant capacity of coenzyme Q10 Ubiquinone, by increasing the ubiquinol synthesis, attenuation of α-synuclein nitration, as well as suppression of proinflammatory cytokines and TNFα [50]. Similarly, *in vivo* and *in vitro* studies examined the peroxynitrite ion generator (ONOO-) 3-morpholinosidnonimine (SIN-1) and observed that the lipid peroxidation induced by SIN-1, reactive oxygen species synthesis, caspase-3, and apoptosis were attenuated by overexpression of the MT gene and increased by MT gene regulation [83]. Coenzyme Q participates in the generation of a proton gradient through the membranes to lead to the formation of ATP; their identification as an important production of antioxidants through cell membranes as well as participation in other redox membrane systems introduces new functions [51], and through these results, the authors have been able to establish dopaminergic neuroprotective mechanisms of MT in PD [83] as well as their new role as a possible biomarker to confirm disease [20].

8. Cerebral Ischemia

Ischemic stroke is the second leading cause of death worldwide, and the absolute number of stroke patients continues to rise despite a decline in standardized rates of age-related mortality from stroke in the past two decades [84]. Focal cerebral ischemia has been shown to be a passive, acute event, in which energy failure leads to damage in the cerebral tissue causing necrosis and cell death (Figure 4); studies in experimental animals have shown that subsequent events involve expression of genes (including MT) which are related to inflammatory processes and apoptosis and contribute significantly to cell death after the ischemic event [85]. An important factor for cell death after cerebral ischemia is the increase of free intracellular Zn; when ischemic damage produces this accumulation of Zn, it is likely that MT is saturated, and together with the production of hydrogen peroxide, they are able to induce the expression of the MT gene by activating the transcription factor-1 and other promoters of the gene [86]. Transient focal ischemia of 1-2 hours in duration induces an increase in the transcription of certain genes that serve as a protection system against cell damage; the increase gene expression is found in the infarct border area while in the central zones of the cerebral infarction, the processes of transcription and translation of genes are suppressed so that cells die rapidly [87]. Experimental evidence also has reported that after mild cerebral ischemia, there is a rapid induction of MT-I in endothelial cells of cerebral capillaries and astrocytes, showing that it is one of the first detoxification genes in response to damage in brain tissue [88, 89]. Studies in mice with serial gene expression analysis (SAGE) showed that MT-II mRNA was identified as the most significant induced transcript in the early phase of ischemic stroke in the ischemic hemisphere, demonstrating that the maximal

concentration of MT occurs at 12 and 24 hours after ischemic damage, expressed almost exclusively in astrocytes of the periphery of the infarcted zone, in the hippocampus, corpus callosum, and striatum regions indicating that the MT gene is overregulated during cerebral ischemia [90].

Although the role of MT-III in the ischemic event remains unknown, some studies have reported an increase in MT-III concentration supporting its neuroprotective role in cerebral ischemia. MT-III mRNA levels were found upregulated in brains after ischemic stress; immunohistochemical analysis showed an increase in immunoreaction in the neurons of the CA1–3 regions of the hippocampus, dentate gyrus, cerebral cortex, olfactory bulb, and Purkinje cells in the cerebellum distributed in the same regions of a normal brain [91]. In knockout mice M-III and wild-type, no significant differences were found after to the 24 h permanent middle cerebral artery occlusion (MCAO). MCAO-induced cerebral infarction in MT-III KO mice was aggravated in comparison to wild-type mice; neurological deficits were found at 5 and 7 days after the cerebral ischemia model. TUNEL tests and markers of oxidative stress were performed on brain tissue and were observed to improve at 24 h after transient MCAO, suggesting that MT-III also has a neuroprotective and antioxidative role in transient cerebral ischemia [92].

The MT-I and MT-II are induced by the inflammatory response generated during cerebral ischemia, mediated by microglial cells, macrophages, and astrocytes with reactive gliosis, which activate proinflammatory mediators that form ROS and oxidative stress that result in the induction of neuronal damage and death [93]. One of the causes of overexpression of MT in ischemia is the association that exists between the factors related to the inflammation process and the response elements which, as mentioned above, are capable of inducing MT expression. It has been reported in *in vitro* and *in vivo* studies that MT promotes the regeneration of neuronal axons as a response to brain damage and that extracellular MT plays an important role in this process and was proposed to the extracellular MT-II as a mediator of its effects on neuronal cells, since through membrane receptors of the low-density lipoprotein family, mostly lipoprotein 1 receptors (megalin). It transfers MT-II from the extracellular medium to the neuronal cytoplasm; this is an important advance in the knowledge of the functions of MT, since extracellular functions of MT do not exclude intracellular functions in damage brains [35, 94, 95].

Diaz-Ruiz et al. [84] reported a protective effect of the application of exogenous MT in rats exposed to events of transient cerebral ischemia and reperfusion; the animals received exogenous MT, and a decrease in lipid peroxidation levels in hippocampus and frontal cortex brain regions as well as a decrease in the area of damaged tissue in the hippocampus after cerebral ischemia was found. According to these results, we observed by immunohistochemical studies in reactive astrocytes a marked increase in the MT immunoreactivity in the peripheral ischemic damage area; the histopathological aspects of the reactive astrocytes and their high immunoreactivity provide evidence of an overexpression of MT in the area of ischemic damage (Figure 4).

On the other hand, several authors have used *in vitro* and *in vivo* models of middle cerebral artery occlusion (MCAO) to analyze the effect of the exogenous application of MT. The results show that there is no protection at 60 min in the MCAO model; however, there was a significant reduction in the volumes of direct and indirect infarction and neurological deficit in the MT-II-treated animal. In addition, MT-II also improved the survival of mice after MCAO, probably due to the suppression of induction of TNF-mRNA in ischemic brain tissue, protecting primary neuronal cells against oxygen-glucose deprivation *in vitro*, supporting the potential therapeutic of exogenous MT administration in experimental stroke models [96, 97].

9. Epilepsy

Epilepsy is the most common neurodegenerative disease after stroke, and, according to epidemiological studies, approximately 70–80% of epilepsy patients achieve remission and approximately 30% of these patients are resistant to pharmacological treatment [98]. Epilepsy is characterized by a large number of syndromes that vary between its clinical characteristics, treatment, and prognosis; over time, the various classifications of the disease grouping the seizures (symptoms) and the syndromes of epilepsy have been improving. Epilepsy is a neurological disorder in which it has been suggested that oxidative and nitrosative stress are contributing factors due to prolonged neuronal hyperexcitation and loss of neurons during seizures causing cellular dysfunction. Several studies conducted in humans and experimental animals have demonstrated the relationship between epilepsy and oxidative stress; the generation of free radicals can lead to prolonged seizures that can lead to mitochondrial dysfunction in the hippocampus that precedes neuronal cell death causing subsequent epileptogenesis, although it is not yet known whether oxidative stress is a cause or consequence of this pathology [99, 100].

Dalton et al. [17] were the first to determine that rats treated with kainic acid (KA) as a model of epilepsy showed severe brain damage partly caused by oxidative stress. They found that KA induced an increase in mRNA of MT-I and heme oxygenase-I, as well as the induction of interleukin-1β gene expression, triggering an inflammatory response in damaged brain regions, suggesting that oxidative stress produced by KA is able to induce seizures. Since then, experimental models of epilepsy have proven to be useful tools and complementary strategies to advance our understanding of this disease and the molecular mechanisms associated with the role of MT. In the KA model, excessive activation of excitatory glutamate receptors results in sustained epileptic activity; pyramidal neurons of the hippocampus are particularly vulnerable to the neuroexcitatory actions of KA, showing that the vulnerability of neurons to oxidative stress varies from one brain region to another [100, 101].

We have previously reported immunohistochemical studies in animals exposed to KA, and an increase in the immunoreactivity of MT in the cytoplasm of astrocytes CA1, CA3, and dentate gyrus of the hippocampus was observed (Figure 5), as well as a significant increase in

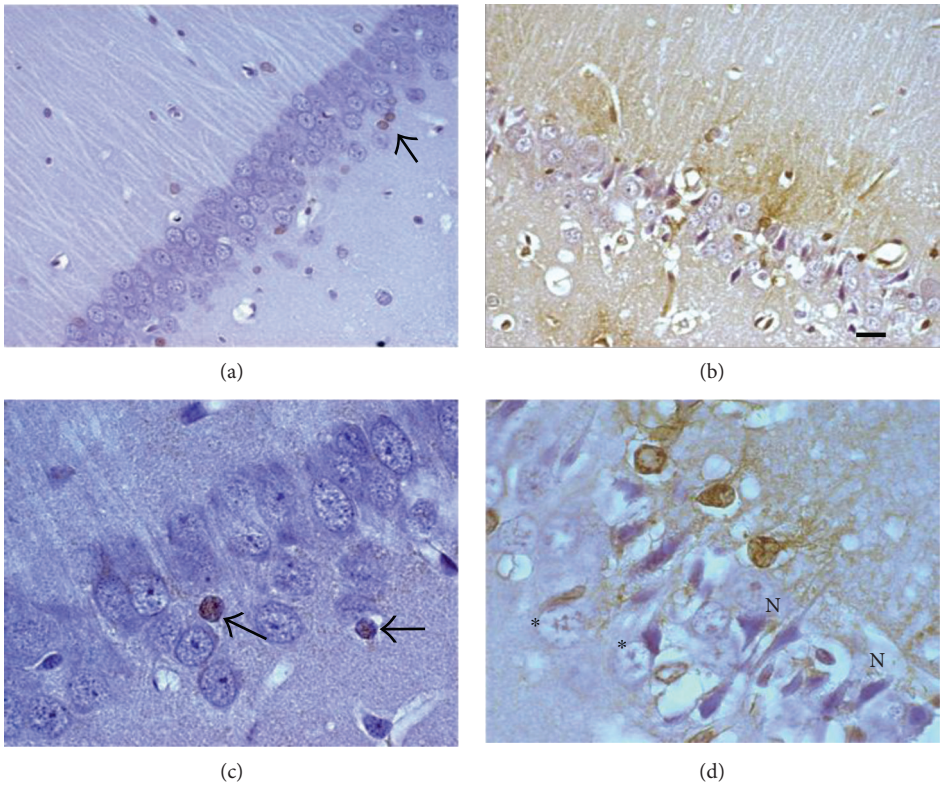

Figure 5: Representative images of the immunohistochemical study of MT in the hippocampus of rats treated with KA. Control rats (a, c) and rats treated with KA (b, d). (a, c) A normal structure of pyramidal cells and astrocytes (↑) in untreated animals. (b, d) Neurons with pyrolysis (∗), pyknotic (N), and neuropil neurons are observed with the presence of interstitial edema in the hippocampus of experimental rats. In the astrocytes (↑) of the rats treated with KA, an increase of the immunoreactivity of the MT (brown color) can be observed. Magnification (a, b) 400x and (c, d) 1000x.

expression of MT-I and MT-II between 6 and 24 h after KA injection; these results contributed by our group strongly support the evidence that MT plays a major role as an early expression protein in epileptic seizure models [101]. Similar results were observed by Kim et al. [102]. They reported an increase in MT-I and MT-II mRNA expression at 2 and 6 h in the rat brain after treatment with KA, and MT-I appeared to be increased in neuron more evidently in glia, whereas the opposite profile was observed in the case of MT-II; although both MT-I and MT-II expressions showed similar sequential changes (first in neurons and then in glia) after treatment with KA, there is a substantial difference in expression between these two genes. Several studies have clearly demonstrated that high levels of MT-I and MT-II are associated with reduced neuronal death following an induced seizure attack during excitotoxicity. MT-I and MT-II knockout mice show increased levels of oxidative stress, neuronal death, and epileptic seizures after being treated with KA [103]. Likewise, studies carried out on transgenic mice with MT-I overexpression showed reduction of hippocampal inflammation and delayed neuronal degeneration 3 days after exposure to KA; a significant reduction in the proinflammatory response and a delay in the presence of cellular damage were also observed [104]. Other studies with neuronal MT-III have suggested that in special circumstances, the MTs could contribute to the neuronal death; administration of KA in the ventricles also caused a positive regulation of MT-III in reactive astrocytes around the degenerate neurons of the CA3 region of Ammon's horn 3 days after injury, acting as an acute phase protein in reactive astrocytes [105]. Contradictory results have been reported in wild-type mice treated with KA; it was observed that MT-III protects against seizures induced by KA and decreasing neuronal damage likely due to protection against oxidative stress associated with neuronal excitation [106].

On the other hand, similar results have been reported in patients with MTLE. Peixoto-Santos et al. [107] reported that both MTLE and TLE patients increase MT-I and MT-II expressions in all subfields of the hippocampus and in the *dentata fascia* associated with increased astrogliosis; MT-I and MT-II expressions correlate with the astroglial population but not with neuronal population. According to these results, our group reported through a histopathological study that a marked expression of MT-I and MT-II was present in the cytoplasm and neuropile in brain regions of patients with ETL, providing more evidence about the role that MT plays in epilepsy [108].

10. Conclusion

This article provides evidence and an overview of the role of MT in normal and pathological conditions in the CNS.

Recent research has provided information about the physiological function of MT-I and MT-II in processes of oxidative stress, apoptosis, inflammation, detoxification, and homeostasis of essential metals which are widely related in the pathophysiology of neurodegenerative diseases and other brain disorders. MTs are very important proteins in brain functioning mainly located in astrocytes which can protect neurons against exitotoxicity induced by experimental models of epileptic seizures or in damaged brain tissue as a result of focal cerebral ischemia through modulation of events such as inflammation, oxidative stress, and apoptosis. On the other hand, MT-III found mainly in neurons seems to be involved in the control of amyloid plaque aggregation proteins or α-synuclein and neurofilaments, which may lead to the development of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. In conclusion, each of the MT isoforms present in the brain may function differently, indicating a multifunctional role over a wide range of cellular processes. However, it is necessary to carry out future studies on the expression and regulation of MT genes to conclusively elucidate and detail the molecular mechanisms involving MT in the nervous system and what their role is in the pathophysiology of brain disorders.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

Cardiovascular Protective Effects of Salvianic Acid A on *db*/*db* Mice with Elevated Homocysteine Level

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The onsets of left ventricular hypertrophy (LVH) and endothelial dysfunction (ED) in diabetics, especially in those with elevated homocysteine (Hcy), precede the development of cardiovascular (CV) events. Salvianic acid A (SAA) is a renowned Traditional Chinese Medicine (TCM) that has been applied in the treatment of cardiovascular disease for many decades. In this study, we aimed (1) to investigate the CV protective effects of SAA on ameliorating LVH and ED in *db*/*db* mice with elevated blood Hcy level and (2) to decipher whether the observed CV protective effects of SAA are associated with Hcy metabolism by modulating the methylation potential and redox status in the liver of the *db*/*db* mice with elevated blood Hcy level. Our results found that the administration of SAA could significantly slow down the build-up of left ventricular mass and ameliorate ED. Immunological assay analysis on the mouse liver tissue also indicated that SAA treatment on *db*/*db* mice with elevated Hcy was associated with reduced methylation potential but improved redox status. In conclusion, we revealed that SAA has the potential to protect against the hyperglycemia- and hyperhomocysteinemia-induced oxidative stress on diabetic mice via modulation in Hcy metabolism.

1. Introduction

In 2013, 382 million people in the world were living with diabetes mellitus (DM), and the figure is expected to rise drastically to about 600 million by 2035 [1]. There is a growing evidence suggesting that DM is coupled with increased oxidative stress and altered redox state and leads to subsequent onsets of cardiomyopathy [2, 3] and endothelial dysfunction (ED) [4, 5]. Remarkably, diabetic patients are exposed to a higher risk to develop hyperhomocysteinemia (HHcy) when compared to nondiabetic subjects, particularly in those taking metformin antidiabetic medication [6, 7]. According to a previous cohort study in 122 type 2 DM patients, the prevalence of HHcy in type 2 diabetes was 31% [8]. Recent evidences have shown that both long-term and short-term metformin treatments could lead to elevation of Hcy in type 2 DM patients due to reduction of serum folate and Vitamin B12 [9–11]. Today, both hyperglycemia and HHcy status have been recognized as strong cardiovascular disease (CVD) risk factors or predictors [12, 13], and HHcy status was strongly associated with the development of diabetes and diabetic CV complications [11, 14, 15].

Diabetic cardiomyopathy is one of the common diabetic heart complications characterized by left ventricular hypertrophy (LVH), fibrotic changes in the myocardium, and thickening of intramural arterioles in the absence of hypertension and atherosclerosis [16]. Patients with diabetes are well known to have increased risk in the development of LVH owing to the clustering of several CV risk factors, including hypertension, hypercholesterolemia, HHcy, and endothelial dysfunction (ED). It has been reported that HHcy triggers oxidative stress in the heart and leads to cardiac hypertrophy and the subsequent cardiac diastolic

function disorder via diminished activity of peroxisome proliferator-activated receptor [17, 18]. Apart from the adverse effects on the heart, extensive clinical and review studies have reported that HHcy status could also impair endothelium-dependent vasodilation in diabetics [19–22]. Interestingly, several previous studies have shown that a short-term Hcy-lowering therapy could improve ED in hypertensive patients [23], and a lower methylation potential (S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) ratio) played a significant role in CVD [24]. These findings therefore suggest that Hcy metabolism might be involved and could serve as a potential treatment pathway to reduce CVD burden in diabetes.

Methylation potential (S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) ratio) and redox status (glutathione (GSH) to glutathione disulfide (GSSG) ratio) are closely linked to Hcy metabolism and are responsible for the clearance of elevated Hcy via the remethylation and transsulfuration pathways. Remethylation converts methionine via SAM and SAH into Hcy in a reversible cycle. Meanwhile, Hcy could also irreversibly transform to GSH via the transsulfuration pathway. As an important intracellular antioxidant, GSH plays a significant role in the redox balance and cell apoptosis [25–28]. GSH reduction was reported as a crucial cause of cardiac cell apoptotic death in diabetic rats, and supplement of GSH could prevent the diabetic rats from heart disease effectively by reversing cell apoptosis and mitochondrial oxidative stress [29]. Generally, SAH level is usually high in subjects with elevated Hcy level because the process of remethylation is a reversible process that catalyzed by SAM and is always at equilibrium due to the high activity of SAH [30]. Also, GSSG is the oxidized form of GSH, and the ratio between GSH and GSSG is fundamental for the maintenance of redox status balance [31, 32].

Danshen is a typical Traditional Chinese Medicine (TCM). It is the dried root of *Salvia miltiorrhizae* that has been used in the treatment of CVD for a long period of time [33]. The earliest use of it was first recorded in the books "Shen Nong's Herbal Classic" (First century B. C. - First century A.D. Eastern Han dynasty). According to the TCM theory, Danshen can "remove blood stasis." Salvianic acid A (SAA), or *Danshensu* with the chemical name of 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid (Figure 1), is one of the major active ingredients in the water-soluble components that extracted from Danshen. It has been proven to possess diverse pharmacological effects such as improving blood circulation, inhibiting platelet adhesion and aggregation, acting against oxidative stress by suppressing the production of reactive oxygen species (ROS), preventing the onset of myocardial ischemia, and ameliorating endothelial cell dysfunction [34–38]. Today, the effects of SAA on ameliorating the progression of LVH and ED are poorly studied, particularly in diabetes with elevated Hcy level. A previous similar study reported that SAA treatment could markedly attenuate HHcy in nondiabetic rats through enhanced production of GSH via activated transsulfuration pathway [39], but whether SAA could exert similar effects on attenuating elevated Hcy level and CVD burdens in a diabetic animal with HHcy is still not clearly understood. Therefore, in the present study, we proposed (1) to investigate the CV protective effects of SAA on ameliorating LVH and ED in *db*/*db* mice with elevated blood Hcy level and (2) to decipher whether the observed CV protective effects of SAA are associated with the modulation of methylation potential and redox status in the liver of the *db*/*db* mice with elevated blood Hcy level.

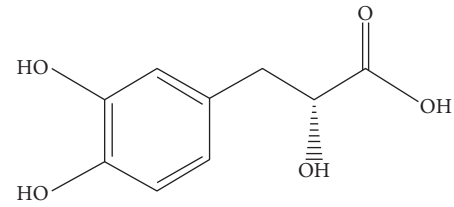

Figure 1: The chemical structure of SAA.

2. Materials and Methods

2.1. Animals and Intervention. Female diabetic homozygous *db*/*db* mice and nondiabetic heterozygous *db*/*m* mice of 10 wks old were housed in the Central Animal Facilities of the Hong Kong Polytechnic University, in a 12 h light/darkness cycle, at a temperature of 20–25°C and humidity of 50–60%. They were allowed regular laboratory chow and tap water ad libitum throughout the experimental period. All the *db*/*db* mice were then divided into four groups ($n = 6$–9): (G1) DM (control); (G2) DM (SAA); (G3) DM (methionine); and (G4) DM (methionine + SAA). Nondiabetic *db*/*m* mice ($n = 10$) were grouped as the non-DM group (G5). During the intervention period of 8 wks, all mice were treated according to the following schedule: G1 and G5 received no treatment; G2 received SAA (purity ≥ 98%, Nanjing Zelang Pharmaceutical Technology Co. Ltd., Nanjing, China) at dose of 60 mg·kg^{-1}·day^{-1} by gastric intubation; G3 received methionine (purity ≥ 98%, Sigma-Aldrich, St. Louis, Missouri, USA) that was dissolved in tap water as 1% (*w*/*v*), and the water was refreshed every 3 days; and G4 received both SAA (60 mg·kg^{-1}·day^{-1} by gastric intubation) and methionine in water (1% (*w*/*v*)). Experimental protocols were performed in accordance with the approved license granted under the Department of Health and approved by the Animal Subjects Ethics Sub-Committee (ASESC) of Hong Kong Polytechnic University.

Fasting blood glucose, echocardiographic, and biochemical parameters were assessed at baseline and at the end of the intervention period. After an overnight fasting for 10 hrs, blood samples were collected from the tail vein of mice at baseline and after intervention for the determination of fasting blood glucose level using a test strip and a glucose meter (Bayer Contour TS, Bayer Inc., Leverkusen, Germany). Then, at the end of an 8 wk intervention, all mice were euthanized. Whole blood was collected by heart puncture and centrifuged at 13,000 rpm in 4°C for 15 min. The serum was then moved to new tubes, snap frozen by liquid nitrogen, and stored under −80°C for later analysis. The liver

tissues of the mice were rapidly excised, weighted and washed with phosphate buffered saline, then maintained in centrifuge tubes and snap frozen in liquid nitrogen and stored under −80°C for later analysis. Finally, dissected thoracic aorta tissues were collected carefully and isolated from the surrounding connective tissues and fat and were then cut into aorta rings with a length of around 2 mm for later analysis using myography. This procedure was performed under a dissection microscope with the dissected aorta immersed in Krebs' buffer.

2.2. Transthoracic Echocardiography Assessment. At baseline and the end of intervention, all mice were arranged to undergo transthoracic echocardiographic assessment using an ultrasound system (model: HD11 XE, Philips Medical Systems, Bothell, WA, USA) with a high-resolution broadband compact linear array transducer (model: L15-7io, Philips Medical Systems, Bothell, WA, USA) according to the protocol of our previous work [40]. Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg)/xylazine (10 mg/kg) mixture and rested on a heating pad to maintain their body temperature during the whole scanning procedure. Then, the hair on their ventral thorax was carefully shaved. During echocardiography, the mice were positioned in prone decubitus position, and the cardiac structure in parasternal short-axis view at the papillary level was determined using B-mode. Then, the left ventricular (LV) interventricular septal thicknesses (IVS), LV internal dimensions (LVID), and posterior wall thicknesses (PW) at the diastole and systole were carefully measured using M-mode at the level of the papillary muscles (Figure 2). Finally, LV ejection fraction (EF), LV fractional shortening (FS), and LV mass were calculated using the following [41]:

$$\begin{aligned} \mathrm{EF}\% &= 100 * \left[\frac{\mathrm{LVIDd}^3 - \mathrm{LVIDs}^3}{\mathrm{LVIDd}^3}\right], \\ \mathrm{FS}\% &= 100 * \left[\frac{\mathrm{LVIDd} - \mathrm{LVIDs}}{\mathrm{LVIDd}}\right], \\ \mathrm{LV\ mass} &= 1.05 * \left[(\mathrm{IVSd} + \mathrm{LVIDd} + \mathrm{PWd})^3 - \mathrm{LVIDd}^3\right]. \end{aligned} \tag{1}$$

2.3. Endothelial Function Assessment. Immediately after scarification, the mouse aortas were extracted, cut into pieces as aortic rings, and mounted in a Wire Myograph System (610M, Danish Myo Technology, Aarhus, Denmark) using two parallel L-shaped metal prongs that connected with a force transducer. The change in isometric tension of the dissected aortic ring was monitored and recorded by a real-time force acquisition programme, LabChart Pro software (AD Instruments, Sydney, Australia). During the whole procedure, the prepared aortic rings were bathed with 5 mL Krebs' buffer at a temperature of 37°C and with a continuous supply of 95% O_2 and 5% CO_2 mixture. After 1 hr of equilibrium, the prepared aortic rings were challenged with 60 mM potassium chloride to confirm its bioactivity. Then, the aorta rings were washed again and challenged by phenylephrine (10^{-6} M) for precontraction

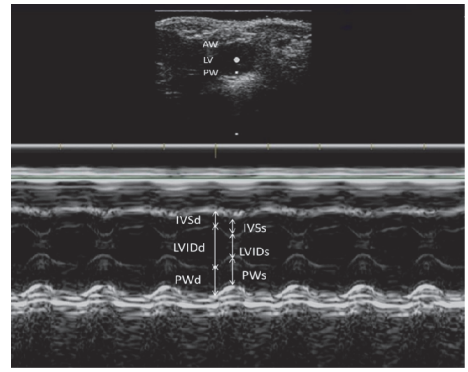

Figure 2: A sample echocardiograph showing the measurement of different parameters of the left ventricle of mice. Upper part of the image showing the cardiac structure of a mouse in parasternal short-axis view at the papillary level in B-mode ultrasound; lower part of the image showing the corresponding left ventricular (LV) and interventricular septal thicknesses (IVS) and LV internal dimensions (LVID) and posterior wall thicknesses (PW) at the diastole and systole of the mice using M-mode ultrasound in a given time period. AW: anterior wall; LV: left ventricular; PW: posterior wall; LVIDd: left ventricular internal dimensions (diastole); LVIDs: left ventricular internal dimensions (systole); PWd: posterior wall thicknesses (diastole); PWs: posterior wall thicknesses (systole); IVSd: interventricular septal thicknesses (diastole); IVSs: interventricular septal thicknesses (systole).

until stabilized. Acetylcholine (Ach) at an accumulative concentration from 10^{-9} M to 10^{-6} M was then added to determine endothelium-dependent dilation, and the whole process was then repeated by adding sodium nitroprusside (SNP) at accumulative concentration from 10^{-10} M to 10^{-5} M to determine endothelium-independent dilation. Cumulative concentration-response curves (from 1/10 nM to 100 mM) were constructed, and the vasorelaxation capacity in response to Ach or SNP was expressed as % at different contractions, where 100% relaxation was considered when the active tone had returned to the baseline level. Krebs' buffer, potassium chloride, acetylcholine, phenylephrine, and sodium nitroprusside were all obtained from the Sigma Chemical Company (Poole, Dorset, U.K.).

2.4. Biochemical Measurement. Serum Hcy level was determined according to a previous study with minor modification [42]. In brief, 10 μL of serum sample was injected into a high-performance liquid chromatography system which consisted of a Waters 2695 Separation Module (Waters Corporation, Milford, MA, USA), a Waters 474 Detector (Waters Corporation, Milford, MA, USA), and an analytical column (Agilent Zorbax Eclipse XDB-C18, 5 μm, 4.6 × 150 mm, Agilent Technologies, Santa Clara, CA, USA) with a guard column (Waters Symmetry C18, 5 μm, 3.9 × 20 mm, Waters Corporation, Milford, MA, USA). Mobile phase A was set at 50 mM KH_2PO_4 (pH = 5.0), and mobile phase B was set at 50 mM KH_2PO_4-methanol 1 : 1(v/v) (pH = 5.0). The flow rate in the column was set at 1.2 mL/min for 10 min. Finally, the concentration of Hcy was determined by measuring the

Table 1: Fasting blood glucose level and body weight at the beginning and end of the experiment.

	DM (control)	DM (SAA)	DM (methionine)	DM (methionine + SAA)	Non-DM
Baseline					
Fasting blood glucose (mmo/L)	27.73 ± 2.08*	22.72 ± 1.50*	27.72 ± 2.14*	23.99 ± 1.71*	5.94 ± 0.50
Body weight (g)	42.95 ± 2.33*	44.63 ± 1.22*	44.76 ± 1.43*	48.42 ± 1.39*	22.14 ± 0.58
After 8 wks					
Fasting blood glucose (mmo/L)	29.18 ± 1.67*	27.08 ± 1.23*	25.77 ± 0.75*	25.64 ± 1.65*	5.43 ± 0.45
Body weight (g)	49.56 ± 4.83*	56.82 ± 2.68*	49.06 ± 3.16*	53.12 ± 2.78*	25.36 ± 0.53

*$P < 0.001$ when compared to the non-DM group.

fluorescence signal captured at an excitation wavelength of 385 nm and an emission wavelength of 515 nm.

SAM and SAH levels in liver tissues were determined following the instruction of commercial ELISA kits (item numbers IK00202S and IK00302S, Arthus Biosystems, LLC, Richmond, CA, USA). The SAM/SAH ratio was calculated to denote methylation potential. Glutathione (GSH) and glutathione disulfide (GSSG) levels in the liver tissue were determined using GSH assay kit (item number 703002, Cayman Chemical, Ann Arbor, Michigan, USA). The GSH/GSSG ratio was calculated to denote redox status.

2.5. Statistical Analysis. All data were analyzed using IBM SPSS Statistics (version 21.0, IBM SPSS Inc., Chicago, IL, USA) and represented as mean ± SEM. Comparison among the multiple groups was conducted by one-way ANOVA followed by post hoc Bonferroni test, while the mean differences between baseline and post measurements were analyzed by paired *t*-test. A *P* value of less than 0.05 was considered significant. All figures were generated using GraphPad Prism software (version 5.01, GraphPad software Inc., San Diego, CA, USA).

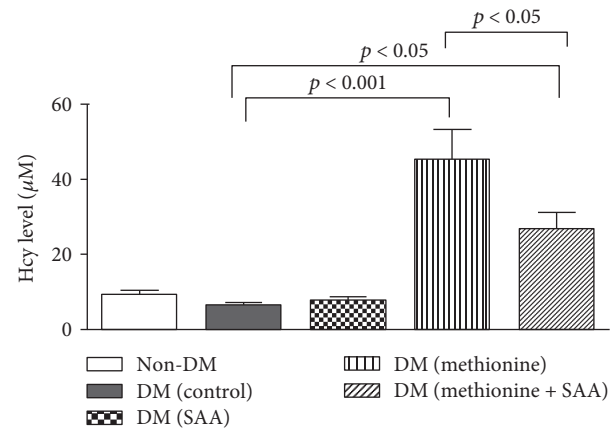

Figure 3: Serum homocysteine (Hcy) level at the end of the experiment. Both DM (methionine) and DM (methionine + SAA) groups exhibit higher Hcy level when compared to the DM (control) group. Besides, the Hcy level in the DM (methionine + SAA) group was significantly reduced to a level lower than that in the DM (methionine) group, probably due to the Hcy-lowering effect induced by the SAA treatment for 8 wks.

3. Results

3.1. Fasting Blood Glucose, Body Weight, and Serum Hcy Level. All the *db*/*db* mice exhibited significantly higher fasting blood glucose level and were more obese than the *db*/*m* mice both at the beginning and at the end of the intervention. However, the fasting blood glucose level in each group was similar before and after 8 wks of intervention period (Table 1). Elevated Hcy levels were successfully induced by using 1% *w*/*v* of methionine in water for 8 wks in the *db*/*db* mice of the DM (methionine) and DM (methionine + SAA) groups and were evidenced by a 6-fold of increment when compared to the other groups without taking methionine in water (Figure 3). Specifically, consumption of methionine in water significantly elevated the serum Hcy levels in both DM (methionine) group (45.39 ± 7.95 μM) and DM (methionine + SAA) group (26.89 ± 4.17 μM) when compared to the DM (control) group (6.70 ± 0.47 μM) that is taking normal tap water. Besides, the expected elevation of Hcy level after 8 wk of SAA cotreatment in the DM (methionine + SAA) group was significantly reduced when compared to the DM (methionine) group (Figure 3), implicating that DSS has a Hcy-lowering effect on this group. Interestingly, the observed Hcy-lowering effect of SAA was not found in the DM (SAA) group, suggesting that SAA might selectively exert its Hcy-lowering effect on diabetic mice with elevated Hcy level only and have little or no effect on those *db*/*db* mice with normal Hcy level.

3.2. Echocardiographic Assessment. We found that the two diabetic groups without taking SAA treatment (DM (control) and DM (methionine)) exhibited increased PWd (58.5%, $P < 0.01$; 58.2%, $P < 0.05$), PWs (31.6%, $P < 0.05$; 32.5%, $P < 0.05$), LVSd (38.1%, $P < 0.05$; 25.4%, $P < 0.001$), and LV mass (49.0%, $P < 0.001$; 31.7%, $P < 0.01$) at the end of intervention period when compared to their baseline values (Table 2). On the contrary, the LV mass in the two SAA-treated diabetic groups (DM (SAA) and DM (methionine + SAA)) was significantly lower than that in the DM (control) group, suggesting a delay in the progression of LVH in the *db*/*db* mice treated with SAA for 8 wks (Figure 4). Nevertheless, the cardiac function as represented by EF and DS before and after 8 wks was similar in all groups, implicating that SAA might have limited impacts on improving cardiac function, or the intervention period of 8 wks was

Table 2: Summary of echocardiography parameters.

Echocardiography parameters	DM (control)		DM (SAA)		DM (methionine)		DM (methionine + SAA)		Non-DM	
	Baseline	Post	Baseline	Post	Baseline	Post	Baseline	Post	Baseline	Post
HR (bpm)	265.78 ± 14.22	249.93 ± 14.23	257.65 ± 12.47	263.32 ± 5.81	263.77 ± 14.00	241.28 ± 7.96	261.90 ± 9.85	238.63 ± 4.75	242.75 ± 6.53	242.26 ± 5.32
Morphological changes										
LVIDd (mm)	3.79 ± 0.12	3.54 ± 0.20	3.96 ± 0.12	4.04 ± 0.11	3.80 ± 0.07	3.45 ± 0.22	3.90 ± 0.06	3.75 ± 0.10	3.70 ± 0.07	3.46 ± 0.78*
LVIDs (mm)	2.08 ± 0.05	2.13 ± 0.16	2.34 ± 0.11	2.25 ± 0.11	2.03 ± 0.08	1.97 ± 0.22	2.15 ± 0.09	2.01 ± 0.10	2.16 ± 0.09	2.07 ± 0.09
PWd (mm)	0.53 ± 0.10	0.84 ± 0.09**	0.49 ± 0.01	0.53 ± 0.02	0.55 ± 0.04	0.87 ± 0.10*	0.56 ± 0.02	0.67 ± 0.05	0.57 ± 0.03	0.60 ± 0.02
PWs (mm)	0.79 ± 0.04	1.04 ± 0.08*	0.80 ± 0.02	0.87 ± 0.03	0.83 ± 0.04	1.10 ± 0.09*	0.94 ± 0.03	1.03 ± 0.06	0.87 ± 0.04	0.88 ± 0.03
IVSd (mm)	0.63 ± 0.06	0.87 ± 0.07*	0.62 ± 0.02	0.58 ± 0.03	0.67 ± 0.02	0.84 ± 0.04***	0.65 ± 0.04	0.65 ± 0.03	0.60 ± 0.02	0.65 ± 0.03
IVSs (mm)	1.08 ± 0.05	1.16 ± 0.09	1.06 ± 0.06	1.02 ± 0.05	1.02 ± 0.03	1.16 ± 0.07	1.02 ± 0.04	1.10 ± 0.05	0.96 ± 0.03	1.07 ± 0.04*
LV mass (mg)	69.98 ± 2.54	104.29 ± 4.63***	71.54 ± 2.27	73.63 ± 2.86	75.66 ± 2.40	99.64 ± 5.30**	78.27 ± 2.07	81.65 ± 3.82	67.91 ± 2.07	65.74 ± 1.85
Functional changes										
LV EF (%)	83.11 ± 1.29	78.02 ± 2.10	78.77 ± 2.00	82.33 ± 1.42	84.25 ± 1.50	80.68 ± 3.26	83.04 ± 1.35	84.10 ± 1.97	79.72 ± 1.72	78.04 ± 1.89
LV FS (%)	45.11 ± 1.45	40.08 ± 1.86	40.77 ± 2.03	44.30 ± 1.57	46.67 ± 1.71	44.34 ± 3.70	45.08 ± 1.49	46.53 ± 2.08	41.93 ± 1.83	40.23 ± 1.84

HR: heart rate; LVIDd: left ventricular internal dimensions (diastole); LVIDs: left ventricular internal dimensions (systole); PWd: posterior wall thicknesses (diastole); PWs: posterior wall thicknesses (systole); IVSd: interventricular septal thicknesses (diastole); IVSs: interventricular septal thicknesses (systole); EF: left ventricular ejection fraction; FS: left ventricular fractional shortening; LV mass: left ventricular mass. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ when compared to its corresponding baseline values.

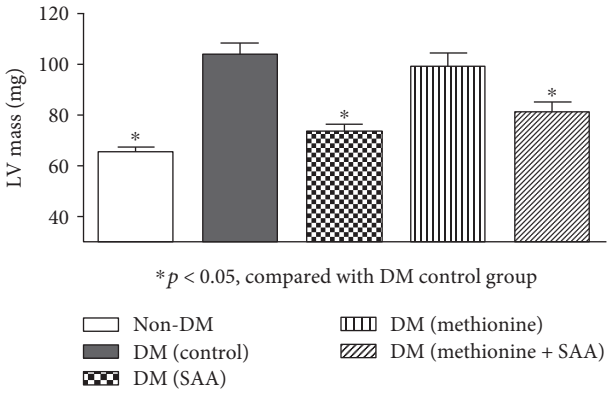

Figure 4: Left ventricular mass (mg) at the end of experiment as estimated by echocardiography. The LV mass in the non-DM, DM (methionine + SAA), and DM (SAA) groups after 8 wks of intervention period was significantly lower when compared to that in the DM (control) group, suggesting that the SAA treatment could delay the progress of the diabetic left ventricular hypertrophy.

too short for the potential beneficial effects of SAA on cardiac function to be observed.

3.3. GSH, GSSG, SAM, and SAH Levels in Mouse Liver. In the present study, SAM level in the liver was higher in the DM (methionine) group ($0.133\,\mu g/g \pm 0.021$) when compared to the non-DM ($0.062\,\mu g/g \pm 0.007$) and DM (SAA) ($0.051\,\mu g/g \pm 0.003$) groups, while the level of SAH in the liver in the DM (methionine + SAA) group ($0.212\,\mu g/g \pm 0.024$) was found significantly higher when compared to that in the DM (SAA) ($0.100\,\mu g/g \pm 0.027$) and non-DM ($0.098\,\mu g/g \pm 0.025$) groups. Besides, the methylation potential (SAM/SAH ratio) in the DM (methionine + SAA) group (0.46 ± 0.08) was significantly lower when compared to that in the DM (control) group (1.57 ± 0.75). All results were summarized in Table 3.

Meanwhile, GSH level in the livers was lower in the DM (methionine) ($3.06\,mg/g \pm 0.17$) and DM (control) ($3.04\,mg/g \pm 0.13$) groups when compared to the non-DM group ($4.28\,mg/g \pm 0.18$). A higher GSH level was also observed in the DM (methionine + SAA) group ($3.77\,mg/g \pm 0.10$) when compared to the DM (methionine) ($3.06\,mg/g \pm 0.17$) group. In addition, reduced GSSG level in the liver was observed in both DM (SAA) ($0.62\,mg/g \pm 0.21$) and DM (methionine) ($0.57\,mg/g \pm 0.06$) groups when compared to the non-DM group ($1.14\,mg/g \pm 0.12$). Of note, the redox status as denoted by GSH/GSSG level was significantly improved and elevated in the DM (SAA) group (9.13 ± 2.24) when compared to the DM (control) (4.05 ± 0.47) and non-DM (4.11 ± 0.45) groups. All results were summarized in Table 4.

3.4. Endothelial Function Assessment. The results of vascular endothelial function assessments were summarized using concentration-dependent relaxation curves and expressed in Figures 5 and 6. At the concentration of 10^{-9} M–10^{-7} M of Ach, significant reduction in the percentage of Ach-induced relaxation was observed in all the diabetic groups when compared to the non-DM group. From $10^{-6.5}$M to 10^{-6} M, the reductions in endothelium-dependent relaxations in DM (control) and DM (SAA) were gradually attenuated or improved to an extent similar to the non-DM group. For the DM (SAA + methionine) group, the percentage of relaxation induced by Ach was not improved to the extent similar to the non-DM group until the Ach concentration reached 10^{-6} M. However, the reduction in the percentage of Ach-induced relaxation persists in the DM (methionine) group at all concentrations of Ach. Conversely, the percentage of relaxation induced by SNP in all diabetic groups was similar when compared to that in the non-DM group, except at the concentrations from $10^{-8.5}$ M to 10^{-7} M where significantly diminished relaxations were observed in the DM (methionine) group when compared to the non-DM group.

4. Discussion

Diabetes is a multifactorial disease that leads to various forms of CVD complications. In this study, we collected evidences that SAA, apart from being a potent antioxidant, could possibly serve as a Hcy-lowering drug in diabetes with HHcy and at the same time exert multiple beneficial effects on the cardiovascular system. The present study indicated that oral administration of SAA for 8 wks could delay the progression of LVH and ameliorates ED in *db*/*db* mice, in particular to those with elevated Hcy level. These observed beneficial cardiovascular effects by SAA in our studied diabetic mice model with elevated Hcy level might be due to an improved redox status by the antioxidant effect of SAA itself and by the increased production of GSH via activated transsulfuration pathway.

Besides, our results indicated that SAA treatment did not significantly affect the serum Hcy level in the diabetic mice with a normal level of Hcy, while clearly lowering down the serum Hcy level in the diabetic mice with elevated Hcy level. This observation might suggest that the beneficial effect of SAA in lowering Hcy level largely depends on the baseline initial value of serum Hcy level of the *db*/*db* mice. We found that a previous study also exhibited a similar bidirectional Hcy-lowering effect of SAA in the nondiabetic rats [39]. Interestingly, the Hcy level was found lowered in *db*/*db* mice when compared to *db*/*m* mice in our study, though it has not reached a significant difference, probably due to (1) a decreased level of methionine in the liver, (2) the sulfur-containing byproduct generated during the methionine metabolism, and (3) a diminished transmethylation activity in the methionine metabolism [43]. Remarkably, as a transgenic diabetic mouse model, *db*/*db* mice were reported previously as possessing a lower Hcy level when compared to *db*/+ mice [43].

5. The CV Protective Effects of SAA on Ameliorating LVH and ED in *db*/*db* Mice with Elevated Blood Hcy Level

As reported in the previous studies, SAA has been indicated to inhibit platelet adhesion and aggregation, act against oxidative stress by suppressing the production of ROS, protect

Table 3: The methylation status (SAM/SAH ratio) in the liver of the mice at the end of the experiment.

	DM (control)	DM (SAA)	DM (methionine)	DM (methionine + SAA)	Non-DM
SAM (μg/g)	0.101 ± 0.01	0.051 ± 0.003	$0.133 \pm 0.021^{*}$	0.089 ± 0.011	0.062 ± 0.007
SAH (μg/g)	0.112 ± 0.027	$0.100 \pm 0.027^{**}$	0.156 ± 0.010	0.212 ± 0.024	$0.098 \pm 0.025^{***}$
SAM/SAH ratio	$1.57 \pm 0.75^{***}$	0.94 ± 0.37	0.86 ± 0.13	0.46 ± 0.08	0.93 ± 0.19

SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine. All values are expressed as concentration (μg/g) per 1 g of liver tissue. $^{*}P = 0.01$ versus the non-DM and DM (SAA) groups. $^{**}P < 0.05$ versus the DM (methionine + SAA) group. $^{***}P < 0.01$ versus the DM (methionine + SAA) group.

Table 4: GSH, GSSG, and oxidative stress (GSH/GSSG ratio) in the liver tissue at the end of the experiment.

	DM (control)	DM (SAA)	DM (methionine)	DM (methionine + SAA)	Non-DM
GSH (mg/g)	$3.04 \pm 0.13^{a,c}$	3.53 ± 0.17^{b}	$3.06 \pm 0.17^{a,c}$	3.77 ± 0.10	4.28 ± 0.18
GSSG (mg/g)	0.85 ± 0.17	$0.62 \pm 0.21^{*}$	$0.57 \pm 0.06^{*}$	0.78 ± 0.07	1.14 ± 0.12
Oxidative stress (GSH/GSSG ratio)	$4.05 \pm 0.47^{**}$	9.13 ± 2.24	5.60 ± 0.47	5.20 ± 0.58	$4.11 \pm 0.45^{**}$

GSH: glutathione; GSSG: oxidized glutathione. $^{a}P < 0.001$ versus the non-DM group. $^{b}P < 0.05$ versus the non-DM group. $^{c}P < 0.05$ versus the DM (methionine + SAA) group. $^{*}P < 0.05$ versus the non-DM group. $^{**}P < 0.05$ versus the DM (SAA) group.

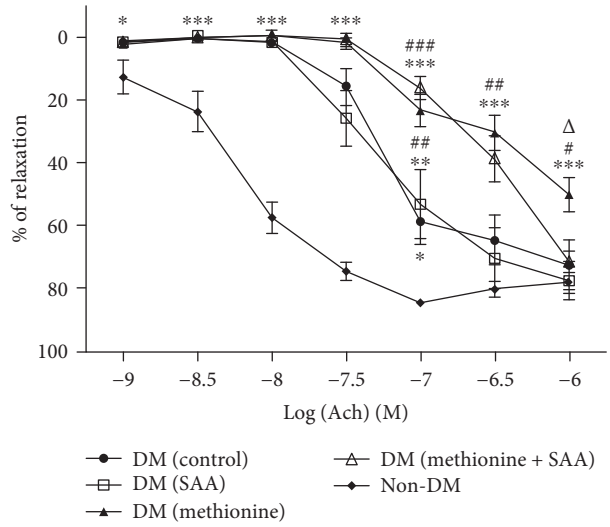

Figure 5: Endothelium-dependent vasorelaxation induced by acetylcholine (Ach). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ when compared to the non-DM group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ when compared to the DM (control) group, $^{\Delta}P < 0.05$ when compared to the DM (SAA) group.

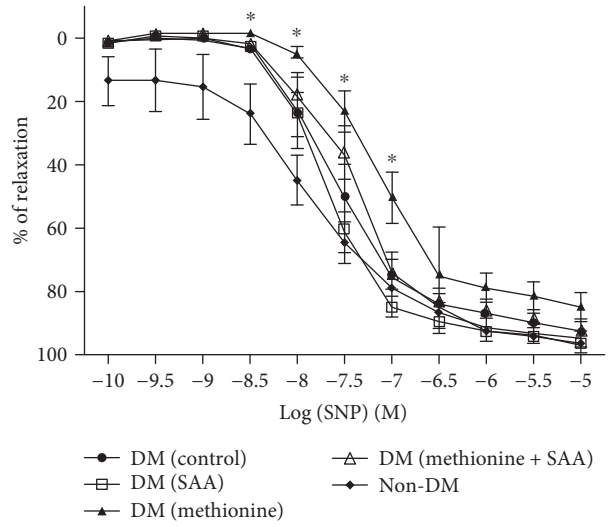

Figure 6: Endothelium-independent vasorelaxation induced by sodium nitroprusside (SNP). $^{*}P < 0.05$ when compared to the non-DM group.

myocardium ischemia, and ameliorate endothelial cells dysfunction via Akt and ERK1/2 phosphorylation [35, 44]. In diabetes, excessive ROS generation could be triggered by hyperglycemia and autoxidation [45]. Elevated ROS was found associated with increased cell death and apoptosis in the heart of the *db*/*db* mice [46], which led to pathological cardiac remodeling and fibrosis [47] and subsequent abnormal cardiac morphological and functional change [48]. Consistent with a previous study [49], the presence of LVH was found in the DM (control) group, as evidenced by the increased LV mass and wall thickness after 8 wks. With the 8 wks SAA treatment in the DM (SAA) and DM (methionine + SAA) groups, we found the LV mass in these 2 groups was similar to the non-DM group and significantly lower than that in the DM (control) group, suggesting that SAA treatment could prevent or slow down the progression of diabetic LVH (Figure 4).

ED is the initial step in the pathogenesis of atherosclerotic CVD. In the endothelium-dependent vasorelaxation test induced by Ach, the diabetic groups with SAA treatment were found capable to ameliorate the detrimental effect by elevated blood glucose and Hcy in a dose-dependent manner (Figure 5). Besides, the additive detrimental effect of both hyperglycemia and HHcy together with the endothelial function in the DM (methionine) group was evidenced by a relatively lower percentage of Ach-induced vasorelaxation at a concentration of $10^{-7.5}$ M to 10^{-6} M when compared to the DM (control) group. On the contrary, the endothelium-independent vasorelaxation induced by SNP in all groups were similar at all concentrations of SNP (Figure 6),

suggesting that the observed impaired Ach-induced vasorelaxation was due to nitric oxide (NO) bioavailability (i.e., proper function of healthy endothelial cells), not due to NO insensitive (i.e., with adequate NO provided by SNP as NO donor, the vessel could be dilated to the full extent).

6. The Methylation Potential and Redox Status in the Liver of the *db*/*db* Mice with Elevated Blood Hcy Level

As a powerful catechol-O-methyltransferase inhibitor, SAH specifically dominates the methylation of polyphenols, such as SAA [39, 50]. Therefore, a high expression of SAH and low methylation potential would inhibit Hcy methylation and hence limiting the Hcy-lowering capability via a remethylation pathway. In our study, the SAH level in the DM (methionine + SAA) group was significantly higher than that in the DM (SAA) and non-DM groups, while the SAH level in the DM (methionine) group was also higher than that in the non-DM group, though the difference did not reach a significant level. Besides, the SAM level was high in the DM (methionine + SAA) group when compared to the DM (SAA) and non-DM groups. At the same time, the SAM/SAH ratio or methylation potential was also found reduced in the DM (methionine + SAA) group when compared to the DM (control) group. These observations suggested that the methylation capacity in the *db*/*db* mice with elevated Hcy level was lowered after SAA treatment. Since, we also found that the treatment of SAA could effectively lower down the Hcy level in the DM (methionine + SAA) group by nearly half when compared to the DM (methionine) group (Figure 3). Thus, it is likely that the remethylation process of Hcy has been largely inhibited by the elevated SAH level in the DM (methionine + SAA) group and that the observed Hcy-lowering effect by SAA treatment on the *db*/*db* mice with elevated Hcy level is most likely through the alternative pathway, that is, the transsulfuration pathway.

Transsulfuration pathway provides an endogenous pathway for the conversion of Hcy into GSH [30, 51]. The significance of the transsulfuration pathway is in the maintenance of the redox homeostasis [52, 53]. In the present study, we found that the GSH level in the DM (SAA + methionine) group was relatively higher when compared to that in the DM (methionine) group after 8 wks of intervention period. This observation further supports our postulation that SAA treatment could normalize Hcy level in the diabetic mice with an elevated Hcy level predominately by activation of transsulfuration pathway and production of more GSH, despite a high expression of SAH and lower methylation potential condition exist after SAA treatment. In addition, the redox imbalance as indicated by reduced GSH/GSSG ratio was significantly elevated in the DM (SAA) group when compared to the DM (control) and non-DM groups, suggesting that SAA is a potent antioxidant. In this study, a higher oxidative stress level was also noted in the *db*/*db* mice, as indicated by a lower GSH pool size in the DM (control) group, when compared to the non-DM group. In the setting of diabetes, GSH depletion caused by disturbed transsulfuration could lead to the oxidant/antioxidant imbalance on its own, and the impaired transsulfuration could cause the loss of the compensatory GSH synthesis in response to oxidative stress, and lead to further oxidative stress [51, 52].

7. Conclusion

From our observations, transsulfuration pathway seems to be activated by SAA treatment and is likely served as a potential target for Hcy-lowering and redox-rebalancing treatments in both hyperglycemic and hyperhomocysteinemic diabetic animals. Although other players in the transsulfuration pathway, such as cystathionine and cysteine levels, were not identified in our experiments, the relatively higher GSH levels in the liver of our diabetic mice with elevated Hcy level after SAA treatment already suggested that SAA could activate the activity of the transsulfuration pathway as a means of lowering Hcy level. We believe that SAA is an important endogenous antioxidant and could work as an exogenous antioxidant against ROS-induced harm in the heart and ameliorate endothelial dysfunction. Besides, the elevated GSH level after SAA treatment provides another explanation for the overall improved antioxidant activity of SAA in protecting against the progression of LVH and ED. Further studies into other key players in the transsulfuration pathway, as well as the underlying mechanism/signaling pathway of CV protective effects by SAA treatment in diabetes with HHcy, are warranted.

Conflicts of Interest

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

Authors' Contributions

Christopher W. K. Lai and Parco M. Siu conceived and designed the experiments. Lei Gao performed the experiments. Lei Gao and Christopher W. K. Lai analyzed the data. Lei Gao and Christopher W. K. Lai wrote the paper. Lei Gao, Shun-wan Chan, Christopher W. K. Lai, and Parco M. Siu approved the paper.

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Research Article

Antioxidant and Antifibrotic Effect of a Herbal Formulation *In Vitro* and in the Experimental Andropause via Nrf2/HO-1 Signaling Pathway

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The Korean herbal formulation Ojayeonjonghwan is used for improving late-onset hypogonadism (LOH) symptoms such as erectile dysfunction (ED). A previous research suggested that a modified Ojayeonjonghwan (KH-204) could be used as an alternative to the treatment for ED. The pharmacological effects were examined in different conditions, including in vitro and in vivo. We measured the survival rate of TM3 Leydig cells under the oxidative stress condition. The s.c. injection of leuprorelin was used to induce androgen deprivation. We measured serum testosterone levels, oxidative stress, and apoptosis. The results of the treatment by KH-204 (1) preserved TM3 cells from oxidative stress by improving the expression of nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1); (2) lowered the expression of transforming growth factor-beta (TGF-β) 1/SMAD; (3) increased the average of serum testosterone in androgen-deprived male rats; (4) kept the activation of spermatogenesis; (5) upgraded the contents of 8-hydroxy-20-deoxyguanosine (8-OHdG) and degraded the contents of superoxide dismutase (SOD); and (6) reduced apoptosis. We studied that KH-204 improved testicular dysfunction in LOH. It is likely, at least in part, to degrade oxidative stress through the Nrf2/HO-1 pathway. These findings may offer credible evidences for the use of new alternative therapies to treat LOH.

1. Introduction

Late-onset hypogonadism (LOH) includes symptoms such as depression, fatigue, low muscle mass, and erectile dysfunction (ED) and occurs in middle-aged males. Many studies have demonstrated that LOH is closely linked to a deficiency in serum testosterone levels [1–3] because the total number of Leydig cells, which are the main source of testosterone in middle-aged men, decreases to about a half of that seen in young men [4, 5]. LOH symptoms are associated with testosterone deficiency and, therefore, can be improved by androgen replacement therapy (ART). ART can be used by men with LOH unless there are contraindications such as unstable cardiovascular disease, prostate cancer, or polycythemia; however, the long-term effects of ART are uncertain. In particular, external testosterone supplementation can contemporaneously increase the incidence of side effects including prostate cancer, benign prostatic hyperplasia (BPH), and cardiovascular events [5, 6]. ART can impair fertility, so men who would like to start a family

in the short run should make sure that they should not undergo ART [7].

Traditional herbal medicine is recognized as an alternative treatment of LOH that avoids the adverse effects of ART and, in some cases, can be of benefit to libido [8, 9]. Now, male infertility and LOH symptoms including ED are usually treated by the herbal formulation Ojayeonjonghwan [10, 11]. Park et al. [10] illuminated that improved Ojayeonjonghwan, known as KH-204, ameliorated ED caused by peripheral neuropathy in aged and diabetic rats. Recently, we also found that KH-204 protected against oxidative stress in rat testis [12]. A major characteristic of aging is the increased oxidative stress. It has been involved in various age-related pathologies [13]. Regarding interacting proteins and regulatory molecules such as heme oxygenase-1 (HO-1), the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling system has become the most significant cellular defense mechanism against oxidative stress, and many reports suggest that the reduction in the adaptive response of the Nrf2 signaling system has had a great influence in the cumulation of oxidative damage in aging [14–16].

Some previous studies demonstrated that progressive fibrosis was recognized as a characteristic in various organs, playing an important role during aging [17, 18]. Transforming growth factor-beta (TGF-β) is considered to have a significant effect in the pathogenic mechanism of diabetes-induced ED. Especially, TGF-β1 has been regarded as one of the most relevant fibrogenic cytokines, which is also high expression in the corpus cavernosum of diabetic rats [19]. Zhang et al. [20] also considered that upregulation of the TGF-β1/SMAD signaling pathway led to structural changes and decline of erectile function in ED. However, the impact of TGF-β1/SMAD signaling pathway on Leydig cells including testicular function is still unidentified.

The present study was intended to assess whether KH-204 could protect TM3 Leydig cells through antioxidant activity in vitro and restore testosterone production in an androgen-deprived animal model. In addition, we attempted to identify a potential mechanism of the protective effect.

2. Materials and Methods

2.1. Preparation of the Herbal Formula (KH-204). The major ingredients of KH-204 include five plants, as previously described: *Cornus officinalis* (32%), *Lycium chinense* (32%), *Rubus coreanus* (16%), *Cuscuta chinensis* (16%), and *Schisandra chinensis* (4%). It was manufactured by a company that makes oriental herbal medicines, KEMIMEDI Co. Ltd. (Seoul, Republic of Korea). The quality of KH-204 was confirmed by its marker compound. In addition, the quality of each extract were identified by using high-performance liquid chromatography (HPLC) as previously described [21]. The marker compounds of *Cornus officinalis*, *Lycium chinense*, *Rubus coreanus*, *Cuscuta chinensi*, and *Schisandra chinensis* are loganin, betain, ellagic acid, hyperoside, and Schizandrin in HPLC chromatogram, respectively. The manufacturing method and the toxicity data of KH-204 were described in a previous report [22].

2.2. In Vitro Cell Viability and Western Blot Testing Using TM3 Mouse Leydig Cells. TM3 mouse Leydig cells (Korean Cell Line Bank, Seoul, Republic of Korea) were cultured in Dulbecco's modified eagle's medium (DMEM)/F-12 medium (GIBCO®, Life Technologies Co., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO) at 37°C. Cells were plated on 96-well plates (Corning) in 10% FBS/DMEM/F-12 and incubated for twenty-four hours. They were pretreated with 50 μg/ml of KH-204 for two hours before treating with hydrogen peroxide (40 μM H_2O_2) for two hours to create oxidative cellular stress. Afterwards, Alamar Blue (Invitrogen, USA) was aseptically added to the cells. The cells were incubated for three hours, and the absorbance of the cells was measured at a wavelength of 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, USA). The background absorbance was measured at 600 nm and was then subtracted. Cell viability treated by ERK inhibitor PD98059 or Akt inhibitor LY294002 was also assessed to identify the activation of ERK and Akt.

After processing, we gathered all cellular proteins, by placing cells in a lysis buffer consisting of 0.1% sodium dodecyl sulfate in phosphate-buffered saline, followed by brief sonication. Protein concentration was identified by a bicinchoninic acid protein assay (Pierce Chemical Co., USA). Thirty micrograms of total cellular protein was separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Blots were probed with an antibody specific for the following proteins: β-actin (1 : 5000 dilution; Assay Designs, USA); phospho-ERK (p-ERK) and total ERK (1 : 1000 dilution; Cell Signaling Technology, USA); phospho-Akt (p-Akt) and total Akt (1 : 500 dilution; Santa Cruz Biotechnology, USA); HO-1 (1 : 1000 dilution; Cell Signaling Technology, USA); Nrf2 (1 : 500 dilution; Santa Cruz Biotechnology, USA); SMAD (1 : 500 dilution; Santa Cruz Biotechnology, USA); and TGF-β (1 : 1000 dilution; Abcam, UK). The binding antibody of each blot was evaluated by enhancing chemiluminescence (Western blot detection kit; Amersham Pharmacia Biotech, USA), which was assessed with horseradish peroxidase-conjugated secondary antibody.

2.3. Animal Groups and Treatment Protocol. We carried out this experiment strictly following the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee in the School of Medicine, The Catholic University of Korea.

Sprague-Dawley (SD) male rats aged 8 weeks were randomly divided into 4 groups (8 rats in each group), which were submitted to (1) sham operation only (normal control), (2) androgen deprived only (Androgen-dep. control), (3) androgen deprived treated by KH-204 200 mg/kg (Androgen-dep. 200), and (4) androgen deprived treated by KH-204 400 mg/kg (Androgen-dep. 400). We administered either distilled water (sham operated) or leuprorelin 0.5 mg/kg subcutaneously once to the backs of androgen-deprived rats. According to previous experimental results, we selected the dosage of leuprorelin [23]. In each group, once-daily oral administration

was lasted for 4 weeks (distilled water or KH-204 dissolved in distilled water). After 4 weeks, the animals in all groups were sacrificed under anesthetic and testes, epididymides, and blood samples were obtained.

2.4. Measurement of Serum Testosterone Level. Before the rats were sacrificed, venous blood samples were collected from the inferior vena cava and were assayed by an ELISA testosterone detection kit (BioVendor, Czech Republic) to determine the serum testosterone level.

2.5. Testicular Histologic Evaluation and Immunohistochemistry. The fixed and embedded testicular tissues were stained with haematoxylin-eosin, and these were examined under a light microscope. Ten representative sites were selected randomly in seminiferous tubules, and spermatogenic cell density was measured as previously described [24].

Leydig cells were identified by histochemically staining for Leydig cell-specific marker 3β-hydroxysteroid dehydrogenase (3β-HSD) [25]. On each occasion, tissue sections in each experimental group were immunostained and the intensity of the immunostaining was scored using a simplified scale ranging from negative (−) through weakly positive (+) to intensely positive (+++), as previously described [26].

2.6. Measurement of Oxidative Stress. Oxidative stress was assessed by measuring the 8-hydroxy-2-deoxyguanosine (8-OHdG) content and superoxide dismutase (SOD) activity quantitatively. Total DNA was extracted from the testis using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). The level of 8-OHdG was measured with a DNA oxidation kit (Highly Sensitive 8-OHdG Check ELISA; Japan Institute for the Control of Aging, Fukuroi, Japan). After the final color was developed with the addition of 3,3′,5,5′-tetramethylbenzidine, absorbance was measured at 450 nm. Tissue sample concentration was measured from a standard curve and corrected for DNA concentration. SOD activity (CuZnSOD and Mn SOD) in tissues was determined using an SOD Assay Kit-WST (Dojindo), and the decrease in the rate of the superoxide-mediated reduction of nitroblue tetrazolium was monitored at 450 nm using a spectrophotometer.

2.7. Assessment of Apoptosis. Testicular tissue sections were washed out with PBS after blocking with 0.1% Triton X-100 last 5 min. Terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL, ApopTag In Situ Apoptosis Detection Kits; Millipore, MA, USA) detection solution was dropped on each section and then incubated at 37°C in the dark which lasted for an hour. Nuclear staining with DAPI was performed last for 5 min after being washed out with PBS, and the sections were fixed with 50% glycerol after being washed out with PBS. For the control sections, the TUNEL solution was replaced with PBS. The sections were observed under a fluorescence microscope.

2.8. Western Blot Testing In Vivo. We gathered testicular tissue proteins of each group, by placing crushed testicular tissues in an ice-cold lysis buffer consisting of 0.1% sodium dodecyl sulfate in phosphate-buffered saline, followed by brief sonication. Protein concentration was identified by a bicinchoninic acid protein assay (Pierce Chemical Co., USA). Thirty micrograms of total testicular tissue protein was separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Blots were probed with an antibody specific for the following proteins: β-actin (1 : 5000 dilution; Assay Designs, USA); HO-1 (1 : 1000 dilution; Cell Signaling Technology, USA); Nrf2 (1 : 500 dilution; Santa Cruz Biotechnology, USA); SMAD (1 : 500 dilution; Santa Cruz Biotechnology, USA); and TGF-β (1 : 1000 dilution; Abcam, UK). The binding antibody of each blot was evaluated by enhancing chemiluminescence (Western blot detection kit; Amersham Pharmacia Biotech, USA), which was assessed with horseradish peroxidase-conjugated secondary antibody.

2.9. Statistical Analysis. Statistical analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, USA). The data was expressed as mean ± standard deviation. Statistical significance was analyzed by the ANOVA test, with group comparisons made by Scheffe's test. $p < 0.05$ was considered significant.

3. Results

3.1. KH-204 Protected TM3 Leydig Cells against Oxidative Stress via Decreased Expression of TGF-β1/SMAD and Increased Nrf2/HO-1 Expression. As shown in Figure 1(a), cell viability significantly decreased under the H_2O_2 incubation, compared to the cells in none H_2O_2 condition ($p < 0.05$). Cell viability was raised to 94% by the addition of KH-204 but was slightly decreased by pretreatment with the ERK inhibitor PD98059 or the Akt inhibitor LY294002.

Western blot analysis was used to assess whether KH-204 could keep TM3 cells from H_2O_2-induced damage. The phosphorylation levels of ERK and Akt were significantly upgraded by KH-204 treatment ($p < 0.05$, Figure 1(b)). However, treatment with the Akt inhibitor LY294002 decreased the phosphorylation levels by inhibiting PI3K in the treatment group. These results showed that KH-204 could effectively protect against H_2O_2-induced damage via ERK and Akt activation. The quantitative result showed that expression of TGF-β1/SMAD was significantly increased and Nrf2/HO-1 was meaningfully decreased after H_2O_2 injury ($p < 0.05$, Figure 2(b)). And this result illuminated that the effect of recovery was according to the dose of KH-204, which means the result is better in the H_2O_2 + KH-204 40 μg group than in the H_2O_2 + KH-204 10 μg group. In the animal experiment, the Western blot results (Figures 2(c) and 2(d)) showed that expression of TGF-β1/SMAD was significantly increased and Nrf2/HO-1 was meaningfully decreased after treatment by leuprorelin ($p < 0.05$). However, with treatment of KH-204, the results of protein expression significantly changed, which turned to normality ($p < 0.05$, Figures 2(c) and 2(d)).

3.2. KH-204 Preserved Testicular Function and Serum Testosterone Levels in an In Vivo Model. There were no significant differences in body weight among the test

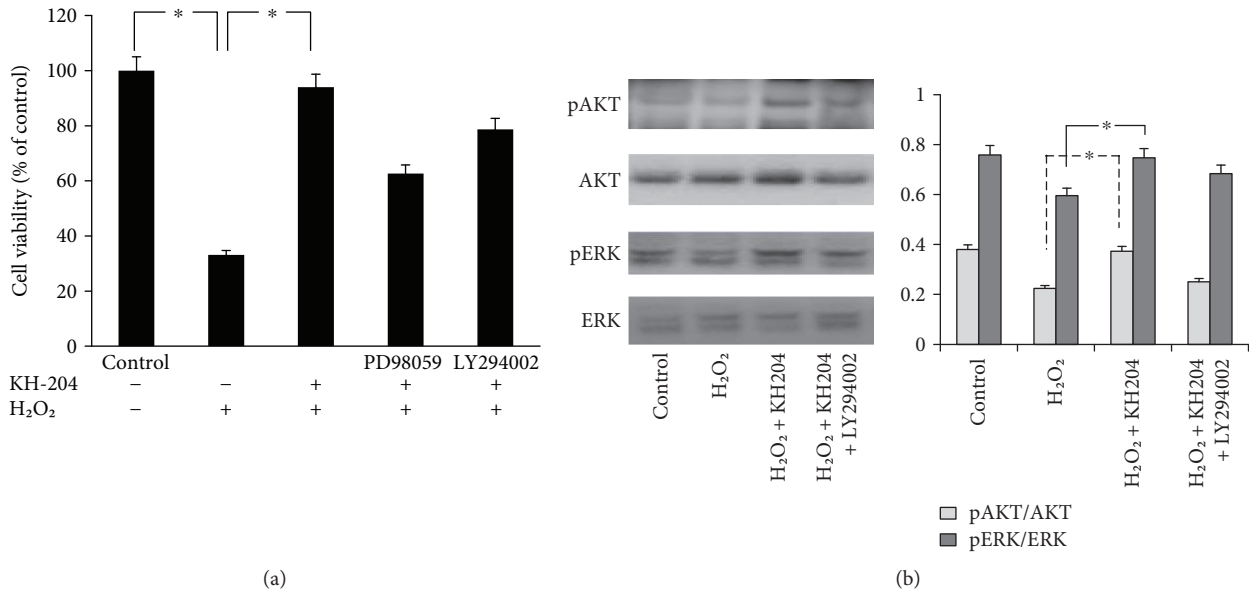

Figure 1: (a) Protective effect of KH-204 against oxidative stress. (b) Enhanced activation of ERK and Akt by KH-204 after 24 h of treatment. The difference was statistically significant ($^*p < 0.05$). And the experiments were repeated for three times.

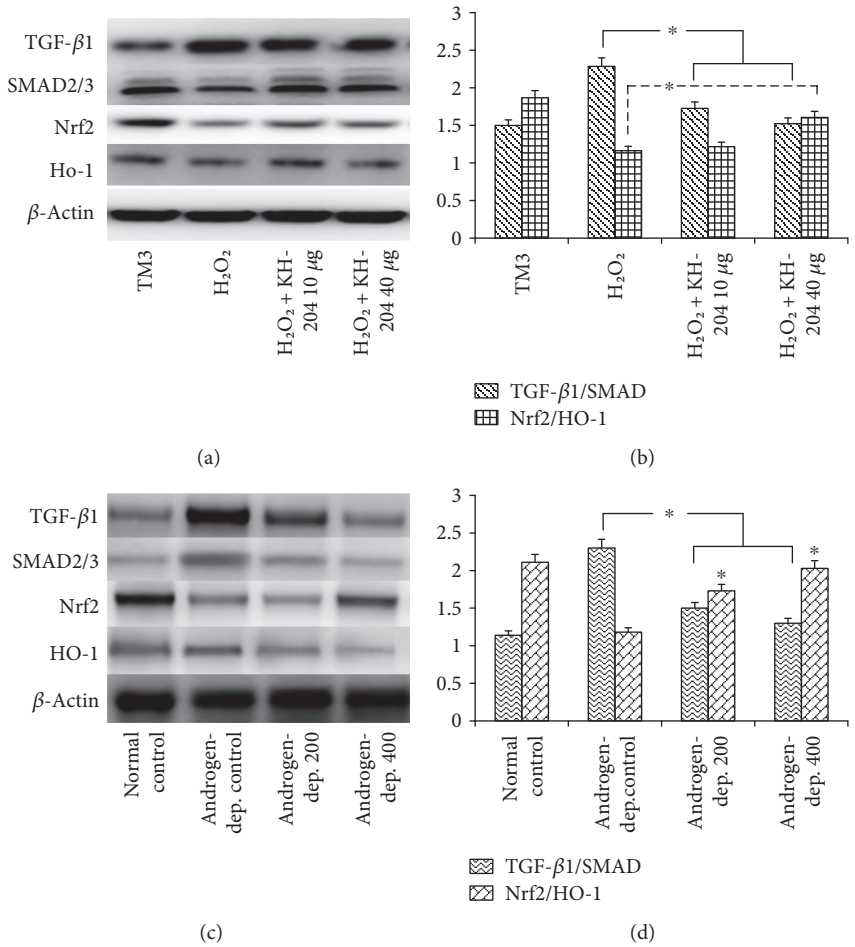

Figure 2: Comparison of the expression levels in vitro and in vivo. (a) is TGF-β1/SMAD and Nrf2/HO-1 in vitro, (b) is densitometric analysis relative to β-actin in vitro, (c) is TGF-β1/SMAD and Nrf2/HO-1 in vivo, and (d) is densitometric analysis relative to in vivo. The difference was statistically significant ($^*p < 0.05$). And the experiments were repeated for three times.

Table 1: Comparisons of parameters of the testicular health.

	Testicular weight (g)	Epididymal weight (g)	Germinal cell layer thickness (μm)	Diameter of seminiferous tubules (μm)	Serum testosterone (ng/ml)
Normal control	1.62 ± 0.19	0.68 ± 0.03	73.22 ± 6.34	297.34 ± 4.33	2.48 ± 0.41
Androgen-dep. control	$1.09 \pm 0.06^{*}$	$0.40 \pm 0.08^{*}$	$42.19 \pm 2.69^{*}$	$251.16 \pm 3.62^{*}$	$1.28 \pm 0.34^{*}$
Androgen-dep. 200	1.15 ± 0.17	0.45 ± 0.06	52.46 ± 3.92	271.31 ± 1.36	1.32 ± 0.52
Androgen-dep. 400	$1.30 \pm 0.05^{**}$	$0.59 \pm 0.01^{**}$	$69.86 \pm 7.10^{**}$	$280.14 \pm 8.32^{**}$	$1.72 \pm 0.13^{**}$

Data show the mean ± s.d. analysis of variance test. *Significant statistical difference ($p < 0.05$) compared with the normal control group. **Significant statistical difference ($p < 0.05$) compared with the androgen-deprived control group.

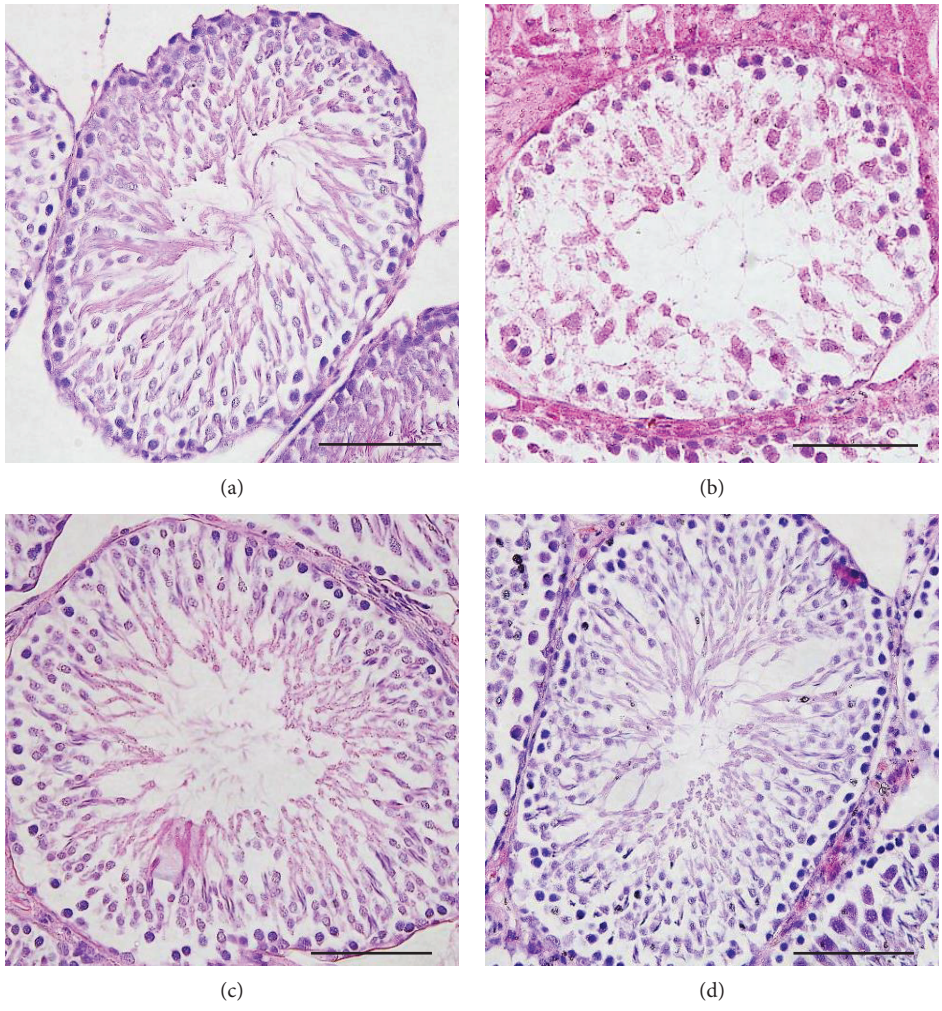

Figure 3: Histopathological findings of the testicular tissues (haematoxylin and eosin stain) in (a) the normal control group ($n = 8$, normal conrtol), (b) the androgen-deprived control group ($n = 8$, Androgen-dep. control), (c) the androgen-deprived 200 mg/kg group ($n = 8$, Androgen-dep. 200), and (d) the androgen-deprived 400 mg/kg group ($n = 8$, Androgen-dep. 400). Compared with the normal control group, a narrow germinal cell layer is observed in the androgen-deprived control group. Scale bars shown in each figure represent 100 μm. And the experiments were repeated for three times.

groups. In Table 1, the mean weights of the testis of each group and the epididymis are listed. There was a sensible difference in the testicular and the epididymal weights between the normal control group and the androgen-deprived control group after 4 weeks ($p < 0.05$). However, treatment by KH-204 caused obvious increases in testicular and epididymal weights in the androgen-deprived 400 mg/kg group when compared with those in the androgen-deprived control group ($p < 0.05$).

The testicular tissues of all groups showed normal structure with mature seminiferous tubules and complete spermatogenic series. However, the spermatogenic cell densities of the testes in the androgen-deprived control group were slightly reduced in comparison with those in the other 3 groups (Figure 3).

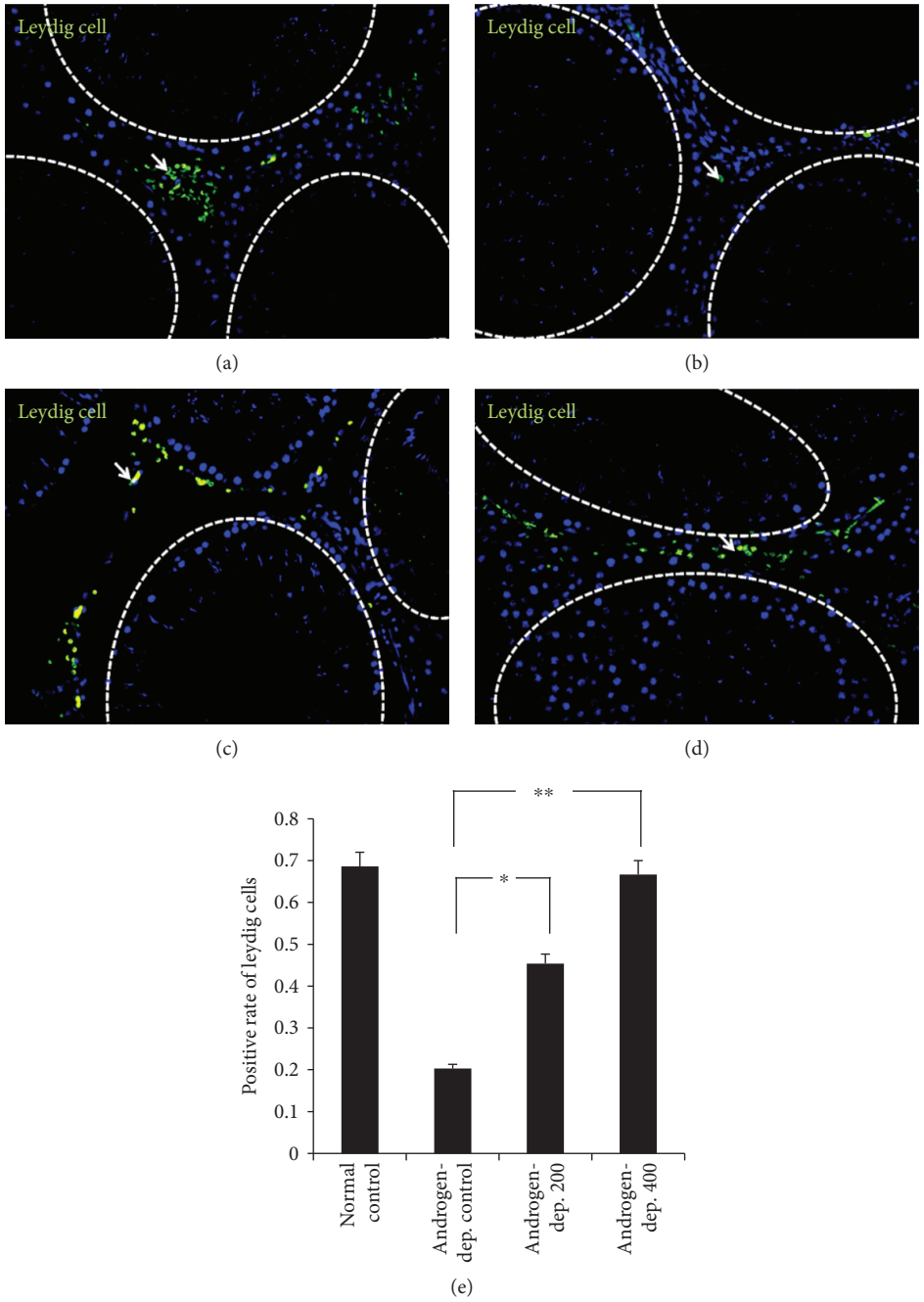

Figure 4: Immunoexpression of 3β-HSD (arrow) in Leydig cells of rat testis after treatment. ×400. (a) The normal control group ($n = 8$, normal control); (b) the androgen-deprived control group ($n = 8$, Androgen-dep. control); (c) the androgen-deprived 200 mg/kg group ($n = 8$, Androgen-dep. 200); (d) the androgen-deprived 400 mg/kg group ($n = 8$, Androgen-dep. 400); and (e) positive rate of 3β-HSD-immunoreactive cells. The difference was statistically significant ($^{*}p < 0.05$ and $^{**}p < 0.01$). And the experiments were repeated for three times.

The tissue was degenerated, and incomplete spermatogenic series were shown in some seminiferous tubules. There were almost normal mature active seminiferous tubules with complete spermatogenic series in the treatment group. The mean thickness of the germinal cell layer and the mean diameter of the seminiferous tubules in the androgen-deprived 400 mg/kg group were significantly increased in comparison with those in the androgen-deprived control group (Table 1).

The serum testosterone levels in the androgen-deprived control group were obviously decreased in comparison with those in the normal control group ($p < 0.05$, Table 1). These values were dose dependently increased after KH-204 treatment, and those from the androgen-deprived 400 mg/kg group were significantly increased compared with those from androgen-deprived control group ($p < 0.05$).

3.3. Immunohistochemistry. Cells positive for 3β-HSD activity are shown in Figures 4(a), 4(b), 4(c), and 4(d). They can be seen in the testicular interstitium, and the intensity was significantly decreased after androgen deprivation.

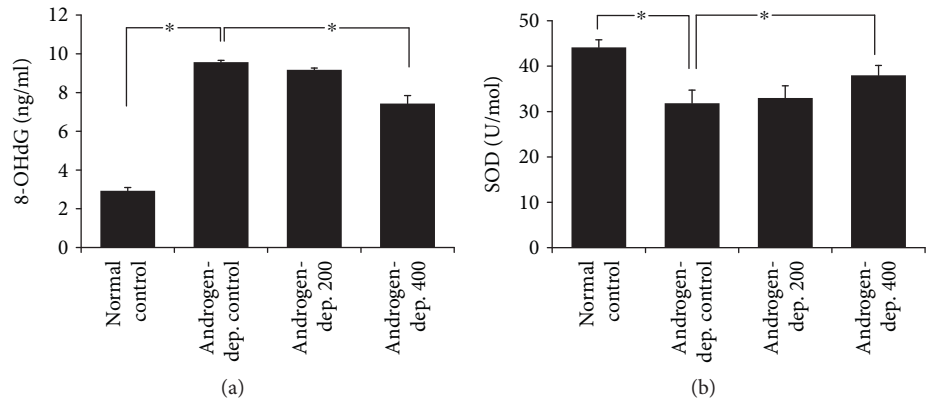

Figure 5: Comparison of the expression levels of 8-OHdG (a) and SOD (b). The difference was statistically significant ($^*p < 0.05$) ($n = 8$, in each group). And the experiments were repeated for three times.

However, the number of 3β-HSD-immunoreactive cells was relatively increased after KH-204 treatment ($p < 0.05$, Figure 4(e)). Moreover, the intensity in the androgen-deprived 400 mg/kg group was comparable to that in the normal control group.

3.4. KH-204 Decreased Oxidative Stress and Apoptosis. The mean expression of 8-OHdG and SOD is shown in Figure 5. After treatment by KH-204, a dose-dependent decrease in oxidative stress was found. Oxidative stress significantly increased in the androgen-deprived control group in comparison with that in the normal control group but obviously reduced in the androgen-deprived 400 mg/kg group after treatment ($p < 0.05$). The apoptotic cells in the testis were observed as being dark red in color in the TUNEL assay (Figure 6). The increased apoptotic cells in the androgen-deprived control group were significantly reduced in the two treatment groups receiving a dose of KH-204 ($p < 0.05$).

4. Discussion

The present study showed that KH-204 increased the viability of TM3 cells in oxidative-stressed conditions. LOH that bothers many middle-aged males is mainly caused by serum levels of testosterone that decline with age. In the aging male, serum testosterone continues to decrease because of reduced function of Leydig cells [27]. Although the age-related mechanism which could induce decreased function of Leydig cells remains unclear, our data suggest that there is a possibility to change the redox balance of the Leydig cells [28]. In this study, we showed that KH-204 increased the viability of TM3 cells under oxidative-stressed conditions in vitro and in vivo, which means KH-204 can protect the Leydig cells against damage from oxidative stress response. What is more, we detected the testicular function, serum testosterone levels, oxidative stress, and apoptosis in androgen-deprived rats, and these results illuminated that KH-204 could improve the survival of Leydig cells under androgen-deprived conditions in vivo.

Some researchers studied the shifts in Nrf2/electrophile response element activity in older organisms. Suh et al. [29] showed that protein expression of Nrf2 was reduced obviously, combined with a decreased GCL expression. The similar results were also observed in another study, which nuclear Nrf2 was reduced in the aorta of elder groups, along with reduced glutamate cysteine ligase, NADPH:quinone oxidoreductase-1, and HO-1 levels [30]. Although there is no report on the shifts in the expression levels of HO-1 with older testis, we found that the levels of Nrf2/HO-1 were obviously reduced after oxidative stress and ameliorated with the treatment of KH-204 in TM3 Leydig cells.

To examine if KH-204 can increase androgen synthesis in the androgen-deprived rat, we analyzed influences of KH-204 on the contents of serum testosterone and 3β-HSD activities. The present data show that the testosterone concentration and the number of 3β-HSD-immunoreactive cells were significantly increased after treatment with KH-204, suggesting a protective effect for KH-204. These findings also support the possibility that an increase in serum testosterone might be induced by the protective actions of KH-204 on Leydig cells which could express testosterone in the testis. KH-204 simultaneously increased spermatogenesis and the germinal cell layer thickness. The active sperm production was influenced by the contents of serum testosterone which could upgrade the efficiency of differentiation and active maturation in germ cell [31]. Therefore, consistent with results from previous studies, we suggest that the improved activity of Leydig cells along with the increased serum testosterone levels by KH-204 might improve spermatogenesis.

Aging is along with reproductive dysfunction where there are significant declines in steroidogenesis, spermatogenesis, and sexual function that might be due to the degradation of testicular function [32, 33]. Although the aging mechanism is confusing, there are commonly held beliefs that two major factors in aging are oxidative stress and apoptosis [34, 35]. Lesniewski et al. [36] found that oxidative stress in cells gradually increased with age in mice. Apoptosis is defined as a kind of biochemical and morphological change in various cellular levels, leading to the removing of unnecessary cells.

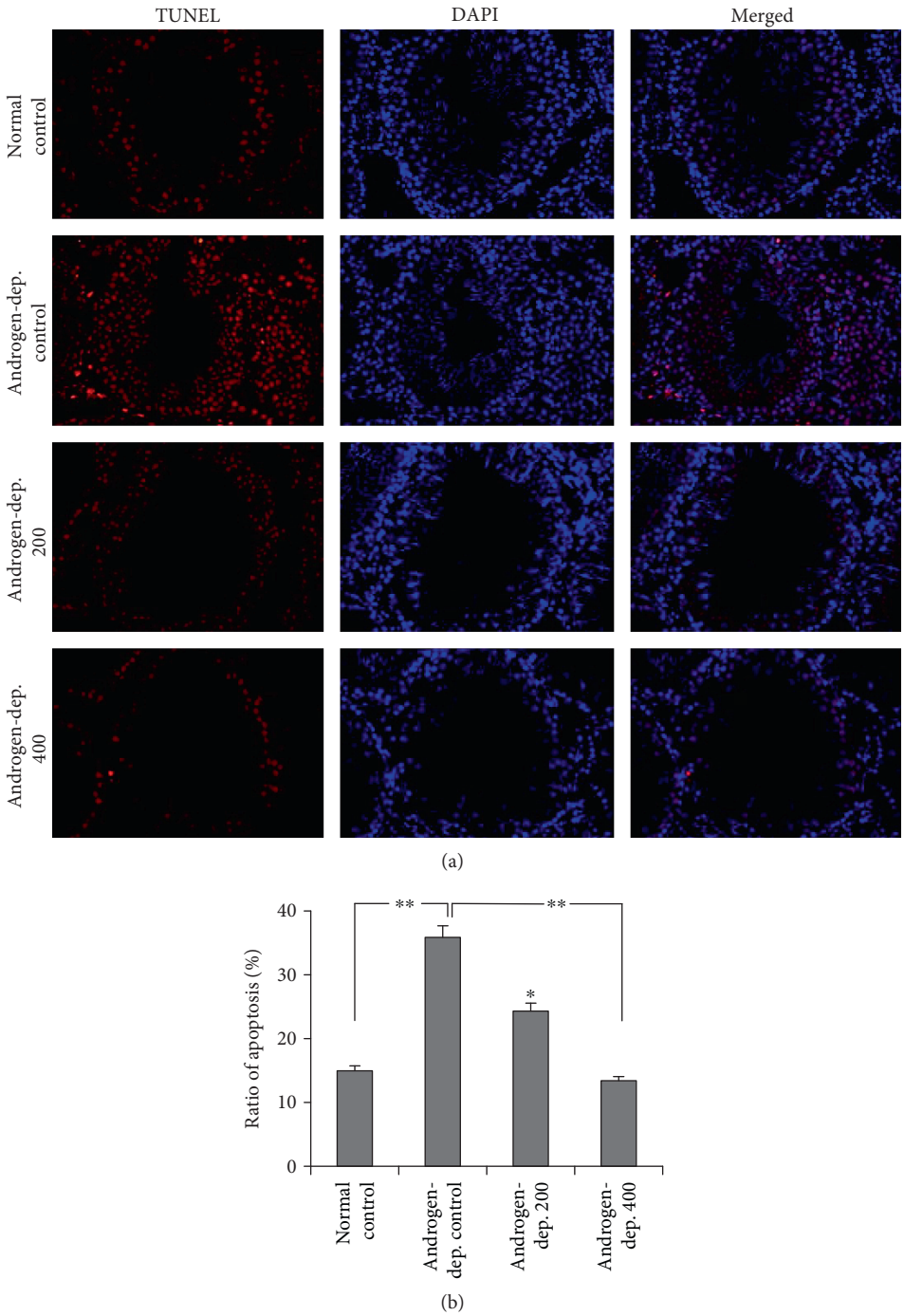

Figure 6: Effect of KH-204 on testicular tissue apoptosis by TUNEL assay. The difference was statistically significant ($^{*}p < 0.05$ and $^{**}p < 0.01$) ($n = 8$, in each group). And the experiments were repeated for three times.

It is an important physiological process [37]. However, apoptosis also has an unwanted influence on aging as it often eliminates important cells that are related to aging [38]. We observed that levels of 8-OHdG degraded and levels of SOD upgraded and that apoptotic cells were significantly decreased following treatment with KH-204 compared with the androgen-deprived group. KH-204 may alleviate testicular dysfunction in androgen-deprived rats via suppression of oxidative stress and apoptosis.

Recent data suggested that there might be a joint pathway for these different tissue damages, which was known as the upregulation of TGF-β1/SMAD signaling pathway [20, 39]. As is known to all, TGF-β can intermediate its fibrotic effects by triggering the receptor-associated SMADs [40]. Although there is no report on the effect of TGF-β1/SMAD pathway on the testis, many reports investigated that it might play a key role in configurational changes for erection [41, 42]. In our study, the expression of TGF-β1/SMAD was higher after

H_2O_2 injury, and these proteins' contents were obviously reduced in the KH-204 treatment groups in comparison with the group without KH-204. It is likely that KH-204 is at least in part attributable to the antifibrotic effect in the testis.

Herbs have been used widely in various urologic diseases, and many researches have been carried out to prove their safety and efficacy [43]. In particular, herbal medicines such as KH-204 are more essential for the treatment of LOH due to the adverse effects of ART. Discovery of natural products or herbs that can protect Leydig cells and restore the production of serum testosterone is very important to improve LOH. Previous studies demonstrated that KH-204 ameliorated ED in aged and diabetic rats [10, 11]. We also found that KH-204 protected against oxidative stress in the cryptorchid testis [12]. However, few studies on the detailed mechanisms against LOH have been reported. The major ingredients in KH-204 were reported to have antioxidant effects in various diseases. Furthermore, it was shown in a previous study that *Cuscuta chinensis* Lam. might improve kidney yang deficiency symptoms by recovering decreased serum testosterone [44]. In this study, we identified that KH-204 treatment improved the viability in oxidative stressed TM3 cells and that the activation of the ERK/Akt-dependent signaling pathways is the main mechanism. Thereafter, we illuminated that treatment with KH-204 can reduce oxidative stress or apoptosis in the androgen-deprived rat model.

Our study does have some limitations. First, the model used is far from a definite model of LOH. Aged or castrated animals have commonly been used as LOH animal models in many studies for oral supplementation [45, 46]. However, we wanted to reproduce a partial androgen deficiency state rather than an entire castration, and low-dose leuprorelin injection was useful for creating this situation. Secondly, while we clarified antioxidant and antifibrotic functions for KH-204, we did not identify the assumed mechanism, through the animal model. In the next work, we should try our best to investigate the accurate mechanism of KH-204 in the LOH animal model to plan for a clinical study.

We studied the efficacy of KH-204 to improve testicular dysfunction in LOH. The efficacies of KH-204 are likely, at least in part, to degrade oxidative stress through the Nrf2/HO-1 pathway. These findings may offer credible evidences for the use of new alternative therapies to treat LOH.

Conflicts of Interest

The authors have no actual or potential conflict of interest associated with this work.

Acknowledgments

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Review Article

Oxidative Stress in Pancreatic Beta Cell Regeneration

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Pancreatic β cell neogenesis and proliferation during the neonatal period are critical for the generation of sufficient pancreatic β cell mass/reserve and have a profound impact on long-term protection against type 2 diabetes (T2D). Oxidative stress plays an important role in β cell neogenesis, proliferation, and survival under both physiological and pathophysiological conditions. Pancreatic β cells are extremely susceptible to oxidative stress due to a high endogenous production of reactive oxygen species (ROS) and a low expression of antioxidative enzymes. In this review, we summarize studies describing the critical roles and the mechanisms of how oxidative stress impacts β cell neogenesis and proliferation. In addition, the effects of antioxidant supplements on reduction of oxidative stress and increase of β cell proliferation are discussed. Exploring the roles and the potential therapeutic effects of antioxidants in the process of β cell regeneration would provide novel perspectives to preserve and/or expand pancreatic β cell mass for the treatment of T2D.

1. Introduction

The prevalence of diabetes mellitus is increasing at an astounding rate worldwide. According to the WHO, the global prevalence of diabetes in 2014 was estimated to be 9% among adults aged 18 years and older. In 2012, an estimated 1.5 million deaths were directly caused by diabetes, and it was projected that diabetes will be the 7th leading cause of death in 2030. Although the etiology differs in the three major types of the disease—type 1 diabetes, type 2 diabetes, and gestational diabetes, all feature a crucial pathological change in the progression of diabetes, which is insufficient numbers of β cells to meet metabolic demand to control blood glucose levels. Pancreatic β cells, located in the islet of Langerhans, are essential for the maintenance of glucose homeostasis via the sensing of elevated blood glucose level and the subsequent production of glucose-lowering hormone insulin. Beta cell regeneration (neogenesis and proliferation) during the neonatal period is critical for the generation of sufficient pancreatic β cell mass/reserve and has a profound impact on long-term protection against T2D [1]. Moreover, under circumstances such as pregnancy or insulin resistance in T2D, enhanced β cell proliferation is present in response to the increased demand of insulin [2]. It is well-established that in response to hyperglycemia in diabetogenic states, β cell proliferation is substantially upregulated to various extents as a compensatory approach before the eventual loss of β cells' mass in later stage of diabetes [2, 3]. Thus, the need for β cell mass to be closely regulated under physiological and pathophysiological conditions on cell replication, size, apoptotic elimination, and, sometimes, neogenesis from progenitor cells is very important.

In T2D, the pathogenic effect of high glucose, possibly accompanied with excessive amount of fatty acids in the case of obesity, is exhibited to a significant extent via imbalanced redox status, through the increased production of reactive oxygen species (ROS) and reactive nitrogen species which results in oxidative stress. Numerous studies observed elevated levels of oxidative stress markers in patients with T2D [4, 5]. Indeed, because of the high demand of insulin, β cells are among the most metabolically active tissues and highly rely on oxidative phosphorylation for the generation of adenosine triphosphate (ATP). Moreover, high oxygen consumption is a key factor for insulin secretion, especially in response to elevated blood glucose levels [6], which renders β cells to higher risk of ROS production and oxidative

stress. On the other hand, β cells are particularly vulnerable to oxidative stress majorly due to the lack of antioxidant enzymes (Figure 1), which further weakened the ability of β cells in defense against oxidative stress.

A number of outstanding review articles have discussed the deleterious effects of oxidative stress on β cell death and dysfunction. During the past two decades, a plethora of evidence showed that oxidative stress is present in β cells while cell growth is most active and tightly controlled, such as during embryogenesis and pathological progressions of obesity and diabetes. These data indicate an important role of oxidative stress in β cell regeneration. Therefore, in this review, we focus on summarizing recent studies reporting the impacts of oxidative stress on β cell regeneration. As such, we do not discuss the impacts of oxidative stress in β cell apoptosis and function. We first overview the susceptibility of β cells to oxidative stress, as well as the molecular mechanisms of β cell regeneration. We then focus on describing recent studies reporting various effects of oxidative stress on β cell regeneration, to deepen our understanding on the broad impacts of oxidative stress on β cells.

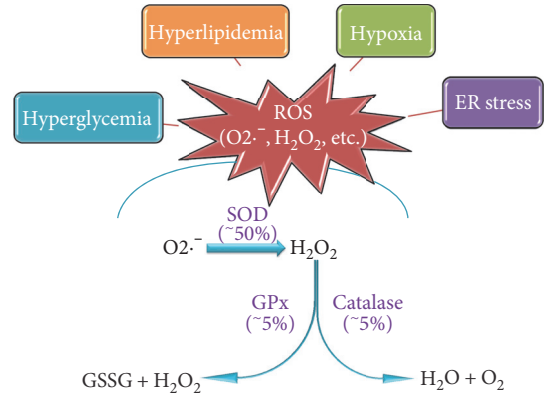

Figure 1: β cells are extremely susceptible to oxidative stress. Two major factors render β cells prone to the risk of oxidative stress: a high endogenous generation of ROS induced by stimuli including hyperglycemia, hyperlipidemia, hypoxia, ER stress, and low expressions of essential antioxidant enzymes such as SOD, catalase, and GPx. Percentages refer to the amount of mRNA expression in pancreatic islets versus liver tissue in rats.

2. Pancreatic β Cells Are Extremely Sensitive to Oxidative Stress

Aerobic cells produce ROS such as superoxide anion ($O2\cdot^{-}$) and H_2O_2 during oxidative phosphorylation in the mitochondria as by-products [7, 8]. Like in other aerobic cell types, mitochondrial electron transport is the main source of superoxide anions of pancreatic β cells. Superoxide anion is a reactive molecule, but it can be converted to H_2O_2 by superoxide dismutase (SOD) isoenzymes and then to oxygen and water by enzymes including catalase (CAT), glutathione peroxidase (GPx), and peroxiredoxin (Prx). Beta cells have lower antioxidative enzymes to combat the continuously generated superoxide anions. They are only equipped with about 50% of the SOD and 5% of H_2O_2-scavenging enzymes GPx and CAT compared to those enzymes found in the liver (Figure 1) [9]. This makes β cells highly sensitive to ROS-related signaling and distinctively susceptible to oxidative and cytotoxicity stress. Indeed, a substantial amount of evidence supports the notion that intrinsically low levels of antioxidant activity of islets render them particularly at risk for ROS-induced damage [10–13].

Several conditions leading to ROS generation in β cells have been proposed, among which are hyperglycemia, hyperlipidemia, hypoxia, and endoplasmic reticulum (ER) stress (Figure 1). Hyperglycemia, defining diabetes, can be directly associated with increased ROS generation through a variety of mechanisms [13]. During chronic hyperglycemia, β cells are exposed to high glucose concentrations for an extended period of time. In this context, the normal route of glycolysis gets saturated and excess glucose is shunted towards alternative ROS-forming pathways including glycosylation (Schiff reaction) [14], glucose autoxidation [15, 16], and glucosamine pathway [17], all of which lead to the accumulation of ROS and induction of oxidative stress.

In addition to hyperglycemia, exposure to excessive lipid (hyperlipidemia) has also been shown to activate cell stress responses including oxidative stress, which contributes to lipotoxicity in β cells in T2D [18]. An *in vitro* study using prolonged exposure to free fatty acid (FFA) exhibited increased islet ROS production in mitochondria, which was prevented by overexpression of the enzyme, GPx4 [19]. The mechanism by which FFA promotes ROS generation in mitochondria remains unclear. One possible explanation is the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase as evidenced by Morgan et al., who reported an increase of the p47 (phox) component of the NADPH oxidase and subsequent production of ROS in β cells after 24 hours incubation with palmitic acid [20]. Koulajian et al. further reported that p47 (phox)-null mice or the treatment of an NADPH oxidase inhibitor protected islets from oleate-induced increased ROS production [21]. Another mechanism that contributes to lipid-induced oxidative stress in β cells is the modulation of respiratory chain by FFA. β cells exposed to FFA exhibited increased ROS production, and respiratory complex I in mitochondria seemed to be the major radical source [22].

In β cells, hypoxia is induced when the cells are exposed to high glucose, with significant upregulations of several hypoxia-related genes including adrenomedullin (*Adm*) and pyruvate dehydrogenase kinase 1 (*Pdk1*) [23]. In addition, prediabetic Zucker rat islets showed increased expression of hypoxia-related genes along with a severely disturbed vascular integrity, strongly suggesting the presence of hypoxia in islets in the development of T2D [24]. It is well-established that hypoxia or low oxygen tension could paradoxically lead to increased ROS generation, majorly within complexes I and III of the mitochondrial electron transport chain [6, 25], making hypoxia a resource for ROS presented in pancreatic β cells.

ER stress is also closely entwined with oxidative stress, especially in insulin-producing β cells. ER stress occurs when the level of misfolded protein, mostly proinsulin in the case of β cells, exceeds ER adaptive capabilities which leads to a

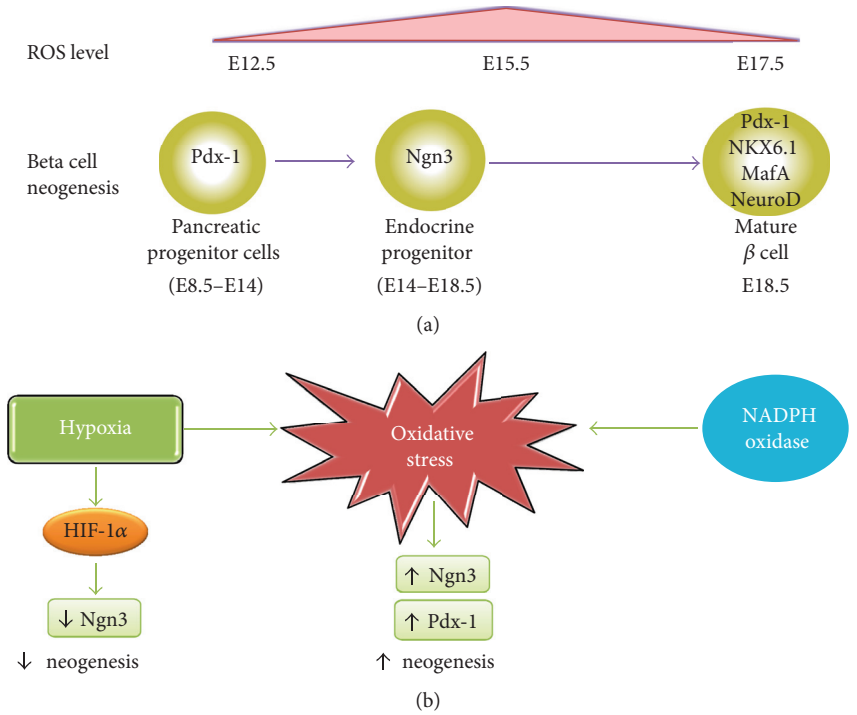

Figure 2: Effects of oxidative stress in β cell neogenesis. (a) An overview of the ROS expression levels at distinct stages of β cell development in mouse embryo. The presence of ROS (E12.5–E17.5) largely overlaps with the Ngn3 expression period (E14–E18.5). (b) Schematic diagram of effects of ROS on β cell neogenesis. On the one hand, hypoxia as an important inducer of ROS activates HIF-1α which suppresses Ngn3 expression, which results in impaired β cell neogenesis. On the other hand, ROS directly upregulates Ngn3 and Pdx-1 in β cell development, and NADPH oxidase seems to be a crucial source of ROS in this process, leading to increased β cell neogenesis.

panel of signaling events leading to reduced insulin transcription and translation [26]. Hydrogen peroxide is generated as a by-product during the formation of oxidized protein in the ER, which is an important source of cellular ROS. Furthermore, under the condition of ER stress, the accumulation of dysregulated disulfide bond formation and breakage results in an excessive amount of ROS which also causes oxidative stress [27]. Additionally, ER stress activates C/EBP homologous protein (CHOP) that is demonstrated to contribute to the induction of oxidative stress during ER stress [28]. In summary, these data suggest that ROS is continuously generated in β cells at a considerable amount, addressing the significance of understanding the impacts of ROS and oxidative stress on β cells.

3. β Cell Neogenesis and Replication

Pancreas provides new β cells through neogenesis (development of β cells from non-β cell precursors) and through replication (mitotic division) of differentiated β cells. Beta cell neogenesis occurs mostly before birth. The neogenesis begins from a pool of pancreatic progenitor cells that express transcription factor pancreatic duodenal homeobox-1 (Pdx-1) starting at embryonic day 8.5 (E8.5) in mice. Later on, a subset of cells that transiently express the transcription factor neurogenin-3 (Ngn-3) between E14 and E18.5 (endocrine progenitor cells) marks the onset of endocrine cell differentiation [29]. Mature β cells eventually express high levels of transcriptional factors including Pdx-1, Nkx6.1, MafA, and NeuroD (Figure 2(a) [30, 31]. The fastest expansion of the β cell mass occurs in late fetal gestation. In rat fetus, β cell population was estimated to be doubled every day starting from 16 days postconception [32]. Similarly, in human fetal pancreas, rapid expansion of the β cell mass has been noted from 20-week fetuses [33]. Although β cell number is usually considered to be stable after birth, accumulating evidence indicates the presence of postnatal β cell replication. Meier et al. observed a 7-fold expansion of β cell mass in human from birth to adulthood [34], and the major mechanism for this is attributable to β cell replication, rather than neogenesis, which is consistent with studies in mice [35]. Beta cell replication rates in adult islets decline substantially (from ~20% per day in pups to ~2% in early adulthood [36] and then approaches 0 in aged animal [37]). Despite the very slow turnover rate of β cells, plenty of evidence indicates that β cell is capable of regeneration under certain physiological or pathological conditions in adults, such as pregnancy, obesity, or diabetes, to meet increased metabolic demands. Significantly, increased number of β cells is observed in response to elevated demand of insulin in several rodent models of obesity and diabetes, and the main mechanism underlying this is the proliferation of fully differentiated β cells [2, 38, 39].

4. The Role of Oxidative Stress in β Cell Neogenesis

Growing evidence indicates that low oxygen tension or hypoxia controls the stemness and lineage commitment of

several precursor cell types during development, including pancreatic β cells. Recently, Liang et al. observed a dynamic change of ROS levels in pancreas development, where ROS was detected in mouse embryonic pancreas as early as E12.5 and peaked at E15.5 (Figure 2(b)) [40]. By culturing embryonic pancreatic cells under controlled O_2 concentrations, researchers reported that β cell neogenesis is positively controlled by oxygen tension in a dose-dependent manner through hypoxia-inducible factor- (HIF-) α. They found that repressed HIF-1α expression led to elevated development of Ngn3-positive endocrine progenitors, which resulted in increased β cell neogenesis and development [41]. The involvement of hypoxia-induced HIF-1α in Ngn3 expression and β cell neogenesis was also confirmed in both human and mice embryonic pancreas [42]. The deletion of von Hippel-Lindau (VHL), a gene encoding a protein necessary for the proteasomal degradation of HIF-1α, led to impaired β cell development, which further confirmed the negative impact of HIF-1α on β cell neogenesis [42]. Interestingly, although oxidative stress was observed in embryonic pancreas under hypoxia condition (3% pO_2), it seemed to have little effect on hypoxia-induced Ngn3 suppression (Figure 2(b)) [42].

Despite the suppressive effect of hypoxia on β cell neogenesis, emerging evidence indicates that ROS by themselves could stimulate β cell regeneration. The inhibition of NADPH oxidase, which is the major source of endogenous ROS, reduced the expression of markers for endocrine cell differentiation including Ngn3 and impaired the differentiation of endocrine progenitors in cultured pancreatic rudiments. Addition of exogenous ROS reversed this effect, which further confirmed that Ngn3 expression is ROS-dependent. Newborn rats exhibit spontaneous remission of β cell shortly after streptozotocin- (STZ-) induced damage. Interestingly, the administration of NADPH oxidase inhibitor attenuated β cell regeneration after STZ injection in rat pups, supporting a positive effect of NADPH oxidase-derived ROS on progenitor cell differentiation [40]. In accordance with this, mouse pancreatic explant treated with angiotensin (1–7) resulted in an increased level of ROS, which is accompanied with elevated expression Ngn3. This was also blocked by the presence of an NADPH oxidase inhibitor [43]. Moreover, Hoarau et al. reported that H_2O_2 enhances the β cell neogenesis in rat embryonic pancreas, and the treatment of pregnant rats with an antioxidant agent, N-acetyl-cysteine (NAC), decreases β cell differentiation in their progeny. They proposed that extracellular signal-regulated kinases 1/2 (ERK1/2) pathway was activated by H_2O_2 and plays a critical role in ROS-induced β cell neogenesis [44]. Recently, Sun et al. discovered that hypoxia condition (2% pO_2) effectively directs mesenchymal stem cell (MSC) differentiation into early β cell progenitors that were further induced into insulin-producing β cells. They detected significantly higher levels of β cell differentiation markers Pdx-1 and HNF6 in MSCs treated with hypoxia compared with the normoxia group; however, Ngn3 expression seemed to be impaired under this condition (Figure 2(b)) [45].

5. Oxidative Stress Influences Cell-Cycle Regulators in β Cell Proliferation

Like in other cells in mammals, cell cycle in β cells is governed intracellularly by various complexes of cyclins and cyclin-dependent kinases (CDKs) and by their inhibitors. Cyclins D1 and D2 are proposed to be essential for postnatal β cell growth in rodents and regulate compensatory β cell replication in response to excessive nutrient or insulin resistance [35, 46, 47]. CDK inhibitors, such as the Cdk-interacting protein (CIP)/the kinase inhibitor protein (KIP) families including p21 and p27, are involved in cyclin binding and kinase inhibitory function. A high-throughput RNAi screening strategy demonstrated that silencing p21 facilitated the cell-cycle entry of quiescent adult human pancreatic β cells. Overexpression of p27 in β cells causes hyperglycemia by four weeks of age and markedly reduced islet mass by 8 weeks of age [48]. These studies demonstrated critical roles of p21 and p27 in β cell proliferation. However, how these cell-cycle regulators are modulated in β cells remains unclear. Emerging evidence suggests that oxidative stress may play a role in regulating cell cycle (Figure 3). Kaneto et al. reported that H_2O_2-induced oxidative stress significantly elevated p21 mRNA in rat islets. Notably, they also found that p21 mRNA increased as hyperglycemia becomes evident in Zucker diabetic fatty (ZDF) rats *in vivo* [49]. In line with this, Zhang et al. observed that intermittent high glucose induced an elevation of intracellular ROS production, leading to decreased cyclin D1 expression as well as increased p21 and p27 expression, and resulted in significantly reduced proliferation rate in the INS-1 β cell line [50]. Nuclear receptor subfamily 2 group E member 1 (NE2E1), an essential regulator of the growth of neural stem cells, has been reported to regulate β cell proliferation. Knockdown of NE2E1 in another β cell line, the MIN6 cells, resulted in decreased proliferation with a partial G0/G1 cell-cycle arrest. Interestingly, NE2E1 deficiency also led to decreased antioxidant enzymes and an augmentation of palmitate-induced oxidative stress in β cells, suggesting a potential role of imbalanced oxidant/antioxidant system in the regulation of β cell proliferation [51].

On the contrary, intermittent hypoxia, which mimics the hypoxic stress present in obstructive sleep apnoea, seemed to trigger β cell proliferation. Yokoe et al. found that mice exposed to intermittent hypoxia exhibited substantially higher proliferation rate in their pancreatic β cells compared with those in the normoxia group [52]. Similarly, Xu et al. applied intermittent hypoxia and observed a significant elevation of β cell proliferation both *in vitro* and *in vivo*, which coincided with increased cyclin D2 translocation to the nucleus. However, overexpression of MnSOD did not affect the mitogenic effect of intermittent hypoxia [53]. Nonetheless, the molecular mechanism underlying intermittent hypoxia-induced β cell proliferation is still indefinite. The experimental differences between intermittent hypoxia and other induction methods of oxidative stress (H_2O_2 treatment or high glucose as mentioned above) may help explain these seemingly contradictory findings.

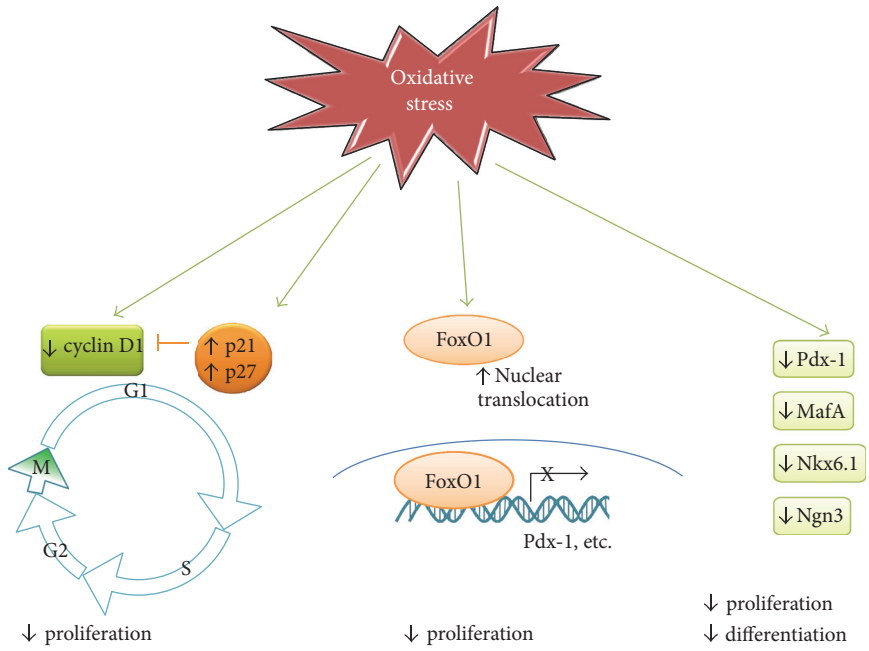

Figure 3: Oxidative stress plays critical roles in β cell cycle. On cell-cycle regulators, accumulated ROS in beta cells suppressed the expression of cyclins D1 and D2, as well as increased cell-cycle inhibitors such as p21 and p27, leading to decreased β cell proliferation rate. Meanwhile, ROS promoted nuclear the translocation/activation of FoxO1, which in turn prevents β cell replication through the inhibition of Pdx-1 and possibly other β cell-related gene transcriptions. Furthermore, ROS also directly downregulates transcription factors such as Pdx-1, MafA, Nkx6.1, and Ngn3 that are crucial in beta cell proliferation and differentiation.

6. Effects of Oxidative Stress on Transcription Factors for β Cell Regeneration

Forkhead box class O family member proteins (FOXOs) are transcription factors that play important roles in β cell differentiation, proliferation, and survival [54]. Among them, FoxO1 is the most predominantly expressed FoxO factor in isolated mouse islets, the insulinoma cell line (βTC3 cells) and human islets [54]. FoxO1 negatively regulates β cell proliferation and differentiation through two mechanisms by the modulation of Pdx-1 gene transcription and nuclear translocation. Firstly, FoxO1 functions as a repressor to compete with the transcription factor FoxA2 for binding to Pdx-1 promoter, leading to the reduction of Pdx-1 transcription and impaired β cell growth [55]. Secondly, the nuclear translocation of FoxO1 accompanies with the nuclear exclusion of Pdx-1, which further suppresses β cell proliferation [55]. In contrast to its inhibitory effects on β cell proliferation, FoxO1 seems to exert a protective effect against oxidative stress-induced damage. It was proposed that FoxO1 facilitated cells that undergo a state of "premature senescence" to protect β cells against hyperglycemia-induced oxidative stress [56, 57]. In cultured β cells, FoxO1 is constitutively phosphorylated and remained inactivated in cytoplasm. Upon exposure to hydrogen peroxide, around 30% FoxO1 translocate into nuclei in βTC3 cells, which resulted in growth arrest [58]. Further studies showed that upon oxidative stress, FoxO1 formed a complex with the promyelocytic leukemia protein (Pml) and the NAD-dependent deacetylase sirtuin-1 (Sirt1), leading to upregulated NeuroD and MafA expression, both of which are important transcription factors for β cell development and Ins2 gene transcription. However, the tradeoff of Pml-mediated transcription activity of FoxO1 accelerated the degradation of this protein [58]. Therefore, it is obvious that the transient effects of FoxO1-induced upregulation of MafA and NeuroD are not sufficient to counteract the replication inhibition of FoxO1 nuclear translocation, resulting in the net effect of growth arrest under oxidative stress in β cells (Figure 2).

In fact, MafA plays a pivotal role in regulating the replication and development of β cells [59] and is exceptionally sensitive to oxidative stress. Under H_2O_2-induced oxidative stress, MafA is inactivated and translocated to cytoplasm within 30 min. The inactivation of Pdx-1 and Nkx6.1, two transcription factors required for β cell differentiation, was observed upon H_2O_2 treatment. Importantly, levels of MafA and Nkx6.1 were also severely compromised in human T2D islets as well as hyperglycemic leptin-receptor-deficient (db/db) mouse islets. Further studies on transgenic db/db mice overexpressing the antioxidant enzyme Gpx1 showed that the nuclear MafA and Nkx6.1 levels were restored, confirming that loss of these important β cell transcription factors in T2D is oxidative stress-dependent [60]. Chiou et al. showed that overexpression of MafA facilitated placenta-derived mesenchymal stem cells to differentiate into mature insulin-producing cells and rendered those insulin positive cells more resistant to H_2O_2-induced oxidative stress [61].

As discussed previously, ER stress contributes to ROS generation and induction of oxidative stress in β cells. Studies have shown that ER stress also significantly impacts β cells'

regeneration. Accumulating evidence suggests that protein kinase-R-like ER kinase (PERK) and its substrate eukaryotic translation initiation factor 2α (eIF2α), major components in ER stress, are critical in the control of β cell proliferation and differentiation [62–66]. Mutations in PERK (EIF2AK3) cause a complex genetic disorder of the Wolcott Rallison syndrome with permanent neonatal diabetes characterized by β cell depletion [67]. Mice lacking PERK or PERK-mediated eIF2α phosphorylation develop a similar phenotype with early onset diabetes and reduced β cell mass, accompanied with decreased expression of Pdx-1 and MafA [63–65]. Detailed studies suggest that in the absence of PERK-mediated eIF2α phosphorylation, islets/β cells exhibited to be under significant oxidative stress as indicated by peroxynitrite modification of tyrosine residues resulted from the reaction between superoxide and nitric oxide. It seemed that the defects of β cell proliferation and differentiation are partly attributable to uncontrolled oxidative stress in the absence of functional PERK/eIF2α pathway [63].

7. Effects of Antioxidants on β Cell Proliferation in T2D

Supplements with antioxidant properties have long been used to decrease oxidative stress and improved pancreatic β cell proliferation and function in T2D [68]. Selenium is a necessary trace element in the body that can act as an antioxidant nutrient in different cell types via incorporation of selenocysteine into selenoproteins through a UGA codon-encoded genetic process. Experimental data suggest that supplementation with selenium as an antioxidant could delay the development of T2D by decreasing oxidative stress [69]. Wang et al. found that long-term administration of dietary selenate supplementation to db/db mice suppressed the elevation of fasting glucose level. Further study showed that selenate supplementation significantly increased islet size, along with the upregulation of genes that encode proteins involved in β cell proliferation and differentiation, suggesting a positive role of antioxidant in the proliferation of β cells in the progression of diabetes [70]. Consistent with this study, Chang et al. discovered that a *Stigmata maydis* (corn silk) fraction exhibited antioxidant effect in hydrogen peroxide- or methylglyoxal-induced oxidative stress in a rat β cell line. They demonstrated that this fraction of corn silk attenuated the suppression of β cell proliferation induced by acute H_2O_2 treatment, indicating a potential benefit of proproliferation in β cells under oxidative stress in T2D [71]. Bitter gourd (*Momordica charantia* Linn.) is a type of well-established remedy food for diabetes patients. The boiled water extraction of sun-dried bitter gourd possesses remarkable free radical scavenging activity, and studies showed that the higher antioxidant activity of the fraction, a corresponding higher proliferation activity was observed on the treated β cells [72]. In addition, a number of natural phenolic compounds, including quercetin, catechin, and ascorbic acid, exhibited great antioxidant properties in various cells [73]. Interestingly, although in normal growth media, these phenolic compounds even amplified H_2O_2-induced proliferation inhibition in β cells at higher concentrations, it was demonstrated that this was caused by a quick generation of H_2O_2 from the incubation of these compounds with the media. Once this is eliminated, the growth inhibition of β cells induced by H_2O_2 treatment was efficiently suppressed by these compounds [74]. These observations strongly suggest that the enhancement of β cell proliferation contributes to the beneficial effects of antioxidants used in T2D.

8. Conclusion

Currently, there is considerable interest in targeting β cell regeneration as an effective approach to replenish insulin for patients with diabetes. Recent insights indicated that oxidative stress and β cell regeneration are highly interrelated biological process, not only demonstrated by the fact that they coexist under several physiological and pathological conditions but also reflected by the profound direct and indirect impacts of oxidative stress on β cell regeneration. The roles of oxidative stress in β cell neogenesis and proliferation are complicated by different stages of cellular growth and different stimuli of the stress. It seems that oxidative stress majorly exerts a negative effect on β cell development and proliferation. However, ROS seems to be temporarily required for β cell neogenesis in embryonic pancreas and during β cell replication shortly after birth. Importantly, several antioxidant supplements increased proliferation rates of pancreatic β cells, which not only confirmed the deleterious effects of oxidative stress on β cell regeneration but also suggested potential benefits of antioxidants as a therapeutic method for T2D patients, in respect of improving β cell regeneration. In summary, oxidative stress plays critical roles during pancreatic β cell neogenesis and proliferation, as well as during the development of T2D.

Abbreviations

T2D: Type 2 diabetes
ROS: Reactive oxygen species
ATP: Adenosine triphosphate
SOD: Superoxide dismutase
CAT: Catalase
Gpx: Glutathione peroxidase
Prx: Peroxiredoxin
ER: Endoplasmic reticulum
FFA: Free fatty acid
CHOP: C/EBP homologous protein
Pdx-1: Pancreatic duodenal homeobox-1
HIF-1α: Hypoxia-inducible factor-1 α
STZ: Streptozotocin
CDK: Cyclin-dependent kinase
FOXO: Forkhead box class O family protein
PERK: Protein kinase-R-like ER kinase.

Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Review Article

Pro- and Antioxidant Functions of the Peroxisome-Mitochondria Connection and Its Impact on Aging and Disease

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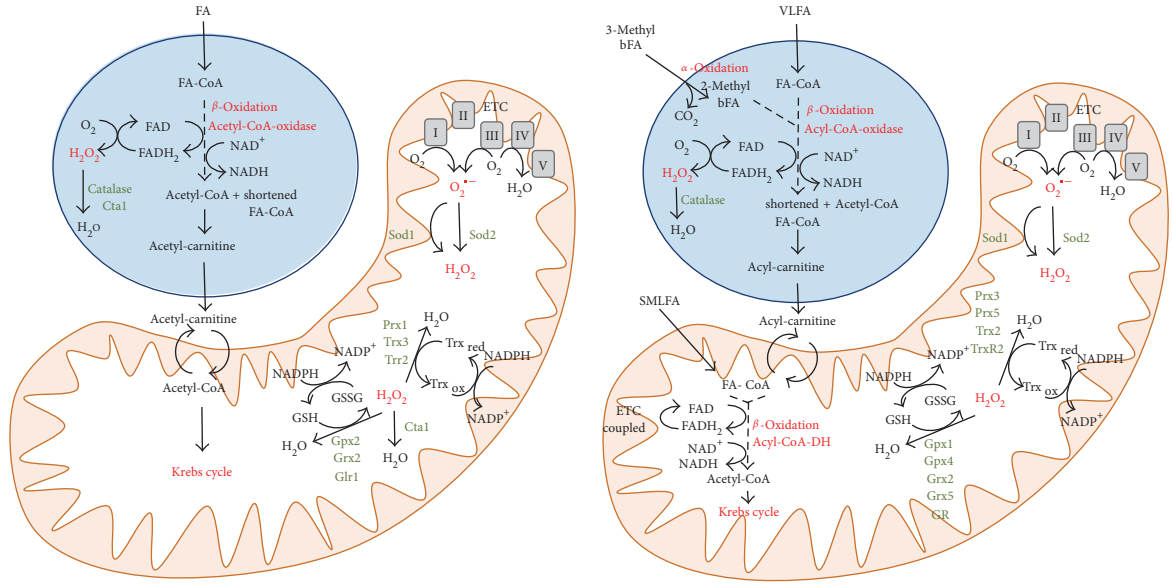
Peroxisomes and mitochondria are the main intracellular sources for reactive oxygen species. At the same time, both organelles are critical for the maintenance of a healthy redox balance in the cell. Consequently, failure in the function of both organelles is causally linked to oxidative stress and accelerated aging. However, it has become clear that peroxisomes and mitochondria are much more intimately connected both physiologically and structurally. Both organelles share common fission components to dynamically respond to environmental cues, and the autophagic turnover of both peroxisomes and mitochondria is decisive for cellular homeostasis. Moreover, peroxisomes can physically associate with mitochondria via specific protein complexes. Therefore, the structural and functional connection of both organelles is a critical and dynamic feature in the regulation of oxidative metabolism, whose dynamic nature will be revealed in the future. In this review, we will focus on fundamental aspects of the peroxisome-mitochondria interplay derived from simple models such as yeast and move onto discussing the impact of an impaired peroxisomal and mitochondrial homeostasis on ROS production, aging, and disease in humans.

1. Introduction

Oxidative stress is causally linked to accelerated aging and aging-related diseases [1]. In eukaryotic cells, mitochondria and peroxisomes are the main ROS contributors [2, 3]. At the same time, both organelles are equipped with their own ROS scavenging repertoire of enzymes. Mitochondria and peroxisomes are metabolically linked because they share common pathways such as the fatty acid β-oxidation. However, both organelles are much more intimately regulated with respect to ROS homeostasis and the clearance of dysfunctional organelles. Here, we will focus on our current knowledge of mitochondrial and peroxisomal pro- and antioxidant functions, their coordinated turnover by autophagic pathways, and their functional and physical interactions. Investigation in the yeast model is contributing fundamental insights into the mechanisms of quality control of peroxisomes and mitochondria. Therefore, we will compare the dynamics of both organelles and its impact on cell survival and health in yeast and mammalian cells throughout the review.

2. Pro- and Antioxidant Functions of Peroxisomes

Peroxisomes are versatile organelles found in most eukaryotic cells. The name peroxisome has been originally introduced for a cellular organelle which contains at least one H_2O_2-producing oxidase and the H_2O_2-detoxifying enzyme catalase [4]. Today, we know that peroxisomal function goes far beyond the metabolism of reactive oxygen species. Peroxisomes contain more than 50 different enzymatic activities, which shape important metabolic and anabolic functions, including fatty acid oxidation and lipid biosynthesis. As they share an oxidative metabolism together with mitochondria, they are key organelles in the cellular homeostasis of ROS



(a) Yeast (b) Mammalian cells

Figure 1: Pro- and antioxidant functions of peroxisomes and mitochondria in yeast and mammalian cells. Peroxisomes play important roles in the oxidative degradation of fatty acids. The peroxisomal fatty acid oxidation pathways are schematically depicted for budding yeast (a) and mammalian cells (b). Mitochondrial pro- and antioxidant functions and their interconnection with peroxisomes are summarized. ROS-generating and ROS-scavenging functions are highlighted for both organelles. FA = fatty acid; bFA = branched fatty acid; VLFA = very long fatty acid; SMLFA = small, medium, and long fatty acids; FA-CoA = fatty acyl coenzyme A; ETC = electron transport chain; Gpx = glutathione peroxidase; Grx = glutaredoxin; GR and Glr = glutathione reductase; Sod = superoxide dismutase; GSH = reduced glutathione; GSSG = oxidized glutathione; Prx = peroxiredoxin; Trx = thioredoxin; Trr and TrxR = thioredoxin reductase; DH = dehydrogenase; I to V = mitochondrial respiratory complexes I–V.

[5, 6]. Although the variability of the metabolic activities contained by peroxisomes of different organisms or tissues is considerable, their universal functions are the oxidative metabolism of fatty acids and the degradation of H_2O_2 by catalases [7]. Thus, peroxisomes are both sources and sinks of ROS and account for a great part of oxygen consumption and ROS production (up to 35%) in metabolically active tissues such as the liver [4, 8]. Peroxisomes are much more dynamic organelles than anticipated, and the impact of the dynamic regulation of their size, abundance, morphology, and function in response to environmental stimuli or during development has only recently been addressed [9–11]. Given the dynamic nature of peroxisomes and their central role in fatty acid oxidation, an efficient quality control of this organelle is required to avoid oxidative damage and premature aging [3, 5, 12].

The oxidative degradation of fatty acids takes place in peroxisomes and mitochondria (Figure 1). However, the compartmentalization of fatty acid oxidation pathways between these two organelles is variable in different organisms. Budding yeast, for example, degrades fatty acids exclusively in peroxisomes via β-oxidation, which implies the removal of two carbons from the fatty acid carboxyl terminus [13]. The first step in this process is catalyzed by acyl-CoA oxidases, which reduce O_2 to H_2O_2. The end product of β-oxidation in yeast, acetyl-CoA, is transformed to acetyl-carnitine by peroxisomal carnitine acetyl-CoA transferases and shuttled into mitochondria to fuel the Krebs cycle and respiration. In human cells, mitochondria and peroxisomes cooperate in different routes of fatty acid oxidation [14]. Here, fatty acids with shorter chain lengths (C_6-C_{20}) are oxidized by β-oxidation in mitochondria, where the first step is catalyzed by acyl-CoA dehydrogenases coupled to the mitochondrial electron transport and not to H_2O_2 production. However, fatty acids with longer chain lengths (>C_{20}) or dicarboxylic fatty acids have to be degraded by the peroxisomal β-oxidation pathway. Additionally, 3-methyl-branched fatty acids are exclusively metabolized in human peroxisomes by the process of α-oxidation, which represents an additional source of the ROS H_2O_2 originating from these organelles. In the human case, β-oxidation is not necessarily carried out to completion in peroxisomes; hence, the final product is a shortened fatty acyl-CoA, which is shuttled to mitochondria in the form of the corresponding fatty acyl carnitine conjugate. It is worth noting that ROS production by mammalian peroxisomes is not limited to the fatty acyl-CoA oxidases of the β-oxidation pathway. Additionally, oxidases participating in glyoxylate metabolism, amino acid catabolism, or the oxidation of polyamines are potential ROS sources of the peroxisome [15].

Peroxisomes contain several antioxidant systems, which are important for ROS homeostasis of this highly oxidative organelle (Figure 1). Since the β-oxidation pathway directly produces H_2O_2, the detoxifying catalase activity is of central importance for the redox balance of the organelle [16, 17]. It has been shown in yeast that peroxisomal catalase is

required also for the tolerance to externally added H_2O_2, which suggests that peroxisomes are generally important for cellular ROS detoxification [18]. An additional peroxisomal antioxidant activity has been described in yeast as the glutathione peroxidase Gpx1, whose activity is generally required for a proper peroxisomal biogenesis [19]. Also in mammalian cells, catalase is the predominant antioxidant activity of peroxisomes. However, several other ROS detoxifying are known in higher eukaryotes to contribute to peroxisomal redox balance, which include superoxide dismutases, peroxiredoxins, glutathione S-transferases, and epoxide hydrolases [5].

The importance of peroxisomes in the maintenance of a healthy redox balance has been derived from situations, which interfere with a normal peroxisomal function and biogenesis. Artificial stimulation of peroxisomal biogenesis by the long-term exposure to peroxisomal proliferators causes oxidative liver damage and ROS imbalances in general in rodents [20, 21], most likely by the uncompensated induction of ROS-generating peroxisomal enzymes [22]. However, stimulated peroxisomal proliferation seems to reduce ROS levels in murine neurons [23]. Moreover, the loss of peroxisomes increases the apoptotic damage in the cerebellum and neurodegeneration [24, 25], and a deficiency in human catalase activity increases the susceptibility to aging-related diseases such as diabetes, cancer, or atherosclerosis [26]. Moreover, catalase mutants show phenotypes of accelerated aging in the Caenorhabditis model [27]. In yeast, loss of catalase function modulates the lifespan depending on the metabolic activity of peroxisomes [18]. Thus, peroxisomal (together with mitochondrial) activity in maintaining the cellular redox state is generally important for cell survival and health [28, 29].

3. Pro- and Antioxidant Functions of Mitochondria

Mitochondria have been traditionally described as the cellular power-house as they are the main contributors of energy through oxidative phosphorylation and the biosynthesis of ATP. Structurally, they contain two biologically different membrane systems: the outer membrane containing the intermembrane space and the inner membrane containing the mitochondrial matrix. The different electron transport chain (ETC) protein complexes are embedded in the inner membrane, which is heavily folded into the cristae structure [30]. Apart from regulating the energy metabolism of the eukaryotic cell, mitochondria are involved in many other fundamental processes, such as the control of the cell cycle or the induction or prevention of cell death [31–33].

Generation of ATP by the mitochondrial oxidative phosphorylation is based on the reduction of molecular oxygen, which can produce toxic byproducts such as the superoxide radical ($O_2^{\cdot-}$) or hydrogen peroxide (H_2O_2). Only complex IV (cytochrome *c* oxidase) of the ETC is able to fully reduce molecular oxygen to water and therefore has an antioxidant function in the ETC, while mainly complex I (NADH ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome *c* oxidoreductase) contribute to superoxide radical formation due to incomplete O_2 reduction [34]. These ROS can trigger the formation of even more harmful secondary ROS such as the hydroxyl radical ($^{\cdot}OH$), which are supposed to be responsible for the main oxidative damage derived from mitochondrial activity [35, 36]. Electron leakage from mitochondrial electron transport chains is now recognized as one of the most important intracellular sources of ROS including hydroxyl radicals [37]. Mitochondria possess their own antioxidant systems, which consist of various ROS-scavenging enzymes and low molecular antioxidants [38, 39]. Primary antioxidant enzymes are superoxide dismutases (SOD), which reduce superoxide radicals to hydrogen peroxide. Two types of SOD can be found in mammalian mitochondria: manganese superoxide dismutase (SOD2) in the mitochondrial matrix and copper/zinc superoxide dismutase (SOD1) in the intermembrane space [40–42]. Detoxification of mitochondrial H_2O_2 can be achieved by the activity of glutathione peroxidases (GPx) and glutaredoxins (GRx). At least two GPx isoforms have been identified in the mammalian mitochondria, GPx1 and GPx4 [43, 44], and two Grx isoenzymes are active in mitochondria, Grx2 and Grx5 [45]. During this reaction, glutathione is oxidized, which has to be reduced by mitochondrial glutathione reductase (GR) with the help of the mitochondrial NADPH pool. Additionally, hydrogen peroxide can be reduced by mitochondrial peroxiredoxins (Prx) coupled to the oxidation of thioredoxins (Trx), which are finally recycled at the expense of NADPH by Trx reductases (TrxR). In the mammalian mitochondria, at least the isoenzymes Prx3, Prx5, and Trx2 have been described [46–50]. Among the nonenzymatic antioxidants found in the mammalian mitochondria, the high NADPH pool, vitamin C, and coenzyme Q have been reported with important functions in ROS elimination [51–53]. As opposed to peroxisomes, significant catalase activity is absent from the mammalian mitochondria. Yeast mitochondria also contain a full set of antioxidant systems based on glutathione or thioredoxin with the enzymes Gpx2, Grx2, Glr1, Prx1, Trx3, and Trr2 [54]. Also in yeast, the Sod1 Cu/Zn- and the Sod2 manganese-superoxide dismutases are the primary ROS-reducing enzymes in mitochondria [55, 56]. Additionally, and different to the situation in mammalian cells, the peroxisomal catalase Cta1 can also be found inside the yeast mitochondria [57].

The free radical theory of aging first postulated that the accumulation of cellular damage caused by free radicals was a decisive driving force in the process of aging and in the determination of a lifespan [58]. A major prediction derived from this theory was that the supplementation with antioxidants or genetic manipulation of endogenous ROS-scavenging systems would have a beneficial effect on the lifespan of the organism. However, no conclusive results were obtained by studies of this type in general [59–64], which led to a revision of the original hypothesis in a form of the mitochondrial-free radical theory [65]. Here, mitochondrially generated free radicals were supposed to have a major effect on aging, and thus it was proposed that reinforcement of mitochondrial antioxidant systems had lifespan extension effects. On the contrary, mitochondrial damage or insufficient ROS-scavenging activity of mitochondria could lead

to lifespan shortening. A large body of evidence has accumulated, which supports this hypothesis [66–68]. Artificial targeting of catalase to mitochondria extends the lifespan of mice [69]. Similarly, the mitochondria-targeted antioxidant SkQ1 has lifespan extension effects in several rodent models [70]. On the contrary, mouse models, with mutations in the mitochondrial DNA polymerase gamma, have a shorter lifespan and manifest several phenotypes of accelerated aging [71]. Furthermore, the accumulation of damage in the mitochondrial DNA (mtDNA) caused in these mouse models has been reported to favor apoptosis and age-related diseases [72] and can be reverted by mitochondria-targeted catalase [73]. Moreover, deficiencies in Mn-superoxide dismutase activity increases oxidative stress and promotes aging in Drosophila [74–76]. Yeast has very efficiently contributed to reveal physiologically relevant determinants of lifespan and health span in higher organisms [77, 78]. Also in this simple model, mitochondria have been identified with an important role in the establishment of lifespan [79]. Specifically, yeast *sod* mutants are known to critically interfere with longevity [80]. Having found that mitochondrial ROS production is a driving factor of aging, a growing number of studies now intend to reduce ROS production by the targeted delivery of antioxidants to the mitochondria [1]. These approaches include the use of mitochondria-directed redox agents to detoxify mitochondrial ROS [81] or modulators of mitochondrial electron transport and leakage to avoid mitochondrial ROS [82]. These strategies are beyond the scope of this review; hence, the interested reader might consult summaries made by excellent recent revisions [68, 83, 84].

It is important to note that ROS might not always trigger fatal oxidative damage within a cell. On the contrary, low ROS concentrations have important biological functions related to signaling and stress resistance [85–87]. It has been increasingly clear in the last years that oxidative stress is able to actually promote longevity, which has led to the theory of mitochondrial hormesis. Here, stimuli such as calorie restriction or exercise are supposed to trigger adaptations, which lead to the reinforcement of endogenous antioxidant systems, increased stress tolerance, and subsequently slow down the aging process [88, 89]. There are many experimental findings in different model systems including yeast, Caenorhabditis or Drosophila, which are in line with the mitohormesis hypothesis [90, 91]. Inhibition of respiration, for example, increases the lifespan of nematodes dependent on the production of mitochondrial ROS [92]. Calorie restriction and especially glucose restriction partially induce mitochondrial metabolism and prevent aging from yeast to flies [93, 94]. It has been demonstrated in Caenorhabditis that reduced sugar intake induces mitochondrial respiration and ROS production, which are necessary for lifespan extension, and this beneficial effect is reverted by antioxidants [95]. In yeast, several interventions including the inhibition of nutrient-sensing protein kinases or dietary restriction cause an extended lifespan [96]. These effects have been shown to depend on mitochondrial activity in general or specific mitochondrial ROS-scavenging activities [97–100]. Taken together, mitochondrial ROS balance is decisive for the modulation of longevity and we will continue to summarize how mitochondrial and peroxisomal activity might change during the process of aging and in age-related diseases.

4. Compromised Function of Peroxisomes and Mitochondria during Aging

The investigation of the detrimental function of peroxisomal dysfunction in the process of aging has traditionally lagged behind mitochondria, which were considered the major source of oxidative stress-related senescence. However, it is much more considered now that a misregulated peroxisomal ROS balance is important in the occurrence of age-related diseases [3, 101, 102]. Recently, it has been shown that a deficiency in peroxisomal ROS clearance actually affects mitochondrial function. Specifically, inhibition of peroxisomal catalase activity negatively modulates the redox balance in mouse mitochondria [103] and human fibroblast [104]. Moreover, a localized oxidative damage to peroxisomes leads to mitochondrial fragmentation and cell death [105, 106]. These results reflect the intimate relation of both organelles in the maintenance of the cellular redox balance and furthermore suggest that peroxisomal dysfunction can subsequently damage mitochondria. Therefore, peroxisomes can act at the forefront in the initiation of cellular oxidative damage and aging [3]. Accordingly, several reports support that peroxisomal function continuously declines during aging. In cultured human cells, it has been shown that catalase is increasingly excluded from peroxisomes after repeated cell passage. At the same time, old cells accumulate more dysfunctional peroxisomes which raise cellular ROS levels and ultimately might accelerate aging [107]. In line with these results is the finding that in Caenorhabditis the abundance of many peroxisomal proteins decays during aging. Importantly, this affects the Pex5 importer of peroxisomal matrix proteins [108]. Pex5 function has been found to be regulated by the redox state of the organelle [109, 110]. Thus, the decline of peroxisomal protein import by oxidative stress, including the import of antioxidants, might be a trigger for senescence. Additionally, it has been shown that yeast peroxisome proliferation is regulated by the redox state of the organelle [19]. Oxidative stress might inhibit yeast peroxisomal biogenesis via multiple targets, such as Pex5 or Pex11 [109, 111–113]. Finally, interference with peroxisomal fission increases the lifespan of yeast cells [114]. Taken together, maintaining functional peroxisomes emerges as an important determinant of the cellular lifespan. The relevant mechanisms of peroxisomal homeostasis will be discussed in the following section.

A large body of experiments has demonstrated that mitochondrial dysfunction is an important trigger for cellular senescence, which contributes to aging in addition to other prosenescence stimuli [115–118]. Although it is clear that dysfunctional mitochondria accumulate in senescent cells and that this is a major driving force for accelerated aging [119–121], it is less understood how mitochondria become dysfunctional during the aging process [122, 123]. Mitochondrial defects which accumulate in older cells range from an increased mitochondrial mass, a decrease of respiratory coupling, less efficient ATP production during respiration, loss

of respiratory complex I function, and increased mitochondrial ROS production [117, 118].

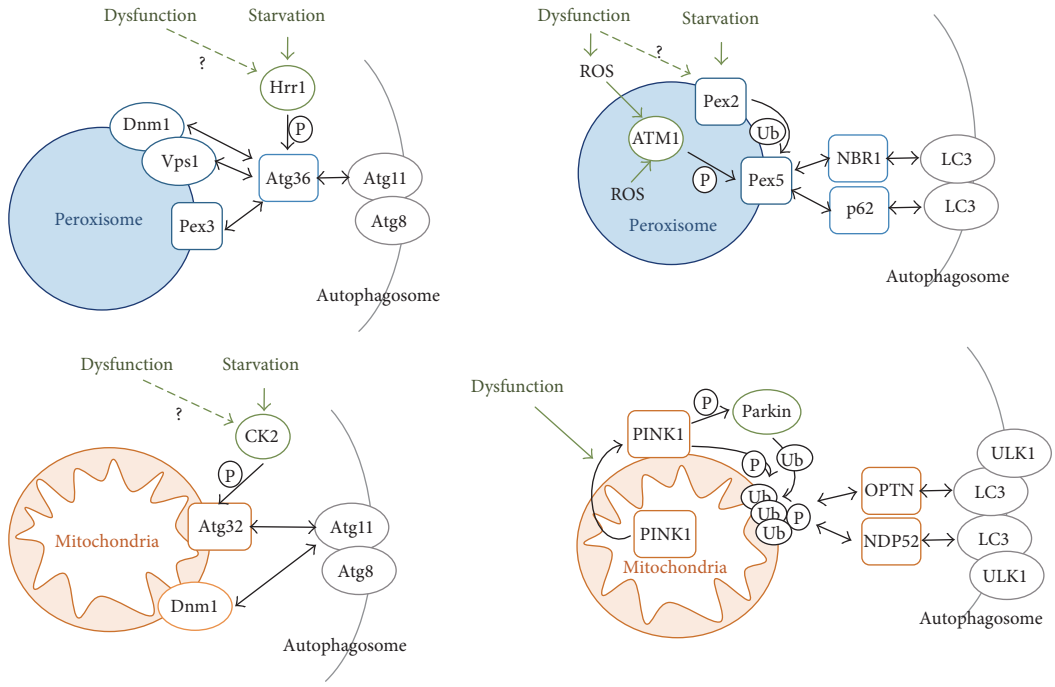
Importantly, there is increasing evidence that misregulated ROS production is an important trigger for mitochondrial dysfunction in the context of several age-related diseases. Cardiomyopathy in the old age, for example, is accompanied by an impairment of oxidative phosphorylation in heart mitochondria [87, 124]. Consequently, the rate of ROS production increases with age especially in cardiac mitochondria [125]. Similarly, an inefficient mitochondrial energy metabolism has been linked to cardiovascular diseases [126, 127]. In order to explain this decline in mitochondrial function, several mechanisms have been suggested, for example, an impaired biogenesis of the organelle, increased mitochondrial uncoupling, or the accumulation of mt-DNA mutations [128–130]. Mitochondrial dysfunction has been broadly implied in age-related neurodegenerative diseases [131–133]. In the particular case of Alzheimer's disease, the accumulation of the toxic aggregates of the unprocessed β-amyloid peptide is known to inhibit the mitochondrial electron transport chain, cause ROS overproduction, and induce mitochondria-mediated cell death [134–138]. Additionally, the accumulation of mt-DNA aberrations and failures in the dynamic regulation of mitochondrial morphology by fission have been linked to Alzheimer's disease [139–141]. Parkinson's disease is another neurodegenerative disorder where mitochondrial dysfunction has been identified as a major driving force. Several genes have been associated with familial Parkinson, and most of these PARK loci are functionally related with mitochondria [142]. PARK1 for example encodes α-synuclein, which can inhibit mitochondrial fusion [143]. Mutations in PARK7 affect DJ-1 function causing defects of mitochondrial respiratory complex I, ROS overproduction, and a loss of mitochondrial membrane potential [144, 145]. PARK8 encodes the LRRK2 protein kinase, whose function is needed for proper oxidative phosphorylation activity and mitochondrial fission [146, 147]. PARK2 and PARK6 encode Parkin and PINK1 (PTEN-induced kinase 1), which are involved in the correct turnover and degradation of dysfunctional mitochondria by mitophagy [148, 149]. The mechanisms of autophagic removal of excess or malfunctioning mitochondria and peroxisomes will be described in the following section.

Mitochondria form dynamic networks, whose morphology changes in response to stress and nutritional stimuli and damage [150, 151]. Several independent experimental evidences exist showing that changes in mitochondrial dynamics are intimately linked to senescence and accelerated aging [152]. During aging, mitochondrial fission is reduced, which leads to mitochondrial elongation [153]. It has been shown that inhibition of the profission protein FIS1 or the E3 ubiquitin ligase MARCH5, which positively regulates mitochondrial fission, induces senescence via mitochondrial elongation [154, 155]. In yeast, the pharmacological repression of mitochondrial fission leads to a lifespan extension [154, 156]. These results indicate that mitochondrial fission is needed for a normal cellular lifespan. This is in agreement with the finding that hyperelongated mitochondria have lower membrane potentials and excessively produce ROS [153, 154]. Additionally, the reinforcement of mitochondrial fission by the overexpression of FIS1 can reduce age-related phenotypes [153]. Importantly, mitochondrial fission is needed for efficient clearance of defective mitochondria via mitophagy, which could additionally explain the antiaging effect of the fission process [157, 158]. In a reverse manner, oxidative stress alters mitochondrial morphology via the modulation of several proteins involved in mitochondrial dynamics and this phenomenon has been linked to cancer progression [159–161]. Exogenous ROS efficiently inhibit mitofusins (Mfn1 and Mfn2) in human fibroblasts and muscle myoblasts inducing mitochondrial fission and membrane depolarization [162, 163]. In yeast, oxidative stress promotes mitochondrial fragmentation via the stimulation of the assembly of the mitochondrial fission machinery composed of Mdv1, the Dnm1 GTPase, and the Fis1 mitochondrial fission proteins [164].

Another important question is whether the asymmetric inheritance of mitochondria of different qualities or activities modulates the aging process. This phenomenon has been extensively studied in the yeast model of replicative aging [165]. During cell divisions, proaging components such as damaged or dysfunctional mitochondria are retained in the older mother cell and not transmitted to the young daughter cell [166]. Specific mitochondrial tether proteins such as Mmr1 have been identified, which actively retain highly oxidative mitochondria in the mother cell. Consequently, interference with this asymmetric mitochondrial distribution by deletion of Mmr1 shortens the yeast lifespan [167]. Additional anchor proteins such as Mfb1 have been identified now, which guarantee the remaining of highly functional mitochondria in the mother cell [168]. Interestingly, the unequal distribution of mitochondria from the mother to daughter cell is conditioned by mitochondrial dynamics since it is impaired in mitochondrial fusion mutants [169]. Taken together, the fusion/fission process of mitochondria (and peroxisomes) is important for the maintenance of a healthy redox balance and lifespan. The quality control of both organelles via autophagic mechanisms is of key importance to maintain both mitochondria and peroxisomes functional.

5. Quality Control of Peroxisomes and Mitochondria by Autophagy and the Impact on Health

The number, activity, and quality of peroxisomes are dynamically adapted and controlled in the cell [170]. An important mechanism to eliminate dysfunctional or superfluous peroxisomes consists in a specific autophagy termed pexophagy [171]. The mechanisms which assure the engulfment of specific peroxisomes in autophagosomes and their subsequent degradation in vacuoles (fungi) or lysosomes (mammals) have been especially elucidated in the yeast model [172]. Here, pexophagy has been intensively studied in response to nutritional changes from respiratory to fermentative growth conditions, which implies the proteolytic removal of excess peroxisomes [172]. The key step in the initiation of



(a) Yeast (b) Mammalian cells

FIGURE 2: Mechanisms of autophagic removal of peroxisomes and mitochondria. In the upper panel, pexophagy mechanisms are depicted for budding yeast (a) and mammalian cells (b). In yeast, pexophagy is induced by the Hrr1 kinase which phosphorylates the Atg36 adaptor. Atg36 contacts the Pex3 peroxisomal receptor, the fission machinery (Dnm1, Vps1), and the autophagosomal adaptor Atg11. In mammalian cells, a dysfunctional peroxisome and general ROS increase activate the ATM kinase, which phoshorylates the Pex5 receptor. Pex5 is additionally targeted by ubiquitination via the starvation-inducible Pex2. Modified Pex5 interacts with the autophagosomal adaptors NBR1 and p62. In the lower panel, mitophagy mechanisms are depicted for budding yeast (left) and mammalian cells (right). In yeast, starvation induces mitophagy via casein kinase 2 (CK2), which phosphorylates the Atg32 receptor. Modified Atg32 interacts with the Atg11 autophagosomal adaptor, which also contacts the mitochondrial fission machinery (Dnm1). In mammalian cells, mitochondrial dysfunction triggers the exposure of PINK1 at the organelle surface. PINK1 phosphorylates and activates the Parkin ubiquitin ligase, which marks outer mitochondrial membrane proteins. PINK1 additionally phosphorylates polyubiquitin chains at mitochondria, which leads to recognition by the autophagosomal adaptor proteins Optineurin (OPTN) and NDP52. P = phosphorylation; Ub = ubiquitination.

pexophagy is the formation of a preautophagosomal structure at the surface of the organelle [173–176] (Figure 2). Several Atg proteins are coordinated in this process termed cytoplasm-to-vacuole targeting; however, the Atg11 cytosolic adaptor protein is the physical link of the organelle to be degraded and the autophagosome in the case of both peroxisomes and mitochondria [175, 176]. Atg11 contacts the peroxisome via a specific adaptor protein at the peroxisomal surface. In budding yeast, the specific adaptor for pexophagy is Atg36, which directly interacts with the peroxisomal Pex3 protein and the core phagosomal components Atg11 and Atg8 [177, 178]. One way to induce pexophagy is through the phosphorylation of Atg36 by the Hrr25 casein kinase homolog [179]. Hrr25 phosphorylates a specific serine residue (S97) in the Atg36 adaptor, which triggers its interaction with Atg11 but not with Pex3 [179]. Therefore, phosphorylation of Atg36 by Hrr25 is a switch in the initiation of pexophagy by allowing the interaction of the peroxisome with the central autophagosomal machinery [179]. Peroxisomes have their own fission system to allow an autonomous proliferation of the organelle. In yeast, the dynamin-related small GTPases Dnm1 and Vps1 together with the fission protein Fis1 are required for peroxisomal fission [180–182]. It has been shown recently that peroxisomal fission is required for pexophagy [183]. Moreover, the Atg11 adaptor protein interacts directly with Dnm1 and Vps1 [183]. Taken together, the Atg11 mediated recruitment of the preautophagosomal structure at strategic sites of the peroxisome via Pex3, and the fission machinery seems to be the initial step for marking peroxisomes for proteolytic degradation. It is less understood whether and how pexophagy serves to eliminate dysfunctional peroxisomes. However, it has been shown in yeast that artificially created catalase aggregates are cleared in a process that depends on peroxisomal fission and pexophagy [184]. Additionally, peroxisomal protein import defects have been shown to increase the rate of pexophagy [185]. These results suggest that the autophagic removal of damaged peroxisomes with increased ROS production could be an important mechanism to maintain a correct redox balance in the cell [172]. Consistent with this idea is the finding that peroxisomal proliferation seems to be regulated by the redox state of the organelle [19] and that abiotic stress such as salt stress induces the number of peroxisomes via Dnm1 and Vps1 [186].

The mechanisms of pexophagy in mammalian cells are only beginning to be unraveled [187, 188]. Here, selective autophagy of peroxisomes relies on cytosolic adaptor proteins such as p62 or NBR1, which recognize ubiquitinated cargo proteins and direct them to the autophagosome [189] (Figure 2). Pex5 is the peroxisomal receptor which upon ubiquitination triggers the process of pexophagy [190, 191]. This process can be stimulated upon external oxidative stress or by the pharmacological induction of peroxisomal fatty acid oxidation and involves the ATM kinase [191, 192]. Activated ATM inhibits signaling through the mammalian TOR pathway and marks Pex5 for pexophagy by phosphorylation [193]. Subsequently, Pex5 is ubiquitinated, and p62-dependent autophagy is initiated [191]. An E3 ubiquitin ligase responsible for Pex5 ubiquitination has been recently found with Pex2 at least upon nutrient starvation conditions [194]. Taken together, these findings suggest that an excess of ROS produced at peroxisomes triggers the process of specific pexophagy to maintain a healthy redox balance in mammalian cells.

The removal of excess or damaged mitochondria by selective autophagy or mitophagy is an essential cellular homeostasis mechanism. In yeast, this process has been heavily studied upon nutrient starvation conditions, for example, upon depletion of a N-source or in the stationary growth phase. These approaches have revealed many molecular insights of mitophagy; however, it remains unclear whether mitophagy in yeast actually degrades dysfunctional organelles [195]. Of central importance is the mitochondrial mitophagy receptor protein Atg32, which is localized at the outer mitochondrial membrane [196, 197]. Upon induction of mitophagy by starvation, Atg32 is phosphorylated by casein kinase 2 (CK2) at a specific serine (S114) residue [198]. Phosphorylation of Atg32 triggers its interaction with the cytosolic Atg11 adaptor protein. Therefore, this step is considered the initial signal in the targeting of mitochondrial to the preautophagosomal structure. Interestingly, there are at least two additional kinases which have been reported to be necessary for Atg32 phosphorylation, the Hog1 and Slt2 MAP kinases [199, 200]. Although both kinases cannot directly phosphorylate, the Atg32 receptor, the involvement of both kinases in stress signaling, could indicate a possible regulation of mitophagy by cellular stress stimuli additionally to nutrient shortage. Although still a matter of debate, there are experimental indications which suggest that mitophagy in yeast depends on mitochondrial fission. Generally, the mitochondrial fission proteins Fis1, Dnm1, Mdv1, and Caf4 seem to require for efficient mitophagy [157, 158, 201]. Specifically, during mitophagy, Atg11 directly targets the mitochondrial Dnm1 fission protein [158]. This suggests that mitophagy takes place close to the mitochondrial fission apparatus which would be necessary to separate the mitophagic cargo from the rest of the mitochondrial network [157]. Additional insights into the specific origins of mitophagy have been recently revealed by the finding that specific contact sites between the ER and mitochondria, the so called ERMES (ER mitochondria encounter structure), are necessary for mitophagy [202]. Thus, the initiation of mitophagy might require a close contact between the mitochondrial fission apparatus and the ER at strategic sites. It is less understood whether mitophagy in yeast is necessary to avoid cellular damage by dysfunctional mitochondria or whether it is activated upon oxidative stress. However, it has been reported that autophagic removal of excess mitochondria upon nutrient starvation is required to decrease intracellular ROS levels [203].

In mammalian cells, mitophagy has been intensively studied upon conditions which damage mitochondria by counteracting and eliminating their normal membrane potential [204] (Figure 2). In fact, loss of the mitochondrial membrane potential is one of the main triggers of mitophagy in mammalian models. Modulation of the mitochondrial shape is another important step in mitophagy, and inhibition of mitochondrial fission impairs the sequestration of damaged mitochondria by autophagosomes [205, 206]. This suggests that fission of the organelle, which is often induced by cellular stress, is an important mechanism to segregate and eliminate damaged mitochondria from the otherwise healthy network. Failure in the execution of mitophagy surveillance pathways clearly impairs the cellular homeostasis. This has been demonstrated for the key regulators of mitophagy, PINK1 (PTEN-induced kinase), and Parkin, whose mutation can cause Parkinson's disease [207, 208]. PINK1 is a mitochondrial serine/threonine kinase encoded by the *PARK6* locus [208], while Parkin is a cytosolic E3 ubiquitin ligase encoded by *PARK2* [209]. Both proteins function in the same mitophagic pathway with PINK1 acting upstream of Parkin [210, 211]. Upon normal cell homeostasis, PINK1 is imported into mitochondria dependent on the mitochondrial membrane potential. In healthy mitochondria, it is cleaved by PARL at the inner mitochondrial membrane, thereby restricting its activity [212–214]. Loss of the mitochondrial membrane potential inhibits PINK1 import and provokes its accumulation at the outer mitochondrial membrane, where it recruits Parkin [215–217]. This is considered the key step to mark dysfunctional mitochondria for selective degradation. Once exposed at the mitochondrial surface, PINK1 forms larger aggregates, which stimulate PINK1 activity by autophosphorylation [218, 219]. Subsequently, the mitophagic pathway is initiated by PINK1 phosphorylation of both Parkin and ubiquitin in a positive feedback regulation. The phosphorylation of Parkin by PINK1 is necessary for its mitochondrial targeting and for its E3 ubiquitin ligase activity [215, 216, 220–223]. Furthermore, PINK1 phosphorylates ubiquitin, and phosphorylated ubiquitin seems to be the true targeting signal for Parkin [224–226]. Indeed, it has been recently revealed that Parkin accumulation at damaged mitochondria requires phosphorylated ubiquitin chains at the mitochondrial surface [227–229]. Ubiquitination of outer mitochondrial membrane proteins, most importantly of the mitofusins Mfn1/2, finally triggers the physical segregation of the damaged mitochondrial parts and degradation via autophagosomes [230–232]. The nature of the autophagy receptors which would recognize ubiquitinated damaged mitochondria has been controversial. However, a recent study discards the pexophagy receptors p62 or NBR1 and specifically implies Optineurin and NDP52 in PINK1/Parkin-mediated mitophagy [233].

Thus, phospho-ubiquitin at the surface of damaged mitochondria is the signal to recruit the NDP52 and Optineurin receptors, which in turn contact with components of the autophagy pathway such as ULK1 or LC3 to engage in the autophagic degradation of the dysfunctional mitochondria [234, 235].

It is important to note that mammalian cells apparently use different mitophagy pathways upon different stimuli. It has been demonstrated in mouse and human cells that oxidative stress is a sensitive mitophagy trigger, which reinforces the idea that the autophagic removal of damaged mitochondria is a physiologically relevant mechanism of cell homeostasis [236]. Apart from the PINK1/Parkin system, which responds to a loss of mitochondrial membrane potential, the Bcl2-L-13 protein (yeast Atg32 ortholog), the NIX/BNIP3L, and the FUNDC1 mitochondrial outer membrane proteins have been involved in different types of mitophagy. Bcl2-L-13 seems to mediate mitophagy and mitochondrial fragmentation upon ETC damage [237], NIX/BNIP3L is involved in mitochondrial clearance during erythrocyte differentiation [238, 239], while FUNDC1 responds to hypoxic conditions [240]. It remains unclear to what degree these different mitophagy receptors act separately in the process of mitochondrial homeostasis.

6. Physiological and Physical Interaction of Peroxisomes and Mitochondria

In mammalian cells, peroxisomes and mitochondria functionally cooperate in the oxidative degradation of fatty acids by β-oxidation (see Figure 1). Both organelles have different but partially overlapping substrate specificities with regard to the chemical structure of the fatty acid to be metabolized. Therefore, peroxisomes and mitochondria cooperate in the homeostasis of lipids. The details of this metabolic cooperation have been summarized in excellent recent reviews [241, 242]. It is important to note that both organelles share metabolic pathways apart from β-oxidation, for example in the detoxification of glyoxylate or in the degradation of special fatty acids via α-oxidation [241, 243]. Also, it can be assumed that extensive peroxisomal α-oxidation depends on the ATP supply from the mitochondrial respiratory chain. More importantly, it has been recently shown that there is an intimate crosstalk between peroxisomes and mitochondria in the homeostasis of intracellular ROS beyond the fact that both organelles are important sources and sinks for ROS as detailed above. It had been known that weakening the peroxisomal catalase activity either by pharmacological treatment or caused by the decreased protein import capacity triggered elevated oxidative stress and reduced enzyme activity at mitochondria [104, 107, 244]. The use of peroxisomally directed inducers of oxidative stress has then revealed that mitochondria indeed are downstream of peroxisomally generated ROS [101], which is manifested by mitochondrial fragmentation and cell death [105, 106]. It has been suggested that mitochondrial oxidative damage is caused by lipid peroxidation originated in peroxisomes arguing against a simple diffusion of peroxisomal ROS to mitochondria. The mechanisms of the peroxisome-mitochondria ROS interplay

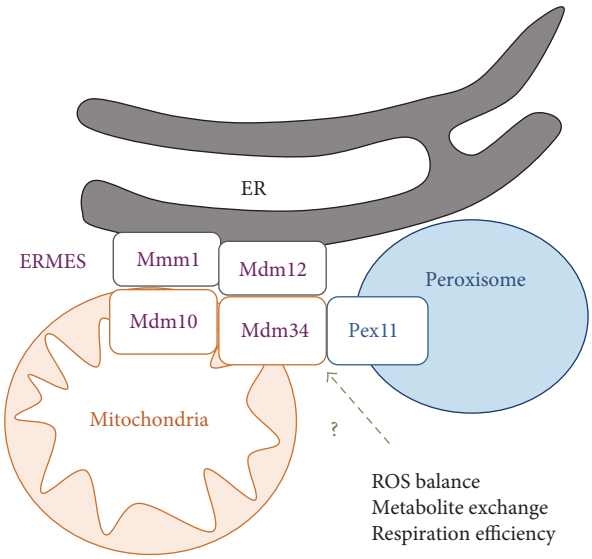


Figure 3: Overview of the yeast peroxisome-mitochondria contact site. The ERMES (endoplasmic reticulum mitochondria encounter structure) tethers mitochondria to the ER, but additionally establishes a contact between mitochondria and peroxisomes through the Mdm34-Pex11 interaction. The mitochondria-peroxisome tether might have dynamic functions in the regulation of ROS homeostasis, metabolite exchange between the two organelles, or the modulation of respiratory efficiency.

are currently unknown; however, interorganelle contact sites could explain this much more intimate relation of peroxisomes and mitochondria.

In the yeast model, the functional and physical interactions of peroxisomes with mitochondria have been studied intensively in recent years. As outlined above, fatty acid β-oxidation occurs exclusively in peroxisomes of budding yeast. Therefore, acetyl-CoA from peroxisomes has to be channeled into mitochondria via the carnitine shuttle and thus a direct contact of both organelles should favor this exchange of metabolites. Indeed, it has been shown that yeast peroxisomes preferentially localize close to specific mitochondrial sites which also contain contacts to the ER [245]. Recently, a possible physical tether between peroxisomes and mitochondria has been suggested by the interaction of the Pex11 protein involved in the biogenesis of the organelle with the mitochondrial Mdm34 protein [246] (Figure 3). Since Mdm34 is one of the structural components of the mitochondria-ER tether ERMES [247], this finding opens up the possibility that at least in yeast peroxisomes and mitochondria are physically connected at specific contact sites with the ER [248]. Recent evidence suggests that peroxisome-mitochondria contact sites might also exist in mammalian cells [249]. Since peroxisomes and mitochondria share components of their fission machineries and the fission of both organelles is important for their autophagic turnover, it is possible that this three-way contact is structurally important for the regulated turnover and therefore the ROS homeostasis of both organelles. Additionally, the mitochondria peroxisome interaction might be regulated dynamically to modulate ROS homeostasis, the interchange of metabolites or the respiratory efficiency. In this respect, it has been

recently shown that the number of peroxisomes attached to the mitochondrial network increases upon cellular stress such as high salinity, which is known to also increase cellular ROS levels [186].

7. Conclusions

We know that peroxisomes and mitochondria are the crucial organelles in the homeostasis of eukaryotic ROS levels. Peroxisomes have traditionally lagged behind the importance of mitochondria in the production and detoxification of intracellular oxidative stress; however, this difference has largely disappeared in the past years. Current investigation is now deciphering the functional interplay of both organelles in the propagation and avoidance of oxidative damage in the cell. Peroxisomal and mitochondrial oxidative stress are intimately linked. Moreover, the dynamic fission of both organelles shares structural components, and the turnover of damaged peroxisomes and mitochondria as a mechanism to reduce intracellular oxidative stress will still be an important field of investigation. From a structural viewpoint, we are only beginning to understand how molecular tethers between peroxisomes and mitochondria, most likely via the ER, can contribute to interorganelle communication and coordinated quality control of both organelles. Therefore, investigation of the peroxisome-mitochondria connection and its regulation by stress will continue to contribute essential knowledge of how the redox homeostasis is maintained in young, environmentally challenged and aged eukaryotic cells.

Conflicts of Interest

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Cardioprotective Effect of Selective Estrogen Receptor Modulator Raloxifene Are Mediated by Heme Oxygenase in Estrogen-Deficient Rat

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Estrogens and raloxifene (RAL) have beneficial effects on certain cardiovascular indices in postmenopausal women characterized by estrogen deficiency. Heme oxygenase (HO) activity is increased by 17β-estradiol (E_2) and RAL in estrogen-deficient rat resulting in vasorelaxation mediated by carbon monoxide. We determined the expressions of HO in cardiac and aortic tissues after ovariectomy (OVX) and subsequent RAL or E_2 treatment. We investigated the effects of pharmacological inhibition of HO enzyme on the arginine vasopressin- (AVP-) induced blood pressure in vivo, the epinephrine- and phentolamine-induced electrocardiogram ST segment changes in vivo, and the myeloperoxidase (MPO) enzyme activity. When compared with intact females, OVX decreased the HO-1 and HO-2 expression, aggravated the electrocardiogram signs of heart ischemia and the blood pressure response to AVP, and increased the cardiac MPO. E_2 and RAL are largely protected against these negative impacts induced by OVX. The pharmacological inhibition of HO in E_2- or RAL-treated OVX animals, however, restored the cardiovascular status close to that observed in nontreated OVX animals. The decreased expression of HO enzymes and the changes in blood pressure ischemia susceptibility and inflammatory state in OVX rat can be reverted by the administration of E_2 or RAL partly through its antioxidant and anti-inflammatory roles.

1. Introduction

Although the clinical cardiovascular outcome study results in postmenopausal women are inconsistent and disappointing so far [1], estrogens demonstrated cardiovascular protective effects in various conditions and play an important role in the sex-related differences of hypertension in experimental models. Estrogen receptor-dependent and independent pathways result in favourable changes in plasma lipoproteins, haemostatic factors, glucose metabolism, and endothelium-derived factors as well as in the inhibition of smooth muscle cell migration and proliferation. Estrogen reduces both the myocardial infarct size and the occurrence of ischemia-reperfusion-induced damage and neutrophil infiltration in cardiac muscle [2]. In addition to their specific, receptor-mediated effects, estrogens have antioxidant properties also related to their aromatic/phenolic chemical structure: ovariectomy results in increased myeloperoxidase (MPO) enzyme activity [3, 4]. MPO acts as a master enzyme in the generation of reactive oxygen species (ROS) which promotes endothelial dysfunction by generating atherogenic-oxidized low-density lipoprotein OxLDL [5]. Elevated circulating MPO levels have been found to be associated with the presence of coronary artery disease (CAD) [6]. Estrogen replacement therapy has antioxidant properties and attenuates neutrophil infiltration and myeloperoxidase (MPO) activity in the heart [2].

Numerous studies prove that the cardioprotective effects of estrogens are mediated by the nitric oxide- (NO-)

dependent pathway [7]. Similarly to NO, carbon monoxide (CO) also plays an important role in the estrogen-mediated cardioprotection. Equimolar amount of CO is generated during the catabolism of heme by the heme oxygenase (HO) enzymes. CO activates soluble guanylyl cyclase (sGC) by a mechanism similar to that for NO leading to smooth muscle relaxation. Three isoforms of HO have been characterized: HO-1, HO-2, and HO-3. HO-1 is widely expressed and can be induced by a host of stimuli that produces oxidative stress [8] and confers protection against vascular injury through its effects on constriction and proliferation against heart failure [9] and may play an important beneficial role in conditions such as hypertension and acute renal and lung injury [10, 11]. HO-2 occurs widely, including neuronal populations and vascular endothelial cells [12], and it is induced by glucocorticoids and probably estrogens [13, 14].

To overcome the adverse proliferative effects of estrogens on breast and endometrial tissues in the clinical practice, selective estrogen receptor modulators (SERMs) have been developed. Preclinical and clinical studies with RAL, a second-generation SERM, used for the prevention and treatment of postmenopausal osteoporosis, indicate its estrogen-like effects on the cardiovascular system. RAL improves the endothelial function in ovariectomized (OVX), aged, or hypertensive rats, ameliorates the hypertension-induced endothelial dysfunction by reducing the production of reactive oxygen species, and enhances endothelial nitric oxide-(NO-) dependent vasodilatation in vitro. Moreover, RAL causes direct vasodilatation [15]. It reduces the increased cardiovascular risk in patients with osteoporosis, although the outcomes of the RUTH trial showed that RAL did not affect the overall risk of coronary heart disease in elderly women. However, the incidence of coronary events was significantly lower in women < 60 years assigned to RAL compared with placebo. Measurements of cardiometabolic risk factors show that women assigned to RAL had greater increases in HDL cholesterol and greater reductions in LDL cholesterol, non-HDL lipoprotein levels, and the ratio of cholesterol to HDL, and fibrinogen levels. Moreover, a meta-analysis recently confirmed the beneficial effect of RAL administration on Lp(a) level [16].

The aim of this current study was to verify the extensive estrogen-agonist properties of RAL in cardiovascular system with analyzing of HO-1 and HO-2 isoforms. Therefore, we determined the effects of E_2 and RAL treatments on the changes of blood pressure in vivo and ischemia susceptibility of the heart in adrenalin and phentolamine models. With pharmacological inhibition of HO, we evaluated its mediating role on these cardiac outcomes.

Epidemiological and clinical studies have shown a strong relationship between inflammatory markers and risk of future cardiovascular events. To examine how E_2 replacement and RAL treatment change the inflammatory status of OVX rats, MPO activity was measured in myocardial tissue.

2. Materials and Methods

2.1. Examined Groups. 4-month-old female Wistar rats (Laboratory Animals Producing Institute, Gödöllő, Hungary) were anesthetized and subjected to ovariectomy surgery (OVX). During OVX, the ovaries were clamped bilaterally and removed. After a 6-week resting period to verify the surgically induced menopause, the estrogen levels were checked by enzyme-linked immunosorbent assay according to the manufacturer's directions (Quantikine rat Estrogen ELISA kit, R&D Systems Inc.) [4]. Moreover, Giemsa staining was used to ensure that all animals were killed at the proestrus stage of the estrus phase. In separate groups of OVX animals, estrogen (estrofem, E_2, 0.10 mg/kg/day, orally, once daily) or RAL (RAL 0.33: 0.33 mg/kg/day, RAL 1: 1.0 mg/kg/day, orally, once daily) replacement therapy was used for a 2-week period. HO activity was inhibited by tin protoporphyrin IX (SnPP, 30.0 μg/kg, pH 7.4, s.c., 24 h and 1 h pretreatment). Each group consisted of at least 10 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. The experimental design of the study is presented in Figure 1.

2.2. HO-1 and HO-2 Protein Expression. The aorta and cardiac left ventricle (LV) were homogenized in ice-cold Tris-mannitol and centrifuged for 20 min at 12,000 g at 4°C. Protein content was measured by spectrophotometric assay. Aliquots of 25.0 μg of total cellular protein were denatured and electrophoresed (100 V, 50 mA) on 10.0% polyacrylamide gel, transferred (100 V, 100 mA, 2 h) to nitrocellulose membrane, and then determined by staining the blot with 0.10% Ponceau red in 5.0% acetic acid. Two hours after blocking, the membranes were incubated with anti-HO-1 mouse monoclonal antibody (final dilution 1 : 1000) or anti-HO-2 monoclonal antibody (final dilution 1 : 1000) (Stress-Gen Biotechnologies Corp., Victoria, Canada) for 2 h at room temperature, washed 3 times with PBS-Tween 20, and then exposed with horseradish peroxidase-conjugated bovine anti-mouse antibody (final dilution 1 : 2000; for 1 h at room temperature). Membranes were developed by using an enhanced chemiluminescence system and exposed to Hyperfilm. Films were analyzed by using ImageQuant Software after scanning with GelAnalyst 3.01 Software. The description of homogenization procedure, the content of solutions, as well as the producers of antibodies and equipment are detailed previously [17].

2.3. The Response of the Blood Pressure to AVP. Rats were anesthetized with 30.0% urethane and then pretreated with phentolamine (P, 10.0 mg/kg, i.p). After a stable baseline measurement, a single bolus injection of arginine vasopressin (AVP; 0.02, 0.06 or 0.18 μg/kg) was infused intravenously to tail vein of rats. The first step of the procedure was to separate the right carotid artery, along with the vagus nerve, from the connective tissue. Then, the right carotid vessel was cannulated and the elevation of blood pressure was measured [18]. The cannula was connected to the pressor transducer, which converted the blood pressure into an electrical signal. To avoid a thrombotic process, the cannula was filled with 10.0% heparin. The changes in blood pressure were analysed

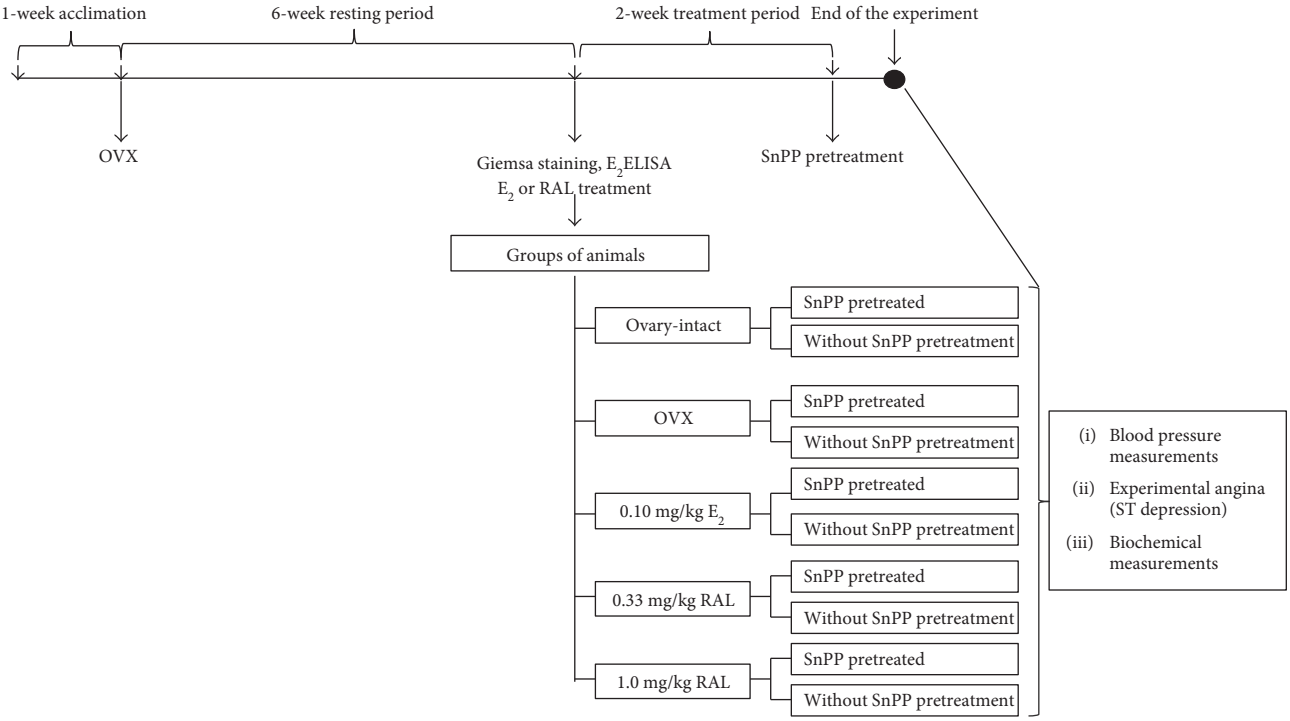

Figure 1: Experimental design of the study. OVX = ovariectomy, E_2 = estrogen, RAL = raloxifene, SnPP = tin protoporphyrin IX.

by HAEMOSYS analysis system and expressed as a percentage of the maximal increase relative to basal value. We followed the methods of Posa et al. [17].

2.4. Experimental Angina Provoked by Epinephrine and Phentolamine. The standard limb lead II of the surface electrocardiogram (ECG) was recorded to measure the changes of ST segment by the HAEMOSYS system [19]. The changes in ST segment were used as the index of angina severity. During the specific experimental procedure, a single dose of epinephrine (10.0 μg/kg) and 30 s later α-adrenoceptor antagonist P (15.0 mg/kg) were infused intravenously for 2 sec into the tail vein. After the administration of angina-provoking agents, the ST segment depression was calculated from the ECG waveform as a change in mV relative to the baseline level. We followed the methods of Posa et al. 2013 [17].

2.5. Cardiac MPO Activity. The cardiac tissues were homogenized in ice-cold PBS (pH 6.0), freeze-thawed three times, and then centrifuged twice at 15000 g for 15 min at 4°C. The supernatant was discarded, and a 12 μL aliquot was added to a mixture of 280 μL of PBS (pH 6) and 0.167 mg mL^{-1} of O-dianisidine dihydrochloride. The reaction was started with 10 μL of 0.03% hydrogen peroxide and assayed spectrophotometrically at 490 nm after 90 s of shaking. Cardiac MPO activity was expressed as mU/mg protein [20].

2.6. Chemicals. RAL (Eli Lilly and Company USA), AVP (Organon, The Netherlands), E_2 (Novo Nordisc, Denmark), urethane (Reanal, Hungary), P (Ciba-Geigy, Switzerland), and SnPP (Frontier Scientific Europe, UK) were the chemicals used in this study. All compounds not specified above were derived from Sigma International.

2.7. Statistical Analysis. The results are expressed as means ± S.E.M. Western blots are shown as representative photographs of 3 independent experiments. Differences between groups were performed using ANOVA test, and $p \leq 0.05$ was taken as significant.

3. Results

3.1. Actions of RAL or E_2 Treatment on HO-1 and HO-2 Expression of LV and Aortic Tissues in Ovariectomized Rat. Ovariectomy was found to lead to significantly decreased cardiac HO expression (HO-1: 39.86 ± 4.79%; HO-2: 48.0 ± 2.76%), and E_2 (HO-1: 95.14 ± 4.11%; HO-2: 100.14 ± 4.02%) or RAL (RAL 0.33, HO-1: 79.5 ± 3.42%; HO-2: 87.55 ± 3.85%, RAL 1, HO-1: 90.29 ± 4.43%; HO-2: 95.86 ± 4.03%) supplementation in the OVX rats completely restored the HO expression to the level observed in the heart of the ovary-intact females. Data are shown in Figures 2(a) and 2(b).

Ovariectomy significantly decreased the aortic HO enzyme expression (HO-1: 49.86 ± 2.59%; HO-2: 53.0 ± 3.76%), and E_2 (HO-1: 90.21 ± 7.41%; HO-2: 94.14 ± 5.02%) or RAL (RAL 0.33, HO-1: 72.34 ± 7.45%; HO-2: 77.55 ± 4.85%, RAL 1, HO-1: 85.31 ± 2.14%; HO-2: 92.46 ± 6.03%) supplementation in the OVX rats restored the HO expression. Data are shown in Figures 3(a) and 3(b).

3.2. The Effect of HO Inhibition on Blood Pressure as a Response to AVP. The arterial blood pressure was measured in the right carotid artery, and an increase was

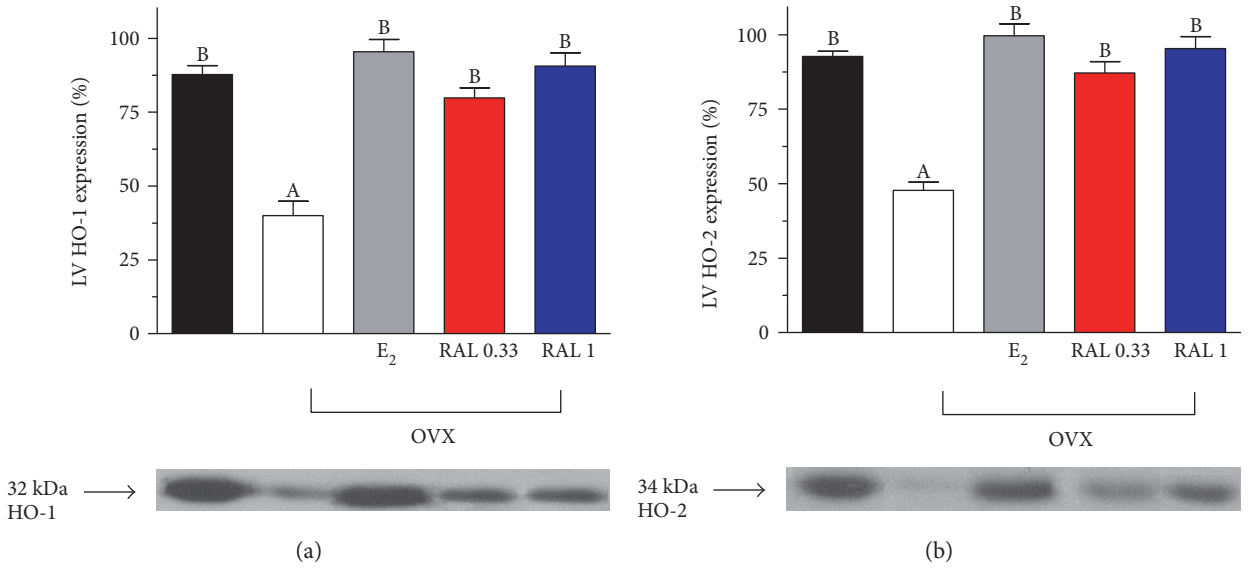

Figure 2: Heme oxygenase-1 and heme oxygenase-2 expression in the cardiac left ventricle. HO-1 (a) and HO-2 (b) expression (expressed as %) in the cardiac left ventricle (LV) of ovary-intact (black bar), ovariectomized (OVX (white bar)), and estrogen- (E_2: (gray bar); 0.10 mg/kg/day, 2 weeks orally) or RAL-treated (RAL 0.33 (red bar): 0.33 mg/kg/day, RAL 1 (blue bar); 1.0 mg/kg/day, 2 weeks, orally) OVX rats. The diagrams demonstrate the densitometric assessment (means ± S.E.M. expressed as %; 100% is the maximal expression). Data are expressed as means ± S.E.M. of the results of a minimum of 10 rats per group. Statistical significance: (A) $p < 0.001$ as compared with the ovary-intact group. (B) $p < 0.001$ as compared with the OVX group without treatment.

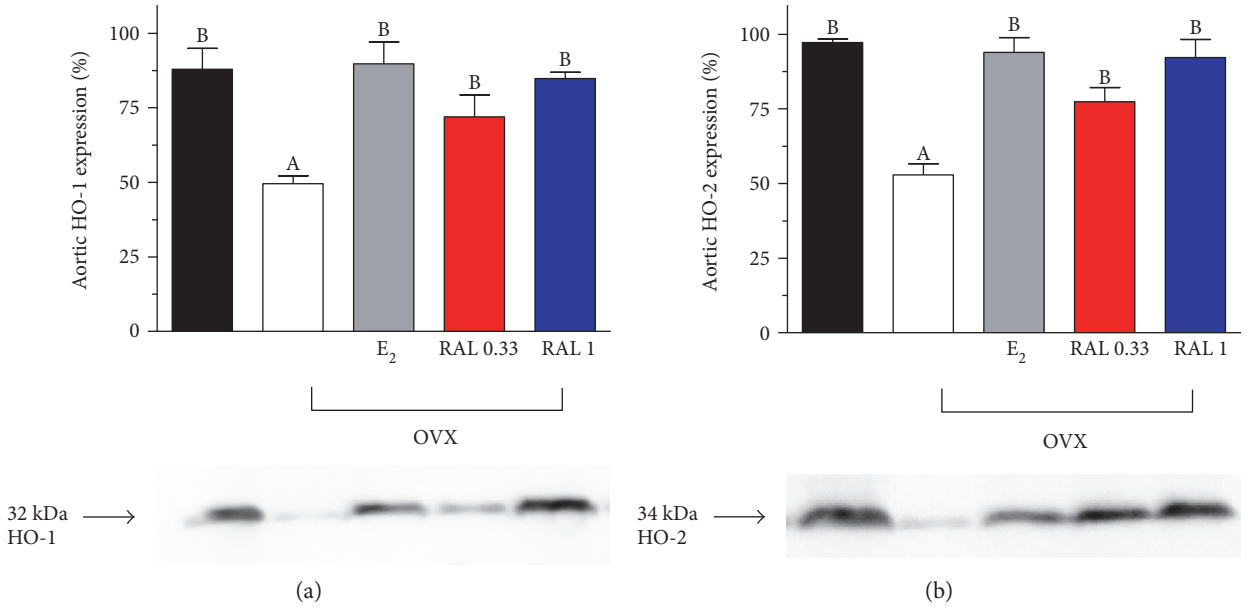

Figure 3: Heme oxygenase-1 and heme oxygenase-2 expression in the aorta. HO-1 (a) and HO-2 (b) expression (expressed as %) in the aortic tissues of ovary-intact (black bar), ovariectomized (OVX (white bar)), and estrogen-treated (E_2: (gray bar); 0.10 mg/kg/day, 2 weeks orally), or RAL-treated (RAL 0.33 (red bar): 0.33 mg/kg/day RAL 1 (blue bar); 1.0 mg/kg/day, 2 weeks, orally) OVX rats. The diagrams demonstrate the densitometric assessment (means ± S.E.M. expressed as %; 100% is the maximal expression). Data are expressed as means ± S.E.M. of the results on a minimum of 10 rats per group. Statistical significance: (A) $p < 0.001$ as compared with the ovary-intact group. (B) $p < 0.001$ as compared with the OVX group without treatment.

induced by i.v. administration of AVP (0.02–0.18 μg/kg) in catecholamine-depleted (P, 10.0 mg/kg i.p.) female rats.

AVP caused a dose-dependent increase in arterial blood pressure in both the ovary-intact and the OVX female rats. In the OVX animals, AVP induced a significantly higher elevation in blood pressure (24.30 ± 1.42 versus 53.60 ± 3.48%) than in the ovary-intact females (9.30 ± 1.62 – 24.0 ± 2.12%). Estrogen replacement (E_2, 0.10 mg/kg,

2 weeks, orally, once daily) (10.20 ± 2.07 versus $27.60 \pm 2.50\%$) abolished the increased blood pressure response, and RAL supplementation (RAL 0.33, 0.33 mg/kg, RAL 1; 1.0 mg/kg, 2 weeks, orally, once daily) (RAL 0.33: 17.6 ± 2.41–$35.40 \pm 2.30\%$, RAL 1: 12.10 ± 1.63–$26.80 \pm 3.45\%$) resulted in a decrease in the blood pressure enhancement provoked by AVP in the OVX rats. The inhibition of HO activity caused significant augmentation in all groups (ovary-intact: $33.10 \pm 2.23\%$ – $49.50 \pm 2.77\%$; OVX group: 29.30 ± 0.56 – $66.10 \pm 1.07\%$; E_2-treated group: 22.60 ± 1.46 – $54.50 \pm 4.50\%$; RAL-treated group: RAL 0.33: $24.0 \pm 3.70\%$ – $49.20 \pm 5.78\%$ RAL 1: $23.40 \pm 1.60\%$ – $55.60 \pm 3.45\%$). Data are shown in Figure 4(a).

3.3. The Effect of Inhibition of HO on Cardiac Ischemia. ST segment changes were measured in a lead II standard surface ECG following i.v. injection of epinephrine (10.0 μg/kg) and 30 s later phentolamine (15.0 mg/kg) in OVX female rats. The administration of phentolamine 30 s after epinephrine caused a significant ST segment depression only in the OVX rats (-0.13 ± 0.038 mV). In the ovary-intact females and in the E_2- (0.10 mg/kg, 2 weeks, orally, once daily) or RAL-treated (1.0 mg/kg, 2 weeks, orally, once daily) OVX groups, an ST segment depression did not develop. Pretreatment with SnPP (30.0 μg/kg, 24 h and 1 h prior to the measurement) caused a ST depression in the intact (-0.20 ± 0.03 mV) and E_2 (-0.16 ± 0.04 mV) or RAL-treated (RAL 0.33: -0.11 ± 0.06 mV, RAL 1: -0.17 ± 0.04 mV) groups and augmented the ST depression in the OVX females (ST segment change: -0.34 ± 0.045 mV). Data are shown in Figure 4(b).

3.4. Cardiac Activity of MPO. MPO activity was measured spectrophotometrically using *o*-dianisidine and hydrogen peroxide. In the OVX hearts, a significant increase in MPO activity was observed when compared with the ovary-intact females (75.0 ± 8.42–59.0 ± 4.37 mU/mg protein). Estrogen replacement therapy (E_2, 0.10 mg/kg, 2 weeks, orally, once daily) and RAL treatment (RAL 0.33, 0.33 mg/kg, RAL 1; 1.0 mg/kg, 2 weeks, orally, once daily) caused a reduction in MPO activity of OVX groups (E_2-treated group: 61.2 ± 4.69 mU/mg protein, RAL-treated group, RAL 0.33: 58.65 ± 5.63 mU/mg protein, RAL 1: 55.53 ± 2.64 mU/mg protein). Pretreatment with SnPP (30.0 μg/kg, 24 h and 1 h prior to the measurement) significantly increased the MPO activity in the ovary-intact (59.0 ± 4.37–73.0 ± 6.34 mU/mg protein), E_2-treated (61.2 ± 4.69–79.35 ± 5.86 mU/mg protein), and RAL-treated (RAL 0.33: 58.65 ± 5.63–82.56 ± 3.7 mU/mg protein, RAL 1: 55.53 ± 2.64–69.46 ± 4.24 mU/mg protein) groups. Data are shown in Figure 5.

4. Discussion

We have demonstrated cardiovascular protective features of E_2 and RAL mediated by the HO system in OVX female rats. Estrogen depletion caused by ovariectomy was accompanied by a decreased expression of HO-1 and HO-2, elevated blood pressure, marked a ST segment depression, and increased MPO activity. These adverse effects could be markedly reversed by the exogenous administration of the E_2 or RAL. These protections by E_2 and RAL were partially offset by a pharmacological HO inhibitor, suggesting an important role of HO system in these findings.

Ovariectomy resulted in reduced HO-1 and HO-2 expression both in the LV of the heart and in the aorta. These data are in line with previous observations on the stimulatory effects of estradiol on the HO system [21]. E_2 treatment elevated HO-1 protein levels and HO activity in trauma-hemorrhage male rats, resulting in the prevention of shock-induced organ damage [22]. Interestingly, in agreement with the present findings, HO-2, which is considered to be constitutively expressed, was also stimulated by E_2 through an estrogen receptor-dependent mechanism in human endothelial cells [14]. Our results suggest that, together with glucocorticoids, E_2 and RAL may belong to the few inducers of HO-2 [13]. Most inducers specifically act on HO-1. For example, hemin, a potent inducer of HO activity, increased HO-1, but not HO-2 expression in the mesenteric artery of young spontaneously hypertensive rats [23]. Similarly, lipopolysaccharides-induced HO-1, but not HO-2 mRNA expression in aortic tissues in rats [24]. We have previously reported that estrogen replacement and RAL treatment cause an increase in HO activity in OVX rat hearts and aorta [21]. In our recent study, we demonstrated that while estrogen deficiency reduces, estrogen supplementation restores HO expression in vivo.

We have found that, similarly to E_2, RAL restores the HO expression in the heart and aorta of OVX rats. RAL induces HO-1 expression in mouse macrophages, resulting in inhibition of inducible NO synthase (iNOS) expression and the subsequent inflammatory reactions. However, these effects of RAL were not mediated by the estrogen receptor [25]. The inhibitory effect of RAL and estradiol on carrageenan-induced iNOS and acute inflammation in normal and OVX rats described earlier could probably also be mediated by HO induction [26]. Our results are the first demonstration that RAL also increases HO expression and activity in the cardiovascular system.

We found that OVX augmented the AVP-induced dose-dependent increase in blood pressure, as reported previously [18, 27]. E_2 or RAL administration to OVX animals restored the blood pressure increase as compared with the control levels, irrespective of the AVP dose. These effects are at least partially mediated by the increased production of NO due to the constitutive nitric oxide synthase (cNOS) activity being elevated close to the pre-OVX level [18, 28]. In addition to the cNOS stimulation, the elevated HO activity induced by E_2 or RAL also plays a role in the attenuated blood pressure response to AVP. The pretreatment with SnPP, a HO activity inhibitor, prevented the reduced blood pressure response by E_2 or RAL. It is possible that the blood pressure responses in our model result from the interplay between the NOS and HO systems. Indeed, HO-1 overexpression restored endothelial NOS (eNOS) activity in endothelial cells under oxidative stress [29]. A low concentration of CO induced NO release, while a high concentration inhibited eNOS activity and NO generation [30]. Moreover, the potential

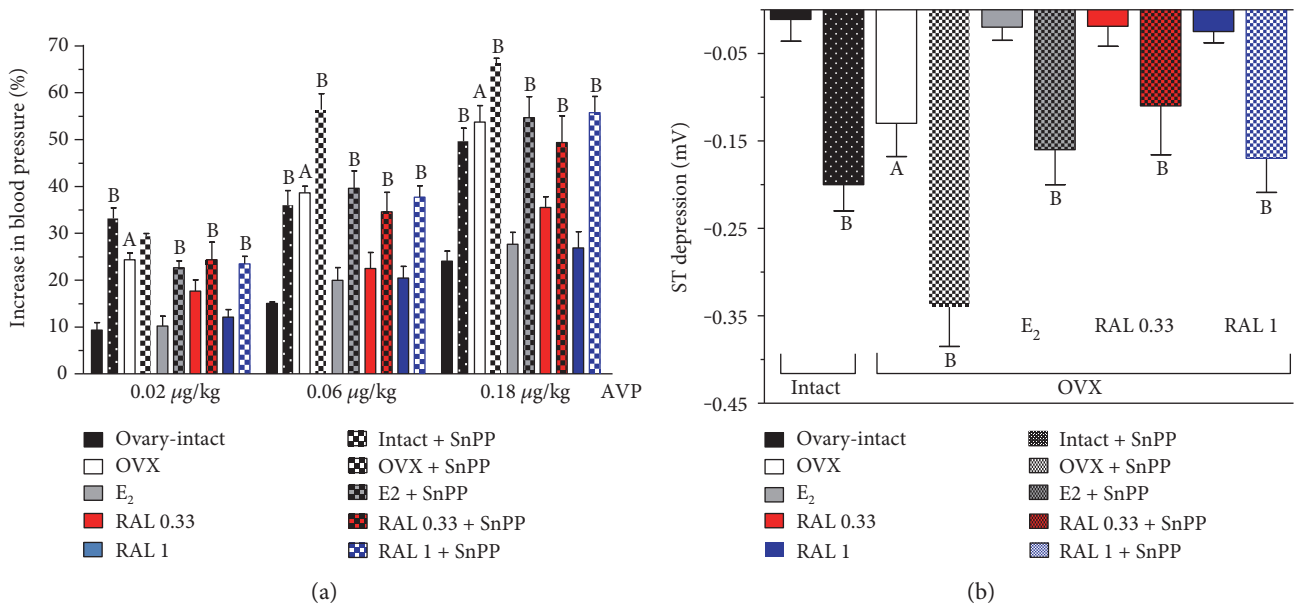

Figure 4: (a) The effect of HO inhibition on blood pressure as a response to AVP. The effects of HO inhibition by tin protoporphyrin IX (SnPP: 30.0 μg/kg, pretreatment 24 h and 1 h prior to the measurement) on the increase in arterial blood pressure measured on administration of arginine vasopressin (0.02, 0.06, or 0.18 μg/kg) in ovary-intact, ovariectomized (OVX), and estrogen- (E_2: 0.10 mg/kg/day, 2 weeks orally, once daily) or RAL-treated (RAL 0.33: 0.33 mg/kg/day, RAL 1: 1.0 mg/kg/day, 2 weeks, orally, once daily) OVX rats. The intact + SnPP, OVX + SnPP, E2 + SnPP, RAL 0.33 + SnPP, and RAL 1 + SnPP columns show the actions of SnPP pretreatment (30 μg/kg 24 h and 1 h prior to the measurement). Results are shown as means ± S.E.M. for 10 animals in each group. Statistical significance: (A) $p < 0.05$ compared with the ovary-intact group, and (B) $p < 0.05$ a significant difference between the groups with and without SnPP pretreatment. (b) The effect of inhibition of HO on ST depression. The effects of the heme oxygenase inhibitor tin protoporphyrin (SnPP) on the ST segment changes (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) in ovary-intact, ovariectomized (OVX), and estrogen- (E_2: 0.10 mg/kg/day, 2 weeks orally, once daily) or RAL-treated (RAL 0.33: 0.33 mg/kg/day, RAL 1: 1.0 mg/kg/day, 2 weeks, orally, once daily) OVX rats. The intact + SnPP, OVX + SnPP, E2 + SnPP, RAL 0.33 + SnPP, and RAL 1 + SnPP columns show the actions of SnPP pretreatment (30 μg/kg 24 h and 1 h prior to the measurement). Results are shown as means ± S.E.M. for 10 animals in each group. Statistical significance: (A) $p < 0.05$ as compared with the ovary-intact group, and (B) $p < 0.05$ a significant difference between the groups with and without SnPP pretreatment.

nonspecific role of the selective HO inhibitor metalloporphyrins in vasoconstriction is also not fully elucidated. Certain other metalloporphyrins similar to SnPP may possess nonspecific vasoconstrictor effects in the rat small cerebral arteries, and SnPP could therefore possibly potentiate the blood pressure increase caused by HO inhibition [31]. In contrast, the metalloporphyrin, chromium mesoporphyrin, has been shown to increase the myogenic tone only of the small muscular branch of rat femoral arteries and not of large arterial vessels such as the aorta or the femoral artery [32]. Thus, the effects of the AVP-induced blood pressure increase in our experiments may largely represent reduced HO-1 and HO-2 activities, but we cannot exclude some additional direct effect by SnPP and the contribution of the interplay between the HO and NO systems. According to Ikeno et al., OVX caused significantly increased blood pressure response to AVP [27]. In our study, we found similar results. The AVP-induced blood pressure response in the presence of SnPP was also augmented in the sham-operated control animals. This in vivo finding supports the role of the basal, constitutive HO activity in the protection against vascular constriction found ex vivo; HO-1 knockout mice exhibited an impaired relaxation of the superior mesenteric arteries and an increased contractility to phenylephrine as compared with the vessels from wild-type animals [33, 34]. The HO inhibitor chromium mesoporphyrin increased the blood pressure in young spontaneously hypertensive rats [23]. Moreover, treatment with lipopolysaccharide induced the HO-1 and significantly reduced the blood pressure in rats, whereas pretreatment with the HO inhibitor zinc protoporphyrin-IX (ZnPP) prevented the fall in blood pressure [24]. Similarly, under stress conditions, while ZnPP decreased the aortic CO and cGMP levels, the acute vasoconstrictor effects of either $\alpha\alpha$Hb or NG-nitro-L-arginine methyl ester were restored in the rat after surgical intervention [35]. Previous studies have also demonstrated that either acute or chronic administration of various inducers of HO-1 to spontaneously hypertensive rats led to a normalization of the blood pressure [36]. In another model, the overexpression of HO-1 was associated with an increase in HO activity and a decrease in the blood pressure in spontaneously hypertensive rats [37]. In addition, Vera et al. demonstrated that induction of HO-1 decreases the blood pressure in angiotensin-II-dependent hypertension [38, 39].

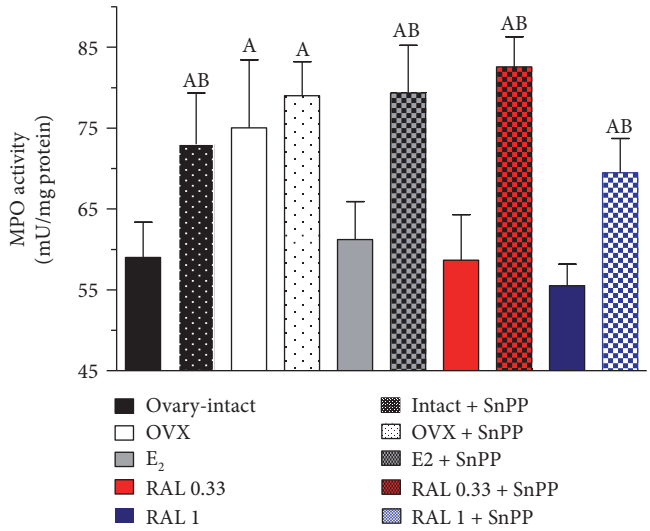

Figure 5: Myeloperoxidase activity in the cardiac left ventricle. Myeloperoxidase activity (MPO; expressed as mU/mg protein) in the cardiac left ventricle (LV) in ovary-intact, ovariectomized (OVX), and estrogen- (E_2: 0.10 mg/kg/day, 2 weeks orally, once daily) or RAL-treated (RAL 0.33: 0.33 mg/kg/day, RAL 1: 1.0 mg/kg/day, 2 weeks, orally, once daily) OVX rats. The intact + SnPP, OVX + SnPP, E2 + SnPP, RAL 0.33 + SnPP, and RAL 1 + SnPP columns show the actions of SnPP pretreatment (30 μg/kg 24 h and 1 h prior to the measurement). Data are expressed as means ± S.E.M. of the results on a minimum of 10 rats per group. Statistical significance: (A) $p < 0.05$ as compared with the ovary-intact group. (B) $p < 0.05$ as compared with the OVX group without treatment.

In our study, estrogen deficiency increased the level of MPO. E_2 substitution and RAL treatment, on the other hand, proved effective to attenuate the MPO activity in OVX rats. The connection between the MPO level and cardiac parameter or tissue HO expression suggests a preventive role of estrogen therapy in cardiovascular pathological processes. Similarly to our results, Chung et al. demonstrated that long-term treatment with RAL significantly decreased the cardiac activity of MPO in OVX rat [40]. While OVX increases the inflammation processes, the elevated levels of inflammatory markers can be decreased with hormone replacement therapy [41]. Oxidative stress plays a critical pathophysiological role during aging and after OVX. MPO is a major component of the oxidative system and displays potent proatherogenic properties. MPO can oxidize LDL cholesterol and reduces NO bioavailability, thereby impairing its vasodilatory and anti-inflammatory functions [42]. In our earlier study, we reported that higher levels of MPO have higher risk of cardiovascular events. The elevated level of the marker of leukocyte activation MPO correlated negatively with the tissue availability of cNOS and the indices of microvascular patency [43].

In conclusion, we have demonstrated that E_2 supplementation and RAL treatment in OVX rats present beneficial effects on cardiovascular system, thereby increasing the HO-1 and HO-2 enzyme expression, decreasing the AVP-induced blood pressure, and attenuating the cardiovascular ischemia susceptibility. Estrogen administration has been shown to attenuate MPO activity in OVX rats.

Our study has several important limitations. Our experiments were performed in young OVX female rats. While this is a widely accepted estrogen deficient, "menopausal" rat model for the investigations of various conditions (hormone replacement therapies, cardiovascular health, osteoporosis, and so forth), our findings may not reflect adequately the situation in aging female rats and their relevance to postmenopausal women is even more limited. All cardiovascular changes and vasoconstrictions investigated in the present acute experiment reflect short-term alterations.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Research Article

Flavonoids as Putative Inducers of the Transcription Factors Nrf2, FoxO, and PPARγ

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Dietary flavonoids have been shown to extend the lifespan of some model organisms and may delay the onset of chronic ageing-related diseases. Mechanistically, the effects could be explained by the compounds scavenging free radicals or modulating signalling pathways. Transcription factors Nrf2, FoxO, and PPARγ possibly affect ageing by regulating stress response, adipogenesis, and insulin sensitivity. Using Hek-293 cells transfected with luciferase reporter constructs, we tested the potency of flavonoids from different subclasses (flavonols, flavones, flavanols, and isoflavones) to activate these transcription factors. Under cell-free conditions (ABTS and FRAP assays), we tested their free radical scavenging activities and used α-tocopherol and ascorbic acid as positive controls. Most of the tested flavonoids, but not the antioxidant vitamins, stimulated Nrf2-, FoxO-, and PPARγ-dependent promoter activities. Flavonoids activating Nrf2 also tended to induce a FoxO and PPARγ response. Interestingly, activation patterns of cellular stress response by flavonoids were not mirrored by their activities in ABTS and FRAP assays, which depended mostly on hydroxylation in the flavonoid B ring and, in some cases, extended that of the vitamins. In conclusion, the free radical scavenging properties of flavonoids do not predict whether these molecules can stimulate a cellular response linked to activation of longevity-associated transcription factors.

1. Introduction

Flavonoid consumption via diet may benefit cardiovascular health in humans [1, 2], and in some cases, flavonoid supplementation prolonged lifespan of lower model organisms such as flies and worms [3]. Since many flavonoids are known to act as free radical scavengers, putative health benefits were partly attributed to their direct antioxidant capacity. However, it has become apparent that flavonoids modulate signalling processes in cultured cells and possibly also in vivo [4]. By inducing redox-sensitive transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) or forkhead box O (FoxOs), these polyphenols could prevent oxidative damage. While Nrf2 controls genes encoding proteins that counteract oxidative stress and detoxify xenobiotics [5, 6], it may also regulate genes involved in cell survival, metabolism, and adipocyte differentiation [7]. There are four FoxOs (FoxO1, FoxO3, FoxO4, and FoxO6) in humans which all bind to the same consensus sequence. FoxOs are important for cellular homeostasis and can induce cell survival or cell death [8]. They appear to be central for stress response [9] and may affect longevity [10]. Another transcription factor that might be modulated by flavonoids is peroxisome proliferator-activated receptor gamma (PPARγ). It is crucial for adipocyte differentiation [11], and regulation of PPARγ by polyphenols may ameliorate diabetes [12]. Interestingly, FoxO1 was shown to repress adipocyte differentiation via PPARγ [13]. In vitro experiments have demonstrated the activation of Nrf2, PPARγ, and FoxOs by flavonoids [11, 14-16]. In various cell models, the flavonol quercetin induced all three transcription factors [16-18]. Moreover, in vivo data points to the notion that flavonols and flavones may exert their health-benefitting effects via these transcription factors [19-21].

To study and compare the activation of Nrf2, FoxO, and PPARγ by flavonoids belonging to different flavonoid

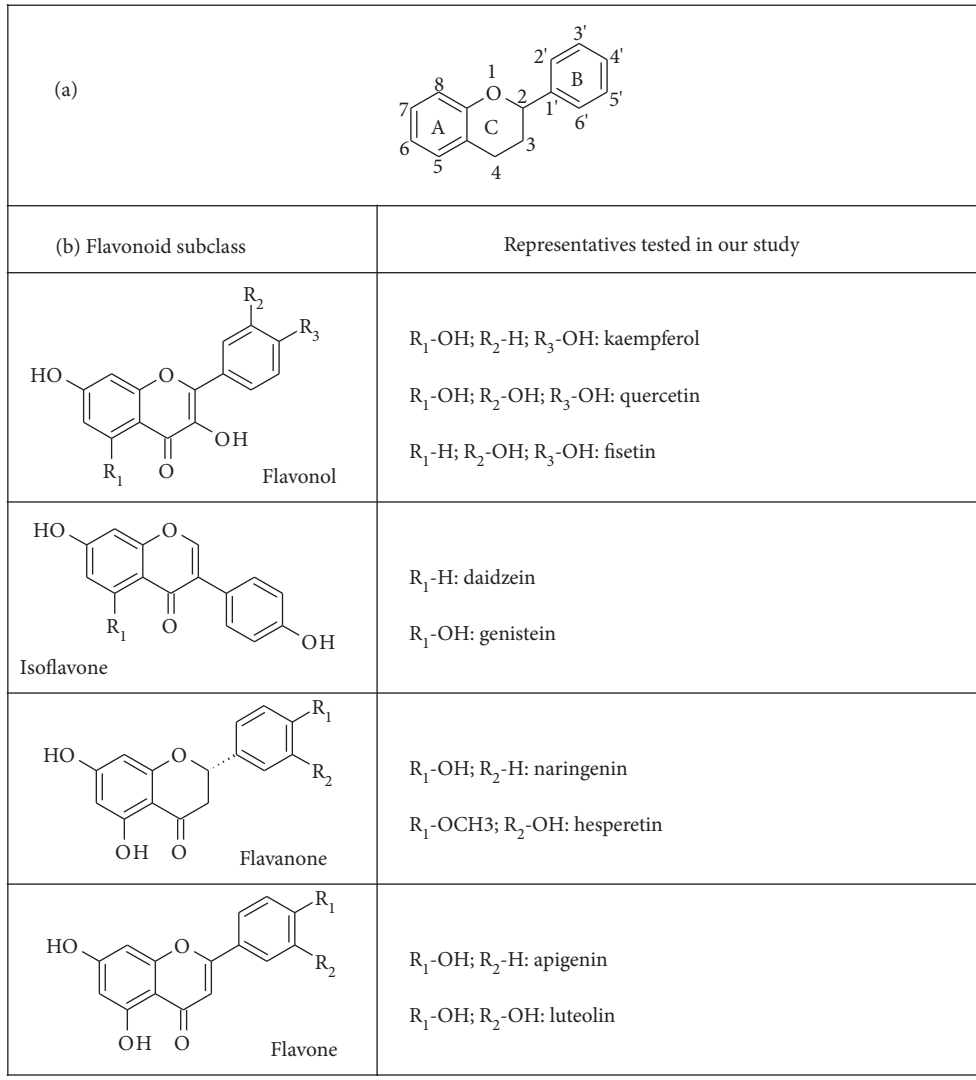

Figure 1: (a) Flavan structure (b) flavonoids used in this study.

subclasses, we tested various flavonols, flavones, isoflavones, and flavanones (Figure 1) in cultured Hek-293 cells transfected with suitable reporter gene constructs. We measured the activity of the flavonols kaempferol and quercetin, which have two hydroxy groups at the A ring and one or two hydroxy groups, respectively, at the B ring. Furthermore, we tested fisetin, which differs from the aforementioned flavonols in having only one hydroxy group at the A ring, as well as apigenin and luteolin, which are the flavone counterparts to kaempferol and quercetin, respectively. From the flavanone subclass of flavonoids, we picked naringenin with a para hydroxy group at the B ring and hesperetin with a meta hydroxy group and a para methoxy group at the B ring. The isoflavones we used, genistein and daidzein, differ in their number of hydroxy groups at the A ring (genistein has two and daidzein has one) while both have one para hydroxy group at the B ring.

In addition to the activation of the transcription factors, we assessed flavonoid antioxidant activity by analysing their ability to reduce the organic radical derived from 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS assay) or a Fe(III) complex (FRAP assay) under cell-free conditions and using the water-soluble vitamin E orthologue trolox (Figure 2) as a control. For both the analyses of the transcription factor activation and the antioxidant activity, we used the antioxidants ascorbic acid and α-tocopherol as comparisons (Figure 2).

2. Materials and Methods

2.1. Cell Culture. Hek-293 cells (German collection of microorganisms and cell cultures, Braunschweig, Germany) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 4 mM L-glutamine, 1 mM sodium pyruvate (PAN Biotech, Aidenbach, Germany), 10% fetal calf serum (Gibco, via Thermo Fisher, Darmstadt, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAN Biotech, Aidenbach, Germany). Cells

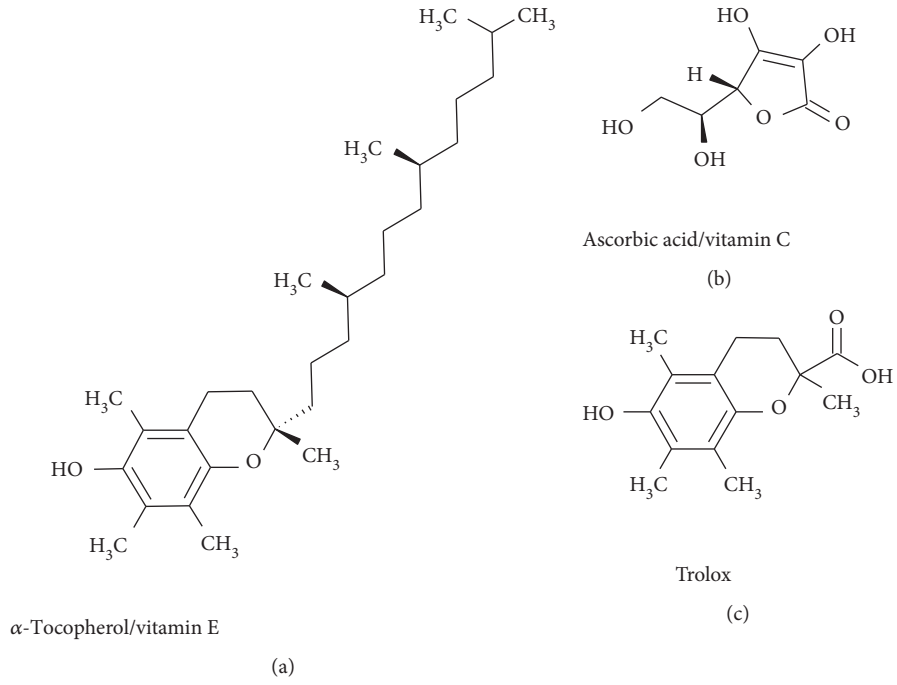

Figure 2: Chemical structure of ascorbic acid (b), α-tocopherol (a), and trolox (c).

were grown in 5% CO_2 at 37°C under a humidified atmosphere. All cell-culture plasticware was purchased from Sarstedt (Nuembrecht, Germany). For all cell culture assays, vehicle controls were performed and did not affect any of the parameters measured.

2.2. Transient Transfection and Luciferase Reporter Gene Assay. Hek-293 cells were grown to 60% confluence in 24-well plates for 24 h. The cells were transiently transfected with a firefly luciferase expression vector or expression system (pGl3-NQO1-ARE, pGl3-FHREΔXRE, or pUAS(4*)-TK-Luc together with pM1-hPPARγ-LBD). For normalization, a renilla-expressing plasmid was cotransfected. Transfection was performed using JetPei transfection reagent (Polyplus transfection, Illkirch Cedex, France) according to the manufacturer's instructions. Following 24 h of transfection, cells were incubated with the test compounds for 24 h in 10% serum-containing medium. Subsequently, cells were lysed and luciferase activity was measured using the dual-luciferase reporter gene assay system (Promega, Mannheim, Germany) in a Tecan Infinite 200 microplate reader (Tecan Group Ltd., Crailsheim, Germany) according to the manufacturer's protocol. A minimum of three independent experiments was performed.

2.3. Plasmids. pGl3-NQO1-ARE and pGl3-FHREΔXRE have a pGl3 backbone (Promega, Mannheim, Germany) which contains a multiple cloning site and a SV40 promoter upstream of a firefly luciferase gene that functions as reporter gene. Fragments containing the binding sites for Nrf2 (pGl3-NQO1-ARE) and FoxO (pGl3-FHREΔXRE) were inserted using the multiple cloning sites to precede the SV40 promoter, thereby functioning as enhancers for luciferase expression. pGl3-NQO1-ARE had a 32 bp oligonucleotide derived from rat NAD(P)H:quinone reductase mRNA that contains an ARE-motif inserted into the pGl3 backbone [22]. pGL3-FHREΔXRE was constructed from addgene plasmid 1789 [8] by removing a xenobiotic response element (XRE) [23].

For measuring PPARγ activation, we used Gal4-directed gene transcription which is widely employed to measure gene expression [24]. The Gal4 (yeast transcription activator protein) fused to the PPARγ ligand-binding domain (LBD) and a firefly luciferase construct under the control of UAS (upstream activating sequence) were used. Upon ligand binding to PPARγ-LBD, the fused Gal4 can bind to UAS and thereby induce luciferase expression. In our model, overexpression of PPARγ was advantageous because PPARγ expression in cells other than adipocytes is rather low [25]. pM1-hPPARγ-LBD and the pUAS(4*)-TK-Luc vector were a kind gift from Karsten Kristiansen (Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark) [26, 27].

The normalization vector phRL-TK was from Promega, Mannheim, Germany.

2.4. Flavonoids and Vitamins. Quercetin, fisetin, hesperetin, naringenin, and α-tocopherol were from Sigma-Aldrich, Darmstadt, Germany; daidzein and kaempferol from Biorbyt, Cambridge, UK; genistein and ascorbic acid from Carl Roth, Karlsruhe, Germany; luteolin from Cayman Chemicals, Ann Arbor, Michigan, US; apigenin from Selleck Chemicals, Munich, Germany; and trolox from Fluka via Sigma-Aldrich. Ascorbic acid and trolox were dissolved in water, α-tocopherol in ethanol (Normapur®, VWR, Darmstadt, Germany), and the flavonoids in DMSO (Carl Roth) at 100 mmol/L for preparing stock solutions.

2.5. Neutral Red Assay. Cytotoxicity was determined via the neutral red assay [28, 29]. Hek-293 cells were seeded in 24-well plates (Fisher Scientific, Schwerte, Germany) at a density of 120,000 cells/well, precultured for 24 h, and treated with the flavonoids, ascorbic acid, or α-tocopherol at concentrations ranging from 1 to 200 μM for 24 h in 10% serum-containing DMEM. Then, the culture medium containing the test substances was replaced with fresh serum-containing medium including 50 μg/mL of neutral red (Carl Roth). After incubation for 3 h, the medium was removed and the cells were extracted using a solution comprising 50 : 49 : 1 (*v*/*v*/*v*) ethanol, water, and glacial acetic acid (Carl Roth). The absorbance was measured in a plate reader (Labsystems, Helsinki, Finland) at 540 nm. Based on these toxicity tests, we chose the highest nontoxic concentration of the most toxic compound for the luciferase assays (20 μM for the flavonoids).

2.6. Antioxidant Capacity Assays

2.6.1. ABTS Assay. The ABTS assay measures how well a test compound can reduce 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals which are formed by oxidation of ABTS with potassium persulfate. Antioxidants can scavenge this blue green radical and thereby decolour the test solution which can be measured photometrically [30].

A 7 mM ABTS and 2.45 mM potassium persulfate (both Sigma-Aldrich, Darmstadt, Germany) solution was diluted with water to give an absorbance of 0.7 at 690 nm. Following the addition of the test compound (or the vehicle control) to yield a total volume of 310 μL, and 6 min of incubation at room temperature, absorbance at 690 nm was measured in a Tecan Infinite 200 microplate reader (Tecan Group Ltd., Crailsheim, Germany). The results were plotted as the differences in absorbance relative to the vehicle control against the concentrations of the tested compound (the larger the difference, i.e., the greater the loss of absorbance, the more extensive is the reduction of the ABTS radical).

2.6.2. FRAP Assay. The ferric-reducing ability of plasma (FRAP) assay measures how well a test compound can reduce ferric (i.e., iron-III) to ferrous (i.e., iron-II). Ferric ion (iron-III) is turned into ferrous ion (iron-II) at low pH upon addition of a reducing agent. The formation of ferrous iron can be measured photometrically in a 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution since iron (II) forms a coloured complex with TPTZ [31].

Following addition of the to-be-tested compound (or the vehicle control) to an iron (III) chloride solution (1.7 mM) with TPTZ (1.67 mM) in acetate-buffered solution (228 mM) at pH 3.6 and 15 minutes of incubation, absorbance at 620 nm was measured. The absorbances resulting from ferrous ion/TPTZ complex formation were plotted against the concentrations of the tested compound.

FRAP and ABTS measurements were carried out in a Tecan Infinite 200 microplate reader (Tecan Group Ltd., Crailsheim, Germany). The final concentrations of the flavonoids and vitamins measured were 645, 323, 161, 65, 32, and 0 (solvent control) nM. In order to calculate the gradient relative to trolox, linear regressions were carried out and the gradient from the plotted flavonoid/vitamin curve was divided by the trolox gradient.

All experiments were carried out a minimum of three times (different days).

2.7. Statistics. The statistical software R [32] was used to evaluate the data. Data evaluation started with the definition of an appropriate mixed model [33, 34]. The data was assumed to be approximately normally distributed. These assumptions are based on a graphical residual analysis. For the reporter gene assays, the treatment was regarded as a fixed factor and the day as a random factor. Based on this model, a pseudo R^2 was calculated [35] and an analysis of variances (ANOVA) was conducted, followed by multiple contrast tests (Dunnet) [36] to compare the firefly/renilla ratios.

For the FRAP and ABTS assays, the treatment and the concentration were regarded as fixed factors and the day as random factor.

Based on this model, an analysis of variances (ANOVA) was conducted, followed by multiple contrast tests (Dunnet) [36] to compare the Δ absorbance at 690 nm for the ABTS assay and the absorbance at 620 nm for the FRAP assay.

Correlations and p values were calculated using "rcorr" type "Pearson" (ABTS, FRAP, and reporter gene assays with each other) or "Spearman" (number of hydroxy groups with assay outcome) from the package "Hmisc."

3. Results and Discussion

3.1. Flavonoids but Not Antioxidative Vitamins Activate Longevity-Associated Transcription Factors in Hek-293 Cells. To analyse Nrf2 activation, we measured antioxidant response element- (ARE-) driven firefly luciferase expression (pGl3-ARE) in Hek-293 cells cotransfected with the plasmid phRL-TK constitutively expressing renilla luciferase (Figure 3(a)). ARE is a binding site in the promotor region of Nrf2 target genes [6]. Similarly, FoxO transcription factors bind the forkhead responsive element (FHRE) [9] and we used a pGl3-FHRE firefly luciferase construct to measure FoxO activation (Figure 3(b)). To measure PPARγ activation, we used the Gal4 (yeast transcription activator protein) bound to the PPARγ-LBD and a firefly luciferase construct that was under the control of UAS (Figure 3(c)).

The flavonol quercetin gave positive results in all our experiments, and this is in accordance with various reports on the induction of redox-sensitive transcription factors [16, 29, 30]. Therefore, we included quercetin and the vehicle control as positive and negative control, respectively, in every set of luciferase assays.

The flavones luteolin and apigenin, which differ from quercetin by lacking the hydroxyl group in the C ring (luteolin) as well as the meta hydroxylation in the B ring (apigenin) (Figure 1), appeared to be the most active flavonoids tested. Interestingly, the flavonol fisetin, which differs from quercetin in lacking one hydroxy group in the A ring, also appeared very potent in the ARE assay but did not reach significance in the PPARγ assay.

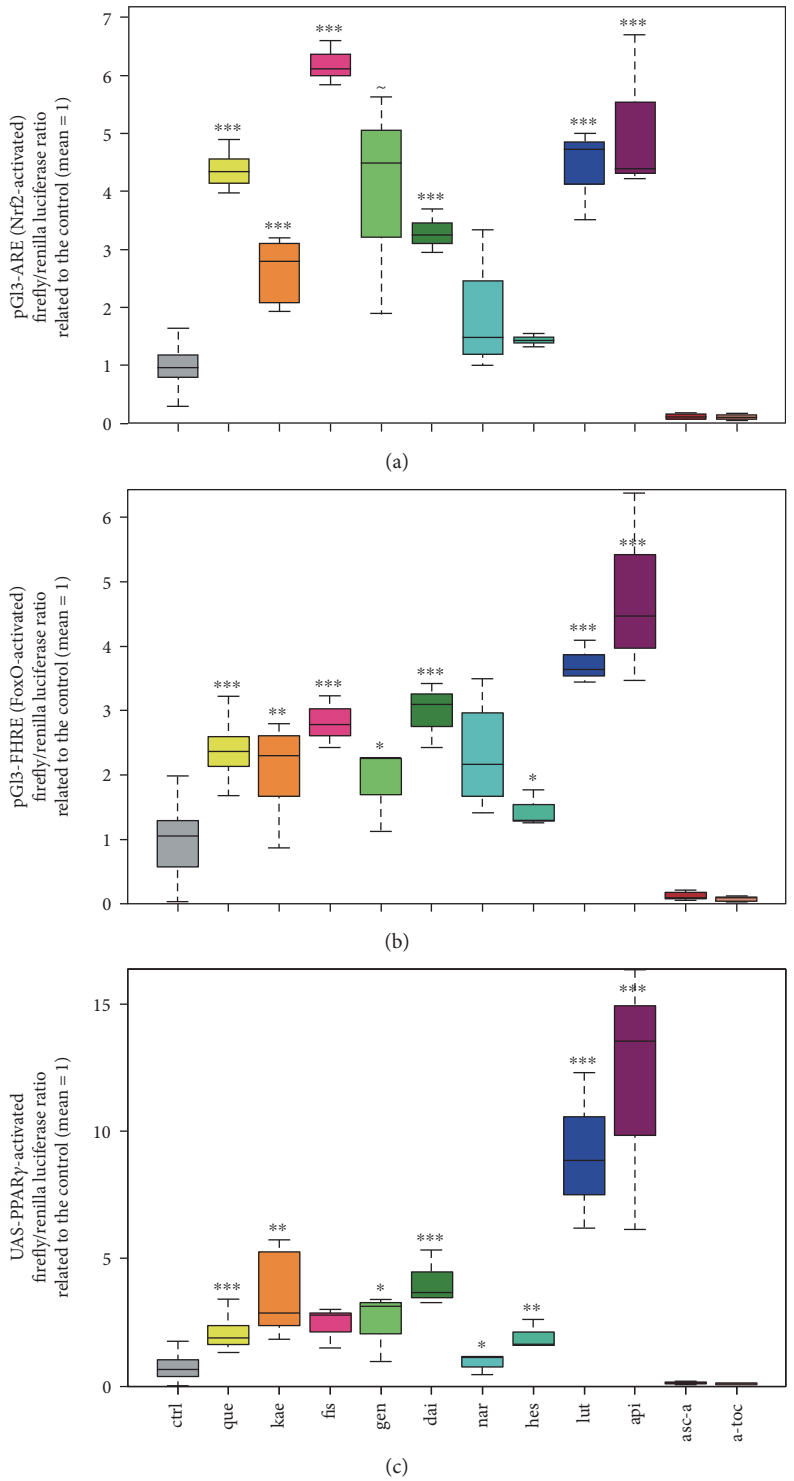

Figure 3: Hek-293 cells were transfected with firefly luciferase constructs controlled by elements responding to Nrf2 (a), FoxO (b), or PPARγ (c) activation. Constitutively expressed renilla luciferase was cotransfected to obtain firefly/renilla ratios. The vehicle for the tested flavonoid or vitamin and quercetin as a positive control were included in every experiment. In order to show all experiments in one plot, the firefly/renilla luciferase ratios were normalised to the difference between the control and quercetin and the mean of the control was set to be 1. Concentrations of flavonoids and vitamins were 20 μM and 100 μM, respectively. ctrl: vehicle control; que: quercetin; kae: kaempferol; fis: fisetin; gen: genistein; dai: daidzein; nar: naringenin; hes: hesperetin; lut: luteolin; api: apigenin; asc-a: ascorbic acid; a-toc: α-tocopherol; ARE: antioxidant response element; FHRE: forkhead responsive element; PPAR: peroxisome proliferator-activated receptor; UAS: upstream activating sequence. $\sim p < 0.1$ compared to the control, $^{*}p < 0.05$ compared to the control, $^{**}p < 0.01$ compared to the control, and $^{***}p < 0.005$ compared to the control. For the statistics, the non-normalised firefly/renilla ratios were used. A minimum of three independent experiments was performed.

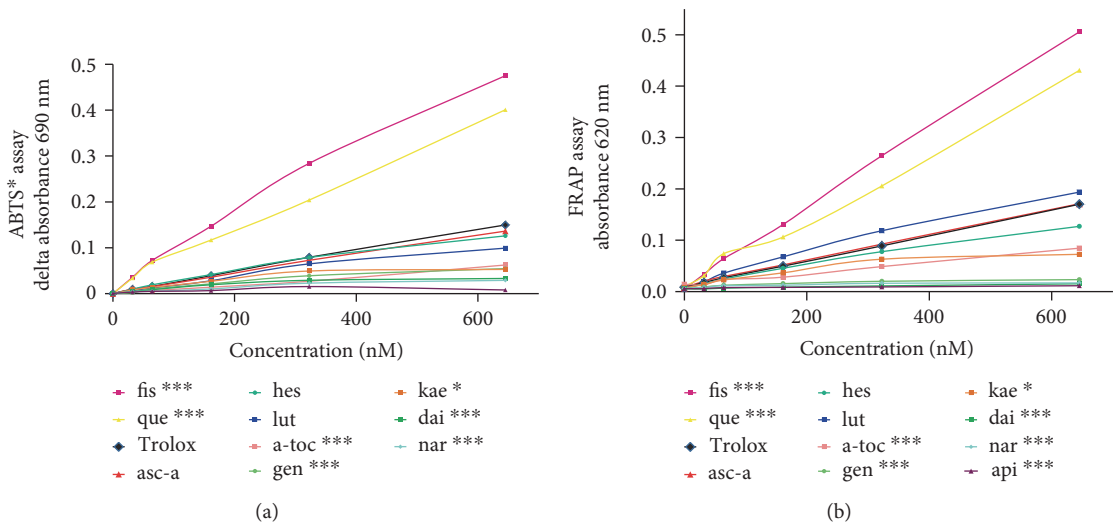

Figure 4: Graph showing the antioxidant capacity of the flavonoids and vitamins tested compared to trolox in assays using the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical (a) and the ferric-reducing ability of plasma (FRAP) (b). (a) The reduction of the coloured ABTS radical is plotted as the difference in absorbance to the vehicle control against the concentration of the compound that was added to the reaction. Absorbance was measured at 690 nm after 6 minutes. (b) The absorbance of a solution containing a known concentration of ferrous ions is plotted against the flavonoid or vitamin concentration. Absorbance was measured at 620 nm after 15 minutes incubation. The legends show the flavonoid with the steepest curve first and the shallowest last. que: quercetin; kae: kaempferol; fis: fisetin; gen: genistein; dai: daidzein; nar: naringenin; hes: hesperetin; lut: luteolin; api: apigenin; asc-a: ascorbic acid, a-toc: α-tocopherol. $^{*}p < 0.05$ compared to trolox, $^{***}p < 0.005$ compared to trolox.

In contrast, luteolin and apigenin were highly active in all three reporter gene assays. Remarkably, most of the compounds tested showed similar potencies to activate all three transcription factor-responsive assays (Figure 3).

Kaempferol, which is a flavonol-like quercetin but with one hydroxy group less in the B ring (Figure 1, Table 1), also showed induction in all three assays. While kaempferol appeared a weaker inducer than quercetin in the Nrf2- and FoxO-responsive assays, it seemed stronger than quercetin in the PPARγ-responsive assay (Figure 3).

The isoflavonoids daidzein and genistein seem to be moderate and weak inducers, respectively, in all three assays. These two isoflavonoids differ from each other in having one (daidzein) or two (genistein) hydroxy groups in the A ring. The flavanone hesperetin appears to be a weak inducer of FoxO- and PPARγ-driven reporters but showed no significant effect on Nrf2. Naringenin, which, compared to hesperetin, has a demethylated para hydroxy group and no meta hydroxy group in the B ring, seemed slightly less active than hesperetin and only showed significant activation in the PPARγ assay (Figures 1 and 3).

Consistent with the data from lifespan studies showing that ascorbic acid and α-tocopherol do not extend lifespan in model organisms [37, 38], neither vitamin C nor vitamin E induced PPARγ, FoxO, or Nrf2-driven luciferase expression (Figure 3).

Wang et al., Bumke-Vogt et al., Lee et al., Saw et al., and Paredes-Gonzalez et al. [39–43] reported that flavonols and flavones were relatively potent inducers of the longevity-associated transcription factors Nrf2, FoxO, and PPARγ. Moreover, it was reported that fisetin stimulated Nrf2 signalling, ERK/MAPK signalling, and kinases involved in cell cycle regulation in vitro [44]. Of interest, flavonoids were shown to influence various cyclin-dependent kinases [45], mitogen-activated protein kinases (MAPK), protein kinase Akt, and FoxO signalling [46] and to counteract inflammation [47-49]. In the roundworm, *Caenorhabditis elegans*, quercetin, kaempferol, fisetin, and naringenin supplementation induced nuclear translocation of the *C. elegans* FoxO orthologue [50, 51]. While FoxO3 single nucleotide polymorphisms have been associated with longevity [52], it remains unclear whether flavonoids exert their lifespan-extending effects observed in model organisms via FoxO. On the one hand, only few flavonoids depend on worm FoxO to exert lifespan extension [53]. On the other hand, in a transgenic mouse model for prostate cancer (TRAMP mice), apigenin inhibited cancer, in part, via FoxO [19].

These effects on cellular signalling pathways may contribute to the health-benefitting findings from epidemiological studies [54]. However, when evaluating the effect of dietary flavonoids, it should be kept in mind that different flavonoids, besides affecting numerous signalling pathways, [55] may act synergistically or antagonistically. Furthermore, when comparing data from in vivo studies with our results, it is important to keep in mind that we were working with flavonoid aglycons at supraphysiological concentrations. Most flavonoids in vegetables and fruits are glycosylated and they may be transported by the SGLT1 or hydrolysed and absorbed as aglycons [56]. Yet, once absorbed, they are readily metabolised by methylation, glucuronidation, and sulfation [56], which is why aglycon concentration in tissues or plasma is very low.

Our data obtained does not indicate obvious structure-activity relationships for the induction of Nrf2, FoxO, or PPARγ by flavonoids. The flavone to flavonol counterparts apigenin to kaempferol and luteolin to quercetin have one

Table 1: Correlation coefficients with p values. Significant correlations are printed in bold ($p < 0.05$) and correlations printed in italics show a trend ($p < 0.1$). We could not find any correlation between the antioxidant capacity as measured by the FRAP or ABTS assay and the luciferase assays. The total number of hydroxy groups in the molecule and especially the number of hydroxy groups in the flavonoid B-ring correlated strongly with the outcome in the FRAP and ABTS assays. Furthermore, ARE- and FHRE-activation correlated strongly with each other, and, albeit to a lesser extent, with PPARγ-activation.

Pearson correlation coefficients (p values)	Total OH	OH in A ring	OH in B ring	OH in C ring	PPAR-UAS activation	FHRE activation	ARE activation	FRAP
ABTS	*0.59* (0.06)	−0.21 (0.59)	**0.73** (0.03)	0.52 (0.15)	0.05 (0.88)	0.09 (0.79)	0.45 (0.16)	**0.98** (<0.0001)
FRAP	**0.73** (0.01)	−0.21 (0.59)	**0.82** (0.001)	0.52 (0.15)	0.12 (0.72)	0.14 (0.70)	0.46 (0.16)	
ARE activation	0.43 (0.19)	−0.31 (0.42)	0.64 (0.06)	0.35 (0.36)	**0.64** (0.04)	**0.85** (0.001)		
FHRE activation	0.20 (0.56)	−0.31 (0.42)	0.37 (0.33)	−0.35 (0.36)	**0.67** (0.03)			
PPAR-UAS activation	0.26 (0.44)	−0.31 (0.42)	0.27 (0.48)	0.17 (0.66)				

or two hydroxy groups, respectively, in the B ring and were active in all luciferase assays. While in the Nrf2-induced luciferase assay, quercetin showed more activity than kaempferol; the flavone with two hydroxy groups in the B ring (luteolin) did not show stronger induction than apigenin (Figure 1). Consistently, we did not find significant correlations between the number of hydroxy groups and activities in the reporter gene assays (Spearman correlation Table 2). However, the differences in luciferase assay activation by flavonoids could also be due to different degrees of protein binding, stability, or flavonoid concentrations in the cell [57].

3.2. Patterns of Flavonoid-Induced Activation Are Similar for Nrf2-, FoxO-, and PPARγ-Dependent Reporters. Interestingly, the different flavonoids showed similar capacities to induce all three transcription factors. This can be seen by looking at the box plots (Figure 3) and the correlation coefficients ARE versus FHRE: $R = 0.85$; PPARγ versus ARE: $R = 0.64$; and PPARγ versus FHRE: $R = 0.67$ (Table 1). Since Nrf2 and FoxOs are redox-sensitive transcription factors, it seemed somewhat plausible that their responsive elements ARE and FHRE were activated by similar stimuli. However, ARE and FHRE activation also correlated with PPARγ activation.

PPARγ controls adipocyte differentiation and is activated by endogenous agonists such as fatty acids [58] and xenobiotics such as rosiglitazone [59]. The thiazolidinedione was used as an antidiabetic drug until it became evident that its use was associated with increased risk of myocardial infarction [60]. Examples of flavonoids that were shown to be agonists or partial agonists of PPARγ are the flavonols kaempferol and quercetin [61], the flavones luteolin [62] and apigenin [63], and the isoflavones daidzein [64] and genistein [65]. It has been hypothesized that plant-derived PPARγ modulators may be able to improve insulin sensitivity without detrimental side effects. Of interest, dietary supplementation of high-fat-fed mice with luteolin ameliorated insulin resistance [62].

Furthermore, PPARγ may participate in antioxidant response since it shares target genes such as those coding for heme oxygenase 1 and catalase with Nrf2 [13] and was shown to be regulated by Nrf2 and FoxO [66-70]. However, in our model, we overexpressed the PPARγ-LBD fused to Gal4 which would induce luciferase expression via activation of the UAS. Thus, flavonoids stimulating this assay would be expected to fit into the PPARγ-LBD (functioning as agonists or partial agonists) rather than to induce transcription or translational modifications of PPARγ or its cofactors [71]. Even so, putative positive feedback loops in between FoxO or Nrf2 and PPARγ-signalling [13] could have contributed to our experimental outcome. Consistent with an interaction between Nrf2 and PPARγ to combat oxidative stress, genistein-mediated protection from stress-induced cell injury depended on both transcription factors [72]. Moreover, flavonoids seem to activate upstream signalling molecules such as PKC which phosphorylates Nrf2 thus enabling its further activation [41, 73]. Stimulation of molecular targets upstream of Nrf2, FoxO, and PPARγ could in part explain why the flavonoids we tested activated all three transcription factors to a similar extent.

3.3. Reporter Gene Activation Patterns Elicited by Flavonoids Do Not Correlate with Their Antioxidant Activities. Previous reports have found that flavonoids and vitamins showed antioxidant capacity in FRAP and ABTS assays [74]. Interestingly, in our ABTS and FRAP assays, the flavonoids quercetin and fisetin (and in the FRAP assay luteolin) showed higher values than the water-soluble vitamin E analogue trolox while apigenin, daidzein, and naringenin showed very low values (Figure 4). By correlating ABTS and FRAP values with the total number of hydroxy groups in the molecule as well as the number of hydroxy groups in the B ring (Table 1), our results reflect previous observations describing structure-activity relationships for radical scavenging polyphenols [75]. The presence of a catechol structure in the B ring could further explain why fisetin with 4 hydroxy groups is more potent in the ABTS and FRAP assays than kaempferol and ascorbic acid which also have 4 hydroxy groups.

By carrying out correlation analyses, we found a very strong correlation between FRAP and ABTS ($R = 0.98$, Table 1). However, while it seemed that FRAP and ABTS values correlated strongly ($R = 0.73$ and 0.59, resp.) with the total number of hydroxy groups in the molecule and even stronger with the number of hydroxy groups in the B ring ($R = 0.82$ and 0.73, resp.), the presence of hydroxy groups did not correlate with transcription factor-induced reporter gene activation (Table 1).

3.4. Pro- and Antioxidant Potential of Flavonoids. Paradoxically, flavonoids may protect from oxidative stress by acting as pro-oxidants. Although in cell-free in vitro assays they show radical scavenging and reducing activity (Figure 4), they are unlikely to exert a protective role towards antioxidative stress by scavenging radicals in vivo. Their low concentration inside the body and the slow reaction rate constants of such nonenzymatic (as compared to enzyme-catalysed) radical scavenging point to the notion that flavonoids stimulate endogenous (e.g., enzymatic) antioxidant defence [76–78]. Kelch-like ECH-associated protein 1 (Keap1) binds to Nrf2 in the cytosol which leads to proteasomal degradation of Nrf2 and prevents transcription of its target genes [79]. After oxidation to quinones, flavonoids could—either directly or via the formation of reactive oxygen species—cause dissociation of Nrf2 from its inhibitor Keap1, inducing nuclear translocation of the transcription factor, leading to expression of genes coding for antioxidant enzymes [77]. In our cell culture model reporting Nrf2-binding to ARE, flavonoids causing such a pro-oxidative, Nrf2-activating effect may have induced ARE-driven luciferase expression. The "xenohormesis hypothesis" postulates that induction of stress signalling pathways by subtoxic doses of a stressor such as a dietary flavonoid may prepare the organism to better defend itself from stress arising during ageing and thus extend lifespan [80].

4. Conclusion

While free radical scavenging properties of flavonoids do not correlate with the capability of these secondary plant metabolites to induce longevity-associated transcription factors,

more in vivo research is needed to understand how flavonoids may benefit longevity. Because of the possibly interconnected signalling pathways that are induced and the impact of metabolism on the bioavailability of these compounds, further research in more complex models is desirable.

Disclosure

Part of this work was presented at the 54th Congress of the German Nutrition Society (DGE).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

The Preconditioning of Berberine Suppresses Hydrogen Peroxide-Induced Premature Senescence via Regulation of Sirtuin 1

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With a long history of application in Chinese traditional medicine, berberine (BBR) was reported to exhibit healthspan-extending properties in some age-related diseases, such as type 2 diabetes and atherosclerosis. However, the antiaging mechanism of BBR is not completely clear. By means of hydrogen peroxide- (H_2O_2-) induced premature cellular senescence model, we found that a low-concentration preconditioning of BBR could resist premature senescence in human diploid fibroblasts (HDFs) measured by senescence-associated β-galactosidase (SA-β-gal), accompanied by a decrease in loss of mitochondrial membrane potential and production of intracellular reactive oxygen species (ROS). Moreover, the low-concentration preconditioning of BBR could make cells less susceptible to subsequent H_2O_2-induced cell cycle arrest and growth inhibition. Experimental results further showed that the low concentration of BBR could induce a slight increase of ROS and upregulate the expression level of sirtuin 1 (SIRT1), an important longevity regulator. H_2O_2-induced activation of checkpoint kinase 2 (Chk2) was significantly attenuated after the preconditioning of BBR. The present findings implied that the low-concentration preconditioning of BBR could have a mitohormetic effect against cellular senescence triggered by oxidative stress in some age-related diseases through the regulation of SIRT1.

1. Introduction

Since the "free radical theory" was proposed by Harman in 1956, it is gradually recognized that excessive ROS, a potent inducer of cellular senescence, are implicated in a variety of age-related diseases, including cardiovascular disease, type 2 diabetes and Alzheimer's disease [1, 2]. Mitochondria are not only a major resource of cellular ROS generation but also a sensitive target for oxidative damage. Mitochondria damage induced by ROS could be indicated by loss of electron transport, mitochondrial membrane potential, and ATP generation, which could suggest respiratory dysfunction that exacerbate ROS generation and lead to the "vicious cycle" [3]. As the major ROS within cells, H_2O_2 is regarded as a key source of hydroxyl radical modifying and mutating mitochondrial DNA (mtDNA), which was proved to be associated closely with premature aging. Experimental evidence also proved that exposure of young HDFs to subcytotoxic concentration of H_2O_2 accelerated cellular senescence, which displays several replicative senescent-like features including senescence-associated β-galactosidase (SA-β-gal) activity, an irreversible growth arrest in G1 and change in expression level of many genes [4–6].

Berberine (BBR) is a botanical alkaloid and the major bioactive compound in the Chinese's herb *Rhizoma coptidis*, which had been utilized to treat diabetes and infection for decades in traditional Chinese medicine [7, 8]. Recently, it was reported that BBR could exhibit a lifespan-prolonged function by influencing the conversion of tryptophan into kynurenine in *Drosophila* [9]. Meanwhile, BBR was also proposed as a potential antiaging agent in oxidative DNA damage and could affect mitoxantrone-induced cellular senescence [10, 11]. But the antisenescence mechanism of BBR is still not very clear. Previously, we observed that BBR was able to protect hepatocytes from H_2O_2-induced cellular apoptosis by SIRT1 [12], which is NAD^+-dependent histone deacetylase, and plays an important role in the lifespan-extending effect of caloric restriction and antiaging as the longevity regulator [13, 14]. Indeed, excessive ROS, especially H_2O_2, could accelerate cellular senescence by affecting the function and expression level of SIRT1 [15, 16]. In this study, we wanted to confirm the hypothesis that BBR may be against H_2O_2-induced premature senescence by SIRT1 and explore the possible pharmacological mechanism of BBR involved with the regulation of SIRT1 in order to expand its potential application for age-related diseases.

2. Materials and Methods

2.1. Materials. Human fetal lung diploid fibroblast cell line 2BS was purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Berberine and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemical Inc. (St. Louis, MO, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Promega (Beijing) Biotech Co., Ltd. ROS Detection Reagents H_2DCFDA was from Molecular Probes (Molecular Probes, Eugene, OR, USA). Hydrogen peroxide was a product of Beijing Chemicals Reagent Co., Ltd (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) with high glucose and fetal bovine serum were from Invitrogen (Carlsbad, CA, USA). Antibodies against Phospho-Chk2(#2661), Chk2(#3440), Sirt1(#2493), and HRP-linked anti-Rabbit IgG(#7074) and anti-Mouse IgG(#7076) were obtained from Cell Signaling Technology (Beverly, MA, USA), anti-actin antibody (sc-1616R) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and ECL plus Western blotting kit was from GE Healthcare Bio-Sciences (Marlborough, MA, USA).

2.2. Cell Culture. The characters and application of human diploid fibroblasts for cellular senescence research have been reported [16]. Briefly, early passage 2BS in 20 population doublings (PDs) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. According to different experiment requirements, 2BS cells were seeded into plates at an appropriate density. When confluent, 2BS cells were preincubated with the indicated concentrations of BBR for 12 hr before H_2O_2 was added to the medium. The H_2O_2 was freshly prepared from 30% stock solution and added to the culture medium at a final concentration of 200 μM.

2.3. Cell Cycle Assay. 20 PDs 2BS cells were seeded into 6-well plates at a density of 4×10^5/well and cultured till confluence. After being synchronized by serum deprivation, cells were pretreated with or without BBR (12 μM) for 12 hr under normal serum conditions and exposed to 200 μM H_2O_2 for 2 hr. After that, cells were switched to fresh DMEM medium for 12 hr, digested by trypsin-EDTA, and collected by centrifuging at 1000 rpm for 5 min. Supernatant was discarded and cells were fixed in ice-cold 70% ethanol for 24 hr. At the time of analysis, cells were washed twice with PBS and then resuspended in 500 μL PBS with 50 μg/mL propidium iodide and 100 μg/mL RNase A. After being incubated at 37°C for 30 min in the dark, DNA contents were analyzed for fluorescence with a BD FACSCalibur™ flow cytometer.

2.4. Senescence-Associated β-Galactosidase Staining. Cellular senescence was identified with β-galactosidase staining as described previously [6]. Briefly, after confluence, 2BS cells were pretreated with 6, 12, and 20 μM BBR for 12 hr before a 2 hr exposure of 200 μM H_2O_2. Next, cells were passaged at a ratio of 1 : 3 to 6-well plates with fresh DMEM medium for four days and then washed twice in PBS, fixed for 3~5 min with 3% formaldehyde, and washed with PBS again. After that, cells were incubated overnight at 37°C without CO_2 in a freshly prepared staining buffer (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$).

2.5. Intracellular Reactive Oxygen Species (ROS) Assay. 20 PDs 2BS cells were cultured to reach confluence in 6-well plates and treated with 12 μM BBR for 12 hr prior to a 2 hr exposure of 200 μM H_2O_2, or only treated with BBR. Then, cells were stained with 10 μM H_2DCFDA for 30 min according to the manufacturer's manual. Oxidation of the probes to 2′,7′-dichlorofluorescein (DCF) was measured by BD FACSCalibur flow cytometry. Mean fluorescence intensity (MFI) was calculated and analyzed by CellQuest software. The percentage of ROS generation was calculated according to MFI value of DCF.

2.6. Measurement of Mitochondrial Membrane Potential ($\Delta\Psi m$). To study the $\Delta\Psi$m changes, cells were stained with Rh123 at a final concentration of 10 μg/mL for 30 min in the dark, washed with PBS twice, and then centrifuged at 500 × g for 10 min. Finally, fluorescence of Rh123 was immediately detected by BD FACSCalibur flow cytometry. Mean fluorescence intensity (MFI) was calculated and analyzed using CellQuest software. The percentage of mitochondrial membrane potential loss was calculated according to MFI value.

2.7. Western Blot. 2BS cells were washed with ice-cold PBS and collected by scraping, immediately lysed in RIPA buffer (10 mM Tris-HCl pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 160 mM NaCl, 5 mM EDTA,

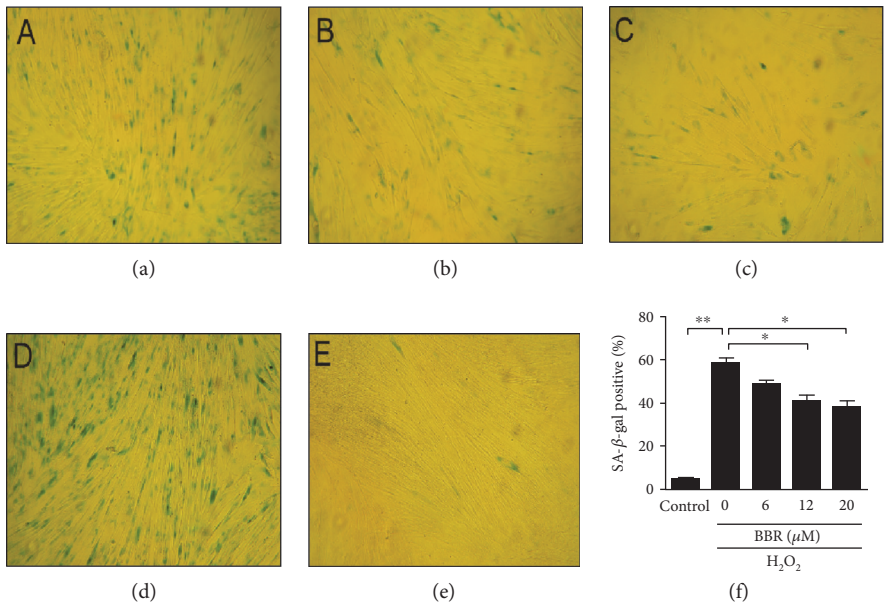

Figure 1: Antisenescence effect of BBR on H_2O_2-induced premature senescence in human diploid fibroblasts by β-galactosidase staining. 2BS cells were pretreated with different concentrations of BBR for 12 hr before exposure to 200 μmol/L H_2O_2. For senescence assay, cells were cultured for four days with new fresh DMEM medium. On the fifth day, the blue precipitate can be seen by X-gal dye in senescent cells as the Materials and Methods mentioned. (a), (b), and (c) Pretreatment with 6, 12, and 20 μmol/L BBR, respectively. (d) H_2O_2 treatment alone. (e) Untreated control. (f) The positive rates of SA-β-gal were calculated by counting the blue-dyed cells with a total of 200 cells at each visual field. $^*p < 0.05$, $^{**}p < 0.01$. The results are representative of three separate experiments.

50 mM NaF, 10 % glycerol, 1 mM Na_3VO_4, and 5 mM sodium pyrophosphate) supplemented with 1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. The protein concentration of extracts was determined by Bradford assay. According to the relative molecular weight of interest proteins, equal amounts of protein samples were subjected to 6–15% SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with various antibodies at 1 : 1000 dilutions. Finally, they were incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 2000). Visualization was detected with ECL Plus Western blotting Detection System according to the manufacturer's recommendation.

2.8. Statistical Analysis. Data are expressed as mean ± SEM, and differences between two groups were assessed by the Student *t*-test. Differences between multiple groups were assessed by one-way ANOVA (Tukey's test). $p < 0.05$ was considered significant.

3. Results

3.1. Berberine Attenuate H_2O_2-Induced Premature Senescence. Sublethal concentration H_2O_2 (<300 μM) could cause growth arrest and promote cellular senescence but no adverse effect on human diploid fibroblasts survival [4, 6]. To investigate the effect of BBR on cellular senescence, we constructed the model of premature senescence via human fetal lung fibroblasts 2BS exposed to 200 μM H_2O_2. As showed in Figure 1(d), H_2O_2 obviously induced premature cellular senescence indicated by a 58.6 ± 2.4% of SA-β-gal-positive cells compared with control group indicated by 4.8% ± 0.8% (Figure 1(e)). Meanwhile, H_2O_2 also induced significantly 92.2 ± 1.29% G1 phase arrest compared with 68.4 ± 2.5% of control (Figures 2(a) and 2(b)). However, the preconditioning of different concentrations of BBR (6–20) exhibited a significant reduction in the proportion of SA-β-gal-positive cells from 58.6 ± 2.4% to 48.8 ± 1.9%, 40.8 ± 3.0, and 38.1 ± 3.15, respectively (Figures 1(a), 1(b), 1(c), and 1(f)). In addition, the preconditioning of BBR at 12 μM partially rescued H_2O_2-induced change of cell cycle from 92.2 ± 1.29% to 80.6 ± 2.5% in G1 phase, 2.2 ± 0.52% to 8.5 ± 1.1% in S phase, and 5.5 ± 0.78% to 8.8 ± 1.5% in G2/M phase (Figures 2(a) and 2(b)). Also, the preconditioning of BBR at 12 μM significantly prevented H_2O_2-induced inhibition of cell growth from 41.8 ± 1.7% to 21.1 ± 4.5%. Interesting, when the concentration of BBR pretreated was 20 μM, the cytoprotective effect of BBR was absent (Figure S1 available online at https://doi.org/10.1155/2017/2391820). These results suggested that low-concentration preconditioning of BBR had a beneficial effect on fibroblasts for resisting H_2O_2-induced cellular senescence.

3.2. Berberine Protect H_2O_2-Induced Damage of Mitochondrial Membrane. To examine the protective effect of BBR on mitochondrial membrane, Rhodamin123 was utilized for staining cells, which could selectively be retained in the mitochondria with an intact membrane potential, and be washed out of cells when mitochondrial membrane potential is lost [17, 18]. As shown in Figure 3, H_2O_2 obviously induced an increase of 2BS cells with $\Delta\Psi$m loss from 6.7 ± 0.64% to 36.6 ± 4.5%. Indeed, pretreatment of BBR at 12 μM decreased the percentage of 2BS cells with $\Delta\Psi$m loss from

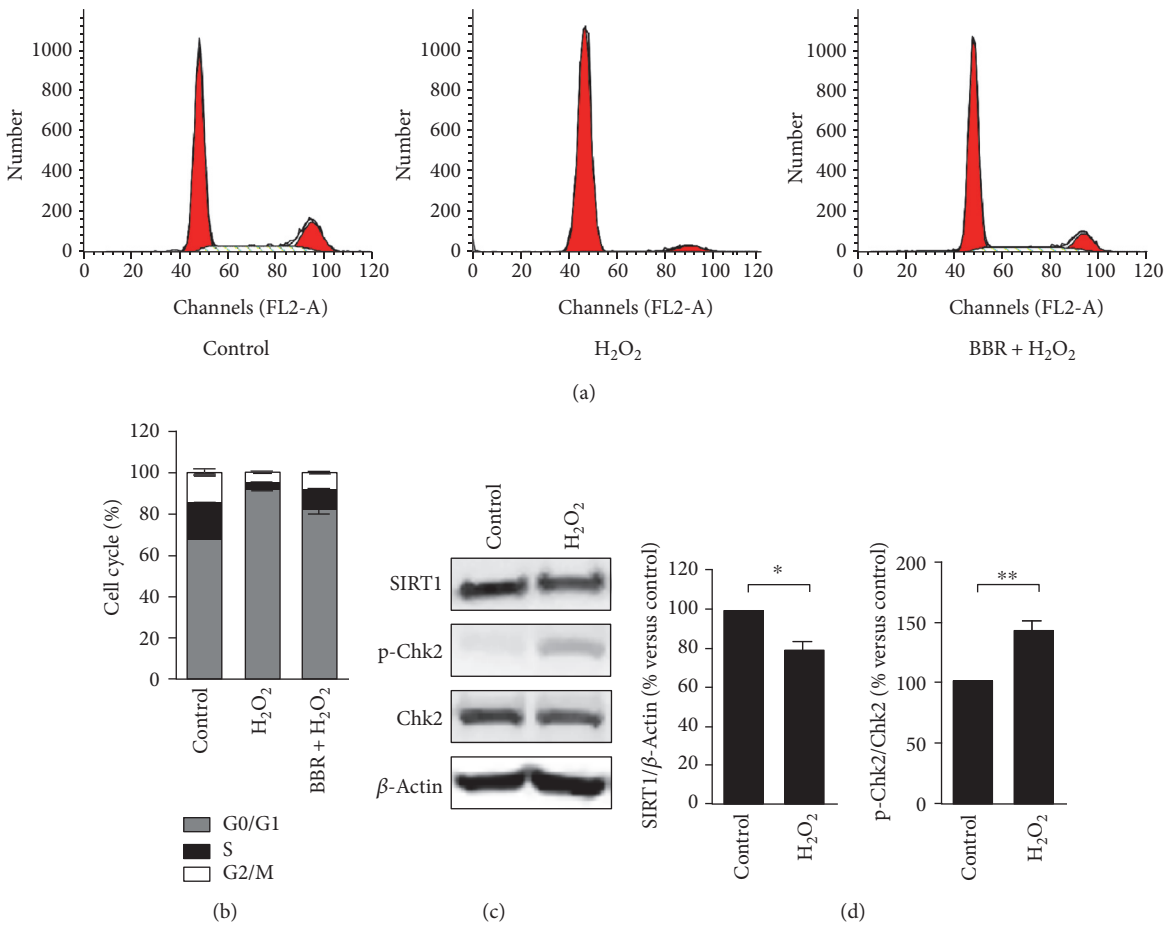

Figure 2: Cell cycle arrest and protein expression of H_2O_2-induced premature senescence in human diploid fibroblasts. 2BS cells were exposed to a sublethal concentration of 200 μmol/L H_2O_2, or pretreated with 12 μmol/L BBR for 12 hr before exposure. (a), (b) The influence of BBR in H_2O_2-induced cell cycle distribution. (c) The changes of SIRT1 expression and phosphorylation of Chk2 in H_2O_2-exposed human diploid fibroblasts. (d) Relative expression levels of Sirt1 and phospho-Chk2 in H_2O_2-exposed human diploid fibroblasts by gray analysis. $^*p < 0.05$, $^{**}p < 0.01$. The results are representative of three separate experiments.

36.6 ± 4.5% to 13 ± 2.7%. Moreover, H_2O_2 exposure led to a significant increase to 21.1 ± 1.8% in intracellular ROS of 2BS cells by fluorescent probe DCF, compared with 3.4 ± 0.57% of untreated control. The percentage of intracellular ROS induced by H_2O_2 reduced obviously from 21.1 ± 1.8% to 9.4 ± 0.9% after the preconditioning of BBR at 12 μM. Interestingly, BBR itself caused a slight increase in intracellular ROS generation (4 ± 1.6%), but nonsignificantly compared with untreated control (Figure 4). These data implied that the low-concentration preconditioning of BBR against H_2O_2-induced cellular senescence could be closely associated with BBR-induced mitochondrial stress, which subsequently plays an important role in the resistance of sublethal H_2O_2-induced damage.

3.3. Berberine Upregulates SIRT1 Expression and Reduces H_2O_2-Induced Chk2 Activation. As our previous report, BBR could influence the survival of cells by an important longevity regulator SIRT1 [12]. Therefore, we examined the role of SIRT1 in H_2O_2-induced premature senescence of human diploid fibroblasts 2BS. As shown in Figure 2, H_2O_2 exposure for 2 hr induced a moderate decrease of SIRT1 expression in 2BS cells. When cells were exposed to H_2O_2 for 4 hr, the expression level of SIRT1 exhibited a significant reduction (Figure 5). It was interesting that BBR at 12 μM alone could upregulate SIRT1 expression of fibroblasts 2BS in a time-dependent manner (Figure S2). H_2O_2 exposure also activated checkpoint kinase 2 significantly in a time-dependent manner (Figure 5), which is an event of the molecular signalling in H_2O_2-induced DNA damage response [19]. However, the preconditioning of BBR could not only protect from reduction of expression level of SIRT1 as same as did in our previous report, but also reduce phosphorylation of Chk2 (Figure 5). Meaningfully, the changes between SIRT1 expression and Chk2 phosphorylation by the preconditioning of BBR were inverse. These results hinted that the protective effect of low-concentration preconditioning of BBR on mitochondria or cellular senescence may be related to the upregulation of SIRT1 and deactivation of Chk2.

4. Discussion

Despite organismal complexity, the knowledge of basic aging or senescence mechanisms had been updated constantly for

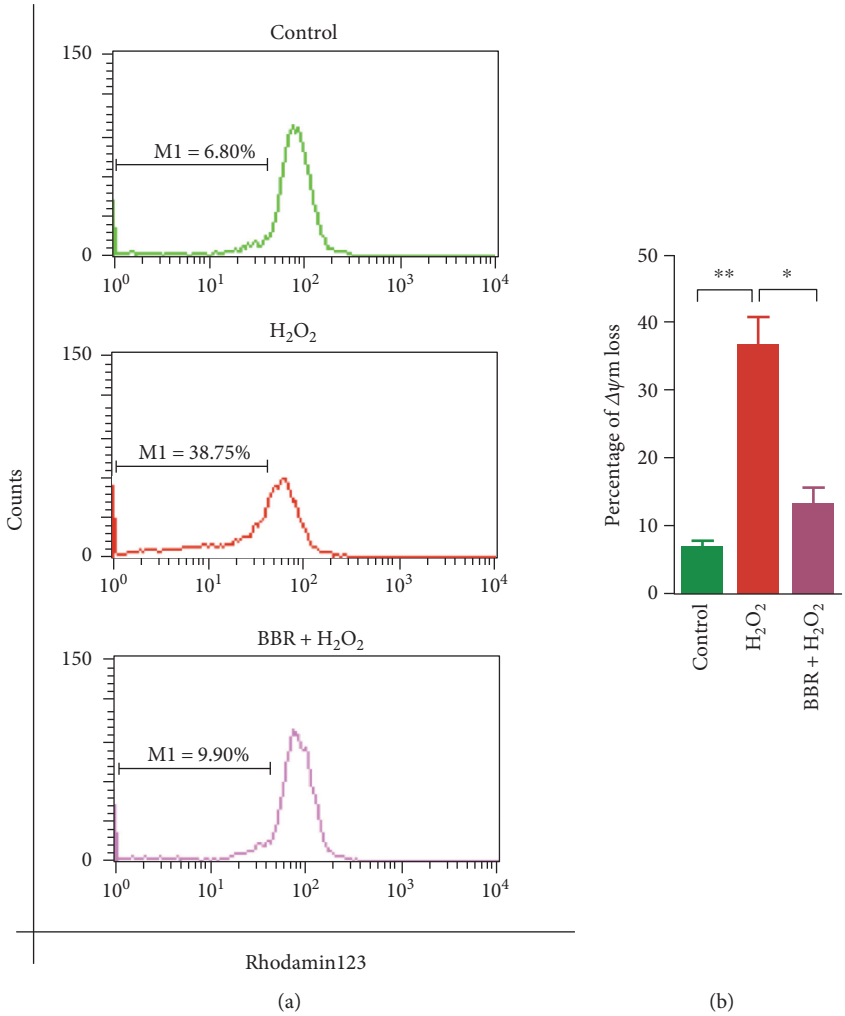

Figure 3: Protective effect of BBR on H_2O_2-induced mitochondrial membrane potential ($\Delta\psi$m) loss in human diploid fibroblasts. 2BS cells were pretreated with or without 12 μmol/L BBR for 12 hr before a 2 hr exposure to 200 μmol/L H_2O_2 and then were stained with Rhodamin123. (a) The diagram from flow cytometry test. M1 represents the percentage of $\Delta\psi$m loss. (b) Histogram of the mean fluorescence intensity of M1. $^*p < 0.05$, $^{**}p < 0.01$. The results are representative of three separate experiments.

decades of research from cell-tissue culture to model organisms. Extending lifespan by biomedical interventions are scientifically plausible, such as calorie restriction and pharmacologically proaging pathway-targeted drugs [20, 21]. Here, we showed that a low concentration of BBR at 12 μM could upregulate the expression level of SIRT1, an important longevity regulator, in human diploid fibroblasts, and the preconditioning of BBR at 12 μM could suppress H_2O_2-induced cellular senescence by deactivating checkpoint kinase 2, which may subsequently prevent the downregulation of SIRT1.

Excessive ROS produced from the mitochondria is still regarded as one of the major factors for cumulative DNA damage to promoting aging. This mitochondrial free radical theory of cellular senescence is partly supported by the model of sublethal H_2O_2-induced replicative senescence in human diploid fibroblast [2, 3, 22]. Indeed, in our experiments, sublethal H_2O_2 increased significantly the number of senescent cells (Figures 1(d) and 1(f)), induced checkpoint kinase 2 activation (Figure 2(c)), and subsequently caused an irreversible growth arrest in G1 phase (Figures 2(a) and 2(b)). As a major and stable product formed by mitochondrial respiratory chain, excessive H_2O_2 can not only directly damage the integrity of DNA to cause genome instability but it can also be converted into hydroxyl radical, which injure mitochondrial membrane and lead to loss of $\Delta\Psi$m. Consequently, loss of $\Delta\Psi$m further can cause dysfunction of the mitochondrial electron transport chain and result in generation of more intracellular ROS [3, 22, 23]. Indeed, as shown in our experiments, H_2O_2 induced loss of $\Delta\Psi$m (Figure 3) and caused generation of more intracellular ROS (Figure 4) in human diploid fibroblast. This suggested that mitochondrial dysfunction may be one of the reasons for acceleration of cellular senescence induced by sublethal H_2O_2.

In some studies, mitochondria-associated pathway can be manipulated to extend lifespan by pharmacological drugs, such as antioxidant and SIRT1-activtor resveratrol [24], antilipolytic acipimox [25], and antidiabetic metformin [26]. Berberine (BBR), the bioactive compound in the Chinese herb *Rhizoma coptidis*, had been reported as an

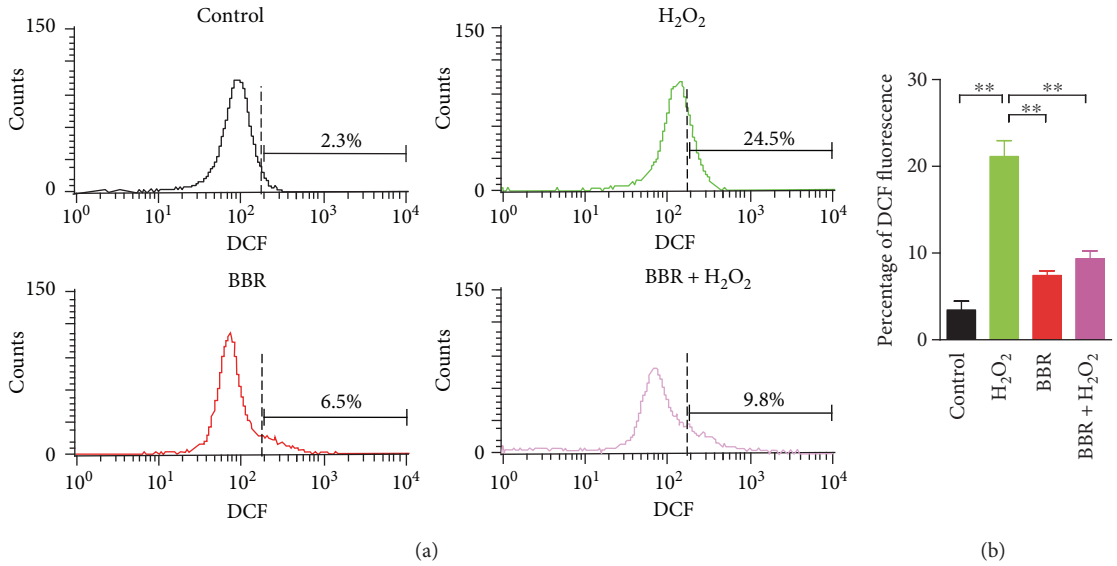

Figure 4: Protective effect of BBR on H_2O_2-induced intracellular ROS production in human diploid fibroblasts. 2BS cells were pretreated with or without 12 μmol/L BBR prior to a 2 hr exposure of 200 μmol/L H_2O_2, or treated with BBR alone as the Materials and Methods mentioned. (a) The diagram of fluorescence DCF detection with different treatments from flow cytometry analysis. (b) Histogram of the mean fluorescence intensity of DCF, reflecting the percentages of ROS generation in different treatment group. $^{**}p < 0.01$. The results are representative of three separate experiments.

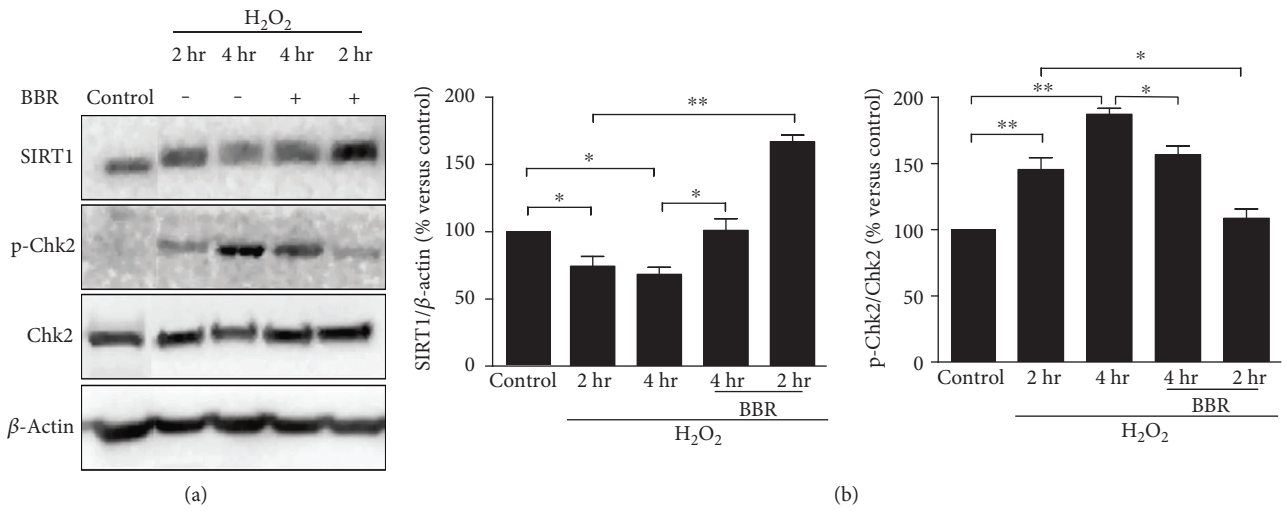

Figure 5: Protective effect of BBR on H_2O_2-induced reduction of SIRT1 expression and augment of phosphorylation of Chk2 in human diploid fibroblasts. 2BS cells were pretreated with 12 μmol/L BBR for 12 hr before a 2 hr or 4 hr exposure to 200 μmol/L H_2O_2. (a) Sirt1 and phospho-Chk2 was detected by western blotting. (b) Relative expression levels of Sirt1 and phospho-Chk2 in cells with different treatment by gray analysis. $^{*}p < 0.05$, $^{**}p < 0.01$. The results are representative of three separate experiments.

antidiabetic drug with a long history in china [8, 27]. Recently, it also was found that BBR had an antiaging effect by AMPK-mediated inhibition of mTOR signaling [11]. However, AMPK activation by BBR may be attributed to its inhibition of mitochondrial respiratory electron transport chain, which causes a decrease of ATP production and leads to an increase of AMP to ATP ratio [28, 29]. AMPK activation also enhance SIRT1 activity by NAD^+ levels [30]. Intriguingly, in our study, the expression level of SIRT1 was increased in a time-dependent manner when fibroblasts were treated at a low concentration (12 μM) of BBR (Figure S2). Moreover, the preconditioning of BBR at 12 μM obviously decreased the amount of senescent cells in a dose-dependent manner measured by the activity of senescence-associated β-galactosidase (Figures 1(a), 1(b), 1(c), and 1(f)), and partly reversed H_2O_2-induced G1 phase growth arrest (Figure 2(a)). These data suggested that upregulation of SIRT1 may be another antiaging mechanism of BBR. Actually, AMPK and SIRT1 are vital participators in cellular senescence by reciprocal regulation [29–32].

However, it was reported in many literature that BBR had a diphasic effect on different type of cells, such as protective effect on endothelial cells [33] and β cells [34] or apoptosis-induced effect on cancer cells [35, 36]. By MTT assay, we observed that BBR could protect cell from H_2O_2-induced growth inhibition at a low concentration ($\leq 12\,\mu$M), and at high concentration ($\geq 20\,\mu$M), BBR had no protective effect (Figure S1). Actually, this could be correlated with the concentration and intracellular distribution of BBR. BBR is a mitochondria-targeted cationic probe and can gather at the inner mitochondrial membrane by electrostatic interaction [37]. At a low concentration, BBR could mostly accumulate in the mitochondria and exhibit no cytotoxicity [38]. With an increase of concentration of BBR, the cellular localization of BBR distributed from the mitochondria to the cytoplasm and nuclei, which may inhibit cell growth and even induce cell death [39]. When targeted mitochondria are at low concentration, BBR could convert energy metabolism of cells into glycolysis by inhibition of mitochondrial respiratory chain [28, 38], which was indirectly reflected by a slight increase of intracellular ROS (Figure 4). Recently, evidence indicated the beneficial role of mitochondrial ROS in lifespan at physiological or low concentration under stress conditions [40, 41]. It implied that low concentration of BBR may alert the mitochondria to trigger stress-mediated SIRT1 expression by an appropriate amount increase of intracellular ROS, and benefit fibroblasts to resist subsequent sublethal H_2O_2-induced stress.

SIRT1 is a master metabolic sensor and a mitochondria function protector by regulating some elements of proaging pathways, and delays the onset of age-related diseases [13, 14, 42–45]. Under oxidative stress, SIRT1 could exhibit antioxidative activity by upregulating expression of antioxidant enzymes [46, 47]. However, H_2O_2 could downregulate the expression level of SIRT1 by Chk2-HuR-mediated instability of SIRT1 mRNA [16]. Indeed, in our study, exposure to sublethal H_2O_2 induced mild downregulation of SIRT1 and Chk2 activation (Figures 2(c), 2(d), and 5(a)). But the H_2O_2-induced Chk2 activation was reduced significantly by pretreatment of BBR, accompanied by an obviously retard in downregulation of SIRT1 expression (Figure 5). These data implied that low concentration of BBR may also protect from reduction of SIRT1 expression by deactivating Chk2, which may be associated with BBR-induced upregulation of SIRT1.

Mitohormesis could be triggered by any insults including pharmacological intervention and induce the cellular stress response, and thereby protect against larger subsequent stresses [48]. More importantly, it was proposed that mitohormesis is a reason for benefits of ROS produced by lifespan-promoting interventions in cellular physiology under stress [49]. Recently, metformin, an antidiabetic drug, shows lifespan-extending properties by peroxiredoxin PRDX-2 related to ROS-mediated mitohormetic signalling pathway [26]. Based on our data, we speculate that the mitohormetic effect of BBR may be associated with the upregulation of SIRT1, which possibly is associated with a BBR-induced appropriate amount of increase of intracellular ROS. However, what is the mitochondrial retrograde signalling that induces SIRT1 expression during mitohormesis? Is ROS produced by low concentration of BBR really involved in mitohormesis as a signalling molecular? All of these questions will need to be answered by further deliberate experiments.

5. Conclusions

Our work revealed that a low-concentration preconditioning of BBR may exhibit antisenescence effect on H_2O_2-induced premature senescence of human diploid fibroblasts through the process of mitohormesis involved with SIRT1 upregulation, which elicits cytoprotective responses to resist subsequent H_2O_2-induced stress by decreasing activation of Chk2. In turn, this could protect against reduction of SIRT1 expression. This could give a hint that mitochondrial-targeted drugs like BBR can be potentially applied for aging-related diseases through mitohormesis.

Conflicts of Interest

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

Acknowledgments

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Research Article

LC-QTOF-MS Analysis and Activity Profiles of Popular Antioxidant Dietary Supplements in Terms of Quality Control

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The dietary supplements with claimed antioxidant activity constitute a substantial part of the dietary supplement market. In this study, we performed the LC-QTOF-MS analysis and investigated the activity profiles of popular antioxidant dietary supplements from different chemical groups in terms of quality control. The commonly used antioxidant tests and statistical analysis revealed that substantial part of the results was comparable if 1 g sample was considered, but while comparing single and daily doses, significant differences in antioxidant values were noticed in all assays. The best antioxidant activity was obtained in ORAC assay (from 142 to 13814 μM of Trolox equivalents per 1 g of sample), and the strongest correlation occurred between TPC and ORAC. The LC-QTOF-MS analysis revealed that catechins were present in samples having the best antioxidant activity and that dietary supplements showing the weakest activity contained very small amount of any chemical constituents.

1. Introduction

Dietary supplements are nowadays a big branch of food industry, and their consumption was reported to increase in the recent years [1]. They are claimed to provide health benefits and prevent numerous chronic diseases. Dietary supplements containing antioxidants (vitamins, glutathione, selenium, and plant polyphenols) are recommended to diminish oxidative stress occurring in the human body and causing diseases such as atherosclerosis, arthritis, cancer, cardiovascular disease, and inflammation [1, 2]. Because of the growing interest in natural antioxidants, it is possible to find in the market more and more new products containing mainly plant extracts, their blends, isolated plant secondary metabolites, or algae with declared antioxidant properties. The diversity of active compounds is very large; however, most of them belong to polyphenols. The antioxidant assays comparing different foods, beverages, or herbs use 1 g or 100 g of sample as a unit of measure. However, for finished products (available as dietary supplements), antioxidant activity should be rather described for a single dose (one tablet/capsule) or recommended by a producer daily dose (number of tablets/capsules taken per day). This new approach enables the proper comparison of antiradical power of dietary supplements used to enrich diet in antioxidants. The assessment of activity of a dose taken is important in dietary supplement industry because it also enables to guarantee a safety of supplemented compounds.

In this paper, we aimed to characterize dietary supplements with claimed antiradical activity containing compounds from different chemical groups (anthocyanins, biflavonoids, catechins, curcuminoids, flavonoids, phenolic

acids, phlorotannins, proanthocyanidins, and stilbenoids). The performed commonly used antioxidant tests (ORAC, ABTS, and DPPH) as well as the measurement of total phenolics with Folin–Ciocalteu reagent enabled to compare for the first time the activity of single dose and daily dose of each formulation and to draw the correlations between tests used. In terms of quality control, the LC-QTOF-MS analysis was used for a chemical characterization of the studied dietary supplements.

2. Experimental

2.1. Sample Preparation. All dietary supplements (DS) were commercially available and were bought in the pharmacy store. The active ingredients declared by the manufacturer along with recommended daily dose are listed in the Table 1. Ten capsules or tablets were taken from each package of examined DS. The content of the capsules was quantitatively transferred into the conical flask, whereas tablets were placed in a flask without grinding. Bidistilled water (100 mL) was added to the flask, and the content of the vessel was heated for 1 hour at 37°C and mixed using a magnetic stirrer. Then, the mixture was centrifuged at a speed of 3300 rpm for 10 min, and precipitate was removed by filtration. The obtained aqueous filtrate was freeze-dried (Freeze Dryer, Christ Alpha 2–4 LD, Germany). A similar procedure was repeated for each DS. The lyophilisates were weighed and stored in a vacuum-sealed containers until used.

2.2. Determination of Antioxidant Activity. The oxygen radical absorbance capacity assay (ORAC) was adopted from [3], and the α,α-diphenyl-β-picrylhydrazyl (DPPH) assay was adopted from [4]. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay followed the method of [5] with modifications for microscale measurements. All reactions were done in 3 replicates for each sample (standard and DS samples). Trolox used in the abovementioned tests was dissolved in methanol and diluted to obtain the following concentrations: 3.125, 6.25, 12.0, 25.0, 50.0, 100.0, and 200.0 μM. The samples of DS were prepared by dissolution of exactly weighted 1 mg of each lyophilisate in deionized water, and the dilutions of obtained stock solutions were in the range from 0.01 to 0.1 mg/mL depending on the sample activity. The results obtained for ORAC, DPPH, and ABTS assays were expressed as Trolox equivalents per 1 g of sample, per single dose, and per daily dose of investigated dietary supplements.

The total phenolic content assay (TPC) was adopted from [6] with modifications for microscale measurements. The working solution of Folin–Ciocalteu reagent (FCR) was prepared by 1 : 10 (v/v) dilution of the stock solution with deionized water. The caffeic acid was dissolved in deionized water and diluted to obtain the following concentrations: 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 μg/mL. The samples of DS were prepared by dissolution of exactly weighted 1 mg of each lyophilisate in deionized water to obtain concentrations in the range from 2.0 to 0.5 mg/mL depending on the sample activity. The experimental wells in the 96-well plate were filled with 10 μL of samples or caffeic acid concentrations, whereas the blank wells received 10 μL of deionized water. Then, 50 μL of FCR was added to every well, and after 30 seconds, all wells were filled with 150 μL of saturated sodium carbonate solution. The plate was incubated in the 40°C for 30 minutes. Next, the absorbance was measured at 740 nm using the microplate reader. The plate reader was controlled by Gen5™ Data Analysis software, which was used also to obtain standard curve by plotting the absorbance of caffeic acid against its various concentrations. All reactions were done in 3 replicates for each sample (standard and antioxidant samples). The results obtained for TPC assay were expressed as caffeic acid equivalents per 1 g of sample, per single dose, and per daily dose of investigated DS.

2.3. LC-QTOF-MS Analysis. The LC-QTOF-MS analysis was performed on Agilent 1200 Infinity HPLC chromatograph hyphenated with QTOF 6530B Accurate-Mass QTOF LC/MS system equipped with Dual Agilent Jet Stream spray source (ESI) (Agilent Technologies, Santa Clara, CA, USA) connected with N2 generator (Parker Hannifin Corporation, Haverhill, MA; generating N2 at purities > 99%). HPLC was performed on Gemini® 3 μm i.d. C18 with TMS endcapping, 110 Å, LC Column 100 × 2 mm, and mobile phase was a gradient of 5% acetonitrile in water (A) and 95% acetonitrile in water (B); both phases have a pH value of 4.5 with addition of 10 mM of ammonium formate. A mobile phase gradient was optimized according to the polarity of compounds present in the samples. Samples S1, S10, and S14 were analyzed in the gradients 0–45 min from 5 to 60% of B, 46–55 min from 61 to 90% of B, and 56–60 min 90% of B. For samples S3–S7, S9, S13, and S15, the gradients 0–45 min from 5 to 40% of B, 46–55 min from 41 to 80% of B, and 56–60 min from 81 to 90% of B were applied. Samples S2, S8, S11–12, and S16 were separated in the gradients 0–45 min from 0 to 20% of B, 46–55 min from 21 to 60% of B, and 56–60 min from 61 to 90% of B. The flow rate was 0.1 mL/min. Total analysis time was 75 min. ESI-QTOF-MS analysis was performed in 2 GHz extended dynamic range, negative ionization mode, with fragmentor energy of 160 V, drying gas temp: 350°C, drying gas flow: 12 l/min, sheath gas temp: 400 °C, sheath gas flow: 12 l/min; nebulizer pressure: 35 psig, capillary V (+): 4000 V, and skimmer: 65 V. The acquisition parameters are as follows: auto MS/MS mode; mass range 50–1000 amu for MS and 50–1000 amu for MS/MS experiment; and 1 spectra/s acquisition. The collision-induced dissociation (CID) energy was optimized in the range 10–40 V. The identification of compounds present in samples was performed by comparison of MS/MS spectra with literature data and records from METLIN database. The tentative identification of some derivatives was based on the fragmentation patterns of known compounds.

2.4. Statistical Analysis. Each antioxidant activity assay was done in 3 replicates from the same sample in order to determine the precision of method used. The arithmetic mean and median, standard deviation, and coefficient of variation were calculated for the results obtained by various methods. Correlations among data obtained using different assays were

Table 1: The declared ingredients of investigated dietary supplements.

	Composition of the supplement (1 capsule/tablet)			
	Ingredients*	Mass [mg]	Formulation	Recommended daily dose
S1 (turmeric)	Turmeric rhizome powder	720	Capsule	2
S2 (bilberry)	Powdered bilberry fruit	250	Tablet	2
	Extract of bilberry including the following:	40		
	(i) Anthocyanins	10		
	(ii) Vit. C	40		
	(iii) Vit. E	6		
	(iv) Lutein	3		
	(v) Beta carotene	83.5		
S3 (blend of extracts)	Extract from the root of Baikal skullcap	150	Capsule	2
	Powdered cinnamon bark	60		
	Cranberry fruit extract	50		
	Extract from green tea leaves	50		
	Extract of the herb of horsetail	45		
	Vit. C	40		
	Extract of chokeberry	20		
	Extract from rhizomes of ginger	12		
	Extract of bilberry fruit	10		
	Extract of grape fruit	3		
S4 (acai)	Extract of acai berry	300	Capsule	1
S5 (grapes)	Grape skin extract including	400	Capsule	1
	(i) Trans-resveratrol	200		
	Grape seed extract including	100		
	(i) Proanthocyanidins	95		
S6 (resveratrol)	Resveratrol (from an extract of *Reynoutria japonica*)	50	Capsule	1
S7 (*Schisandra*)	Powdered fruits of the *Schisandra chinensis*	525	Capsule	
S8 (goji)	Extract of goji fruit including	300	Capsule	1
	(i) Polysaccharides	150		
S9 (pomegranate)	Pomegranate peel extract including	300	Capsule	1
	(i) Elagic acid	120		
S10 (spirulin)	Spirulin powder	450	Capsule	3–6
S11 (green tea)	55% green tea extract including the following:	250	Capsule	1-2
	(i) EGCG (epigallocatechin gallate)	137.5		
	(ii) Polyphenols	249		
	(iii) Catechins	200		
S12 (green coffee)	Extract of green coffee including the following:	800	Capsule	1
	(i) Caffeine	34.8		
	(ii) Chlorogenic acid	400		
S13 (hawthorn)	Powdered fruit of hawthorn	565	Capsule	1-2
S14 (OXXYNNEA and blend of extracts)	OXXYNNEA**	200	Capsule	1
	Grape seed extract (95% of proanthocyanidins)	150		
	Extract from green tea leaves (55% of EGCG)	150		
	Citrus bioflavonoids 40%	150		
	Trans-resveratrol	100		
	Extract of *Rhodiola rosea* root (4% of rosavins)	100		
	Quercetin	100		
	Extract from the leaves of artichoke (5% of cynarin)	50		

Table 1: Continued.

	Composition of the supplement (1 capsule/tablet) Ingredients*	Mass [mg]	Formulation	Recommended daily dose
	Extract of cranberry fruit (10% of proanthocyanidins)	40		
	Alpha-lipoic acid	30		
	Coenzyme Q10	15		
	Astaxanthin	5		
	Lycopene	1		
	Beta carotene	1		
S15 (green coffee)	An extract of green coffee beans (50% of chlorogenic acid) including	400	Capsule	2
	(i) Caffeine	20		
S16 (Ecklonia)	*Ecklonia cava* extract 25 : 1 (98.8% pure *Ecklonia cava*—the stem and leaves standardized to 15% polyphenols and phlorotannins)	53	Capsule	1

*The fillers and additives forming capsule/tablet were omitted; **OXXYNNEA—the blend of extracts of fruits and vegetables: white and red grapes, oranges, grapefruit, blueberry, papaya, pineapple, strawberries, apples, apricots, cherries, black currants, tomato, carrot, green tea, broccoli, cabbage, onions, garlic oil, wheat germ, cucumber, and asparagus.

calculated using Pearson's correlation coefficient. Statistical calculations were performed using the Statistica software version 10 (StatSoft Inc., Tulsa, OK, USA).

3. Results and Discussion

Dietary supplements are a big part of food and pharmaceutical market worldwide. They do not undergo quality controls; hence, sometimes the obtained activity may be different than expected. In this study, dietary supplements with claimed antioxidant activity were tested to check their real value. Preparations consisted of substances from different chemical groups (Table 1) (catechins, curcuminoids, flavonoids, phenolic acids, phlorotannins, proanthocyanidins, and stilbenoids) and showed both very high and negligible antioxidant activities. The water extracts used in this study were prepared during one hour stirring of dietary supplement content in water in 37°C. These conditions were applied to simulate the release from formulation matrix of the compounds soluble in gastrointestinal fluids.

The results obtained in activity assays (Figures 1 and 2) indicated that the activity of standard unit of measure (1 g/100 g) does not correspond to the activity of the dose recommended for diet supplementation. The highest discrepancies among the doses were noticed for ABTS assay, where substantial part of the results was comparable if we take into account the 1 g sample, but totally different pattern was visible for single and daily doses. Smaller differences were observed for ORAC and DPPH assays, where the best activities were obtained for the same samples. S14 gave the best result as a single dose, but S11 was the most active as a daily dose (with exception of ABTS assay).

Catechins were the major compounds in the samples which showed the best antioxidant activity (S11 and S14). The supplement with an extract of green tea (S11) contained mainly epi- and gallocatechin gallate as well as epi- and catechin gallate. The ORAC value obtained for S11 (13814.2 μmol Trolox/g) corresponds to the highest result obtained by Seeram et al. who in their study examined supplements with green tea and described results in the range from 218.7 to 13690.7 μmol Trolox/g [7]. It also should be noted that the antioxidant activity of green tea supplement measured in the ORAC assay was 1.4 times greater than that measured in the DPPH assay and up to 8.3 times higher than that measured in the ABTS method, which can be explained by the different reaction mechanisms of catechins and used reagents. This observation is consistent with the results obtained by Tabart et al. who showed that flavan-3-ols such as catechins, epicatechins, and epigallocatechins give a much higher antioxidant activity values in the ORAC method than in ABTS and DPPH assays [8]. The second potent preparation was S14 (OXXYNNEA® and blend of extracts). According to the manufacturer, the preparation should contain grape seed extract (95% of proanthocyanidins), extract from green tea leaves (55% of EGCG), citrus bioflavonoids 40%, extract of *Rhodiola rosea* root (4% of rosavins), extract from the leaves of artichoke (5% of cynarin), extract of cranberry fruit (10% of proanthocyanidins), trans-resveratrol, quercetin, alpha-lipoic acid, coenzyme Q10, astaxanthin, and lycopene. The performed LC-QTOF-MS analysis (Figure 3, Table S2 available online at https://doi.org/10.1155/2017/8692516) confirmed the presence of catechins, with epigallocatechin gallate as a predominant compound followed by gallocatechin gallate and epicatechin gallate. The other detected compounds were gallic acid, flavonoids, and resveratrol. The proanthocyanidins, rosavins, and cynarin were not detected. From the chemical profile of this sample, it can be seen that catechins are responsible for the quite high antioxidant value obtained in the ORAC assay (5513.39 μmol Trolox/g), but the declared content of preparation was not assured. The sample following catechins in activity was S15. Between the two supplements with green coffee extract, only S15 exerted quite high antioxidant activity and this was consistent with total phenolic content which reached 304.62 and 34.67 mg of caffeic acid equivalents/g for S15 and S12, respectively. Based on these data, it can be concluded that the tested supplements of green tea had significantly different quality in terms of quantity of

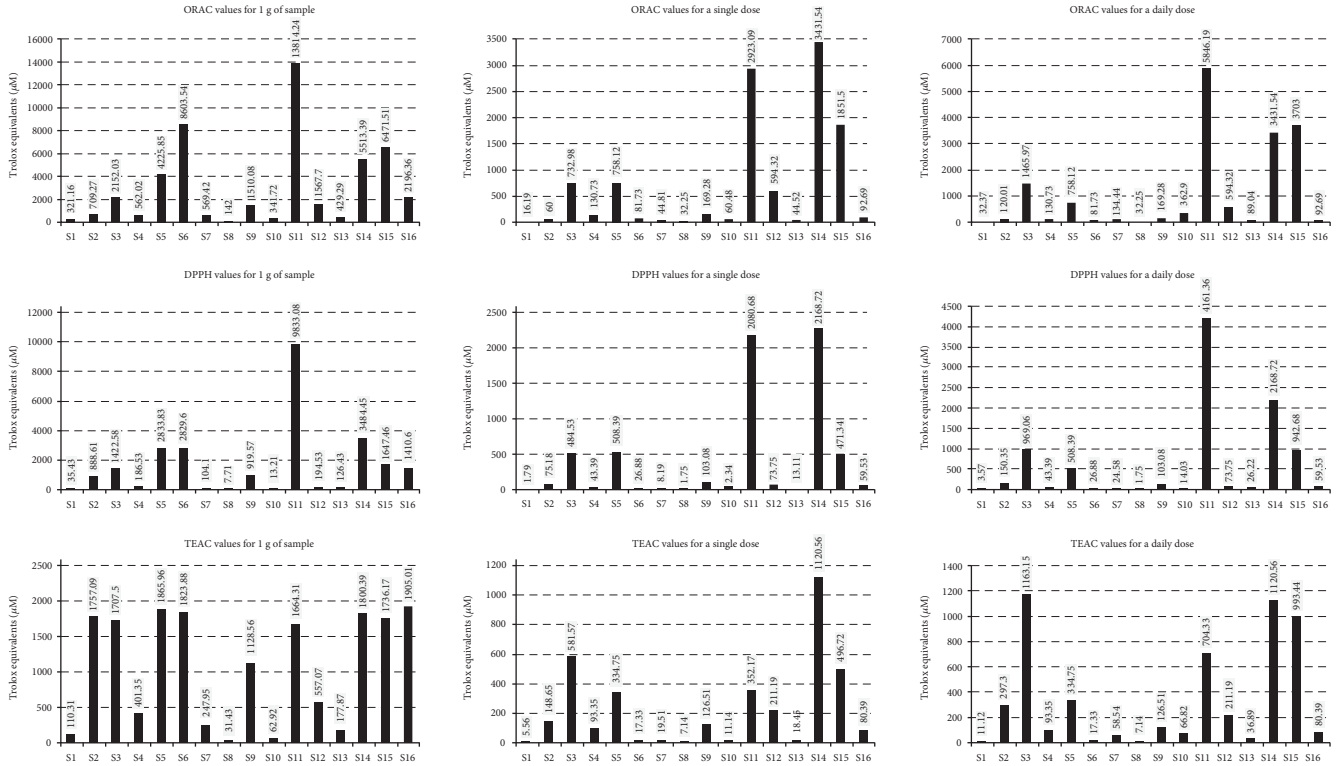


Figure 1: The results obtained for antioxidant capacity assays (ORAC, DPPH, and ABTS) expressed as Trolox equivalents per 1 g of sample, per single dose, and per daily dose of investigated dietary supplements.

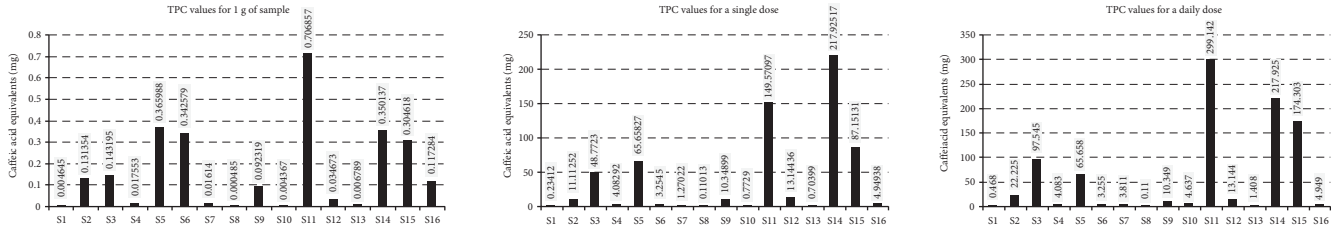


Figure 2: The results obtained for total polyphenol content (TPC) expressed as caffeic acid equivalents per 1 g of sample, per single dose, and per daily dose of investigated dietary supplements.

active compounds in formulation, because the chemical profile of both samples was quite similar with 5-O-caffeoylquinic acid as the main component, followed by other caffeoylquinic acids, 5-O-feruloylquinic acid, dicaffeoylquinic acids, and their derivatives. High antioxidant activity was also showed by extract from the skins and seeds of grapes (S5) and extract from the root of Japanese knotweed (S6). According to the manufacturer's declaration, the main ingredient of these preparations should be resveratrol, and the LC-QTOF-MS analysis revealed the presence of resveratrol; however, in S5, the main compounds were proanthocyanidins A and A-type trimeric proanthocyanidins, while gallic acid, piceid, emodic acid, and emodin were among the major compounds of S6 (Table S2). Although these two supplements were proven to be good antioxidants in terms of 1 g of sample, the daily dose was too low and these samples were placed among the weakest preparations in terms of daily dose activity. Formulations containing goji berry extract (S8), acai berry extract (S4), and powdered fruits of Chinese magnolia (*Schisandra chinensis*) (S7) were proved to be weak antioxidants; however, due to their high nutritional value and high content of polyphenols, they are so-called "superfruits" [9]. Although the literature data refer to high ORAC values for the mentioned fruits, the results obtained in our studies were in the range from 142 to 569.42 μmol Trolox/g, which is in agreement with the studies performed by Henning et al., which showed very low antioxidant values for supplements with acai and goji fruits [10]. The possible reason of low antioxidant activity of these preparations is that the producers use substances of poor quality, or that during treatment process, a loss of active ingredients occurs, because among the identified compounds in S4 were only catechins and quinic acid, S7 contained organic and phenolic acids, while in S8, cabrohydrates and phenolic glycosides were present. Supplement with another "superfruit," a pomegranate in the form of

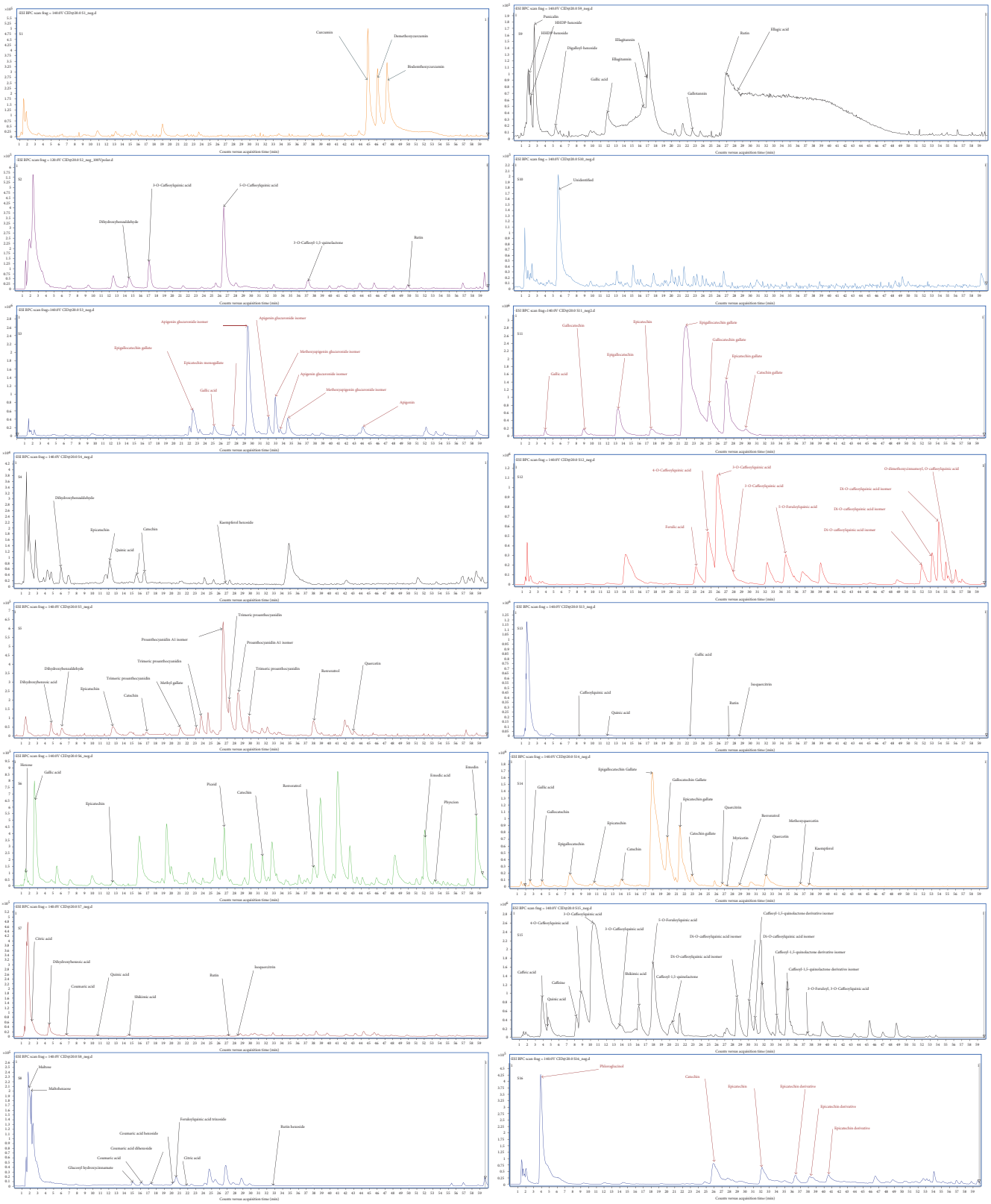


Figure 3: The chemical profiles of studied dietary supplements.

an extract, showed moderate antioxidant activity at the level of 1510.08 μmol Trolox/g in ORAC assay. This result was placed in the middle comparing to results obtained by Madrigal-Carballo et al. in an ORAC method [11]. Madrigal-Carballo et al. evaluated supplements with pomegranate, which showed antioxidant activity values between

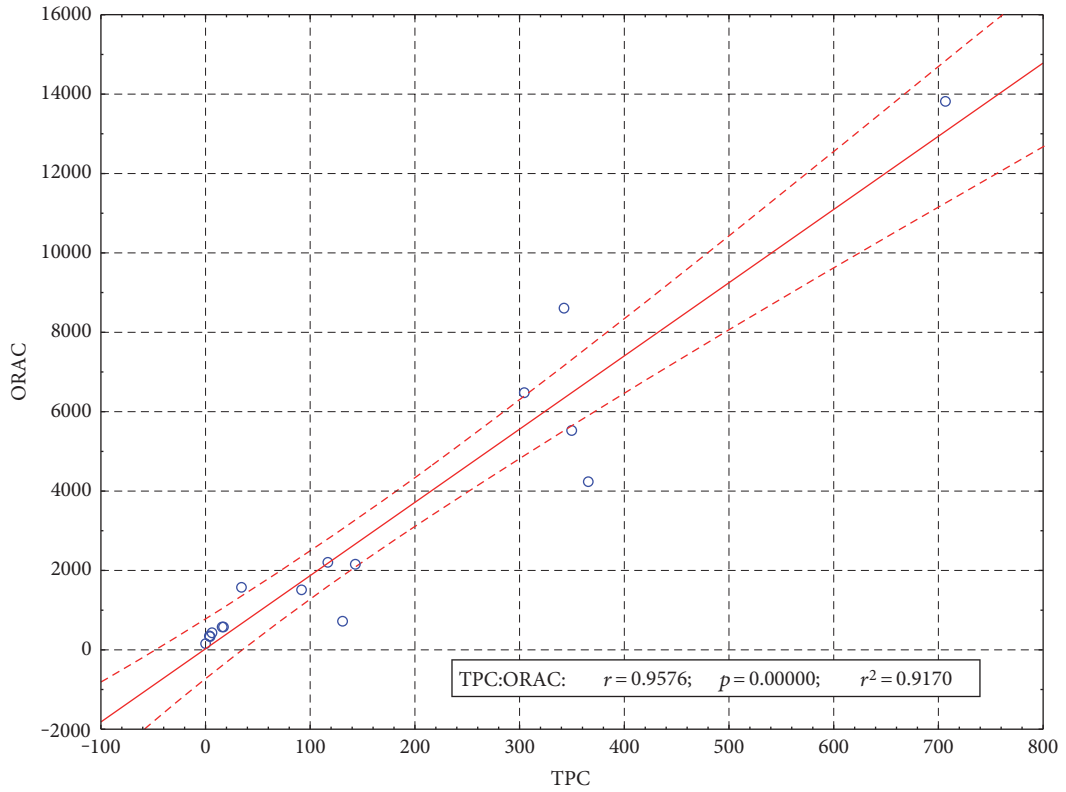
59 and 3210 μmol Trolox/g with an average of 1127 μmol Trolox/g. As was reported, supplements with pomegranate are often falsified, which can be detected by analyzing the qualitative composition of polyphenol fraction. True skin extract and pomegranate seeds contain a high amount of punicalagin, punicalin, and gallo- and elagotanins [11]. The LC-QTOF-MS analysis performed in our study confirmed the authenticity of the extract (Table S2). The lack of punicalagin may be explained by used scan range which was between 50 and 1000 m/z. Low antioxidant activity results were obtained for preparations with powdered rhizome of turmeric (S1) and powdered fruits of hawthorn (S13), although these substances are considered to be valuable antioxidants. According to Wu et al., the total antioxidant capacity of 1 g of powdered rhizome of turmeric is 1592.77 μmol Trolox/g [12]. It is the sum of the values obtained for lipophilic (lipophilic ORAC (L-ORAC)) and hydrophilic compounds (hydrophilic ORAC (H-ORAC)), which are 1193.46 μmol Trolox/g (L-ORAC) and 399.31 μmol Trolox/g (H-ORAC) [12]. The high value of L-ORAC confirms that compounds responsible for antioxidant properties of curcumin: curcuminoids and volatile oil, are contained in the lipophilic fraction [13] and that curcuminoids detected in the studied water extract had to lower concentration to exert a potent antioxidant effect (321.16 μmol Trolox/g). According to the data obtained by Kratchanova et al. also, antioxidants of the hawthorn fruit are lipophilic [14]. The ORAC value described by these authors for an aqueous hawthorn extract was 364 μmol Trolox/g, whereas 2163 μmol Trolox/g for acetone hawthorn extract [14]. In our study, the chromatographic profile of the aqueous extract of supplement containing hawthorn was poor explaining the low antioxidant activity (428.29 μmol Trolox/g). Two of the tested supplements contained ingredients different than plants. Supplement S10 contained spirulina or powdered fronds of freshwater algae, and supplement S16 contained an extract from brown algae *Ecklonia cava*. Despite the antioxidant properties of spirulina, proven by Abd El-Baky et al. [15] in our study, formulation with spirulina had the weakest antioxidant activity and the LC-QTOF-MS analysis confirmed poor extract composition with main, unidentified compound having parent ion at 218 m/z $(M\text{-}H)^-$. Preparation with *Ecklonia cava* showed moderate antioxidant activity and interestingly gave the highest result in the ABTS method suggesting good affinity of detected phloroglucinol to ABTS reagent. It also should be noted that despite many scientific reports on the antioxidant activity of these algae, as well as other species of freshwater and saltwater algae, the antioxidant properties of these species are much less frequently investigated than activity of higher plants.

The results obtained in different assays ranged several orders. The ORAC value for sample S10 was 341.72 μmol Trolox/g, whereas the same sample gave only 13.21 μmol Trolox/g in the DPPH test. Similar differences were shown for S11: 13814.24 and 1664.31 μmol Trolox/g were obtained in ORAC and ABTS assays, respectively. This confirms that each method for antioxidant activity testing is specific and in some terms imperfect. Despite the use of Trolox as a universal reference and calculating the results on the Trolox equivalents, comparing the results obtained using different methods may lead to different conclusions. Antioxidants react differently with reagents used in the determination of the antioxidant capacity, and this gives inconsistent results. Our findings are supported by the literature data. In the study conducted by Zulueta et al., antioxidant activity of ascorbic acid was twice higher in ABTS than in the ORAC method although the results obtained for gallic acid were comparable in both methods [16]. Tabart et al. compared antioxidant activity of 22 various phenolic compounds by three methods: ABTS, DPPH, and ORAC. Significant differences in the results expressed as Trolox equivalents were observed for 16 samples in ABTS, for 11 in DPPH, and for 21 in ORAC method [8]. In addition, the antioxidant activity measured by ORAC was the highest and that measured by DPPH was the lowest [8]. A similar relationship exists also between our results. The arithmetic mean of the different methods gave the highest average value in the ORAC method, while the lowest in ABTS, but calculated median value was highest in the ORAC method and lowest in the DPPH method. The discrepancies between the various methods may be caused by the different physicochemical properties of the reagents (including standards) and samples (the color, dispersion), the mechanism of reaction of the reagents with the tested compounds, and assay conditions—the type of solvent, pH, temperature, time of the measurement, and the type of measuring apparatus [17]. Depending on the structure of the antioxidants tested, the reaction with the reagent may require different time. ABTS reacts with the antioxidants immediately, in less than mixing time, for example, with Trolox or chlorogenic acid. However, some antioxidants do not react with ABTS or react slowly in low concentrations of a more rapidly at higher concentrations; this includes quercetin and curcumin [17]. The reaction of ABTS and DPPH radicals with the sample components is difficult because of their steric hindrance and difficult access to the atom with an unpaired electron. The reaction with DPPH is also very sensitive to the type of solvent, pH, oxygen, and light. Furthermore, these methods measure only the end result of the reaction, neglecting the kinetics and the effect of antioxidant concentration [17]. It appears that the most reliable method of determining antioxidant activity is the ORAC method because the measurements are based on the continuous generation of radicals in real time, as it occurs in the living body [2]. The specificity of the methods also affects the correlations between the results obtained from different methods. It is difficult to clearly define the strength of the correlations because, depending on the sample matrix, the type of antioxidants, and their physicochemical properties, the correlation

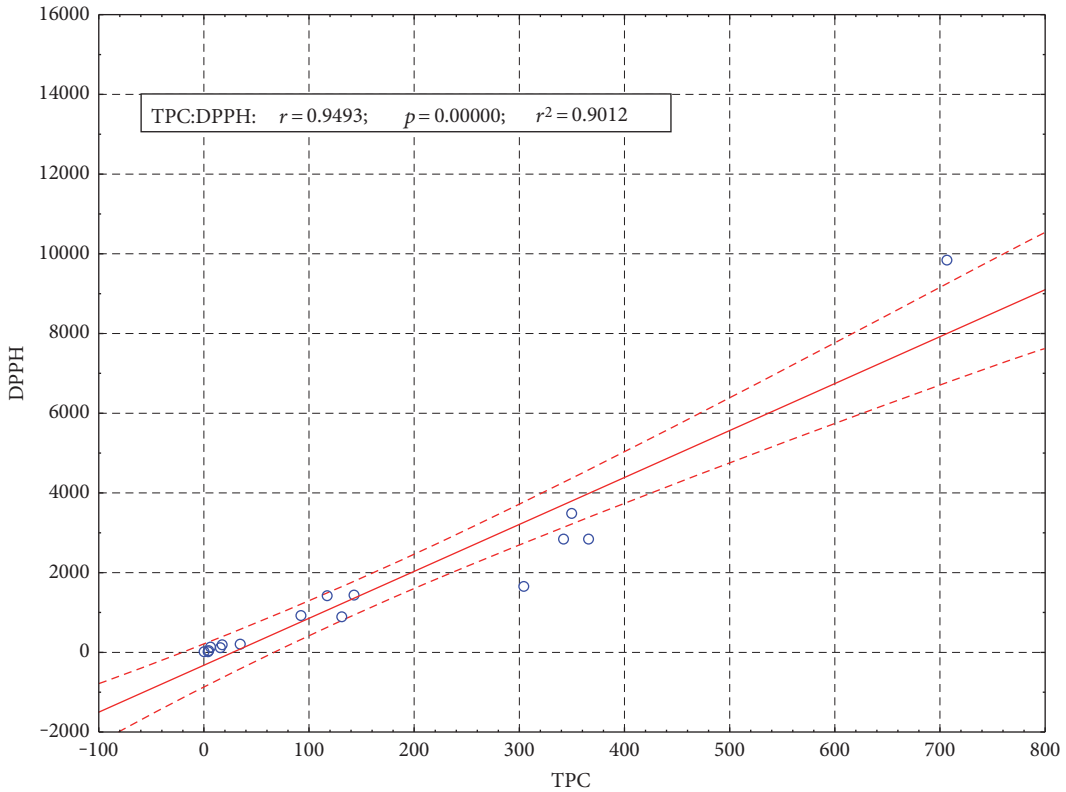
Table 2: Values of Pearson's r correlation coefficients for the antioxidant assays used.

	ABTS	ORAC	DPPH	TPC
ABTS	—	0.6142	0.5619	0.7145
ORAC	0.6142	—	0.9254	0.9576
DPPH	0.5619	0.9254	—	0.9493
TPC	0.7145	0.9576	0.9493	—

All results were statistically significant for $p < 0.05$.



(a)



(b)

Figure 4: Continued.

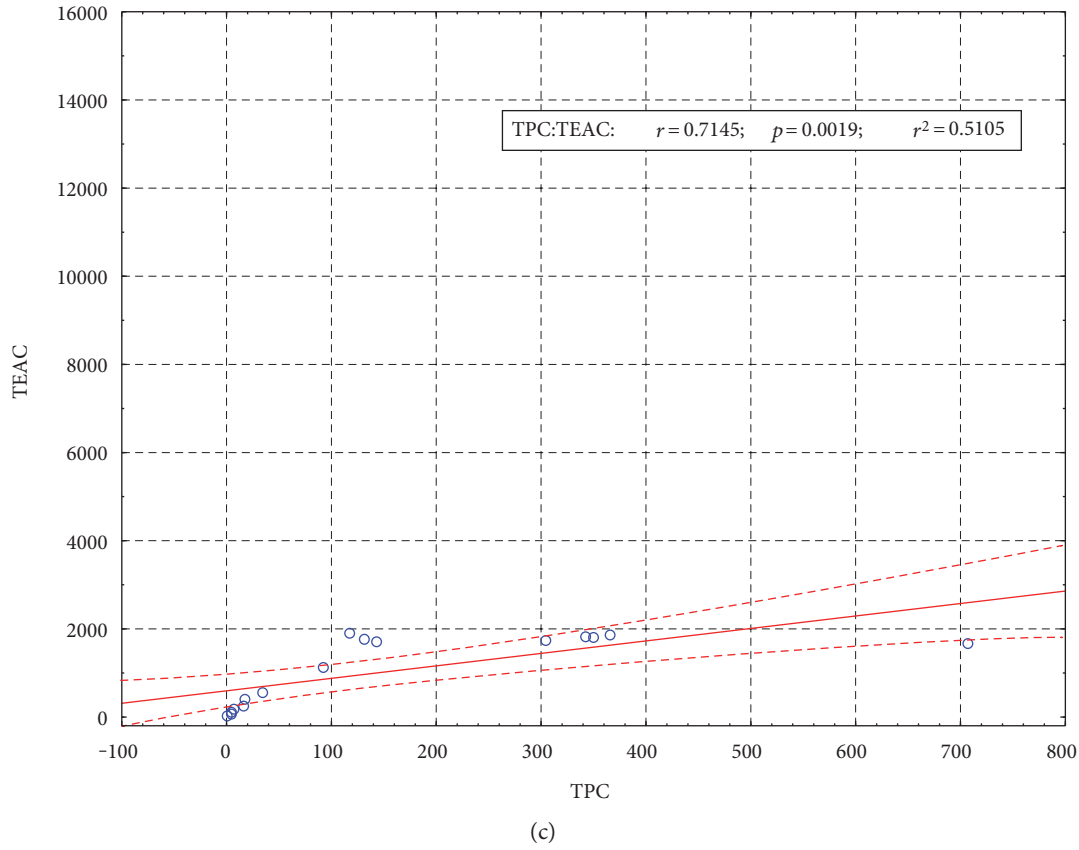


Figure 4: The Pearson's correlations between results obtained using different antioxidant activity tests and total polyphenol content (TPC). (a) ORAC versus TPC, (b) DPPH versus TPC, and (c) ABTS versus TPC.

between various methods can be very strong or may not exist at all. The samples examined in this study were not uniform in terms of quality, formulation, and chemical composition; however, statistically significant correlations between the different methods were observed (Table 2, Figure 4). The strongest correlation occurred between the ORAC and DPPH tests, while the weakest correlations with the other assays gave ABTS method. Based on the calculated correlation coefficients, it can also be said that there were a strong correlation between the antioxidant activity and total polyphenol content measured by the Folin–Ciocalteu assay. The strongest correlation of TFC occurred with ORAC results while the weakest with ABTS. The literature data are partially in opposition with the obtained results. Stintzing et al. who studied the antioxidant activity of prickly pear juice demonstrated a strong correlation between the results obtained by ORAC and using ABTS ($r = 0.974$) [18], while Silva et al. showed only a weak correlation ($r = 0.551$) between results of these antioxidant tests conducted for 15 plants grown in Brazil [19]. In turn, the study of white and red wines did not show any significant correlation between the ORAC, TRAP, and ABTS methods [20] and no correlation was found for the results of antioxidant capacity of human plasma tested using ORAC and ABTS assays [21]. Murillo and coworkers studied 39 exotic fruits for the content of polyphenolic compounds and their antioxidant properties. The correlation between the results of the ABTS and the TPC in the tested fruits was $r = 0.89$ [22]. On the other hand, research on the species of medicinal plants from Nepal showed a weak correlation between phenolic content and the inhibition of DPPH ($r = 0.3004$) [23]. The described literature data demonstrate that it is not possible to compare different antioxidant assays because of the different chemistries involved with the different methods. Also, the requirement for equivalency between assays was revised and removed [24].

The above-discussed activity in correlation with sample chemical composition is in agreement with scatterplots describing the correlation between TPC and the type of DS (Figure 5). The highest activity and TPC value for a daily dose obtained for green tea placed this supplement as a separate group on the cluster graph indicating good quality and high antioxidant potential of tested preparation. In the second group, DS containing catechins, proanthocyanidines, resveratrol, and caffeoylquinic acids were found showing their quite good quality. The remaining preparations formed the biggest cluster characterized by the weakest activity and the very poor composition in some cases. These results prove that because quality control is not applied during the production of dietary supplements, many of the preparations investigated in this study are of uncertain quality. The desired antioxidant effect may not be obtained in the case of these samples because the single dose and the daily dose contain

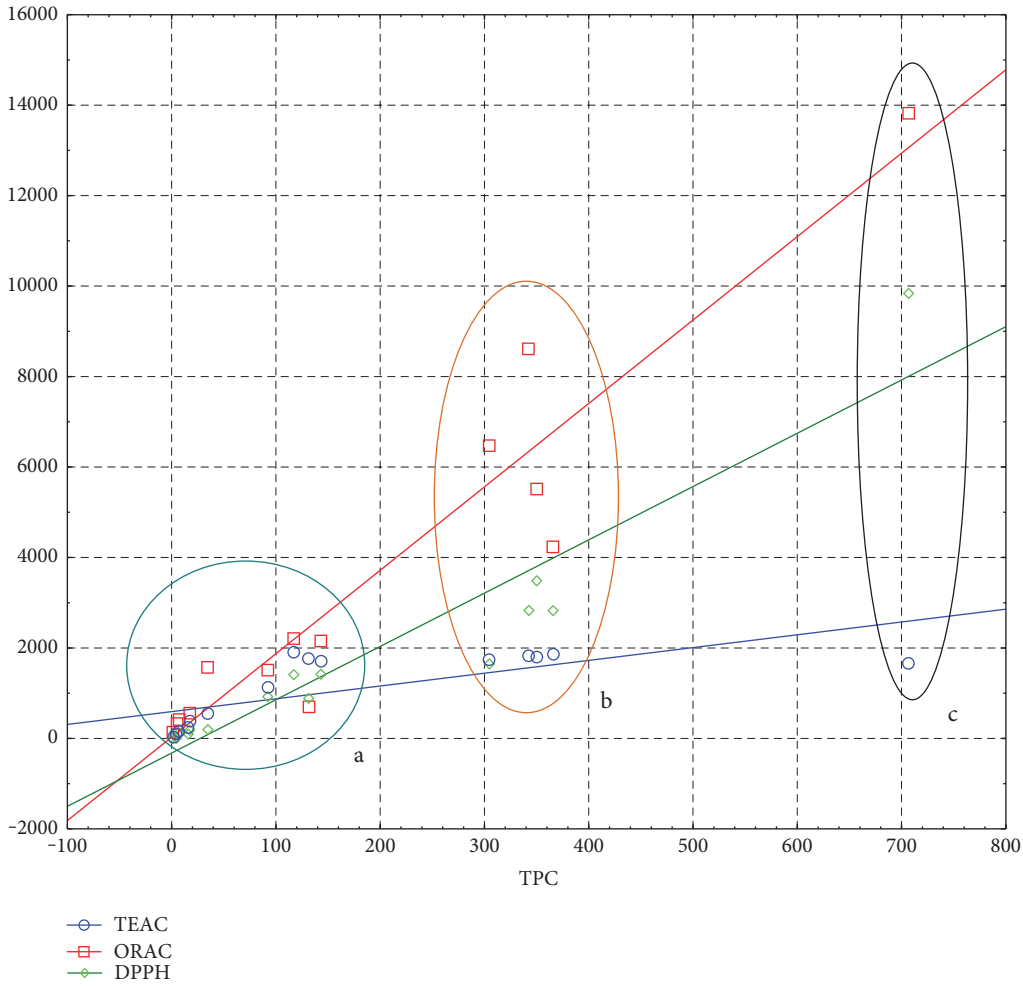


Figure 5: The scatterplots showing correlation between TPC and activity (ABTS, ORAC, and ABTS) and the number of supplement. (a) S1-S4, S7-S10, and S12-S13; (b) S5-S6 and S14-S15; and (c) S11.

small amount of compounds exerting antioxidant activity. Currently, in many countries, the status of dietary supplements is discussed. These products are popular and used among people of all ages. Some countries seek to restrict the sale of supplements in pharmacies and directing them mainly to shopping areas. However, it is worth to remember that dietary supplement is not only a food but also a mixture of a number of biologically active components that have an impact on our body. Therefore, a greater supervision over the composition of these kinds of products is necessary, and the manufacturers should be more responsible when declaring their qualitative and quantitative composition.

4. Conclusions

In this study, we showed that while investigating the functional products such as preparations of dietary supplements, the criterion of a single and a daily dose should not be omitted. The results obtained in activity assays and calculated on the standard unit of measure (1 g/100 g) do not correspond to the activity of the dose suggested for diet supplementation. A substantial part of the results is comparable if we take into account the 1 g sample, while comparing single and daily doses, significant differences in the antioxidant value of preparations are noticed in all assays. The highest antioxidant values were obtained in the ORAC assay, and the strongest correlation occurred between total phenol content and ORAC results suggesting that the most reliable method of determining antioxidant activity is ORAC. The best antioxidant activity was obtained for preparations containing catechins, suggesting these compounds may be responsible for a beneficial effect of dietary supplements. The performed analyses revealed that the majority of studied dietary supplements contain pharmacologically active ingredients. However, some products had poor quality, resulting in the need to increase the number of doses taken. Consumers who purchase dietary supplements to maintain a good health have no knowledge of the effective dose. Hence, the whole responsibility for the therapeutic effect of dietary supplement lies on the manufacturer who should assure the safety and quality of the product.

Conflicts of Interest

The authors declare no competing financial interests.

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Research Article

Evaluating the Risk of Tumors Diseases Based on Measurement of Urinary and Serumal Antioxidants Using the New Agar Diffusion Methods

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Objectives. To discuss the characteristics of the amount of urinary total antioxidants in tumor diseases and the possibility of utilizing the changing regulation of urinary antioxidants to diagnose tumor diseases. *Method*. Urine and serum specimens from 130 healthy people were used to investigate the variation of antioxidant capacity against age. Urine and serum specimens from 44 unselected patients with tumors and 44 healthy people with same age background were used to explore the significance of urinary antioxidant capacity in clinic to diagnose tumor diseases. Potassium permanganate agar method and iodine starch method were used to determine the amount of total antioxidants. *Results*. In healthy people, more antioxidants in urine were measured in older people, while the results were opposite in serum. More antioxidants were found in urine of tumor patients than in healthy people with same age-range. *Conclusions*. According to the results of 130 measurements, the amount of antioxidants in urine varies by age. By using agar methods to measure antioxidants, the effect of age is required to be considered. Antioxidants levels from tumor patients were significantly higher than healthy individuals in urine. The combination of urine and serum to determine total antioxidants can better diagnose tumor diseases based on iodine starch method, with area under the receiver operating characteristics curve at 0.787.

1. Introduction

Biological free radicals are general products of metabolism, mainly containing reactive oxygen species (ROS) and reactive nitrogen species (RNS). In common condition, the free radicals producing and removing are kept balanced, which play an important role in biological system. Once the balance is disturbed, free radicals in body can damage cells, tissues, and organs and further cause aging, cardiac diseases, brain diseases, and cancer [1, 2]. Thus, it is of great importance to know the total antioxidant capacity (TAC) of an organism to evaluate its free radicals producing/removing balance.

Nowadays, many researches are investigating the serum TAC [3], but few are focused on the urinary TAC. Human urine metabolome [4] has pointed out that thousands of compounds are detected in urine, including wide-ranged concentrations of urea, urobilinogen, inorganic salts, creatinine, ammonia, organic acids, and water-soluble toxins. As an important and easily accessible biological fluid, urine reflects the continuously changing environment of an organism [5]. Nevertheless, it has been proved that the level of urea [6], bilirubin [7, 8], and creatinine [9] in serum is associated with the TAC of an organism. So the measurement of antioxidants in urine is important; the variation of urinary metabolite profiling especially the TAC profiling may reveal specific disease.

In tumor patients, obvious oxidative stress is observed, indicating the balance between oxidants and antioxidants is broken up in each studied kind of tumors. In the development of tumors, there exists superfluous generation of ROS and RNS in organism [10]. ROS and RNS can cause DNA damage, protein damage, and lipid peroxidation [11–14]. ROS causes overexpression of Jun gene in lung cancer [15], while the increase of RNS causes protein damage in liver cancer [16]. Free radicals are involved in the initial, enhancement, and accumulation stage of tumor cells developing [17]. And the antioxidants in vivo can fight against with the surplus of

free radicals. Low level of antioxidants bilirubin increases the risk of tumor related with smoking and alcohol [18, 19], while antioxidants uric acid is associated with DNA damage [20]. The amount of all the antioxidants in vivo reflects the capacity to react with the surplus of free radicals. However, it is unable to measure all the antioxidants because how many compounds are included in the antioxidants list is still unclear. Now, any proposed methods only cover a subset of the total antioxidants, which cannot truly reflect the redox state of an organism. A method that can measure the amount of total antioxidants in vivo is necessary.

Total antioxidants were previously investigated based on potassium permanganate agar and iodine starch agar method [21, 22]. In both methods, the diffusion area reflects TAC in an organism, and larger diffusion area represents better TAC. Both methods can better reflect the state of TAC than other methods such as ferric reducing antioxidant power assay [23, 24], cupric ion reducing antioxidant capacity assay [25, 26], and 2,2-diphenyl-1-picrylhydrazyl assay [27, 28].

In this work, potassium permanganate agar method and iodine starch agar method were used to determine the antioxidants in urine and serum, with the aim of applying antioxidant capacity to distinguish healthy people and tumor patients and to evaluate the risk of tumor diseases in clinic in a simpler way.

2. Materials and Method

2.1. The Variation of Total Antioxidants Measured in Urine and Serum in Healthy People

2.1.1. Specimens. Urine specimens from 130 healthy people were collected from the first hospital affiliated with Dalian Medical University and the second hospital affiliated with Dalian Medical University. The urine specimens were divided into 13 groups equally for every five years; each group contained 5 males and 5 females; the ages of subjects ranged from 20 to 85 years. Serum specimens were also collected from the above. Urine and serum specimens were stored at −20°C after being collected and melted in room temperature before experiment.

2.1.2. Method. The amount of total antioxidants in urine was determined by potassium permanganate agar method [21] and iodine starch agar method [22], respectively. Serum specimens were prepared and measured in the same way.

2.2. The Clinical Significance in Measuring the Amount of Total Antioxidants in Urine and Serum

2.2.1. Specimens. Urine specimens were collected from the first hospital affiliated with Dalian Medical University and the second hospital affiliated with Dalian Medical University. Forty-four unselected patients with tumors were regarded as experimental group, including 19 males and 25 females, mean age at 57.84 ± 10.94 years. Forty-four healthy people were collected as control group, including 31 males and 13 females, mean age at 57.80 ± 11.45 years. The diagnoses of experimental group include gastric cancer, intestinal cancer, lung cancer, and breast cancer. Serum specimens were also collected from the above. Urine and serum specimens were stored at −20°C after being collected and restored in room temperature before experiment.

2.2.2. Method. This section was the same as Section 2.1.2.

2.3. Statistical Analysis. Nonparametric correction was used to analyze the variation of antioxidant capacity in urine as well as serum. Nonparametric test was used to compare the results of urine diffusion. T-test was used to compare the results of serum diffusion. Receiver operating characteristics (ROC) curves were constructed to assess sensitivity, specificity, and respective areas under the curves (AUCs) with 95% confidence interval (CI). A value of $p < 0.05$ (two tailed) was considered significant. Statistical software package SPSS 13.0 was used to evaluate the results.

2.4. Ethical Approval. This article does not contain any studies with human participants or animals performed. The protocol has been approved by the Ethical Committee of Dalian Medical University.

3. Results and Discussion

In our previous publications, potassium permanganate agar method and iodine starch agar method were used to determine TAC in urine and serum [21, 22]. The diffusion area was dependent on the amount of antioxidants in urine and serum, in which larger diffusion area represents the fact that more antioxidants were measured. So, the amount of antioxidants reflects the antioxidant capacity in urine and serum; more antioxidants represent better antioxidant capacity. Both methods have good linearity and precision and can better reflect the state of TAC than reported methods in urine [22–26]. Neutral environment required in our methods is closer to physiological pH, high standard electrode potential of MnO_4^-/MnO_2 can oxidize most antioxidants, and indicator starch-iodine is highly sensitive to judge the end point and the covering of liquid paraffin on the surface of the agar can exclude the interference of external O_2.

3.1. The Variation of Total Antioxidants Measured in Healthy People by Potassium Permanganate Agar Method and Iodine Starch Agar Method. In Table 1, the amount of antioxidants was measured in both urine and serum of 130 healthy people. In urine, positive correction coefficients ($p = 0.025$ in potassium permanganate agar method and $p = 0.016$ in iodine starch agar method) between age and urine diffusion area represent the fact that more urinary antioxidants exist in older people than the younger age. Contrastingly, in serum, negative correction coefficients ($p < 0.001$ in potassium permanganate agar and $p = 0.014$ in iodine starch agar method) represent the fact that less antioxidants exist in sera from older people. Similar results were observed in both methods, which ensure the accuracy of the results that older people has more antioxidants in urine and less antioxidant in serum comparing with the younger ones.

Table 1: The diffusion area (cm^2) of urine and serum in healthy people in different groups (mean ± S).

Age groups	Urine		Serum	
	Potassium permanganate agar	Iodine starch agar	Potassium permanganate agar	Iodine starch agar
20~	3.35 ± 0.93	1.21 ± 0.25	2.52 ± 0.18	3.48 ± 0.51
25~	1.99 ± 1.42	1.19 ± 0.48	2.34 ± 0.15	3.15 ± 0.42
30~	2.24 ± 1.57	1.48 ± 0.51	2.41 ± 0.16	3.41 ± 0.39
35~	2.14 ± 0.96	1.22 ± 0.47	2.45 ± 0.16	3.36 ± 0.42
40~	2.15 ± 1.59	1.27 ± 0.32	2.39 ± 0.18	3.08 ± 0.30
45~	2.04 ± 1.17	1.10 ± 0.31	2.37 ± 0.21	3.37 ± 0.60
50~	2.33 ± 1.43	1.25 ± 0.44	2.37 ± 0.14	3.25 ± 0.47
55~	1.72 ± 0.98	1.38 ± 0.53	2.39 ± 0.13	3.29 ± 0.33
60~	3.10 ± 0.88	1.52 ± 0.50	2.26 ± 0.21	3.33 ± 0.48
65~	2.47 ± 1.29	1.57 ± 0.49	2.35 ± 0.19	3.44 ± 0.46
70~	2.28 ± 1.07	1.30 ± 0.51	2.28 ± 0.12	3.04 ± 0.27
75~	2.71 ± 1.73	1.22 ± 0.37	2.27 ± 0.33	3.24 ± 0.49
80~85	3.85 ± 1.27	1.47 ± 0.23	2.39 ± 0.22	3.03 ± 0.39
Correlation coefficient	0.139	0.150	−0.239	−0.152
p	0.025	0.016	<0.001	0.014

Table 2: Urine and serum diffusion area (cm^2) of tumor patients and healthy people in the two methods.

Diffusion area (cm^2)		Urine			Serum		
		Median	Z	p	Mean ± SD	t	p
$KMnO_4$	Tumor patients	1.83	−2.076	0.038	2.02 ± 0.17	−1.424	0.158
	Healthy people	1.54			1.97 ± 0.17		
I_2	Tumor patients	1.54	−2.399	0.016	3.20 ± 0.40	−2.867	0.005
	Healthy people	1.13			2.94 ± 0.46		

Because urinary diffusion areas in both methods were in nonnormal distribution, nonparametric test was used to compare the results in the two groups. While the serumal diffusion areas in both methods were in normal distribution, t-test was used to compare the results in the two groups. SD, standard deviation.

The contrasting variations of antioxidants in urine and serum might be caused by the different antioxidant mechanisms. Future work is deserved to compare antioxidants profiling and fluid pathway in serum and urine. The question is whether the antioxidants in urine are derived from blood through glomerular filtration and tubular secretion.

The reason why we consider the age-background factor of healthy people is that, in previous reports, the serumal antioxidant capacity of the elderly will decline, but it is still unclear whether it is the same situation in urine. Thus, we measured the urinary antioxidants of healthy people to eliminate the age influence. Because there is age dependent trend observed between TAC and both urine and serum, the ages of tumor patients group and healthy group were designed to be matched to eliminate the influence.

3.2. The Total Antioxidants in Urine and Serum Were Measured in Unselected Patients with Tumors by Potassium Permanganate Agar Method and Iodine Starch Agar Method. Obvious oxidative stress is observed in patients with tumor. The balance between oxidants and antioxidants was broken up, regardless of the kinds of tumor. Here, clinical specimens of urine and serum were collected from patients with tumors without specifying the kind of tumors, which were labeled as "unselected patients with tumors" group.

In Table 2, because the urinary diffusion areas were not normally distributed in both methods, median diffusion areas were compared between the two groups. In potassium permanganate agar, the median diffusion area in tumor patients was 1.83 cm^2, while it was 1.54 cm^2 in healthy group; more antioxidants were measured in tumor patients, where $p = 0.038$; in iodine starch agar method, the median diffusion area in tumor patients was 1.54 cm^2, while it was 1.13 cm^2 in healthy group; more antioxidants were measured in tumor patients, where $p = 0.016$. While in serum the serum diffusion areas were normally distributed in both methods, average diffusion areas were compared between the two groups. In potassium permanganate agar, the average diffusion area in tumor patients was 2.02 ± 0.17 cm^2, while it was 1.97 ± 0.17 cm^2 in healthy group, because $p = 0.158$; no evidence showed that more antioxidants were measured in tumor patients; in iodine starch agar method, the average diffusion area in tumor patients was 3.20 ± 0.40 cm^2, while it was 2.94 ± 0.46 cm^2 in healthy group; more antioxidants were measured in tumor patients, where $p = 0.005$.

Table 3: Variables in binary logistic regression model.

Variables	B	SE	Wald	Sig.
U_I	0.850	0.280	9.192	0.002
S_I	2.029	0.651	9.702	0.002
Constant	−7.691	2.199	12.230	<0.001

U_I, urine diffusion area in iodine starch agar; S_I, serum diffusion area in iodine starch agar.

Table 4: Results for the measurement of total antioxidants in urine and serum in the diagnosis of tumor diseases.

Variable	AUC	Std. error	Asymptotic Sig.	95% CI
U_{Mn}	0.626	0.060	0.043	0.509~0.742
U_I	0.629	0.061	0.037	0.509~0.749
S_{Mn}	0.503	0.064	0.963	0.377~0.629
S_I	0.669	0.058	0.006	0.556~0.782
Logistical regression model	0.787	0.047	<0.001	0.694~0.880

U_{Mn}, urine diffusion area in potassium permanganate agar; U_I, urine diffusion area in iodine starch agar; S_{Mn}, serum diffusion area in potassium permanganate agar; S_I, serum diffusion area in iodine starch agar; AUC, area under curve; CI, confidence interval.

To ensure the accuracy of the results, only the results were significant in both methods; the amount of antioxidants was considered different between the two groups. Thus, more urinary antioxidants were observed in tumor patients, because the results were significant in both methods. However, there was no difference in the serum antioxidants between the two groups; the result was significant only in iodine starch method. From the data presented in Table 1, it can be seen in older healthy people that less serumal antioxidants were measured. This is opposite to the results presented in Table 2, where no more serumal antioxidants were measured in tumor patients. However, in urine, more antioxidants were measured in the both older group and tumor patients. Thus, it may indicate a fact that the occurrence of tumor diseases is related to not only aging but also other factors. Obviously, the antioxidant mechanism of aging and tumor diseases has its own characteristic, and the mechanism of tumor diseases is still to be explored. Thus, we can conclude that, in tumor diseases, the change of antioxidants in urine was more sensitive than that in serum. Compared with serum, urine is a better specimen to evaluate the risk of tumor diseases. Exploring the diagnosis value of measuring the total antioxidants in urine to evaluate the risk of tumor diseases was deserved.

3.3. Measuring the Urinary Total Antioxidants Can Improve the Diagnosis Efficiency of Tumor Diseases Based on the Measurement of Antioxidants in an Organism to Diagnose Tumor Diseases. In Table 4, four ROC curves for urine and serum in both methods were constructed, but all AUCs at either urine or serum were small. So the combination of them was in consideration. To assess the combined use of the measurement of urine diffusion area in potassium permanganate agar (U_{Mn}), serum diffusion area in potassium permanganate agar (S_{Mn}), urine diffusion area in iodine starch agar (U_I), and serum diffusion area in iodine starch agar (S_I), binary logistic regression was conducted. In Table 3, only U_I and S_I were significant in the regression model, where

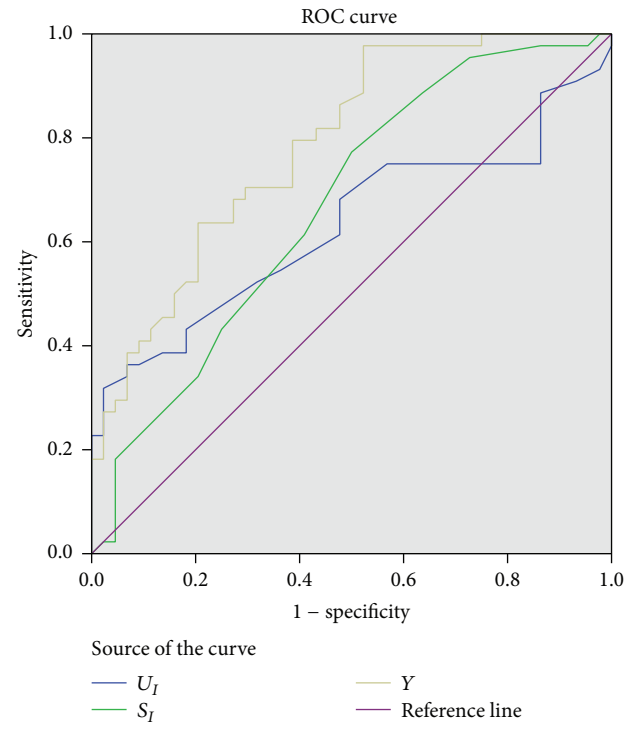

Figure 1: ROC curves for urine and serum in both potassium permanganate agar and iodine starch agar method.

the p values were both 0.002. U_{Mn} and S_{Mn} were excluded; the logistical regression model was $Y = 0.85 * U_I + 2.029 * S_I - 7.691$. Thus, U_I, S_I, and Y were chosen to build ROC, and it can be seen from Figure 1 that the AUC of Y was 0.787, which is larger than the use of U_I and S_I alone (Figure 1).

The reason why U_{Mn} and S_{Mn} were not selected in the regression model is that the sensitivity of potassium permanganate method is relatively lower than the iodine starch agar method; only few antioxidants that represent the

differences between tumor diseases and healthy subjects were measured, but with the use of indicator starch, the sensitivity of iodine starch method was good; it can measure most antioxidants that represent the differences between tumor diseases and healthy subjects. And it also can be inferred that the electric potential of antioxidants in tumor patients was not high.

In all, the measurement of the urinary total antioxidants can improve the diagnosis efficiency of tumor diseases.

4. Conclusions

The antioxidant capacity of organism varies by age. More urinary antioxidants were measured in older people, while less antioxidants exist in serum of older people. The influence of age should be taken into consideration when discussing the TAC of an organism.

In patients with tumors, more antioxidants were found in urine with potassium permanganate agar method ($p = 0.038$) and iodine starch agar method ($p = 0.016$). More antioxidants were found in serum only in iodine starch agar method ($p = 0.005$). The change of the total amounts in urine was more sensitive than that in serum in tumor diseases.

The combined measurement of antioxidants in urine and serum could improve the diagnostic ability of tumor diseases, where AUC = 0.787. The measurement of total antioxidants in urine should be applied alone or in combination in clinic to evaluate the risk of tumor diseases.

Conflicts of Interest

No conflicts of interest exist in this manuscript.

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