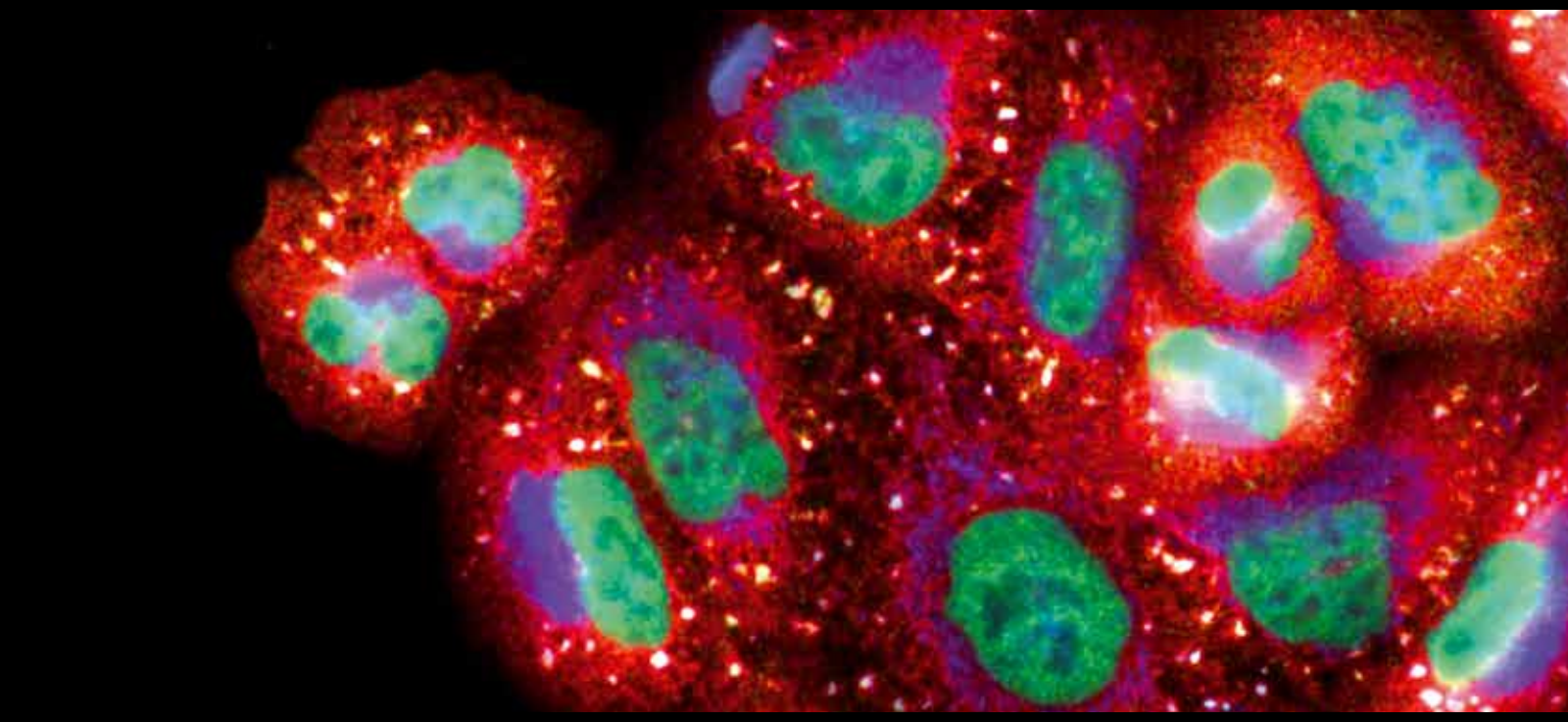
Oxidative Medicine and Cellular Longevity

Dietary Polyphenols and Their Effects on Cell Biochemistry and Pathophysiology 2013

Guest Editors: Tullia Maraldi, David Vauzour, and Cristina Angeloni



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Editorial

Dietary Polyphenols and Their Effects on Cell Biochemistry and Pathophysiology 2013

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1. Introduction

Dietary polyphenols, along with other natural compounds occurring in fruits and vegetables, have been reported to exert beneficial effects in a multitude of disease states, including cancer, cardiovascular disease, and neurodegenerative disorders. Many of the biological actions of polyphenols have been attributed to their antioxidant properties; however, during the last years, a new realization of how nutritional antioxidants may function has been envisaged, and recent findings have suggested that they affect several cellular pathways exerting a pleiotropic effect.

This special issue analyzes and expands our knowledge on the new mechanisms of actions of polyphenols and other natural compounds with the aim to better understand what could be defined as "the network" of their different biological effects.

2. Role of Polyphenols in Redox Modulation and Inflammatory Processes

Although moderate physical exercise is considered an essential component of a healthy lifestyle that leads the organism to adapt itself to different stresses, strenuous exercise is however known to induce oxidative stress, inflammation, and muscle damage. The review of M. Malaguti et al. focused on polyphenols, present in the plant kingdom, that have been recently suggested to exert some positive effects on muscle damage and oxidative stress induced by vigorous physical exercise.

In the study of L. D'evoli et al., the nutraceutical effects of red chicory extracts were evaluated in various intestinal models. Compared to red chicory whole leaf extracts, the red part of leaf extracts had a significantly higher content of both total phenolics and anthocyanins, leading to an increase of antioxidant, cytoprotective, and antiproliferative activities. The study of I. Chkhikvishvili et al. analyzed the effects of summer savory extracts in counteracting H_2O_2-induced oxidative stress in human lymphoblastoid Jurkat T cells. In particular they demonstrated that summer savory extracts protective effect may be attributed to the direct radical-scavenging activity of rosmarinic acid and other phenolic compounds, as well as to indirect mechanisms such as the enhancement of antioxidant enzymes and the release of anti-inflammatory signaling molecules, such as IL-10.

C. Wall et al. demonstrated that luteolin and kaempferol decrease IL-1β-induced NF-κB p65 DNA binding activity and nuclear c-Jun expression in human gestational tissues, suggesting a potential role of these compounds in counteracting infection/inflammation, commonly associated with preterm birth, initiating uterine contractions and rupture of fetal membranes.

Neutrophils are capable of releasing cytotoxic substances and inflammatory mediators, which, along with their delayed apoptosis, have a potential to maintain permanent inflammation.

V. Jancinova et al. investigated the ability of piceatannol, a naturally occurring stilbenoid, in reducing the toxic effect of neutrophils. This stilbenoid elevates the percentage of early apoptotic neutrophils and inhibits the activity of protein kinase C (PKC), the main regulatory enzyme in neutrophils, indicating that piceatannol may be useful as a complementary medicine in states associated with persisting neutrophil activation and with oxidative damage of tissues.

In the study of M.-J. Bak et al., the anti-inflammatory effect and underlying mechanisms of wild grape seeds procyanidins (WGP) were examined in RAW 264.7 cells. They demonstrated that WGP exerts potent anti-inflammatory activity through the inhibition of iNOS and COX-2 and by regulating NFκB and p38 MAPK pathways. R. Nosàl et al. showed that resveratrol represents an effective naturally occurring substance with potent pharmacological effect on oxidative burst of human neutrophils and nitric oxide production by macrophages.

K. Drábiková et al., with the aim to specify the site of action of the synthetic coumarin derivatives 7-hydroxy-3-(4′-hydroxyphenyl) coumarin (HHC) and 7-hydroxy-3-(4′-hydroxyphenyl) dihydrocoumarin (HHDC), evaluated their effects on extra- and intracellular ROS formation in phorbol-myristate-13-acetate (PMA) stimulated human neutrophils. Without affecting cytotoxicity, both tested coumarins were effective inhibitors/scavengers of ROS produced by neutrophils on extracellular level. HHC markedly diminished oxidant production and also decreased both PKC activity and phosphorylation of PKCα, βII intracellularly.

Oxidative stress plays a major role in the pathophysiology of chronic inflammatory disease and it has also been linked to accelerated telomere shortening. Research has shown that poly(ADP-ribose) polymerase-1 (PARP-1) and subtelomeric methylation play a role in telomere stability. D. M. P. H. J. Boesten et al., evaluating the effect of chronic PARP-1 inhibition (by fisetin and minocycline) in human fibroblasts exposed to chronic oxidative stress, demonstrated that PARP-1 plays a role in accelerated aging in chronic inflammatory diseases due to its role as coactivator of NF-κB and AP-1.

3. Role of Polyphenols in Molecular Signaling

The NADPH oxidase (NOX) physiological functions concern host defense, cellular signaling, regulation of gene expression, and cell differentiation. On the other hand, increased NOX activity contributes to a wide range of pathological processes, including cardiovascular diseases, neurodegeneration, organ failure, and cancer. The review by I. Maraldi summarizes the current state of knowledge of the role of NOX enzymes in physiology and pathology and provides an overview of the currently available NOX inhibitors derived from natural extracts such as polyphenols.

In several species, the polyphenol resveratrol activates sirtuins (SIRT). SIRT1, a NAD^+-dependent deacetylase, was identified as one of the molecules through which calorie restriction extends the lifespan or delays age-related diseases, regulating adaptations to cellular stress, through the deacetylation of target proteins. F. Pifferi et al. showed a reduction in locomotor activity onset and changes in body temperature rhythm in resveratrol-supplemented aged animals suggesting an improved synchronisation on the light-dark cycle through SIRT-regulation of energy balance and biological clock processes.

Moreover the review by M. Kitada and D. Koya focused on resveratrol and its beneficial effects on renal diseases. They evidenced that resveratrol can exert protective effects against both acute and chronic kidney injuries through its antioxidant effects and ability to activate SIRT1. The regulation of SIRT1 was proposed also by K. Pallauf et al. suggesting that the so-called "MediterrAsian" diet combining sirtuin-activating foods of the Asian as well as Mediterranean diet may be a promising dietary strategy in preventing chronic diseases, thereby ensuring health and healthy ageing.

4. Role of Polyphenols in Stem Cell Proliferation and Differentiation

Human stem cells with multilineage differentiation potential are studied for cell therapy, but *in vitro* expansion leads to senescence affecting differentiation and proliferative capacities. M. Guida et al. demonstrated that with a decrease of Nox4 activity in stem cells, obtained with plumbagin, a decline of nuclear ROS production and of DNA damage occurs. They suggest that nuclear Nox4 regulation may have important pathophysiologic effects in stem cell proliferation through modulation of nuclear signaling and DNA damage.

5. Role of Polyphenols in Metabolism Regulation

Extracts from *Stevia rebaudiana* Bertoni are largely used as a noncaloric, high-potency biosweetener alternative to sugar, due to the growing incidence of type 2 diabetes mellitus, obesity, and metabolic disorders worldwide. B. Rizzo et al. showed that these extracts are able to enhance glucose uptake in different cell lines and were as efficient as insulin at modulating PI3K/Akt pathway.

Nonalcoholic fatty liver disease, defined by excessive lipid accumulation in the liver, is the hepatic manifestation of insulin resistance and the metabolic syndrome. L. Valenti et al. summarize the evidence evaluating the mechanisms of action of anthocyanins on hepatic lipid metabolism in different experimental settings including cells, animals, and human trials. Data shown in the paper by E. Giaretta et al. demonstrate that resveratrol supplementation in various phases of *in vitro* oocyte maturation and vitrification/warming procedure can modulate the apoptotic process, improving the resistance of porcine oocytes to cryopreservation-induced damage.

6. Potential Effect of Polyphenols in Cancer

M. Del Carmen García-Rodríguez et al. investigated the modulating effects of green tea polyphenols on genotoxic damage and apoptotic activity induced by hexavalent chromium (Cr (VI)) in mice. Their findings supported the protective effects of green tea polyphenols by inducing apoptosis that could

contribute to elimination of the DNA damaged cells induced by Cr (VI).

Y. Ke et al. evaluated the potential protective effects of Fructus rhodomyrti (FR) extracts against oxidative DNA damage. Their findings suggested that FR might act as a chemopreventive agent with antioxidant properties offering effective protection against oxidative DNA damage in a concentration-dependent manner *in vitro* and *in vivo*. Quercetin is a dietary flavonoid with known antitumor effects against several types of cancers by promoting apoptotic cell death and inducing cell cycle arrest. H. Kim et al. showed that quercetin is significantly effective in inhibiting cell proliferation through mitochondrial apoptosis pathway but also inducing autophagy.

7. Potential Effect of Polyphenols in Cardiovascular Diseases

Epigallocatechin gallate (EGCG) is known to exhibit antioxidant, antiproliferative, and antithrombogenic effects and reduce the risk of cardiovascular diseases. Key events in the development of cardiovascular disease are hypertrophy and hyperplasia according to vascular smooth muscle cell proliferation. M. H. Lee et al. suggest that EGCG can inhibit PDGF-BB stimulated mitogenesis by indirectly and directly interrupting PDGF-BB pathway. Therefore, EGCG may be used for treatment and prevention of cardiovascular disease through blocking of PDGF signaling.

8. Potential Effect of Polyphenols in Neurodegenerative Diseases

The role of oxidative stress in age-related dementia and in stroke pathophysiology is crucial; therefore the use of antioxidant plant derivatives is under investigation. In the studies by C. Sutalangka et al. and W. Kirisattayakul et al. the neuroprotective effect of *Moringa oleifera*, a plant possessing potent antioxidant activity, was assessed in animal models of age-related dementia and focal stroke. Their data showed that *M. oleifera* leaves extract is neuroprotectant and cognitive enhancer partly via the decreased oxidative stress and the enhanced cholinergic function.

Moreover they showed that all extract doses decreased infarction volume in both cortex and subcortex. The protective effect of low extract doses in all areas occurs mainly via the decreased oxidative stress. The protective effect of the high dose extract in striatum and hippocampus occurs via the same mechanism, whereas other mechanisms might play a crucial role in cortex.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an environmental toxin which selectively induces oxidative damage and mitochondrial and proteasomal dysfunctions to dopaminergic neurons in the substantia nigra leading to Parkinsonian syndrome in animal models and humans. N. Braidy et al. investigated the therapeutic effect of different varieties of pomegranate juice extracts (PJE), Helow, Malasi, Qusum, and Hamadh, against MPTP-induced neurotoxicity in primary human neurons. Helow and Malasi show the best effect in attenuating the observed changes in redox function thus ameliorating MPTP-induced neurotoxicity.

Rotenone, a widely used pesticide that inhibits mitochondrial complex I, has been used to investigate the pathobiology of Parkinson's disease. Studies have shown that the neurotoxicity of rotenone may be related to its ability to generate ROS, leading to neuronal apoptosis. The study of K. Tamilselvam et al. was carried out to investigate the neuroprotective effects of hesperidin, a citrus fruit flavanol, against rotenone-induced apoptosis in human neuroblastoma cells. The data suggested that hesperidin exerts its neuroprotective effect against rotenone due to its antioxidant, maintenance of mitochondrial function, and antiapoptotic properties.

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Research Article

On the Molecular Pharmacology of Resveratrol on Oxidative Burst Inhibition in Professional Phagocytes

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Resveratrol—3,5,4′-trihydroxystilbene—possesses antioxidant activities *in vitro*. It dose-dependently inhibited the generation of peroxyl, hydroxyl, peroxides, and lipid peroxidation products in cell free systems. Oxidative burst of whole human blood stimulated with PMA, fMLP, OpZ, and A23187 was inhibited in a concentration-dependent way, indicating suppression of both receptor and nonreceptor activated chemiluminescence by resveratrol. Results from isolated human neutrophils revealed that resveratrol was active extracellularly as well as intracellularly in inhibiting the generation of reactive oxygen species. Liberation of ATP and analysis of apoptosis showed that in the concentration of 100 μM, resveratrol did not change the viability and integrity of isolated neutrophils. Western blot analysis documented that resveratrol in concentrations of 10 and 100 μM significantly decreased PMA-induced phosphorylation of PKC α/βII. Dose-dependent inhibition of nitrite production and iNOS protein expression in RAW 264.7 cells indicated possible interference of resveratrol with reactive nitrogen radical generation in professional phagocytes. The results suggest that resveratrol represents an effective naturally occurring substance with potent pharmacological effect on oxidative burst of human neutrophils and nitric oxide production by macrophages. It should be further investigated for its pharmacological activity against oxidative stress in ischaemia reperfusion, inflammation, and other pathological conditions, particularly neoplasia.

1. Introduction

Neutrophils are present in high numbers in areas of inflammation, where they constitute an important source of reactive oxygen species (ROS). The massive production of antimicrobial and tumoricidal ROS in an inflammatory environment is called "oxidative burst" and plays an important role as the first line of defense against environmental pathogens. Paradoxically, however, neutrophils are also implicated in tissue-damaging inflammatory reactions that underlie the pathogenesis and exacerbation of many inflammatory diseases [1, 2]. There are at least two signalling pathways responsible for induction of neutrophil activation: one is the protein kinase C (PKC) mediated pathway, which can be driven by stimulation with phorbol-4β-12β-myristate-13α-acetate (PMA), and the other is the *Src* family protein tyrosine kinase mediated pathway [3].

Apoptosis is critical for the regulation of life span of circulating as well as emigrated neutrophils. Accumulating evidence indicates that neutrophil apoptosis is one of the critical determinants of the outcome of the inflammatory response and is a potential target for therapeutic interventions. A delay of neutrophil apoptosis exacerbates and prolongs inflammation or even prevents spontaneous resolution of inflammation [4]. The apoptotic neutrophil and the process of cell death exert anti-inflammatory effects that have been shown to be of therapeutic value in inflammatory diseases [5, 6].

A wide array of phenolic substances, particularly those present in edible and medicinal plants, have been reported to possess substantial antioxidative, anticarcinogenic, and antimutagenic activities by modulating important cellular signalling processes [7–10]. Natural polyphenols suppressed oxidative burst of stimulated human neutrophils by enhancing their apoptosis and decreasing protein kinase C activation [11–17].

Resveratrol (RES), a polyphenolic phytoalexin, is one of the most extensively studied natural products, with wide-ranging biological activities and tremendous clinical potential [18]. RES has been shown to have antioxidant, antiinflammatory, antiproliferative, and anti-angiogenic effects, while those on oxidative stress are presumably the most important [19].

In spite of the fact that almost 5000 papers are evidenced in PubMed database, there is a lack of evidence about the mechanism of the effect of resveratrol on oxidative burst in human professional phagocytes at molecular level. In this study, we investigated the effect of RES on the mechanism of oxidative burst in human whole blood, isolated neutrophils at extra- and intracellular level, activation of protein kinase C, caspase-3 activity and cellular viability and on free radical scavenging activity in cell free systems (oxygen radical absorption capacity—ORAC, hydroxyl radical averting capacity—HORAC, scavenging of ROS generation, nitric oxide production, and inhibition of lipid peroxidation). Moreover, we studied the effect of resveratrol on nitrite production and iNOS protein expression in murine RAW 264.7 macrophage cell line.

2. Methods and Materials

2.1. Chemicals. Luminol, isoluminol, PMA (phorbol-4β-12β-myristate-13α-acetate), Ca^{2+}-ionophore A23187, superoxide dismutase, dextran (average MW 464,000), zymosan A (from *Saccharomyces cerevisiae*), luciferase (from firefly *Photinus pyralis*), and D-luciferin sodium salt from Sigma-Aldrich Chemie (Deisenhofen, Germany). HRP (horseradish peroxidase), catalase, and Folin-Ciocalteu's phenol reagent were purchased from Merck (Darmstadt, Germany) and Lymphoprep (density 1.077 g/mL) from Nycomed Pharma AS (Oslo Norway). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein disodium salt, cobalt(II) fluoride tetrahydrate, and gallic acid were obtained from Sigma-Aldrich (Steinheim, Germany). Picolinic acid was purchased from Fluka (Deisenhofen, Germany), and human purified caspase-3 was from Enzo Life Sciences, Lausen, Switzerland. All other chemicals used were of analytical grade and obtained from commercial sources.

ORAC and HORAC analyses were carried out on a FLUOstar Galaxy plate reader (BMG Labtechnology, Offenburg, Germany).

The phosphate buffered saline solution (PBS) used in this study contained 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.8 mM $CaCl_2$, and 0.5 mM $MgCl_2 \times 6H_2O$ and had a pH of 7.4. Tyrode's solution used in this study consisted of 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM NaH_2CO_3, 0.4 mM $NaH_2PO_4 \times 2H_2O$, 1 mM $MgCl_2 \times 6H_2O$, and 5.6 mM glucose, pH of 7.4.

Resveratrol (RES) was prepared by targeted regioselective synthesis purely as transisomer [20] and was diluted in 1 : 50 (v/v) of 1 M NaOH in water.

2.2. Blood Collection and Neutrophil Separation. Fresh human blood was obtained at the blood bank by venipuncture from healthy male volunteers (20–50 years) who had not received any medication for at least 7 days. It was anticoagulated with 3.8% trisodium citrate (blood : citrate ratio = 9 : 1). The Ethical Committee license for blood sampling at the National Transfusion Service NTS-KRA/2012/SVI was registered. Human neutrophils were isolated from whole blood, as described previously [21, 22]. The final suspension of neutrophils contained more than 96% of viable cells, as evaluated by trypan blue exclusion and was used within 2 h, as long as the control chemiluminescence remained constant.

2.3. Chemiluminescence (CL) Assay of Whole Blood and Isolated Neutrophils. The oxidative burst in whole blood was stimulated with phorbol myristate acetate (PMA 0.05 μM), opsonized zymosan (OpZ; 0.5 mg/mL), fMLP (1 μM), or Ca ionophore A23187 (1 μM). CL was measured in 250 μL samples consisting of 50 μL aliquots that contained blood (50× diluted), luminol (200 μM), RES (0.01–100 μM), and phosphate buffer [11]. The effect of RES on extra- and intracellular ROS production was measured in unstimulated and PMA (0.05 μM) stimulated neutrophils (5×10^5 per sample) by isoluminol/luminol-enhanced CL. The CL of both whole blood and isolated neutrophils was evaluated and measured in a microplate luminometer Immunotech LM-01T (Czech Republic) at 37°C [23].

2.4. Analysis of Apoptosis. Citrated whole human blood was collected as described above. Dextran (3%) was added (blood : dextran = 2 : 1) and centrifuged at 10 ×g at room temperature [16]. Before use, 1 mL of buffy coat that contained leukocytes was collected and stored on ice. The cells were counted on the hemocytometer (Coulter Counter), which focused on granulocytes. The cell suspension was adjusted to get 2×10^5 neutrophils per sample. Three different concentrations of RES (1, 10, and 100 μM) were applied and incubated with a control sample at 37°C for 10 min. The cells were stained with Annexin V, conjugated with FITC (BenderMedSystems) in the dark at 4°C for 10 min, followed by staining with propidium iodide (1 μg/mL), and then analysed immediately by the Beckman Coulter Cytomics FC500 cytometer (for details, see Perečko et al. [16]).

2.5. Neutrophil Integrity. The cytotoxic effect of RES was evaluated by means of ATP liberation by luciferin-luciferase chemiluminescence [11]. The neutrophil suspension (30 μL; 30 000 cells/sample) and 20 μL of Tyrode's solution were incubated with 50 μL of RES (1 to 100 μM) for 15 min at

37°C. The total ATP content was assessed immediately after sonication of neutrophils for 10 s.

2.6. Recombinant Caspase-3 Activity. To determine the caspase-3 activity, a modified method was applied [16]. The final reaction with luciferase was detected by CL. The light production was measured in the Luminometer Immunotech LM-01T. The reagent was added and the mixture was measured for 60 min to determine caspase-3 activity. The solvent for RES, containing NaOH, was also evaluated.

2.7. Protein Kinase C Activation. Phosphorylation of protein kinase (PKC) isoenzymes α and βII was detected [11]. Isolated human neutrophils (5×10^6) were incubated at 37°C with RES for 1 min, stimulated with PMA (0.15 μM, 1 min), and lysed by the addition of solubilisation buffer. After sonication on ice, samples were centrifuged to remove unbroken cells, the supernatant was boiled for 5 min with sample buffer, and samples were loaded on 9.8% SDS polyacrylamide gels. Membrane strips were blocked for 60 min with 1% bovine serum albumin in Tris buffered saline. This was followed by 60 min incubation in the presence of the phospho-PKC α and βII (Thr638/641) antibody (rabbit anti-human, 1 : 8000, Cell Signaling Technology) or β-actin antibody (rabbit anti-human, 1 : 4000, Cell Signaling Technology, Danvers, MA, USA). The membranes were subsequently washed six times with TBS and incubated 60 min with the secondary antibody conjugated to horseradish peroxidase (anti-rabbit from donkey, 1 : 10,000, Amersham, UK). The optical density of each PKC band was corrected by the optical density of the corresponding β-actin band.

2.8. Cell Culture. Murine peritoneal macrophage cell line RAW 264.7 (American Type Culture Collection, USA) was cultivated in Dulbecco's Modified Eagle Medium (PAN, Germany) supplemented with 10% foetal bovine serum (PAN, Germany) and 1% gentamycin (Sigma, USA). Cells were maintained at 37°C and 5% CO_2. After reaching confluence, cells were harvested and washed. Cell numbers and viability were determined by ATP test [24].

2.9. Formation of Nitric Oxide. Generation of reactive nitrogen species was determined indirectly as the accumulation of nitrites in the supernatant of murine macrophages RAW 264.7 [12]. Control cells were incubated with LPS without RES treatment. At the end of the incubation period, culture media were collected from wells and centrifuged at 5000 ×g and 4°C for 5 min. Then 150 μL of supernatant was mixed with equal volume of Griess reagent (Sigma, USA) in a 96-well plate, and the mixture was incubated at room temperature and in the dark for 30 min. The cell fractions of these samples were used for the detection of inducible NO synthase expression by Western blot.

2.10. Western Blot Analysis of iNOS Expression. After removing the supernatant for nitrite measurement, the remaining cells were washed with cold phosphate buffered saline (PBS) and lysed in the lysis buffer (1% sodium dodecyl sulphate—SDS, 10^{-1} M Tris pH 7.4, 10% glycerol, 10^{-3} M sodium orthovanadate, 10^{-3} M phenylmethanesulfonyl fluoride). Protein concentrations were determined by using BCATM protein assay (Pierce, USA), with bovine serum albumin as standard. Equal amounts of protein were then subjected to SDS-polyacrylamide gel electrophoresis using 7.5% running gel. The expression of iNOS protein was quantified by Western blot analysis [25]. Relative protein levels were quantified by scanning densitometry using the Image JTM programme, and the individual band density value was expressed in arbitrary units.

2.11. Antioxidant Activity Assays (Free Radical Scavenging Activity in Cell Free Systems)

2.11.1. ORAC Assay. The ORAC method measures the antioxidant scavenging activity against peroxyl radical induced by 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) at 37°C [26, 27]. Fluorescein (FL) was used as the fluorescent probe. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC). The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the curve.

2.11.2. HORAC Assay. HORAC measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions employing a Co(II) complex and hence the protecting ability against formation of hydroxyl radical [26, 27]. The initial fluorescence was measured, after which the readings were taken every minute after shaking in the presence of 100, 200, 400, 500, and 600 μM gallic acid solutions (in phosphate buffer 75 mM, pH 7.4). The final HORAC values were calculated using a regression equation between gallic acid concentration and the net area under the curve.

2.12. ROS Scavenging in Luminol-Horseradish Peroxidase (HRP)-H_2O_2 Cell Free System. Aliquots of 50 μL of RES solutions, HRP (2 U/mL), and luminol (10 μM) were mixed in a 96-well luminescence plate to yield final concentrations of RES 1, 10, and 100 μM. The reaction was started by adding hydrogen peroxide at the final concentration of 100 μM (final volume of the sample was 200 μL). Chemiluminescence was measured for 10 minutes at 37°C with Luminometer Immunotech LM-01T (Beckman Coulter).

2.13. NO Scavenging Activity. The potential ability of extracts to scavenge NO in chemical systems was tested by electrochemical measurement of NO [28]. NO was measured using three electrode systems: a porphyrinic microsensor working electrode, counter electrode, and a reference electrode were connected to the ISO-NO MARK II potentiostat (WPI, USA) [28]. The injection of the 2 μL NO-saturated water into the measurement glass vial (final concentration of NO = 2.38 μM) caused a rapid increase with subsequent gradual decrease of the NO induced signal until it reached the background current [29]. The scavenging properties of the

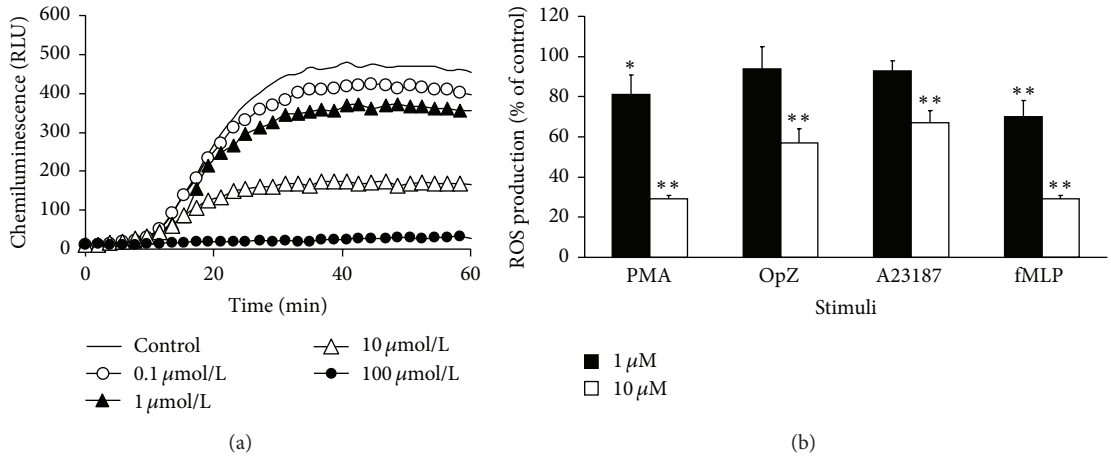

Figure 1: (a) Resveratrol dose-dependently decreased luminol-enhanced representative chemiluminescence curves of human whole blood stimulated with phorbol myristate acetate (PMA = 0.05 μM) at 37°C. (b) Effect of resveratrol in 1 and 10 μM concentration on PMA (0.05 μM), OpZ (0.5 mg/mL), fMLP (1 μM), and A23187 (1 μM) stimulated chemiluminescence. n = 6–8, Mean ± SEM, $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

extracts tested were evaluated as the time needed for reaching again the background current.

2.14. Lipid Peroxidation. The amount of 0.9 mL of 0.5 mM α-linolenic acid (Sigma-Aldrich, Steinheim, Germany) was mixed with 0.1 mL sample. Then, a system generating hydroxyl radical (0.1 mL Co(II) and 0.1 mL hydrogen peroxide for details, see section HORAC) was added for the induction of lipid peroxidation and the mixture was incubated for 2 h in 37°C. The concentration of thiobarbituric acid-reactive substances (TBARS) was measured as the index of lipid peroxidation [30]. The absorbance of the upper layer was measured at 532 nm. 1,1,3,3-Tetraethoxypropane (Sigma-Aldrich, Steinheim, Germany) in the final concentration of 0.1 μM was used as standard. Lipid peroxidation was expressed in nM of TBARS per 1 mL of the mixture α-linolenic acid/analysed sample.

2.15. Statistical Analysis. Data represent the mean ± SEM, unless stated otherwise. Statistical analysis was performed using the ANOVA paired test to examine differences between the treatments and control. Differences were considered to be statistically significant when $P < 0.05$ (*) or $P < 0.01$ (**).

3. Results

Figure 1(a) demonstrates representative dose-dependent CL curves of whole blood treated with RES and stimulated with PMA (0.05 μM). Figure 1(b) shows the effect of RES in 1 and 10 μM concentration on whole human blood CL stimulated with PMA (0.05 μM), OpZ (0.5 mg/mL), A23187 (1 μM), and fMLP (1 μM). In 1 μM concentration, RES significantly decreased CL for PMA and fMLP stimuli to 19 and 30 per cent of control value (=100%), respectively. RES in 10 μM concentration significantly inhibited CL with all stimuli applied in the rank order of potency PMA = fMLP > OPZ > A23187 demonstrating an evident difference in resveratrol activity to decrease stimulated chemiluminescence of whole blood.

Table 1: Effect of resveratrol in concentrations of 1 to 100 μM on viability of isolated neutrophils. Cells were incubated with resveratrol at 37°C for 10 min, stained with Annexin V, subsequently conjugated with FITC in the dark at 4°C for 10 min, followed by staining with propidium iodide (1 μg/mL), and then analysed immediately by the Beckman Coulter Cy. n = 4–6, mean ± SEM.

Resveratrol (μM)	Live cells	Apoptotic cells	Dead cells
0	91.90 ± 1.02	7.90 ± 1.00	0.20 ± 0.04
1	92.20 ± 1.31	7.60 ± 1.30	0.20 ± 0.05
10	92.10 ± 1.16	7.80 ± 1.60	0.10 ± 0.03
100	81.20 ± 2.73**	18.60 ± 2.70**	0.20 ± 0.03

$^{**}P \leq 0.01$.

The effect of RES on extra- and intracellular CL of isolated neutrophils stimulated with PMA is demonstrated in Figure 2. It is evident that RES dose-dependently decreased extracellular CL, significantly starting at 0.1 μM concentration. At 100 μM concentration (Figure 2(a)), there was complete inhibition of stimulated CL due to PMA. At intracellular level (Figure 2(b)), RES significantly decreased CL at 10 and 100 μM concentrations to 26 and 0.33 percent of the control value, respectively (Figure 2(c)).

Isolated intact neutrophils liberate 18 ± 3.8 nM ATP, which represents 3.2% from the total ATP amount (548 ± 112 nM/3 × 10^4 cells). RES in any concentration used did not liberate ATP from isolated neutrophils (results not shown), indicating that RES did not disintegrate isolated neutrophils in any concentration used.

Table 1 demonstrates the effect of RES in concentrations of 1 to 100 μM on viability of isolated neutrophils. In concentrations of 1 and 10 μM, RES did not change significantly the amount of dead cells as compared with control cells. In 100 μM, concentration RES increased the number of apoptotic cells from 7.9% (controls) to 18.6% of the total viable

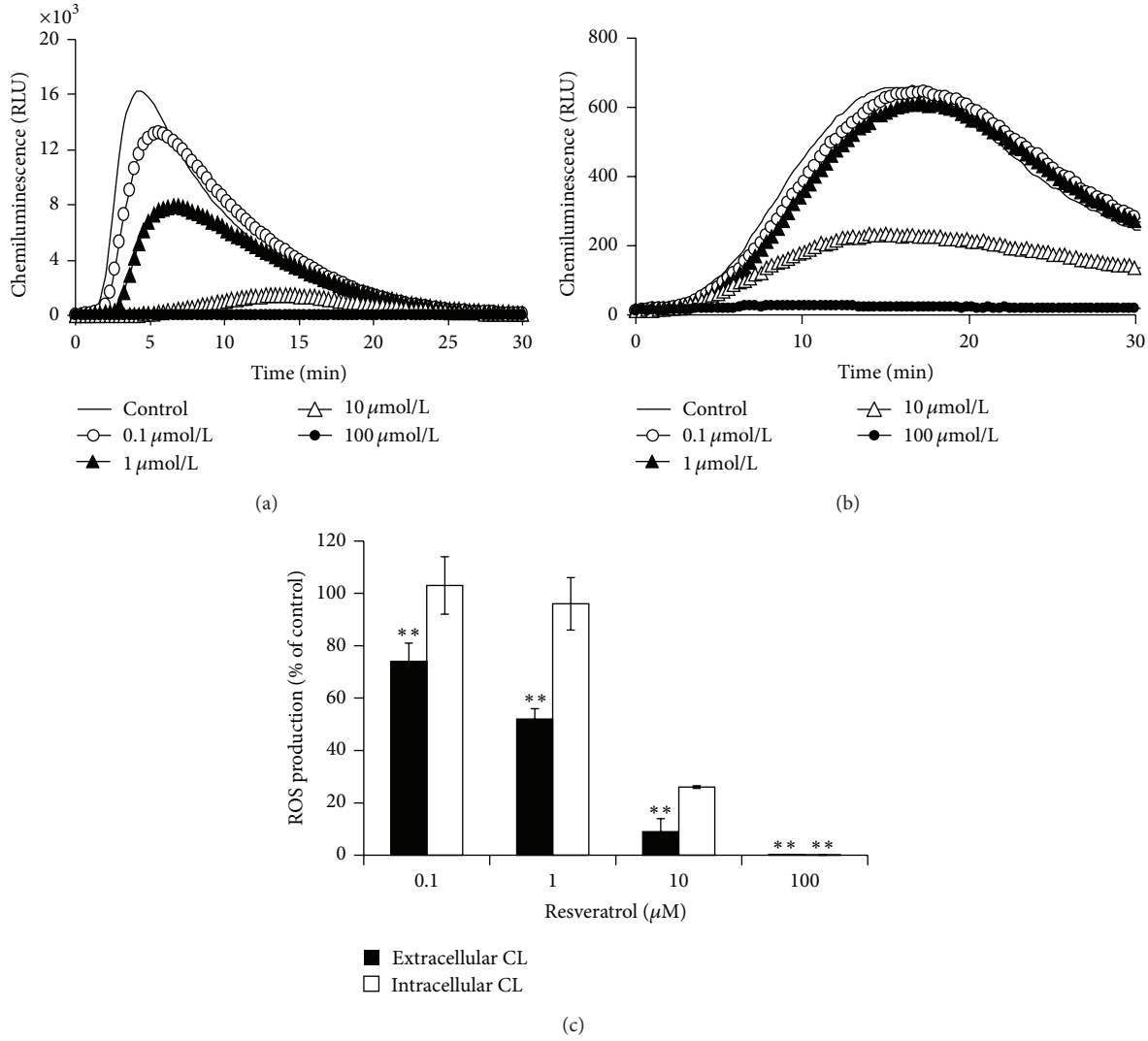

Figure 2: Chemiluminescence of isolated neutrophils. (a) Dose-dependent extracellular representative chemiluminescence (luminol + peroxidase) curves of human isolated neutrophils pretreated with resveratrol and stimulated with PMA (0.05 μM). (b) Intracellular dose-dependent representative chemiluminescence (isoluminol + catalase + superoxide dismutase) curves of isolated human neutrophils pretreated with resveratrol and stimulated with PMA (0.05 μM). (c) Dose-dependent effect of resveratrol on PMA (0.05 μM) stimulated extracellular and intracellular chemiluminescence. n = 6–8; mean ± SEM, $^{**}P \leq 0.01$.

cells. The number of dead cells did not increase. These results show that RES in the optimal concentrations used (1 and 10 μM) did not change apoptosis of isolated human neutrophils.

Figure 3 shows the result of RES on radical scavenging activity in cell free system. RES in concentrations 10 and 100 μM increased hydroxyl scavenging activity (HORAC) to 88 and 114 μM of gallic acid equivalents, respectively, and peroxyl scavenging activity (ORAC) to 34 and 29 of Trolox equivalents, respectively. This effect demonstrates effective scavenging activity of RES on hydroxyl and peroxyl radicals *in vitro*.

Figure 4 shows the inhibitory effects of different concentrations (1, 10, and 100 μM) of RES on production of ROS in cell free system generated by means of luminol + hydrogen peroxide + HRP. RES in 1 μmol/L concentration significantly ($P < 0.01$) inhibited and in 10 μM concentration totally blocked the chemiluminescence of 100 μM hydrogen peroxide in the samples.

Since lipids are very susceptible to lipid peroxidation, we tested also the ability of RES to prevent the peroxidation of polyunsaturated fatty acids induced by hydroxyl radical. It is evident from Figure 5 that RES in all tested concentrations significantly and dose-dependently inhibited lipid peroxidation.

The activation of protein kinase C in isolated neutrophils stimulated with PMA (0.15 μM) in the presence of 10 and 100 μM RES is demonstrated in Figure 6. In both concentrations applied, RES reversed PMA stimulated PKC activation

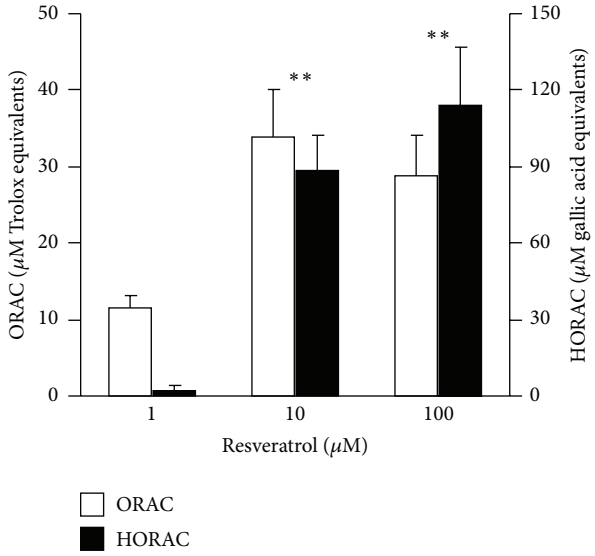

Figure 3: Effect of resveratrol on hydroxyl (HORAC) and peroxyl (ORAC) radical scavenging activity in cell free system expressed as gallic acid and Trolox equivalents, $n = 4$, mean ± SEM, $^{**}P \geq 0.01$.

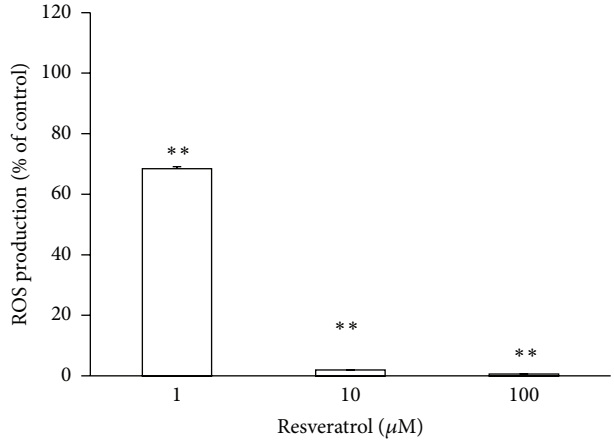

Figure 4: Dose-dependent effect of resveratrol on reactive oxygen species generated in cell free system by means of luminol + hydrogen peroxide + horseradish peroxidase. $n = 3$, mean ± SEM, $^{**}P \geq 0.01$.

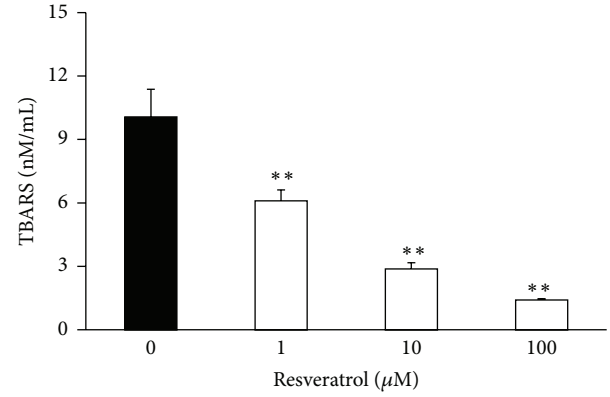

Figure 5: Effect of resveratrol on lipid peroxidation of α-linolenic acid expressed as thiobarbituric acid-reactive substances (TBARS), induced by hydroxyl radicals. $n = 6$, mean ± SEM, $^{**}P \geq 0.01$.

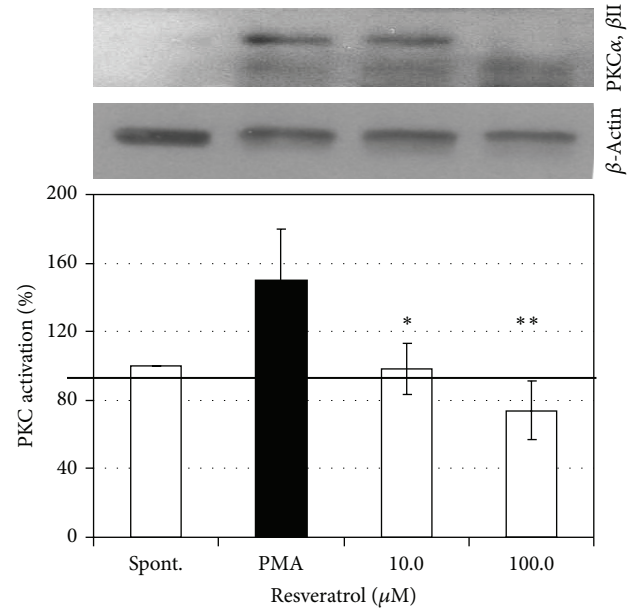

Figure 6: Western blotting analysis of protein kinase C activation in isolated human neutrophils pretreated with resveratrol (10 and 100 μM) and stimulated with PMA (0.15 μM). $n = 3$-4, mean ± SEM; $^{*}P \leq 0.05$.

to spontaneous (control) values indicating a suppressive effect of RES on the activity of protein kinase C, one of the essential regulatory enzymes in reactive oxygen generation.

The effect of RES on nitrite production and iNOS expression in RAW 264.7 cell culture after LPS activation is demonstrated in Figure 7. Figure 7(a) demonstrates that RES in concentrations of 1, 10, and 100 M decreased nitrite concentration in cell supernatants to 82, 65, and 6 percent of the control value, respectively.

Figure 7(b) shows the effect of RES on iNOS expression determined by Western blot analysis. In comparison with the iNOS protein level in the control sample, iNOS protein expression was significantly inhibited only by the highest concentration (100 μM) of RES to 60% of the control value.

4. Discussion

Resveratrol dose-dependently inhibited oxidative burst in human whole blood stimulated with two membrane-bypassing (PMA, A23187) and two membrane-operating stimuli (OpZ, fMLP). There was no significant difference between the stimuli applied and chemiluminescence decrease of whole blood indicating that RES may not act only as an extracellular scavenger but suppresses oxidative burst also intracellularly. This suggestion was confirmed on isolated neutrophils (Figure 2) stimulated with PMA demonstrating that RES in a concentration dependent way inhibited not only extracellularly determined chemiluminescence but effectively suppressed formation of intracellularly generated

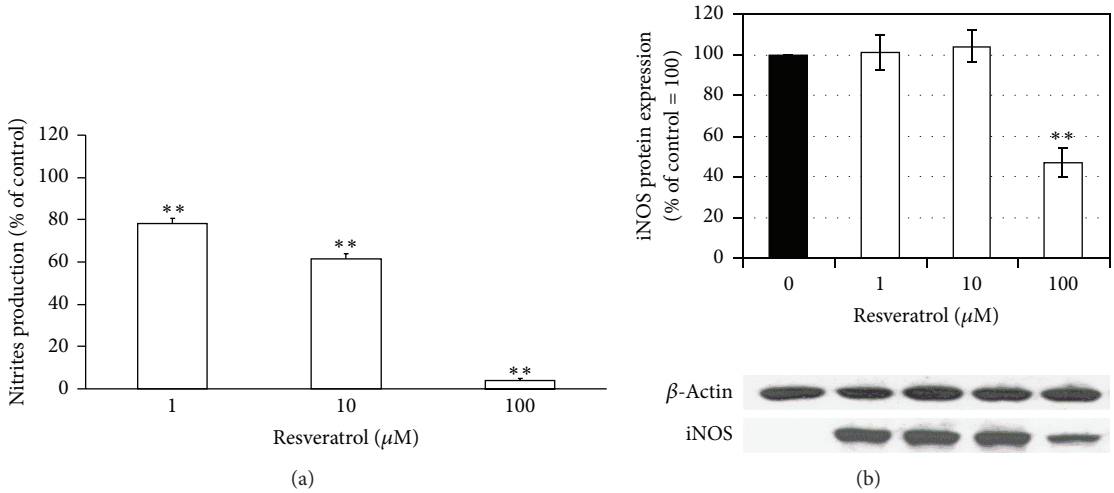

Figure 7: Nitrite production and iNOS expression in RAW 264.7 culture cells. Nitrite production (a) and densitometric analysis together with representative Western blot of iNOS protein expression (b) in LPS stimulated RAW 264.7 cells treated with resveratrol. $n = 3$, mean ± SEM, $^{**}P < 0.01$.

ROS (Figure 2(b)). The difference was evident; RES started to inhibit extracellular chemiluminescence at 0.1 μM, intracellularly at 10 μM concentration (Figure 2(c)).

The stimulated generation of ROS in whole blood and isolated neutrophils was decreased by many polyphenolic compounds like curcumin, pterostilbene, pinosylvin, and N-feruloyl serotonin [11, 14, 31]. Inhibition of fMLP-activated human neutrophil chemiluminescence was accompanied by inhibition of elastase and β-glucuronidase secretion and production of 5-lipoxygenase metabolites leukotriene B4, 6-trans-LTB4, and 12-trans-epi-LTB4 after stimulation with calcium ionophore, indicating that transresveratrol interferes with the release of inflammatory mediators in activated polymorphonuclear leukocytes [32].

Since RES did not liberate ATP from isolated neutrophils, it is evident that even in higher concentrations used (up to 100 μM) there was no disintegration of cells. Moreover, RES dose-dependently decreased spontaneous ATP liberation.

RES did not change significantly the number of dead cells even in the highest concentration used, decreasing the number of live cells by 10.7% (Table 1). In contrast, RES decreased the activity of recombinant caspase-3 activity in cell free system, significantly at 10 μM concentration. This result has to be verified in the cellular model since apoptosis, a programmed cell death, appears to be the most frequent fate of cells treated with RES [33]. Moreover, RES induced autophagy in human U251 glioma cells [34], decreased the intracellular reactive oxygen species level, which correlated with the induction of caspase-8 and caspase-3 cleavage in human colon cancer cells [35] and induced apoptosis in patients with chronic myeloid leukemia cells [36].

The antioxidant properties of RES were analysed via five different methods: ORAC(peroxyl), HORAC(hydroxyl), hydrogen peroxide-peroxidase dependent chemiluminescence, NO scavenging, and lipid peroxidation inhibition. The chosen methods embrace different aspects of the antioxidant action and give a comprehensive view on the antioxidant potential of the sample investigated.

In the following experiments, we tested also the scavenging properties of RES against NO, using electrochemical analysis which is considered to be a reliable method for verifying NO scavenging. However, no scavenging properties of resveratrol against NO were found in any concentration used.

These observations confirmed previous findings that RES is both a free radical scavenger and a potent antioxidant because of its ability to promote the activities of a variety of antioxidant enzymes [37]. The result of decreasing dose-dependently LDL oxidation in cell free system is supportive of its effect on lipid peroxidation and atherosclerotic lesion formation in animal hypercholesterolemic models [38].

RES decreased protein kinase C activation in PMA-stimulated neutrophils, indicating its interference with oxidative burst in neutrophils. Similar results were demonstrated in human gastric adenocarcinoma and CaSki cells [39, 40] and for the polyphenolic compound N-feruloyl serotonin in human neutrophils [14]. By inhibiting the activation of PKC [41], RES may interfere with modulation of intracellular signalling pathways involved in downregulation of COX-2 and iNOS expression and NF-κB activation [10, 42]. Nitric oxide, a member of reactive nitrogen species, is an important molecule involved in the regulation of many physiological and microbicidal processes. RES markedly inhibited NO production by LPS stimulated macrophages. This finding corresponds with the latest results of other authors [43, 44] who also reported suppression of inducible nitric oxide synthase expression and NO production in macrophages after RES administration. Our results showed that resveratrol reduced nitrite accumulation more effectively than it reduced iNOS protein expression in stimulated macrophages through a mechanism which is at least partially independent of the regulation of iNOS protein expression. Nevertheless, in

electrochemical measurements we showed that RES was not able to scavenge NO, suggesting that the direct scavenging activity against NO resulting from the inhibitory action of RES can be excluded.

In conclusion, RES possesses antioxidant activities *in vitro* inhibiting generation of ROS in cell free systems, oxidative burst of stimulated blood and isolated neutrophils both at extracellular and intracellular level, as measured by chemiluminescence.

Oxidative burst inhibition of human blood and isolated neutrophils, suppression of free radical generation and NO formation in cell free system confirmed the antioxidative properties and supported the effort to enlarge clinical studies with RES.

Conflict of Interests

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

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Research Article

Inhibition of Nuclear Nox4 Activity by Plumbagin: Effect on Proliferative Capacity in Human Amniotic Stem Cells

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Human amniotic fluid stem cells (AFSC) with multilineage differentiation potential are novel source for cell therapy. However, *in vitro* expansion leads to senescence affecting differentiation and proliferative capacities. Reactive oxygen species (ROS) have been involved in the regulation of stem cell pluripotency, proliferation, and differentiation. Redox-regulated signal transduction is coordinated by spatially controlled production of ROS within subcellular compartments. NAD(P)H oxidase family, in particular Nox4, has been known to produce ROS in the nucleus; however, the mechanisms and the meaning of this function remain largely unknown. In the present study, we show that Nox4 nuclear expression (nNox4) increases during culture passages up to cell cycle arrest and the serum starvation causes the same effect. With the decrease of Nox4 activity, obtained with plumbagin, a decline of nuclear ROS production and of DNA damage occurs. Moreover, plumbagin exposure reduces the binding between nNox4 and nucleoskeleton components, as Matrin 3. The same effect was observed also for the binding with phospho-ERK, although nuclear ERK and P-ERK are unchanged. Taken together, we suggest that nNox4 regulation may have important pathophysiologic effects in stem cell proliferation through modulation of nuclear signaling and DNA damage.

1. Introduction

Stem cells are characterized by a high capacity of self-renewal and differentiation. Through self-renewal, stem cells maintain the homeostasis of a stem cell pool; through differentiation, stem cells can give rise to terminal cells with diverse morphology and functions [1]. In tissues, most stem cells are in the quiescent state, and they are protected by special microenvironments (niches) [2]. The quiescence of stem cells may prevent the accumulation of DNA replication errors [3] and may facilitate resistance to many stressors [4]. The intracellular ROS level is a critical factor that regulates the quiescent status of mesenchymal stem cells (MSC) [5]. Similar to the low partial pressure of oxygen, low levels of ROS in niches are important for the stemness of MSC [6]. However, *in vitro* expansion of stem cells implies normoxic culture condition.

Indeed, MSC proliferative and colony formation capacity is significantly increased in normoxia. However, MSC expanded under normoxia show a threefold to fourfold increase in senescence, suggesting that hypoxia prevents oxidative stress-induced senescence and preserves MSC long-term self-renewal [7].

Accumulation of ROS is a common occurrence in senescent cells. Studies have shown that induction of ROS in senescent cells is involved in inhibiting proliferation [8]. On the other hand, intracellular accumulation of H_2O_2 in senescent human fetal MSCs termed placenta-derived multipotent cells (PDMCs) has been found, but the accumulation was not involved in inhibiting proliferation. Rather, H_2O_2 was involved in altering the differentiation potential of senescent PDMCs [9].

Various ROS-generating and ROS-degrading systems in different compartments of the cell seem to play an important

role. The nucleus itself contains a number of proteins with oxidizable thiols that are essential for transcription, chromatin stability, and nuclear protein import and export, as well as DNA replication and repair [10]. Specific isoforms of glutathione peroxidases, glutathione S-transferases, and peroxiredoxins are enriched in nuclei, further supporting the interpretation that functions of the thiol-dependent systems in nuclei are at least quantitatively and probably also qualitatively distinct from similar processes in the cytoplasm [11].

ROS generation within the nucleus may have several important effects on cellular function. ROS can inactivate nuclear-localized phosphatases and thereby enhance kinase activation. For example, the oxidative inactivation of the nuclear phosphatase mitogen-activated kinase phosphatase 1 regulates ERK1/2 activation [12]. Excessive production of ROS could also lead to oxidative DNA damage.

In this point of view, the subcellular localization of NADPH oxidase isoform 4 (Nox4) is likely to be especially important, given its constitutive activity, unlike isoforms, such as Nox1 or Nox2, that requires agonist activation. However, its subcellular distribution remains controversial, at least in part attributable to the lack of sufficiently specific or characterized antibodies. Nox4 has been reported to be variably present in the ER [13, 14], mitochondria [15], cytoskeleton [16], plasma membrane [17], and nucleus [18] in different cell types.

Other unresolved questions include whether Nox4 utilizes NADPH or NADH as a substrate to produce $O_2^{\cdot}$ [18, 19] and whether it primarily produces superoxide or hydrogen peroxide [18, 20].

More recently, endothelial nuclei have been shown to produce ROS that are, at least in part, Nox4 dependent [18, 21], but its subnuclear localization (within specific nuclear membranes) remains unclear [22]. Nuclear Nox4 has also been implicated in DNA damage resulting from both hemangioendothelioma formation [23] and hepatitis C infection [24].

NADPH oxidase Nox4 is a critical mediator in oncogenic H-RasV12-induced DNA damage response [25]. DNA damage response, detected by c-H2A.X foci analysis, leads to cell aging and subsequent senescence [26].

Anilkumar et al. [27] found that there is a nuclear-localized and functionally active splice variant of Nox4 (Nox4D) that may have important pathophysiologic effects through modulation of nuclear signaling and DNA damage. Interestingly, a significant proportion of nuclear Nox4D was localized to the nucleolus of vascular cells.

In this study, we investigated the role of Nox4-derived nuclear ROS on proliferative capacity of amniotic fluid stem cells (AFSC) since they can be considered representative of human stem cells, in view of their characteristics, such as the intermediate status between embryonic stem cells and adult stem cells.

Moreover, De Coppi and colleagues [28] described that AFSC can be directed into a wide range of cell types representing the three primary embryonic lineages of mesoderm, ectoderm, and definitive endoderm [29, 30]. Amniotic fluid is known to contain a heterogeneous population of cell types derived from fetal tissues and the amnion [31]. *In vitro* expansion of human stem cells is necessary to obtain a sufficient cell number to *in vivo* implant purpose, but it leads to senescence affecting proliferative and differentiation capacities. Thus, if the function of ROS is to enforce irreversible cellular senescence, the NADPH oxidase Nox4, as ROS-generating system, appears to be a potential nuclear ROS source.

Here, we show that a part of Nox4 localizes to the nucleus of AFSC, where the oxidase likely forms a functional complex with p22phox and produces ROS in the nucleoplasm. In addition, Nox4 seems to regulate DNA damage, constituting a part of oxidative stress. Here, we assessed the effect of plumbagin, a plant-derived naphthoquinone directly inhibiting Nox4 activity [32], on DNA damage and Nox4 nuclear interactions.

2. Materials and Methods

2.1. Cell Culture. Amniocentesis samples (5 back up flasks obtained from different donors) were provided by the Laboratorio di Genetica, Ospedale Santa Maria Nuova (Reggio Emilia, Italy). All samples were collected with informed consent of the patients (mother's age ≥ 35) according to Italian law and ethical committee guideline.

Human AFSC were isolated as previously described by De Coppi et al. [28]. Human amniocentesis cultures were harvested by trypsinization and subjected to c-Kit immunoselection by MACS technology (Miltenyi Biotec, Germany). AFSC were subcultured routinely at 1 : 3 dilution and not allowed to expand beyond the 70% of confluence. AFSC were grown in culture medium (αMEM supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) (all from EuroClone Spa, Italy) [33].

Cells were treated with plumbagin (5-hydroxy-2-methyl-1,4 naphthoquinone) or diphenyleneiodonium (Sigma Aldrich, St. Louis, MO,USA).

2.2. Cell Viability and Proliferation Assay. Viable cells were evaluated by the MTT assay, since the reduction of tetrazolium salts is widely accepted as a reliable way to examine cell viability/proliferation. Cells were incubated with 0.5 mg/mL MTT for 4 h at 37°C, as previously reported [34]. At the end of the incubation, purple formazan salt crystals were dissolved by adding the solubilization solution (isopropanol, 0.01 M HCl). The absorption at 570 nm was measured on a multiwell plate reader (Appliskan, Thermo-Fisher Scientific, Vantaa, Finland).

2.3. Cell Cycle Analysis. For detection and quantification of cell cycle distribution, samples containing $2–5 \times 10^5$ cells were harvested by centrifugation, fixed in cold ethanol, and subjected to propidium iodide (Sigma Aldrich, St. Louis, MO, USA) flow cytometric assay. Total lysates, obtained as reported below, were subjected to Western blotting and revealed for anticyclin E (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4. Preparation of Cell Extracts. Cell extracts were obtained as described by Maraldi et al. [35]. Briefly, subconfluent cells were extracted by addition of AT lysis buffer (20 mM Tris-Cl, pH 7.0; 1% nonidet P-40; 150 mM NaCl; 10% glycerol; 10 mM EDTA; 20 mM NaF; 5 mM sodium pyrophosphate; and 1 mM Na_3VO_4) and freshly added Sigma Aldrich protease inhibitor cocktail at 4°C for 30 min. Lysates were sonicated, cleared by centrifugation, and immediately boiled in SDS sample buffer or used for immunoprecipitation experiments, as described below.

2.5. Immunoprecipitation and Electrophoresis. Immunoprecipitation was performed as reported by Cenni et al. [36]. Equal amounts of precleared lysates, whose protein concentration was determined by the Bradford method, were incubated overnight with anti-NOX4 (Novus Biologicals, CO, USA), antipan 14.3.3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (3 μg all). Then, the two samples were treated with 30 μL of 50% (v/v) of protein A/G agarose slurry (GE Healthcare Bio-sciences, Uppsala, Sweden) at 4°C with gentle rocking for 1 h. Pellets were washed twice with 20 mM Tris-Cl, pH 7.0; 1% Nonidet P-40; 150 mM NaCl; 10% glycerol; 10 mM EDTA; 20 mM NaF; and 5 mM sodium pyrophosphate and once with 10 mM Tris-Cl, pH 7.4, boiled in SDS sample buffer, and centrifuged. Supernatants were loaded onto SDS-polyacrylamide gel, blotted on Immobilon-P membranes (Millipore, Waltham, MA, USA), and processed by Western blot with the indicated antibodies, detected by Supersignal substrate chemiluminescence detection kit (Pierce, Rockford, IL, USA). Quantitation of the signal was obtained by chemiluminescence detection on a Kodak Image Station 440CF and analysis with the Kodak 1D Image software.

2.6. Nuclei Purification. Human AFSC nuclei were purified as reported by Cenni et al. [37]. Briefly, to 5×10^6 cells 400 μL of nuclear isolation buffer was added (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin and leupeptin, and 5 mM NaF) for 8 min on ice. MilliQ water (400 μL) was then added to swell cells for 3 min. Cells were sheared by passages through a 22-gauge needle. Nuclei were recovered by centrifugation at 400 ×g at 4°C for 6 min and washed once in 400 μL of washing buffer (10 mM Tris-HCl, pH 7.4, and 2 mM $MgCl_2$, plus inhibitors as described earlier in the text). Supernatants (containing the cytosolic fractions) were further centrifuged for 30 min at 4000 ×g. Isolated nuclear and cytoplasmic extracts were finally lysed in AT lysis buffer, sonicated, and cleared by centrifugation.

2.7. Western Blot. The protocols of the western blot were performed as described by Hanson et al. [38].

Protein extracts, quantified by a Bradford Protein Assay (Bio-Rad Laboratories, CA, USA), underwent to SDS-polyacrylamide gel electrophoresis and were transferred to Immobilon-P membranes. The following antibodies were used: rabbit antipan 14.3.3, rabbit anticyclin E, rabbit anti-Nox4, rabbit anti-Ki67, Rabbit anti-ERK1/2, goat anti-Matrin 3, goat anti-actin, anti-p22phox (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 500, rabbit anti-p44/42 ERK1/2 (Cell Signalling Technology, Beverly, MA, USA), mouse anti-Tubulin (Sigma Aldrich St. Louis, MO, USA), rabbit anti-Nox4 (Abcam, Cambridge, UK), rabbit anti-Nox4 (Novus Biologicals, CO, USA), and mouse anti-pH2A (Ser139) (Millipore, Billerica, MA, USA) diluted 1 : 1000; peroxidase-labelled anti-rabbit, mouse, and goat secondary antibodies diluted 1 : 3000 (Pierce Antibodies, Thermo Scientific; Rockford, IL, USA). Ab dilution was performed in TBS-T pH 7.6 containing 3% BSA. The membranes were visualized using Supersignal substrate chemiluminescence detection kit (Pierce, Rockford, IL, USA). Antiactin antibody was used as control of protein loading.

2.8. Confocal Microscopy. Undifferentiated AFSC were fixed for 20 min in 4% ice-cold paraformaldehyde and then permeabilized with 0.1% Triton X-100 in ice-cold phosphate-buffered saline (PBS) for 5 min. Permeabilized samples were then blocked with 3% of bovine serum albumin (BSA) in PBS for 30 min at room temperature and incubated with primary antibodies (Abs). Rabbit anti-Nox4, goat anti-Matrin 3, rabbit anti-Ki67 (Santa Cruz, CA, USA) (diluted 1 : 50), and mouse anti-pH2A (Ser139) (Millipore, Billerica, MA, USA) (diluted 1 : 100), in PBS containing 3% BSA for 1 h at RT were used as primary antibodies (Ab). Secondary Ab was diluted 1 : 200 in PBS containing 3% BSA (goat anti-mouse Alexa 647, goat anti-rabbit Alexa 488, and donkey anti-goat Alexa 488). After washing in PBS, samples were stained with 1 μg/mL DAPI in H_2O for 1 min and then mounted with antifading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH 8.0). Negative controls consisted of samples not incubated with the primary antibody, but only with the secondary antibody.

Confocal imaging was performed on a Nikon A1 confocal laser scanning microscope as previously described [39].

Spectral analysis was carried out to exclude overlapping between two signals or the influence of autofluorescence background on the fluorochrome signals, as previously shown [40]. The confocal serial sections were processed with ImageJ software to obtain three-dimensional projections, as previously described [41]. The image rendering was performed by Adobe Photoshop software.

2.9. Nuclear ROS Imaging. Nuclear ROS were detected with nuclear-localized fluorescent probe for H_2O_2, Nuclear Peroxy Emerald 1 (NucPE1) [42–45]. For all experiments, 5 μM solutions of NucPE1 (from 5 mM stocks in DMSO) were made in PBS/glucose. The cells were then kept in an incubator (37°C, 5% CO_2) during the course of all experiments. The probes were incubated for total 30 min. Fluorescence was measured on a multiwell plate reader (Appliskan, Thermo Scientific) using 488 nm filter for excitation and 535 nm filter for emission.

Confocal fluorescence imaging studies were performed with a Nikon A1 confocal laser scanning microscope. Excitation of NucPE1-loaded cells at 488 nm was carried out with an Ar laser and emission was collected at 535 nm. All images in an experiment were collected simultaneously using identical

microscope settings. Image analysis was performed in Image J.

2.10. Statistical Analysis. *In vitro* experiments were performed in triplicate. For quantitative comparisons, values were reported as mean ± SD based on triplicate analysis for each sample. To test the significance of observed differences between the study groups, unpaired Student's t-test was applied. A P value < 0.05 was considered to be statistically significant.

3. Results and Discussion

3.1. Nox4 Expression in AFSC. At first we noticed that amniotic fluid stem cells express NADPH isoform 4 Nox4 both into the nucleus and in the cytoplasm, unlike Nox1 and Nox2 (data not shown). We tested different antibodies directed to Nox4 protein in order to verify this observation. In fact, in the literature, a homemade antibody anti-Nox4 is often used (polyclonal Nox4 antibodies by the Lambeth and Shah groups are the most frequently used) (see review [46]). On the other hand, all the employed antibodies revealed a variable percentage of cells with a nuclear localization of Nox4 (Figure 1(a)) and this data is consistent with other studies in which Nox4 has been found in nuclear or perinuclear area [18, 25, 47, 48]. By using antibodies from Santa Cruz, Abcam, or Novus, we can see a signal mostly localized inside the nuclei, not in the nuclear envelope, as demonstrated in mouse liver cells [48]. Regarding the staining obtained with Abcam antibody, all the nucleoplasm is marked except for nucleoli. Novus antibody binds other perinucleolar domains, while Santa Cruz antibody shows a more punctate pattern.

In order to investigate the NADPH oxidase activity inside nuclei, we used a nuclear selective probe for H_2O_2, nuclear Peroxy Emerald 1 (Figure 1(b)). Detection of nuclear ROS production with this probe demonstrates that ROS sources inside the nuclei have a punctate pattern similar to the one of Nox4 from Santa Cruz. Therefore, for immunofluorescence analysis, we utilized this antibody.

The same analysis has been performed for Western blot experiments (Figure 2(a)). Total lysates, cytosol, and nuclear fractions and the three antibodies reported above were tested.

WB analysis with all the antibodies of nuclear and cytoplasmic fractions confirms Nox4 presence both in cytosol and nuclei. Incubation with all the three antibodies reveals a triple band between 50 and 75 kDa, even if the band intensity is different depending on the antibody. In WB, the antibody from Novus seems to recognize the nuclear Nox4 (nNox4) better than others, since the bands in nuclear protein fraction are the most intense (Figure 2(a)).

The Novus antibody works also in immunoprecipitation experiment. Figure 2(b) shows nuclear extracts (NL) used for coimmunoprecipitation analysis with anti-Nox4 (IPNox4). This experiment confirms the presence of Nox4 in nuclear proteins showing the interaction between Nox4 and the modulatory subunit p22phox. Furthermore, Nox4 seems to be linked with nuclear matrix protein, Matrin3, and with the mitogen-activated protein kinase ERK1/2, suggesting

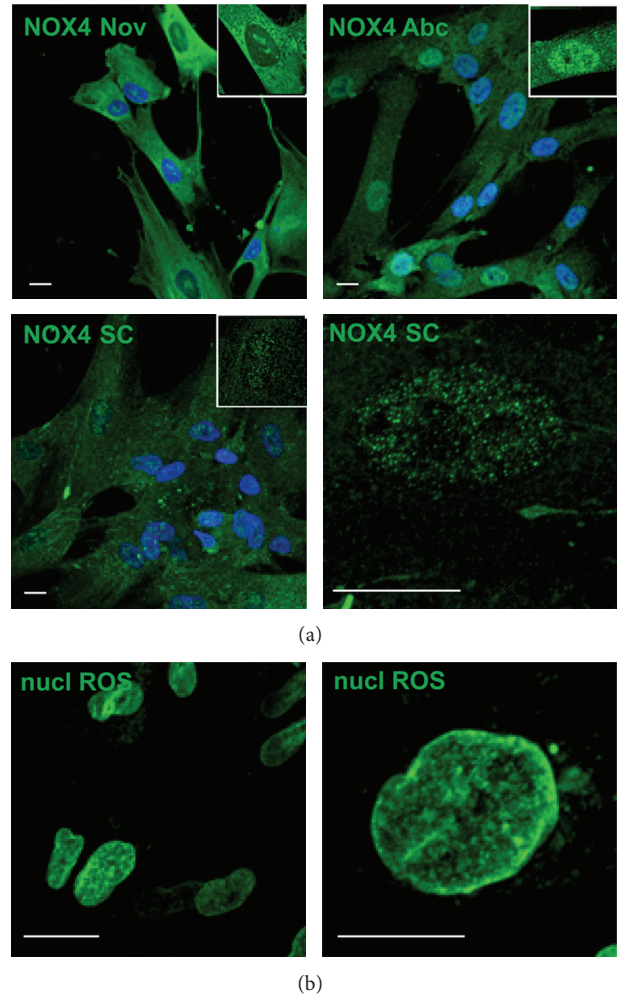

Figure 1: Immunofluorescence analysis of Nox4 expression and nuclear ROS in AFSC. (a) Representative images showing superimposing between DAPI (blue) and Nox4 (green) signals obtained with antibody from Novus (Nov), Abcam (Abc), and Santa Cruz (SC). Inside all the three images, a square showing magnification of a cell with only the Nox4 signal in green is present. The highest magnification of one nucleus is shown for Santa Cruz labelling. (b) Representative images showing staining with nuclear ROS probe (Nuclear peroxy Emerald 1). Scale bar: 10 μm.

a direct role in nuclear MAPK signaling regulation. It has been previously demonstrated that ERK activation (phosphorylation) occurs downstream from the Nox4 pathway: in particular through the Ras activation in endoplasmic reticulum of Human umbilical vein endothelial cells [49] or by a Src/caveolin-mediated activation in renal tubular cells [50].

3.2. Modulation of Nox4 Presence into the Nuclei. The ERK cascade is involved in cellular proliferation, differentiation, and survival. In fact nuclear translocation of ERK1 and ERK2 is critical for both gene expression and DNA replication induced by growth factors [51]. Moreover, in the nucleus, ERK phosphorylates an array of targets, including transcription

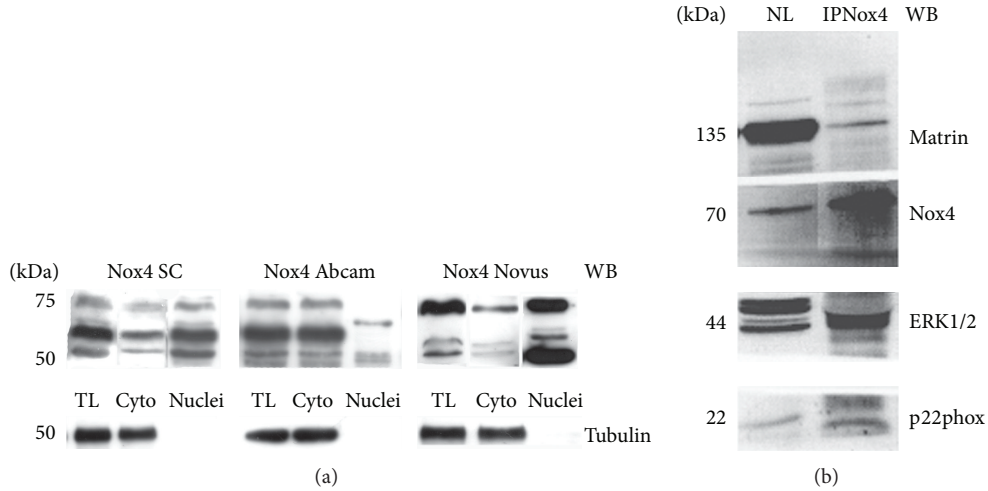

Figure 2: Nox4 expression in subcellular compartment of AFSC. (a) Representative images of Western blot analysis of total lysates (TL), cytosol (cyto) and nuclear fractions (nuclei) of AFSC revealed with different Nox4 antibodies: Santa Cruz (SC), Abcam (Abc), and Novus (Nov). Tubulin is shown as marker of nuclei purification. (b) Western blot analysis of nuclear lysate (NL) and immunoprecipitation experiment of NL with Nox4 antibody from Novus revealed with anti-Matrin, anti-Nox4, anti-ERK1/2, and anti-p22phox. Presented data are representative of three independent experiments.

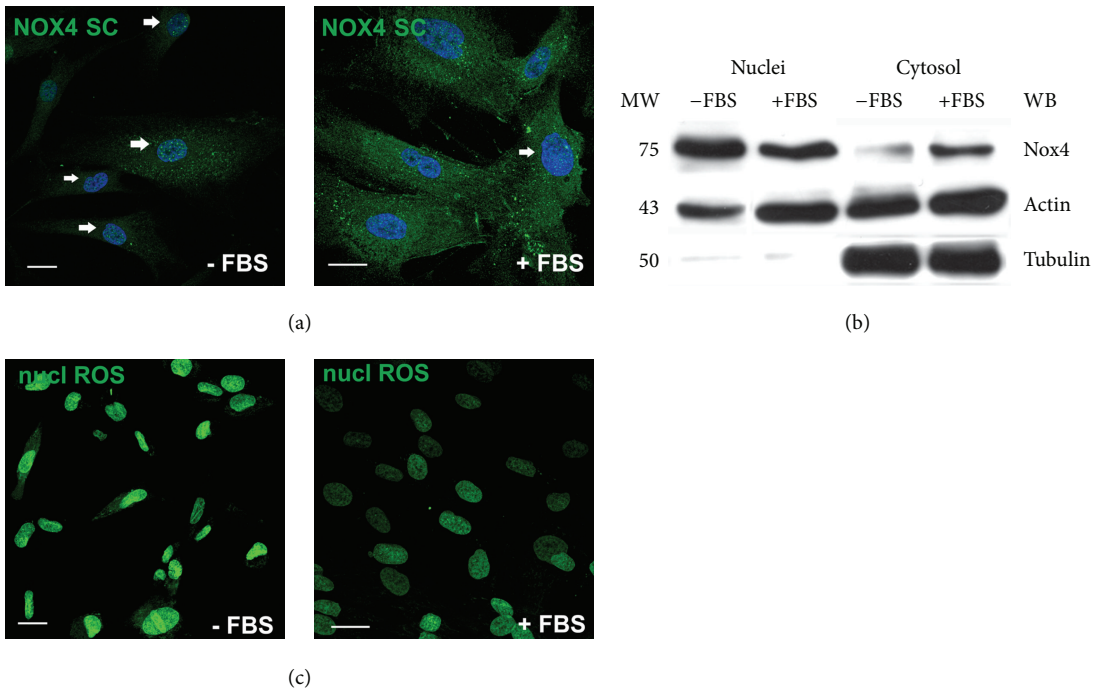

Figure 3: Effect of serum presence on Nox4 localisation end nuclear ROS production. (a) Representative images showing superimposing between DAPI (blue) and Nox4 SC (green) signals of AFSC cultures in the presence of absence of serum (FBS). Arrows indicate Nox4 staining in the nuclei. (b) Representative images of Western blot analysis revealed with Nox4 from Novus of cytosol and nuclei fractions of AFSC grown in the presence or absence (for 24 hours) of FBS. Actin and tubulin detection were performed in order to show the amount of protein loaded in each line and the nuclear fraction purity, respectively. Presented data are representative of three independent experiments. (c) Representative images showing staining with nuclear ROS probe (Nuclear peroxy Emerald 1) of AFSC with or without serum. Scale bar: 10 μm.

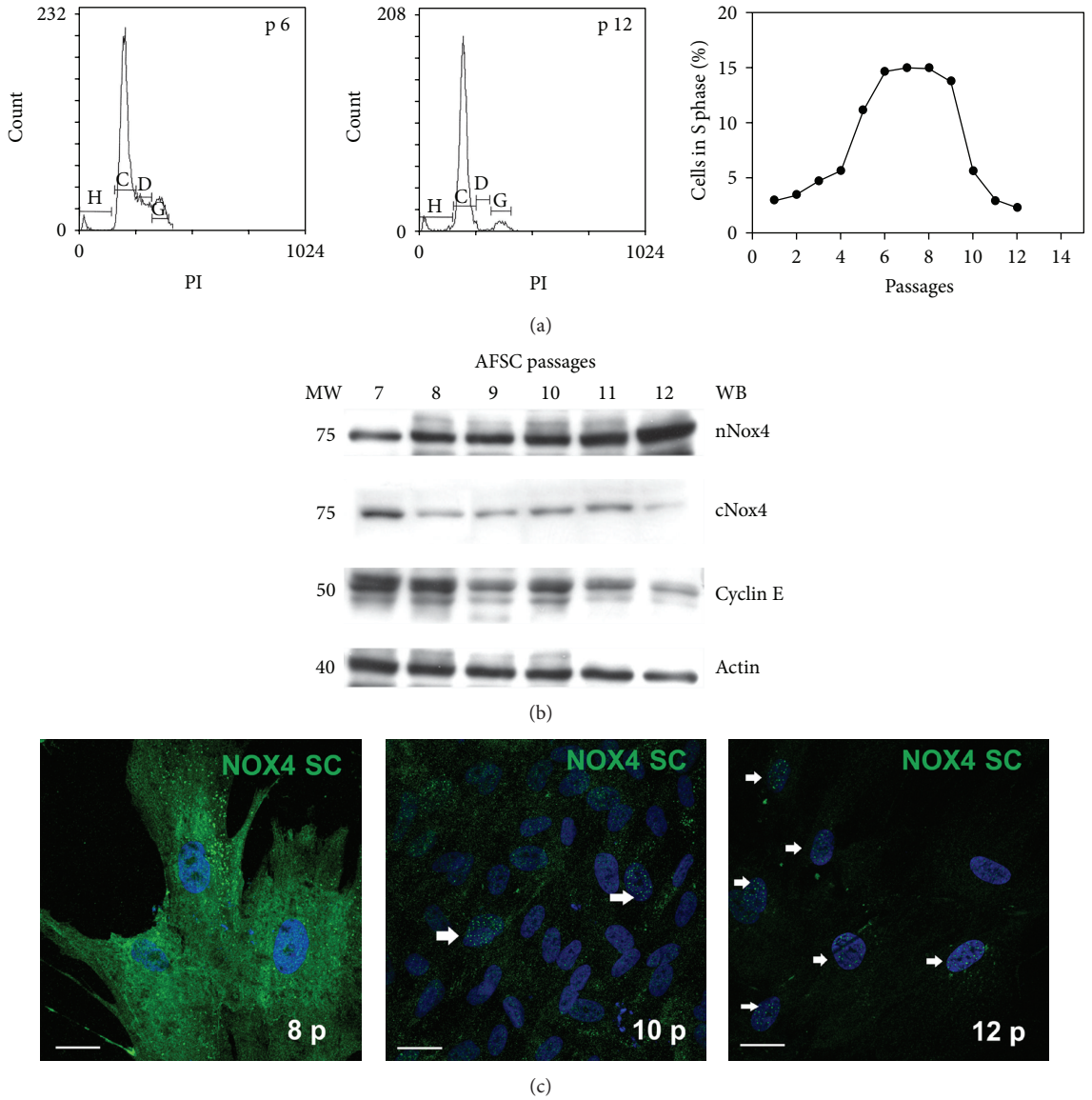

Figure 4: Nox4 localisation during culture passages. (a) Cytofluorimetric analysis with propidium iodide (PI) of AFSC in culture from passage 1 to 12. Representative FACS analysis of cells at passages 6 and 12. The graph shows the percentage of cells on S phase. (b) Representative images of Western blot analysis of cytosol and nuclei of AFSC from passages 7 to 12 and revealed with Nox4 from Novus, Cyclin E, and actin. Presented data are representative of three independent experiments. (c) Representative images showing superimposing between DAPI (blue) and Nox4 SC (green) signals of AFSC cultures at passages 8, 10, and 12. Arrows indicate Nox4 staining in the nuclei. Scale bar: 10 μm.

factors involved in multiple aspects of growth control. Based on these considerations, we investigated the effect of proliferation stimuli on Nox4 localization in AFSC. AFSC are usually grown in the presence of 20% serum in order to induce the best proliferation rate. Serum starvation (−FBS) in fact affects Nox4 distribution within the cell. As demonstrated by WB and IF analysis shown in Figures 3(a) and 3(b), the nuclear portion of Nox4 (nNox4) increases in AFSC cultured without FBS for 24 hours. In fact, confocal images show the Nox4 punctate pattern in the majority of nuclei in−FBS sample. Serum deprivation induces also a decrease in Nox4 staining in the cytosol (Figures 3(a) and 3(b)), compared to +FBS sample.

This observation is consistent with data obtained with the fluorogenic probe assay for nuclear ROS. In fact the number of nuclei strongly stained with the probe is higher in culture −FBS (Figure 3(c)). The nuclear ROS quantification shows that the increase of fluorescence intensity in the absence of FBS is around 50%.

Amniotic fluid stem cells, after a week of slow proliferation in culture, can be passaged at 70% confluence every two to three days. As mesechymal stem cells, they can rapidly react to changes of the culture environment during expansion. In particular, MSC have been proved to gradually become senescent and decrease their proliferative capacity while in culture. The proliferation curve of AFSC in our

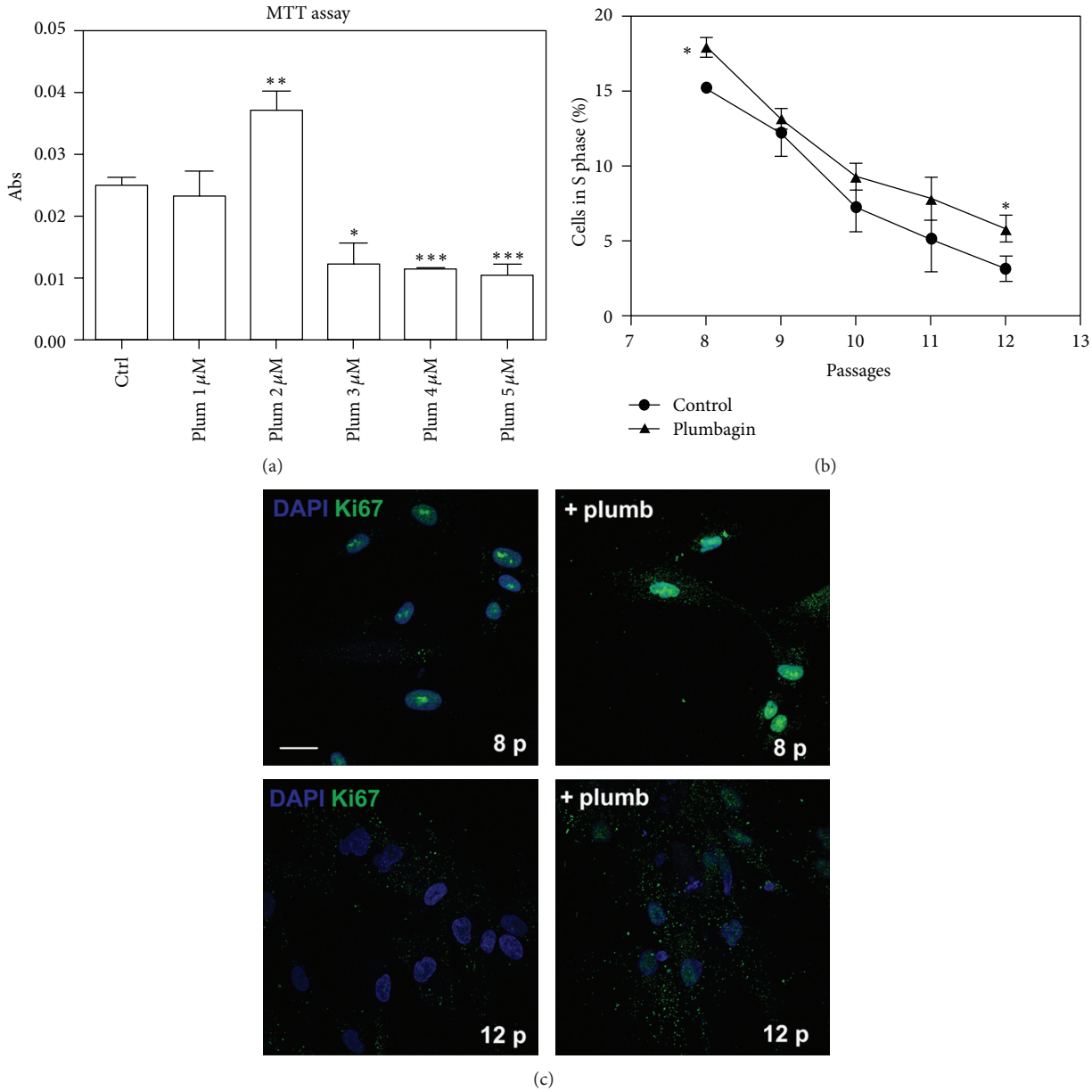

Figure 5: Effect of plumbagin on AFSC proliferation. (a) AFSC (between the 6th and at 8th passages) were incubated for 24 hours with increasing concentration of plumbagin (0–5 μM). Cell viability was determined with the MTT test, as described in the Material and Methods section. $^{***}P < 0.0001$; $^{**}P < 0.01$; $^{*}P < 0.05$ significantly different from control cells. (b) Cytofluorimetric analysis with propidium iodide (PI) of AFSC in culture from passages 8 to 12 in the presence or absence of 2 μM plumbagin. The graph shows the percentage of cells on S phase. Presented data are representative of three independent experiments. $^{*}P < 0.05$ significantly different from control cells. (c) Representative images showing superimposing between DAPI (blue) and Ki67 (green) signals of AFSC cultures at passages 8 and 12 in the presence or absence of 2 μM plumbagin for 24 hours. Scale bar: 10 μm.

experimental condition is shown in Figure 4(a), where the percentage of cells in S phase is graphed. After the first 3-4 passages of slow growth, an increase in proliferation occurs up to a plateau state around the 8th passage; hence, the number of cells able to divide decreases quickly.

Western blot analysis of AFSC during late passages (Figure 4(b)) shows that nuclear Nox4 presence dramatically increases at cell cycle arrest, decreasing the cytosol. Indeed confocal images show the Nox4 punctate pattern in the majority of nuclei in sample at 12th passage. In fact, while Cyclin E expression decreases at 12th passage, indicating a stop in proliferation, Nox4 raises in nuclear fraction. The same trend can be observed by IF analysis, as shown in Figure 4(c).

3.3. Effect of Nox4 Inhibition on AFSC Proliferation. In order to evaluate the effects of Nox4 inhibition on cell viability/proliferation, we used plumbagin, a plant-derived naph-

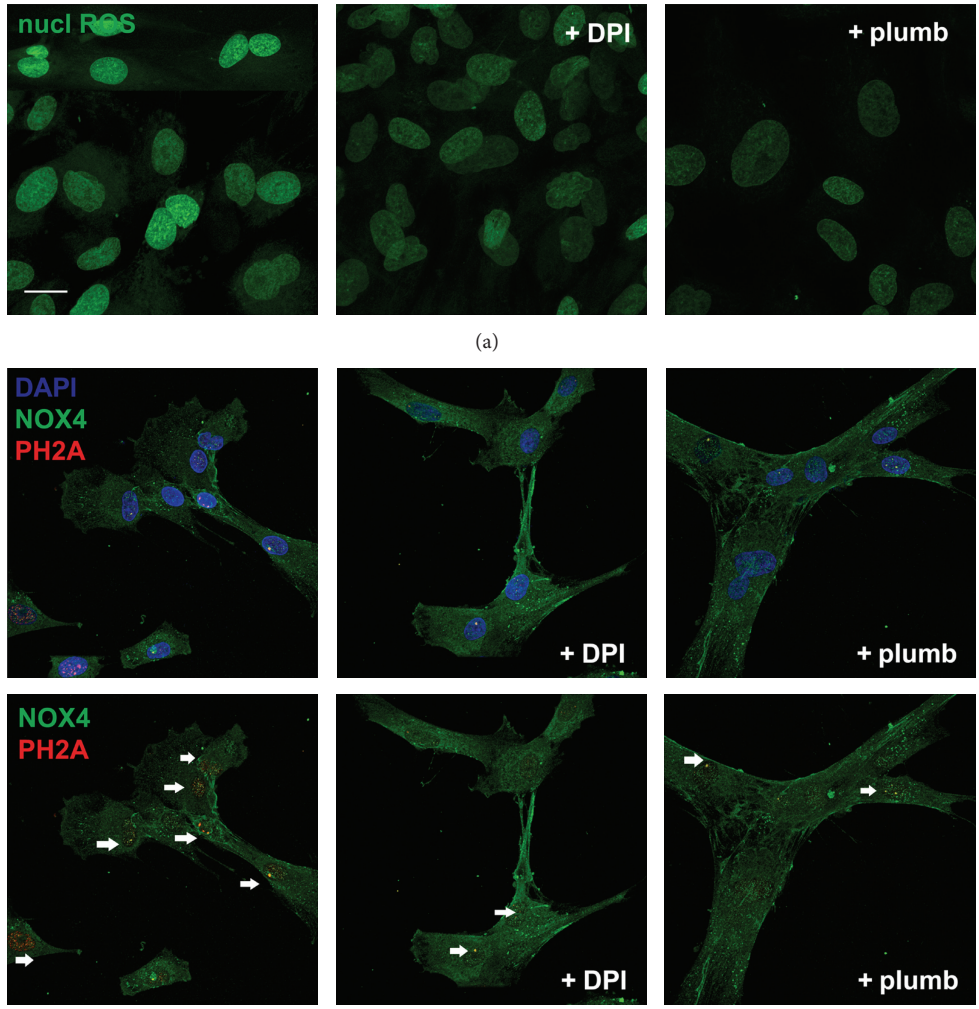

Figure 6: Effect of Nox4 inhibition on nuclear ROS production and DNA damage. (a) Representative images showing staining with nuclear ROS probe (Nuclear peroxy Emerald 1) of AFSC in the presence or absence of 2 μM plumbagin or 1 μM DPI for 24 hours. Scale bar: 10 μm. (b) Representative images showing superimposing between Nox4 SC (green) and PH2A (red) signals with or without DAPI (blue) of AFSC in the presence or absence of 2 μM plumbagin or 1 μM DPI for 24 hours. Arrows indicate the PH2A staining in the nuclei. Scale bar: 10 μm.

thoquinone, directly interacting with Nox4 and inhibiting its activity [31]. MTT test shows that 1-2 μM plumbagin does not affect negatively cell viability, but rather, at 2 μM concentration plumbagin is able to significantly improve cell proliferation. At higher concentrations, plumbagin decreases AFSC viability around 50–60% after 1 day of incubation (Figure 5(a)); therefore, these concentrations were not chosen for the reported experiments.

Moreover, late passages (8–12 p) AFSC cultured in the presence of 2 μM plumbagin display a better proliferation trend, as shown in graph of phase S cells (Figure 5(b)), even if not always in a significant manner. The same indication is provided by IF experiment of Ki-67 staining during culture passages (Figure 5(c)). The expression of the human Ki-67 protein is strictly associated with cell proliferation. The number of nuclei positive for Ki-67 is dramatically reduced in sample of late passage. In the presence of plumbagin, the staining of Ki-67 is higher both at 8th and 12th passages, compared to control samples.

Even if the use of Nox4 synthetic or natural inhibitors, diphenyleneiodonium (DPI) and Plumbagin, is not directed to the nuclear part of Nox4, as demonstrated by the fluorogenic probe assay, the Nox4 activity inhibition reduces the nuclear ROS production (Figure 6(a)). The nuclear ROS quantification shows that the decrease of fluorescence intensity in the presence of DPI or plumbagin is around 50% for both. This effect can regulate cell proliferation also through a modulation of DNA stability. In fact, ROS can also activate both cell survival and senescence pathways depending on its concentration and localization [52–54]. To assess whether nNox4-generated ROS can induce nuclear DNA damage, we studied nuclear H2A foci. It has been shown recently that the status of H2AX phosphorylation is crucial to determine whether cells will survive after DNA damage [55].

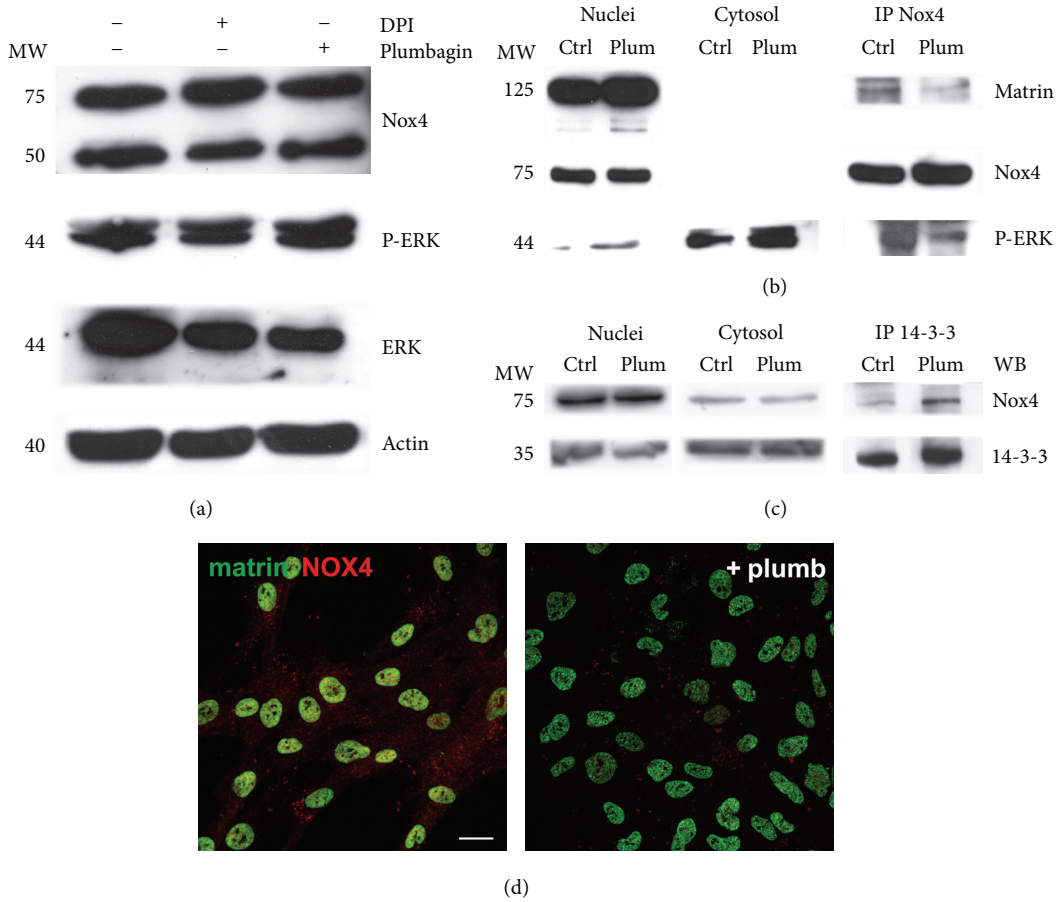

Figure 7: Effect of plumbagin on Nox4 nuclear interaction. (a) Representative images of Western blot analysis of total lysates of AFSC treated with 2 μM plumbagin or 1 μM DPI for 24 hours. Membranes were revealed with anti-Nox4, anti-PERK1/2, and antiactin. (b) Subcellular fractions obtained with the plumbagin treatment were prepared for immunoprecipitation analysis. Nuclear lysate (NL) were immunoprecipitated with Nox4 antibody from Novus then revealed with anti-Matrin, anti-Nox4, and anti-PERK1/2. (c) Nuclear lysate (NL) were immunoprecipitated with anti-14-3-3 then revealed with anti-Nox4 and anti-14-3-3. All presented data are representative of three independent experiments. (d) Representative images showing superimposing between Matrin 3 (green) and Nox4 (red) signals of AFSC in the presence or absence of 2 μM plumbagin for 72 hours. Scale bar: 10 μm.

The ROS production decrease, obtained with synthetic or natural Nox4 inhibitors (DPI and Plumbagin), reduces the staining for the phosphorylated form of histone H2AX, marker of oxidative stress derived-DNA damage (Figure 6(b)).

3.4. Effect of Plumbagin on Nox4 Nuclear Interaction. We investigated the effect of Nox4 inhibition on the previously observed nuclear Nox4 binding network (Figure 2(b)). The Nox4-derived ROS decrease, obtained with DPI or plumbagin incubated for 24 hours, reduces the expression of ERK in total lysates, but the P-ERK level remains unchanged (Figure 7(a)). Coimmunoprecipitation experiment for Nox4 shows that, in the presence of plumbagin, Nox4 binding with P-ERK decreases (Figure 7(b)). This data confirms the link between Nox4 and ERK1/2 within the nucleus, as shown also in VSMC where Nox4 specifically increased nuclear phosphorylated ERK1/2 [27] but emphasizes the direct association and the modulation of this nuclear kinase in the active form by the ROS source, Nox4.

Also the interaction with some nucleoskeleton molecules seems to be modulable by Nox4 inhibition. In fact, CoIP for Nox4 shows a decrease in matrin 3 binding in sample treated with plumbagin (Figure 7(b)). Matrin 3, an abundant protein of the internal nuclear matrix, has been linked to a variety of functional events. While many sites occurred proximal to nucleoli, no significant staining was detected within the nucleolar interior. On the other hand, matrin 3 has been demonstrated to bind DNA at sites termed scaffold/matrix attachment regions to regulate gene expression through interactions with chromatin remodeling. Matrin 3 has been involved also in transcriptome machinery through RNA processing and structural organization [56]. Matrin 3 could be a docking site where nuclear ROS signaling may exert its function on transcription/pre-mRNA modulation in specific nuclear domains.

14-3-3 proteins have the ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, playing important roles in a wide range of vital regulatory processes, such as mitogenic signal transduction and cell cycle control.

There are common themes by which 14-3-3 proteins regulate different signaling pathways. 14-3-3 can control the location of proteins by preventing nuclear import/export or membrane translocation or both. Moreover, 14-3-3 may compete with other signaling proteins for binding on the target protein and may modulate its substrate from ubiquitination and degradation [57].

The effect of Nox4 activity inhibition by plumbagin could cause a Nox4 sequestration by 14-3-3 binding, as shown by CoIP experiment for 14-3-3 (Figure 7(c)). In fact, a longer incubation (72 h) with plumbagin induces a decrease in Nox4 presence inside nuclei, while matrin 3 pattern is unchanged (Figure 7(d)). Indeed, the image shows the double staining for matrin3 (green) and Nox4 (red). In the control case, the superimposing produces an orange staining, indicating the presence of high red signal, while in the sample with plumbagin the superimposing causes only a green staining, due to the anti-Matrin 3 labeling.

4. Conclusions

We observed that Nox4 isoform is expressed in human AFSC and, interestingly, it localizes into the nucleus. Confocal analysis demonstrates Nox4 presence in nucleoplasm domains, not only in nuclear membranes, suggesting that Nox4 could be involved in regulating DNA-mRNA processing machinery by ROS production in specific nuclear area. During culture passages up to cell cycle arrest, AFSC exhibit a proliferation rate inversely coupled with Nox4 presence into the nuclei. Furthermore, the serum starvation causes the same effect. Moreover, immunoprecipitation analysis demonstrated that Nox4 interacts with ERK signaling, suggesting a role in nuclear signaling pathways.

Inhibition of Nox4 activity, obtained with plumbagin, induces a decline of nuclear ROS production and of DNA damage. Moreover, plumbagin exposure reduces the binding between nNox4 nucleoskeleton components. The same effect was observed also for the binding with phospho-ERK, although nuclear ERK and P-ERK are unchanged. A longer incubation with plumbagin may modulate Nox4 nuclear expression, by controlling the protein localization or/and a degradation pathway involvement. Taken together, we suggest that nNox4 regulation may have important pathophysiologic effects in stem cell proliferation through modulation of nuclear signaling and DNA damage.

Abbreviations

AFSC: Amniotic fluid stem cells
BSA: Bovine serum albumin
DABCO: 1,4-Diazabicyclo(2.2.2)octane
DAPI: 4′,6-Diamidino-2-phenylindole
DPI: Diphenyleneiodonium
EDTA: Ethylenediaminetetraacetic acid
MSCs: Mesenchymal stem cells
PBS: Phosphate buffered saline
TBS: Tris-buffered saline
TxTBS: Triton-X-100.

Conflict of Interests

The authors report no conflict of interests.

Authors' Contribution

Marianna Guida prepared *in vitro* culture and carried out almost all the experiment; Tullia Maraldi designed the experiment, carried out experiments, and drafted the paper; Elisa Resca participated in IF experiments; Francesca Beretti carried out IP experiments; Manuela Zavatti carried out statistical analysis; Laura Bertoni participated in the analysis of all the experiments; Giovanni B. La Sala collected amniotic fluids and the informed consents of patients; Anto De Pol participated in drafting the manuscript.

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Research Article

Moringa oleifera Mitigates Memory Impairment and Neurodegeneration in Animal Model of Age-Related Dementia

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To date, the preventive strategy against dementia is still essential due to the rapid growth of its prevalence and the limited therapeutic efficacy. Based on the crucial role of oxidative stress in age-related dementia and the antioxidant and nootropic activities of *Moringa oleifera*, the enhancement of spatial memory and neuroprotection of *M. oleifera* leaves extract in animal model of age-related dementia was determined. The possible underlying mechanism was also investigated. Male Wistar rats, weighing 180–220 g, were orally given *M. oleifera* leaves extract at doses of 100, 200, and 400 mg/kg at a period of 7 days before and 7 days after the intracerebroventricular administration of AF64A bilaterally. Then, they were assessed memory, neuron density, MDA level, and the activities of SOD, CAT, GSH-Px, and AChE in hippocampus. The results showed that the extract improved spatial memory and neurodegeneration in CA1, CA2, CA3, and dentate gyrus of hippocampus together with the decreased MDA level and AChE activity but increased SOD and CAT activities. Therefore, our data suggest that *M. oleifera* leaves extract is the potential cognitive enhancer and neuroprotectant. The possible mechanism might occur partly via the decreased oxidative stress and the enhanced cholinergic function. However, further explorations concerning active ingredient(s) are still required.

1. Background

Dementia, a serious loss of global cognitive ability including the impairments of memory, attention, language, and problem solving, is continually growing worldwide accompanied with the increased elderly population. It has been estimated that there are approximately 35.6 million people with dementia worldwide [1]. Due to the rapidly growth of prevalence, high expenditure cost, and unsatisfactory outcomes of therapeutic strategy, dementia has been recognized as one of the major medical and social challenges especially in developing countries [2].

Recent findings have shown that the age-related cognitive dysfunction occurs as a result of oxidative stress elevation in the brain [3], hippocampal atrophy [4], and the disturbances of neurotransmission, especially cholinergic transmission. Therefore, the modulation of cholinergic function becomes the approach to dementia treatment. However, most drugs still induce adverse effects [5]. This disadvantage consequently motivates research effort to find out novel protective agent against dementia.

Herbal medicine has long been used to treat numerous ailments. Moreover, the "Green" movement has driven the attitude changes of the general population to prefer naturally derived substances and extracts as being inherently safer and more desirable than synthetic chemical products. Accumulative lines of evidence have demonstrated that consumption of antioxidant-rich foods and polyphenol treatment can enhance cognitive performance in elderly subjects [6–8].

Moringa oleifera, a plant in the family of Moringaceae, is an edible plant which has been used both as food and as medicine in many Asian countries including Thailand for

centuries. The leaves of this plant have been reported to be a rich source of potassium, calcium, phosphorous, iron, vitamins A and D, essential amino acids, and antioxidants such as β-carotene, vitamin C, and flavonoids [9–13]. In addition, antinutrients such as alkaloids, tannins, phenolics, saponins, and steroids were also observed [14]. The leaves extract also exhibits antioxidant activity [15]. Recently, it has been shown that *M. oleifera* leaves extract at doses more than 3000 mg/kg show genotoxicity effect. In addition, LD50 of the alcoholic extract of *M. oleifera* leaves is reported to be more than 2800 mg/kg. [16]. Therefore, the leaves extract intake is safe at dose ≤ 1000 mg kg-1 BW [17]. The extract also possesses antioxidant and nootropic effects. Moreover, it has been reported to combat oxidative stress in rat model of Alzheimer's disease induced by colchicines such as vitamin C and vitamin E [18, 19]. However, the scientific evidence concerning the effect of *M. oleifera* leaves extract on cognitive dysfunction induced by hypocholinergic function, the important cause of memory impairment in dementia, is limited until now. Therefore, this study aimed to investigate the memory enhancing effect, neuroprotective effect, and possible underlying mechanism of *M. oleifera* leaves extract in animal model of dementia induced by AF64A, a cholinotoxin.

2. Materials and Methods

2.1. Plant Materials and Preparation. Fresh leaves of *M. oleifera* were collected during November–December 2010 from Khon Kaen province, Thailand. After the authentication, the herbarium specimen was kept at Integrative Complementary Alternative Medicine, Khon Kaen University (voucher specimen 2010001). The fresh leaves were immediately cleaned, cut into small pieces, and dried in oven at 40°C. The dried plant material was ground into powder and extracted with 50% hydroalcohol (50% water : 50% alcohol) in a flat bottom flask at room temperature and allowed to stand for several days with occasional shaking. When the solvent becomes concentrated, the content is then filtered through Whatman number 1 filter paper and then concentrated with rotator evaporator at 45°C, dried, and kept at 4°C till used for further study. The yielded extract was 17.49% and contained total phenolic compounds and flavonoids at concentrations of 62 ± 0.08 mg of GAE·g^{-1} extract (milligram of Gallic acid equivalents) and 29.9 ± 0.02 mg QE/g extract (milligrams of Quercetin), respectively.

2.2. Animals. Male Wistar rats, weighing 180–220 g, were used as experimental animals. They were obtained from National Animal Center, Salaya. They were housed 6 per cage and maintained in 12 : 12 light : dark cycle and given access to food and water ad libitum. The experiments were performed to minimize animal suffering and the experimental protocols were approved by the Institutional Animal Care and Use Committee, Khon Kaen University, Thailand (AEKKU 41/2554).

2.3. Surgical Procedures. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital at dose of 60 mg/kg BW. Then, AF64A (2 nmol/2 μL) was infused bilaterally via intracerebroventricular (i.c.v.) route with a 30-gauge needle inserted through a burr hole drilled into the skull into both the right and left lateral ventricles. Stereotaxic coordinates were measured (from the bregma): posterior 0.8 mm, lateral ±1.5 mm, and ventral (from dura) 3.6 mm. The rate of infusion is 1.0 μL/min. The needle were left in place for 5 min after infusion and then slowly withdrawn [20].

2.4. AF64A Administration. AF64A was prepared as described previously [20, 21]. Briefly, an aqueous solution of acetylethylcholine mustard HCl (Sigma, St. Louis, MO) was adjusted to pH 11.3 with NaOH. After stirring for 30 min at room temperature, the pH was lowered to 7.4 with the gradual addition of HCl and stirred for 60 min. The amount of AF64A was then adjusted to 2 nmol/2 μL. The vehicle of AF64A was distilled water prepared in the same manner as the AF64A and recognized as artificial cerebrospinal fluid (ACSF) [20, 21].

2.5. Experimental Protocol. All rats were randomly assigned to 7 groups of 6 animals each as follows: (1) vehicle + ACSF: rats received vehicle via oral route and received artificial cerebrospinal fluid (ACSF) via intracerebroventricular (i.c.v.) route bilaterally; (2) Vehicle + AF64A: rats received distilled water (vehicle) via oral route and received AF64A, a cholinotoxin, via the intracerebroventricular route bilaterally; (3) Donepezil + AF64A: rats had been given Donepezil (1 mg/kg BW) via oral route 7 days prior to the administration of AF64A bilaterally via intracerebroventricular route and 7 days after AF64A administration via the intracerebroventricular route bilaterally; (4) Vitamin C + AF64A: rats received Vitamin C (250 mg/kg BW) at a period of 7 days before and 7 days after AF64A administration via the intracerebroventricular route bilaterally; (5)–(7) *M. oleifera* 100, 200, and 400 mg/kg: rats in these groups were given the extract at one of the following doses of 100, 200, and 400 mg/kg via oral route 7 days before AF64A administration bilaterally via intracerebroventricular route and the extract was continually administered for 7 days after AF64A administration. All rats were determined spatial memory using Morris water maze test at the end of experiment. Then, they were sacrificed and brains were isolated to determine the neurons density in various sub regions of hippocampus. In addition, brain oxidative stress markers and the suppression activities of acetylcholinesterase (AChE) in hippocampus were also evaluated as shown in Figure 1.

2.6. Determination of Spatial Memory. Spatial memory was evaluated via the Morris water maze. The water maze consists of a metal pool (170 cm in diameter × 58 cm tall) filled with tap water (25°C, 40 cm deep). The pool was divided into 4 quadrants (Northeast, Southeast, Southwest, and Northwest). The water surface was covered with nontoxic milk. The removable platform was placed below the water level at the center of one quadrant. For each animal, the location

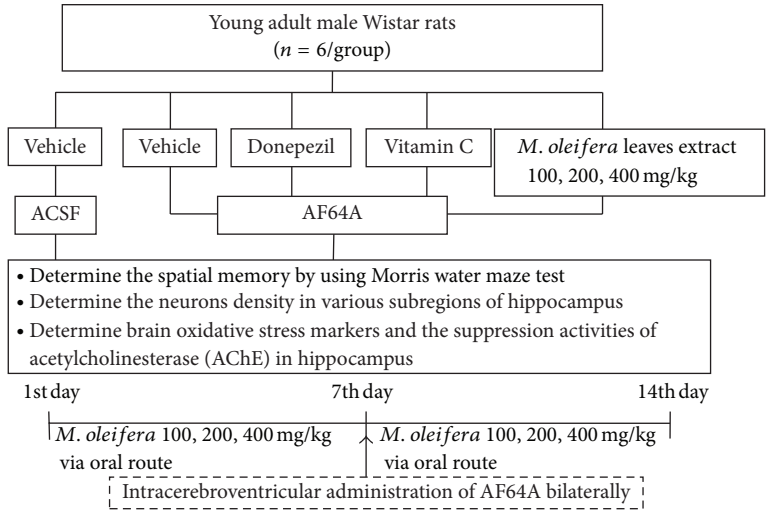

Figure 1: Schematic diagram shows the experimental protocol.

of the invisible platform was placed at the center of one quadrant and remained there throughout training. The times for animals to climb on the hidden platform were recorded as escape latency. In order to determine the capability of the animals to retrieve and retain information, the platform was removed 24 hr later and the rats were released into the quadrant diagonally opposite to that which contained the platform. Time spent in the region that previously contained the platform was recorded as retention time [22].

2.7. Histological Procedure. After the anesthesia with sodium pentobarbital (60 mg/kg BW), brains were subjected to transcardial perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. After the perfusion, brains were removed and stored over a night in a fixative solution used for perfusion. Then, they were infiltrated with 30% sucrose solution at 4°C. The specimens were frozen rapidly and 30 μM thick sections were cut on cryostat. The selected sections were rinsed in the phosphate buffer and picked up on slides coated with 0.01% of aqueous solution of a high molecular weight poly-L-lysine.

2.8. Morphological Analysis. Five coronal sections of each rat in each group were studied quantitatively. Neuronal counts in hippocampus were performed by eye using a 40x magnification with final field 255 μm^2 according to the following stereotaxic coordinates: AP −4.8 mm, lateral ±2.4–6 mm, and depth 3–8 mm. The observer was blind to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give total number of neurons per 255 μm^2. All data are represented as number of neurons per 255 μm^2.

2.9. Determination of Malondialdehyde Level and Acetylcholinesterase Activity. Hippocampus was isolated and prepared as hippocampal homogenate and the determination of the malondialdehyde (MDA) level and acetylcholinesterase (AChE) activity in hippocampus were performed. Malondialdehyde was indirectly estimated by determining the accumulation of thiobarbituric acid reactive substances (TBARS) [23] while the activity of AChE was determined using Ellman method [24].

2.10. Determination of Scavenging Enzymes Activities. In order to determine the activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), the brains tissues were weighed and homogenized with a buffer consisting of 10 mM sucrose, 10 mM Tris-HCl, and 0.1 mM EDTA (pH 7.4). Then, the brain homogenates were centrifuged at 3000 g for 15 min at 4°C. The supernatant was used for bioassays. The activity of SOD was determined using a xanthine/xanthine oxidase system for the production of superoxide radical and subsequent measurement of cytochrome *c* as a scavenger of the radicals. Optical density was determined using a spectrometer (UV-1601, Shimadzu) at 550 nm [25]. One unit of enzyme activity was defined as the quantity of SOD required to inhibit the reduction rate of cytochrome *c* by 50%. SOD activity was presented as units per milligram of protein (U mg^{-1} protein). CAT activity in the supernatant was measured by recording the reduction rate of H_2O_2 absorbance at 240 nm [26]. The activity of CAT was expressed as μmol H_2O_2/min/mg protein. GSH-Px was determined using *t*-butyl hydro peroxide as a substrate. The optical density was spectrophotometrically recorded at 340 nm [27]. One unit of the enzyme was defined as micromole (μmol) of reduced nicotinamide adeninedinucleotide phosphate (NADPH) oxidized per minute. GSH-Px activity was expressed as U/mg protein.

2.11. Statistic Analysis. Data were expressed as means ± S.E.M. and analyzed statistically by one-way ANOVA, followed by post hoc (LSD) test. The results were considered statistically significant at P-value < .05.

3. Results

3.1. Effect of M. oleifera Leaves Extract on Spatial Memory. Based on the crucial role of cholinergic function and hippocampus on learning and memory mentioned earlier, we have induced memory impairment as that observed in dementia by using the bilateral administration of AF64A, a cholinotoxin, into lateral ventricle via intracerebroventricular route in order to induce cholinergic damage in the area around lateral ventricle especially hippocampus which in turn induces spatial memory impairment. In this study, we evaluated the spatial memory by using escape latency and retention time in Morris water maze as indices. The results were shown in Figure 2. It was found that AF64A administration significantly increased the escape latency but decreased retention time (P-value < .001 compared to vehicle + ACSF group). Both Donepezil and Vitamin C treatments significantly mitigated the enhanced escape latency (P-value < .01 and .001, respectively, compared to vehicle + AF64A group) and the decreased retention time (P < .01 all compared to vehicle + AF64A group) induced by AF64A. All doses of *M. oleifera* leaves extract also significantly mitigated the enhanced escape latency (P-value < .001 compared to vehicle + AF64A group) and the decreased retention time induced by AF64A (P-value < .001 compared to vehicle + AF64A group).

3.2. Effect of M. oleifera Leaves Extract on Hippocampal Neurodegeneration. Since memory impairment is associated with the neurodegeneration in hippocampus [28, 29], we also determined the effect of *M. oleifera* leaves extract on neurons density in various sub-regions of hippocampus. The results were shown in Figure 3. It was found that AF64A significantly decreased neurons density in CA1, CA2, CA3, and dentate gyrus (P-value < .001 all; compared to vehicle + ACSF group). Both Donepezil and Vitamin C could mitigate the decreased neurons density in all areas mentioned earlier (P-value < .001 all compared to vehicle + AF64A group). The crude extract of *M. oleifera* at high concentration (400 mg kg^{-1} resp.) significantly attenuated the reduction of neurons density in CA1, CA2, CA3 and dentate gyrus (P-value < .05, .05, .01 and .05 respectively; compared to vehicle + AF64A group) while the medium concentration (200 mg kg^{-1}) produced a significant attenuation effect on the decreased neurons density in CA1, CA3 and dentate gyrus (P-value < .05 all compared to vehicle + AF64A group) and low dose concentration produced an attenuation effect on the decreased neurons density in CA2, CA3, and dentate gyrus (P-value < .05, .01, and .05, respectively, compared to vehicle + AF64A group).

3.3. Effect of M. oleifera Leaves Extraction Oxidative Stress Markers and AChE Enzyme Activity. Based on the crucial role of oxidative stress and cholinergic system function on memory impairment previously mentioned, this part of study was focused on the effect of *M. oleifera* on oxidative stress markers including MDA level and the activities of scavenger enzymes including SOD, CAT, and GSH-Px and on the activity of AChE, an indirect indicator reflecting the available acetylcholine, in hippocampus. The results were shown in Figures 4 and 5. It was demonstrated that AF64A injection significantly increased MDA level as shown in Figure 4 (P < .05 compared to ACSF + vehicle group) but decreased the activities of SOD (P < .05 compared to vehicle + ACSF) and CAT (P < .001 compared to vehicle + ACSF group) as shown in Figure 5. Interestingly, the elevation of MDA level in hippocampus was mitigated by Donepezil, Vitamin C, and all doses of *M. oleifera* leaves extract (P < .01 all compared to vehicle + ACSF group).

We also determined the effect of *M. oleifera* extract on the activities of main scavenger enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in hippocampus. Figure 5 showed that AF64A significantly decreased SOD and CAT activities whereas no change of GSH-Px activity was observed (P-value < .05 and .001, respectively, compared to vehicle + ACSF group). Vitamin C treatment could attenuate the reduction of SOD and CAT activities induced by AF64A (P-value < .05 all compared to vehicle + AF64A group). High dose of *M. oleifera* significantly attenuated the decreased activities of SOD and CAT induced by AF64A (P-value < .05 all compared to vehicle + AF64A group) whereas the low dose of extract could only produce a significant modulation effect to attenuate the decreased of SOD activity in the mentioned area. No other significant changes were observed as shown in Figure 5.

The effect of *M. oleifera* leaves extract on the activity of AChE in hippocampus was also investigated. The results were

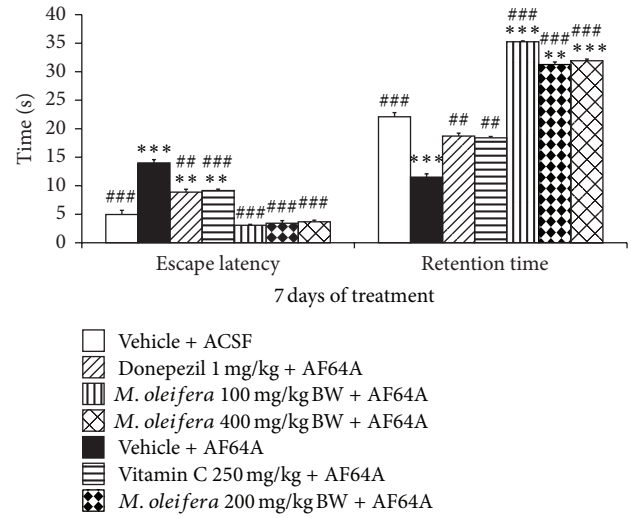

Figure 2: Effect of *M. oleifera* leaves extract on escape latency and retention time of memory deficit rats induced by AF64A, a cholinotoxin, in Morris water maze test. Each column and bar represent mean ± S.E.M. (n = 6/group). $^{\#\#}P$ value< .01, $^{\#\#\#}P$ value< .001 compared to vehicle + AF64A. $^{**}P$ value < .01, $^{***}P$ value < .001 compared to vehicle + ACSF.

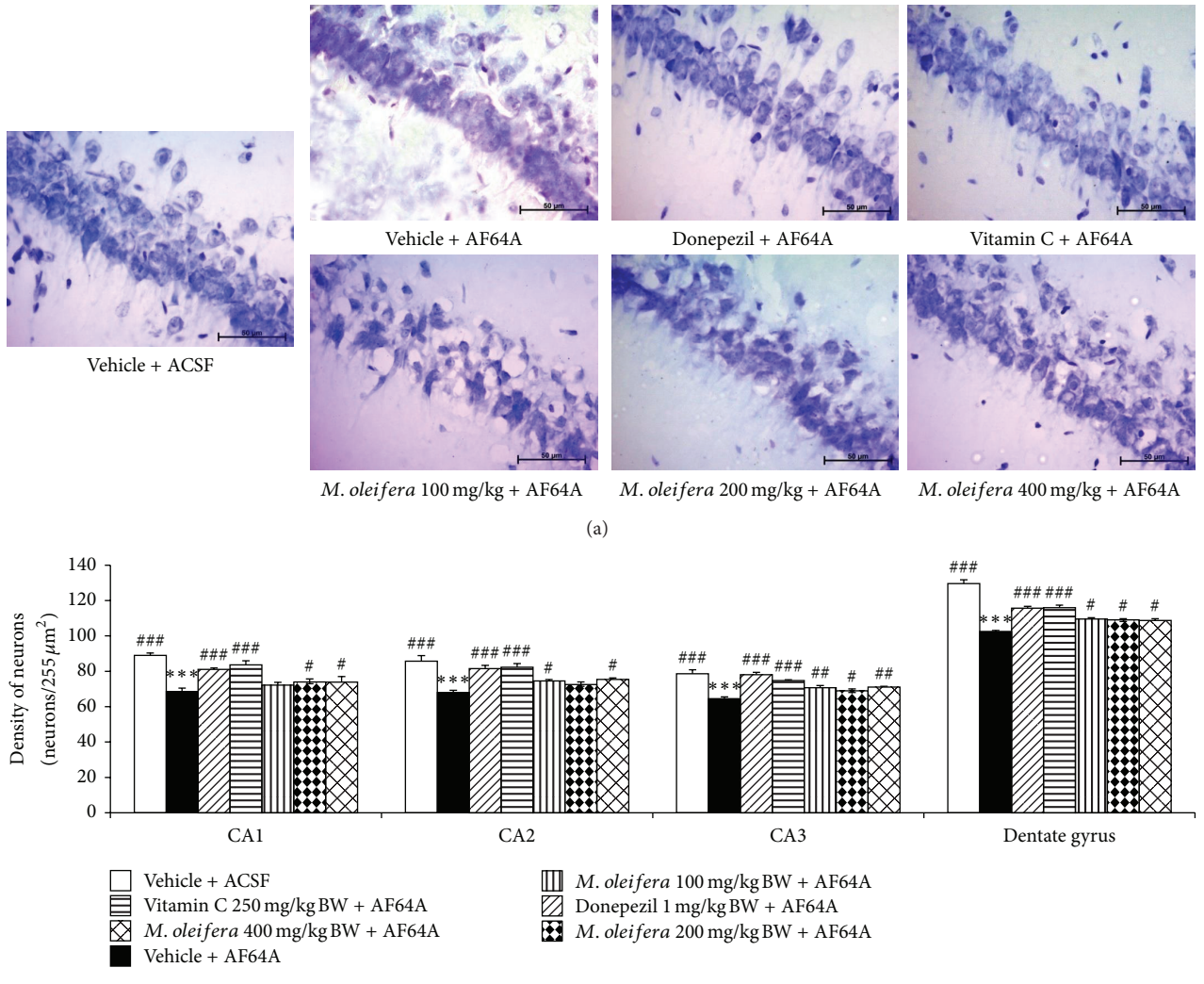

FIGURE 3: Effect of *M. oleifera* leaves extract on neurons density in various subregions of hippocampus of memory deficits rats induced by AF64A. (a) Image of neurons in CA1 of hippocampus stained with cresyl violet. (b) Neurons density in various sub-regions of hippocampus after various treatments including vehicle, Donepezil, Vitamin C, and various *M. oleifera* leaves extract at doses of 100, 200, and 400 mg·kg^{-1} BW. The column and bar represent mean ± S.E.M. (n = 6/group) $^{***}P < .001$ compared to vehicle + ACSF group; $^{\#}$, $^{\#\#}$, $^{\#\#\#}P < .05$, .01, and .001, respectively, compared to vehicle + AF64A group.

shown in Figure 4. It was found that AF64A enhanced AChE but it failed to show the significant effect. *M. oleifera* leaves extract at doses of 100 and 200 mg·kg^{-1} BW significantly decreased AChE activity in hippocampus ($P < .001$ and .01, resp. compared to vehicle + AF64A group).

4. Discussion

The current study has investigated the effect of *M. oleifera* leaves extract on spatial memory and on the neurodegeneration, oxidative stress markers, and the alteration of AChE activity in hippocampus. The results clearly demonstrated that *M. oleifera* leaves extract significantly improved spatial memory and decreased neurodegeneration in CA1, CA2, CA3, and dentate gyrus of hippocampus together with the decreased MDA level but increased SOD, CAT, and AChE activities.

Recent studies have demonstrated that dorsal hippocampus provides animals with a spatial map of their environment [30]. It makes use of reference and working memory and has an important role in information processing which involves spatial locations [31]. Lesion in this region results in problems concerning goal-directed navigation and impairs the ability to remember precise locations [32]. Various subregions of hippocampus play different roles in spatial memory. It has been demonstrated that lesion to the ventral hippocampus produces no effect in spatial memory and the dorsal hippocampus plays essential role in retrieval, processing short-term memory and transferring memory from the short term to longer delay periods [33–35]. The memory encoding process of spatial memory is associated with the major

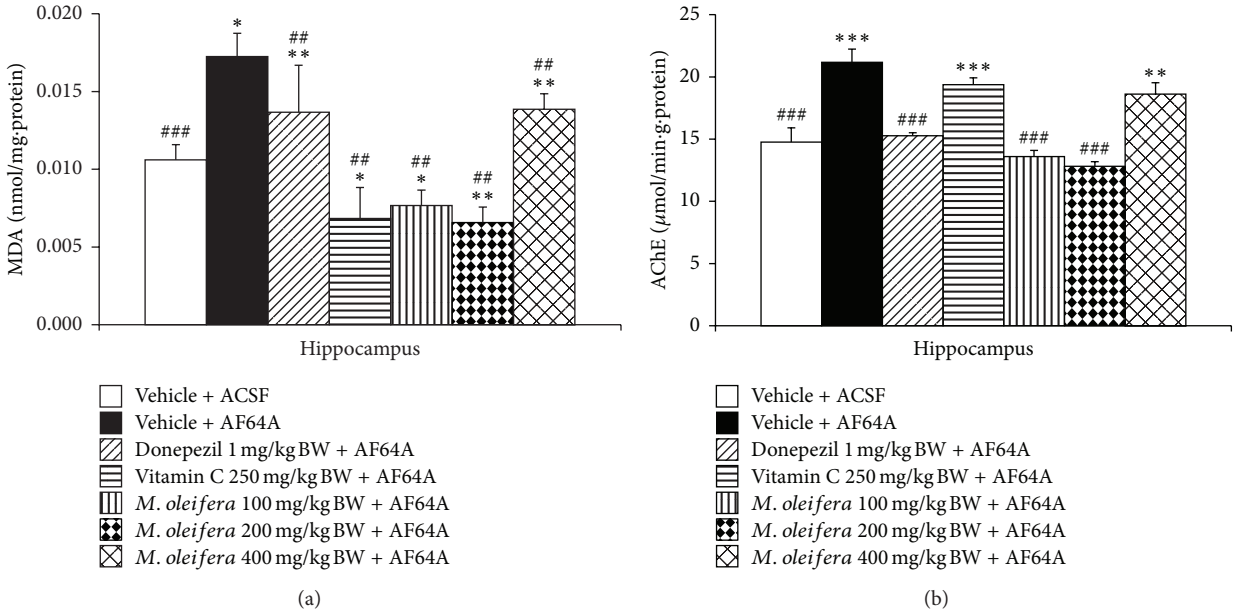

Figure 4: Effect of *M. oleifera* leaves extract on the level of malondialdehyde (MDA), a lipid peroxidation product, and the activity of acetylcholinesterase AChE enzyme in hippocampus. (a) Effect of *M. oleifera* leaves extract on malondialdehyde (MDA) level. (b) Effect of *M. oleifera* leaves extract on the activity of AChE. The column and bar represent mean ± S.E.M. (n = 6/group). *, **, $^{***}P < .05$, .01, and .001, respectively, compared to ACSF group. $^{\#\#}$, $^{\#\#\#}P < .01$ and .001, respectively, compared to vehicle + AF64A group.

hippocampal subregions, CA1, CA3, and dentate gyrus (DG), especially in dorsal hippocampus [36], whereas the recall process is associated with CA3 [37, 38]. Unfortunately, the role of CA2 is still unclearly understood.

The data obtained from this study showed that *M. oleifera* treated groups showed the decreased escape latency but increased retention time. The changes were higher than those observed in Vitamin C and Donepezil treated groups. The possible explanation might be related to the multitarget sites of action of *M. oleifera*. Numerous factors are contributing to the important roles in memory retention. Besides cholinergic function and the density of neuronal cells which contribute to the crucial role in memory retention circuit in cerebral cortex, the increased cerebral blood flow and the increased dopaminergic function also contribute to the role in memory retention [39–41]. It was found that *M. oleifera* did not only suppress acetylcholinesterase (AChE) activity but also increased neurons density. In addition, *M. oleifera* also exhibits vasodilation effect [42, 43] and modulates the function of monoamines transmitters such as dopamine [43, 44]. Since *M. oleifera* exerts its effect on multi-target sites which contribute to the crucial role in memory retention, it could exert its influence more than Donepezil or Vitamin C treatments.

Recently, it has been reported that the neurodegeneration of hippocampal neurons is under the influence of oxidative stress [45, 46], calcium homeostasis disturbance [47], apoptosis [48], and the decreased vascular supply [49]. Since the interaction between various constituents in *M. oleifera* leaves extract could modify the bioavailability and signal transduction pathway of active substances in the extract, no dose dependent response manners of neuronal density changes, oxidative stress markers, and AChE activity were observed in hippocampus.

This study demonstrated that the memory enhancing effect of *M. oleifera* leaves extract might occur partly via the decreased oxidative stress and the enhanced cholinergic function. These effects have been shown in Figure 6. However, other mechanisms concerning the vasodilation effect [42] which in turn increased regional blood flow and the suppression of monoamine oxidase (MAO) which gave rise to the enhanced dopaminergic function [43] induced by *M. oleifera* leaves extract might also play the pivotal role in the cognitive enhancing effect of *M. oleifera* leaves extract. Based on the previous finding that the neuronal dysfunctions and neurodegeneration could be improved by flavonoids [50], we did suggest that the neuroprotective and cognitive enhancing effects of *M. oleifera* leaves extract might occur partly via the flavonoids in the extract. However, this still requires further investigation.

5. Conclusion

The current results suggest that *M. oleifera* leaves extract possesses the neuroprotective and memory enhancing effects. The possible underlying mechanisms appear to depend on application doses. Low and medium doses seem to provide beneficial effects just mentioned via the decreased oxidative stress and the suppression of AChE activity whereas high dose of extract appears to induce the beneficial effects primarily via the decreased oxidative stress. Since the effective doses are very much less than LD50, *M. oleifera* leaves extract may be served as the potential medicinal food against dementia. However, further explorations concerning active

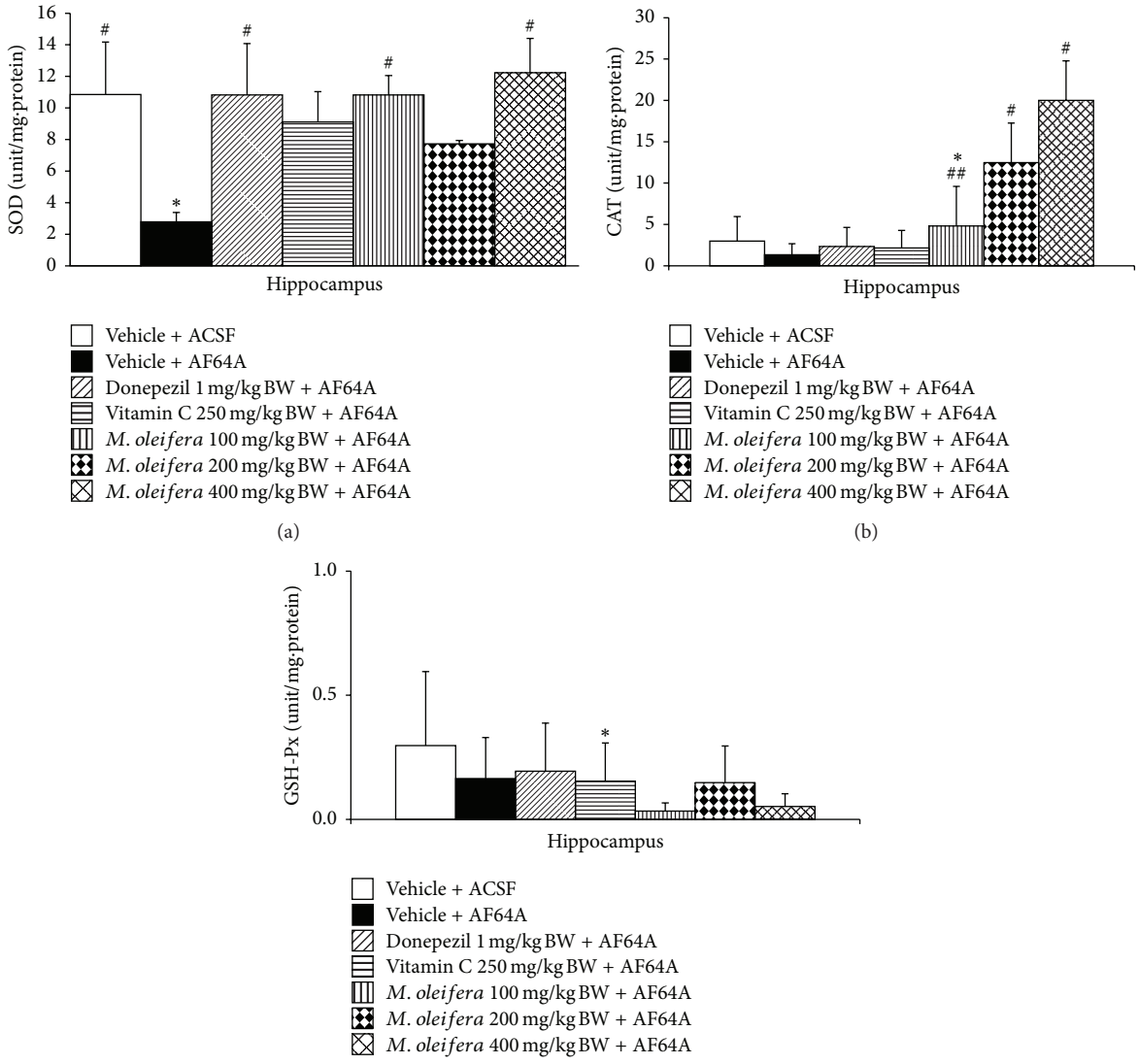

Figure 5: Effect of *M. oleifera* leaves extract on the activities of scavenger enzymes in hippocampus. (a) Effect of *M. oleifera* leaves extract on superoxide dismutase (SOD) activity. (b) Effect of *M. oleifera* leaves extract on catalase (CAT) activity. (c) Effect of *M. oleifera* leaves extract on glutathione peroxidase (GSH-Px) activity. The column and bar represent mean ± S.E.M. (n = 6/group). *, **, $^{***}P < .05$, .01, and .001, respectively, compared to ACSF group; $^{\#}$, $^{\#\#}P < .05$ and .01, respectively, compared to vehicle + AF64A group.

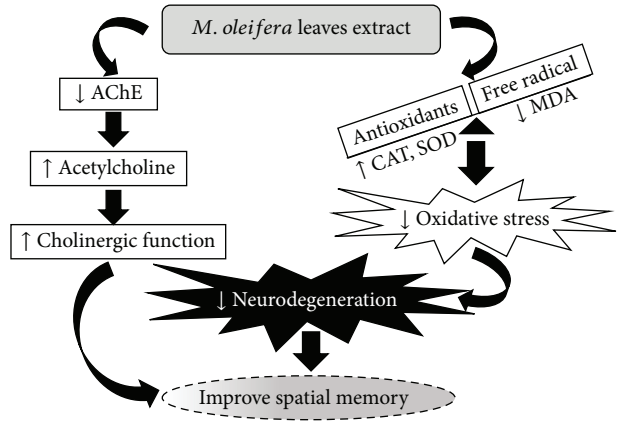

Figure 6: Schematic diagram shows the possible underlying mechanism of memory enhancing effect of *M. oleifera* leaves extract.

ingredient(s) and the detail of underlying mechanism of the extract are still required.

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Conflict of Interests

The authors declare they have no conflict of interests.

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Research Article

Cerebroprotective Effect of *Moringa oleifera* against Focal Ischemic Stroke Induced by Middle Cerebral Artery Occlusion

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The protection against ischemic stroke is still required due to the limitation of therapeutic efficacy. Based on the role of oxidative stress in stroke pathophysiology, we determined whether *Moringa oleifera*, a plant possessing potent antioxidant activity, protected against brain damage and oxidative stress in animal model of focal stroke. *M. oleifera* leaves extract at doses of 100, 200 and 400 mg·kg^{-1} was orally given to male Wistar rats (300–350 g) once daily at a period of 2 weeks before the occlusion of right middle cerebral artery (Rt.MCAO) and 3 weeks after Rt.MCAO. The determinations of neurological score and temperature sensation were performed every 7 days throughout the study period, while the determinations of brain infarction volume, MDA level, and the activities of SOD, CAT, and GSH-Px were performed 24 hr after Rt.MCAO. The results showed that all doses of extract decreased infarction volume in both cortex and subcortex. The protective effect of medium and low doses of extract in all areas occurred mainly via the decreased oxidative stress. The protective effect of the high dose extract in striatum and hippocampus occurred via the same mechanism, whereas other mechanisms might play a crucial role in cortex. The detailed mechanism required further exploration.

1. Introduction

Stroke, the major cause of death and disability, is regarded as the important problem in developing countries [1]. Despite the importance of stroke and the advances of technologies nowadays, clinical therapy of the deliberating disorder is still not in the satisfaction level. Therefore, the prophylactic protection against stroke with neuroprotective agent has gained much attention.

Cerebral ischemia is characterized by a rapid onset of neurological injury due to interruption of blood flow to the brain [2]. This injury has been reported to be associated with the action and interaction of many factors such as excitatory amino acids, calcium overloading, oxidative stress damage, periphery depolarization of infarction, neuroinflammation, and apoptosis [3–5]. However, accumulative lines of evidence in this decade point out to the crucial role of oxidative stress. It has been reported that the reduction of cerebral blood flow and the reperfusion period induce the elevation of oxidative stress and lipid peroxidation [6–9]. Interestingly, both in vitro and in vivo data have demonstrated that this injury can be protected by polyphenolics including flavonoids [7, 8, 10–13].

Moringa oleifera Lam. or Marum or Drumstick, a member of Moringaceae family, is widely cultivated in Asia, Polynesia, and the West Indies. In Thailand, leaves of *Moringa oleifera* have been consumed as vegetables for more than 100 years. *M. oleifera* leaves can also serve as a rich source of substance possessing antioxidant activity such as betacarotene,

vitamin C, vitamin E, and polyphenolics [14, 15]. Many reports have described the potential therapeutic values of *M. oleifera* including anticancer, antidiabetes, anti-rheumatoid arthritis, anti-fungal, anti-microbial [16], anti-atherosclerotic [17], antifertility, pain relief, depressant [18], diuretic and thyroid regulation effects [19]. Recent findings have shown that the leaves extract also exhibits antioxidant effect and can protect against oxidative damage [20, 21]. In addition, it has been reported that LD_{50} of alcoholic extract of *M. oleifera* leaf is approximately 2.8 g·kg^{-1} BW [22], and this information suggests that it is quite safe even when consumed in a higher quantity due to its high LD_{50}. Based on the crucial role of oxidative stress on the pathophysiology of cerebral ischemia and antioxidant effect of *M. oleifera* leaves, the cerebroprotective effect of *M. oleifera* leaves extract against focal ischemic stroke has been focused on. Since no scientific evidence concerning this issue was available, this study was carried out to determine the cerebroprotective effect of the mentioned extract against brain damage and oxidative stress in animal model of focal ischemic stroke.

2. Materials and Methods

2.1. Plant Material Preparation. The fresh *Moringa oleifera* Lam (Moringaceae) leaves were harvested during November to December, 2010, with the permission from the owners of the land, Mr. Chalerm Pattum, Mr. Padungkiet Jutakanchana, and Mrs. Oranuch Boonlue, in Khon Kaen province, Thailand. The plant specimen was authenticated by Associate Professor Dr. Panee Sirisa-ard, Faculty of Pharmacy, Chiangmai University, Thailand. The voucher specimen was kept at the Integrative Complementary Alternative Medicine Research and Development Center (voucher specimen 2010002), Khon Kaen University, Khon Kaen, Thailand.

2.2. Plant Material Preparation. The fresh leaves of *M. oleifera* were immediately cleaned, cut in to small pieces, and dried in oven at 40°C. The dried plant material was ground into powder and extracted with 50% hydroalcohol using maceration technique. Then, the extract was filtered through Whatman filter paper number 1 and evaporated to dryness using rotator evaporator. The yielded extract was kept at 4°C in a dark bottle until used. The percent yield of extract was 17.49%. The extract contained total phenolic compounds at concentration of 86.73–93.6 ± 0.51 mg of GAE·g^{-1} extract. The crude extract was suspended in 1% CMC (sodium carboxymethylcellulose) to the desired concentration during the experiment.

2.3. Experimental Animals. Healthy male Wistar rats (300–350 g) were obtained from the National Laboratory Animal Center, Salaya, Nakorn Pathom. They were randomly housed 5 per cage, maintained in 12 : 12 light:dark cycle, and given access to food and water ad libitum. The experiments were strictly performed in accordance with the internationally accepted principles for laboratory use and care of the European Community (EEC directive of 1986; 86/609/EEC). The experiment protocols were approved by the Institutional Animal Care and Unit Committee Khon Kaen University, Thailand (Record no. AEKKU 51/2553). All surgery was performed under the pentobarbital sodium anesthesia in order to minimize animal suffering.

2.4. Experimental Protocols. Animals were divided into 7 groups as follows.

Group I: vehicle plus sham operation group; animals in this group were orally given 1% carboxymethylcellulose and received sham operation.

Group II: vehicle plus Rt.MCAO group; all rats were orally given 1% carboxymethylcellulose and subjected to the occlusion of right middle cerebral artery.

Group III: piracetam plus MCAO; rats in this group received Piracetam, a standard drug claiming for the enhanced cerebral blood flow, via oral route at dose of 250 mg·kg^{-1} BW and were exposed to the occlusion of right middle cerebral artery.

Group IV: vitamin C plus MCAO; the animals in this group received Vitamin C, a well-known antioxidant, via oral route at dose of 250 mg·kg^{-1} BW and were exposed to the occlusion of right middle cerebral artery.

Group V–VII: *M. oleifera* extract plus MCAO treated groups; rats in these groups were orally given the extract at doses of 100, 200, and 400 mg·kg^{-1} BW and subjected to the occlusion of right middle cerebral artery.

The animals in groups II–VII were orally given the assigned substances at a period of 14 days and subjected to the occlusion of right middle cerebral artery (RT.MCAO), whereas animals in group I were treated with vehicle at the same period and exposed to sham operation. All substances treatments were continually performed throughout a 21-day study period. The assessment of motor and sensory function recovery was performed every 7 days throughout the study period, while the biochemical assays and the determination of histological changes were performed at the end of study.

2.5. Focal Cerebral Ischemic Induction. Animals were deprived of food but water was allowed to be assessed 12 hours prior to the surgery. Then, they were anesthetized by injecting thiopental sodium at dose of 60 mg·kg^{-1} body weight via intraperitoneal route. After the anesthetization, the focal cerebral ischemic induction was performed [23]. In brief, the bifurcation of right common carotid artery was exposed through a ventral midline incision. The internal carotid artery and external carotid artery were distally dissected, free from the adjacent tissues, and ligated. The monofilament nylon coated with silicone was gently inserted into the internal carotid artery and the filament was advanced up to 17 mm into the middle cerebral artery (MCA) from carotid bifurcation. Then, the distal end of monofilament was tied up and the wound was sewed using the surgical suture. Rats were cared of until full recovery from anesthesia and returned to cage.

2.6. Determination of Neurological Score. The sensorimotor performance of rats following cerebral ischemia induced by right middle cerebral artery occlusion (Rt.MCAO) was evaluated using modified neurological score of Bederson [24] by a "blinded" coworker. Normal rats could extend both forelimbs toward the floor. Rats that extended both forelimbs toward the floor were assigned grade 0 or no spontaneous activity. Cerebral ischemic rats usually flexed the forelimb contralateral to the injured hemisphere. Moreover, it was found that the severely dysfunctional rats had consistently reduced resistance to the gentle lateral push toward the paretic side when the stimulus was applied behind the rats' shoulder. When rats were allowed to move freely, the severely dysfunctional rats usually showed the circling behaviors toward the paretic side. Based on the changes mentioned earlier, the modified neurological score was graded as 6-point neurological function score. In brief, the scoring was performed as follows:

0: no spontaneous activity,

1: spontaneous circling,

2: circling if push tail,

3: lower resistance to lateral push,

4: contralateral forelimb flexion,

5: no apparent deficit.

2.7. Hot Plate Test. Hot plate test was used to assess the sensory function recovery of rat by measuring the latency of foot withdrawal reflex in response to temperature stimuli. Rats were placed on the heat surface of hot plate which was maintained at 50°C. Then, the paw withdrawal latency was recorded and used as index indicating the recovery of sensory response to heat stimuli.

2.8. Infarction Volume Evaluation. Rats were anesthetized with thiopental sodium at dose of 60 mg/kg BW and transcardially perfused with phosphate buffer solution (PBS). After brain removal, it was coronally cut at 2 mm thick with brain slicer and immersed in 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) solution at room temperature at a 15-minute period. The staining brain sections were photographed, and the infarction area was determined by measuring the white area of brain section with computer software (Image Tool for Window version 3).

2.9. Determination of Oxidative Stress Markers. Since the oxidative stresses and free radicals, one of the key factors which induced brain damage in cerebral ischemia, are produced every day via the function of mitochondria [25, 26], polymorphonuclear neutrophils, macrophages, and endothelial cells [27], the alteration of oxidative stress markers has gained much attention. According to the functions of the aforementioned organelle and cells, superoxide was generated and normally it could be detoxified to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 in turn was converted to water (H_2O) by catalase (CAT) and glutathione peroxidase (GSH-Px) enzymes. The excess oxidative stress either via the increased production or via the decreased inactivation process of scavenger enzymes could attack the lipid component of cellular membrane and gave rise to the increased lipid peroxidation and neuronal death. Based on the oxidative stress changes mentioned earlier, the lipid peroxidation product such as malondialdehyde (MDA) and the activities of SOD, CAT, and GSH-Px were used as indices to reflect the oxidative stress markers.

To determine the oxidative stress markers, rats were divided into various groups as previously described in the experimental protocol. After the last dose of administration, all rats were sacrificed by the cervical dislocation. Right hippocampus, cortex, and striatum of each rat were isolated and prepared as homogenate for the determination of the oxidative stress markers including malondialdehyde (MDA) level and the activities of the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). MDA level was estimated by determining the accumulation of thiobarbituric acid reactive substances (TBARS) in the brain homogenate [28]. The activities of SOD, CAT, and GSH-Px were determined by recording the ability to inhibit cytochrome C [29], the rate of decrease in H_2O_2 [30], and the amount of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per minute [31], respectively.

2.10. Statistical Analysis. Data were presented as mean ± standard error of mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by LSD posthoc test. Probability levels less than 0.05 were regarded as significant.

3. Results

3.1. Effect of M. oleifera Leaves Extract on the Functional Recovery of Brain Dysfunction. Based on the fact that hemispheric cerebral ischemia due to the occlusion of middle cerebral artery develops contralateral paralysis and sensory loss, the effect of *M. oleifera* leaves extract on the motor and sensory recovery function after Rt.MCAO was determined using neurological examination and hot plate test.

The effect of *M. oleifera* leaves extract on the neurological score had been shown in Table 1. The results showed that rats which received vehicle plus MCAO showed a significant reduction of neurological scores throughout the 21-day experimental period (P value < .001 all, compared to vehicle + sham operation). This reduction was reversed by Piracetam throughout the experimental period (P value < .05, .001, and .001, resp., compared with vehicle plus MCAO group). Although Vitamin C treated group showed an increased neurological score, the significant effect was observed only at 14 and 21 days after MCAO (P value < .01 all, compared with vehicle plus MCAO group). *M. oleifera* leaves extract at doses of 200 and 400 mg/kg BW significantly improved neurological score at the 14 and 21 days after Rt. MCAO, respectively (P value < .05 all, compared with vehicle plus MCAO group), whereas *M. oleifera* leaves extract at dose of

Table 1: Effect of *Moringa oleifera* leaves extract on neurological score in animal model of focal ischemic stroke ($n = 5$).

Treatment	Neurological score Days after MCAO		
	7 days	14 days	21 days
Vehicle + Sham operation	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
Vehicle + MCAO	3.33 ± 0.33^{aaa}	3.33 ± 0.33^{aaa}	3.67 ± 0.33^{aaa}
Piracetam + MCAO	4.33 ± 0.33*	4.83 ± 0.17***	5.0 ± 0.0***
Vitamin C + MCAO	3.83 ± 0.40	4.67 ± 0.21**	4.83 ± 0.17**
M. oleifera 100 mg/kg + MCAO	3.83 ± 0.40	3.67 ± 0.42	4.17 ± 0.40
M. oleifera 200 mg/kg + MCAO	4.17 ± 0.31	4.17 ± 0.31*	4.20 ± 0.37
M. oleifera 400 mg/kg + MCAO	3.83 ± 0.40	4.00 ± 0.45	4.40 ± 0.40*

Data were expressed as mean ± SEM. ^{aaa}P value < .001, compared to vehicle plus sham operation. $^{*,**,***}P$ value < .05, .01, and .001, respectively, compared to vehicle plus MCAO.

Table 2: Effect of *Moringa oleifera* leaves extract on foot withdrawal time in animal model of focal ischemic stroke ($n = 5$).

Treatment	Foot withdrawal time (seconds) Days after MCAO		
	7 days	14 days	21 days
Vehicle + Sham operation	1.84 ± 0.17	1.70 ± 0.15	1.88 ± 0.16
Vehicle + MCAO	4.30 ± 0.63^{aaa}	3.79 ± 0.55^{aaa}	3.76 ± 0.20^{aaa}
Piracetam + MCAO	3.42 ± 0.35	3.12 ± 0.30	2.73 ± 0.07***
Vitamin C + MCAO	2.48 ± 0.29**	2.98 ± 0.27	2.71 ± 0.08***
M. oleifera 100 mg/kg + MCAO	3.40 ± 0.27	2.60 ± 0.49*	3.08 ± 0.20**
M. oleifera 200 mg/kg + MCAO	3.17 ± 0.24*	2.12 ± 0.33**	2.27 ± 0.11***
M. oleifera 400 mg/kg + MCAO	3.43 ± 0.38	2.42 ± 0.28**	2.72 ± 0.24***

Data were expressed as mean ± SEM. ^{aaa}P value < .001, compared to vehicle plus sham operation. $^{*,**,***}P$ value < .05, .01, and .001, respectively, compared to vehicle plus MCAO.

100 mg/kg BW treated group failed to produce the significant change.

In addition to the motor performance, the recovery of sensory function after Rt.MCAO was also determined using hot plate test. Data were shown in Table 2. The results showed that vehicle plus MCAO markedly enhanced the foot withdrawal reflex time in hot plate test (P value < .001 all, compared to vehicle plus sham operation). Rats exposed to Piracetam plus MCAO showed the significant decreased foot withdrawal reflex time in hot plate test (P value < .001, compared to vehicle plus MCAO) at 21-day period after MCAO, whereas Vitamin C treatment showed the significant reduction of this parameter at 7 and 21 days after MCAO (P value < .01 and .001, resp., compared to vehicle plus MCAO). It was found that rats which were exposed to the extract at dose of 200 mg/kg BW decreased the elevation of foot withdrawal reflex time induced by MCAO throughout the 21-day period after MCAO (P value < .05, .01 and .001, resp., compared to vehicle plus MCAO group), while rats exposed to the extract at doses of 100 and 400 mg·kg^{-1} BW significantly decreased the mentioned parameter at 14 days (P value < .05, and .01 resp., compared to vehicle + MCAO) and 21 days after MCAO (P value < .01 and .001, compared to vehicle MCAO).

3.2. Effect of M. oleifera Leaves Extract on Brain Infarction. Figure 1 showed that both rats treated with Piracetam and Vitamin C attenuated brain infarction volume both in cortex and subcortex (P value < .01 all, compared to vehicle plus MCAO group). Interestingly, *M. oleifera* leaves extract at doses of 100, 200, and 400 mg·kg^{-1} also decreased brain infarction volume in cortex (P value < .01, .05, and .05, resp., compared to vehicle plus MCAO group) and subcortex (P value < .01, .05, and .01, resp., compared to vehicle plus MCAO group).

3.3. Effect of M. oleifera Leaves Extract on Oxidative Stress. The effect of *M. oleifera* leaves extract on MDA levels in cortex, striatum, and hippocampus was shown in Figure 2. It was found that Rt.MCAO significantly increased the level of MDA in all areas (P value < .01, .01, and .05, resp., compared to naïve control group). Piracetam treatment significantly attenuated the elevation of MDA levels induced by MCAO in cortex and hippocampus (P value < .05 and .01 resp.; compared to vehicle plus MCAO), whereas Vitamin C treatment significantly alleviated the elevation of MDA levels induced by MCAO in cortex, hippocampus, and striatum (P value < .01, .01, and .05, resp., compared to vehicle plus MCAO). *M. oleifera* leaves extract at doses of 200 and 400 mg·kg^{-1}BW

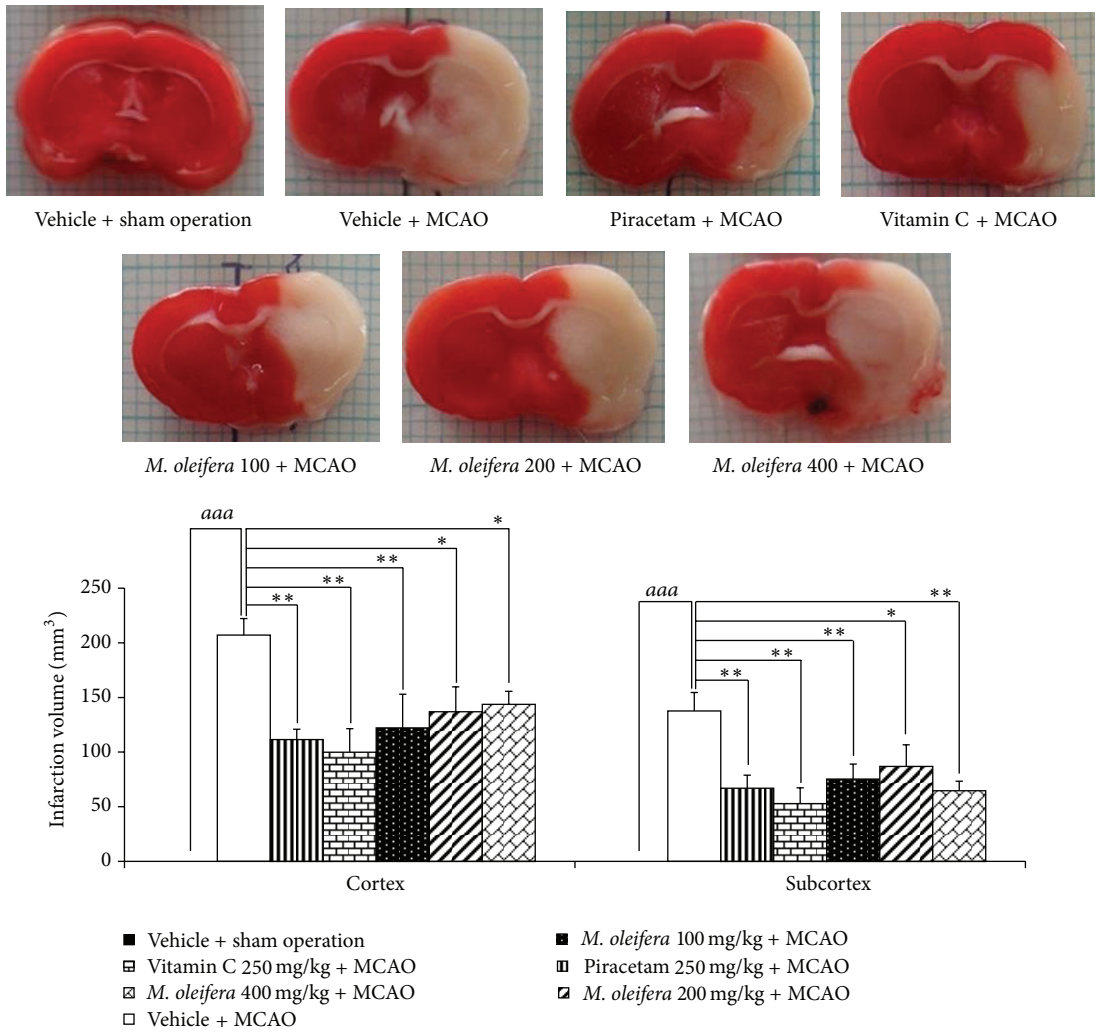

FIGURE 1: Effect of *Moringa oleifera* leaves extract on brain infarction volume in animal model of focal ischemic stroke. ($n = 5$) Data were expressed as mean ± SEM. $^{*,**}P$-value < .05 and .01 respectively; compared to vehicle plus MCAO.

induced the significant reduction of MDA levels in cortex (P value < .001 and .05, resp., compared to vehicle plus MCAO group), hippocampus (P value < .01 and .05, resp., compared to vehicle plus MCAO group) and striatum (P value < .05 and .01 resp.; compared to vehicle plus MCAO group). However, *M. oleifera* leaves extract at dose of 100 mg·kg^{-1}BW significantly attenuated the elevation of MDA levels induced by MCAO only in hippocampus and striatum (P value < .05 all, compared to vehicle plus MCAO).

In addition to MDA level, the effects of *M. oleifera* leaves extract on endogenous scavenging enzymes mentioned above in ischemic hemisphere were also determined and data were shown in Figure 3. It was found that Piracetam, Vitamin C, and all doses of *M. oleifera* leaves extract treated groups significantly increased the reduction of SOD activity in hippocampus (P value < .05, .01, .001, .001, and .001, resp., compared to vehicle plus MCAO) and in striatum (P value < .05, .01, .05, .05, and .001, resp., compared to vehicle plus MCAO). Unfortunately, no significant changes of SOD were observed in cortex as shown in Figure 3.

Figure 4 showed the effect of *M. oleifera* leaves extract on GSHPx activities in cortex, hippocampus, and striatum. MCAO treated group showed the decreased GSH-Px activity in cortex and hippocampus, but no change was observed in striatum (P value < .001 and .01, resp., compared to vehicle plus MCAO). Piracetam treatment significantly attenuated the decreased GSH-Px activity only in hippocampus (P value < .001, compared to vehicle plus MCAO group), whereas Vitamin C treatment produced significant attenuation on the reduction of this enzyme in cortex and hippocampus (P value < .01 and .001, resp., compared to vehicle plus MCAO). It was found that *M. oleifera* leaves extract at doses of 100 and 400 mg·kg^{-1} BW significantly mitigated the decreased GSH-Px activity induced by MCAO only in hippocampus (P value < .01 and .001, resp., compared to vehicle plus MCAO). No changes were observed in other treatments.

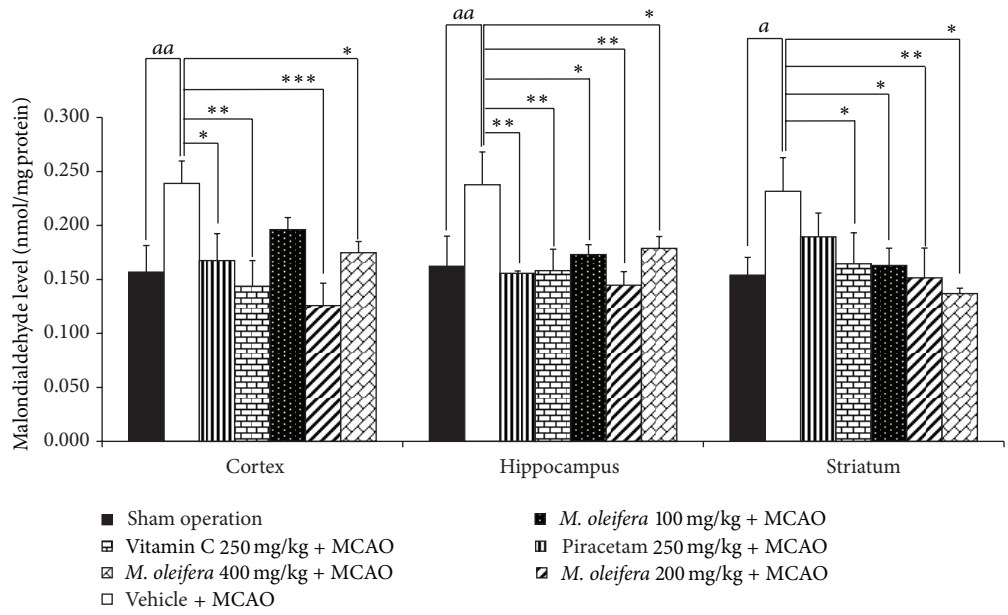

Figure 2: Effect of *Moringa oleifera* leaves extract on malondialdehyde (MDA) level in cerebral cortex, hippocampus and striatum of animal model of focal ischemic stroke. ($n = 5$) Data were expressed as mean ± SEM. $^{a,aa}P$-value < .05 and .01 respectively; compared to vehicle plus sham operation. $^{*,**,***}P$-value < .05, .01 and .001 respectively; compared to vehicle plus MCAO.

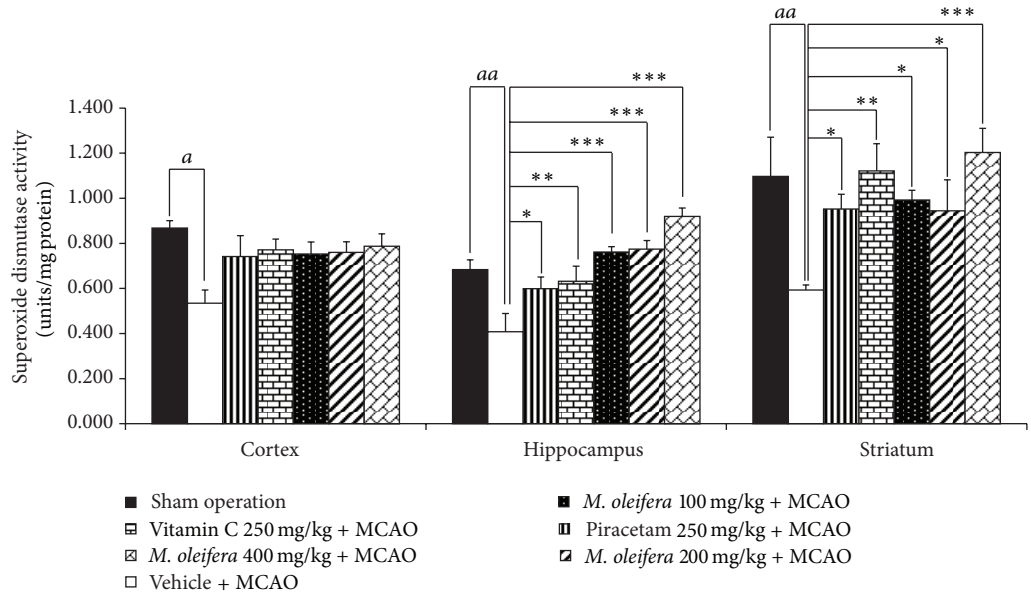

Figure 3: Effect of *Moringa oleifera* leaves extract on superoxide dismutase (SOD) activity in cerebral cortex, hippocampus and striatum of animal model of focal ischemic stroke. ($n = 5$) Data were expressed as mean ± SEM. $^{a,aa}P$-value < .05 and .01 respectively; compared to vehicle plus sham operation. $^{*,**,***}P$-value < .05, .01 and .001 respectively; compared to vehicle plus MCAO.

The effect of *M. oleifera* leaves extract on CAT activity was also investigated and data were shown in Figure 5. MCAO treatment significantly decreased CAT activity in cortex, hippocampus, and striatum (P value < .05 all, compared to vehicle treated group). Piracetam treatment failed to show positive modulation effect on CAT activity induced by MCAO, whereas Vitamin C treatment significantly mitigated the decreased CAT activity induced by MCAO in cortex and striatum (P value < .05 all, compared to vehicle plus MCAO). However, no significant changes were observed in *M. oleifera* leaves extract treated group.

4. Discussion

In this study, we have demonstrated that *M. oleifera* leaves extract attenuated brain dysfunction and brain damage

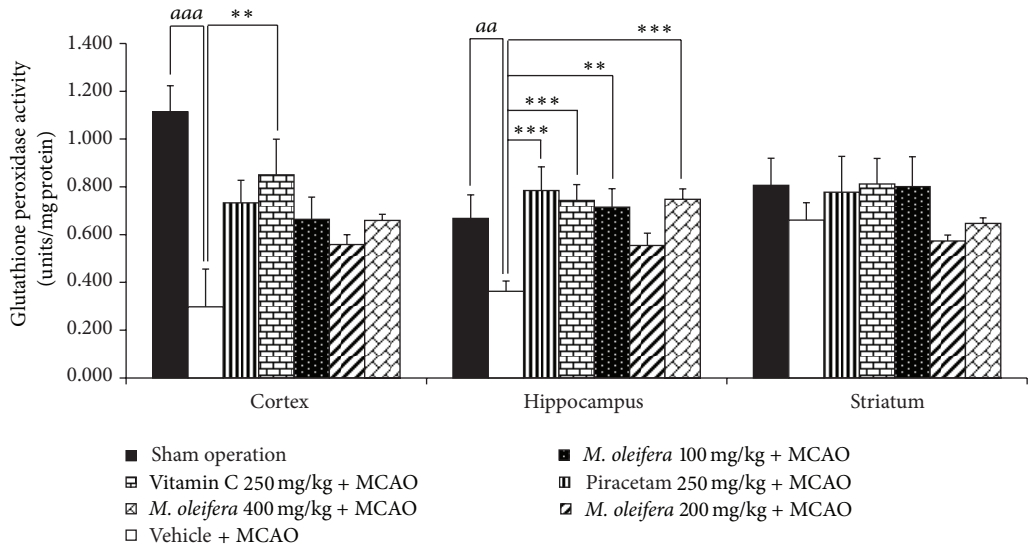

Figure 4: Effect of *Moringa oleifera* leaves extract on glutathione peroxidase (GSH-Px) activity in cerebral cortex, hippocampus and striatum of animal model of focal ischemic stroke. ($n = 5$) Data were expressed as mean ± SEM. $^{aa,aaa}P$-value < .01 and .001 respectively; compared to vehicle plus sham operation. $^{*,**,***}P$-value < .05, .01 and .001 respectively; compared to vehicle plus MCAO.

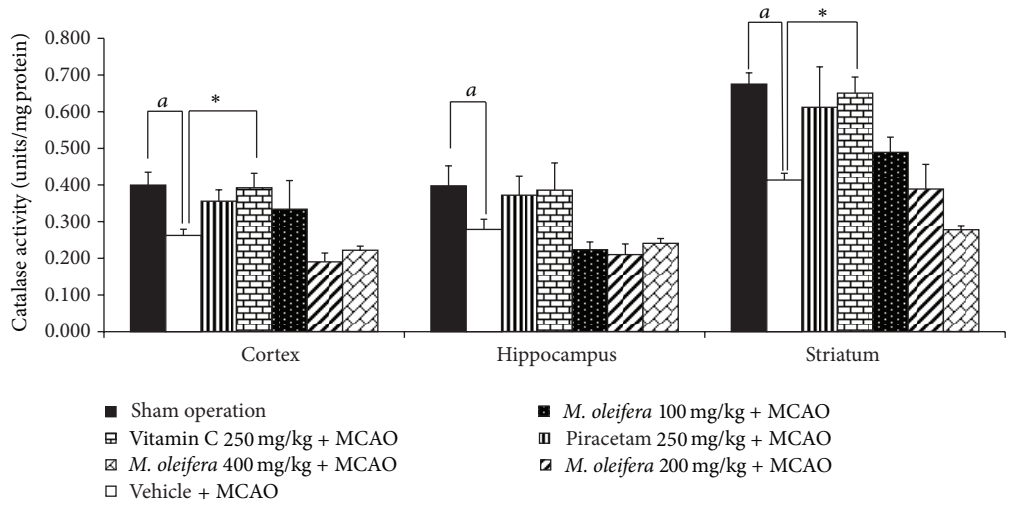

Figure 5: Effect of *Moringa oleifera* leaves extract on catalase (CAT) activity in cerebral cortex, hippocampus and striatum of animal model of focal ischemic stroke. ($n = 5$) Data were expressed as mean ± SEM. $^{a,aa}P$-value < .05 and .01 respectively; compared to vehicle plus sham operation. $^{*}P$-value < .05; compared to vehicle plus MCAO.

together with the decreased oxidative stress especially in hippocampus and striatum.

It has been well known that oxidative stress is one of the most important factors that exacerbate brain damage induced by cerebral ischemia. Among various brain regions, cortex, striatum, and hippocampus are more susceptible to brain ischemia [32] due to the high rate of oxidative metabolic activity, intense production of reactive oxygen metabolites, high content of polyunsaturated fatty acids, relatively low antioxidant capacity, low repair mechanism activity, and the poor plasticity in the areas just mentioned [33]. Our data clearly demonstrated that the permanent occlusion of MCA produced the decreased SOD and CAT activities in cortex, striatum, and hippocampus, while it produced the significant decreased GSH-Px activity in cortex and hippocampus. These changes in turn decreased the buffering capacity of antioxidant activities and gave rise to the excess oxidative stress and free radicals and resulted in the increased lipid peroxidation indicated by the increased MDA level. Then, the neurodegeneration and brain infarction occurred and finally led to brain dysfunctions of the suffered areas. Vitamin C and Piracetam treatments could protect against brain damage and dysfunctions induced by cerebral ischemia. Previous study demonstrated that Vitamin C could be metabolized to dehydroascorbic acid (DHA) and transported across blood-brain-barrier (BBB) via facilitated transport. DHA could

provide the beneficial effect both via its antioxidant effect and its effect to enhance cerebral blood flow [34]. Piracetam was reported to improve mitochondria function and decreased oxidative stress [35]. In addition, Piracetam also enhanced cerebral blood flow [36]. Therefore, both Vitamin C and Piracetam could provide cerebroprotective effect via the antioxidant and the enhanced cerebral blood flow. Our data also demonstrated the decreased oxidative stress by both substances. Therefore, our data are in agreement with the previous data. Both Vitamin C and the plant extract showed a better improvement in foot withdrawal reflex than Piracetam, while Piracetam produced better improvement in neurological score than Vitamin C and the leaves extract Although the precise understanding is still unknown, we did suggest that the recovery of both sensory-motor performance and foot withdrawal reflex might be associated with the different severity of damage and factors which play an important role in brain damage and brain plasticity among various areas. In addition, these factors might also have different vulnerability to the substances such as Vitamin C, the plant extract, and Piracetam. Moreover, the difference in bioavailability of the active form of Vitamin C and Piracetam and active ingredient of the plant extract in the targeted areas might also play an important role in different vulnerability to the effect of the mentioned substance and the different improvement of various brain areas which in turn are possibly to induce different improvement in performance of neurological score and foot withdrawal reflex.

The present study has demonstrated that *M. oleifera* leaves extract treatment at dose of 200 $mg \cdot kg^{-1}$ BW produced optimum changes of MDA level in all areas, whereas the optimum changes of antioxidant enzymes such as SOD and CAT were observed in rats treated with *M. oleifera* leaves extract at dose of 400 $mg \cdot kg^{-1}$ BW. Therefore, our data have shown that no close association between the decreased MDA level and the elevation of antioxidant enzymes was observed. This suggests that *M. oleifera* may decrease oxidative stress especially in cerebral cortex by other mechanisms such as the decreased oxidative stress generation capacity either via mitochondria or via the inflammatory cells [37]. Since *M. oleifera* leaves extract exhibits potent anti-inflammatory activity [38, 39], we suggest that the decreased oxidative stress reflected by the decreased MDA level occurs partly via the anti-inflammatory effect of the plant extract.

It has been found that *M. oleifera* decreases brain infarction volume in cortex without the decreased MDA level. Therefore, our data suggest that other mechanisms also contribute to the role on the neuroprotective effect of *M. oleifera* leaves extract. In addition to oxidative stress, calcium ion over load, excitatory amino acid toxicity, and apoptosis also contribute to the role on neuronal cell death and brain infarction volume [40, 41]. Moreover, the increased blood flow may possibly play a role in the decreased brain infarction volume following stroke [42]. Therefore, the decreased brain infarction volume induced by the extract may be associated with the factors just mentioned.

According to the focal cerebral ischemic stroke model induced by the occlusion of middle cerebral artery occlusion (MCAO), the affected areas are caudate, putamen, parietal cortex, neocortex, and entorhinal cortex [40]. Therefore, the occlusion of MCAO induces both sensory and motor impairments. Since *M. oleifera* leaves extract improved brain infarction both in cortex and striatum, the improvement of both sensory and motor impairments was observed. However, the low dose of extract improved brain infarction volume without the improved neurological score. This suggested that the magnitude of improvement of infarction area was not high enough to improve neurological score. This might be associated with the lack of no significant improvement in striatum.

In this study, no dose dependent response was observed. The possible explanation might be associated with the masking effect of other ingredients in the crude extract of *M. oleifera* leaves. In addition, all parameters investigated in this study appeared to be under the influence of numerous factors. Therefore, no simple relationships between the concentration of extract and the magnitude of changes of the interested parameters were observed.

Although *M. oleifera* leaves extract could not provide better cerebroprotective effect than Vitamin C, it appeared to have low risk of toxicity. It has been shown that *M. oleifera* leaves extract at the dose of more than 3000 $mg \cdot kg^{-1}$ BW is genotoxic, and LD_{50} of alcoholic extract of *M. oleifera* leaves is more than 2800 but less than 5000 $mg \cdot kg^{-1}$ BW [43], whereas the toxicity of Vitamin C was reported at 3000 $mg \cdot day^{-1}$. When compared to Piracetam, *M. oleifera* leaves extract was more easy to approach and cheaper. Therefore, *M. oleifera* leaves extract is an interesting resource for developing functional food and worthy of further study.

5. Conclusion

This study has demonstrated that *Moringa oleifera* leaves extract is the potential neuroprotectant which is cheap and easy to approach. The possible underlying mechanism may occur partly via the decreased oxidative stress. Other mechanisms may be also involved and required further exploration.

Acknowledgments

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Research Article

Antigenotoxic and Apoptotic Activity of Green Tea Polyphenol Extracts on Hexavalent Chromium-Induced DNA Damage in Peripheral Blood of CD-1 Mice: Analysis with Differential Acridine Orange/Ethidium Bromide Staining

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This study was conducted to investigate the modulating effects of green tea polyphenols on genotoxic damage and apoptotic activity induced by hexavalent chromium [Cr (VI)] in CD-1 mice. Animals were divided into the following groups: (i) injected with vehicle; (ii) treated with green tea polyphenols (30 mg/kg) via gavage; (iii) injected with CrO_3 (20 mg/kg) intraperitoneally; (iv) treated with green tea polyphenols in addition to CrO_3. Genotoxic damage was evaluated by examining micronucleated polychromatic erythrocytes (MN-PCEs) obtained from peripheral blood at 0, 24, 48, and 72 h after treatment. Induction of apoptosis and cell viability were assessed by differential acridine orange/ethidium bromide (AO/EB) staining. Treatment of green tea polyphenols led to no significant changes in the MN-PCEs. However, CrO_3 treatment significantly increased MN-PCEs at 24 and 48 h after injection. Green tea polyphenols treatment prior to CrO_3 injection led to a decrease in MN-PCEs compared to the group treated with CrO_3 only. The average of apoptotic cells was increased at 48 h after treatment compared to control mice, suggesting that apoptosis could contribute to eliminate the DNA damaged cells induced by Cr (VI). Our findings support the proposed protective effects of green tea polyphenols against the genotoxic damage induced by Cr (VI).

1. Introduction

Green tea (*Camellia sinensis*) is one of the most ancient beverages, consumed by over two-thirds of the world's population. The principal constituents are caffeine, tannins, and essential oils. The tannins encompass a variety of polyphenolic compounds, including important flavonoids such as catechins: (−)-epicatechin (EC), (−)-epigallocatechin (EGC) and their gallate forms (+) gallocatechin (GC), (−) epicatechin-3-gallate (ECG) and (−) epigallocatechin-3-gallate (EGCG) [1, 2]. These compounds are chemically classified as dibenzpyrans, pyrones, and their derivatives. The core structure contains a diphenylpropane skeleton (Figure 1(a)). The primary flavonoids found in fresh green tea leaves are catechins (flavan-3-ols or flavanols) and the flavonols (Figures 1(b) and 1(c), resp.) [1]. In addition, green tea contains other polyphenols such as theaflavins (Figure 1(d)) but at lower concentration than catechins. The polyphenols are also naturally found in fruits and vegetables, as well as in drinks such as red wine and beer [1, 2].

Green tea has attracted significant attention recently, both in the scientific and in consumer communities for its health benefits for a variety of diseases associated with oxidative stress such as cancer, cardiovascular, and neurodegenerative diseases [3, 4]. The beneficial effects of green tea are attributed to the antioxidant properties of the polyphenolic compounds. In addition to the cancer chemopreventive properties, green

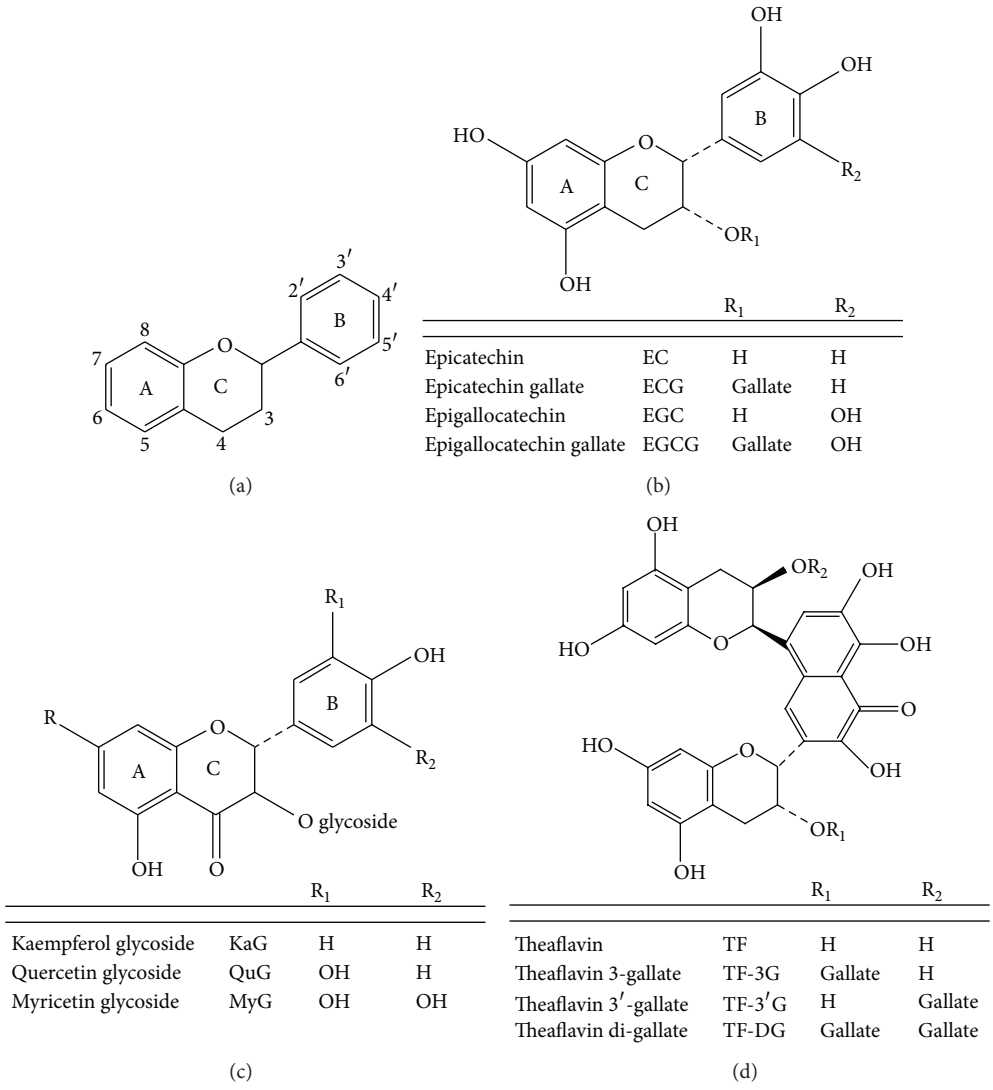

Figure 1: Classification of flavonoids. (a) Flavonoid diphenylpropane skeleton; (b) tea flavanols (flavan-3-ols); (c) tea flavonols; (d) tea theaflavins.

tea polyphenols have shown anti-inflammatory, antiallergenic, antibacterial, and antiviral properties [4–6], as well as antimutagenic activity [7].

The polyphenol compounds have shown direct effects as radical scavengers and metal chelators and indirect effects via the modulation of transcription factors and enzymes [8, 9]. In fact, these antioxidants can inhibit the formation of 8-hydroxydeoxyguanosine (8-OH-dG, 7,8-dihydro-8-oxodeoxyguanosine) *in vivo* [10, 11]. Thus, substances with antioxidant properties have emerged as putative preventives and coadjuvants in the treatment of chronic degenerative diseases related to oxidative stress and DNA damage.

In contrast, hexavalent chromium [Cr (VI)] compounds are particularly effective at inducing genotoxicity by causing several types of DNA lesions and gene mutations. Cr (VI) compounds have been widely studied because they have various industrial applications, including chromium plating, metallurgy, pigment manufacturing, leather tanning, and wood preservation, and because they are associated with the induction of cancer [12]. Cr usually exists in various oxidation states, primarily Cr (III) and Cr (VI). Nonetheless, Cr (III) is an essential micronutrient and plays an important role in protein, sugar, and fat metabolism [13]. Cr (VI) is particularly effective at inducing genotoxicity by causing several types of DNA lesions and gene mutations. Cr (VI)-induced DNA-DNA interstrand cross-links, oxidative DNA damage, and mutations in the tumor suppressor gene *p53* are some of the major factors that may play a significant role in determining cellular genotoxicity [14, 15]. According to previous studies, Cr-induced genomic DNA damage includes 8-hydroxydeoxyguanosine (8-OH-dG, 7,8-dihydro-8-oxodeoxyguanosine), which is a form of oxidative DNA damage [16]. Maeng et al. [17] observed changes in 8-OH-dG levels in DNA when rats were exposed to Cr (VI) and

suggest that DNA damage caused by Cr (VI) compounds may be partially associated with oxidative stress. Cr (VI) generates reactive oxygen species (ROS) and free radicals (FRs) via its intracellular reduction to Cr (III) via the Fenton and Haber-Weiss reaction [15, 18, 19]. Although the direct relationship between DNA-ROS and chromium-induced DNA damage is heavily debated and unclear, there have been several studies supporting the role of ROS in Cr (VI)-induced genotoxicity and cytotoxicity [20]. Moreover, it has been observed that Cr (VI) given orally to mice could induce dose- and time-dependent effects on hepatic oxidative stress and hepatocyte apoptosis [21]. Apoptosis is a process in which cell death is initiated and completed in an orderly manner through the activation and/or synthesis of gene products necessary for cell destruction [22]. Apoptosis plays a crucial role in a number of physiological and pathological processes and is accompanied by characteristic morphological changes, which include cytoplasmic shrinkage, plasma membrane blebbing, condensation or fragmentation of nuclei, and extensive degradation of chromosomal DNA. Indeed, many chemopreventive agents act by inducing apoptosis as a mechanism to suppress carcinogenesis [23]. Roy et al. [24] observed that EGCG not only protects normal cells against genotoxic alterations induced by MNNG but also eliminates cancer cells via the induction of apoptosis *in vitro*. We previously observed that *in vivo* administration of green tea (*ad libitum* for 10 days) decreased the induction of MN-PCEs upon treatment with CrO_3. This result supports the protective effects of green tea against the genotoxic damage induced by metal compounds such as Cr (VI). However, the MN-PCEs induced by CrO_3 were only partially blocked by the addition of green tea (approximately 42%) at days 1 and 2 [25]. This finding may be related to factors such as the origin of the tea because it has been observed that the amount of polyphenols in tea plants is influenced by environmental factors (i.e., weather, light, nutrients, preparation process, storage, horticulture leaf age, etc.). Also, it has been reported that polyphenols make up more than 30% of the dry weight of tea leaves; 90% of these compounds are catechins, and 10% are flavonols [26–28]. Therefore, as part of our research program that evaluates chemopreventive and chemoprotective components in the diet, to obtain a more efficient modulation of the genotoxic damage induced by Cr (VI) *in vivo*, we directly studied green tea polyphenol extracts that contain a mixture of polyphenolic compounds (minimum 60% total catechins with higher antioxidant activity [Polyphenon 60]), and we analyzed its apoptotic activity in the peripheral blood of CD-1 mice using analysis with differential acridine orange/ethidium bromide staining.

2. Materials and Methods

2.1. Chemicals. The following test chemicals and reagents were obtained from Sigma Chemicals Co. (St. Louis, MO, USA): CrO_3 [CAS no. 1333-82-0], acridine orange (AO) [CAS no. 10127-02-3], ethidium bromide (EB) [CAS no. 1239-45-8], and green tea polyphenol extracts (Polyphenon 60) [CAS no. 138988-88-2].

2.2. Animals. Two- to three-month-old CD-1 male mice (28–35 g) were used in the experiments. The animals were kept under controlled temperature (22°C) with a 12-12 h light-dark period (light 07:00–19:00 h). Mice had free access to food (Purina-México chow for small rodents) and water. All of the mice were obtained from Harlan at "Facultad de Química, Universidad Nacional Autónoma de México" (UNAM) and were acclimated for a two-week period. The Bioethics Committee of the "Facultad de Estudios Superiores-Zaragoza", UNAM approved the experimental protocols used in this study.

2.3. Experimental Design. The dosage of green tea polyphenols extract (Polyphenon 60) was based on results obtained in previous studies, which utilized other commercially available Polyphenon 60 (0.625–1.25% body weight) [29] and our preliminary studies to determine the maximum tolerated dose (MTD) that did not induce MN-PCEs. The CrO_3 dose was selected according to previous studies that intraperitoneally (i.p.) administered 20 mg/kg. This CrO_3 dosage induced MN-PCEs in the peripheral blood of mice [30].

The green tea polyphenol extracts and CrO_3 were prepared in solution by dissolving the dry compounds in sterile distilled water. Following preparation of the compounds, the solutions (0.25 mL) were administered immediately. The control group was treated in an identical manner with vehicle only. The evaluation criteria and work conditions were set up according to the OECD guideline (474), Food and Drug Administration (FDA) guidelines, Environmental Protection Agency (EPA) guidelines, and guidelines for the testing of chemicals specified by the Collaborative Study Group for the Micronucleus Test (CSGMT) and the Mammalian Mutagenesis Study Group of the Environmental Society of Japan (JEMS.MMS) for the short-term mouse peripheral blood micronucleus test [31–35].

After establishing treatment doses, the effects of green tea polyphenol extracts on genotoxic damage in CrO_3-treated mice were evaluated. This assessment was performed using MN-PCEs kinetic analysis [31]. Mice were assigned at random to one of the following groups ($n = 5$ mice per group).

(1) Animals injected with vehicle (control group).

(2) Animals treated with green tea polyphenol extract (30 mg/kg) by gavage.

(3) Animals injected with CrO_3 (20 mg/kg).

(4) Animals treated with green tea polyphenol extract (30 mg/kg) by gavage and then (4 h later) injected with CrO_3 (20 mg/kg).

2.4. Micronucleus Assay. Slides were covered with AO and prepared according to the technique described by Hayashi et al. [36]. Briefly, AO was dissolved in distilled water at a concentration of 1 mg/mL, and 10 μL of this solution was placed on a preheated (approximately 70°C) clean glass slide. The AO was spread evenly on the slide by moving a glass rod back and forth over the slide, which was then air-dried.

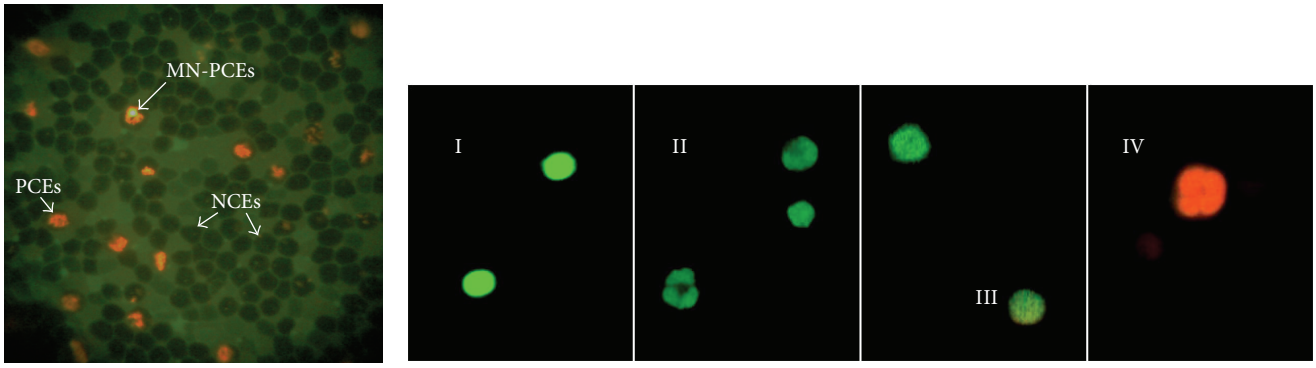

Figure 2: (a) Fluorescent microphotograph of CD-1 mouse peripheral blood cells using the AO coating method (NCEs, PCEs, and MN-PCEs). (b) Morphology of viable cells (apoptotic and nonapoptotic cells) and nonviable cells (apoptotic and nonapoptotic cells) as assessed by acridine orange/ethidium bromide staining. Viable cells stain uniformly green (I), early apoptotic cells with intact plasma membranes appear green, with "dots" of condensed chromatin that are highly visible within (II), late apoptotic cells are stained bright green-orange because membrane blebbing starts to occur, EB can enter the cell (III), and apoptotic nonviable cells are stained bright orange because of the entry of ethidium bromide into these cells (VI).

The AO-coated glass slides were stored in a dark, dry location at room temperature prior to experimental use.

To evaluate MN after treatment, 5 μL peripheral blood samples were collected by piercing a tail blood vessel of the mice every 24 h during a four-day period (0 to 72 h). The samples were placed directly on slides previously treated with AO according to Hayashi et al. [36]. After the sample was placed on the slide, a coverslip (24 × 50 mm) was immediately placed on the slide, and its edges were sealed with rubber cement. All of the slide preparations were kept in plastic boxes in the dark at 4°C. These slide preparations cannot be stored permanently, but they can be stored for several days in a refrigerator if the coverslip has been sealed. Two slides were prepared for each mouse, and analysis of the slides was conducted after 12 h.

PCEs, NCEs, and MN-PCEs were identified under a fluorescent microscope (Nikon OPTIPHOT-2) using a blue excitation filter and a yellow barrier filter. The differential AO staining distinguished PCEs from NCEs, as PCEs were stained a fluorescent red-orange color due to the presence of ribosomal RNA. The AO staining also identified MN-PCEs, which were stained a fluorescent green color due to their DNA content (Figure 2(a)). The MN-PCEs analysis was based on the results from 2,000 cells per mouse, and the presence of MN-PCEs was used as a marker for genotoxic damage [36].

2.5. Apoptosis and Cell Viability Analyses. To evaluate apoptosis and cell viability we used the differential acridine orange/ethidium bromide (AO/EB) staining. Blood samples (100 μL) were collected by piercing a tail blood vessel of the mice prior to treatment and 48 h after treatment. Heparin (10 μL) was added to the blood samples, and 20 μL of AO/EB dye mix (100 μL/mL AO and 100 μL/mL EB, both prepared in PBS) was then added. The suspension was concentrated via centrifugation (5,000 rpm), and the cell pellet was resuspended in 10 μL and plated on a clean slide; a coverslip (24 × 24 mm) was immediately placed on the slide. Two slides were prepared per mouse, and the analysis was conducted immediately.

Apoptosis was assessed by identifying apoptotic, viable and nonviable cells under a fluorescent microscope (Nikon OPTIPHOT-2) with a blue excitation (480 nm) and a barrier filter (515–530 nm). The differential AO/EB staining is capable of distinguishing between viable and nonviable cells based on membrane integrity. When the cell is viable, the AO intercalates into the DNA, giving the cell a green appearance. Conversely, when the cell is nonviable, the EB also intercalates into the DNA, making the cell appear orange. Thus, a nonviable cell will contain a bright orange nucleus as EB overwhelms AO staining. Both healthy and apoptotic nuclei in viable cells will fluoresce bright green. In contrast, healthy or apoptotic nuclei in nonviable cells will fluoresce bright orange [33]. The apoptotic and cell viability assessments were based on 200 cells per mouse.

2.6. Statistical Analysis. The MN-PCEs induction results and the viable cells (apoptotic and nonapoptotic) and nonviable cells (apoptotic and nonapoptotic) data are expressed as the mean ± standard deviation (S.D.), and results from the various treatment groups were compared by an ANOVA test followed by a Tukey test. The net induction frequency (NIF) of MN-PCEs was analyzed using a Chi-square test [30, 37]. SPSS/PC V18TM and Statistica/PC V 6.0TM software were used for the statistical analyses. For all of the analyses, $P < 0.05$ was considered to be significant.

3. Results

The results obtained in the present study are shown in Table 1. Vehicle and green tea polyphenol extract did not modify the average number of induced MN-PCEs in the treated mice. CrO_3 treatment increased the average number of MN-PCEs in all of the samples, but statistical significance was only

Table 1: Averages of the induction MN-PCEs in peripheral blood of mice treated with polyphenol extracts of green tea and CrO_3.

Treatment	Dose (mg/kg)	n	Time analysis (hours)	MN-PCEs 2,000 cells (mean ± S.D.)	ANOVA
Control	0	5	0	0.6 ± 0.9	
			24	0.8 ± 0.8	
			48	1.2 ± 0.8	
			72	1.6 ± 1.5	
Polyphenol extracts of green tea	30	5	0	1.0 ± 1.2	
			24	1.8 ± 1.9	
			48	1.2 ± 1.3	
			72	1.4 ± 0.5	
CrO_3	20	5	0	0.2 ± 0.5	
			24	3.0 ± 1.6	
			48	13.2 ± 3.8	a, b, c, d
			72	3.4 ± 1.1	
Polyphenol extracts of green tea–CrO_3	30–20	5	0	1.2 ± 1.3	
			24	0.6 ± 0.5	
			48	5.2 ± 1.8	a, b, e
			72	1.2 ± 1.1	

[a]$P <$ versuscontrol 48 h; [b]$P <$ versus polyphenol extracts of green tea 48 h; [c]$P <$ versus CrO_3 0 h; [d]$P <$ versus polyphenol extracts of green tea–CrO_3 48 h; [e]$P <$ versus polyphenol extracts of green tea–CrO_3 0 h.

observed at the 48 h time point when compared to the 0 h samples and the control group. Furthermore, CrO_3 treatment increased the average number of MN-PCEs (approximately 13 MN-PCEs) compared to vehicle-treated mice. When the treatment included both green tea polyphenol extract and CrO_3, we observed a decrease in the average number of MN-PCEs at 24, 48, and 72 h after treatment when compared to MN-PCEs induction in the CrO_3 only treatment group, but the MN-PCEs induction observed at 48 remains statistically significant compared with the control group (Table 1). To compare the kinetics of MN-PCE induction in the various treatment groups, the data were analyzed by calculating the NIF value, which was calculated as follows [30]:

$$\text{NIF} = \text{number of MN-PCEs measured at time } x_i - \text{number of MN-PCEs measured at time 0}, \quad (1)$$

where x_i = evaluation at 24, 48, or 72 h. Time 0 = evaluation at 0 h (before treatment).

When the NIF is calculated, the net MN-PCEs induction can be more readily observed. This calculation subtracts the frequency of MN-PCEs prior to treatment from the frequency following treatment, thereby eliminating the baseline MN-PCEs variability that occurs between the treatment groups at time 0 (see S.D. Table 1). Figure 3 presents the NIF values for all treatments at 24, 48, and 72 h after treatment. The groups treated with green tea polyphenol flavonoid extracts exhibited a principal MN-PCEs reduction at 24 and 72 h after treatment (approximately 121% and 100%, resp.).

Apoptosis and cell viability were evaluated directly in the peripheral blood of mice before (0 h) and after (48 h) treatment. Induction of apoptosis and cell viability were assessed via AO/EB staining of peripheral blood collected from treated mice. This technique shows the differential uptake of the fluorescent DNA-binding dyes AO and EB to determine viable and nonviable cells. These dyes were used to identify cells that have undergone apoptosis and to distinguish between cells in the early or late stages of apoptosis based on membrane integrity (Figure 2(b)). AO intercalates into the DNA, giving it a green appearance. This dye also binds to RNA, but because it cannot intercalate, the RNA stains red orange. Thus, a viable cell will have a bright green appearance. EB is only taken up by nonviable cells. This dye also intercalates into DNA, making it appear orange; however, EB only binds weakly to RNA, which may appear slightly red. Thus, a nonviable cell will have a bright orange nucleus, as EB overwhelms AO staining, and its cytoplasm will appear dark red (if any content remains). Both normal and apoptotic nuclei in viable cells will fluoresce bright green (Figure 2(b), I–III). In contrast, normal or apoptotic nuclei in nonviable cells will fluoresce bright orange (Figure 2(b), IV). Therefore, one can differentiate between early and late apoptotic cells using this system. Viable cells with intact membranes will have a uniformly stained green nucleus (Figure 2(b), I). Early apoptotic cells with intact membranes but that have started to fragment their DNA will still have green nuclei because EB cannot enter the cell, but chromatin condensation can be visualized as bright green patches in the nuclei (Figure 2(b), II). As the cell progresses through the apoptotic pathway and membrane blebbing starts to occur, EB can enter the cell, causing the cell to stain green orange (Figure 2, III). Late apoptotic cells will have bright orange patches of condensed chromatin in the nucleus that will distinguish these cells from necrotic cells, which will be uniformly stained orange (Figure 2, IV).

All of the treatments increased the average number of apoptotic viable cells, but statistical significance was achieved only in the CrO_3 treatment group and the combined green

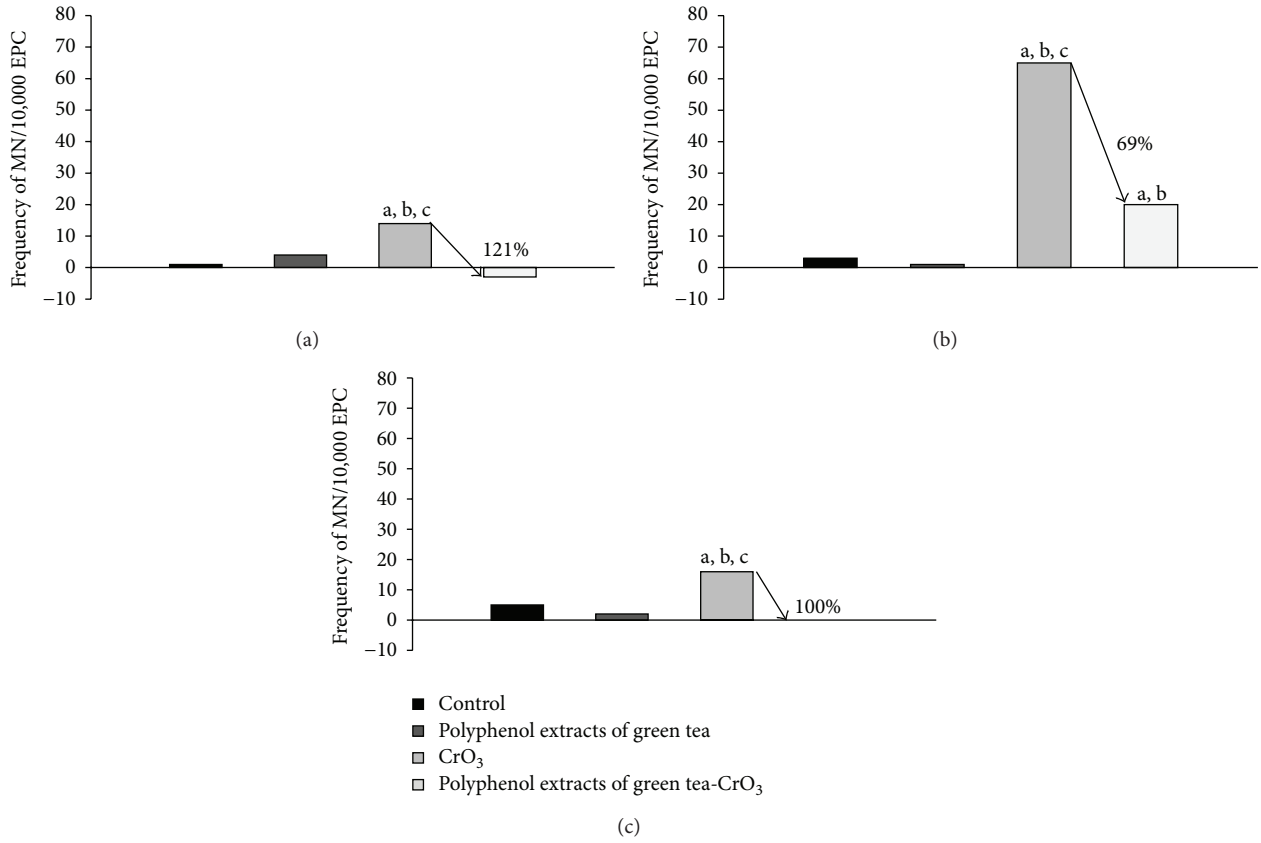

Figure 3: Effects of polyphenol extracts of green tea on the MN-PCEs frequency (% reduction) in mice peripheral blood at different times: (a) 24 h, (b) 48 h, and (c) 72 h after treatment with CrO_3. Data represent MN-PCEs frequency obtained at 24, 48, and 72 h minus the MN-PCEs frequency at 0 h (NIF). [a]$P < 0.05$ versus control group; [b]$P < 0.05$ versus polyphenol extracts of green tea group; [c]$P < 0.05$ versus CrO_3-polyphenol extracts of green tea group. $n = 5$ (2000 cells for mouse).

Table 2: Averages of viable cells (apoptotic and nonapoptotic) and nonviable cells (apoptotic and nonapoptotic) in peripheral blood of mice treated with polyphenol extract of green tea and CrO_3.

Treatment	Dose(mg/kg)	n	Nonapoptotic viable cells* (mean ± S.D.)	Apoptotic viable cells* (mean ± S.D.)	Apoptotic nonviable cells* (mean ± S.D.)	Nonapoptotic nonviable cells* (mean ± S.D.)
Control	0	5	197.2 ± 2.2	2.4 ± 1.8	0.0 ± 0.0	0.4 ± 0.9
Polyphenol extracts of green tea	30	5	192.8 ± 4.9	6.0 ± 4.1	0.8 ± 1.3	0.4 ± 0.6
CrO_3	20	5	189.2 ± 3.3[a]	10.2 ± 3.6[c]	0.2 ± 0.5	0.4 ± 0.6
Polyphenol extracts of green tea–CrO_3	30–20	5	187.2 ± 6.3[b]	12.8 ± 6.3[d]	0.0 ± 0.0	0.0 ± 0.0

[a]$P < 0.05$ versus control; [b]$P < 0.01$ versus control; [c]$P < 0.04$ versus control; [d]$P < 0.007$ versus control.
*Evaluation in 200 cells.

tea polyphenol extract-CrO_3 group compared to the control group. The increase in apoptosis was greater in the combined green tea polyphenol extract-CrO_3 group than in the CrO_3 group (12.8 versus 10.2 cells, resp.). The green tea polyphenol extract only treatment group had an increase of approximately four apoptotic viable cells compared to the control group. The average numbers of apoptotic nonviable cells and nonapoptotic cells were unchanged in all of the treatment groups (Table 2).

4. Discussion

The antioxidant properties present in polyphenols make them potentially useful for counteracting the DNA damage induced by oxidative stress agents such as Cr (VI) compounds. In this study, we evaluated the capability of polyphenol extracts from green tea to inhibit the genotoxic damage induced by Cr (VI) *in vivo* and analyzed apoptosis in peripheral blood of CD-1 mice.

The genotoxicity of Cr (VI) was demonstrated by the observation of a significant increase in MN-PCEs at 24 and 48 h after treatment in the CrO_3-treated group (Table 1). This increase was clearly observed in all samples compared to the negative control, and this finding corroborates the previously reported genotoxicity of Cr (VI) and particularly CrO_3 [15, 30, 38]. The mechanism of genotoxicity for Cr (VI) compounds has been linked to the intracellular reduction of Cr (VI) to Cr (III). Cr (VI) compounds can cross cell membranes via nonspecific anion transporters and are reduced by their interaction with intracellular cytoplasmic molecules. During this process, FRs are generated, which are capable of inducing genotoxic alterations [15, 18, 19]. Hence, we suggest that these effects can lead to the formation of MN-PCEs.

However, the average number of MN-PCEs revealed an increase of less than 1 MN-PCEs at 24 h after treatment in the group treated with green tea polyphenol extracts (Table 1), and the calculated NIF value corroborates the nongenotoxic effect of these extracts (Figure 3). It has been reported that the administration of polyphenol or flavonoids from green tea does not induce genotoxicity; additionally, the administration of these compounds over a long period of time at high doses in experimental animals has no effect on genotoxicity [4, 39, 40].

The *in vivo* administration of green tea polyphenol extracts prior to CrO_3 injection decreased MN-PCEs formation by 121%, 69% and 100% at 24, 48, and 72 h after treatment, respectively, compared to MN-PCEs formation in the group treated with CrO_3 alone (Figure 3). This result demonstrates that green tea polyphenol extracts protected cells against Cr (VI)-induced genetic damage more effectively than the administration of green tea *ad libitum* [25]. Due to the phenolic structure of green tea polyphenol extracts (Figure 1), it is possible that these flavonoids may act as hydrogen donors to suppress the formation of lipid radicals and FRs, including the superoxide and hydroxyl radicals generated by the Fenton reaction. These flavonoids may also chelate metals via their ortho-hydroxy-phenolic groups [41, 42]. Therefore, therapeutic agents that enhance intra- and extracellular antioxidant levels and block the Cr (VI)-mediated generation of ROS and FRs may prevent or attenuate Cr (VI)-induced genotoxicity.

In our experiments, the average number of apoptotic viable cells was increased at 48 h after treatment (Table 2). This increase was observed in all treatment groups compared to the negative control, and this finding supports the previously reported observations following Cr (VI) treatment [43–45]. The alteration of intracellular oxidative states has a potential to trigger or sensitize a cell to undergo apoptosis; thus, the ROS generated from Cr (VI) during its reduction plays an important role in the apoptotic signaling pathway [45, 46]. The administration of green tea polyphenol extracts alone led to an increase in the average number of apoptotic viable cells (approximately 4), but this increase was not statistically significant. Other studies have shown that green tea polyphenols such as EGCG and ECG inhibit the growth of the human lung cancer (cell line PC-9) [47]. The study further demonstrated that growth inhibition was accompanied by cell cycle arrest at the G2/M phase [48], which could be related to the apoptotic activity of these polyphenols. The induction of apoptosis by catechins has also been demonstrated in human lymphoid leukemia cells, human epidermoid carcinoma cells, human carcinoma keratinocytes, and human prostrate carcinoma cells [3, 49, 50]; thus, apoptosis plays an essential role as a protective mechanism against carcinogenesis by eliminating genetically damaged cells.

When the green tea polyphenol extracts were administered prior to the injection of CrO_3, the average number of apoptotic viable cells was increased to a level that was higher than that observed following CrO_3 treatment alone, but these interactions have shown that the effects are not additive or antagonistic (Table 2). Gao et al. [51] observed that ascorbic acid could enhance the EGCG and theaflavin-3-30-digallate induced apoptosis in human lung adenocarcinoma SPC-A-1 cells and esophageal carcinoma Eca-109 cells. Other studies have shown that the apoptosis-inducing activity of EGCG in human lung adenocarcinoma (PC-9 cells) can be synergistically enhanced by combined treatment with chemopreventive agents (including sulindac, cisplatin, and tamoxifen) [3, 52, 53]. The enhanced induction of apoptosis following a combined treatment suggests that this process may contribute to eliminate the cells with damaged DNA induced by Cr (VI).

In summary, the current study demonstrates that administration of CrO_3 via i.p. injection of mice could induce DNA damage in peripheral blood and apoptosis, and these effects were time dependent. ROS and FRs formation may play an essential role in Cr (VI)-mediated DNA damage and apoptosis *in vivo*. Moreover, the polyphenol extracts derived from green tea are capable of reducing genotoxic damage induced by Cr (VI). The greatest degree of protection was observed at 24 h after injection of CrO_3. The magnitude of protection is given in the following order: 121% at 24 h, >69% at 48 h, and >100% at 72 h after injection. Based on these results, polyphenol extracts from green tea can effectively protect against genotoxic damage in mice treated with Cr (VI). The beneficial effects of green tea polyphenol extracts could result from the inhibition of ROS and FRs chain reactions generated by the oxidative stress caused by Cr (VI) and by the extract's apoptotic activity.

There is limited evidence demonstrating that the regular consumption of green tea may reduce genotoxic damage. Therefore, this study contributes *in vivo* evidence showing that green tea polyphenol extracts can protect against genotoxic damage induced by carcinogens related to oxidative stress, such as Cr (VI) compounds.

Abbreviations

AO: Acridine orange
Cr (VI): Hexavalent chromium
EB: Ethidium bromide
EC: Epicatechin
ECG: Epicatechin-3-gallate
EGC: Epigallocatechin
EGCG: (−)-epigallocatechin-3-gallate
FDA: Food and Drug Administration
FRs: Free radicals

GC: (+) gallocatechin
i.p.: Intraperitoneal
MNNG: N-methyl-N'-nitro-N-nitrosoguanidine
MN-PCEs: Micronucleated polychromatic erythrocytes
NCEs: Normochromatic erythrocytes
NIF: Net induction frequency
PCEs: Polychromatic erythrocytes
ROS: Reactive oxygen species.

Conflict of Interests

The authors of the paper declare that they have no direct financial relationship with the commercial identities mentioned in the present paper that might lead to a conflict of interests.

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Research Article

Quercetin Induces Mitochondrial Mediated Apoptosis and Protective Autophagy in Human Glioblastoma U373MG Cells

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Quercetin is a dietary flavonoid with known antitumor effects against several types of cancers by promoting apoptotic cell death and inducing cell cycle arrest. However, U373MG malignant glioma cells expressing mutant p53 are resistant to a 24 h quercetin treatment. In this study, the anticancer effect of quercetin was reevaluated in U373MG cells, and quercetin was found to be significantly effective in inhibiting proliferation of U373MG cells in a concentration-dependent manner after 48 and 72 h of incubation. Quercetin induced U373MG cell death through apoptosis, as evidenced by the increased number of cells in the sub-G1 phase, the appearance of fragmented nuclei, decreased mitochondrial membrane potential, proteolytic activation of caspase-3 and caspase-7, an increase in caspase-3 and 9 activities, and degradation of poly(ADP-ribose) polymerase protein. Furthermore, quercetin activated JNK and increased the expression of p53, which translocated to the mitochondria and simultaneously led to the release of cytochrome c from mitochondria to the cytosol. We also found that quercetin induced autophagy. Pretreatment with chloroquine, an autophagy inhibitor, strongly augmented apoptosis in U373MG cells, indicating that quercetin induced protective autopagy in U373MG cells.

1. Introduction

Glioblastoma is the most common type of primary brain tumor in adults and the most lethal and least successfully treated tumor. The low absolute incidence combined with high morbidity, poor response rate, and short survival time poses practical problems for clinical trial execution [1]. Less than 30% of patients suffering from this devastating disease survive 12–15 months, even after receiving multimodal treatments such as surgical resection, combined chemotherapy and radiotherapy, and adjuvant chemotherapy [2]. These observations underscore the need for alternative therapies to prevent and effectively treat glioblastoma.

Quercetin is an antioxidative flavonoid ubiquitously distributed in plants. Its anticancer effects have been attributed to antioxidative activity, inhibition of enzymes activating carcinogens, modification of signal transduction pathways, and interactions with receptors and other proteins [3]. Quercetin is an anticancer agent in many cancer models [4–12]. Several studies have reported that quercetin increases the efficacy of glioblastoma treatment by suppressing the PI-3-kinase-Akt pathway [13], inducing apoptosis by reducing X-linked inhibitor of apoptosis protein (XIAP) [14], blocking signal transducer and activator of transcription 3 (STAT3) [15], arresting cells at the G2 checkpoint of the cell cycle, and decreasing the mitotic index in glioma cells [16]. Such effects of quercetin in glioblastoma cells seem to be dependent on cell type because combined application of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and quercetin strongly reduces viability of U87MG, U251, A172, and LN229 glioma cells but fails to reduce the viability of U373MG cells [17]. The cause of U373MG cell resistance to quercetin-TRAIL-mediated apoptosis is not fully understood. Inactivating p53 is not important in quercetin-TRAIL-mediated

apoptosis, as both sensitive U251 and the completely resistant U373MG cells have p53 mutations.

Apoptosis is programmed cell death mediated by caspases, which are cysteine proteases that cleave target proteins at aspartic acid. p53 is a transcription factor that induces the expression of proapoptotic genes [18, 19], and activating apoptosis is an important mechanism in p53-induced tumor suppression. Mitochondrial localization of mutant p53 and evidence linking p53 transcription-independent or mitochondria-targeted apoptosis has received considerable attention. A fraction of p53 translocates to mitochondria prior to changes in the mitochondrial membrane potential, cytochrome c release, and activation of caspases [20–22]. A previous study suggested that cellular cross-talk may occur between mitochondrial and nuclear p53. Heyne et al. [23] suggested that mutant p53 exists as a monomeric protein in mitochondria, and Tang et al. [24] demonstrated that mutant p53 translocates to mitochondria in UVB-irradiated murine skin carcinoma cells. Mahyar-Roemer et al. [25] suggested that mutant p53 is present in mitochondria independent of apoptotic signals. Mihara et al. [26] reported that p53 binds to Bcl-xL via its DNA-binding domain and that mutant p53 R273H cannot bind Bcl-xL and is therefore unable to activate the direct mitochondrial pathway of apoptosis despite being localized in or at the mitochondria. However, mutant p53 can also induce apoptosis through a transcription-independent pathway, in which wild-type p53 and p53 mutants are transiently located to the mitochondria with changes in the mitochondrial membrane potential [27]. Furthermore, a few such transcriptionally impaired p53 mutants (e.g., the structural mutant R175H and the DNA contact mutants R273H and C277F) bind Bak in vitro, and this is correlated with their ability to oligomerize Bak and induce cytochrome c release from isolated mitochondria [28]. We questioned whether mutant p53 R273H affects mitochondrial functions in human glioma blastoma cells.

Here, we addressed the question of whether autophagy is intimately linked with apoptosis induced by quercetin in U373MG cells. Autophagy is an evolutionarily conserved and genetically programmed process that degrades long-lived cellular proteins and organelles. The role of autophagy in cancer is quite complicated and controversial. Autophagy is assumed to be tumor suppressive during cancer development but to contribute to tumor cell survival during cancer progression [29]. Alternatively, autophagy prevents tumor cells from dying by inhibiting apoptosis during nutritional deprivation, and the cells undergo apoptosis when autophagy is prevented [30–32]. Regardless of whether they promote cell survival or cell death, the two processes engage in complex and poorly understood molecular cross-talk [33] and inducing apoptosis and inhibiting protective autophagy have become an effective means of cancer therapy. With the aim of exploring the effective anticancer activity of quercetin, we reevaluated the induction of cell death by quercetin at various concentrations and incubation times and examined the role of autophagy in quercetin-induced apoptosis in p53-mutant U373MG cells. Our results demonstrated for the first time that quercetin suppresses the proliferation of U373MG cells by modulating apoptotic and autophagic fluxes. Quercetin-induced cytotoxicity of U373MG cells was enhanced when autophagy was blocked before quercetin treatment. Thus, inhibiting autophagy must be considered a therapeutic approach to reduce U373MG proliferation and as a promising strategy to sensitize cells to quercetin treatment.

2. Materials and Methods

2.1. Reagents. Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), penicillin, streptomycin, Alexa Fluor 488 Goat anti-rabbit IgG(H+L), and Hoechst 33342 dye were purchased from Invitrogen Life Technologies, Inc. (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) and MTT were obtained from Amresco (Cleveland, OH, USA). Quercetin, chloroquine, propidium iodide (PI), acridine orange, and LC3II antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). JNK, phospho-JNK, p53, Beclin-1, cytochrome c, cleaved poly(ADP-ribose) polymerase (PARP), and caspase-3, -7, and -9 antibodies were acquired from Cell Signaling Technology (Beverly, MA, USA). Polyclonal anti-caspase-8 was purchased from R&D Systems (Minneapolis, MN, USA). The BD Mitoscreen (JC-1) kit was acquired from BD Biosciences (Franklin Lakes, NJ, USA). A BCA protein assay kit was obtained from Pierce (Rockford, IL, USA), and polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). Fluorescence Mounting Medium was purchased from DAKO (Ely, United Kingdom). All other reagents used were of analytical grade.

2.2. Cell Culture. Human glioblastoma U373MG cells were kindly provided by Professor Tae-Hoo Yi at the Department of Biotechnology, Kyunghee University, Republic of Korea [34]. The U373MG cells were cultured in DMEM containing 10% (v/v) heat inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained in a humidified 5% CO_2 incubator at 37°C.

2.3. Cell Viability. The effect of quercetin on U373MG cell viability was determined by an MTT-based assay [35]. Briefly, exponential-phase cells were collected and transferred to a microtiter plate (1×10^4 cells/mL). The cells were incubated with various concentrations of quercetin and/or chloroquine for 24, 48, and 72 h. Then, 0.1 mg of MTT was added to each well and incubated for 4 h at 37°C. The medium was removed, and DMSO (150 μL) was added to each well to dissolve the formazan crystals. The plates were read immediately at 570 nm on a Sunrise microplate reader (Tecan, Salzburg, Austria). The percentage of viable cells was calculated based on the following formula: mean value of (control group – treated group/control group) × 100%. All results were assessed in triplicate at each concentration.

2.4. Cellular Morphology, Nuclear Fragmentation, and Acidic Vesicular Organelles. U373MG cells were placed in 12-well plates at 3×10^4 cells/mL and treated with different concentrations of quercetin and/or pretreated with chloroquine. After

48 h, 10 μM Hoechst 33342, a DNA-specific fluorescent dye or acridine orange, a lysotropic dye, was added to each well, and the plates were incubated for 10 min at 37°C. The stained cells were observed under an Olympus fluorescence microscope (Tokyo, Japan).

2.5. Flow Cytometric Analysis. Cells (1×10^4 cells/mL) were plated in 60 mm plates and treated with quercetin (0–100 μM) for 48 h. Then, the cells were harvested, washed with phosphate-buffered saline (PBS), fixed in 70% ethanol, rehydrated in 2 mM EDTA-PBS, treated with RNase A (25 ng/mL), and stained with propidium iodide (40 μg/mL) for flow cytometry. The cells were stained with 10 μM acridine orange, harvested, and maintained in 2 mM EDTA-PBS containing 10% FBS to detect autophagy. We followed the manufacturer's protocol for JC-1 mitochondrial membrane detection. In brief, treated cells were trypsinized and washed with 1× assay buffer, stained with JC-1 for 15 min at 37°C in a CO_2 incubator, and washed twice with 1× assay buffer at room temperature. All analyses were performed using a FACScaliber flow cytometer (BD Biosciences). Data from 10,000 cells per sample were analyzed with CellQuest software (BD Biosciences). Each experiment was repeated at least three times.

2.6. Caspase Activity. Caspase-3 and -9 activities were measured using a colorimetric assay following the protocol of the commercially available kit from Sigma Chemical Co. and Biovision (San Jose, CA, USA), respectively. Briefly, cells were lysed after a 48 h quercetin treatment with or without chloroquine, and aliquots (10 μL) of the supernatant were placed in a 96-well microplate containing reaction buffer. Substrate was added, and the microplate was incubated at 37°C overnight. Activity was monitored as the linear release of *p*-nitroaniline from the substrate and compared with a linear standard curve generated on the same microplate.

2.7. Cellular Fraction and Immunoblot Analyses. U373MG cells were collected and washed twice with cold PBS after treatment with various quercetin concentrations. The cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) and kept in ice for 30 min. The lysates were centrifuged for 30 min at 13,000 rpm and 4°C, and the supernatants were stored at –70°C until use. Cytosolic and mitochondrial extracts were prepared using fraction lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM $Na_2H_2PO_4$, 250 mM sucrose, 1 mM EDTA, and 350 μg/mL digitonin). Lysed cells were kept in ice for 10 min and then centrifuged for 15 min at 15,000 rpm and 4°C. The supernatant was the cytosolic fraction. After the pellet was washed with lysis buffer, it was lysed in lysis buffer to prepare whole lysates. Protein concentration was measured using a BCA Protein Assay kit. Aliquots of the lysates (30–70 μg protein) were separated via 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane using glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.8, and 20% methanol, v/v). After blocking with 5% nonfat dried milk, the membranes were incubated for 4 h with primary antibodies, followed by an additional 30 min incubation with secondary antibodies in milk containing Tris-buffered saline and 0.1% Tween 20. Human anti-caspase-3, -caspase-7, -caspase-8, cleaved PARP, cytochrome c, JNK, phospho-JNK, p53, HSP60, LC3II, and Beclin-1 antibodies were used at a 1 : 1,000 dilution as the primary antibodies, and horseradish peroxidase-conjugated goat antihuman IgG was utilized as the secondary antibody at a 1 : 5,000 dilution. The membranes were then exposed to X-ray film. Protein bands were detected using the WEST-ZOL plus Western blot detection system (Intron, Gyeonggi-do, Republic of Korea).

2.8. Statistical Analysis. All results are expressed as means ± standard deviations. A one-way analysis of variance was conducted using the SPSS ver. 12.0 for Windows, 2004 package (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant. All assays were performed in triplicate.

3. Results and Discussion

3.1. Quercetin Induces Apoptotic U373MG Cell Death. The MTT assay was performed to investigate the cytotoxic effects of quercetin on human glioblastoma U373MG cells. As shown in Figure 1(a), a 48 or 72 h incubation with quercetin reduced U373MG cell viability in a dose-dependent manner. A significant decrease in cell viability was observed after 48 (27.63%) and 72 h (20.52%) of incubation at a concentration of 200 μM, whereas cell viability after a 24 h incubation was 59.17% (Figure 1(b)). Various morphological changes such as cell shrinkage and condensation as well as chromatin fragmentation were clearly noted during apoptotic cell death. U373MG cells treated with various concentrations of quercetin were examined by fluorescence microscopy after Hoechst 33342 staining to evaluate the effect of quercetin on the induction of apoptosis. As depicted in Figure 1(b), cells showed marked morphological changes such as condensed and fragmented chromatin and the formation of apoptotic bodies after treatment with 25, 50, 75, and 100 μM quercetin. Furthermore, the induction of apoptosis was indicated by the accumulation of sub-G1-phase U373MG cells after quercetin treatment. A significant increase in sub-G1-phase cells was not observed in the 24 h treatment (from 1.02% at 0 μM to 2.05% at 100 μM). However, quercetin significantly increased the sub-G1 population concentration dependently after the 48 h treatment (from 0.46% at 0 μM to 8.48% at 100 μM; Table 1). The mitochondrial membrane potential was disrupted as a result of apoptosis (Figure 1(c)) in dose-dependent manner. Because caspases-9 and -3 are key executioners of apoptosis, we quantified their enzymatic activities using a commercially available kit. As a result, quercetin dose-dependently increased caspase-9 and -3 activities (Figures 1(d) and 1(e)). These results indicate that quercetin induces apoptosis in U373MG cells via the intrinsic pathway by activating caspase-3 and caspase-9.

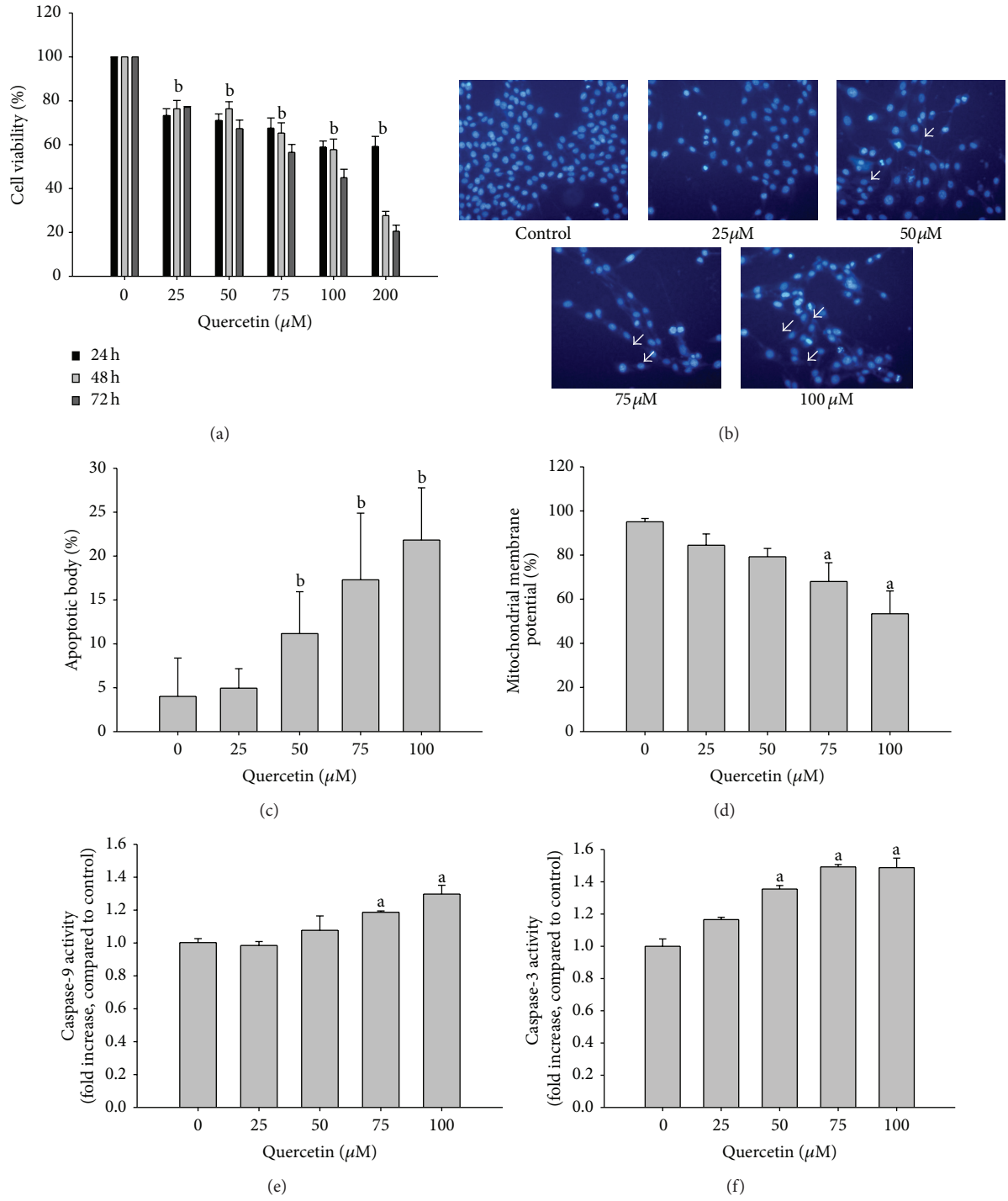

Figure 1: Quercetin inhibits cell growth and induces apoptosis. (a) Cell viability was determined by MTT reduction. U373MG human glioblastoma cells were treated with increasing doses of quercetin for different lengths of time (24–72 h). Data are the mean ± standard error for one experiment performed in triplicate. Values are the mean ± standard deviation (SD) of three independent experiments. $^{b}P < 0.01$ compared to the control. (b) Treated cells were stained with nuclear Hoechst 33342 and visualized under a fluorescence microscope after a 48 h treatment. White arrows indicate apoptotic bodies. Representative areas were photographed with 200X magnification. (c) Graph for quantification of number of apoptotic bodies. Data are the mean ± standard error for one experiment performed in triplicate. $^{b}P < 0.01$ compared to the control. (d) Flow cytometry analysis of JC-1 staining. Cells were trypsinized, stained with JC-1, washed, and analyzed by flow cytometry. The decrease in mitochondrial membrane potential percentage indicates that cells were undergoing mitochondrial dysfunction. (e) Caspase-9 and (f) caspase-3 activities in U373MG cells. Caspase activities were measured by a colorimetric assay and calculated as fold changes compared to the same control. The cells were treated with quercetin for 48 h ((b)–(f)). All data are the mean ± SD of three independent experiments. a: significantly different from the control, $P < 0.05$.

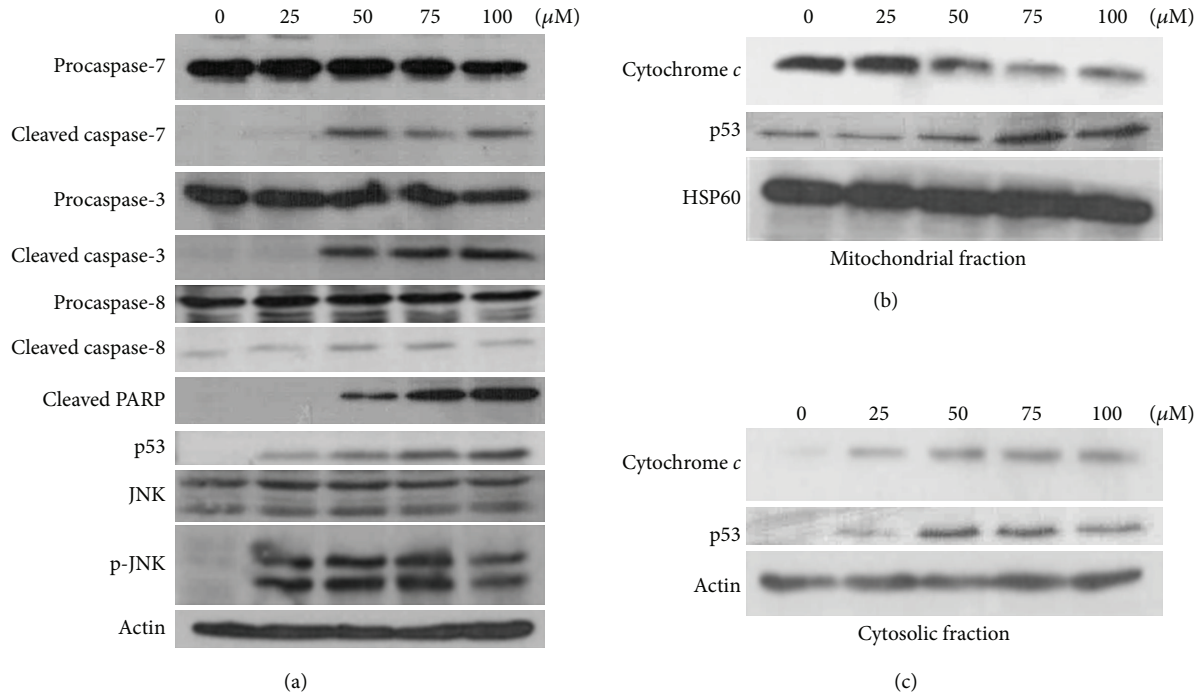

Figure 2: Expression levels of apoptosis-related proteins were analyzed by immunoblotting. (a) Cellular proteins, (b) mitochondrial fraction, and (c) cytosolic fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were probed with the indicated primary antibodies and then with horseradish peroxidase conjugated goat anti-rabbit IgG. Heat shock protein (HSP)60 was used as the loading control for the mitochondrial proteins (b). Actin was used as the internal control (a) and loading control for the cytosolic proteins (c).

Table 1: The percentage of U373MG cells in the sub-G1 fraction after treatment with different doses of quercetin for 24 and 48 h.

Concentration (μM)	24 h Sub-G1 phase (%)	48 h Sub-G1 phase (%)
Control	1.02 ± 0.35	0.5 ± 0.31
25	1.78 ± 0.89	1.71 ± 0.53
50	2.33 ± 1.17	2.26 ± 0.86
75	1.65 ± 0.65	6.66 ± 0.79[a]
100	2.05 ± 0.93	8.48 ± 0.40[a]

All data are the mean ± SD of three independent experiments. [a]Significantly different from the control, $P < 0.05$.

3.2. Effects of Quercetin on Apoptosis-Related Protein Expression. The expression of apoptosis-related proteins and activation of caspases following quercetin treatment were examined by Western blotting to further determine the mechanism of quercetin-induced apoptosis. The protein level of the caspase-7 and -3 precursor decreased, and proteolytically cleaved caspase-7 and -3 as well as PARP levels increased, although not much change was observed in the caspase-8 level (Figure 2(a)). JNK was activated, and p53 protein and phosphorylation levels dose-dependently increased following quercetin treatment (Figure 2(a)). JNK is one of the mitogen-activated protein kinases (MAPKs) that regulate cell proliferation, differentiation, and apoptosis [36]. JNK directly or indirectly modulates the expression levels of p53 and its target genes [37]. Evidence suggests that p53 induces cell death by several molecular pathways involving the activation of target genes and transcriptionally independent direct signaling [21]. In our study, p53 expression increased concentration dependently, but the level of the p53 downstream protein, PUMA, remained unchanged (data not shown) because U373MG is a p53 mutant cell line. Our result suggests that mutant p53 in U373MG cells can induce apoptosis through a transcription-independent pathway, as the level of cytochrome c increased in the cytosolic fraction, whereas the level of cytochrome c decreased in the mitochondrial fraction (Figures 2(b) and 2(c)) with a decrease in mitochondrial membrane potential (Figure 1(c)). Although additional experiments are required to determine whether the p53-Bcl-2/xL interaction occurs to fully understand quercetin-induced death of the p53 mutant glioblastoma-derived cell U373MG cells, we speculate that our results are similar to previous data in C33A cells in which the p53 mutant was transiently located to the mitochondria with changes in the mitochondrial membrane potential [27].

3.3. Quercetin Induces Autophagy in U373MG Cells. We examined the effects of quercetin on other cellular responses associated with cell death to better understand its anticancer effect. Acridine orange staining was used to analyze the formation of acidic vesicular organelles (AVOs) or autophagolysosome vacuoles, which occur as a result of fusion between autophagosomes and lysosomes as a key feature of autophagy [38]. Large numbers of AVOs were detected in

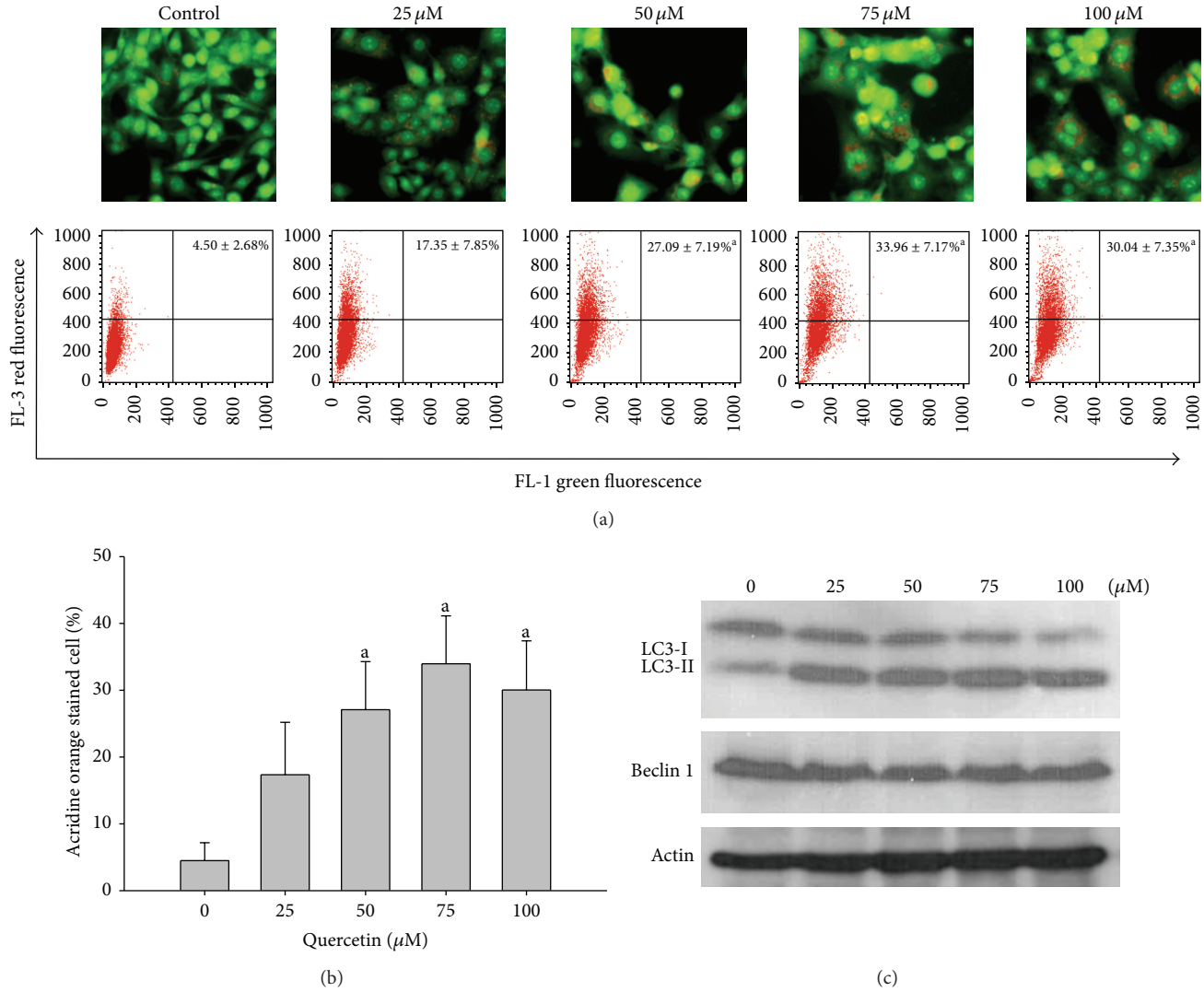

FIGURE 3: Quercetin induces autophagy in U373MG cells. The cells were treated with various concentrations of quercetin for 48 h. (a) Analysis of the formation of autophagosomes and autolysosomes by fluorescence microscopy and flow cytometry. Representative areas were photographed with 200X magnification. (b) Quantification of acridine orange-positive cells. (c) Western blotting using antibodies specific for LC3, Beclin-1, and actin. Results shown are representative of three independent experiments. Images were captured using a fluorescence microscope. Results shown are representative of at least three replicates. All data are the mean ± standard deviation of three independent experiments. a, significantly different from the control, $P < 0.05$.

U373MG cells treated with quercetin (Figure 3(a)). The FACS analysis showed that AVOs formed in 17.35% of U373MG cells treated with 25 μM quercetin and in 33.9% of U373MG cells treated with 75 μM quercetin and that a slight decrease (30.04%) occurred in U373MG cells treated with 100 μM quercetin (Figure 3(b)). These data are consistent with the results of our Western blot analysis of the autophagy marker protein LC3. Conversion of the lipidated form of LC3 (LC3-I) to LC3-II, which is an autophagosomal marker, is due to localization and aggregation of LC3-II in autophagosomes [39]. Quercetin induced the processing of full-length LC3-I (18 kDa) to LC3-II (16 kDa) dose-dependently at concentrations up to 75 μM, but a slight decrease was observed in 100 μM treated cells (Figure 3(c)). These results indicate that autophagy is less predominant in U373MG cells treated with a high concentration of quercetin. In contrast to LC3, Beclin-1 expression remained unchanged (Figure 3(c)). Although Beclin-1 plays an important role in autophagy, several studies have revealed that autophagy can occur in a Beclin-1-independent manner [40, 41]. We analyzed the cells stained with fluorescent LC3 antibodies to confirm that quercetin induced autophagy. Quercetin reportedly can induce autophagy in many different cell lines such as gastric, bladder, and colon cancer cell lines [6, 42, 43]. However, a recent study reported that quercetin significantly induces apoptosis but has no effect on inducing autophagy in the T98G glioma cell line [44], highlighting the heterogeneity of glioma cell biology; therefore, comprehensive multicell line studies are needed. P53 typically regulates autophagy via transcription-dependent and -independent mechanisms. A

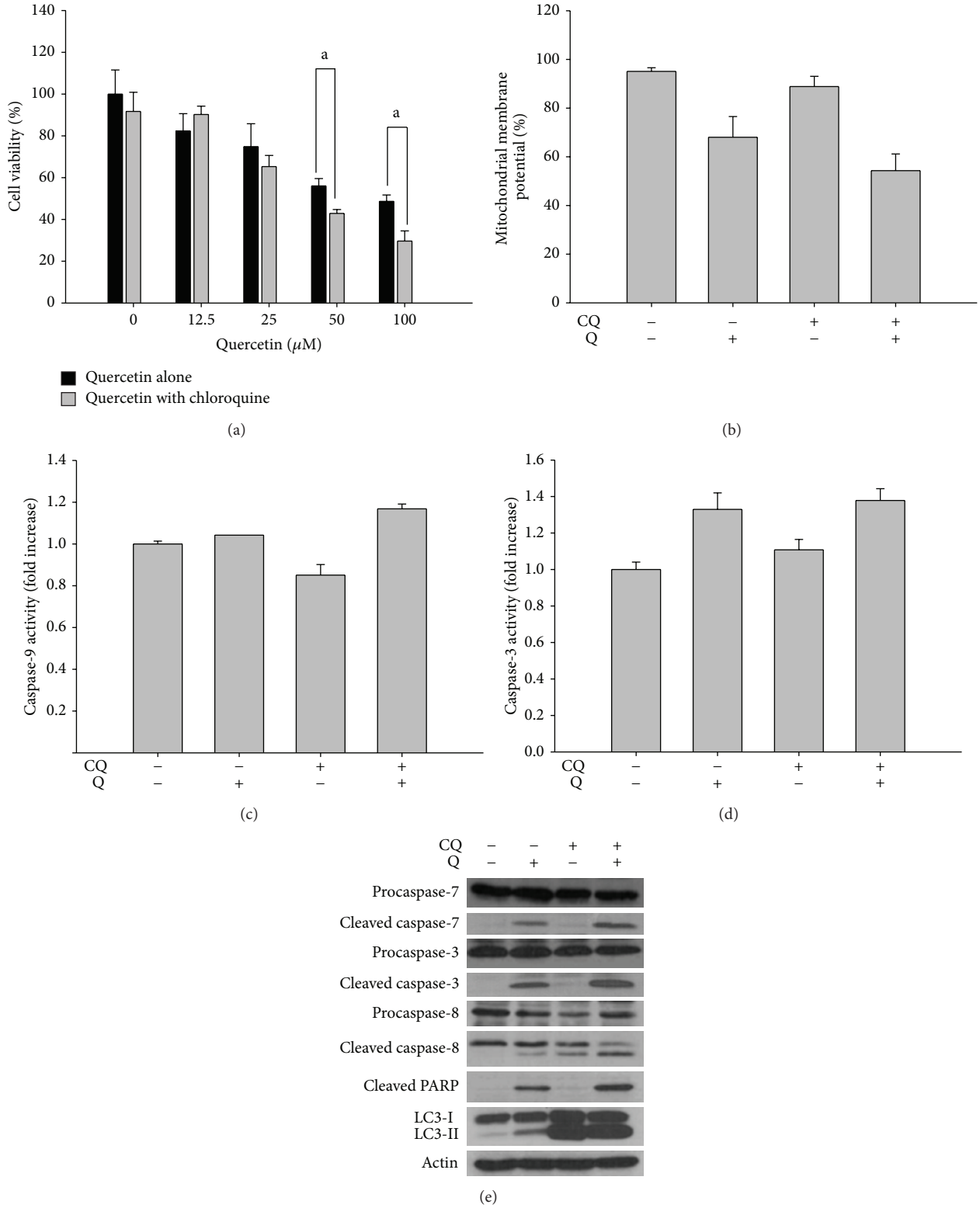

Figure 4: Inhibition of autophagy by chloroquine enhances apoptotic cell death. Cells were treated with 50 μM chloroquine for 2 h before quercetin treatment. (a) Cell viability was measured by the MTT assay. Values are the mean ± standard deviation (SD) of three independent experiments. a: significantly different from the control, $P < 0.05$; b: significantly different from the control, $P < 0.01$. (b) The percentage of cells undergoing mitochondrial dysfunction. Treated cells were trypsinized, stained with JC-1, washed, and analyzed by flow cytometry. (c) Caspase-9 and (d) caspase-3 activities. All data are the mean ± SD of three independent experiments. a: significantly different from the control, $P < 0.05$. (e) Analysis of apoptosis-related protein and LC3II expression by Western blotting. CQ: 50 μM chloroquine; Q: 75 μM quercetin.

Table 2: The percentage of U373MG cells in the sub-G1 fraction after treatment with 75 μM quercetin alone for 48 h, 50 μM chloroquine alone for 2 h, or quercetin for 48 h plus pretreatment with chloroquine for 2 h.

Treatment	Sub-G1 phase (%)
Control	2.21 ± 1.1
Quercetin alone	1.45 ± 0.21
Chloroquine alone	5.18 ± 0.96
Chloroquine + quercetin	18.68 ± 3.02

spectrum of p53 target genes such as DRAM and AMPK positively regulates autophagy in a transcription-dependent manner [45]. However, several studies have reported that cytoplasmic p53 inhibits autophagy in a transcription-independent manner through a poorly investigated mechanism [46–48]. Further investigation is required to determine whether increase in the cytosolic mutant p53 following quercetin treatment in U373MG cells represses autophagy and promotes apoptosis through translocation to mitochondria.

3.4. Inhibition of Autophagy by Chloroquine Promotes U373MG Cell Apoptosis. Several studies have shown that autophagy and apoptosis interact. Some proteins, already known as autophagy proteins, have dual functions in autophagy and apoptosis [4, 35]. To further analyze whether the autophagy signal induced by quercetin is prosurvival or prodeath, we treated U373MG cells with chloroquine, an inhibitor of autophagy, for 2 h before quercetin treatment. Chloroquine induces apoptosis in glioma cell lines via the p53 pathway [49]. The chloroquine treatment alone showed no effect on cell viability but a combined treatment with quercetin decreased cell viability compared to that of quercetin treatment alone (Figure 4(a)). Furthermore, pretreatment with chloroquine plus quercetin resulted in significant sub-G1 phase cell cycle arrest; the percentage of sub-G1 cells was 1.45%, 5.18%, and 18.68%, in U373MG cells treated with quercetin alone, chloroquine alone, and pretreated with chloroquine plus quercetin, respectively (Table 2). The mitochondrial membrane potential values were 68.0% and 54.3% following treatment with 75 μM quercetin alone and after pretreatment with chloroquine plus quercetin, respectively (Figure 4(b)). Caspase-9 and -3 activities increased slightly in the combined treatment compared to those of quercetin treatment alone (Figures 4(c) and 4(d)). Western blot results showed a similar tendency in the cell cycle distribution. We also confirmed that inhibiting autophagy promoted apoptosis by increasing cleavage of caspases-3 and -7 and PARP (Figure 4(e)). In particular, the proteolytic cleavage of procaspase-8, which was not detected following quercetin treatment alone, rose after chloroquine pretreatment. Taken together, these data strongly suggest that inhibiting autophagy enhances apoptotic cell death induced by quercetin in U373MG cells.

4. Conclusion

We demonstrated that quercetin induced cell death in the human glioblastoma U373MG cell line through an apoptotic pathway, which was confirmed by cleavage of caspases, PARP, and sub-G1 phase cell cycle arrest. Quercetin also activated JNK and modulated p53 expression accompanied by increased translocation of p53 to the mitochondria. The induction of autophagy in U373MG glioblastoma cells by quercetin was confirmed through acridine orange staining and conversion of LC3II. Furthermore, pretreatment with chloroquine enhanced intrinsic and extrinsic apoptotic cell death induced by quercetin, indicating that quercetin induced protective autophagy in U373MG cells. These results suggest that quercetin, in a combined treatment with an autophagy inhibitor, may be an excellent therapeutic approach to reduce U373MG proliferation and could be a promising strategy to sensitize cells to quercetin treatment.

Acknowledgments

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Review Article

Renal Protective Effects of Resveratrol

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Resveratrol (3,5,4′-trihydroxystilbene), a natural polyphenolic compound found in grapes and red wine, is reported to have beneficial effects on cardiovascular diseases, including renal diseases. These beneficial effects are thought to be due to this compound's antioxidative properties: resveratrol is known to be a robust scavenger of reactive oxygen species (ROS). In addition to scavenging ROS, resveratrol may have numerous protective effects against age-related disorders, including renal diseases, through the activation of SIRT1. SIRT1, an NAD^+-dependent deacetylase, was identified as one of the molecules through which calorie restriction extends the lifespan or delays age-related diseases, and this protein may regulate multiple cellular functions, including apoptosis, mitochondrial biogenesis, inflammation, glucose/lipid metabolism, autophagy, and adaptations to cellular stress, through the deacetylation of target proteins. Previous reports have shown that resveratrol can ameliorate several types of renal injury, such as diabetic nephropathy, drug-induced injury, aldosterone-induced injury, ischemia-reperfusion injury, sepsis-related injury, and unilateral ureteral obstruction, in animal models through its antioxidant effect or SIRT1 activation. Therefore, resveratrol may be a useful supplemental treatment for preventing renal injury.

1. Introduction

Chronic kidney disease (CKD), which is characterized by a chronic reduction in the glomerular filtration rate (GFR) and the presence of proteinuria or albuminuria, is recognized as an independent risk factor for both end-stage renal disease (ESRD) and cardiovascular disease, leading to a decrease in quality of life and an increased risk of mortality [1]. Acute kidney injury (AKI) is common in the setting of critical illness and is associated with a high risk of death [2]. In addition, AKI can directly cause ESRD and can increase the risks of the development of incident CKD and the worsening of underlying CKD [3]. Therefore, additional treatment to prevent both chronic and acute kidney injury is necessary.

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenolic phytoalexin that occurs naturally in many plant parts and products, such as grapes, berries, red wine, and peanut skins [4], and has numerous beneficial health effects. Previous epidemiological studies have revealed an inverse correlation between red wine consumption and the incidence of cardiovascular disease, a phenomenon known as the "French Paradox." The French population has relatively low rates of cardiovascular disease despite traditionally eating a diet rich in saturated fat [5]. Resveratrol, which is present in red wine, has been postulated to explain the protective effects on the cardiovascular system observed in the French Paradox, and the effects of this compound are exerted through several mechanisms, including antioxidant effects [6]. SIRT1, an NAD^+-dependent deacetylase, has been identified as one of the molecules through which calorie restriction (CR) extends the lifespan and delays age-related diseases [7–9]. The activation of SIRT1 exerts cytoprotective effects through multiple mechanisms, such as antiapoptosis, antioxidative, and anti-inflammation effects and the regulation of mitochondrial biogenesis, autophagy, and metabolism in response to the cellular energy and redox status [10]. Resveratrol has been shown to be a SIRT1 activator [11], and numerous previous studies have shown that the administration of resveratrol can prevent many diseases, such as diabetes, neurodegenerative disorders, cognitive disorders, cancer, kidney diseases, and cardiovascular disease through SIRT1 activation [9, 10, 12]. Thus, resveratrol exerts its cytoprotective effects through at least two mechanisms, antioxidant activity and SIRT1 activation (Figure 1). In the present review, we summarize the

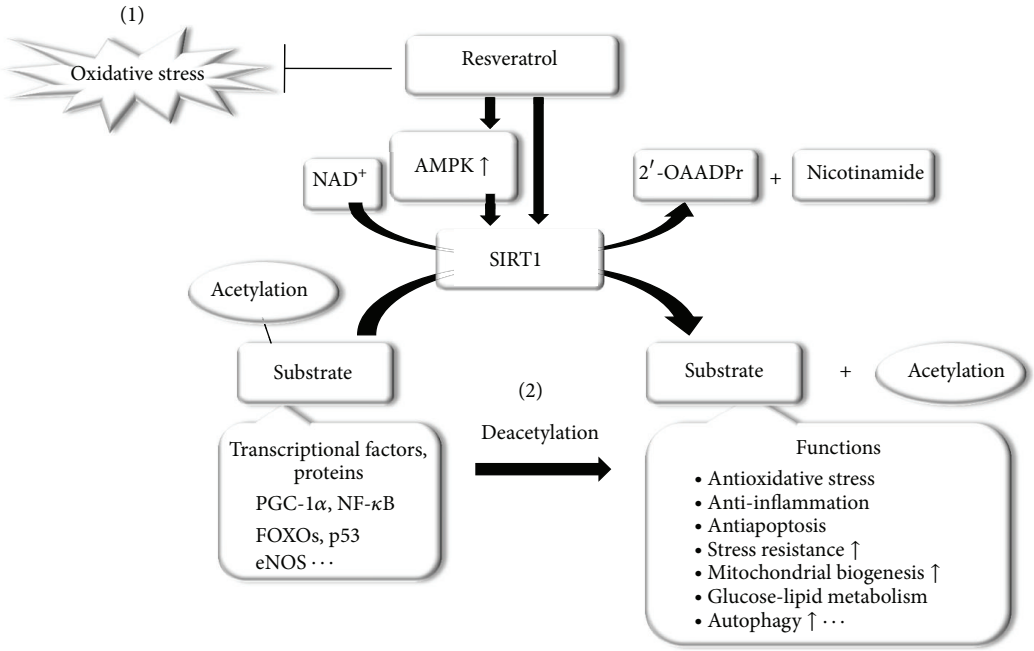

Figure 1: Proposed mechanisms by which resveratrol exerts cytoprotection. (1) Resveratrol attenuates oxidative stress. (2) Resveratrol activates SIRT1, which is an NAD^+-dependent deacetylase, directly or indirectly via AMPK activation. SIRT1 plays an important role in the regulation of oxidative stress, inflammation, apoptosis, stress resistance, mitochondrial biogenesis, autophagy, and glucose-lipid metabolism, via the deacetylation of many substrates.

protective effects of resveratrol against several types of renal injury and discuss the mechanisms involved.

2. Mechanisms of the Cytoprotective Effects of Resveratrol

2.1. Resveratrol as an Antioxidant. An excess of reactive oxygen species (ROS) is involved in a variety of diseases, the aging process, and numerous cellular response pathways [13, 14]. ROS include superoxide (O_2^-), the hydroxyl radical ($OH^\bullet$), and peroxynitrite ($ONOO^-$), and these compounds attack cellular proteins and DNA. Oxidative stress is induced by an imbalance between ROS production and antioxidant defenses; therefore, exogenous antioxidants or the modulation of antioxidant enzymes can be expected to reduce oxidative stress. Resveratrol is a natural antioxidant. Previous studies have shown that resveratrol can directly scavenge ROS, such as O_2^-, $OH^\bullet$, and $ONOO^-$ [15, 16]. In addition to scavenging ROS, exogenously administered resveratrol modulates the expression and activity of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase (GPx), and catalase, through transcriptional regulation via nuclear factor E2-related factor 2 (Nrf2), activator proteins (AP)-1, forkhead box O (FOXO), and SP-1 or through enzymatic modification [17–20].

2.2. Resveratrol Activates SIRT1. Aging is a universal process that affects all organs, and age-related disruptions in cellular homeostasis result in a reduction in responsiveness to physiological stress and organ dysfunction. Numerous studies have revealed that CR retards aging or extends the lifespans of yeast, worms, flies, and rodents [7]. Colman et al. also reported that CR delayed the onset of age-associated pathologies, including diabetes, cancer, cardiovascular disease, and brain atrophy and decreased mortality in rhesus monkeys [21]. In addition, Fontana et al. showed that CR for an average of 6 years improved metabolism in humans, as measured by the levels of serum insulin, cholesterol, C-reactive protein (CRP), and tumor necrosis factor (TNF)-α as well as the thickness of the carotid intima media [22]. This group also observed that long-term CR ameliorated declines in left ventricular diastolic function and decreased the levels of serum tumor growth factor (TGF)-β1, TNF-α, and high-sensitivity CRP [23]. Thus, CR induces these antiaging effects by improving insulin sensitivity and reducing inflammation and oxidative stress, and CR is accepted as the only established experimental antiaging paradigm.

Based on initial studies on aging in yeast, silent information regulator 2 (Sir2), an NAD^+-dependent deacetylase, was identified as one of the molecules through which CR extends the lifespan and delays age-related diseases [24]. Homologues of Sir2 in higher eukaryotic organisms are known as sirtuins. SIRT1, the sirtuin most closely related to Sir2, is one of seven sirtuins in mammals [9]. The beneficial effects of CR involve the function of SIRT1, which is induced by CR in various tissues [21]. The importance of SIRT1 in the effects of CR has been demonstrated using genetically altered mice [25, 26]. SIRT1 is an important regulator of a wide variety of cellular processes, including stress responses, cell survival, mitochondrial biogenesis, and metabolism in

response to the cellular energy, as well as the redox status, via the deacetylation of many substrates [9, 12]. Therefore, SIRT1 activators are expected to function as CR mimetics, and the screening of compounds for their ability to activate SIRT1 led to the discovery of 18 small molecules, including resveratrol [11]. Resveratrol can activate SIRT1 through multiple mechanisms. Although resveratrol was originally thought to directly activate SIRT1 through an allosteric effect, AMPK is required for the activation of SIRT1 by resveratrol. AMPK plays an important role in the regulation of metabolism in response to the energy balance [27]. In addition, Park et al. found that resveratrol activates SIRT1 through the activation of AMPK via the inhibition of phosphodiesterase 4 (PDE 4) and the elevation of cAMP in cells, thereby providing a new mechanism to explain SIRT1 activation by resveratrol [28]. A recent study reported by Price et al. also demonstrated a direct link between SIRT1 and the metabolic benefits of resveratrol [29]. These authors reported that a moderate dose of resveratrol (25–30 mg/kg/day to mice treated with high fat diet) first activated SIRT1 and then induced the deacetylation of liver kinase B (LKB) 1 and the activation of AMPK, leading to increased mitochondrial biogenesis and function. In addition, a high dose of resveratrol (215–235 mg/kg/day to mice treated with high fat diet) may directly activate AMPK, independently of SIRT1. Moreover, Hubbard et al. demonstrated that sirtuin-activating compounds (STACs), including resveratrol, can increase the catalytic activity of SIRT1 toward certain substrates through an allosteric mechanism involving an amino terminal domain near the catalytic core and through direct binding to SIRT1 [30].

3. Renal Protective Effects of Resveratrol

3.1. Diabetic Nephropathy. Diabetic nephropathy is one of the more serious complications of diabetes and is the most common cause of ESRD. Oxidative stress has been implicated in the pathogenesis of diabetic vascular complications, including nephropathy [31]. Previous studies have clearly demonstrated that resveratrol can improve diabetic nephropathy in several animal models of types 1 and 2 diabetes through its antioxidative effects resulting from direct radical scavenging or the modulation of antioxidant enzymes.

Sharma et al. reported that treatment with resveratrol (5 mg or 10 mg/kg orally) for 2 weeks improved urinary protein excretion, renal dysfunction, and renal oxidative stress in streptozotocin- (STZ-) induced diabetic rats [32]. In addition, Palsamy and Subramanian reported that resveratrol treatment (5 mg/kg orally for 30 days) resulted in significant normalization of the creatinine clearance and the levels of plasma adiponectin, C-peptide, and renal oxidative stress and inflammation in STZ-nicotinamide-induced diabetic rats [33]. Furthermore, resveratrol treatment ameliorated the dysfunction of antioxidant enzymes, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione reductase (GR), and the reduction in the levels of vitamin C, vitamin E and reduced glutathione (GSH) in diabetic kidneys. In addition, they found that the expression levels of Nrf2 and its downstream enzyme, including γ-glutamyl cysteine synthetase (GCS), m-GST, and hemoxygenase-1 (HO-1) were significantly decreased in the renal tissues of diabetic rats. However, the administration of resveratrol modulates the expression of Nrf2 in the context of diabetes-induced oxidative stress by upregulating γ-GCS, m-GST, and HO-1. We also reported that resveratrol treatment (400 mg/kg, orally, administered at concentration of 0.3% resveratrol) alleviated albuminuria and histological mesangial expansion and reduced the increased levels of renal oxidative stress and inflammation in the kidneys of db/db mice through the scavenging of ROS and normalizing manganese (Mn)-SOD function by decreasing its levels of nitrosative modification [20]. Kim et al. demonstrated that resveratrol prevents diabetic nephropathy in db/db mice by the phosphorylation of AMPK and SIRT1-peroxisome proliferator-activated receptor γ coactivator (PGC)-1α signaling, which appear to prevent lipotoxicity-related mesangial cell apoptosis and oxidative stress in the kidney [34]. Zhang et al. showed that high glucose levels enhance mesangial cell proliferation and fibronectin expression through the c-Jun N-terminal kinase (JNK)/nuclear factor κB (NF-κB)/NADPH oxidase/ROS pathway, which was inhibited by resveratrol in cultured mesangial cells [35].

In addition to antioxidant effects, resveratrol has other properties that can ameliorate diabetes or high glucose-induced kidney injury by activating AMPK or SIRT1. Ding et al. reported that resveratrol treatment attenuates renal hypertrophy and urinary albumin excretion in the early stage of diabetes in STZ-induced diabetic rats without affecting the blood glucose levels [36]. They found that resveratrol activates AMPK and inhibits the phosphorylation of 4E-BP1 and S6 in diabetic kidneys. Moreover, in cultured mesangial cells, resveratrol has been shown to block the high glucose-induced dephosphorylation of AMPK and the phosphorylation of 4E-BP1 and S6, inhibiting both the DNA synthesis and proliferation. In addition, Lee et al. reported that resveratrol ameliorates high glucose-induced protein synthesis in glomerular epithelial cells [37]. Resveratrol increases AMPK phosphorylation and abolishes high glucose-induced reductions in the AMPK phosphorylation level. In addition, resveratrol inhibits the high glucose-induced phosphorylation of eIF4E, eEF2, eEF2 kinase, and p70S6 kinase, which have significant roles in the initiation and elongation steps of mRNA translation. Resveratrol prevents the high glucose-induced hyperacetylation of LKB-1, which is an upstream regulator of AMPK, leading to AMPK activation, and the deacetylation of LKB-1 is independent of SIRT1. However, Tikoo et al. showed that resveratrol (55 mg/kg, intraperitoneal injection) prevents the decrease in SIR2 expression and the increases in the p38MAPK and p53 levels and the dephosphorylation of histone H3 in the kidney of STZ-induced diabetic rats, suggesting that SIR2 is involved in the beneficial effects of resveratrol in the kidneys [38]. Furthermore, Wu et al. demonstrated that resveratrol has protective effects on diabetic kidneys by modulating the SIRT1/FOXO1 pathway [39]. They demonstrated that FOXO1 activity is reduced, with a concomitant decrease in the expression of catalase, a FOXO1 target gene, and that

SIRT1 expression decreased in the renal cortex of STZ-induced diabetic rats, resulting in enhanced renal oxidative stress. Treatment with resveratrol increased the renal FOXO1 activity, catalase expression, and SIRT1 expression, leading to a reduction in oxidative stress. Moreover, in cultured mesangial cells, Xu et al. demonstrated that resveratrol exerts protective effects on high glucose-induced mitochondrial oxidative stress and mitochondrial dysfunction [40]. All of these protective effects of resveratrol were blocked by the knockdown of SIRT1 and by EX-527, a specific inhibitor of SIRT1.

Chen et al. reported that resveratrol treatment improved diabetes-induced glomerular hypertrophy and urinary albumin excretion; reduced the expression of glomerular fibronectin, collagen IV, and transforming growth factor (TGF)-β; reduced the thickness of the glomerular basement membrane; and reduced nephrin expression in the kidneys of STZ-induced diabetic rats, possibly through the inhibition of the phosphorylation of Smad2, Smad3, and ERK1/2 [41]. However, the mechanism by which resveratrol inhibits Smad2, Smad3, and ERK1/2 phosphorylation remains unknown.

3.2. Drug-Induced Renal Injury Model. Cisplatin is a chemotherapeutic agent that is widely used to treat malignant tumors. As the most common adverse effect of cisplatin, nephrotoxicity is an important dose-limiting factor in cisplatin treatment. The nephrotoxicity of cisplatin is induced directly by DNA damage, inflammation, and oxidative stress in the proximal tubules ofthe S3 segment in the outer medulla and the corticomedullary region of the kidney [42]. Amaral et al. reported that pretreatment with resveratrol (25 mg/kg, intraperitoneal injection) attenuated signs of cisplatin-induced renal injury, such as tubular cell apoptosis and inflammation and renal dysfunction, by reducing the level of oxidative stress and inhibiting inflammation [43]. In addition, Kim et al. showed that SIRT1 activation by resveratrol reduces the cisplatin-induced acetylation of p53, apoptosis, and cytotoxicity in cultured mouse proximal tubular cells [44]. SIRT1 expression and activity after 3 days of cisplatin treatment have been shown to decrease in the kidneys; however, the administration of resveratrol ameliorated the decreases of SIRT1 activation and the glomerular filtration rate and the increases of tubular cell apoptosis and urinary Kim-1 excretion, which is induced by cisplatin.

Other studies have shown that resveratrol attenuated renal injury caused by several drugs, including glycerol [45, 46], gentamicin [47, 48], and cyclosporine [49], by reducing oxidative stress, as one of the mechanisms of the renal protective effect of resveratrol.

3.3. Aldosterone-Induced Kidney Injury. Aldosterone and its activation pathway through mineralocorticoid receptor contribute to podocytes injuries and progression of proteinuric kidney disease. Yuan et al. reported that SIRT1/PGC-1α axis in mitochondria ameliorated aldosterone-induced podocytes injuries [50]. They found that aldosterone suppressed SIRT1 and PGC-1α activation in cultured podocytes, resulting in increased podocytes apoptosis and the loss of slit diaphragm proteins, including nephrin and podocin, accompanied with mitochondrial dysfunction. SIRT1 activation protected against aldosterone-induced podocytes injuries with mitochondrial dysfunction, by inhibiting both apoptosis and loss of slit diaphragm proteins, through deacetylation and activation of PGC-1α. Treatment with resveratrol prevented aldosterone-induced podocytes apoptosis and mitochondrial dysfunction and restored expression of nephrin and podocin in vitro and vivo model, through activation of the SIRT1/PGC-1α axis.

3.4. Ischemia-Reperfusion and Sepsis-Induced Kidney Injuries. Renal ischemia is a common course of AKI. Reperfusion is essential for the survival of ischemic renal tissue; however, reperfusion also contributes to additional renal damages [51]. Oxidative stress plays a crucial role in ischemia-reperfusion injury of the kidney. Several studies have demonstrated that resveratrol exerts protective effects against ischemia-reperfusion injury in the kidneys, as well as the heart and brain injury, by reducing oxidative stress and several other mechanisms. Giovannini et al. reported that the pretreatment of rats with resveratrol (0.23 μg/kg) reduced the mortality rate of ischemic rats from 50% to 10% and reduced the extent of renal damage, as reflected by glomerular dysfunction, tubular cell necrosis, inflammatory cell infiltration, glomerular thrombosis, urinary IL-6 excretion, and oxidative stress [52]. Peroxynitrite ($ONOO^-$), which is generated by the reaction of NO with superoxide, is a powerful oxidizing RNS and causes protein nitration, DNA damage, and mitochondrial dysfunction, leading to endothelial and epithelial dysfunction. Treatment with NG-nitro-L-arginine methyl ester (L-NAME), which is a nitric oxide synthase inhibitor, abolished the effects of resveratrol on ischemic kidneys, suggesting that resveratrol protects the kidneys from ischemia-reperfusion injury through a nitric oxide-dependent mechanism. Chander and Chopra also showed that pretreatment with resveratrol (5 mg/kg) attenuates renal ischemia-reperfusion injury through NO release in rats [53]. Resveratrol may enhance the enzymatic activity of endothelial NOS (eNOS) through phosphorylation by AMPK [54] or deacetylation by SIRT1 [55], possibly leading to the production of NO and protecting vascular tissues, including the kidneys. The transcriptional activity of eNOS is also increased by resveratrol-induced FOXO activation via SIRT1 [56].

The development of AKI is a common complication during severe sepsis and more than doubles the mortality rate to nearly 75% [57]. When severe sepsis develops, the dysfunction of the renal microcirculation, which is induced by increased oxidative stress, especially as the result of reactive nitrogen species (RNS), contributes to the progression of AKI [58–60]. Holthoff et al. investigated the effects of resveratrol on sepsis-induced AKI using the cecal ligation and puncture (CLP) murine model [61]. Resveratrol restored the renal microcirculation and scavenged reactive nitrogen species, thus protecting the tubular cells in the kidney during sepsis. Furthermore, the administration of resveratrol to septic mice

at 6, 12, and 18 hr resulted in a significant improvement in survival compared with that of the vehicle-treated mice.

3.5. Obstructed Kidneys. Renal fibrosis is the hallmark of progressive renal disease and is recognized as the final common pathway of glomerular sclerosis and tubule-interstitial fibrosis. The unilateral ureteral obstruction (UUO) model is widely used to investigate the mechanisms of renal fibrosis [62]. The TGF-β/Smad3 signaling pathway plays a central role in the pathogenesis of renal fibrosis. Li et al. reported that resveratrol reversed the acetylation of Smad3 and inhibited the TGF-β-induced upregulation of fibrosis-related genes, such as collagen IV and fibronectin, through SIRT1 activation in the interstitial lesion of the obstructed kidney [63].

3.6. Aging Kidney. Aging causes progressive postmaturational deterioration of tissues and organs, leading to impaired tissue function, increased vulnerability to stress, and death. Kidney is one of the typical target organs of age-associated tissue damage, and the high incidence of CKD in the elderly is a health problem worldwide.

Kume et al. found that mitochondrial damage in aged kidneys is associated with a decrease in SIRT1 activation [64]. In the renal proximal tubular cells of aged mice, autophagy in response to renal hypoxia is decreased, resulting in renal dysfunction and histological renal fibrosis. CR-mediated renal SIRT1 activation deacetylates and activates FOXO3a transcriptional activity, leading to the recovery of Bnip3-mediated autophagy, even in aged kidneys. These findings indicate that SIRT1 is a crucial target in aging kidneys; therefore, resveratrol is expected to prevent renal aging.

4. Conclusions

Resveratrol can exert protective effects against both acute and chronic kidney injuries through its antioxidant effects and ability to activate SIRT1 (Figure 2). Therefore, resveratrol should be a useful additional treatment for preventing renal injury. However, it remains unclear whether resveratrol has beneficial effects on kidney diseases in humans and other animal models of renal diseases. In addition, a number of recent studies indicate that many of the protective effects of resveratrol could be mediated by SIRT1-independent mechanisms. Among them, the activation of mammalian target of rapamycin (mTOR) signaling pathway is involved in the pathogenesis for several kidney diseases, such as diabetic nephropathy [65–67] and the autosomal dominant polycystic kidney disease [68]. Liu et al. reported that RSV increases the association between mTOR and the DEP-domain-containing and mTOR-interactive protein (DEPTOR), an identified negative regulator of mTOR [69]. Therefore, resveratrol is expected to protect the kidney by the inhibition of mTOR pathway. Further studies are necessary to verify the beneficial effects of this compound in humans and other animals of kidney diseases and to clarify the detailed mechanism for the renal protective effect of resveratrol.

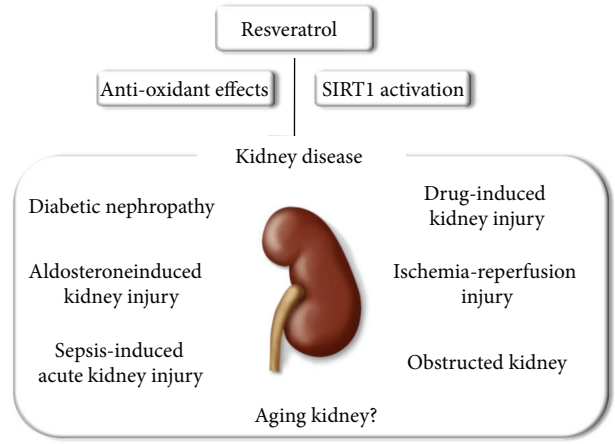

Figure 2: Resveratrol can prevent renal injury, including diabetic nephropathy, drug-induced renal injury, aldosterone-induced renal injury, ischemia-reperfusion injury, sepsis-induced kidney injury, and obstructed kidney, through its antioxidant effects and SIRT1 activation.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Natural Compounds as Modulators of NADPH Oxidases

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Reactive oxygen species (ROS) are cellular signals generated ubiquitously by all mammalian cells, but their relative unbalance triggers also diseases through intracellular damage to DNA, RNA, proteins, and lipids. NADPH oxidases (NOX) are the only known enzyme family with the sole function to produce ROS. The NOX physiological functions concern host defence, cellular signaling, regulation of gene expression, and cell differentiation. On the other hand, increased NOX activity contributes to a wide range of pathological processes, including cardiovascular diseases, neurodegeneration, organ failure, and cancer. Therefore targeting these enzymatic ROS sources by natural compounds, without affecting the physiological redox state, may be an important tool. This review summarizes the current state of knowledge of the role of NOX enzymes in physiology and pathology and provides an overview of the currently available NADPH oxidase inhibitors derived from natural extracts such as polyphenols.

1. ROS Involvement in Cell Pathophysiology

Oxidative stress is a molecular deregulation in reactive oxygen species (ROS) metabolism involved in the pathogenesis of several diseases. Oxidative stress is no longer considered as a simple imbalance between the production and scavenging of ROS, but as a dysfunction of enzymes involved in ROS production [1].

Reactive oxygen species such as superoxide, hydrogen peroxide, and peroxynitrite are generated by all mammalian cells and have been recognized for many decades as causing cell damage by oxidation and nitration of macromolecules, such as DNA, RNA, proteins, and lipids. Moreover, ROS can also promote cell signaling pathways modulated by growth factors and transcription factors, therefore regulating cell proliferation, differentiation, and apoptosis [2], which are important processes for proper cell functioning [3]. At physiological concentrations they facilitate the signal transduction derived from receptor tyrosine kinases and transcriptional factors such as NF-E2-related factor-2 (Nrf-2) leading to antioxidant gene expression [4].

The instability of an unpaired electron in its valence shell causes the high reactivity of superoxide. Superoxide has been implicated in numerous pathological processes, including cancer, cardiovascular disease (e.g., atherosclerosis and stroke), and acute and chronic diseases due to microbial infections. Superoxide can directly or indirectly damage DNA through oxidation [5], directly inactivate cellular antioxidants enzymes such as catalase and glutathione peroxidase [6], and activate proinflammatory nuclear factor jB (NF-jB) [7].

However, superoxide gives rise to other ROS that possess different redox chemistries, and, thus, different physiological and pathophysiological effects. For example, superoxide is rapidly reduced, both spontaneously and enzymatically, to H_2O_2. Unlike superoxide, H_2O_2 has no net charge; so, it is more lipid-soluble, with the potential to diffuse through organelles and cellular membranes reaching sites distant from its source. H_2O_2 modifies cellular proteins via oxidation of cysteine, methionine [8], and genetic material [9]. However, perhaps the major dangerous properties of H_2O_2 are in its ability to generate more reactive molecules. For instance, in the presence of transition metals, H_2O_2 can generate the highly reactive OH•. The OH• is highly reactive and will indiscriminately oxidize the nucleotides causing breaks and lesions of DNA [for review see [10]], which are processes involved in carcinogenesis. The oxidation of lipids by OH• may influence many physiological processes and contribute to cellular dysfunction, such as oxidation of lipids by peroxidation [11], during cardiovascular disease [12].

One of the most important and fast redox reactions in biology is between superoxide and the nitric oxide (NO) radical giving rise to $ONOO^-$. $ONOO^-$ is an oxidizing and nitrating molecule that has been implicated in cancer [13] and other acute [14] and chronic [15] diseases. ROS levels in tumor cells are controlled in a particular way, which stresses the importance of the development of novel ROS-targeted anticancer therapies.

As with every mechanism involved in both normal cell function and the development of disease, strategies to counteract ROS must take into account their critical importance in the normal functioning of the organism [1].

Further understanding of the biological mechanisms among oxidative stress, tumor growth, and metastasis could contribute to the advancement of cancer treatment.

For example, angiogenesis is another important factor for tumor growth and metastasis, and ROS has a key role in angiogenesis regulation [16].

After all, an emerging concept suggests that ROS modulate the immune cells functions that infiltrate the tumor environment and stimulate angiogenesis [2].

These oxidative processes have been implicated in many diseases in addition to cancer.

Overproduction of ROS is involved in the development of a number of diseases, which range from neurological such as Parkinson's [17] and Alzheimer's disease [18], to psychiatric disorders such as schizophrenia [19] and bipolar disorder [20], and to a majority of cardiovascular diseases [21].

2. NADPH Oxidases as ROS Sources

Several enzymes produce ROS, including the mitochondrial electron transport chain, nitric oxide synthases (NOSs), cytochrome P450 reductase, and xanthine oxidase. However, for all of these systems, ROS production takes place as a byproduct of the main catalytic function of the enzyme/system or from a dysfunctional variant of the enzyme. In contrast, NADPH oxidases are the only enzymes whose primary function is to generate superoxide/ROS [2]. NOX family proteins are the catalytic, electrontransporting subunits of the NADPH oxidase enzyme complex. The NOX family consists of seven members, NOX1–5, and two dual oxidases (Duox), Duox1 and Duox2 (Table 1). The NOX isoforms contain FAD and NADPH binding sites, two heme molecules and six transmembrane [22] spanning alpha helices with cytosolic N and C-termini. The Duox isoforms also contain the same domains; however, a seventh transmembrane domain and peroxidase homology are present.

They are differentially expressed and regulated in various tissues and have different subcellular localizations (reviewed in [23]). NOX1, NOX2, and NOX5 appear to produce mainly superoxide NOX4, mainly H_2O_2 [24]. All NOX isoforms have been reported to bind to one or more additional components. p22phox appears to be a general binding partner for NOX1–4. NOX1 and 2 also bind the small GTPase, Rac. Moreover, NOX1 binds the cytosolic subunits, NOX organizer 1 (NOXO1), and NOX activator 1 (NOXA1). NOX2 binds the respective homologues, p47phox and p67phox, and also the cytosolic protein, p40phox [22, 25]. NOX4 was reported to bind to the polymerase (DNA-directed) delta-interacting protein 2 (PolDip2) [26].

In addition to these established NOX binding partners, the tyrosine kinase substrate with 4/5 SH3 domains (Tks4/5) [27] and protein disulfide isomerase (PDI) were recently suggested to bind to both NOX1 and 4 [28]. Two maturation factors specific for Duox (DuoxA1 and DuoxA2) have also been described [22].

NOX catalytic subunits are differently regulated: NOXA1 plays a key role for NOX1 activation [29], p67phox for NOX2 [30], and calmodulin for NOX5 [31].

In contrast, NOX4 is constitutively active, and modulation of its expression may thus be a major activity regulator.

ROS produced by NOXs have been shown to affect all other possible sources of ROS, leading to their dysfunction and to a further increase in ROS generation, forming a vicious cycle of oxidative stress. For example, increased $O_2^{\bullet-}$ generation by NADPH oxidase induces mitochondrial oxidative damage via structural changes to the inner mitochondrial membrane and disturbs flow in the electron transport chain which enhances ROS production [32].

3. NOX and Pathophysiology

The oxidant signaling involving NADPH oxidase has important roles in cell biology participating in intracellular signaling of cell differentiation and proliferation. These mechanisms are important in tissue repair and tumorigenesis, processes where cell proliferation occurs, but when poorly controlled the generation of ROS is dangerous. Indeed, NADPH oxidase-mediated cell proliferation has been observed in a wide range of cell types including those found in blood vessels, kidney, liver, skeletal muscle precursors, neonatal cardiac myocytes, lung epithelial cells, gastric mucosa, brain microglia, and a variety of cancer cells. For example, NOX may stimulate Akt activation also by inactivating the phosphatase PTEN, a direct negative regulator of the PI3K/Akt pathway [33]. Therefore, NADPH oxidase-mediated redox signaling may amplify diverse signaling pathways triggered in tissue repair processes such as cell proliferation, wound healing, angiogenesis, or fibrosis. Recent studies also suggest that NADPH oxidase is involved in differentiation and proliferation of stem cells. Currently, little is known about whether ROS regulate different signaling pathways in stem cells and differentiated cells and whether ROS play a different role in these cells [16]. Thus, modulating NADPH oxidase may have significant impacts on regenerative medicine and tissue engineering, such as growing heart muscle.

At first, cellular stresses may induce NOX-dependent ROS generation as an alert system that drives the cells into a relatively stress-resistant status by integrating and amplifying the stress signals, as preconditioning against further cellular challenges [34]. The double-edged sword property of NOX adds another level of complexity for therapeutic targeting of this enzyme. The mechanisms of NOX-mediated redox signaling may be either enhancing stress resistance through prevention of ischemia-reperfusion injuries and accelerating

Table 1: NOX isoforms and pathology [modified from [22, 23]].

Characteristic	Binding partners	Intracellular localization	Tissue distribution	Implication in pathology
NOX1	$p22^{phox}$, NOXO1, NoxA1, Rac1, PDI, TKS4/5*	Caveolae on the plasma membrane, redoxisomes	Colon epithelia VSMCs, endothelial cells, uterus, placenta, prostate, osteoclasts, retinal pericytes, neurons, astrocytes, microglia	Colon cancer, prostate cancer, gastrointestinal inflammation, hypertension, restenosis after angioplasty
NOX2	$p22^{phox}$, $p67^{phox}$, $p40^{phox}$, $p47^{phox}$, Rac1/2	Phagosomes, cytoskeleton, lamellipodia, redoxisomes	Phagocytes, CNS, endothelium, VSMCs, fibroblasts, cardiomyocytes, skeletal muscle, hepatocytes, hematopoietic stem cells	Gastrointestinal inflammation, hypertension, myocardial injury, restenosis after angioplasty, melanoma, diabetes, neurodegenerative diseases
NOX3	$p22^{phox}$, NOXO1	Plasma membrane	Inner ear, lung endothelial cells, fetal tissues	Hearing loss, pancreatic cancer
NOX4	$p22^{phox}$, PDI, TKS4/5, Poldip2*	Focal adhesions, nucleus, endoplasmic reticulum, mitochondria	Ubiquitously expressed but highly in the kidney	Pancreatic cancer, melanoma, diabetes
NOX5	Ca^{2+}, Hsp90, CaM#	Internal membranes, plasma membrane	Lymphatic tissue, testis, VSMCs, endothelial cells, spleen, uterus, and prostate	Atherosclerosis prostate cancer, pancreatic cancer
Duox1	Ca^{2+}, DuoxA1	Plasma membrane	Thyroid, respiratory epithelium	Thyroid dysfunction, cystic fibrosis
Duox2	Ca^{2+}, DuoxA2	Plasma membrane	Airway epithelial, colon, salivary gland	Thyroid dysfunction, cystic fibrosis

*Recently, the protein polymerase (DNA-directed) delta-interacting protein 2 (Poldip2) was identified to bind and to increase the activity of NOX4. Further, protein disulfide isomerase (PDI) and a p47phox analogue tyrosine kinase substrate with 4/5 SH3 domains (Tks4/5) have been reported to bind and activate NOX1 and NOX4. NOX4 is the only isoform that produces hydrogen peroxide instead of superoxide.
#The NOX5 protein contains four N-terminal calcium-binding sites that regulate activation of the enzyme. Activity of NOX5 can be further supported by the binding of Hsp90 or Calmodulin to the C-terminus of the protein.

wound healing, or avoiding the stress-induced cytotoxicity for iper-proliferative diseases, such as cancer. Therefore, a therapeutic modulation of NOX activity has to be developed taking in account the disease, the stage of disease, the tissue localization area, the NOX isoforms, and the NOX intracellular localization.

4. NOXs and Diseases

NADPH oxidase has received much attention as a major cause of oxidative stress leading to vascular disease. Moreover different NOX subunits have been suggested to play a role in cancer, lung fibrosis, stroke, heart failure, diabetes, and neurodegenerative diseases [22]. NOX-derived ROS can lead to pathology through differential ways: for example, spatially confined levels of ROS that interfere with a particular signaling pathway, and high levels (local or systemic) that are directly cytotoxic, causing apoptosis, or disturbing redox-sensitive signaling cascades.

4.1. Vascular Diseases. NADPH oxidases are ubiquitously expressed in tissues but are the major source of superoxide anions observed in the vasculature [1]. Thus, similarly to ROS, NOX proteins have both beneficial and harmful effects. They are important signaling molecules which regulate vascular tone, expression, proliferation, migration, and differentiation. On the other hand, cardiovascular risk factors and vascular diseases increase ROS and contribute to atherosclerosis, vascular dysfunction, hypertension, vascular hypertrophy, and thrombosis.

With respect to low and spatially confined ROS overproduction, NOX1 is a good candidate to migrate into caveolae and there causes eNOS uncoupling and endothelial dysfunction, which is often associated with increased blood pressure and enhanced platelet aggregation as an early step in the development of atherosclerosis [35].

4.2. Cancer. NOX1, NOX2, and NOX4 are known to be expressed in multiple tumor cell types (see Table 1). Tumor cells produce high amounts of ROS [36, 37] by NADPH oxidases to promote their own proliferation, through regulation of proliferative signaling kinases, such as cell survival factors, such as Akt and MEK-ERK pathway [38].

Recently, the role of cancer stem cells (CSCs) in cancer progression and metastasis has attracted much attention since CSCs are integral parts of pathophysiologic mechanisms of metastasis and chemo/radioresistance [16]. To date, molecular events that govern the survival and self-renewal of CSCs are poorly defined, but a link between ROS modulation and cancer stem cell metabolism seems to have a basis. Stem cells and also CSCs are known to reside in niches characterized by low ROS, a critical factor in maintaining

stem cell properties such as self-renewal. Multiple signaling pathways in normal stem cells and CSCs can regulate ROS level and could be exploited against CSCs. Elucidation of ROS function in CSCs will enrich our knowledge of cancer development and metastasis.

Moreover, ROS have also been shown to regulate angiogenesis through the release and actions of tumor-derived growth factors that induce endothelial cell proliferation. In fact, ROS production within tumor cells dramatically promotes the release of paracrine growth factors such as VEGF and the expression of its receptor, VEGF receptor-1 which, in turn, stimulate proliferation, migration, and tube formation in nearby endothelial cells [34]. NADPH oxidase within endothelial cells cooperates with growth factors, such as VEGF released by tumors, to stimulate endothelial cell proliferation and then angiogenesis. Thus, NOXs as ROS source in tumors and in endothelium may be considered novel targets for antiangiogenic treatment.

4.3. Inflammation. The oxidative burst of phagocytes has long been considered proinflammatory, causing cell and tissue destruction. Recent findings have challenged this inflammatory role of ROS, and now ROS are also known to regulate immune responses and cell proliferation and to determine T-cell autoreactivity. NOX2-derived ROS have been shown to suppress antigen-dependent T-cell reactivity and remarkably to reduce the severity of experimental arthritis in both rats and mice [39]. In fact, regarding these chronic inflammatory diseases (rheumatoid arthritis) there is increasing evidence that ROS can often help to modulate inflammation, ensuring that it does not become too prolonged [see review [40]].

In retrospect, this is also suggested by the pathology of chronic granulomatous disease (a condition characterized by inborn defects in the phagocyte $O_2^{\bullet-}$ generating NADPH oxidase), there is an increased risk of infection due to an inability to kill certain microorganisms; this is in addition to a severe dysregulation and prolongation of inflammation. Thus, stopping ROS production can be deleterious. On the other hand, ROS can cause severe cartilage damage and the ability of nuclear factor erythroid 2 p4-5-related factor 2 to enhance endogenous antioxidant defenses in response to the inflammation may play a significant ameliorative role. The same may be true in sepsis; some ROS may help but too many can cause harm [see review [40]].

Finally, NADPH oxidase-derived ROS are also crucial players of tumor anti-immunity regulating specialized subsets of immune cells such as macrophages and T lymphocytes. Thus, NOXs could represent a novel molecular link between chronic inflammation and angiogenesis during cancer [2].

NOX2 is connected to the innate immune response [41], including to fungal infections [42] and adaptive immune response at the level of both T cells and antigen-presenting cells [43]. Thus, NOX2 inhibition leads to an improvement of diseases involving a significant inflammatory response. On the other hand, the essential immune functions of NOX2 have to be preserved [35].

4.4. Organ Failure. ROS are generally thought to play an important role in the pathophysiology of organ failure [22]. For example, in liver and intestinal tissue injury, there is some indication for a pathogenetic role of NOX2-derived ROS by neutrophils [44, 45].

With respect to high levels of ROS produced by NOX4, they can be directly cytotoxic or cause apoptosis inducing heart ischemic stroke. On the other hand, regarding the NOX4 role on pressure overload of the heart, NOX4 might be responsible of both acute damage of the cardiomyocyte and subacute protection of the heart by promoting angiogenesis [35].

4.5. CNS Diseases

4.5.1. Ischemic Stroke. In a gerbil model of global cerebral ischemia-reperfusion injury, NOX inhibition by apocynin strongly diminishes damage to the hippocampus [46]. Stroke size was markedly reduced in NOX2-deficient mice [47], while increased NOX2 expression in diabetic rats was associated with an aggravated ischemic brain injury [48].

4.5.2. Alzheimer's Disease, Parkinson's Disease, and HIV Dementia. NOX2 seems to have a role in inflammatory neurodegeneration diseases, including Alzheimer's disease and Parkinson's disease [49, 50]. In the case of Alzheimer's disease, amyloid precursor protein fragments released from neurons activate NOX2 -dependent ROS generation by microglia cells leading to death of neighboring neurons [51]. Several studies suggest similar mechanisms, involving NOX2, in Parkinson's disease [52, 53].

Microglia activation is also thought to be a key element in the development of dementia [54], and a role of NOX2 activation in animal model of dementia has been suggested [55].

Microglial NOX2-derived ROS have also been implicated in the progression of the demyelinating disease through phagocytosis of myelin and damage to the myelin sheath [56]. In periventricular leukomalacia, the combination of NOX2-derived superoxide and inducible nitric oxide synthase-derived nitric oxide leads to the formation of peroxynitrate and thereby to the killing of oligodendrites [57].

5. Antioxidant Therapy

Classically, oxidative stress has been defined as an imbalance between the endogenous production of reactive oxygen compounds and the antioxidative potential of cells [58].

But the low or apparent lack of clinical effectiveness of ROS-scavenging approaches is not entirely explained. It can be due to the partial removal of selected harmful endproducts by ROS-scavengers. Furthermore, antioxidants, including vitamins, reaction with superoxide anions is slower than NO. Moreover, it does not take into account that cellular events leading to disease primarily occur in individual cellular compartments [3].

5.1. NOX Inhibition (See Table 2). The main sources of ROS include redox enzymes such as the respiratory chain,

Table 2: NOX inhibitors.

Name and origin	Mechanism of action	NOX isoform selectivity	Other pharmacological effects	References
AEBSF synthetic	Inhibits $p47^{phox}$ assembly with oxidase subunit	NOX2	Proteases inhibitor	[64]
Apocynin picrorhiza kurroa	Inhibits $p47^{phox}$ assembly with membrane	NOX2	H_2O_2 scavenging	[65]
Berberine Berberis	Inhibition of $gp91^{phox}$ expression	NOX2	Enhancement of SOD activity	[72]
Blueberry derived polyphenols	Disrupts NOX assembly in lipid rafts	NOX2	Minimal if any ROS scavenging capacity	[82]
Celastrol Tripterygium wilfordii	Inhibition of association between cytosolic subunits and the membrane subunit	Mostly Nox1 and NOX2	None reported	[96]
DPI	Flavoprotein inhibitor	No selectivity	Inhibits NOS, xanthine oxidase, NADH ubiquinone oxidoreductase, NADH dehydrogenase, cytocrome P450 oxidoreducatese	[60–63]
EGCG green tea	Inhibits the expression of NADPH oxidase subunits	No selectivity	ROS scavenging capacity and ENOX proteins function as terminal oxidases of plasma membrane electron transport (PMET)	[87]
Emodin and rhein Rhubarb	NADPH oxidase $p47^{phox}$ activation	NOX2?	Interfere with electron transport process and in altering cellular redox status	[74]
Ginko biloba	Inhibition of Rac1- and $p47^{phox}$-mediated NADPH oxidase activation	NOX2?	Increases the expression of Cu-Zn superoxide dismutase heat shock protein 70	[79]
HDMPPA Fruits and nuts kimchi	Downregulates expression of $p47^{phox}$ and Rac1	NOX2?	Preservation of NO bioavailability	[75]
Magnolol and honokiol magnolia	Inhibit ERK pathway	unknown	Inhibit NO production	[81]
Plumbagin	Unknown	Nox4	ROS scavenger	[90–95]
Prodigiosin microbial pigment	Inhibits the binding of $p47^{phox}$ and Rac to the membrane components	NOX2?	Reduces gp91(phox) and iNOS expression	[82–84]
Resveratrol red wine	Decreases NADPH oxidase expression ($p47^{phox}$)	NOX2?	Free-radicals-scavenging	[88, 89]
S17834	Unknown	NOX2 NOX4	None reported	[90]
Sinomenine Sinomenium acutum	Inhibits $p47^{phox}$ translocation to the cell membrane	NOX2?	Minimal interaction with opiate receptors	[85]

xanthine oxidase, lipooxygenase, cyclooxygenase, and NADPH oxidases, and these systems are continuously interacting with each other. Due to the complex mechanisms involved in the activation of NADPH oxidases, these enzymes can be targeted on several different levels of their activity. Firstly, decreasing NADPH oxidase expression can inhibit them. Also, the activation of NADPH oxidase can be decreased by blocking the translocation of its cytosolic subunits, when present, to the membrane.

Another possibility is inhibition of the p47phox subunit, either by preventing its phosphorylation using PKC inhibitors or by blocking its binding to other subunits. A decrease of signal transduction and inhibition of Rac 1 translocation has also been demonstrated to decrease ROS generation [3].

Several compounds have been used, including apocynin, diphenylene iodonium (DPI), and 4-(2-aminoethyl)-benzensulfonylfluorid (AEBSF). However, it has become apparent that these inhibitors are not specific for NOX [59] and not selective for single NOX isoforms.

One of the first inhibitors used in model studies was diphenyliodonium (DPI), which is very potent (although in micromolar range) but lacks specificity. DPI is a general flavoprotein inhibitor, also inhibiting, for example, xanthine oxidase and eNOS [60–62], as well as cholinesterases and a calcium pump [63].

Later studies involved apocynin, a naturally occurring NADPH oxidase inhibitor originally isolated from the roots of Picrorhiza kurroa. Apocynin cannot be used as selective NADPH oxidase inhibitor due to its direct antioxidant and several off-target effects [60]. Apocynin is an orally active agent that can block NADPH oxidase assembly in membrane but requires a reaction with peroxidase for its activation, and therefore does not work immediately [65]. Apocynin reduces ROS production in models of arthritis, asthma, and hypertension, abolishing the increase in vascular $O_2^{\bullet-}$ and preventing endothelial dysfunction [66]. However, effects of apocynin are not specific, as it has been reported to affect arachidonic acid metabolism [67], to increase glutathione synthesis, and to activate the AP-1 transcription factor [68]. In addition, it has recently been shown to be a direct ROS scavenger in certain experimental conditions [69].

The use of natural antioxidants represents a promising new approach for NOX inhibition. Polyphenols represent more than 10000 compounds occurring naturally in foods and the recommendation for a polyphenol rich (green tea/red wine/fruits/vegetables/whole grain foods) diet in the prevention of cardiovascular disease is still valid [70]. It was found that polyphenols, apart from their well-known superoxide radical scavenging abilities, decrease NADPH oxidase activity [71] in a number of tissues including vessels and platelets [72]. Moreover, novel polyphenolic compounds are being investigated which lack typical superoxide scavenging properties and directly inhibit NADPH oxidase.

Recent studies with berberine, a plant alkaloid [73], revealed an inhibition of NADPH oxidase activity and reduction of gp91phox mRNA expression in macrophages. Also, emodin, an active component extracted from rhubarb and rhein, reduced ROS generation [74]. Similar effects were observed by treatment with 3-(4′-hydroxyl-3′,5′-dimethoxyphenyl)propionic acid (HDMPPA), the active ingredient in kimchi, ellagic acid, a polyphenol present in fruits and nuts [75], and dihomo-γ-linolenic (ω-6) acid [76].

The inhibitory effect of flavonoids (kaempferol, morin, quercetin, and fisetin) on the respiratory burst of neutrophils was observed by Pagonis et al. [77] as early as in 1986.

A Ginkgo biloba extract containing flavonoids, among other compounds, was tested by Pincemail et al. [78] for its effect on the release of ROS (superoxide anion radical, hydrogen peroxide, and hydroxyl radical) during the stimulation of human neutrophils by a soluble agonist. The extract slowed down the oxygen consumption (respiratory burst) of the stimulated cells by its inhibitory action on NADPH oxidase [79]. The extract was also able to reduce the activity of myeloperoxidase contained in neutrophils. Moreover, it had free radical scavenging activity.

A higher number of hydroxyl substituents are an important structural feature of flavonoids in respect to their scavenging activity against ROS, while C-2,3 double bond (present in quercetin and resveratrol) might be important for the inhibition of ROS production by phagocytes [80].

The bark of magnolia has been used in oriental medicine to treat a variety of remedies, including some neurological disorders [81]. Magnolol (Mag) and honokiol (Hon) are isomers of polyphenolic compounds from the bark of Magnolia officinalis, and have been identified as major active components exhibiting antioxidative, anti-inflammatory, and neuroprotective effects. It has been reported that exposure of Hon and Mag to neurons for 24 h did not alter neuronal viability, but both compounds inhibited superoxide production, a pathway known to involve NADPH oxidase. This study highlighted the important role of NADPH oxidase in mediating oxidative stress in neurons and microglial cells and has unveiled the role of IFNγ in stimulating the MAPK/ERK1/2 signaling pathway for activation of NADPH oxidase in microglial cells. Hon and Mag offer anti-oxidative or anti-inflammatory effects, at least in part, through suppressing IFNγ-induced p-ERK1/2 and its downstream pathway [81].

Incubation of human neuroblastoma cells with nonpolar blueberry fractions obstructed the coalescing of lipid rafts into large domains disrupting NOX assembly therein and abolishing ROS production [82]. In fact, this NOX inhibiting bioactivity in crude blueberry extracts partitioned into a polyphenol-devoid fraction lacking virtually any antioxidant capacity and prevented proper assembly of the multisubunit NOX complex interfering with the coalescence of large lipid raft domains.

Prodigiosin, a microbial pigment, and some derivatives suppressed NOX activity most likely by disrupting Rac function [83, 84], and inhibition of NOX by the alkaloid sinomenine is unclear at best [85].

In an oxygen-glucose deprivation and reoxygenation (OGD/R) model, pretreatment with green tea polyphenols (GTPP) and their active ingredient, epigallocatechin-3-gallate (EGCG), protects PC12 cells from subsequent OGD/R-induced cell death [86, 87]. GTPP stimulates laminin receptor and thereby induces NADPH oxidase-dependent generation of ROS, which in turn induces activation of PKC resulting in preconditioning against cell death induced by OGD/R.

Resveratrol is a naturally occurring polyphenol, which has vasoprotective effects in diabetic animal models and inhibits high glucose (HG-) induced oxidative stress in endothelial cells. It has been reported that HG induces endothelial cell apoptosis through NF-κB/NADPH oxidase/ROS pathway, which was inhibited by resveratrol [88, 89].

Other NOX inhibitors are VAS 2870, VAS 3947, GK-136901, plumbagin, and polyphenolic derivative S17834 [90].

Plumbagin, a plant-derived naphthoquinone, has been shown to exert anticarcinogenic and antiatherosclerosis effects in animals. Plumbagin inhibits NADPH-dependent superoxide production in cell lines that express NOX4 oxidase [91]. Although its exact mechanism of action remains unclear, the inclusion of a napthoquinone structure within the molecule may be responsible for its ROS scavenging abilities [90]. Plumbagin inhibited the activity of NOX4 in a time- and dose-dependent manner in HEK293 and LN229 cells directly interacting with NOX4 and inhibiting its activity [91].

Indeed, plumbagin has been reported to exert anticancer activity on osteosarcoma cells by inducing proapoptotic signaling and modulating the intracellular ROS that causes

induction of apoptosis [92]. Moreover plumbagin-induced AMPK activation might be the key mediator of plumbagin's antitumor activity [93].

Furthermore, PI5K-1B plays a crucial role in ROS generation and could be a new molecular target of plumbagin [94]. At last, plumbagin can exert its function in depleting glutathione (GSH) levels that led to increase in ROS generation [95].

Celastrol is one of several bioactive compounds extracted from the medicinal plant Tripterygium wilfordii. Celastrol is used to treat inflammatory conditions and shows benefits in models of neurodegenerative disease, cancer, and arthritis, although its mechanism of action is incompletely understood. Authors demonstrated that celastrol is a potent inhibitor of NOX enzymes in general with increased potency against NOX1 and NOX2. Furthermore, inhibition of NOX1 and NOX2 was mediated via a novel mode of action, namely, inhibition of a functional association between cytosolic subunits and the membrane flavocytochrome [96].

6. Conclusions

Accumulating evidence clearly indicates that NADPH oxidases are critical molecular targets for dietary bioactive agents for prevention and therapy of different pathologies.

The development of specific and not toxic inhibitors of NADPH oxidases and their redox signaling network (kinase, transcription factors, and genes) could provide useful therapeutic strategies for the treatment of oxidative stress dependent processes such as cancer and other degenerative diseases.

In fact, classical antioxidant therapies have been demonstrated inadequate since the importance of ROS in physiology has been ignored leading to the lack of clinical benefits. Indeed, further research into selective molecular inhibitors interfering with NADPH oxidase activation are warranted. The selective targeting of dysfunctional NADPH oxidase homologs appears to be the most suitable approach, with the potential to be far more efficient than the one with nonselective antioxidants having only ROS scavenging properties.

NOX enzymes, however, are very complex with numerous specific targets within each isoform. More information is needed on how these proteins are targeted to different subcellular compartments and how this transport process is regulated.

It is encouraging, however, that single bioactive dietary agents can directly and indirectly influence most, if not all, of the myriad targets within NOX family. Additionally, many of these dietary agents appear to exhibit some degree of specificity for redox deregulated cells while unaffecting normal cells balance. Moreover, the protective effects of some single agents could be potentiated and/or synergized by other dietary agents. While encouraging, there are many considerations that remain, such as the issue of appropriate dose of each agent, appropriate timing and duration of exposure, importance of cell type specificity, relative bioavailability of each agent, and potentially adverse side effects and interactions.

Conflict of Interests

The author does not have a direct financial relationship with the commercial identities mentioned in the paper that might lead to a conflict of interests.

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Research Article

Rosmarinic Acid-Rich Extracts of Summer Savory (*Satureja hortensis* L.) Protect Jurkat T Cells against Oxidative Stress

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Summer savory (*Satureja hortensis* L., *Lamiaceae*) is used in several regions of the world as a spice and folk medicine. Anti-inflammatory and cytoprotective effects of *S. hortensis* and of its rosmarinic acid-rich phenolic fraction have been demonstrated in animal trials. However, previous studies of rosmarinic acid in cell models have yielded controversial results. In this study, we investigated the effects of summer savory extracts on H_2O_2-challenged human lymphoblastoid Jurkat T cells. LC-MS analysis confirmed the presence of rosmarinic acid and flavonoids such as hesperidin and naringin in the phenolic fraction. Adding 25 or 50 μM of H_2O_2 to the cell culture caused oxidative stress, manifested as generation of superoxide and peroxyl radicals, reduced cell viability, G0/G1 arrest, and enhanced apoptosis. This stress was significantly alleviated by the ethanolic and aqueous extracts of *S. hortensis* and by the partially purified rosmarinic acid fraction. The application of an aqueous *S. hortensis* extract doubled the activity of catalase and superoxide dismutase in the cells. The production of IL-2 and IL-10 interleukins was stimulated by H_2O_2 and was further enhanced by the addition of the *S. hortensis* extract or rosmarinic acid fraction. The H_2O_2-challenged Jurkat cells may serve as a model for investigating cellular mechanisms of cytoprotective phytonutrient effects.

1. Introduction

Summer savory (*Satureja hortensis* L.) is an herb of the *Lamiaceae* family that is used in cooking and folk medicine in several regions of the world [1]. In Georgia, dried and ground summer savory (local name *kondari*) is one of the most popular spices, used either on its own or as an ingredient in spice blends. In addition, from ancient times, it has been known locally as an antimicrobial folk remedy for gastrointestinal problems [2]. Indigenous landraces of summer savory are cultivated in Georgia [3].

The leaves of summer savory are rich in phenolic compounds, particularly rosmarinic acid and flavonoids, which account for the high antioxidant capacity of these leaves [4, 5]. In our previous study of Georgian spices, we found that *kondari* had one of the highest total phenolic content levels and one of the highest hydrophilic antioxidant capacity levels [6]. Rosmarinic (α-O-caffeoyl-3,4-dihydroxy-phenyl lactic) acid was found to be the major compound in ethanolic extracts of summer savory and some other *Lamiaceae* herbs [4]. Rosmarinic acid is a phenylpropanoid derivative that is the second most common ester of caffeic acid in the plant kingdom.

Animal studies have revealed anti-inflammatory activity of *S. hortensis* extract and its polyphenolic fraction, in particular [7, 8]. This activity might be associated, at least partially, with rosmarinic acid, whose antiinflammatory and antiallergic properties have been demonstrated in animal and human trials [9, 10]. Osakabe et al. [10] suggested that the antiallergic effect of rosmarinic acid might be due to two independent

mechanisms: the scavenging of reactive oxygen species and the modulation of the inflammatory response. For example, the nephroprotective effect of rosmarinic acid was associated with improved antioxidant potency, including enhanced glutathione content and activity of antioxidant enzymes [11].

However, the cellular mechanisms by which rosmarinic acid exerts its anti-inflammatory effects are not fully understood and demand further investigation. The human lymphoblastoid T-cell Jurkat line, a constitutive producer of the potent T-cell growth factor interleukin 2 (IL-2), is a popular model for the study of immune signaling [12]. Jurkat cells can imitate both healthy and inflammatory T-cells in their response to oxidative metabolites, such as hydrogen peroxide [13]. Therefore, investigating the effect of *S. hortensis* extract on the proliferation and activity of T-cells may contribute to our understanding of the mechanism(s) of its anti-inflammatory and cytoprotective effects. Although H_2O_2 plays an important role in antigen-dependent lymphocyte activation [14], excessive production of H_2O_2 induces oxidative stress and impairs T-cell activity, leading to chronic inflammation and cell death.

The response of Jurkat cells to H_2O_2 is dose-dependent. Reversible oxidative changes that can be repaired by cellular antioxidant system occur at a H_2O_2 concentration of 20 μM, and the first signs of apoptosis are noted at 50 μM H_2O_2 [15]. Relatively high bolus doses of H_2O_2 (150 μM) induce apoptosis in Jurkat cells, but the continuous presence of a lower concentration of H_2O_2 (2 μM) inhibits the apoptotic process [16]. Both apoptosis and necrosis were observed in the Jurkat cells exposed to 100 μM H_2O_2 [17], while necrosis was far more common at 500 μM H_2O_2 [18]. Despite its well-documented cytoprotective activity in animal trials, concentrations of up to 150 μM of rosmarinic acid failed to prevent the H_2O_2-mediated apoptosis of Jurkat cells and showed no antioxidant properties [19]. Moreover, even in the absence of exogenous hydrogen peroxide, rosmarinic acid was reported to induce the apoptosis of Jurkat cells [19, 20].

The discrepancy between the prooxidant behavior of rosmarinic acid toward Jurkat cells that has been observed in previous studies and its well-known antioxidant and anti-inflammatory properties hamper the use of Jurkat cells as a model for investigating the mode of action of this phytonutrient. In the present work, we reexamined the effects of summer savory extracts and thier rosmarinic acid-rich phenolic fraction on H_2O_2-challenged Jurkat cells.

2. Materials and Methods

2.1. Plant Material. Plants of a local Georgian landrace of *S. hortensis* were grown in an experimental plot near Tbilisi from seeds purchased from commercial supplier. The plants were harvested at their vegetative state (55 days after seed germination), the phenological stage characterized by the highest phenolic compound content, highest flavonoid content, and greatest antioxidant activity (I. Chkhikvishvili, unpublished data). The collected plant material was air-dried in the shade at 25–30°C. The dried matter was stored in a closed glass container in a cool, dry place.

2.2. Extraction and Purification. The dried plant material (1 g samples) was sequentially extracted with chloroform, ethyl acetate, and ethanol at a 1 : 5 w/v ratio of plant material to solvent; the duration of each extraction step was 24 h. The residue was extracted with water by steeping for 20 min at 90°C and subsequent gradual cooling down to room temperature. Direct application of this water extraction technique to the dried plant material produced a "total aqueous extract." The solvents were removed by evaporation under vacuum at a temperature below 40°C, and the extracts were stored at −80°C until use. For the purification of the phenolic fraction, the total aqueous extract was percolated through a polyamide column. The column was washed with water and the purified fraction was eluted with 96% ethanol.

2.3. Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis. The samples of purified phenolic fraction were dissolved in HPLC-grade methanol and filtered through a Millex-HV Durapore (PVDF) membrane (0.22 μm) before being injected into the LC-MS instrument. Mass spectral analyses were carried out using the Ultraperformance LC-Quadruple Time of Flight (UPLC-QTOF) instrument (Waters Premier QTOF, Milford, MA, USA), with the UPLC column connected online to a PDA detector (Waters Acquity), and then to an MS detector equipped with an electrospray ion (ESI) source (used in ESI-negative mode). Separation was performed on a 2.1 × 50 mm i.d., 1.7 μm UPLC BEH C18 column (Waters Acquity).

The chromatographic and MS parameters were as follows: the mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The linear gradient program was as follows: 100% to 95% A over 0.1 min, 95% to 5% A over 9.7 min, held at 5% A over 3.2 min, and then returned to the initial conditions (95% A) in 4.2 min. The flow rate was 0.3 mL min^{-1} and the column was kept at 35°C. Masses of the eluted compounds were detected with a QTOF Premier MS instrument. The UPLC-MS runs were carried out at the following settings: capillary voltage of 2.8 kV, cone voltage of 30 eV, and collision energy of 5 eV. Argon was used as the collision gas. The *m/z* range was 70 to 1,000 D. The MS system was calibrated using sodium formate and Leu-enkephalin was used as the lock mass. The MassLynx software version 4.1 (Waters) was used to control the instrument and calculate accurate masses.

2.4. Cell Culture and Experimental Design. The human T-cell leukemia lymphoblastoid Jurkat cells (DSMZ ACC 282) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The cells were grown in suspension culture at 37°C under 5% humidified CO_2 in bioactive medium RPMI 1640 (Gibco, Grand Island, NY, USA) containing inactivated embryonic bovine serum (Sigma, St. Louis, MO, USA), L-glutamine (4 mM), penicillin (100 U mL^{-1}), and streptomycin (100 U mL^{-1}). The experiments were carried out at cell densities of 0.3 to 0.6 × 10^6 cells mL^{-1}. In order to imitate the oxidative stress conditions, H_2O_2 (Sigma) was added to the Jurkat culture to reach the concentrations of 25

and 50 μM, corresponding to low and intermediate stress severity, respectively [15]. In the unstressed control treatment, water was added to the samples instead of H_2O_2. The *S. hortensis* extracts were added to the cultures at a rate of 2 mg mL^{-1} as the H_2O_2 was added.

In a separate trial, the effect of cell pretreatment with *S. hortensis* extract on their response to subsequent H_2O_2 oxidative stress was investigated. Cell suspensions (2×10^6 cells mL^{-1}) were incubated with *S. hortensis* rosmarinic acid fraction as described above. After the incubation period, the cells were harvested by centrifugation at 1500 g for 5 minutes, washed, resuspended in fresh medium, and exposed to H_2O_2. Cellular responses to oxidative stress were evaluated by free radicals generation and cell viability as described below.

2.5. Hydrogen Peroxide Scavenging Capacity. The ability of *S. hortensis* extracts to scavenge hydrogen peroxide in the absence of cells was tested in order to check possible contribution of this abiotic H_2O_2 decomposition to experimental results. The H_2O_2-scavenging capacity of extracts was tested as described by Ruch et al. [21]. A solution of hydrogen peroxide 50 μM was prepared in phosphate buffer (pH 7.4). Phenolic extracts (2 mg mL^{-1}) in distilled water and 50 μM hydrogen peroxide solution were added to incubation system comprising bioactive medium RPMI 1640 (GIBSO) with inactivated embryonic bovine serum (Sigma), L-glutamine (4 mM), penicillin (100 U mL^{-1}), and streptomycin (100 U mL^{-1}). Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the incubation medium with hydrogen peroxide. The percentage of hydrogen peroxide scavenging by *S. hortensis* extracts was calculated. The trial revealed a 17% reduction of H_2O_2 concentration due to the interaction with *S. hortensis* rosmarinic acid fraction.

2.6. Electron Paramagnetic Resonance (EPR) Spectroscopy. The effect of *S. hortensis* extracts on the generation of free radicals in H_2O_2-challenged and unchallenged cells was studied using the electron paramagnetic resonance (EPR) method. EPR spectra were registered on a radiospectrometer, RE 1307 (EPSI, Chernogolovka, Russia). Peroxyl radicals were detected with spin-trap α-phenyl-tertbutylnitrone (PBN; Sigma) (50 mM on 0.6×10^6 cells in 0.5 mL medium) at room temperature at microwave power (20 mV). Superoxide radicals were detected with a spin-trap 5,5 dimethyl-I-pyrrolyn-IV-oxide (DMPO) (Sigma) (50 mM on 0.6×10^6 cells in 0.5 mL medium) at room temperature at microwave power (20 mV).

2.7. Cell Viability and Proliferation. The viability of the cells was determined using the MTT cell proliferation assay. Cell suspensions (2×10^6 cells mL^{-1}) were incubated with H_2O_2 and *S. hortensis* preparations as described above. After the incubation period, the cells were harvested by centrifugation at 1500 g for 5 minutes, washed, and resuspended in fresh medium. The 8 mg mL^{-1} solution of 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) in buffer (140 mM NaCl, 5 mM HEPES, pH 7.4) was added to the cell suspension at a rate of 30 μL per 100 μL suspension and the mixture was incubated for 4 h at 37°C in a 5% CO_2 atmosphere. After this incubation, the supernatant was carefully removed and the colored formazan crystals produced from the MTT were dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorption values of the solutions were measured at 570 nm. The distribution of the Jurkat cells among the different cell-cycle phases was studied using flow cytometry. Mitochondrial transmembrane potential ($\Delta\Psi$) in the cell culture was determined by flow cytometry using the lipophilic cation test 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) described by Zamzami et al. [22].

2.8. Antioxidant Enzymes. Jurkat cell extract was prepared by centrifuging the cell suspensions at 500 g and then homogenizing the cellular precipitate in a lysis buffer (pH 7.9) that was comprised of 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 1 μg mL^{-1} leupeptin, 1 μg mL^{-1} aprotinin, and 10 mM HEPES. The volume of the buffer was twice the volume of the precipitate. Lysis of the cells was performed by passing the suspension through a 27-gauge needle 10 times. The obtained homogenate was centrifuged for 20 min at 10,000 g. The supernatant was used to determine the levels of enzyme activity. Catalase (EC 1.11.1.6) activity was measured spectrophotometrically as the decomposition of H_2O_2 at 240 nm [23]. One unit of catalase activity was defined as the amount of enzyme decomposing 1 μmol of H_2O_2 per minute. The superoxide dismutase (SOD; EC 1.15.1.1) was assayed using NADPH and phenazine methosulfate (PMS) reagents for the reduction of nitroblue tetrazolium salt (NBT) into blue-colored formazon measured spectrophotometrically at 560 nm [24]. One unit of SOD activity was defined as the amount of enzyme oxidizing 1 nmol NADPH per minute. The activity of both enzymes was expressed in terms of units per mg of protein. A total protein micro Lowry kit (Sigma) was used to determine the protein content.

2.9. Interleukin Analysis. Jurkat cells were prestimulated by incubation with 50 μg/mL phytohemagglutinin (PHA) at 37°C for 5 min and cultured for 24 h with nonstimulated Jurkat cells (40% stimulated and 60% non-stimulated cells). The pro- and anti-inflammatory cytokines IL-2 and IL-10 were assayed using ELISA kits (Bender Medsystems, Vienna, Austria) and the Multiscan microplate reader (LabSystem, Helsinki, Finland).

2.10. Statistics. The trials were performed in five replications. The statistical analysis of the obtained results, including calculation of means and standard deviations, was conducted using the IBM SPSS Statistics program. The statistical significance of the differences between the means was analyzed by pair-wise comparison of treatment results with nontreated control using Student's t-test at $P < 0.05$.

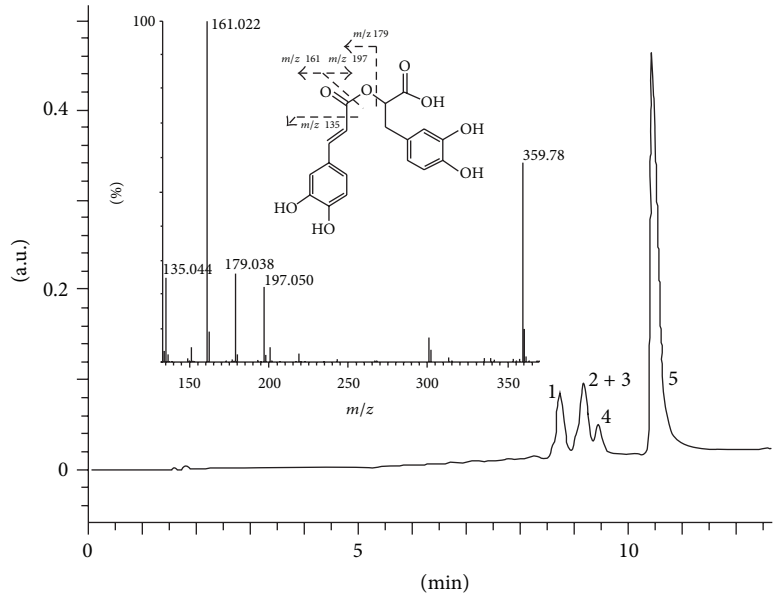

Figure 1: HPLC chromatogram of the *S. hortensis* rosmarinic acid fraction. The peak 5 represents rosmarinic acid and the peak 2 + 3 partially separated naringin and hesperidin. The peaks 1 and 4 were tentatively identified as rutin and apigenin-7-glucoside, respectively. Insert: mass-spectrum of the rosmarinic acid and its fragmentation scheme.

3. Results

3.1. Analysis of S. hortensis Extracts. HPLC analysis revealed a number of phenolic compounds in the ethanolic extract of *S. hortensis*, rosmarinic and ferulic acids being the major compounds. In addition, a number of phenolic acids (caffeic, *p*-coumaric), flavonoid aglycones (catechin, epicatechin, luteolin, apigenin), and glycosides (rutin, hesperidin, apigenin-7-glucoside) were tentatively identified in the ethanolic extract. Partial purification of the rosmarinic acid provided a fraction comprising four major peaks. The tentative identification of the rosmarinic acid as the most abundant component of the fraction was based on its UV absorption spectrum and retention time as compared with those of the authentic standard sample. The identity was confirmed by LC-MS based on the presence of a deprotonated molecular ion $[M-H]^-$ at *m/z* 359 and characteristic fragment ions at *m/z* 123, *m/z* 135, *m/z* 161, *m/z* 179, and *m/z* 197, in accordance with data in the literature [25, 26] and fragmentation scheme (Figure 1). Two flavonoid glycosides were identified by LC-MS through comparisons with standard samples as hesperidin based on a $[M-H]^-$ at *m/z* 609, a characteristic hesperetin fragment ion at *m/z* 301, naringin based on $[M-H]^-$ at *m/z* 579, and a characteristic naringenin fragment ion at *m/z* 271. In addition, two more flavonoid glycosides were tentatively identified in the fraction as rutin and apigenin-7-glucoside.

H_2O_2-Induced Oxidative Stress as Affected by S. hortensis Extracts. The addition of 25 or 50 μM of hydrogen peroxide caused oxidative stress in the Jurkat cells, which was manifested as the generation of superoxide and peroxyl radicals that could be detected by EPR spectroscopy. The amount of radicals formed depended on the concentration of H_2O_2; no radicals were detected in the absence of hydrogen peroxide (Table 1).

Chloroform and ethyl acetate extracts of *S. hortensis* had only limited effects on the oxidative state of the cells, slightly reducing the amount of radicals detected at higher hydrogen peroxide concentrations. On the other hand, considerable alleviation of the oxidative stress and almost complete elimination of the radicals were observed in the presence of the ethanolic *E. hortensis* extract. Significant antioxidant effects were also associated with the aqueous extract and with the partially purified rosmarinic acid fraction, although the efficacy of the latter preparation was markedly lower than that of the crude ethanolic extract. In line with these findings, the total aqueous extract of *S. hortensis* doubled the activity of the antioxidant enzymes catalase and superoxide dismutase in the Jurkat cells, even in the absence of exogenous hydrogen peroxide (Figure 2).

3.2. Effects on Jurkat Cell Viability. In the absence of any exogenous H_2O_2 challenge, adding ethanolic *S. hortensis* extract or the purified phenolic fraction to Jurkat cells slightly improved their viability. Other *S. hortensis* extracts had no significant effects on the viability of unstressed Jurkat cells, as measured by the MTT test (Table 2).

Hydrogen peroxide-induced oxidative stress reduced the viability of Jurkat cells in a dose-dependent manner. This hydrogen peroxide effect was alleviated by the application of ethanolic and aqueous extracts of *S. hortensis* and by the phenolic fraction. The aqueous *S. hortensis* extract was the most effective for restoring cell viability to the level observed in the unstressed control culture (Table 2).

Table 1: Effects of *S. hortensis* extracts and of the partially purified rosmarinic-acid fraction on the generation of superoxide (O_2^-) and peroxyl ($LOO^\bullet$) radicals in Jurkat cells subjected to hydrogen peroxide-induced oxidative stress.

	Hydrogen peroxide concentration, μM					
	0		25		50	
	EPR signal intensity, arbitrary units					
	O_2^-	$LOO^\bullet$	O_2^-	$LOO^\bullet$	O_2^-	$LOO^\bullet$
No additives (control)	0	0	2.4 ± 0.2	3.0 ± 0.2	3.1 ± 0.1	3.8 ± 0.3
Chloroform extract	0	0	2.0 ± 0.2	3.2 ± 0.2	2.1 ± 0.2*	2.8 ± 0.3*
Ethyl acetate extract	0	0	2.0 ± 0.2	3.2 ± 0.2	2.1 ± 0.2*	2.8 ± 0.3*
Ethanolic extract	0	0	0*	0*	0.1 ± 0.1*	0*
Aqueous extract	0	0	0*	0.3 ± 0.1*	0.1 ± 0.1*	0.5 ± 0.1*
Rosmarinic acid fraction	0	0	1.0 ± 0.2*	2.2 ± 0.2*	1.1 ± 0.2*	1.8 ± 0.3*

Values represent averages of five replications ± standard deviations. Values marked with the asterisk are significantly different from the control in the same column at $P \leq 0.05$, according to Student's t-test.

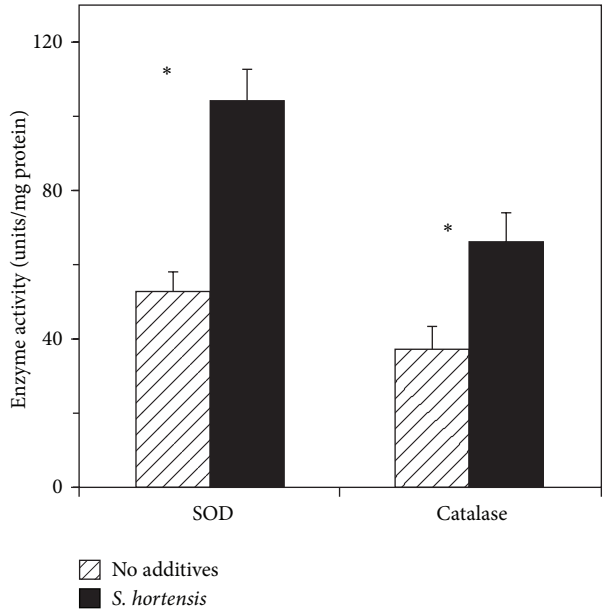

Figure 2: Effect of the total aqueous *S. hortensis* extract on the activities of superoxide dismutase (SOD) and catalase in Jurkat cells. Error bars represent standard deviations of five replications. Bars marked with an asterisk are significantly different from the control at $P \leq 0.05$, according to Student's t-test.

3.3. Effect of Pretreatment of Jurkat Cells with S. hortensis Extract on Subsequent Cellular Sensitivity to Oxidative Stress. The data presented in Table 3 demonstrate that pretreatment of Jurkat cells with the rosmarinic acid fraction significantly alleviated the oxidative stress incurred to cells by subsequent exposure to hydrogen peroxide, as expressed by free radical generation and decline in cell viability. This alleviation could not be attributed to the peroxide-scavenging activity of the extracts because no direct contact of the extracts with the peroxide took place in that case. In addition, the direct peroxide-scavenging capacity of the rosmarinic acid fraction did not exceed 17%, so that its contribution to the cell protection was rather limited.

3.4. Effects on the Cell Cycle. Oxidative stress changed the cell-cycle phase distribution of the Jurkat cells, restricting cell proliferation and increasing the relative proportions of G0/G1 cells (the G0/G1 arrest) and apoptotic cells among the total cell population. These trends were alleviated by the addition of the ethanolic *S. hortensis* extract, so that the amount of apoptotic cells in that treatment was not significantly different from that observed in the unstressed control (Table 4). Adding the *S. hortensis* extract alone, without hydrogen peroxide, had no significant effect on the cell-cycle phase distribution of the Jurkat cells (data not shown). The alleviation of H_2O_2-induced apoptosis by the ethanolic *S. hortensis* extract and by the partially purified rosmarinic acid fraction was also evident from the index of mitochondrial transmembrane potential determined by flow cytometry (Table 5).

3.5. Interleukin Production. The production of both IL-2 and IL-10 interleukins by Jurkat cells was stimulated by hydrogen peroxide and further enhanced by the addition of the *S. hortensis* extract and its phenolic fraction (Table 6).

4. Discussion

Our study has confirmed that rosmarinic acid is an abundant phenylpropanoid compound in summer savory. To the best of our knowledge, hesperidin and naringin have not been previously reported in *S. hortensis*, but they have been found in other *Satureja* species [27] and in other genera of this family, such as *Mentha* [25].

The present research has demonstrated for the first time that *S. hortensis* and its rosmarinic acid-rich fraction can protect Jurkat cells from oxidative stress caused by hydrogen peroxide. These findings are in line with the antioxidant, cytoprotective, and anti-inflammatory activities of *S. hortensis* [7] and rosmarinic acid [9, 10] that have been observed *in vivo* in animals and humans. Similar protective antioxidant properties were exhibited by *S. hortensis* extracts when applied to H_2O_2-stressed lymphocytes isolated from blood taken from healthy rats [28]. In cell cultures, rosmarinic acid protected

Table 2: Effects of *S. hortensis* extracts on the viability of Jurkat cells in the presence or absence of hydrogen peroxide.

	Hydrogen peroxide concentration, μM		
	0	25	50
	MTT test results, A_{570}		
No additives (control)	0.69 ± 0.02	0.36 ± 0.01	0.22 ± 0.01
Chloroform extract	0.63 ± 0.02	0.37 ± 0.01	0.24 ± 0.03
Ethyl acetate extract	0.58 ± 0.05	0.47 ± 0.03	0.42 ± 0.03*
Ethanolic extract	0.74 ± 0.03*	0.56 ± 0.05*	0.46 ± 0.03*
Aqueous extract	0.61 ± 0.01	0.68 ± 0.04*	0.67 ± 0.02*
Rosmarinic acid fraction	0.75 ± 0.04*	0.62 ± 0.03*	0.42 ± 0.04*

Values represent averages of five replications ± standard deviations. Values marked with the asterisk are significantly different from the control in the same column at $P \leq 0.05$, according to Student's t-test.

Table 3: Effect of pretreatment of the Jurkat cells with partially purified *S. hortensis* rosmarinic acid fraction on the cellular response to subsequent hydrogen peroxide-induced oxidative stress.

	Hydrogen peroxide concentration, μM		
	0	25	50
	Peroxyl radicals generation, EPR signal intensity (arbitrary units)		
Nontreated control	0	3.0 ± 0.2	3.8 ± 0.3
Rosmarinic acid fraction	0	1.9 ± 0.2*	2.1 ± 0.3*
	Cell viability (MTT test results, A_{570})		
Nontreated control	0.69 ± 0.02	0.36 ± 0.01	0.22 ± 0.01
Rosmarinic acid fraction	0.75 ± 0.04*	0.59 ± 0.04*	0.37 ± 0.03*

Values represent averages of five replications ± standard deviations. Values marked with the asterisk are significantly different from the control in the same column at $P \leq 0.05$, according to Student's t-test.

Table 4: Effects of hydrogen peroxide and of the ethanolic *S. hortensis* extract on the cell-cycle phase distribution of Jurkat cells.

	Cell-cycle phases, %			
	G0/G1	S	G2/M	G0/Apoptosis
No additives (control)	23.8 ± 3.4	54.5 ± 3.3	19.0 ± 2.9	2.7 ± 3.6
H_2O_2 25 μM	42.3 ± 3.3*	36.7 ± 3.4*	12.5 ± 1.7*	8.5 ± 1.9*
H_2O_2 25 μM + *S. hortensis* (ethanolic extract)	37.5 ± 2.5*	43.0 ± 3.3*	16.0 ± 3.4	3.5 ± 1.3

Values represent averages of five replications ± standard deviations. Values marked with the asterisk are significantly different from the control in the same column at $P \leq 0.05$, according to Student's t-test.

human neuronal cells against hydrogen peroxide-induced apoptosis [29] and inhibited in a dose-dependent manner the formation of reactive oxygen and nitrogen species in RAW264.7 macrophages stimulated with lipopolysaccharide or phorbol 12-myristate 13-acetate [30].

On the other hand, in a previous study, rosmarinic acid failed to protect Jurkat cells from H_2O_2-mediated oxidative damage and actually induced their apoptosis [19, 20]. Such prooxidant cytotoxic reactions in cell cultures are associated with the generation of H_2O_2 through the interaction of phenolic compounds with culture media ingredients (e.g., transient metals) and can, therefore, be considered artifacts [31, 32]. Inclusion of catalase or metmyoglobin in the growth medium negates these reactions and allows the realization of the cytoprotective antioxidant potential of phenolic compounds [31].

One possible explanation for the apparent discrepancy between our results and those of Kolettas et al. [19] might be that the high dose of antioxidant materials used in our study could overcome the influence of H_2O_2, either added exogenously or generated in cell cultures with participation of transient metals. Indeed, in a metal-catalyst system, most phenolic compounds exhibited pro-oxidant effects at low doses and shifted to antioxidant activity at higher concentrations [33]. Furthermore, it was shown recently that high doses (2-3 mM) of caffeic acid and other phenylpropanoids protected Jurkat cells from H_2O_2-induced DNA damage by chelating intracellular labile iron [34]. The presence of the potent flavonoid antioxidants in the phenolic fraction, in addition to rosmarinic acid, might further strengthen its antioxidant capacity. Enhancement of the activity of the antioxidant enzymes by *S. hortensis* (Figure 2) might

Table 5: Effects of ethanolic *S. hortensis* extract and of the partially purified rosmarinic-acid fraction on the incidence of apoptosis in Jurkat cells in the presence of hydrogen peroxide.

	Cell counts		K ratio*
	Healthy	Apoptotic	
No additives (control)	212	8	26.5
H_2O_2 25 μM	268	3519	0.08
H_2O_2 25 μM + *S. hortensis* (ethanolic extract)	2090	539	3.9
H_2O_2 25 μM + rosmarinic acid fraction	1211	108	11.2

* K-ratio of healthy to apoptotic Jurkat cells.

Table 6: Effects of the ethanolic *S. hortensis* extract and of the partially purified rosmarinic-acid fraction on the production of interleukins by Jurkat cells in the presence of hydrogen peroxide.

	IL-2, pg mL^{-1}	IL-10, pg mL^{-1}	IL-2/IL-10
No additives (control)	0.90 ± 0.05	3.21 ± 0.04	0.28 ± 0.04
H_2O_2 25 μM	2.61 ± 0.04*	6.80 ± 0.05*	0.38 ± 0.01
H_2O_2 25 μM + *S. hortensis* (ethanolic extract)	15.30 ± 0.04*	20.01 ± 0.08*	0.76 ± 0.07
H_2O_2 25 μM + *S. hortensis* rosmarinic acid fraction	20.80 ± 0.07*	38.40 ± 0.06*	0.54 ± 0.07

Values represent averages of five replications ± standard deviations. Values marked with the asterisk are significantly different from the control in the same column at $P \leq 0.05$, according to Student's t-test.

also contribute to the neutralization of hydrogen peroxide. Catalase and SOD play important roles in the control of oxidative stress and apoptosis in Jurkat cells [35]. Similar to our findings, an aqueous extract of another rosmarinic acid-containing *Lamiaceae* herb, *Perilla frutescens*, was shown to upregulate the mRNA and protein expression of these antioxidant enzymes in cultured human vein endothelial cells [36].

Another noteworthy phenomenon observed in this work was a parallel increase in the levels of the IL-2 and IL-10 interleukins. Robust production of IL-2 is the major trait of the Jurkat cell line [12]. There is a synergistic interaction between these two interleukins during the immune response [37]. Anti-inflammatory factors such as IL-10 may be released in order to balance the dramatic increase in proinflammatory cytokines in stressful situations, and thereby control the magnitude and duration of the inflammatory response [38]. Interestingly, adding antioxidant-rich plant materials to the diets of animals enduring proinflammatory conditions has been shown to increase the level of IL-10 [39] or the levels of both IL-2 and IL-10 [40] in parallel with a decrease in the levels of pro-inflammatory factors, such as IL-6, TNF-α, and IL-1β. In addition, these dietary interventions preserved normal antioxidant enzyme activity, inhibited lipid peroxidation, and increased the HDL levels in the treated animals, resulting in the alleviation of disorders and enhanced immunity. Rosmarinic acid increased the secretion of IL-10 in a lipopolysaccharide-stimulated macrophage model [41].

Addition of the *S. hortensis* extract or its phenolic fraction restored the viability and proliferation of H_2O_2-challenged Jurkat cells, alleviated the G0/G1 arrest, and controlled the apoptosis of these cells. Altogether, these phenomena were in line with the general scheme of cellular response to oxidative stress, implying that low doses of reactive oxygen species promote cell proliferation, intermediate doses result in growth arrest, and severe oxidative stress ultimately causes cell death via apoptotic or necrotic mechanisms [42]. Apparently, the addition of *S. hortensis* extracts alleviated the oxidative stress exerted on the cells by hydrogen peroxide. These effects may be attributed to the direct radical-scavenging activity of rosmarinic acid and other phenolic compounds, as well as to indirect mechanisms such as the enhancement of antioxidant enzymes and the release of anti-inflammatory signaling molecules, such as IL-10.

5. Conclusions

The present research has demonstrated that rosmarinic acid-rich extract of *S. hortensis* can protect Jurkat cells from oxidative stress caused by hydrogen peroxide. These findings are in line with the antioxidant, cytoprotective, and anti-inflammatory activities of rosmarinic acid that have been observed in animals and humans. Therefore, the H_2O_2-challenged Jurkat cells may serve a model for investigating cellular mechanisms of cytoprotective effects of phytonutrients. It should be kept in mind, however, that these results were achieved with a rather high concentration of rosmarinic acid that supposedly could overcome the culture-associated artifacts. Further research is needed, in order to optimize the experimental system.

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Research Article

Study of Possible Mechanisms Involved in the Inhibitory Effects of Coumarin Derivatives on Neutrophil Activity

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To specify the site of action of the synthetic coumarin derivatives 7-hydroxy-3-(4′-hydroxyphenyl) coumarin (HHC) and 7-hydroxy-3-(4′-hydroxyphenyl) dihydrocoumarin (HHDC), we evaluated their effects on extra- and intracellular reactive oxygen species (ROS) formation in phorbol-myristate-13-acetate (PMA) stimulated human neutrophils. We studied also the effects of HHC and HHDC on possible molecular mechanisms which participate in the activation of NADPH oxidase, that is, on PKC activity, on phosphorylation of some PKC isoforms (α, βII, and δ), and on phosphorylation of the NADPH oxidase subunit $p40^{phox}$. Without affecting cytotoxicity, both coumarines tested were effective inhibitors/scavengers of ROS produced by neutrophils on extracellular level. HHC markedly diminished oxidant production and also, intracellularly, decreased PKC activity and partly phosphorylation of PKCα, βII. On the other hand, we did not observe any effect of coumarin derivatives on phosphorylation of PKCδ and on phosphorylation of the NADPH oxidase subunit $p40^{phox}$, which were suggested to be involved in the PMA-dependent intracellular activation process. In agreement with our previous findings, we assume that the different molecular structures of HHC and HHDC with their different physicochemical and free radical scavenging characteristics are responsible for their diverse effects on the parameters tested.

1. Introduction

Neutrophils are key cells of the first line of defense, but they are also considered potent inflammatory cells causing tissue damage. Thus the ability of compounds which prevent extensive and potentially dangerous activation of neutrophils has been proposed as an important injury-limiting way. Coumarins belong to the group of plant-derived polyphenolic compounds possessing broad biochemical and pharmacological effects, like anti-HIV, anti-inflammatory, antioxidant, antibacterial, anticoagulant, and anticancer activities [1–4]. Over the last years, natural as well as synthetic coumarins were extensively studied and many of them are considered attractive candidates in therapeutic development.

Production of reactive oxygen species (ROS) in neutrophils and other phagocytic cells is linked to the activation of NADPH oxidase, a multiprotein enzyme complex, which plays an essential role in innate immunity. Yet excessive ROS generation by phagocytes is involved in tissue injury associated with a number of chronic inflammatory diseases [5–7]. In resting cells, NADPH oxidase is inactive and its components are distributed between the cytosol and membranes. When cells are activated, the cytosolic components ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac2) migrate to the membranes, where they associate with the membrane-bound component (flavocytochrome b558) to assemble the catalytically active NADPH oxidase, resulting in the delivery of ROS into the extracellular environment or inside into the

phagocytic vesicle [8–10]. NADPH oxidase is also activated within specific intracellular compartments, leading to an intracellular ROS production which may have a direct role in signal transduction. It was reported that the cytosolic component of the NADPH oxidase, $p40^{phox}$, associates with cytochrome b, especially in intracellular membranes [10–13]. Localisation of neutrophil ROS production and its possible regulation thus play an important role in the development of effective treatments to control the damage associated with chronic inflammation [8, 14].

Protein kinase C (PKC) has been shown to activate NADPH oxidase in several types of cells, such as phagocytes, cardiomyocytes, aortic endothelial cells, vascular smooth muscle cells, and renal mesangial cells [15, 16]. Various PKC isoforms have been shown to stimulate superoxide production, including α, β, δ, ε, and ζ [17, 18]. Neutrophils are strongly activated by PKC activating phorbol esters, which together with the expression of several PKC isoforms in neutrophils suggest a role for PKC in neutrophil functions [19]. Human neutrophils contain multiple isotypes of PKC, including Ca^{2+}/DG-dependent isotypes-α; alternatively spliced βI and βII; Ca^{2+}-independent/DG-dependent isotype δ; and phosphatidylserine-dependent Ca^{2+}/DG-independent ξ [19, 20]. In cell-free systems, PKCα, β, and δ are implicated as regulators of NADPH oxidase and superoxide generation [21]. Moreover, PKCδ has been suggested to be responsible for intracellular ROS generation in PMA activated neutrophils [22].

Previously, we compared the effect of 7-hydroxy-3-(4′-hydroxyphenyl) coumarin (HHC) and 7-hydroxy-3-(4′-hydroxyphenyl) dihydrocoumarin (HHDC) on stimulated phagocyte functions with their physicochemical characteristics and free radical scavenging activities in chemical assays [23].

To specify the site of action of the synthetic coumarin derivatives HHC and HHDC (Figure 1), we evaluated their effects on extra- and intracellular ROS formation in PMA stimulated human neutrophils. We also studied the effects of HHC and HHDC on possible molecular mechanisms which participate in the activation of NADPH oxidase: on PKC activity, phosphorylation of some PKC isoforms (α, βII, and δ), and on phosphorylation of the subunit $p40^{phox}$.

2. Material and Method

Phorbol-myristate-13-acetate (PMA), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), superoxide dismutase, dextran (average MW 464.000), and protease inhibitor cocktail: 104 mmol/L AEBSF [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride], 0.085 mmol/L aprotinin, 1.53 mmol/L bestatin hydrochloride, 1.40 mmol/L E-64 [N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide], 1.90 mmol/L leupeptin hemisulfate salt, and 4.22 mmol/L pepstatin in DMSO, were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany), horse radish peroxidase (HRP) and catalase from Merck (Darmstadt, Germany), and Lymphoprep (density 1.077 g/mL) from Nycomed Pharma AS (Oslo, Norway).

HHC and HHDC were kindly provided by Dr. Juraj Harmatha from the Institute of Organic Chemistry and Biochemistry, AV ČR, Prague, Czech Republic, and Professor Jan Šmidrkal from the Institute of Chemical Technology, Department of Dairy and Fat Technology, AV ČR, Prague, Czech Republic.

HHC (1.27 mg) was dissolved in a mixture of 20 μL 1 NaOH and 980 μL Tyrode solution (Tyrode solution consisted of 136.9 mmol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L $NaHCO_3$, 0.4 mmol/L $NaH_2PO_4 \cdot 2H_2O$, 1 mmol/L $MgCl_2 \cdot 6H_2O$, and 5.6 mmol/L glucose, pH 7.4). The stock solution (5 mmol/L) was further diluted with Tyrode solution to give HHC samples, concentrations 0.01–100 μmol/L. The corresponding final concentrations of NaOH were 0.4–400 μmol/L; at these concentrations, the solvent agent alone did not reduce the activity and viability of neutrophils.

All other chemicals of analytical grade were from available commercial sources.

This work was approved by the Local Ethic Committee, Institute of Experimental Pharmacology and Toxicology SAS.

2.1. Blood Collection and Isolation of Human Neutrophils. Fresh blood was taken at the blood bank from healthy volunteers (men, aged 20 to 50 years) by antecubital venipuncture and was immediately mixed with 3.8% v/w trisodium citrate, in the ratio of 9 mL of blood to 1 mL of citrate, in polypropylene centrifugation tubes. Erythrocytes were allowed to sediment in 3% dextran solution (1 ×g, 25 min, 22°C) and neutrophils were separated by gradient centrifugation on Lymphoprep (500 ×g, 30 min, 22°C). After hypotonic lysis of contaminating erythrocytes, neutrophils were washed and resuspended in calcium- and magnesium-free phosphate buffer saline in mmol/L: 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, and pH 7.4, at final concentration of 1×10^7 cells/mL. The obtained cell suspension contained more than 96% viable cells, as evaluated by trypan blue exclusion [24, 25]. Prior to neutrophil activation studies, the cells were resuspended in phosphate buffer saline (PBS) in mmol/L: 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 1.8 $CaCl_2$, 0.5 $MgCl_2 \cdot 6H_2O$, and pH 7.4.

2.2. Chemiluminescence Assay of Isolated Neutrophils. The effect of HHC or HHDC (0.01, 0.1, 1, 10, and 100 μmol/L) on extra- and intracellular ROS production was measured in unstimulated and PMA (0.05 μmol/L) stimulated neutrophils (5×10^5/sample) by isoluminol-/luminol-enhanced chemiluminescence (CL). Extracellular CL was determined in the system containing isoluminol (5 μmol/L) and HRP (8 U/mL). Intracellular CL was measured with luminol (5 μmol/L) in the presence of the extracellular scavengers superoxide dismutase (100 U/mL) and catalase (2 000 U/mL). CL was evaluated in a microplate luminometer Immunotech LM-01T (Czech Republic) at 37°C. Data were based on integral values of CL over 1800 s, (RLU × s; RLU: relative light units) [26, 27].

2.3. Chemiluminescence Assay in Cell-Free System. In the cell-free system, luminol, horseradish peroxidase, and hydrogen peroxide were used for chemiluminescence generation [28]. The 200 μL samples consisted of 50 μL aliquots: HRP (2 U/mL), luminol (10 μmol/L), HHC, or HHDC solutions in the concentrations of 1, 10, or 100 μmnol/L. The reaction was initiated by adding hydrogen peroxide at the final concentration of 100 μmol/L. The chemiluminescence responses were measured for 10 minutes at 37°C in a microplate luminometer Immunotech LM-01T (Czech Republic).

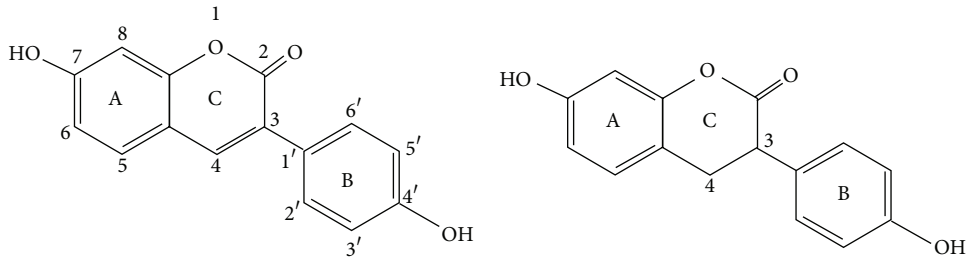
2.4. PKC Activity Assay. PKC activity in the cytosol was detected by the modified method of Varga et al. [29]. Isolated human neutrophils (5×10^5/mL) were incubated 30 min with HHC (1, 10, and 100 μmol/L) or HHDC (1, 10, and 100 μmol/L) at 37°C. Neutrophils were then stimulated with PMA (0.15 μmol/L, final concentration) at 37°C for 3 min. The reaction was stopped by addition of 10 vol of ice-cold PBS. After centrifugation (500 ×g, 4°C, 10 min) the cells were resuspended in sample buffer (20 mmol/L TRIS-HCl, 5 mmol/L EDTA, 1% Triton, 10% glycerol, and protease inhibitor cocktail), sonicated on ice, and centrifuged (14 000 ×g, 5 min, 4°C). The cytosolic fractions were transferred to a prechilled 1.5 mL microcentrifuge tube and stored at –70°C. Protein content of cytosol fraction was measured using Bradford Dye Reagent Detection Kit (Bio-Rad, USA). PKC activity was measured with a Nonradioactive Protein Kinase Assay kit (Assay Designs, Ann Arbor, MI, USA), which is based on a solid-phase enzyme-linked immunoabsorbent assay (ELISA), utilising a specific synthetic peptide as substrate for PKC and a polyclonal antibody recognizing the phosphorylated form of the substrate. The assay is developed with tetramethylbenzidine substrate and a colour develops in proportion to PKC phosphotransferase activity. The colour development was stopped with acid stop solution and the intensity of the colour was measured in a microplate reader at 450 nm. The data were expressed as relative kinase activity per 1 mg of protein.

2.5. Western-Blot Analysis. Phosphorylation of PKC isoenzymes α, βII, and δ, as well as NADPH oxidase subunit $p40^{phox}$, was detected using the method described by Jančinová et al. [24, 27].

The suspension of isolated neutrophils (100 μL) containing 5×10^6 cells was preincubated at 37°C for 60 seconds with different concentrations of HHC or HHDC (10, 100 μmol/L) prior to addition of PMA (0.15 μmol/L). Incubation with PMA (5 min) was stopped by using a solubilisation buffer (20 mmol/L Tris-HCl, 5 mmol/L EDTA, 1% Triton, 10% glycerol, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 208 μmol/L AEBF, 0.17 μmol/L aprotinin, 8 μmol/L bestatin, 2.8 μmol/L E-64, 4 μmol/L leupeptin, and pH 7.4). The suspension was sonicated at 4°C for 20 minutes and centrifuged at 14 000 ×g at 4°C for 5 min to remove unbroken cells. The supernatant was taken for measuring total protein by using Bradford Dye Reagent Detection Kit from Bio-Rad and for blotting assay. The supernatant for blotting assay was boiled for 5 min with sample buffer (50 mmol/L Tris-HCl, 2% SDS, 7.5% glycerol, 2.5% mercaptoethanol, 0.01% bromophenol blue, and pH 6.8) and samples were loaded on 9.8% SDS polyacrylamide gels. Proteins (20 μg per lane) were separated by electrophoresis and transferred to Immobilon-P Transfer Membrane (Millipore Corp., USA). From the two strips taken, one was detected for PKCα, βII (area between 60 and 100 kD) and the second for detection of β-actin (30–60 kD), which represented the internal control. Membrane strips were blocked for 60 min with 1% bovine serum albumin in Tris buffered saline (TBS: 20 mmol/L Tris-HCl, 154 mmol/L NaCl, 0.05% Tween-20, and pH 7.5). This was then followed by 60 min incubation in the presence of the primary antibodies: anti-phospho-PKC-α, βII (Thr638/641) (1 : 5 000) or β-actin (rabbit anti-human, 1 : 4 000) (Cell Signaling Technology, Danvers, MA, USA). The membranes were subsequently washed six times with TBS and incubated 60 min with the secondary antibody conjugated to horseradish peroxidase (anti-rabbit from donkey, 1 : 5 000, GE Healthcare Life Sciences, Little Chalfont, UK), and the activity of horseradish peroxidase of the bands corresponding to individual PKC isoforms was visualised using Enhanced Chemiluminescence Western Blotting Detection Reagents (GE Healthcare Life Sciences, Little Chalfont, UK).

NADPH oxidase subunit $p40^{phox}$ and PKC isoenzyme δ were detected in the same blots after their washing and incubation with 10x diluted stripping buffer (Reblot Plus Mild Solution, Millipore, Temecula, CA, USA) for 15 min and washed overnight in diluent buffer (1% bovine serum albumin in Tris buffered saline). This was followed by 60 min incubation in the presence of primary antibodies: anti-phospho-$p40^{phox}$ (T154) (1 : 5 000) or PKCδ (Thr505) (1 : 1 000) (Cell Signaling Technology, Danvers, MA, USA). The membranes were subsequently washed six times with TBS and incubated 60 min with the secondary antibody conjugated to horseradish peroxidase (anti-rabbit from donkey, 1 : 5 000, GE Healthcare Life Sciences, Little Chalfont, UK), and the activity of horseradish peroxidase of the bands corresponding to the individual NADPH oxidase subunit or to PKC isoform was visualised using Enhanced Chemiluminescence Western Blotting Detection Reagents (GE Healthcare Life Sciences, Little Chalfont, UK). Autoradiogram bands were quantified using the Image J programme. The optical density of each PKC or NADPH oxidase band was corrected by the optical density of the corresponding β-actin band [24, 27].

2.6. Measurement of Cytotoxicity. The cytotoxic effect of HHC and HHDC was evaluated by means of ATP liberation by the luciferin-luciferase chemiluminescence. The neutrophil suspension (30 μL; 30 000 cells/sample) and 20 μL of Tyrode's solution were incubated with 50 μL of HHC or HHDC (1–100 μmol/L) for 15 min at 37°C. Ten microliters of a mixture of luciferin (1.6 μg/sample) and luciferase (45 000 U/sample) was added, and the chemiluminescence was recorded for 60 s. In each experiment, the chemiluminescence of ATP standards (1–500 nmol/L) was measured and the concentrations of ATP in samples were calculated from



(a) (b)

Figure 1: Chemical structure of synthetic phenylcoumarin derivatives related to the natural 7-hydroxycoumarin (umbelliferon). (a) 7-Hydroxy-3-(4′-hydroxyphenyl) coumarin (HHC). (b) 7-Hydroxy-3-(4′-hydroxyphenyl)-3,4-dihydrocoumarin (HHDC).

the calibration curve. The total ATP content was assessed immediately after sonication of neutrophils for 10 s [24, 27].

2.7. Statistical Analyses. All values are given as means of 4–8 experiments ± SEM. Statistical significance of differences between means was established by Student's t-test and P values below 0.05 were considered statistically significant.

3. Results

3.1. Effects of HHC and HHDC on ATP Liberation. To determine cytotoxicity of HHC and HHDC, we tested their effect on ATP liberation (Table 1). In the concentrations of 1, 10, and 100 μmol/L, these compounds did not increase spontaneous ATP liberation (12.6 ± 0.9 nmol/L ATP), representing 7.6% of the total ATP content (158.7 ± 12.1 nmol/L ATP), as determined immediately after complete neutrophil destruction. The results indicate that treatment with increasing concentrations of HHC and HHDC did not cause neutrophil damage.

3.2. Effects of HHC and HHDC on Extra- and Intracellular Chemiluminescence of Neutrophils. For activation of isolated human neutrophils, the soluble stimulus PMA was used, which bypasses receptors and activates NADPH oxidase via redistribution of PKC and phosphorylation of several proteins. As mentioned above, PMA is useful in investigating signal transduction pathways leading to NADPH-oxidase activation in plasma (extracellular) and granule membranes (intracellular) [10, 11]. Figure 2 demonstrates kinetics of extra- and intracellular ROS generation in isolated human neutrophils after PMA stimulation. The extracellular ROS generation was much more intensive and reached the maximum sooner than did the intracellular ROS generation. The ratio between extra- and intracellular ROS generation was approximately 10 : 1 (12 696 RLU : 1 295 RLU).

Figure 3 shows that both compounds, HHC and HHDC, in the concentration scale of 0.01–100 μmol/L, decreased significantly extracellular ROS production, as measured by chemiluminescence. The concentrations of HHC and HHDC producing 50% inhibition (IC_{50}) of control extracellular chemiluminescence were 1.04 ± 0.20 μmol/L and 1.01 ± 0.13 μmol/L, respectively (Table 2).

Table 1: Cytotoxic effect of HHC and HHDC evaluated by means of ATP liberation.

Concentration (μmol/L)	ATP liberation (nmol/L)	
	HHC	HHDC
0	13.53 ± 1.59	12.38 ± 1.22
1	12.63 ± 1.67	11.99 ± 1.24
10	10.25 ± 0.37	12.57 ± 1.14
100	7.91 ± 0.41	12.94 ± 1.01

The data express ATP liberation from 30,000 neutrophils.
0: untreated neutrophils, 1–100 μmol/L: treated by HHC or HHDC, total (159 ± 12 nmol/L) represents ATP content as determined immediately after complete neutrophil destruction. Mean ± SEM, $n = 6$.

Table 2: Doses of the compounds tested producing 50% inhibition of control extracellular CL and intracellular CL of human neutrophils and cell-free CL system.

Chemiluminescence	HHC	HHDC
	EC_{50} (μmol/L)	EC_{50} (μmol/L)
Extracellular	1.04 ± 0.20	1.01 ± 0.13
Intracellular	5.24 ± 0.57	>100
Cell free	0.59 ± 0.01	2.47 ± 0.04

Human isolated neutrophils (5×10^5/sample) were stimulated with PMA (0.05 μmol/L) at 37°C, in the presence of HHC or HHDC (0.01–100 μmol/L). Extra- and intracellular ROS production were measured by isoluminol-/luminol-enhanced CL. In the cell-free system (luminol, horseradish peroxidase, and hydrogen peroxide), CL responses were measured in the presence of HHC or HHDC (1, 10, or 100 μmol/L) at 37°C. Percentage inhibition was calculated on the basis of integrated values of CL over 1800 s (extra- and intracellular CL) and of cell-free CL over 10 minutes. Mean ± SEM, n = 3–8. Control values given in RLU × seconds were $5.8 \times 10^6 \pm 1.4 \times 10^6$ for extracellular CL, $5 \times 10^5 \pm 0.5 \times 10^4$ for intracellular CL, and $3.8 \times 10^5 \pm 0.18 \times 10^4$ for cell-free CL.

While HHC in the concentration scale of 0.01–100 μmol/L decreased significantly intracellular ROS production (IC_{50}: 5.24 ± 0.57 μmol/L), HHDC was slightly effective only in the highest concentration tested (Figure 4 and Table 2).

3.3. Effects of HHC and HHDC on Cell-Free Chemiluminescence System. Further we tested the participation of direct antioxidant activity of HHC and HHDC in a cell-free CL system consisting of luminol, horseradish peroxidase, and

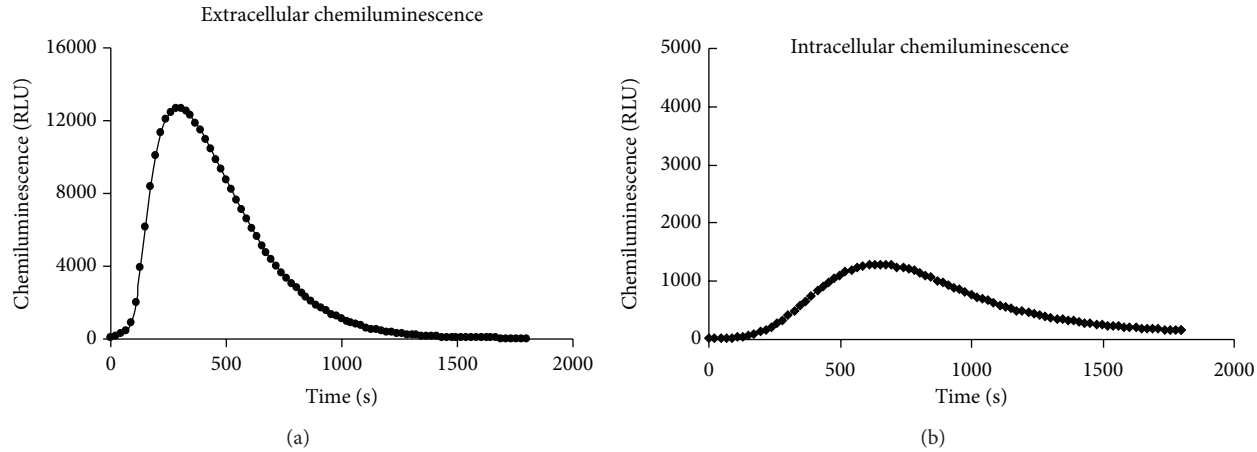


Figure 2: Kinetic curves of extra- and intracellular ROS formation in PMA stimulated human neutrophils. Human isolated neutrophils (5×10^5/sample) were stimulated with PMA (0.05 μmol/L) at 37°C. Extra- and intracellular ROS production were measured by isoluminol-/luminol-enhanced chemiluminescence over 1800 s. Kinetic curves are representative of 6 donors. RLU: relative light units.

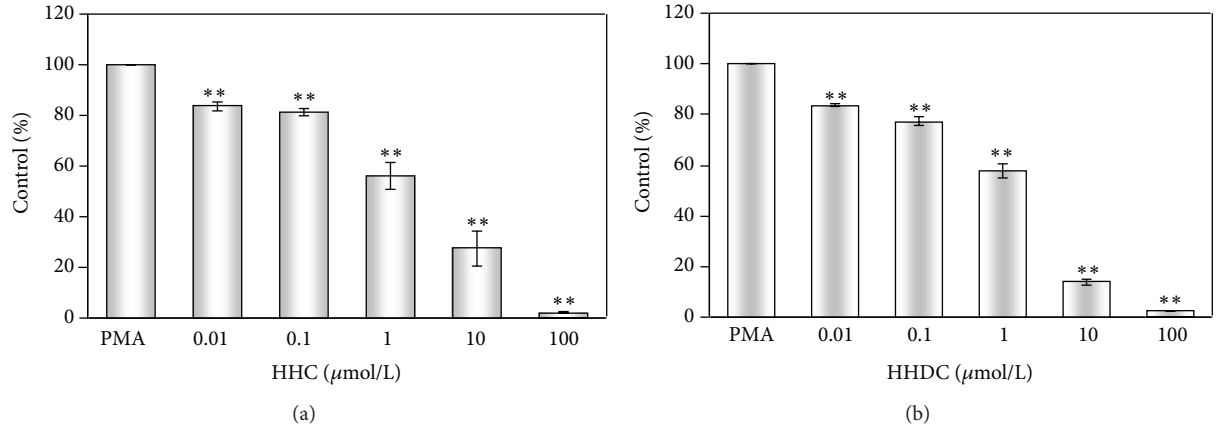


Figure 3: Effect of HHC and HHDC on PMA stimulated extracellular chemiluminescence of human neutrophils. Human isolated neutrophils (5×10^5/sample) were stimulated with PMA (0.05 μmol/L) at 37°C in the presence of HHC or HHDC (0.01–100 μmol/L). Extracellular ROS production was measured in the presence of HRP by isoluminol-enhanced chemiluminescence over 1800 s. The values were calculated as percentage of stimulated (PMA) control, on the basis of integrated values of chemiluminescence over 1800 s. Control value given in RLU × seconds was $5.8 \times 10^6 \pm 1.4 \times 10^6$. Mean ± SEM, $n = 6$. $^{**}P < 0.01$ as compared with the control (PMA) in the absence of the substances tested.

hydrogen peroxide. Our results showed an effective inhibition of chemiluminescence generated by cell-free system with HHC (IC_{50}: 0.59 ± 0.01) and HHDC (IC_{50}: 2.47 ± 0.04) (Table 2).

3.4. Effects of HHC and HHDC on PKC Activity and on the Phosphorylation of the PKC Isoforms (PKCα, βII, and δ). Stimulation of neutrophils with phorbol esters activates the PKC isoforms α, βII, and δ, which are involved in the oxidative burst as key activators of NADPH oxidase. First we analysed whether modulation of PKC activity by HHC or HHDC might mediate their inhibitory effect on PMA stimulated ROS production. HHC in the concentrations of 1, 10, and 100 μmol/L significantly decreased PKC activity to 87.7 ± 4.2%, 74.8 ± 6.3%, and 46.3 ± 1.8%, respectively. On the other hand, HHDC did not significantly influence PKC activity (Figure 5). Since the PKC activity assay determined all PKC isoforms ($\alpha, \beta, \gamma, \delta, \varepsilon, \mu, \zeta$, and θ), we further specified the effect of HHC and HHDC on phosphorylation of PKCα, βII, and δ.

After PMA stimulation, we observed increased phosphorylation of PKCα, βII, and δ in comparison with unstimulated human neutrophils. Treatment with HHC or HHDC in the concentrations of 10 and 100 μmol/L resulted in decrease in the level of PKCα, βII as compared to those of control but did not affect phosphorylation of PKCδ (Figures 6 and 7).

3.5. Effects of HHC and HHDC on the Phosphorylation of the NADPH Oxidase Component p40^{phox}. Recently, the selective role for p40^{phox} in intracellular ROS production was reported [10, 13]. We studied whether the observed inhibition of intracellular ROS production, particularly by HHC, might

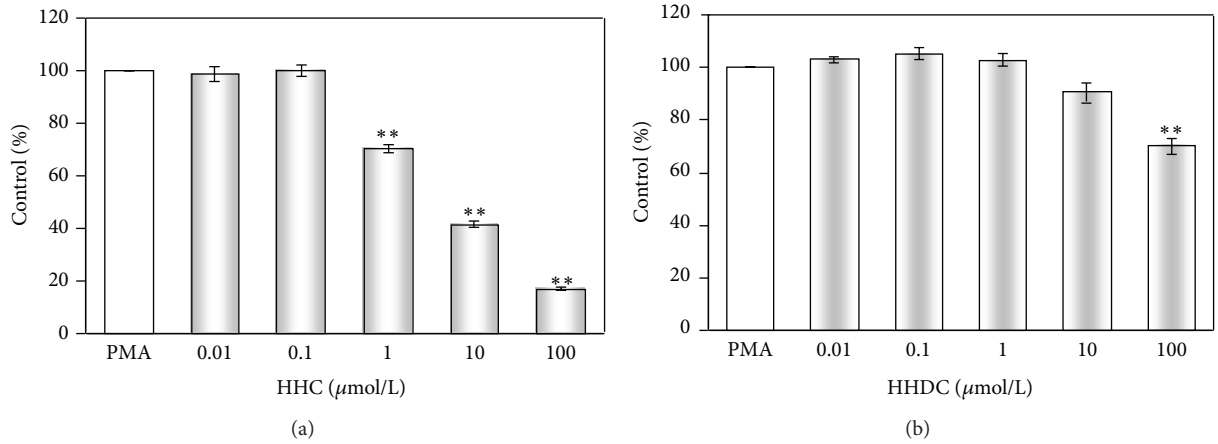


Figure 4: Effect of HHC and HHDC on PMA stimulated intracellular chemiluminescence of human neutrophils. Human isolated neutrophils (5×10^5/sample) were stimulated with PMA (0.05 μmol/L) at 37°C in the presence of HHC or HHDC (0.01–100 μmol/L). Intracellular ROS production was measured in the presence of superoxide dismutase and catalase by luminol-enhanced chemiluminescence over 1800 s. The values were calculated as percentage of stimulated (PMA) control, on the basis of integrated values of chemiluminescence over 1800 s. Control value given in RLU × seconds was $5 \times 10^5 \pm 0.5 \times 10^4$. Mean ± SEM, $n = 6$. $^{**}P < 0.01$, $^{*}P < 0.05$ as compared with the control (PMA) in the absence of the substances tested.

be connected with its effect on phosphorylation of $p40^{phox}$ subunit in PMA stimulated human neutrophils. As shown in Figure 8, PMA increased phosphorylation of $p40^{phox}$ subunit, yet HHC and HHDC failed to influence it.

4. Discussion

Regulation of neutrophil activity is one of the important factors in achieving host defense and avoiding tissue-damaging inflammation. Recently, great attention has been devoted to polyphenols of natural origin as well as to their synthetic derivatives. Many of them were found to possess effects which could be used in prevention and support therapy of chronic inflammatory diseases [30–35]. Previously, we have suggested that different effects of phenylcoumarin derivatives HHC and HHDC on phagocyte functions may be due to their diverse free radical scavenging properties and lipophilicity features. Further, we indicated that the ability of HHC and HHDC to reduce oxidant production in neutrophils might be connected with inhibition of NADPH oxidase activity, via decrease of PKC activation [23]. In the present study we specified the site of HHC and HHDC action on ROS generation and considered some possible molecular mechanisms linking regulation of oxidant production.

Our results showed that neutrophils activated by PMA responded by an oxidant production composed of both extra- and intracellular component of chemiluminescence. We found the extracellular ROS production to be predominant, as also demonstrated by Björnsdottir et al. [13] and Dahlgren and Karlsson [36]; however, in comparison with their results we observed a higher ratio between oxidants produced extra- and intracellularly, in favour of the former. HHC and HHDC were potent inhibitors of extracellular ROS production in human neutrophils stimulated with PMA. While HHDC reduced intracellular ROS generation only in the highest concentration tested, HHC possessed significant inhibitory effect in a concentration-dependent manner. The inhibition of both extra- and intracellular oxidant production indicated a possible interference of HHC and HHDC with ROS (scavenging activity) as well as with signalling events resulting in ROS production.

The effective inhibition of cell-free system chemiluminescence by both compounds tested (Table 2) showed that free radical scavenging properties of HHC and HHDC play an important role in the reduction of both extra- and intracellular oxidant production. The results obtained in cell-free system also suggest they interfere with peroxidase, since luminol reaction is highly dependent on the participation of myeloperoxidase. The possibility of this interaction with peroxidase is supported by findings of Kabeya et al. [37] and Andrade et al. [38] who demonstrated the inhibitory effect of 3-phenylcoumarin hydroxylated derivatives on horseradish peroxidase catalytic activity. The inhibitory effect of stilbene derivatives on myeloperoxidase release was described also by Pečivová et al. [39].

The relationships between antioxidant activities and chemical structures of coumarin derivatives are important factors which may influence the oxidant production *in vitro* and *in vivo* [1, 23, 38, 40–42]. Both coumarines tested, HHC and HHDC, have a similar structure: a hydroxyl group at C7 position at ring A and a hydroxyl group at C4′ position at ring B. While HHC has an unsaturated bond between C3 and C4 at ring C, HHDC fails to possess it (Figure 1). Our results indicate that the inhibitory effect of HHC and HHDC on PMA stimulated extracellular ROS generation in neutrophils is probably not dependent on the presence of a double bond between C3 and C4 at ring C. On the other hand, the more pronounced inhibition of intracellular ROS production by HHC indicates the requirement of an unsaturated 3-, 4-binding site at ring C for this inhibitory effect.

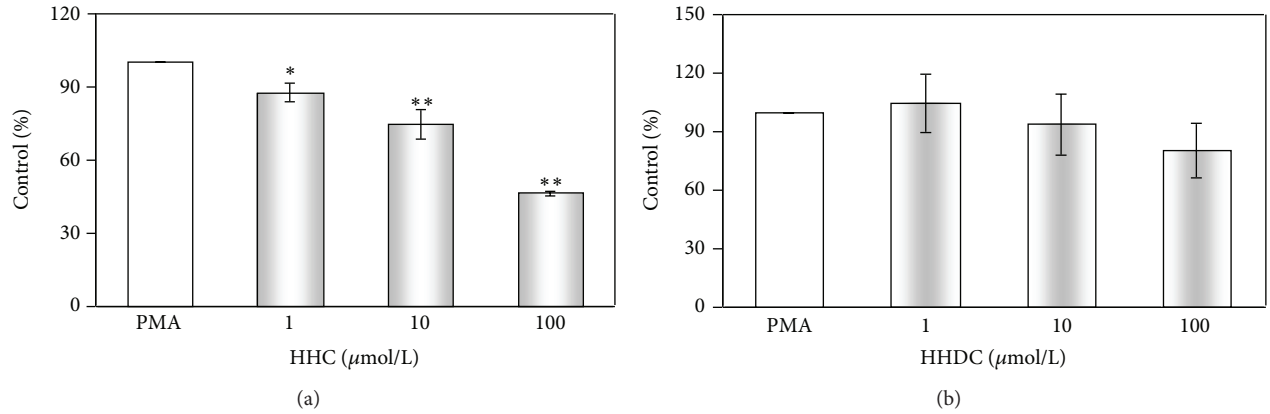


Figure 5: Effect of HHC and HHDC on PKC activity. Human isolated neutrophils (5×10^5/mL) were incubated for 30 min with HHC or HHDC (1, 10, and 100 μmol/L) and stimulated with PMA (0.15 μmol/L) at 37°C for 3 min. PKC activity was measured by ELISA kit in the supernatant of cell lysate. The values were calculated as percentage of stimulated (PMA) control. Control value given as relative kinase activity (450 nm) per 1 mg of protein was 9 708 ± 1 168. Mean ± SEM, $n = 8$. $^{**}P < 0.01$, $^{*}P < 0.05$ as compared with the control (PMA) in the absence of the substances tested.

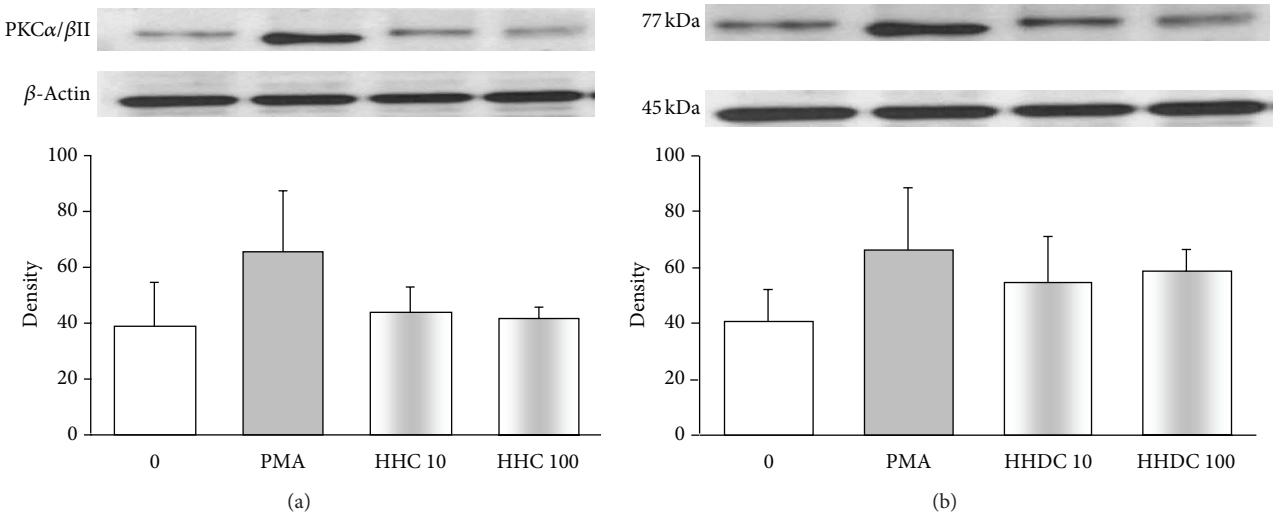


Figure 6: PKC phosphorylation in PMA stimulated human neutrophils treated with 10 and 100 μmol/L HHC or HHDC. Human isolated neutrophils (5×10^6 cells) were incubated at 37°C with HHC or HHDC (10 or 100 μmol/L) for 1 min, prior to addition of PMA (0.15 μmol/L). Cell lysates were prepared, and the protein levels of PKC isoenzymes (α and βII) were analyzed by Western blotting and detected by Phospho-PKCα/βII (Thr638/641) Antibody. The data are evaluated as optical density of PKC corrected to optical density of the corresponding β-actin band. Mean ± SEM, $n = 8$ (the data are representative of 4 donors performed in 2 separate experiments).

Polyphenols were reported to affect cell functions by modifying plasma membrane structure and physical characteristics, such as fluidity and electrical properties. These effects can be observed both when polyphenols are adsorbed on the membrane and when they are inserted into the bilayer [43]. The low effect of HHDC on intracellular ROS generation might be explained by the lower values of the partition coefficient of HHDC compared to that of HHC and thus the more efficient penetration of HHC into the membrane than that of the less lipophilic HHDC, as shown by our earlier published findings [23].

In biological systems, ROS are generated by a number of enzymatic systems, and modifications of plasma membrane structure can result in functional changes, including the activity of membrane-associated enzymes and the modulation of signal transduction [7, 43].

Inhibition of PKC or downregulation of its intracellular expression and activity has been proposed as an important mechanism of the antioxidant effect of polyphenols [7, 27, 35, 44, 45]. We found that HHC, unlike HHDC, effectively decreased intracellular oxidant production involved in the regulation of neutrophil function, and we thus supposed its interaction to occur at PKC level. Since we observed a significant reduction of PKC activity by HHC, we further specified its effect on PKCα, βII phosphorylation. Inhibition of PKC activity and PKCα, βII phosphorylation by HHC only,

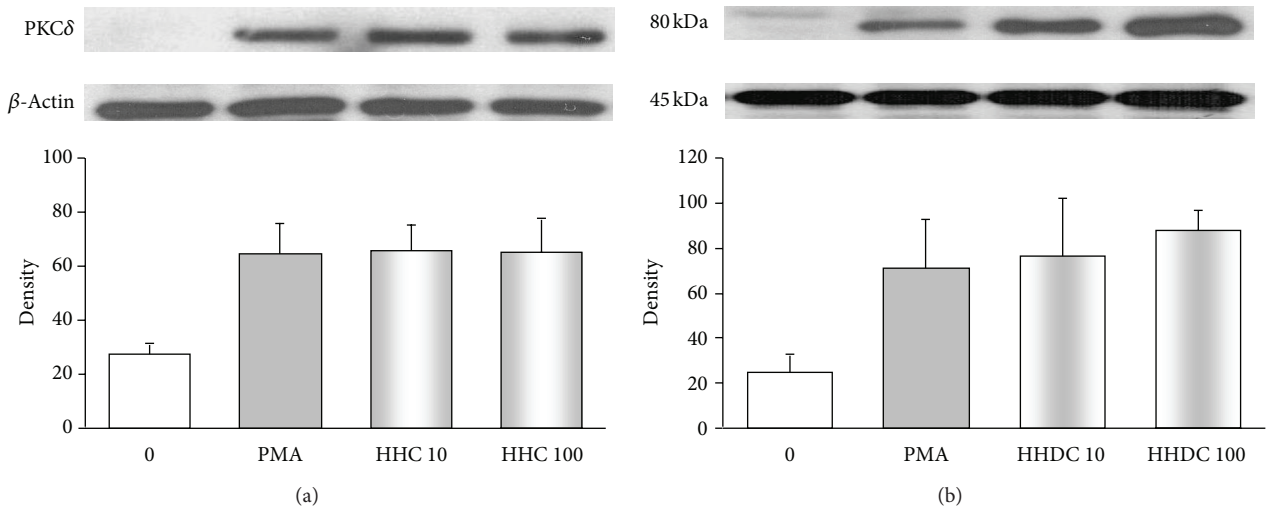


Figure 7: PKC phosphorylation in PMA stimulated human neutrophils treated with 10 and 100 μmol/L HHC or HHDC. Human isolated neutrophils (5×10^6 cells) were incubated at 37°C with HHC or HHDC (10 or 100 μmol/L) for 1 min, prior to addition of PMA (0.15 μmol/L). Cell lysates were prepared, and the protein level of PKC isoenzyme δ was analyzed by Western blotting and detected by Phospho-PKCδ (Thr505) Antibody. The data are evaluated as optical density of PKC corrected to optical density of the corresponding β-actin band. Mean ± SEM, $n = 8$ (the data are representative of 4 donors performed in 2 separate experiments).

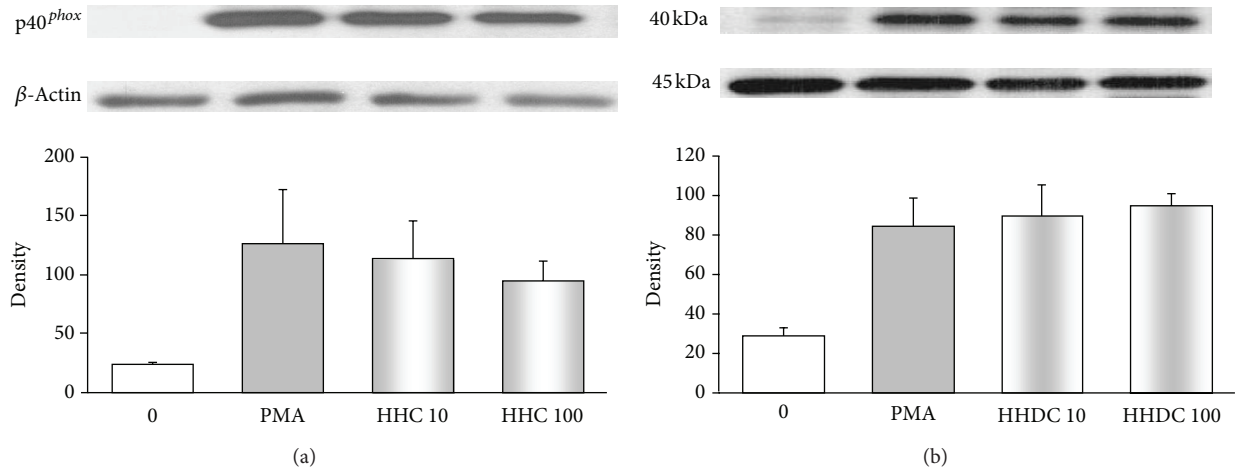


Figure 8: Phosphorylation of the NADPH oxidase component $p40^{phox}$ in PMA stimulated human neutrophils treated with 10 and 100 μmol/L HHC or HHDC. Human isolated neutrophils (5×10^6 cells) were incubated at 37°C with HHC or HHDC (10 or 100 μmol/L) for 1 min, prior to addition of PMA (0.15 μmol/L). Cell lysates were prepared, and the protein level $p40^{phox}$ subunit was analyzed by Western blotting and detected by Phospho-$p40^{phox}$ (Thr 154) Antibody. The results are evaluated as optical density of $p40^{phox}$ corrected to optical density of the corresponding β-actin band. Mean ± SEM, $n = 8$ (the data are representative of 4 donors performed in 2 separate experiments).

indicates that the presence of the double bond between C3 and C4 at ring C is necessary for this effect. Structure dependence of the inhibitory effect of polyphenolic antioxidants on signal transduction enzymes, such as PKC, was found also by Varga et al. [40]. Although a specific role for PKCδ in the PMA-dependent intracellular activation process was reported [22], we did not observe any effect of coumarines tested on phosphorylation of PKCδ.

One of the cytosolic components of the NADPH oxidase, $p40^{phox}$, which is specifically translocated to intracellular phagosomal and granule membranes, was also indicated as a further determining factor for intracellular ROS production [10, 12]. Moreover, a reduction of $p40^{phox}$ phosphorylation by PKC inhibitors was found by Bouin et al. [46] and Someya et al. [47]. Our results, however, showed that in PMA stimulated neutrophils reduction of intracellular ROS generation and PKC activity by HHC was not associated with inhibition of phosphorylation of the NADPH oxidase subunit $p40^{phox}$.

5. Conclusion

We extended our previous findings about the actions of the coumarine derivatives HHC and HHDC on the activity

of human neutrophils by investigating the influence of extra- and intracellular ROS formation and some possible molecular mechanisms affecting the regulation of oxidant production. The presented results suggest that, in pathological processes in which neutrophils are involved, HHDC may act predominantly as a potent inhibitor/scavenger of extracellularly produced ROS. HHC may act both extra- and intracellularly, and, besides its direct interference with ROS, it may interfere also with the PKC signalling pathway. These findings confirmed our previous assumption that the different effects of the coumarine derivatives tested might be due to their diverse molecular structures, which provides them with different physicochemical and free radical scavenging characteristics.

Conflict of Interests

The authors state no conflict of interests.

Acknowledgments

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Research Article

Steviol Glycosides Modulate Glucose Transport in Different Cell Types

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Extracts from *Stevia rebaudiana* Bertoni, a plant native to Central and South America, have been used as a sweetener since ancient times. Currently, *Stevia* extracts are largely used as a noncaloric high-potency biosweetener alternative to sugar, due to the growing incidence of type 2 diabetes mellitus, obesity, and metabolic disorders worldwide. Despite the large number of studies on *Stevia* and steviol glycosides *in vivo*, little is reported concerning the cellular and molecular mechanisms underpinning the beneficial effects on human health. The effect of four commercial *Stevia* extracts on glucose transport activity was evaluated in HL-60 human leukaemia and in SH-SY5Y human neuroblastoma cells. The extracts were able to enhance glucose uptake in both cellular lines, as efficiently as insulin. Our data suggest that steviol glycosides could act by modulating GLUT translocation through the PI3K/Akt pathway since treatments with both insulin and *Stevia* extracts increased the phosphorylation of PI3K and Akt. Furthermore, *Stevia* extracts were able to revert the effect of the reduction of glucose uptake caused by methylglyoxal, an inhibitor of the insulin receptor/PI3K/Akt pathway. These results corroborate the hypothesis that *Stevia* extracts could mimic insulin effects modulating PI3K/Akt pathway.

1. Introduction

Stevia rebaudiana Bertoni is a weak perennial shrub belonging to Asteraceae (Compositae) family, native to subtropical regions of Brazil and Paraguay. Its leaves have been used as a sweetener since ancient times and for many other medicinal purposes in Latin America and the Orient for centuries [1, 2]. The "sweet herb" has gained increasing interest from nutritional researchers and commercial area in the last years, due to the growing need to find new natural calorie-free sweeteners alternative to sugar. Indeed, in both industrialized and developing countries, the incidence of type 2 diabetes mellitus and obesity is sharply increasing as a result of dietary behaviours, reduced physical activities, and ageing. These metabolic disorders have become major public health problems worldwide [3, 4].

Glycemic control is fundamental to the management of diabetes since it is associated with significantly decreased rates of retinopathy, nephropathy, neuropathy, and cardiovascular disease, the most common cause of death in diabetic patients. The effort to achieve near-normoglycemia through the key strategy of glycemic control includes recommendations for prevention and control of diabetes, for example, monitoring carbohydrate intake and limiting the consumption of sugar-sweetened beverages [5].

Stevia leaves and extracts are natural noncaloric sweeteners that can substitute sucrose. The main sweet components in leaves, approximately 200–400 times sweeter than sucrose as shown by organoleptic tests [1, 6], are stevioside and rebaudioside A, steviol glycosides differing only by one glucose moiety. Stevioside is formed by 3 molecules of glucose and one molecule of the aglycone steviol, a diterpenic carboxylic alcohol; rebaudioside A holds one additional glucose molecule [7] (Figure 1).

Steviol glycosides have been recently authorised as commercial sweeteners. US Food and Drug Administration

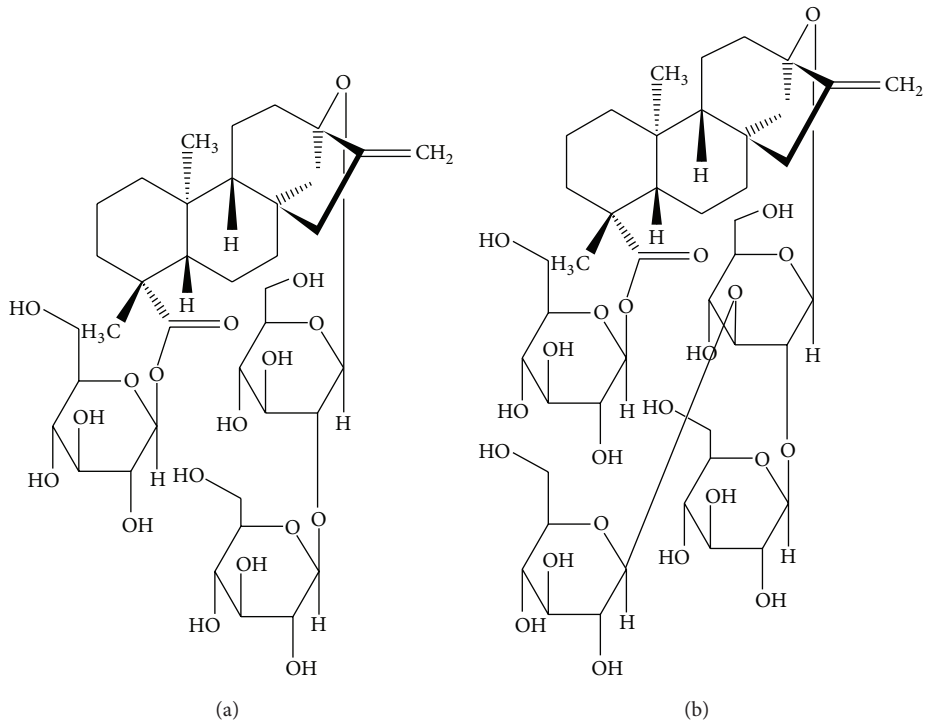

Figure 1: Chemical structure of stevioside (a) and rebaudioside A (b).

(FDA) has allowed the use of *Stevia* extracts containing not less than 95% total steviol glycosides. Recently, the European Food Safety Authority (EFSA) approved the use of steviol glycosides as food additive [8, 9]. Considering the available toxicity data (*in vitro* and *in vivo* animal studies and some human tolerance studies), steviol glycosides are considered not carcinogenic, genotoxic, or associated with any reproductive/developmental toxicity. Joint Expert Committee on Food Additives (JECFA) established an accepted daily intake (ADI) for steviol glycosides (expressed as steviol equivalents) of 4 mg/kg bw/day [10, 11].

Besides sweetness, steviol glycosides, in particular stevioside, have been shown to possess beneficial effects on human health [7, 12, 13]. Briefly, pharmacological activities and therapeutical benefits include antitumour and anticancer, anti-inflammatory, antihyperglycemic, antihypertensive, antidiarrheal, immunomodulatory, diuretic, and enzyme inhibitory actions. *Stevia* has also been used to help control weight in obese subjects [14]; moreover, antioxidant properties have been described [15, 16]. Stevioside, rebaudioside A, and their metabolite steviol have been mostly investigated in *in vivo* animal studies and, at a lesser extent, in humans. Results suggest that stevioside and related compounds affect plasma glucose modulating insulin secretion and sensitivity, which increase glucose removal from the plasma [17, 18]. In addition, it seems likely that stevioside inhibits gluconeogenesis in the liver of diabetic rats [19, 20]. These antihyperglycemic, insulinotropic, and glucagonostatic effects, especially for rebaudioside A, are largely plasma glucose level dependent, requiring high glucose levels [21, 22]. Despite the large number of studies on *Stevia* and steviol glycosides, very little is reported concerning the cellular and molecular mechanisms underpinning these effects.

In the present study, we examined the role of steviol glycosides on cellular glucose transport in cultured cells. Glucose is a polar molecule and requires specific carrier proteins, located in the plasma membrane, to cross the lipid bilayer and enter the cell. Glucose is transported into the cells through two different types of membrane associated carrier proteins, the Na^+-coupled glucose transporters (SGLT) and the facilitative glucose transporters (GLUT). The human GLUT family is integral membrane proteins widely distributed in probably all mammalian cells that regulate the movement of glucose between extracellular and intracellular compartments maintaining a constant supply of glucose available for metabolism [23]. To date, GLUT family is constituted by 14 distinct isoforms, differently distributed in human tissues [23, 24]. GLUT1 is considered responsible for the basal uptake in many cell types, representing the most ubiquitously expressed isoform; GLUT4 is responsible for insulin-stimulated glucose uptake in peripheral tissues, but its expression has also been reported in the brain [25, 26], where glucose is an essential substrate for cerebral oxidative metabolism. It has recently been reported that in a human neuronal cell line, SH-SY5Y, GLUT1 translocation in response to insulin-like growth factor (IGF-I) occurs [27] and, for the first time in a neuronal cell system, also GLUT4 is translocated to the plasma membrane in response to insulin [28]. We have been studying for a long time the glucose transport activity in many leukaemia cell lines expressing mainly GLUT1, demonstrating that, also in these cell types, GLUT1 is recruited on the plasma membrane from

intracellular compartments in response to different stimuli, greatly enhancing the rate of glucose uptake [29, 30]. Moreover, it is well known that impaired GLUT4 translocation is causally linked to insulin resistance and consequently to noninsulin-dependent diabetes mellitus [31, 32].

Starting from this knowledge and this background, we chose the neuroblastoma SH-SY5Y and the promyelocytic leukaemia line HL-60, both expressing insulin and insulin-like growth factor-1 (IGF-I) receptors [28, 33] to test some commercial *Stevia* extracts, in order to evaluate a possible effect of these compounds on glucose transport and to clarify the molecular mechanism of action.

2. Materials and Methods

2.1. Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), penicillin/streptomycin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), staurosporine, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, dichlorofluorescin diacetate), 2-deoxy-glucose (DOG), phloretin, CelLytic M, mammalian protease inhibitor mixture, primary antibody to β-actin, methylglyoxal (MG), hydrogen peroxide, bovine serum albumin (BSA), rebaudioside A standard (StReb), stevioside standard (StStev), and all other chemicals of the highest analytical grade were purchased from Sigma-Aldrich. Roswell Park Memorial Institute (RPMI) 1640 medium (with Hepes, with L-glutamine) was purchased from PAA. 2-Deoxy-D-[2, 3]-glucose and Ultima Gold MV scintillation cocktail were from PerkinElmer. PhosSTOP, a phosphatase inhibitor cocktail, was obtained from Roche Diagnostic. Nitrocellulose membranes and Amersham ECL Advance Western Blotting Detection Reagents were from GE-Healthcare. Primary antibodies against phospho-Akt (Ser473) (no. 4058), total Akt (no. 9272), and horseradish peroxidase-conjugated secondary antibodies anti-rabbit (no. 7074) and anti-mouse (no. 7076) were purchased from Cell Signaling Technologies. Anti-GLUT1 (sc-1603), anti-GLUT4 (sc-1606) antibodies, and anti-goat IgG conjugated to horseradish peroxidase (sc-2020) were obtained from Santa Cruz Biotechnology. Primary antibody anti-phospho-PI3 Kinase p85 pTyr458/p55 pTyr199 (no. PA5-17387) was from Thermo Scientific. Anti-PI3 Kinase (no. 06-195) antibody was purchased from Millipore. PageRuler Prestained protein ladder was from Fermentas—Thermo Fisher Scientific.

Extracts from *Stevia rebaudiana* Bertoni were kindly supplied by Eridania Sadam SpA.

According to FDA and EFSA [8–11], total content of steviol glycosides in commercial *Stevia* extracts has to be at least 95% (w/w), and rebaudioside A plus stevioside must be at least 75%. The four extracts tested differ by the relative content of rebaudioside A and stevioside. In particular, according to the certificates of analysis of each sweetener, Reb A (R97) contains 97-98% rebaudioside A, *Stevia* RA60 (R60) contains about 60% rebaudioside A and about 20% stevioside; Steviol Glycosides SG95 (SG) contains 50% rebaudioside A and at least 25% stevioside; Truvia (TRU) contains a mixture of steviol glycosides not analytically quantified.

2.2. Cell Culture. SH-SY5Y, human neuroblastoma cells, were grown at 37°C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin, as reported in [34]. HL-60, acute myeloid leukaemia cells, were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere maintained at 5% CO_2.

2.3. Cell Viability. Cells were treated with different concentrations of steviol glycosides (0.5 to 5 mg/mL) or 1 mM (corresponding to 1 mg/mL) StReb or 1 mM (corresponding to 0.8 mg/mL) StStev for 24 h. Cell viability was evaluated by the MTT assay as reported in [35]. SH-SY5Y and HL-60 cells were incubated with 0.5 mg/mL MTT for 4 h at 37°C in multiwell plates. At the end of the incubation, blue-violet formazan salt crystals were formed and dissolved by adding the solubilisation solution (10% SDS, 0.01 M HCl); then the plates were incubated overnight in humidified atmosphere (37°C, 5% CO_2) to ensure complete lysis. The absorbance at 570 nm was measured using a multiwell plate reader (Wallac Victor^2, PerkinElmer).

2.4. Lactate Dehydrogenase Assay. SH-SY5Y and HL-60 cells were incubated with 1 mg/mL of each *Stevia* extract for 24 h. Lactate dehydrogenase (LDH) release from cells was monitored by collecting aliquots of medium. LDH activity was assayed by a spectrophotometric method based on the reduction of pyruvate to lactic acid coupled to NADH oxidation. The decrease in absorbance at 340 nm was monitored at 37°C. 100 μM H_2O_2 for 30 minutes was used as a positive control.

2.5. Assay for Caspase 3 Activity. Caspase 3 activity in the cell lysates was measured using a colorimetric assay kit by following the instructions from the manufacturer (Sigma), as described in [36]. Cells were incubated with or without steviol glycosides (1 mg/mL) for 1, 6, or 24 h. After 24 h, cells were collected and lysed using the lysis buffer provided in the kit (250 mM HEPES, pH 7.4 containing 25 mM CHAPS, and 25 mM DTT). The assay was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) by caspase 3, resulting in the release of free AMC moiety. The fluorescence of AMC was read using a multiwell plate reader (Wallac Victor^2, PerkinElmer); excitation and emission wavelengths were 360 nm and 460 nm, respectively.

The concentration of the AMC released was calculated using an AMC standard curve. Caspase 3 activity was expressed in nmole of AMC released per min per mL of cell lysate and normalised for total protein content in the lysate. Results are reported as percentage with respect to the control. Staurosporine (1 μg/mL) was used as an apoptosis inducer (positive control).

2.6. Measurement of Intracellular Reactive Oxygen Species (ROS) Levels. ROS intracellular level was evaluated by using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). SH-SY5Y and HL-60 cells were incubated with 5 mg/mL of each *Stevia* extract for 1 h and then subjected or not to oxidative stress generated by 100 μM H_2O_2 for 30 minutes. Successively, cells were washed twice in PBS and incubated with 5 μM H_2DCFDA for 20 min at 37°C. H_2DCFDA is a small nonpolar, nonfluorescent molecule that diffuses into the cells, where it is enzymatically deacetylated by intracellular esterases to a polar nonfluorescent compound, that is oxidised to the highly green fluorescent 2′,7′-dichlorofluorescein (DCF). The fluorescence of oxidized probe was measured using a multiwell plate reader (Wallac Victor^2, PerkinElmer). Excitation wavelength was 485 nm and emission wavelength was 535 nm. Fluorescence values were reported as the percentage of intracellular ROS with respect to control.

2.7. Glucose Transport Assay. Glucose transport assay was performed as described in [37, 38]. Cells were incubated or not with different compounds (1 mg/mL) for 1 h; then they were washed twice in PBS and treated for 10 min (SH-SY5Y) or 2 min (HL-60) at 37°C with a mixture of 2-deoxy-D-[2, 3] glucose (0.8 μCi/assay) and 1.0 mM unlabeled glucose analogue, under conditions where the uptake was linear at least for 20 min. The transport was stopped by adding phloretin (final concentration 0.3 mM), a potent inhibitor of glucose transport activity. Radioactivity was measured by liquid scintillation counting (Tri-Carb liquid scintillation analyser, PerkinElmer).

2.8. Immunoblotting Analysis. After treatments, cells were washed with ice-cold PBS and lysed on ice using CelLytic M containing mammalian protease and phosphatase inhibitor mixture. The resulting lysed cells were left on ice to solubilize for 45 min. The lysates were centrifuged at 5000 g for 5 min at 4°C to remove unbroken cell debris and nuclei. Cell lysate protein concentration was determined by the Bio-Rad Bradford protein assay (Bio-Rad Laboratories). Samples were kept at 95°C for 5 min prior to separation on 10% SDS-PAGE Mini-Protean TGX precast gels using a Mini-Protean apparatus (Bio-Rad Laboratories). Proteins (15 μg/lane) were electrophoretically transferred to nitrocellulose membrane (Hybond-C; GE Healthcare) in Tris-glycine buffer at 110 V for 90 min. Membranes were then incubated in blocking buffer containing 5% (w/v) albumin in Tris-buffered saline (TBS)/Tween to avoid nonspecific binding and incubated overnight at 4°C with primary antibodies (anti-GLUT1, anti-GLUT4, anti-phospho-Akt, anti-total Akt, anti-phospho-PI3K, anti-total-PI3K, or anti-β-actin as internal normalizer). Nitrocellulose membranes were then washed 3 times with TBS/Tween and incubated with secondary antibodies in TBS/Tween containing 5% albumin for 60 min at room temperature and successively washed with TBS/Tween. The results were visualized by chemiluminescence using ECL Advance reagent according to the manufacturer's protocol (GE Healthcare). Images of the blots were obtained using a CCD imager (ChemiDoc MP System, Bio-Rad). Bands were acquired and analysed by using Image Lab analysis software.

2.9. Statistical Analysis. Results are expressed as means ± SD. Differences among the means were determined by Bonferroni multiple comparison test following one-way ANOVA and were considered significant at $P < 0.05$.

3. Results and Discussion

The effect of different commercial extracts from *Stevia rebaudiana* Bertoni on glucose transport was investigated in both SH-SY5Y neuroblastoma and HL-60 myeloid leukaemia human cells.

Glucose is the primary source of energy used by the brain and it is constantly delivered to individual cells (glial cells and neurons) [39]. In brain, the relationship among glucose metabolism, GLUT isoforms, modulation of glucose uptake, role of insulin, and distribution of insulin receptor (IR) is very complex, being dependent on specific regions of the brain and playing a key role also in cognitive functions. Recent studies report a close correlation between impaired glucose uptake/metabolism and neurodegenerative diseases such as Alzheimer's disease [40–42].

It is also recognised that cancer cells frequently overexpress the GLUT family members, due to the uncontrolled proliferation requiring elevated energy, and they often express GLUT isoforms not present in normal conditions. Moreover, large hypoxic areas into the tumour cause an increase in glucose utilization by cancer cells through glycolysis. The requirement for energy is satisfied by an augmented sugar intake, realised by an increase in GLUT expression and an increment in the translocation of the transporters to the plasma membrane [23]. For these reasons, cancer cells are a useful model system to study the glucose transport activity and its signalling transduction pathway, allowing to clarify the molecular mechanism underlying steviosides biological effects on glucose metabolism.

The first aim of our paper was to evaluate the effect of four different *Stevia* extracts on cellular viability, assessed by MTT assay. SH-SY5Y and HL-60 cells were treated with different concentrations (0.5–5 mg/mL, corresponding to 0.5–5 mM for R97, which can be assumed as a pure compound) of *Stevia* extracts for 24 h. Data reported in Figure 2 show that the extracts did not affect cell viability/proliferation, confirming that they are not cytotoxic within the concentration range tested. Same results were obtained with similar concentrations of StReb or StStev (data not shown).

Cytotoxicity was also evaluated by lactate dehydrogenase (LDH) assay, which indicated that cell membrane integrity was not compromised and excluded cellular necrosis (Figure 3).

In order to evaluate a possible effect on apoptosis, the activity of caspase 3 was measured. Caspases play a central role in mediating various apoptotic responses and are activated in a sequential cascade of cleavages. To detect the enzymatic activity of caspase 3, the fluorogenic substrate Ac-DEVD-AMC was employed. Treatments of cells with *Stevia*

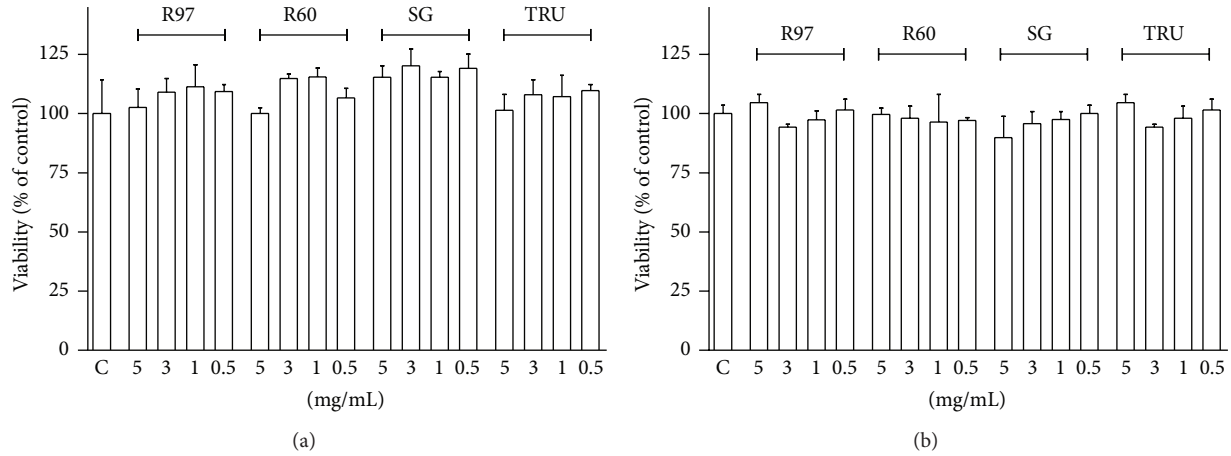

Figure 2: Effect of steviol glycosides on cell viability/proliferation. SH-SY5Y (a) and HL-60 (b) cells were treated for 24 hours with different concentrations of the four compounds (0.1 mg/mL to 5 mg/mL). Viability/proliferation was evaluated by MTT assay as described in Section 2 and compared to control (C). Results are expressed as means ± SD of three independent experiments ($n = 8$). Statistical analysis was performed by Bonferroni multiple comparison test following one-way ANOVA. Significant differences were not revealed.

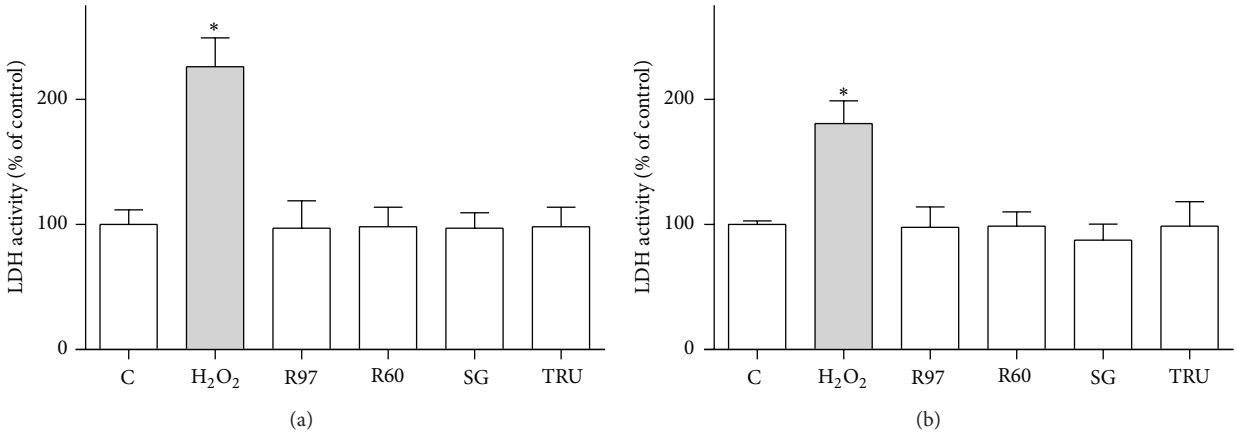

Figure 3: Effect of steviol glycosides treatment on lactate dehydrogenase (LDH) activity. LDH activity was measured by LDH assay as described in Section 2. SH-SY5Y (a) and HL-60 (b) cells were treated with different compounds at 1 mg/mL final concentration for 24 hours, or cells were treated with 100 μM H_2O_2 for 30 min as control of LDH activity. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. Statistical analysis was performed by Bonferroni multiple comparison test following one-way ANOVA. $^*P < 0.05$, significantly different from control cells.

extracts for 1 hour, 6 hours (data not shown), or for 24 hours (Figure 4) did not influence caspase 3 activity, indicating that the compounds did not induce programmed cell death.

Since antioxidant properties of *Stevia* extracts have been described [15, 16], the antioxidant activity of the commercial extracts was investigated in SH-SY5Y and HL-60 cells. Reactive oxygen species (ROS) levels were measured with the cell-permeant probe H_2DCFDA, commonly used to detect free radical/ROS production in cells, owing to the intracellular conversion to the highly green fluorescent DCF [43]. As shown in Figure 5, the compounds did not exhibit any antioxidant activity, since they were neither able to decrease basal ROS level at the highest concentration used nor to counteract intracellular ROS raise due to exogenous oxidative stress (100 μM H_2O_2 for 30 min). This lack of antioxidant activity is in contrast with the data reported by other authors [15, 16], probably because the compounds used in the present study are commercial sweeteners containing 95–98% steviol glycosides with no appraisable amounts of polyphenols, naturally present in *Stevia* leaves, likely responsible for *Stevia* antioxidant activity.

Stevia extracts are largely used as a noncaloric high-potency biosweetener substitute for sugar. The effect of four *Stevia* extracts on glucose transport activity was evaluated in HL-60 human leukaemia cells, expressing principally GLUT1, the basal glucose transporter, and in SH-SY5Y human neuroblastoma cells, expressing also GLUT4, the insulin-sensitive one. Figure 6 shows that all the extracts and the two standard compounds were able to enhance glucose uptake at similar extent after 1-hour incubation in both cellular lines.

Since the increase in glucose uptake obtained with standards is consistent with that shown by the whole extracts, we

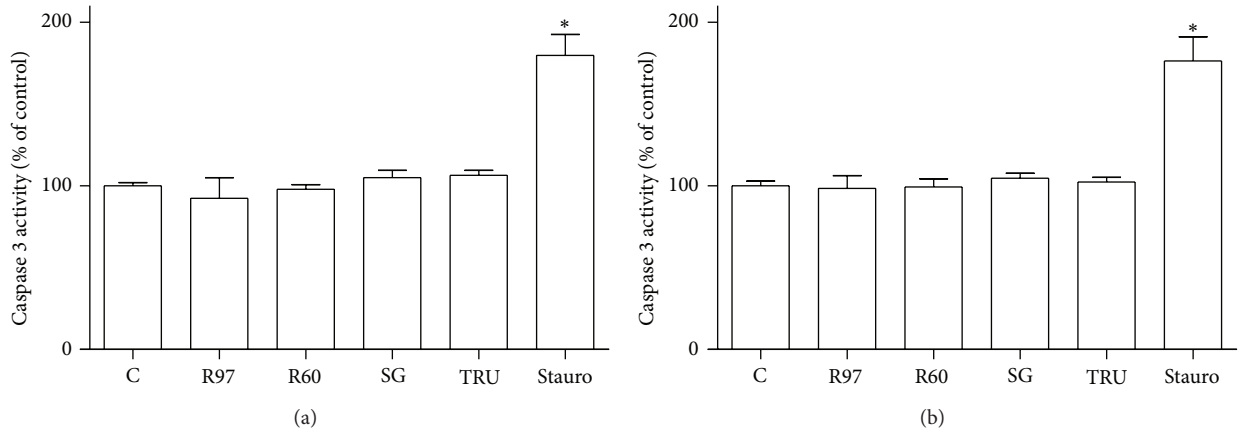

Figure 4: Caspase 3 activity in SH-SY5Y (a) and HL-60 (b) cells was evaluated in the presence of 1 mg/mL steviol glycosides incubated for 24 hours. Staurosporine (Stauro, 1 μg/mL for 4 hours) was used as positive control. Caspase 3 activity was measured spectrofluorimetrically after 24 hours in cell lysates as reported in Section 2. Each column represents the mean ± SD of three independent experiments. Statistical analysis was performed by Bonferroni multiple comparison test following one-way ANOVA. $^{*}P < 0.05$, significantly different from control cells.

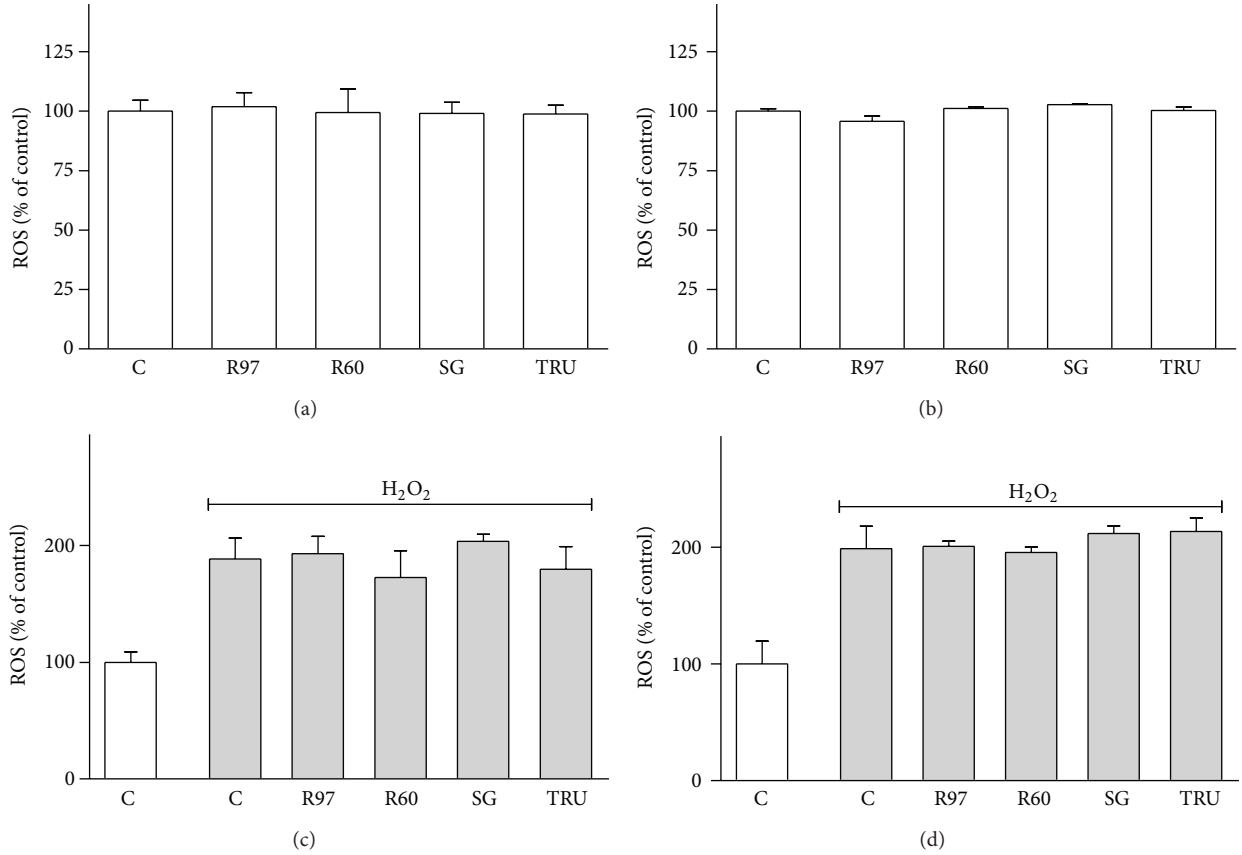

Figure 5: Effect of steviol glycosides on ROS levels in SH-SY5Y and HL-60 cells. SH-SY5Y (a) and HL-60 (b) cells were treated for 1 hour with different compounds (5 mg/mL); then basal ROS levels were measured by means of H_2DCFDA assay as described in Section 2. Results are expressed as means ± SD of four independent experiments ($n = 8$). Statistical analysis was performed by Bonferroni multiple comparison test following one-way ANOVA. Significant differences were not revealed. SH-SY5Y (c) and HL-60 (d) cells were preincubated for 1 hour with different compounds (5 mg/mL) and then exposed to oxidative stress generated by 100 μM H_2O_2 for 30 min. ROS levels were measured by means of H_2DCFDA assay as described in Section 2. Results are expressed as means ± SD of four independent experiments ($n = 8$). Statistical analysis was performed by Bonferroni multiple comparison test following one-way ANOVA. Significant differences were not revealed among H_2O_2 treated cells.

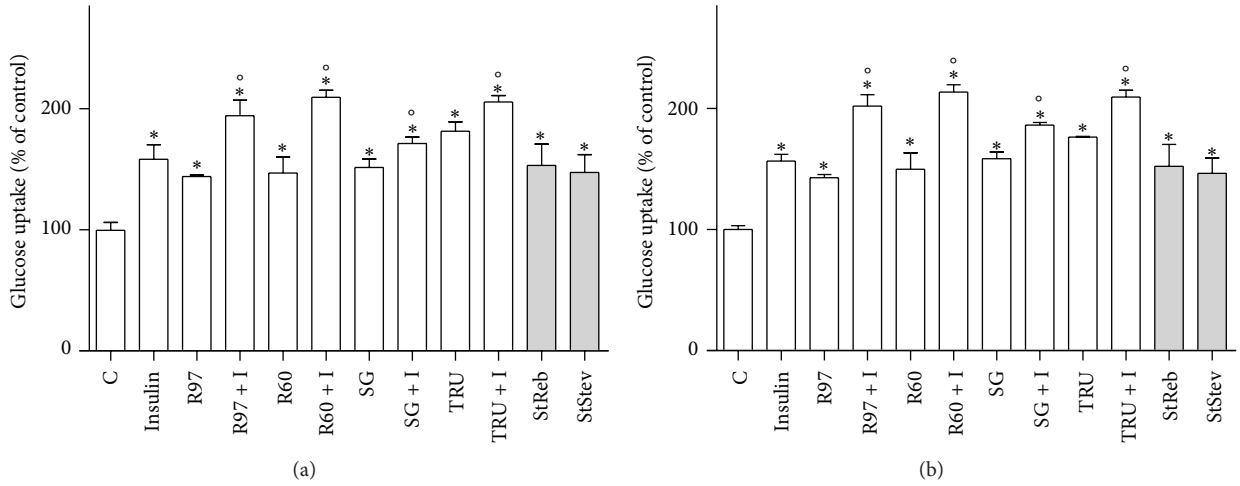

Figure 6: Effects of *Stevia* extracts on glucose transport activity compared to the effect of insulin. SH-SY5Y (a) and HL-60 (b) cells were treated with steviol glycosides (1 mg/mL), with 100 nM insulin (I), with steviol glycosides and insulin simultaneously, or 1 mM standard compounds (StReb, StStev). Glucose uptake was assayed as described in Section 2. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. Statistical analysis was performed by Bonferroni multiple comparison test following one-way ANOVA. $^{*}P < 0.05$, significantly different from control cells; $^{\circ}P < 0.05$ significantly different from the corresponding cells not stimulated with insulin.

can hypothesize that this effect is due to rebaudioside A and stevioside, the two major components of *Stevia* extracts.

Furthermore, the influence of *Stevia* extracts on glucose transport activity was compared to the effect of 100 nM insulin (Figure 6). Results reveal that *Stevia* extracts and insulin behave similarly, being *Stevia* extracts as efficient as insulin in increasing glucose uptake. The cotreatment with insulin and *Stevia* extracts causes a rise of glucose transport significantly higher than the increase due to insulin alone.

It is well known that insulin induces the translocation of GLUT4 from cytosolic storage vesicles to the plasma membrane, enhancing glucose transport. Recently, this phenomenon was observed also for neural cells *in vitro*, in particular the human neuroblastoma SH-SY5Y cells, through a phosphatidylinositol 3-kinase (PI3K)–dependent mechanism similar to the mechanism described in muscle or adipose tissues [28]. Translocation of GLUTs to the membrane has been reported as a consequence of various stimuli in many cellular types; in addition, changes in the expression of GLUTs have been described in response to several metabolic and oxidative stresses and in various physiological or pathological conditions. For example, insulin and ischemia induce GLUT1 movement to the membrane in rat heart [44]; doxorubicin recruits GLUT1 to the plasma membrane by a ROS-mediated mechanism in cardiomyocytes [37]; L-cysteine increases glucose uptake and GLUT3 levels in SH-SY5Y cells [45]; growth factors, cytokines, hydrogen peroxide, and cholesterol depletion are able to increase glucose uptake and GLUT1 translocation in leukaemia cell lines [46–49]. The expression of GLUT1 and GLUT4 in neuroblastoma and leukaemia cells following treatments with *Stevia* extracts or insulin was assessed by Western blot analysis on cell lysates. Figure 7 reports representative immunoblots for GLUT isoforms and the densitometric analysis in both cell lines. It can be seen that the increase in GLUT1 and GLUT4 content obtained following exposure to *Stevia* extracts is similar to that obtained by insulin stimulation. These results are in accordance with those observed in the evaluation of glucose transport activity.

To clarify the molecular mechanism by which *Stevia* extracts enhance glucose transport, the phosphorylation status of PI3K and Akt was evaluated following *Stevia* extract treatment and insulin stimulation. Insulin activates the PI3K/Akt pathway, critical for neuronal survival and growth, synaptic plasticity and development, and learning [50, 51]. Indeed, stimulation of insulin receptor, localized in lipid rafts [52, 53], produces the phosphorylation of tyrosine receptor kinases and the activation of a signal transduction pathway involving PI3K and Akt. The interaction of insulin with its receptor is a regulator of growth and differentiation of leukaemia cells [54, 55]. Highly specific insulin receptors have been identified on human promyelocytic leukaemia cells HL-60 [56]. Immunoblotting results (Figure 8) show an increase of phosphorylated forms of both PI3K and Akt following the treatment with insulin or *Stevia* extracts, indicating a possible similar mechanism of action or, at least, a common signalling pathway.

To better characterize the mechanisms of glucose uptake induction by steviol glycosides, we used methylglyoxal (MG) as an inhibitor of the insulin receptor/PI3K/Akt pathway. MG is a reactive ketoaldehyde, product of many metabolic pathways, primarily glycolysis, which is considered the most relevant and reactive glycation agent *in vivo*. Advanced glycation end products (AGEs) have been implicated in development and progression of several diseases, including diabetes and its associated vascular complications, renal failure, cirrhosis, aging, and recently also in diabetic neuropathy and in Alzheimer's disease [57]. Furthermore, recent studies suggest a correlation between MG and insulin resistance [58].

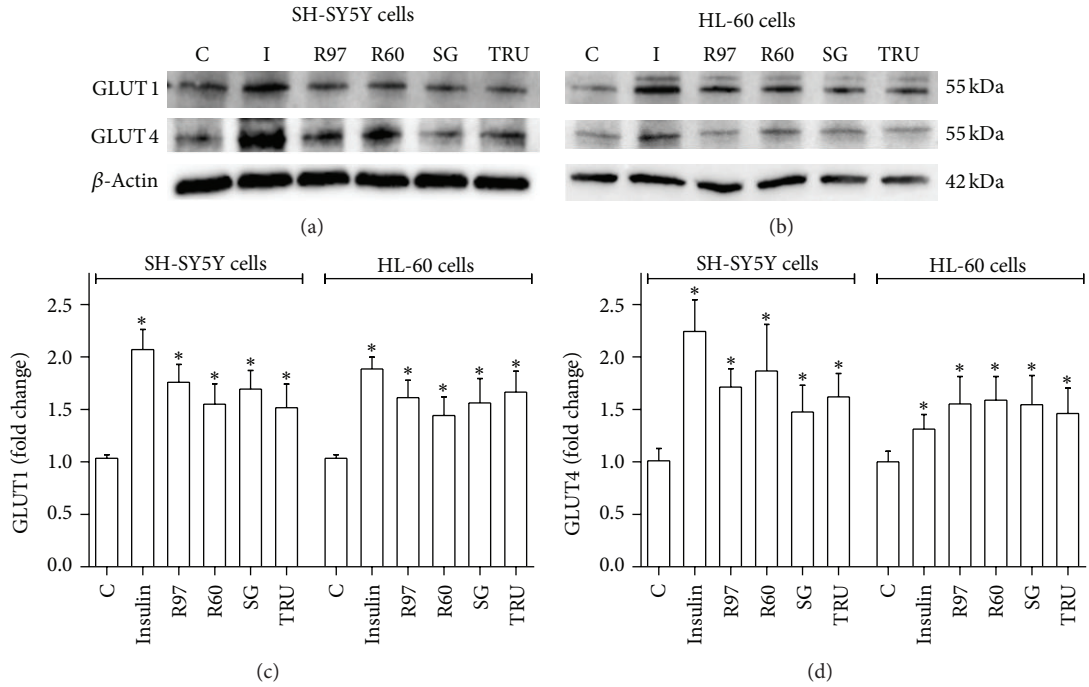

Figure 7: Effects of *Stevia* extracts on GLUT content. SH-SY5Y and HL-60 cells, treated with insulin or with steviol glycosides, were lysed with CelLytic M as described in Section 2. Cell lysates were electrophoresed and immunoblotted with the indicated antibodies, as described in Section 2. β-Actin detection was used as a control. Immunoblots representative of three independent experiments are reported for SH-SY5Y (a) and HL-60 (b) cell lines; densitometric analysis (normalized for β-actin content and expressed as fold of control) is shown for GLUT1 (c) and GLUT4 (d). $^*P < 0.05$, significantly different from control cells.

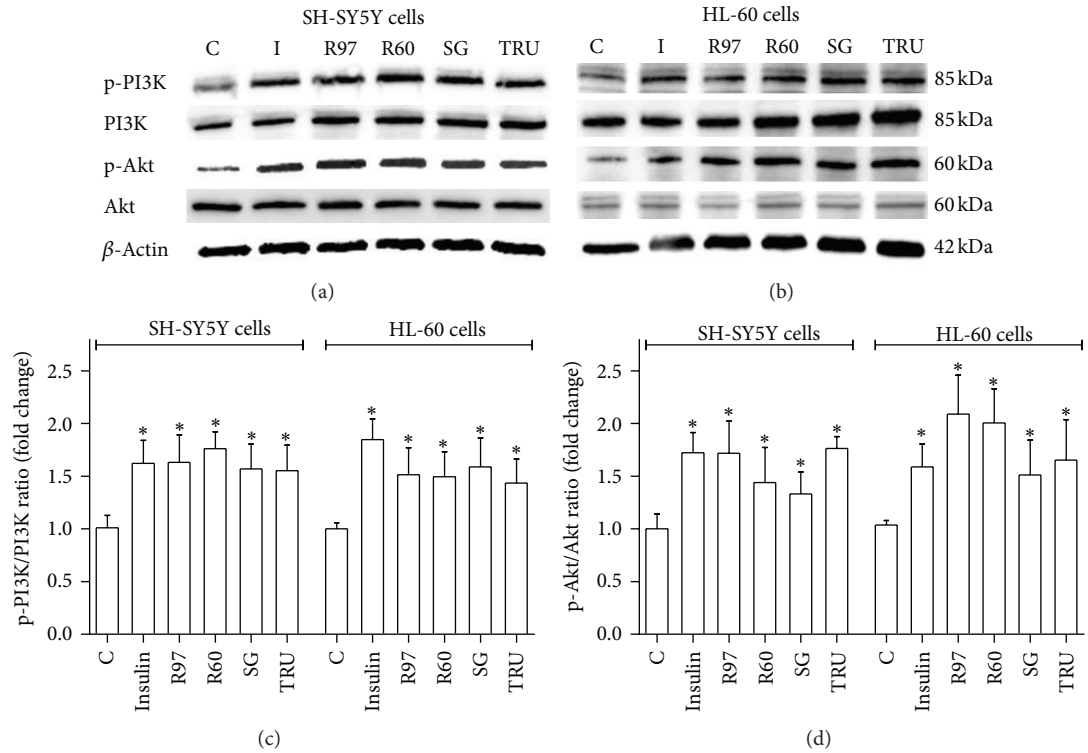

Figure 8: Effects of *Stevia* extracts on PI3K/Akt pathway. SH-SY5Y and HL-60 cells, treated with insulin or with steviol glycosides, were lysed with CelLytic M as described in Section 2. Cell lysates were electrophoresed and immunoblotted with the indicated antibodies, as described in Section 2. β-Actin detection was used as a control. Immunoblots representative of three independent experiments are reported for SH-SY5Y (a) and HL-60 (b) cell lines; densitometric analysis of PI3K phosphorylation status is expressed as phospho-PI3K/total PI3K ratio (c) and densitometric analysis of Akt phosphorylation status is expressed as phospho-Akt/total Akt ratio (d). $^*P < 0.05$, significantly different from control cells.

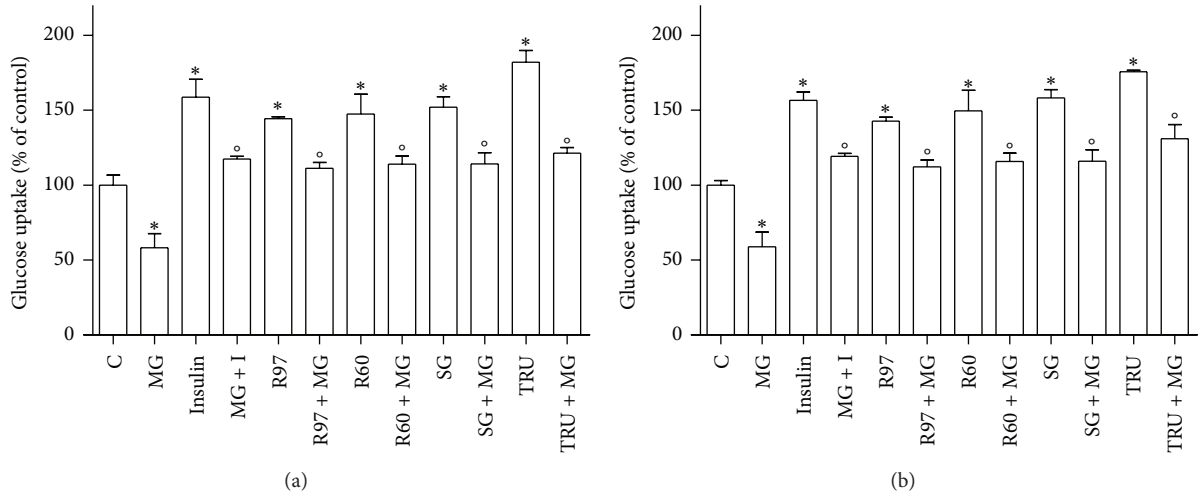

Figure 9: Effects of *Stevia* extracts on glucose transport activity compared to the effect of 0.1 mM of methylglyoxal. SH-SY5Y (a) and HL-60 (b) cells were treated with steviol glycosides, with insulin (I), with methylglyoxal (MG, 2 hours), and with steviol glycosides or insulin in the presence of MG. Glucose uptake was assayed as described in Section 2. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. Statistical analysis was performed by Bonferroni multiple comparison test following one-way ANOVA. $^{*}P < 0.05$, significantly different from control cells; $^{\circ}P < 0.05$, significantly different from cells treated with MG alone.

Figure 9 shows that MG incubation (2 hours) induces a significant decrease in glucose transport and that the subsequent treatment with *Stevia* extracts or insulin is able to raise the glucose uptake, corroborating the hypothesis that *Stevia* extracts could act *via* PI3K/Akt pathway similar to insulin. As MG is a reactive aldehyde toxic to cells, MTT assay was performed to verify that the MG concentration (0.1 mM) used to inhibit glucose transport did not influence cell viability (data not shown).

4. Conclusions

In this study, we evaluated the effects of steviol glycosides, extracted from *Stevia rebaudiana* Bertoni leaves, on glucose transport activity in two different cell lines. We demonstrated, for the first time to our knowledge, that rebaudioside A and stevioside, the major glycosides in *Stevia* extracts, are able to enhance glucose uptake in both SH-SY5Y neuroblastoma and HL-60 myeloid leukaemia human cells, the raise being similar to that induced by insulin. Our data suggest that steviol glycosides act by modulating GLUT translocation through the PI3K/Akt pathway. Although further experiments are needed, these results support the hypothesis that steviol glycosides and insulin could share a similar mechanism in regulating glucose entry into cells. In conclusion, *Stevia* extracts, commercialised as zero-calorie natural sweeteners, are involved in insulin regulated glucose metabolism. These findings suggest that the use of *Stevia* extracts goes beyond their sweetening power and may also offer therapeutic benefits, supporting the use of botanicals dietary supplements to improve the quality of life.

Authors' Contribution

Benedetta Rizzo and Laura Zambonin contributed equally to this paper.

Acknowledgments

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Research Article

Accelerated Aging during Chronic Oxidative Stress: A Role for PARP-1

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Oxidative stress plays a major role in the pathophysiology of chronic inflammatory disease and it has also been linked to accelerated telomere shortening. Telomeres are specialized structures at the ends of linear chromosomes that protect these ends from degradation and fusion. Telomeres shorten with each cell division eventually leading to cellular senescence. Research has shown that poly(ADP-ribose) polymerase-1 (PARP-1) and subtelomeric methylation play a role in telomere stability. We hypothesized that PARP-1 plays a role in accelerated aging in chronic inflammatory diseases due to its role as coactivator of NF-κb and AP-1. Therefore we evaluated the effect of chronic PARP-1 inhibition (by fisetin and minocycline) in human fibroblasts (HF) cultured under normal conditions and under conditions of chronic oxidative stress, induced by *tert*-butyl hydroperoxide (*t*-BHP). Results showed that PARP-1 inhibition under normal culturing conditions accelerated the rate of telomere shortening. However, under conditions of chronic oxidative stress, PARP-1 inhibition did not show accelerated telomere shortening. We also observed a strong correlation between telomere length and subtelomeric methylation status of HF cells. We conclude that chronic PARP-1 inhibition appears to be beneficial in conditions of chronic oxidative stress but may be detrimental under relatively normal conditions.

1. Introduction

Chronic inflammatory diseases afflict millions of people across the world leading to a substantial social and economic burden. From diabetes alone, 366 million people worldwide were suffering in 2011 [1]. It is estimated that by the year of 2030 this number will be almost doubled due to the rapid increase in the incidence of the disease caused by population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity [2]. Chronic inflammatory conditions in diabetes may lead to many serious complications, for example, retinal damage, renal failure, and cardiovascular diseases. Chronic inflammation and chronic oxidative stress, which occur in many chronic diseases, can contribute to the progress of these diseases by accelerating the rate of biological aging [3].

Accelerated biological aging has been associated with telomere shortening [4]. Telomeres are nucleoprotein structures at the end of chromosomes consisting of stretches of a repetitive DNA sequence, TTAGGG in humans. They prevent chromosomal ends from being recognized as double strand breaks and protect them from end-to-end fusions and degradation. In somatic human cells, telomeres shorten with every round of replication (i.e., end replication problem) and cells are triggered into replicative senescence once telomeres shorten to a critical length [3, 5, 6]. However, the end replication problem is not the only factor that contributes to the loss of telomeric DNA. Oxidative stress also appears to play a role in telomere shortening because of the high presence of GGG repeats, which are more readily oxidized compared to a lone guanine in the DNA [7, 8]. Recently, it has been shown that telomeric regions are favoured targets of a persistent DNA damage response induced by genotoxic and oxidative stress, both *in vitro* and *in vivo* [9]. Oxidative stress induces single-strand breaks both directly and indirectly. These are less efficiently repaired in telomeric DNA as compared to genomic DNA and, as a result, increase the rate of telomere shortening due to incomplete replication [10].

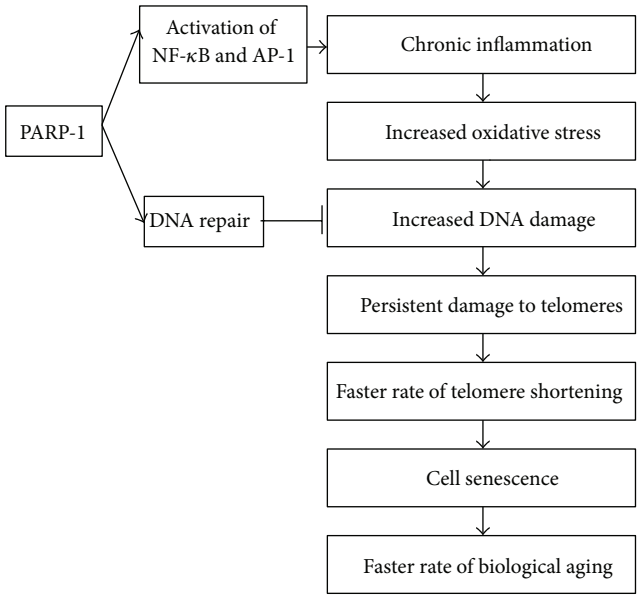

Figure 1: PARP-1 can influence telomere length regulation in two different ways. First, it can enhance DNA repair, protect the telomeres, and contribute to a decrease in the rate of telomere shortening. Second, it can enhance the inflammatory response by activating NF-κB or AP-1, which will lead to more oxidative DNA damage which in turn could accelerate telomere shortening.

Since oxidative stress plays a major role in chronic inflammatory diseases, telomere attrition may be involved in the pathophysiology of these diseases. Several studies have linked telomere shortening to various chronic metabolic and inflammatory diseases such as atherosclerosis, diabetes type 2, inflammatory bowel disease, and chronic obstructive pulmonary disease, conditions that are all characterized by the presence of systemic oxidative stress [11–18]. However, the exact underlying mechanisms of telomere shortening under conditions of chronic oxidative stress still have to be elucidated.

Recent evidence indicates that epigenetic regulation may be important in telomere stability. Telomeres lack CpG dinucleotides which are susceptible to methylation, but the immediately adjacent subtelomeric regions have a high density of CpG sequences [19]. In cells deficient in DNA methyltransferases (DNMTs) an induction of telomere elongation was observed which was associated with subtelomeric DNA hypomethylation [20]. Also other studies have found a link between epigenetic status of subtelomeres and telomere length [21], which suggests a role for subtelomeric DNA methylation in telomere stability.

The activity of the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) has also been reported to play a role in this process of telomere stability. It has been shown that PARP-1 associates with telomere repeat binding factor 2 (TRF2), a telomere-specific DNA binding protein that protects chromosome ends by promoting the formation of the "capped" state [22]. Furthermore, PARP-1 has been implicated in the regulation of multiple physiological cellular functions like DNA repair, gene transcription, cell cycle progression, cell death, chromatin function, and genomic stability [23, 24]. PARP-1 may influence telomere stability under conditions of chronic oxidative stress in two different ways (Figure 1). First, through its function in repair of oxidative stress induced DNA damage. It can enhance repair, protect the telomeres, and contribute to a decrease in the rate of telomere shortening. On the other hand, PARP-1 is also a coactivator of the stress-response related transcription factors nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1), and as a coactivator it can mediate the inflammatory response [25, 26]. In this role it may have a negative effect on telomere shortening, since inflammation will lead to more oxidative stress and oxidative DNA damage which could accelerate telomere shortening. Therefore, it is hypothesized that PARP-1 activity will contribute to accelerated telomere shortening and accelerated aging in chronic inflammatory diseases, through its function as a coactivator of inflammatory responses. The aim of this study was to investigate the effect of chronic PARP-1 inhibition on telomere stability under normal culturing conditions and under conditions of chronic oxidative stress in an *in vitro* model using human fibroblasts (HF). Additionally, the effect of prolonged culturing of HF cells under these conditions on subtelomeric methylation status was studied.

2. Material and Methods

2.1. Chemicals. Minimum essential medium (MEM), Hank's buffered salt solution (HBSS), fetal bovine serum (FCS), trypsin, essential amino acids, nonessential amino acids, vitamins, and penicillin/streptomycin were all obtained from Invitrogen (Breda, The Netherlands). Bovine serum albumin (BSA), minocycline, 4′,6-diamidino-2-phenylindole (DAPI), *tert*-butyl hydroperoxide (*t*-BHP), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The cell supernatant containing mouse monoclonal 10H anti-PAR polymer antibody was produced by Professor W. Buurman (Maastricht University, Maastricht, The Netherlands). FITC-conjugated goat anti-mouse immunoglobulin and fluorescent mounting medium were obtained from DAKO (Glostrup, Denmark). Hydrogen peroxide (H_2O_2) was purchased from Merck (Darmstadt, Germany). Fisetin was obtained from Fit Ingredients (Haibach, Germany). Primary human fibroblast cells (normal nonfetal skin tissue) were acquired from Coriell (Coriell Institute for Medical Research, Camden, USA). Hela cell lines were kindly provided by Professor Alexander Bürkle (University of Konstanz, Germany).

2.2. Cell Culture. HF cells were cultured in minimum essential medium (MEM) + GlutaMAX supplemented with 20% nonheat inactivated FCS, 1% penicillin/streptomycin, 0.5% nonessential amino acids, 0.5% essential amino acids, and 0.03% vitamins. Cells were maintained at 37°C in a 5% CO_2 atmosphere. All cells were passaged at approximately 80% confluency. To induce oxidative stress, parallel cultures were grown with or without exposure to *t*-BHP. To determine the concentration to be used, a concentration series was made

Table 1: Primer sequences and concentrations for telomere length PCR.

Primer	Sequence (5′ to 3′)	Concentration
Telomere 1	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	100
Telomere 2	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	900
HBG forward	GCTTCTGACACAACTGTGTTCACTAGC	300
HBG reverse	CACCAACTTCATCCACGTTCACC	700

from 5 nM to 100 nM *t*-BHP. Cell viability was tested by the trypan blue exclusion test. Cells were viable at concentrations <10 nM. At 5 nM a viability of >80% was found and we used this concentration for our experiments. Additionally, in a separate experiment the effect of PARP-1 inhibition was investigated by supplementation of fisetin (10 mM dissolved in DMSO, further diluted in culture medium to final concentration of 1 μM) or minocycline (10 mM dissolved in DMSO, further diluted in culture medium to final concentration of 100 nM) to the medium of HF cells in the presence or absence of *t*-BHP. All compounds were added to the culture medium every 2-3 days during passaging or medium renewal. The passage at which the experiment was started is called P0.

2.3. Immunohistochemical Staining of PAR Polymers. To verify the PARP-1 inhibiting effects of the selected PARP inhibitors, HF cells were seeded at a density of 15×10^4 cells per well in a six-well plate. The next day, cells were treated with 300 μM H_2O_2 to induce PARP-1 overactivation and PARpolymer formation. Treatment was done for 10 minutes in the presence or absence of fisetin (1 μM) or minocycline (100 nM), which were added 30 minutes before the H_2O_2 treatment. After incubation, the cells were trypsinized, washed with PBS, and fixed in methanol. Fixed cells were put on microscope slides, washed with 0.1% BSA in PBS, and incubated with 100 μL mouse monoclonal 10H anti-PAR polymer antibody for one hour at room temperature. After washing with 0.1% BSA in PBS, cells were incubated with 100 μL polyclonal goat anti-mouse immunoglobulin/FITC for one hour at room temperature. Next, cells were washed again with 0.1% BSA in PBS and incubated for 10 minutes with 100 μL DAPI solution. Subsequently, cells were mounted with fluorescent mounting medium and evaluated using a fluorescence microscope with Lucia GF 4.80 software. At least 100 cells per slide were studied for the presence of PAR polymers in the nucleus.

2.4. DNA Isolation. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol and quantified using a Nanodrop (Isogen Life Science, Belgium).

2.5. Telomere Length Measurement. Telomere length was determined by quantitative PCR as previously described [11, 27]. Two master mixes were prepared, one with telomere primers and one with human β globin (HBG) primers (1x iQ SYBR Green Supermix from Bio-Rad). Sequences and concentrations of the primers are shown in Table 1. Sample DNA was pipetted in a 96-well plate at a final concentration of 10 ng/μL. 20 μL of the mastermix was added and the plate was shortly centrifuged. Each sample was run in triplicate. For the standard curve a reference DNA sample was diluted serially to produce three concentrations of 1.25, 5, and 10 ng/μL. In every run, negative controls (MQ + mastermix) and reference samples were included. The references were derived from two different Hela cell lines, one with relatively short telomeres (Hela S3: 5.5 kb) and one with long telomeres (Hela 229: 14-15 kb). By adding reference DNA of controls to each qPCR, a standard curve could be created and the absolute telomere length of the samples could be calculated as kilo-base pairs (kbp). Hela cell lines were kindly provided by Professor Alexander Bürkle, University of Konstanz, Germany. The PCR was performed using a Bio-Rad MyiQ iCycler Single Color RT-PCR detection system using iQ SYBR Green Supermix, containing iTaq polymerase, dNTPs, SYBR Green I, and buffers (Bio-Rad, CA, USA).

2.6. Telomerase Activity. Telomerase activity was evaluated by telomeric repeat amplification protocol (TRAP) assay using the TeloTAGGG Telomerase PCR ELISAPLUS (Roche Diagnostics, Milan, Italy) which is an extension of the original method described by Kim et al. [28]. Cell extracts ($1–3 \times 10^5$ cell equivalents) were employed in the first step, in which telomerase adds telomeric repeats (TTAGGG) to the 3′-end of the biotin-labeled synthetic P1-TS primer. These elongation products, as well as the internal standard (IS), were amplified by PCR. In the second step the PCR products were split into two aliquots, denatured, denatured and hybridized separately to digoxigenin-(DIG)-labeled detection probes, specific for the telomeric repeats and for the IS, respectively. Results obtained by densitometric analysis were normalized upon the data of IS and expressed as relative telomerase activities (RTA).

2.7. Subtelomere Methylation. Bisulfite treatment of genomic DNA was performed with an EZ DNA methylation kit (Zymo Research, CA, USA). The bisulfite-treated DNA was subjected to a polymerase chain reaction (PCR) to amplify the subtelomere region with the primers specific for chromosome arm 2p. Primer sequences were obtained from Lee et al. [29]. Methylation specific primers were: forward ATGATTAGCGAGTTCGGTTTTAAC and reverse: GAATCGCGCCAAATATATACG, and primers specific for unmethylated DNA were forward: GATGATTAGTGAGTTTGGTTTTAATG and reverse: ACAAATCACACCAAATATATACAAA. PCR reactions were performed in a total volume of 25 μL containing 1x Taq polymerase buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 μM of each primer, 1 U

Table 2: Average time span to reach 80% confluency in HF cultures (moment of passage). Mean ± SD are shown.

Condition	Days between passage (average P0–P11)
Control	5.5 ± 1.0
t-BHP	6.1 ± 1.2
Fisetin	5.6 ± 1.0
Fisetin + *t*-BHP	6.7 ± 2.4
Minocycline	6.2 ± 2.2
Minocycline + *t*-BHP	6.1 ± 1.0

Taq polymerase, and 500 ng bisulfite-treated DNA. PCR amplification was conducted as follows: initial denaturation of 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds, and ending with an extension at 72°C for 10 minutes. Amplified products were run on an ethidium bromide stained with 2% agarose gel. Quantification was done by measuring grey values with the program ImageJ (http://rsbweb.nih.gov/ij/).

2.8. Statistical Analysis. Differences between groups for telomere length and PAR polymer staining were tested using the Mann-Whitney *U* test. Effects of PARP-1 inhibition were tested using a Wilcoxon signed-rank test. The association between telomere length and subtelomeric methylation status was evaluated using the nonparametric Spearman's rank correlation coefficient. P values < 0.05 were considered statistically significant and P values < 0.1 were considered statistical trends. Statistical analyses were analyzed with SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL, USA).

3. Results

To determine whether chronic exposure of HF cells to oxidative stress induces a faster rate of telomere shortening, we determined telomere length of HF cells exposed to 5 nM *t*-BHP. HF cells exposed to *t*-BHP showed significantly shorter telomeres than nonexposed cells of the same passage number ($P < 0.01$) (Figure 2(a)). It was also observed that the telomere length significantly decreased over time in nonexposed cells as well as in exposed cells ($P < 0.001$) (Figure 2(a)). In addition, the population doubling time was increased in the exposed cells compared to nonexposed cells. Telomere length decreased with approximately 1490 bp after 45 population doublings in the nonexposed cells and with 1938 bp after 45 population doublings in the exposed cells. In the nonexposed cells, at several time points the telomere length appeared to increase instead of decreasing. To be able to explain this phenomenon we measured the telomerase activity in the cells. As expected (as we used a primary cell line), no detectable telomerase activity was measured (Figure 2(b)).

To confirm the PARP-1 inhibiting effect of fisetin and minocycline, HF cells were treated with H_2O_2 to induce PARP activity. The formation of PAR polymers in these cells was evaluated using immunohistochemical staining. In nontreated cells, no PAR polymer formation was observed. Treatment with H_2O_2 induced an increase in the number of PAR polymer positive cells ($P < 0.01$). Preincubation with fisetin decreased the number of PAR polymer positive cells with 40% indicating that 1 μM fisetin mildly inhibits PARP-1. Preincubation with 100 nM minocycline resulted in a 90% reduction in the number of PAR polymer positive cells ($P < 0.001$), indicating that minocycline is a strong inhibitor of PARP-1 (Figure 3).

To investigate the effect of chronic PARP-1 inhibition on telomere length regulation under conditions of chronic oxidative stress, HF cells were cultured with 1 μM fisetin or 100 nM minocycline in the presence or absence of *t*-BHP. After 10 passages, telomeres in all culturing conditions were shorter compared to telomere length at the start of the experiment (Figure 4). In addition, culturing the cells in presence of *t*-BHP, fisetin, and minocycline ($P < 0.1$) resulted in shorter telomeres compared to untreated cells. However, culturing them in presence of minocycline or fisetin in combination with *t*-BHP did not result in accelerated telomere shortening when compared to untreated cells (Figure 4). Additionally, at the end of the experiment, cells treated with *t*-BHP, minocycline and *t*-BHP, and fisetin showed a senescence-like phenotype (flattened, contracted, and detached cells [30]). Cells cultured under the other conditions had a normal appearance. Also, cells cultured with *t*-BHP, *t*-BHP and fisetin, and minocycline alone showed a decreased growth rate, resulting in a longer period before passaging (Table 2).

To examine the effect of subtelomeric methylation on telomere stability, the methylation status of chromosome 2p was evaluated at the start and end of the experiment (Figure 5). At the start of the experiment HF cells showed an unmethylated pattern of the subtelomere region of chromosome 2p. At the end of the experiment differences between the conditions were observed. Cells cultured under normal conditions showed a methylation pattern that was similar to the pattern at the start of the experiment, while cells treated with *t*-BHP or minocycline showed an increase in methylation of almost 30%. For cells treated with fisetin this increase was only half (~15%). Spearman's correlation was run to determine the relationship between the level of methylation and telomere length, which revealed a statistically significant correlation ($r_s = 0.668$; $P = 0.018$).

4. Discussion

In this study we investigated the effect of chronic PARP-1 inhibition on telomere stability under conditions of chronic oxidative stress. We used prolonged culturing of HF as a model for development of cellular senescence. To induce oxidative stress, we cultured HF in presence of *t*-BHP, which is a short-chain organic hydroperoxide that produces free radicals after metabolic activation [31].

4.1. Chronic Oxidative Stress Induces Telomere Shortening. Prolonged culturing of HF resulted in telomeres shortening, indicating biological aging of HF. In addition, exposure to chronic oxidative stress significantly increased the rate of

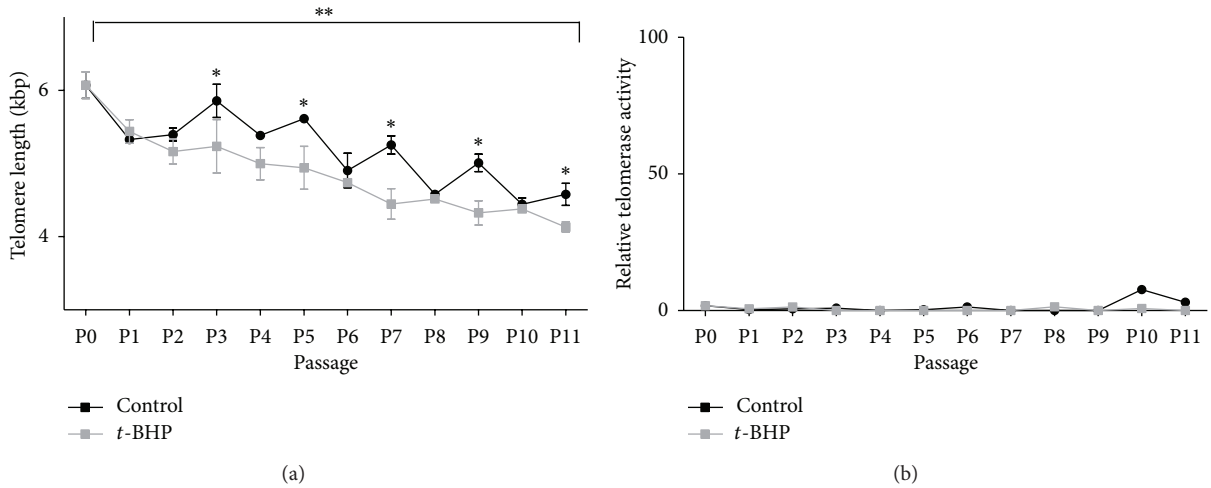

Figure 2: (a) Telomere length and (b) telomerase activity in human fibroblasts during culturing in the absence (black line) or presence (grey line) of 5 nM *tert*-butyl hydroperoxide (*t*-BHP). HF cells exposed to *t*-BHP showed significantly shorter telomeres than nonexposed cells of the same passage number. Telomere length significantly decreased over time in nonexposed cells as well as in exposed cells. $^{**}P < 0.001$ compared to P0 (start of the experiment); $^{*}P < 0.01$ compared to nonexposed cells of the same passage number. Mean ± SD of telomere length is shown. If telomerase activity was present, the relative telomerase activity value would be higher than 100. The values are lower than 10, which indicates that there is no telomerase activity in these cells.

telomere shortening. Because the observed telomere shortening might be caused by an increased rate of cell division, the population doubling times were calculated. We found that the population doubling time was increased in the cells exposed to *t*-BHP, which could be caused by an increased level of apoptosis, leading to a decreased cell proliferation capacity. Unexpectedly, at several time points in the nonexposed cells telomere length was increased. This appeared not to be caused by increased telomerase activity, since telomerase activity was absent or very low in this primary cell line. An alternative mechanism may be involved which can be adopted by yeast and human telomerase-deficient cell lines, the alternative lengthening of telomeres (ALT) for telomere maintenance. ALT appears to be mechanistically related to survival in cells and to involve a homologous recombination based mechanism in which one telomere can be extended using the telomere from a nonhomologous chromosome arm or extrachromosomal telomeric DNA [32–34]. This mechanism has been demonstrated to exist in HF [35, 36]. Other processes may contribute to the observed increase in telomere length as well, such as survival and selection of cells with longer telomeres and with a better adaptation to the culturing conditions.

4.2. Inhibition of PARP-1 by Fisetin and Minocycline. Fisetin and minocycline were used to inhibit PARP-1. Fisetin is a flavonoid that is normally present in dietary sources like fruits and vegetables [37]. Fisetin has been described to have many beneficial health effects, like memory enhancement [38]. It was found to possess anti-inflammatory effects via inhibition of the activation of NF-κB [39] and it has been shown previously that fisetin inhibited PARP-1 in pulmonary epithelial cells [40].

At a concentration of 1 μM, fisetin caused a mild inhibition of PARP-1 activity when cells were exposed to H_2O_2. Chronic treatment of HF cells with fisetin resulted in shorter telomeres compared to control cells. In cells cultured in the presence of both fisetin and *t*-BHP average telomere length was not significantly different compared to that of control cells. This finding appeared to support our hypothesis of the involvement of PARP-1 activity in telomere stability.

A recent study by Sengupta et al. showed fisetin to be a promising ligand for the formation of a four-stranded structure known as a G-quadruplex [41]. Formation of quadruplexes has been shown to decrease the activity of telomerase, but it also inhibits the ALT mechanism [42, 43]. Since this mechanism might play a role in our model, it is possible that quadruplex formation by fisetin under normal situations causes a faster rate of telomere shortening due to impairment of ALT. G-quadruplex formation is beneficial in anti cancer therapy, as it impedes telomere elongation, a mechanism most tumor cells use for unlimited proliferation [44].

Minocycline, also known as minocycline hydrochloride, is a member of the broad spectrum tetracyclines antibiotics. It is primarily used to treat acne and other skin infections and exerts anti-inflammatory effects that are completely separate from its antimicrobial actions [45]. We showed that nanomolar concentrations of minocycline significantly inhibited PARP-1 activity, as was previously reported by Alano et al. [46]. Culturing of cells in the presence of 100 nM minocycline resulted in shorter average telomere length as compared to control cells. On the other hand, when cells were cultured in the presence of minocycline in combination with *t*-BHP, average telomere length was not significantly decreased when compared to control. These findings, together with the findings of inhibition by fisetin,

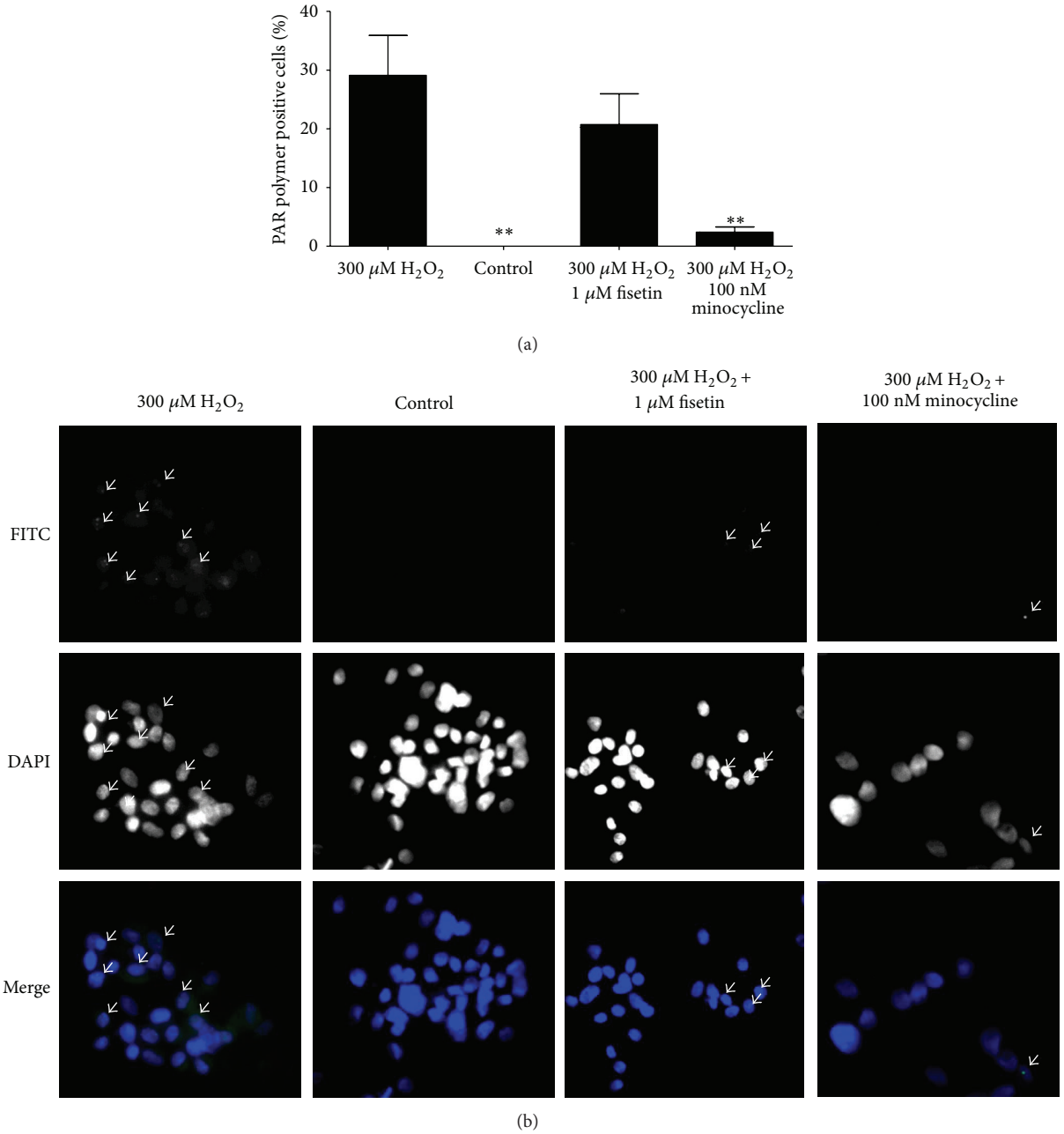

FIGURE 3: (a) PAR polymer formation in human fibroblasts treated with 300 μM H_2O_2 for 10 minutes in the presence or absence of 1 μM fisetin or 100 nM minocycline which were added 30 minutes before the H_2O_2 treatment. $^{**}P < 0.05$ compared to cells treated with 300 μM H_2O_2. (b) Representative photographs of PAR polymer staining. Arrows indicate examples of PAR polymer positive cells. Magnification: 300x.

appeared to support our hypothesis that PARP-1 activity contributes to telomere stability and that inhibition of PARP-1 activity increases the rate of telomere shortening. However, under conditions of chronic oxidative stress, inhibition of PARP-1 appeared to result in a decreased rate of telomere shortening. The anti-inflammatory activity of fisetin and minocycline may also contribute to the stabilizing effect on telomere length under chronic oxidative stress conditions.

The effects of chronic minocycline treatment on mammalian cells are still largely unknown. Research mainly focussed on examining the possible neuroprotective and anti-inflammatory effects of minocycline on progression of neurodegenerative disorders like multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). It has been described that minocycline is effective in various experimental models of ALS, Parkinson and Huntington disease [47–51]. In a study of patients with acute stroke it was found that treatment with minocycline significantly improved the outcome compared to patients treated with placebo [52]. Since oxidative stress is known to play a role in neuronal cell death in these diseases

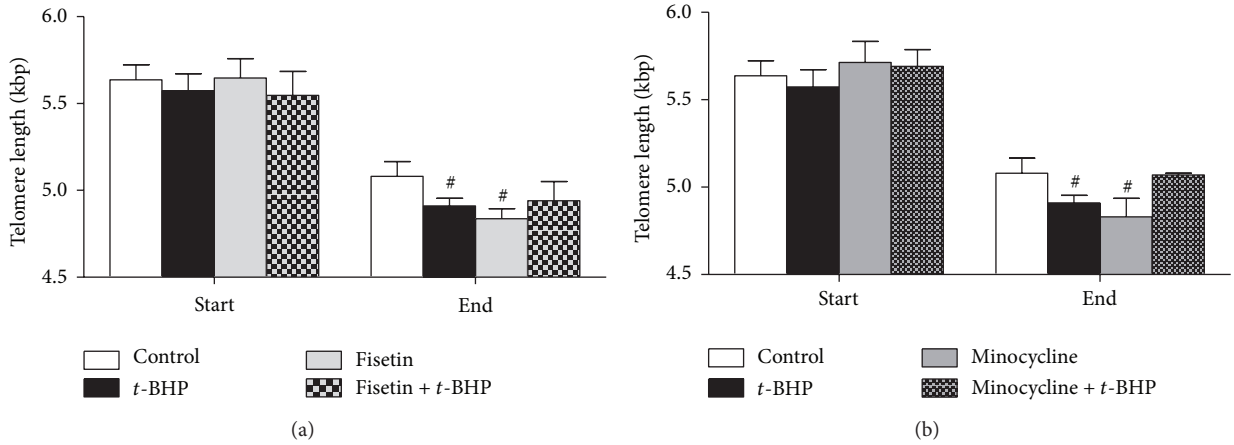

Figure 4: Effect of PARP inhibition by 1 μM fisetin (a) and 100 nM minocycline (b) on telomere length at the start (P0–P2) and end (P9–P11) of the experiment cultured under normal conditions or under conditions of chronic oxidative stress induced by *tert*-butyl hydroperoxide (*t*-BHP). $^{\#}P < 0.1$ compared to control.

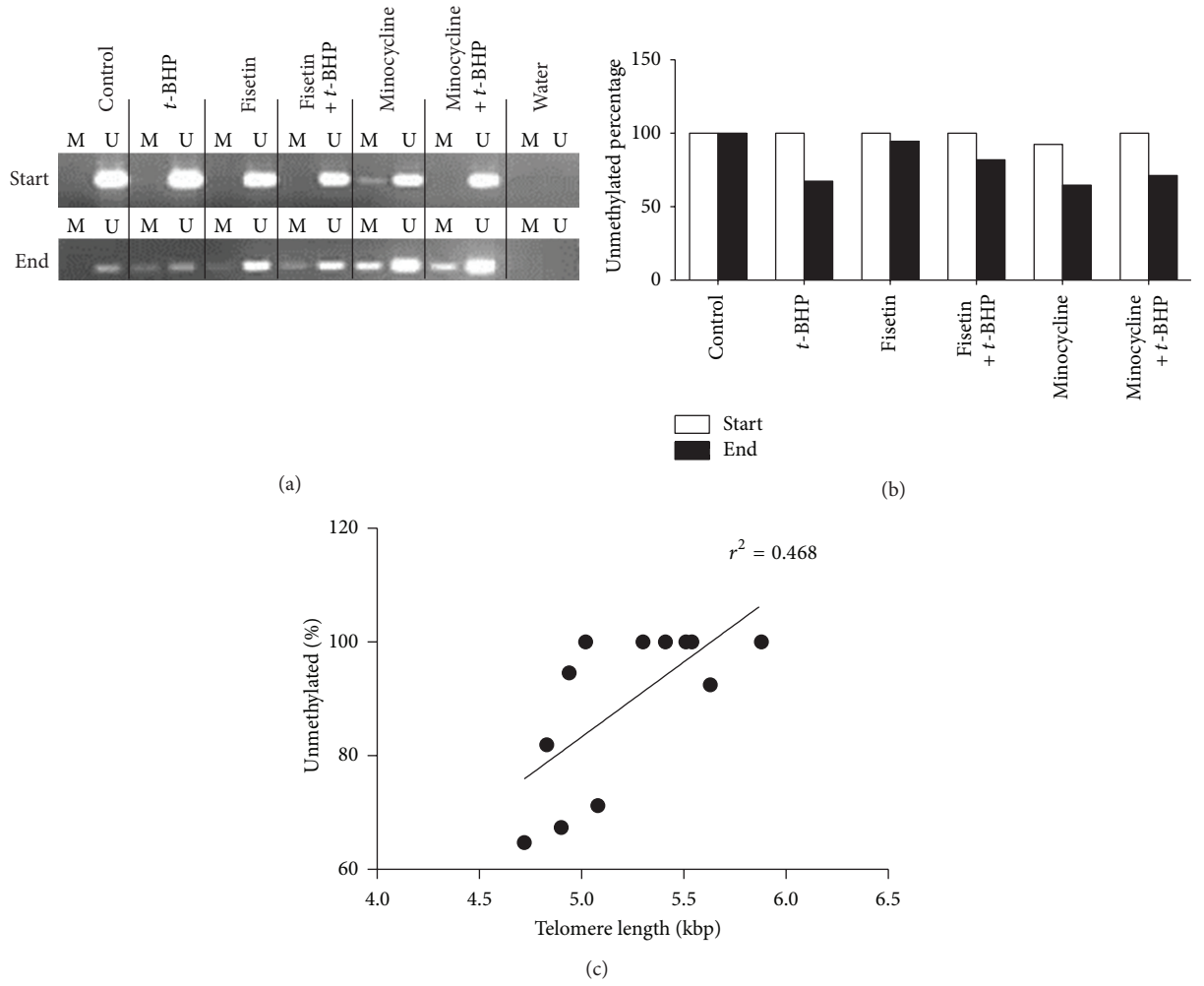

Figure 5: (a) Methylation specific PCR (MSP) result for chromosome 2p at passage 1 (start) and passage 10 (end). M and U indicate amplification from methylation and unmethylation sequence-specific primers. Water indicates MSP result with no template DNA (negative control). Densitometry of the photographs is shown in B. (c) Spearman's correlation revealed a statistically significant correlation ($r_s = 0.668$; $P = 0.018$) between telomere length and the level of methylation.

[53], PARP-1 inhibition might be an underlying mechanism by which minocycline exerts these neuroprotective effects. However, minocycline has also been shown to be effective in other disease models. It was already found that minocycline may prevent blindness in a rat model of diabetic retinopathy [45]. Several studies have suggested an important role of PARP activation in the pathogenesis of diabetic complications like nephropathy, neuropathy, and retinopathy [54–56]. Furthermore, minocycline has been shown to exert *in vivo* cardioprotective effects by suppressing oxidative stress and therefore preventing fetal cardiac myocyte death after prenatal cocaine exposure [57].

A selective PARP-1 inhibitor was not tested in our experiments. However, Beneke et al. described an experiment in which they exposed cells to the well-known PARP-1 inhibitor 3-aminobenzamide (3-AB) and measured telomere length [58]. They found in two mammalian cell systems (hamster and human) that pharmacological inhibition of PARP1 led to a fast, dose-dependent decrease of telomere length. These results are comparable with the results we obtained in our experiment in which fisetin and minocycline were applied as PARP inhibitors.

The effects on telomere length were observed to be similar with both inhibitors. However, minocycline treated cells showed morphological changes at an earlier passage than untreated cells or cells treated with fisetin. This might be explained by the fact that minocycline is known to have strong PARP-1 inhibiting capacity only, whereas fisetin has been reported to exert other effects that may enhance cellular function [37]. Fisetin is an activator of sirtuin 1 (SIRT1), a histone deacetylase [59]. An increased activity of SIRT1 is associated with enhanced survival and longevity. Fisetin has already been shown to increase the lifespan of the yeast *Saccharomyces cerevisiae* [59]. Additionally, fisetin is able to inhibit COX2 expression [60]. It has been shown that selective COX2 inhibitors can modulate cellular senescence in human dermal fibroblasts [61]. Activation of SIRT1 and/or inhibition of COX2 by fisetin could explain why cells treated with fisetin had a normal appearance and cells treated with minocycline, which is not known as a SIRT1 activator, were in a senescence state at passage 11, although they had similar telomere length.

4.3. Subtelomeric Methylation. Mouse models and *in vitro* studies suggest a role for subtelomeric methylation in telomere length regulation [20, 21]. Normally, telomeres have a "closed" conformation which is established by epigenetic markers, including methylation of the subtelomeric region. When telomeres become shorter, the epigenetic markers decrease, which leads to a more "open" confirmation that allows a greater accessibility for telomere-elongating activities [62]. It has been shown that the subtelomeric region of Alzheimer patients with short telomeres was hypermethylated [63]. In contrast, in patients with Parkinson's disease an increase in short telomeres with subtelomeric hypomethylation was found [64]. PARP-1 is also known to be able to influence DNA methylation by regulation the expression and activity of DNMT1 or by direct interaction with DNMT1 [65–67]. We observed a correlation between methylation status and telomere length. We found a change from unmethylated status at the beginning of the experiment to 50% methylated in conditions with the shortest telomeres at the end of the experiment of the subtelomeric region of chromosome 2p. Cells that were chronically treated with fisetin showed less increase in methylation, which could be caused by the ability of fisetin to inhibit SssI DNMT- and DNMT1-mediated DNA methylation [68].

5. Conclusion

Chronic fisetin treatment of HF at physiological concentrations resulted in shorter telomeres compared to control cells, indicating reduced telomere stability and enhanced biological aging of these cells. Under the assumption that it is healthy, fisetin is often added to nutritional supplements at relatively high concentrations. Since the biological effects of regular consumption of high doses of fisetin (and also flavonoids in general) are not known, thorough safety evaluation is warranted with respect to these nutritional supplements. Chronic minocycline treatment also enhanced telomere shortening. This implies that precaution should be taken when minocycline is subscribed as a chronic treatment.

However, under conditions of chronic oxidative stress, both fisetin and minocycline appeared to reduce the rate of telomere shortening. Since our study was limited to testing the effects of fisetin and minocycline in an *in vitro* model with HF cells that were chronically exposed to oxidative stress more research is needed to evaluate possible positive effects of fisetin and minocycline in chronic inflammatory diseases.

It can be concluded that chronic administration of pharmaceuticals or nutraceuticals with PARP inhibiting activity appears to be beneficial in conditions of chronic oxidative stress, but may be detrimental under relatively normal conditions.

Conflict of Interests

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

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Research Article

Mitogenesis of Vascular Smooth Muscle Cell Stimulated by Platelet-Derived Growth Factor-bb Is Inhibited by Blocking of Intracellular Signaling by Epigallocatechin-3-*O*-Gallate

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Epigallocatechin gallate (EGCG) is known to exhibit antioxidant, antiproliferative, and antithrombogenic effects and reduce the risk of cardiovascular diseases. Key events in the development of cardiovascular disease are hypertrophy and hyperplasia according to vascular smooth muscle cell proliferation. In this study, we investigated whether EGCG can interfere with PDGF-bb stimulated proliferation, cell cycle distribution, and the gelatinolytic activity of MMP and signal transduction pathways on RAOSMC when it was treated in two different ways—cotreatment with PDGF-bb and pretreatment of EGCG before addition of PDGF-bb. Both cotreated and pretreated EGCG significantly inhibited PDGF-bb induced proliferation, cell cycle progression of the G0/G1 phase, and the gelatinolytic activity of MMP-2/9 on RAOSMC. Also, EGCG blocked PDGF receptor-β (PDGFR-β) phosphorylation on PDGF-bb stimulated RAOSMC under pretreatment with cells as well as cotreatment with PDGF-bb. The downstream signal transduction pathways of PDGFR-β, including p42/44 MAPK, p38 MAPK, and Akt phosphorylation, were also inhibited by EGCG in a pattern similar to PDGFR-β phosphorylation. These findings suggest that EGCG can inhibit PDGF-bb stimulated mitogenesis by indirectly and directly interrupting PDGF-bb signals and blocking the signaling pathway via PDGFR-β phosphorylation. Furthermore, EGCG may be used for treatment and prevention of cardiovascular disease through blocking of PDGF-bb signaling.

1. Introduction

Several vascular diseases involve vascular smooth muscle cell (VSMC) proliferation as their primary mechanism. Dedifferentiated VSMCs induce cell proliferation and migration, as well as extracellular matrix (ECM) protein deposition [1–4]. Intimal hyperplasia is an excessive tissue ingrowth and chronic structural lesion that can be observed at the site of atherosclerotic lesion formation, arterial angioplasty, vascular graft anastomoses, and so forth. This phenomenon is caused by the phenotype change of VSMCs from a differentiated state to a dedifferentiated one. Several studies have focused on VSMC phenotype switching, decreasing expression of smooth muscle specific contractile markers such as α-smooth muscle actin, smooth muscle myosin heavy chain, and calponin, [5, 6], migration and proliferation from media to the intima, and extracellular matrix remodeling [7, 8]. Vascular proliferation is the most important factor in intimal hyperplasia and is linked to other cellular processes such as migration, inflammation, and extracellular matrix production.

Platelet-derived growth factor-bb (PDGF-bb) is one of the most potent mitogens and chemoattractants for VSMC and plays a central role via simultaneous interactions between itself [9]. In previous studies, it was confirmed that PDGF-bb induced phenotype switching [10, 11], MMP-2 upregulation [12, 13], and migration [14] on VSMCs. PDGF-bb is also known to bind to the PDGR receptor (PDGFR)-β and subsequently activates several intracellular signaling cascades, including the extracellular signal-regulated kinase (ERK),

p38 mitogen-activated protein kinase (p38 MAPK) pathways, and phosphatidylinositol 3-kinase-Akt (PI3 K-Akt), and stimulates VSMC dedifferentiation [15].

Epigallocatechin gallate (EGCG) is the most prevalent polyphenol contained in green tea. This has been reported to have antioxidant, antiproliferative, and antithrombogenic effect. Recent experiments have suggested that green tea catechins can reduce atherosclerotic lesions in various animal models and prevent cardiovascular diseases [16–18]. In addition, EGCG inhibits VSMC invasion by preventing matrix metalloproteinase (MMP) expression and provides a protective effect against atherosclerosis and cancer via matrix degradation [19].

In this study, we investigated the effects of EGCG on proliferation, cell cycle, and the intracellular signal transduction pathway of PDGF-bb in rat aortic vascular smooth muscle cell (RAOSMC) and demonstrated the preventive mechanism of PDGF-bb stimulated RAOSMC dedifferentiation.

2. Materials and Methods

2.1. Cell Culture. Rat aortic smooth muscle cells (RAOSMC) were purchased from Biobud (Seoul, Republic of Korea), and cells at passage 5 to 9 were used. The cells were routinely maintained in Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and a 1% antibiotic-antimycotic solution containing 10,000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin B per mL (Sigma) at 37°C in a humidified atmosphere of 5% CO_2.

2.2. Cell Stimulation by PDGF-bb. EGCG (Teavigo), the major polyphenolic constituent of green tea, was purchased from DSM Nutritional Products Ltd. It was dissolved in 50% DMSO (Sigma) for a stock solution of 100 mM and then diluted to the desired concentrations with media prior to cell treatment. For the experiments, RAOSMCs were routinely incubated. Cells were synchronized in serum-free medium for 24 h before experiments. Prior to the experiments, the cells were incubated with two different methods. With the first method, the synchronized RAOSMCs were preincubated with EGCG in serum-free media for 24 h. Then, EGCG-treated cells were washed twice with PBS and stimulated with serum-free media containing 10 ng/mL of human recombinant PDGF-bb (Sigma) for a desired length of time. For the second method, cells were synchronized in serum-free DMEM medium for an additional 24 h and stimulated with 10 ng/mL PDGF-bb and soluble EGCG.

2.3. Cell Proliferation and DNA Synthesis. Cell proliferation was determined by MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product, Sigma) and a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay (Roche Applied Science, Seoul, Republic of Korea).

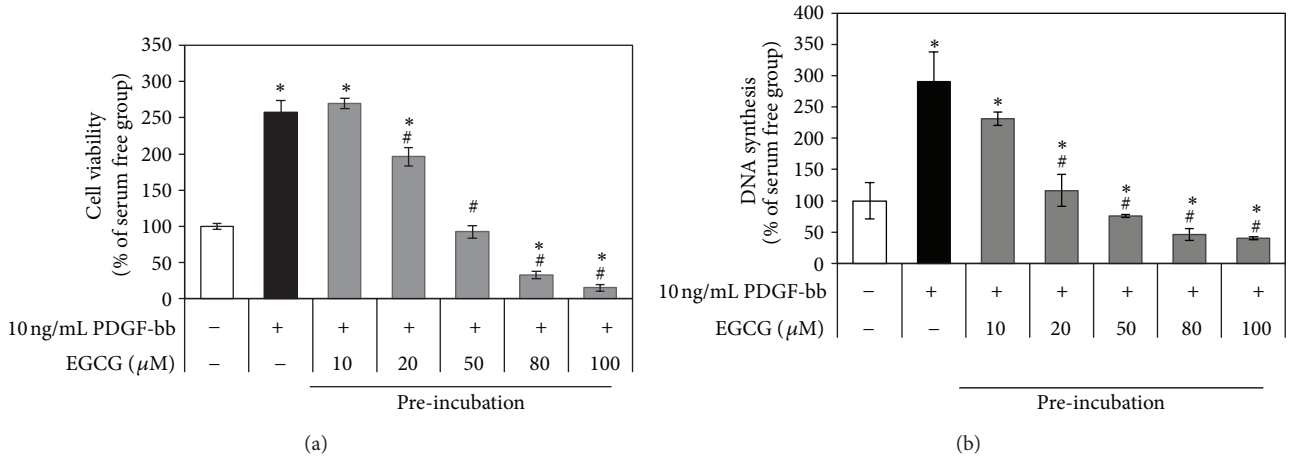
For the MTT assay, the cells incubated with 0.5 mg/mL of MTT in the last 4 h of the culture period were tested at 37°C in the dark. The media were decanted, and the produced formazan salts weredissolved with dimethylsulphoxide, and absorbance was determined at 570 nm by an automatic microplate reader (Spectra Max 340, Molecular Devices Co., Sunnyvale, CA, USA).

For BrdU incorporation assay, BrdU-labeling solution was added to the cells, and it was reincubated for 2 h at 37°C. Labeling medium was then removed, and the cells were incubated with fixated solution for 30 min at room temperature. After fixation of the cells, anti-BrdU-POD working solution was added, and the cells were incubated for 90 min at room temperature. Then, the substrate solution was added, and absorbance was measured at 370 nm with 492 nm reference wavelength by an automatic microplate reader (Spectra Max 340, Molecular Device Co.)

2.4. Cell Cycle Analysis. To analyze the cell cycle, RAOSMCs were collected and washed with cold phosphate-buffered saline (PBS, pH 7.2). The cells were resuspended in 95% cold methanol for 1 h at 4°C and then centrifuged at 120 ×g for 5 min. The resultant pellet was washed twice with cold PBS and incubated with RNase A (20 U/mL final concentration, Sigma) at 37°C for 30 min. Intracellular DNA was labeled with 100 μg/mL propidium iodide (PI, Sigma) for 1 h and then analyzed with a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, San Jose, CA, USA). The cell cycle profile was gained by analyzing at least 20,000 cells with the ModFit LT program written by Mac-App (Becton Dickinson).

2.5. Gelatin Zymography. Gelatinase activity was detected in the conditioned medium of cultured RAOSMC. The conditioned media mixed with Laemmli buffer under nonreducing conditions were loaded onto 10% SDS-polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gels were washed for 20 min at room temperature in 2.5% Triton X-100 and incubated for 18 h at 37°C with reaction buffer (50 mM Tris base (pH7.6), 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35). The gels were stained with Coomassie Brilliant Blue R-2500 (0.1%) and destained. Densitometric analysis was performed with imageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. Western Blot Analysis. After being stimulated with PDGF-bb, the cells were washed twice with cold PBS (10 mM, pH 7.4). Ice-cold RIPA lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added to the cells and incubated for 5 min. The cells were scraped, and the lysate was cleared by centrifugation at 14,000 ×g for 20 min at 4°C. The resultant supernatant (total cell lysate) was collected. Protein concentration was determined by using a DC Bio-Rad assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). For immunoblot analysis, the protein was run on SDS-PAGE and then electrotransferred onto a PVDF membrane. The membrane was blocked with the buffer (5% nonfat dry milk and 1% Tween-20 in 20 mM TBS, pH 7.6) for 1 h at room temperature and then probed overnight with phospho-PDGFR-β (p-PDGFR-β), PDGFR-β, phospho-MEK1/2 (p-MEK1/2), MEK1/2, phospho-p42/44 MAPK (p-p42/44 MAPK), p42/44



EGCG	Cell cycle distribution (%)		
10 ng/mL PDGF-bb, μM	G0/G1	S	G2/M
Serum free	92.83	1.31	5.86
0	78.24	13.27	8.49
10	81.24	12.93	5.83
20	89.27	4.38	6.35
50	89.81	3.73	6.47
80	89.86	3.71	6.43
100	88.70	5.23	6.07

(c)

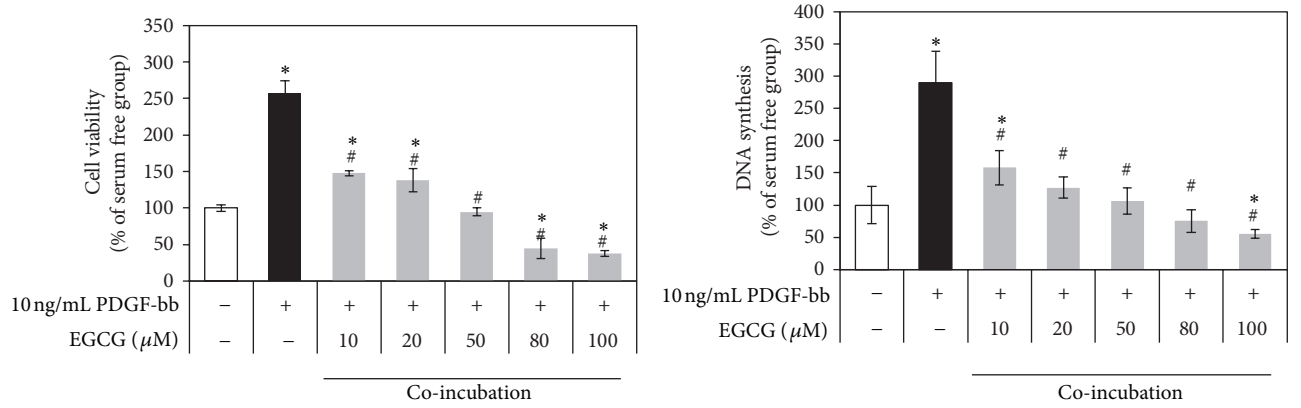
Figure 1: The antiproliferative activity and cell cycle arrest activity by PDGF-bb on EGCG preincubated RAOSMC. After 24 h of starvation with DMEM containing increasing concentrations (10–80 μM) of EGCG, cells at 80% confluence were washed and treated with 10 ng/mL PDGF-bb. (a) The effects of growth inhibition on PDGF-bb stimulation in EGCG preincubated RAOSMC. Cell viability was detected using the MTT assay. $^{*}P < 0.05$ compared with nonstimulation control; $^{\#}P < 0.05$ compared with the 10 ng/mL PDGF-bb stimulated control. (b) The effect of EGCG preincubation on PDGF-bb-induced DNA synthesis in RAOSMC. DNA synthesis was detected using the BrdU incorporation assay. $^{*}P < 0.05$ compared with nonstimulation control; $^{\#}P < 0.05$ compared with 10 ng/mL PDGF-bb stimulated control. (c) EGCG preincubation with PDGF-bb stimulated cell cycle distribution in RAOSMC. Cell cycle distribution was determined by propidium iodide (PI) labeling followed by flow cytometry. The percentages of cells in the G0/G1, S, and G2/M phases were calculated using Modifit computer software and represented within the histograms.

MAPK, phospho-Akt (p-Akt), Akt, phospho-p38 MAPK (p-p38 MAPK), and p38 MAPK used at a 1 : 1,000 dilution from Cell Signaling Technology (Danvers, MA, USA). Detection of horseradish peroxidase-conjugated secondary Ab (e.g., anti-rabbit IgG (1 : 5,000) and anti-mouse IgG (1 : 2,000) from Santa Cruz Biotechnology Inc.) was accomplished using enhanced chemiluminescence using the ECL Plus detection kit (Amersham Biosciences, Buckinghamshire, England). Densitometric analysis was performed with imageJ (National Institutes of Health, Bethesda, MD, USA).

2.7. Statistical Analysis. All variables were tested in three independent cultures for each experiment. The results are reported as a mean ± SD and compared to non-treated controls. Statistical analysis was performed using a one-way (ANOVA), followed by a Tukey HSD test for multiple comparisons using SPSS software. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Inhibitory Effect of Proliferation by PDGF-bb on EGCG Pretreated RAOSMC. To investigate proliferation by PDGF-bb stimulation on RAOSMC pretreated with EGCG, increasing EGCG concentration was treated with serum-free DMEM for 24 h at 70~80% confluence RAOSMC. Cells were then washed twice with PBS and incubated with 10 ng/mL PDGF-bb for 24 h. 10 ng/mL PDGF-bb induced a significant ($P < 0.05$) RAOSMC proliferation as compared to the nonstimulated group as assessed by increased DNA synthesis and increased formazan absorbance. When cells were preincubated with increasing concentrations of EGCG, cell proliferation by 10 ng/mL PDGF-bb was significantly ($P < 0.05$) decreased in a dose-dependent manner of EGCG. Therefore, cell viability (Figure 1(a)) and DNA synthesis (Figure 1(b)) were not significantly affected in concentrations up to 50 μM. To investigate the effects of EGCG pretreatment on cell



EGCG	Cell cycle distribution (%)		
μM	G0/G1	S	G2/M
Serum free	92.83	1.31	5.86
10 ng/mL PDGF-bb 0	78.24	13.27	8.49
10	87.05	8.18	4.76
20	86.08	6.21	7.70
50	88.52	2.95	8.54
80	88.88	2.94	8.18
100	86.83	4.59	8.57

(c)

Figure 2: Antiproliferative activity and cell cycle arrest activity of PDGF-bb with EGCG on RAOSMC. After 24 h of starvation with serum-free DMEM, the cells were treated with 10 ng/mL PDGF-bb and increasing concentrations (10–80 μM) of EGCG for 24 h. (a) The effects of EGCG growth inhibition on PDGF-bb stimulation in RAOSMC. Cell viability was detected using an MTT assay. $^{*}P < 0.05$ compared with nonstimulation control; $^{\#}P < 0.05$ compared with the 10 ng/mL PDGF-bb stimulated control. (b) The effects of EGCG on PDGF-bb-induced DNA synthesis in RAOSMC. DNA synthesis was detected using the BrdU incorporation assay. $^{*}P < 0.05$ compared with nonstimulation control; $^{\#}P < 0.05$ compared with 10 ng/mL PDGF-bb stimulated control. (c) The effect of EGCG on PDGF-bb stimulated cell cycle distribution in RAOSMC. Cell cycle distribution was determined by propidium iodide (PI) labeling followed by flow cytometry. The percentages of cells in the G0/G1, S, and G2/M phases were calculated using Modifit computer software and represented within the histograms.

cycle distribution, DNA cell cycle analysis was performed on RAOSMC stimulated with PDGF-bb. As shown in Figure 1(c), EGCG pretreatment resulted in an appreciable increase in cells in the G0/G1phase, with a decrease in S-phase cells in up to 20 μM EGCG pretreatment. These results indicate that EGCG pretreatment can suppress cell cycle progression and cell growth on RAOSMC with distributed PDGF-bb stimulation.

3.2. Inhibitory Effect of Proliferation by Cotreatment of PDGF-bb and EGCG on RAOSMC. To investigate proliferation by PDGF-bb stimulation with EGCG on RAOSMC, synchronized cells were incubated for 24 h with increasing concentrations of EGCG and 10 ng/mL PDGF-bb. Cotreatment with EGCG and PDGF-bb significantly inhibited the proliferation of RAOSMC by PDGF-bb stimulation (Figure 2(a)). Similarly, Figure 2(b) shows that DNA synthesis is also inhibited. According to BrdU incorporation into RAOSMC, cotreatment of EGCG (10 μM) and PDGF-bb represented more inhibitory effects than pretreatment of EGCG. Proliferation was completely inhibited at a concentration of 50 μM EGCG. EGCG induced a significant accumulation of the cells in the G0/G1 phase of the cell cycle at up to 10 μM. Inhibition of cell growth in RAOSMC may be caused by G0/G1 arrest as EGCG interrupts PDGF-bb stimulated cell cycle progression.

3.3. Preventive Effect of Active MMP-2/9 Production by EGCG on PDGF-bb Stimulated RAOSMC. MMP-2 and MMP-9 were detected in the conditioned media from cultured RAOSMC for 24 h with EGCG and PDGF-bb by gelatin zymography assay. After stimulation with PDGF-bb, RAOSMC showed more pro-MMP conversion into the intermediated and active form of MMP-2, and increased the MMP-9 release. As shown in Figure 3, EGCG pretreated RAOSMC significantly reduced the PDGF-bb-induced gelatinolytic activities of active MMP-2 and MMP-9. Therefore, the stimulatory effect of PDGF-bb also caused a reduction in MMP-2/9 gelatinolytic activity in a

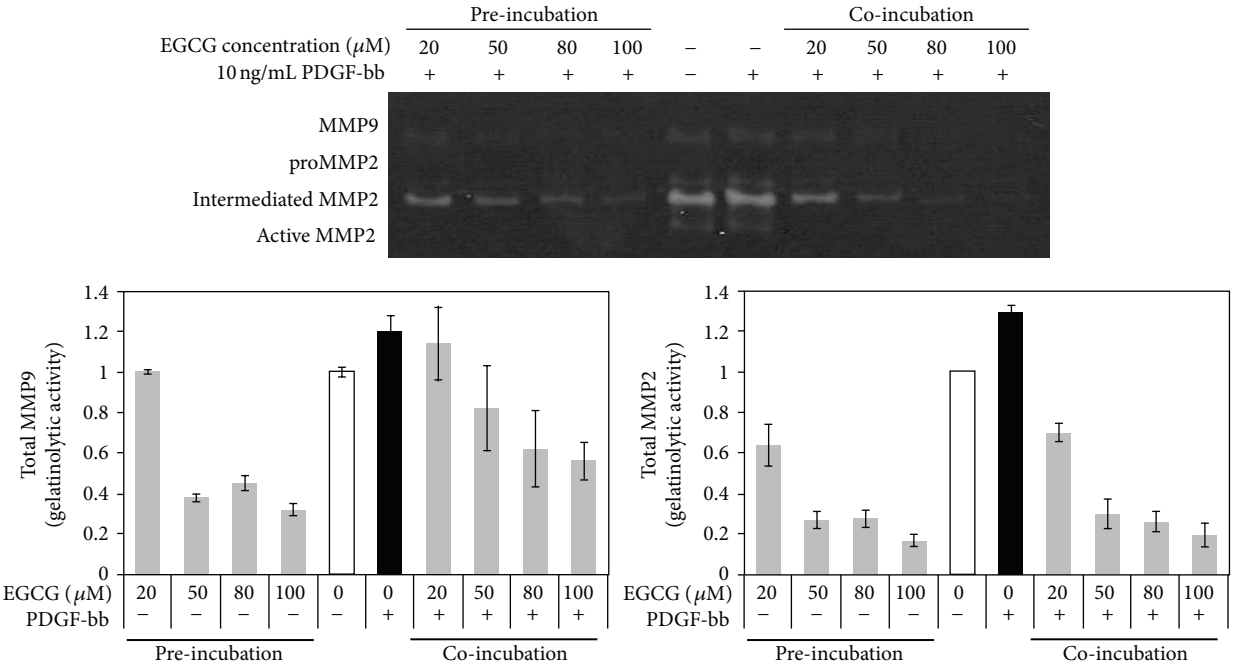


Figure 3: Inhibitory effect of EGCG on PDGF-bbinduced MMP gelatinolytic activity in RAOSMC. Gelatin catalytic activity was analyzed by gelatin zymography using conditioned medium. The band intensity was normalized by densitometry. PDGF-bb induced the gelatinolytic activity of MMP-9 and MMP-2. However, both preincubated and coincubated EGCG inhibited secretion of PDGF-bbinduced MMP-9 and MMP-2 activity.

concentration-dependent manner by treatment of RAOSMC with EGCG. The inhibitory effect of MMPs gelatinolytic activity was dose-dependently expressed on EGCG. The active form of MMP-2 was not detected at up to 20 μM of EGCG.

3.4. Inhibitory Effect of PDGF-bb Stimulated Signal Transduction Pathway in EGCG Preincubated RAOSMC. To define the effects of EGCG pretreatment on signaling pathways of PDGF-stimulated mitogenesis, already synchronized RAOSMCs were incubated with EGCG and serum-free media for 24 h. For PDGF-bb stimulation, the cells were washed using PBS to remove EGCG, incubated for the desired time, and examined for levels of various proteins by Western blot analysis. Addition of 10 ng/mL PDGF-bb to serum-starved RAOSMCs led to complete PDGFR-β phosphorylation, which reached the peak within 10 min and then decreased to nearly baseline levels at 240 min. However, pretreated EGCG suppressed PDGFR-β phosphorylation by PDGF-bb and sustained only baseline level (Figure 4(a)). The phosphorylations of MEK1/2 and p42-44MAPK, downstream proteins of PDGF-induced signaling, were significantly increased between 10 and 30 min and declined over the following 240 min. However, pretreated EGCG inhibited MEK1/2 and p42-44MAPK phosphorylations in a time-dependent manner, similar to PDGFR-β phosphorylation (Figure 4(b)). In the other intracellular signal pathways, phosphorylations of Akt and p38 MAPK were activated by PDGF-bb stimulation. However, the Akt and p38 MAPK phosphorylations induced by PDGF-bb were inhibited in RAOSMCs by being pretreated with EGCG (Figure 4(c)). These results suggest that EGCG can indirectly inhibit the phosphorylation of PDGFR-β by PDGF-bb.

3.5. Inhibitory Effect of Signal Transduction Pathway on RAOSMC by PDGF-bb Stimulation with EGCG. To characterize the signaling pathways by direct interaction between EGCG and PDGF-bb, serum-starved RAOSMCs were incubated with EGCG and PDGF-bb for the desired times. PDGFR-β phosphorylation was completely suppressed and inactivated on PDGF-bb induced RAOSMC by EGCG compared with the PDGF-stimulated samples that were processed on the same blot (Figure 5(a)). Therefore, MEK1/2 and p42/44 MAPK phosphorylations were suppressed and sustained at baseline levels by being cotreated EGCG with PDGF-bb (Figure 5(b)). The phosphorylations of Akt and p38 MAPK were also suppressed by inhibition of PDGF-bb signaling by EGCG. These results reveal that EGCG can directly interrupt PDGF-bb stimulation by inhibiting PDGFR-β phosphorylation.

4. Discussion

PDGF-bb is a major stimulator of VSMC dedifferentiation and is known to play a central role in the pathogenesis of various vascular disorders. Signal transduction pathways involve the activation of mitogen-activated protein kinases (MAPKs) on PDGF-induced responses. MAPK is a family of serine/threonine protein kinases with 3 subfamilies named c-jun-N-terminal kinase 1/2 (JNK1/2), ERK1/2, and p38 MAPK.

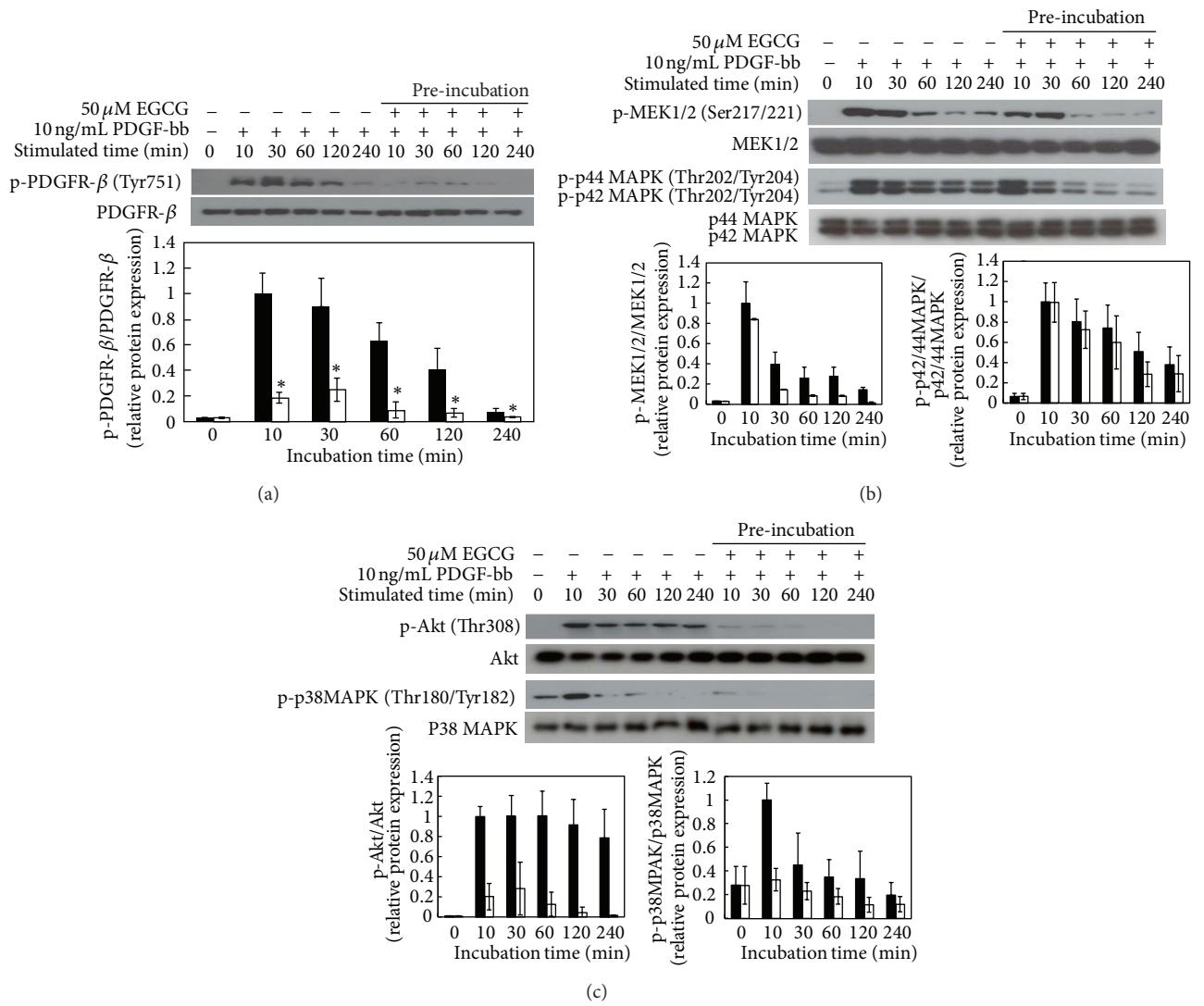


Figure 4: Modulation of PDGF-bb stimulatory signal pathways on EGCG preincubated RAOSMC. RAOSMC preincubated with EGCG was stimulated with 10 ng/mL PDGF-bb for the desired time (10 m, 30 m, 1 h, 2 h, and 4 h, resp.), lysed, and lysates were immunoblotted with antibodies. After densitometric quantification, data were each expressed as the mean ± SD from three independent experiments. The black bars indicate expression by PDGF-bb stimulation. The white bars indicate expression by PDGF-bb stimulation on EGCGpretreated RAOSMC. (a) The expression of phospho-PDGFR-β (Tyr751) in a time-dependent manner. The band intensity was normalized to total PDGFR-β expression. (b) The expression of phospho-MEK1/2 (Ser217/221) and phospho-p42/44 MAPK (Thr202/Tyr204) in a time-dependent manner. The band intensity was normalized to total MEK1/2 and p42/44 MAPK expression. (c) The expression of phospho-Akt (Thr308) and phospho-p38 MAPK (thr180/Tyr182) in time-dependent manner. The band intensity was normalized to total Akt and p38 MAPK expression.

PDGF stimulated rapid and significant activation of Akt, ERK1/2, and p38 MAPK in cultured VSMC. MAPKs are proposed to play a major role in the activation of various transcription factors [20, 21]. PDGF-bb binds with PDGFR-β and triggers receptor dimerization and autophosphorylation at tyrosine residues that activate the kinase and serve as recruitment sites for SH2 domain-containing proteins. Within minutes, many signaling modules are engaged, including Ras, Src, phosphoinositide 3′-kinase (PI3 K), SHP2, and phospholipase Cγ (PLCγ) [9, 22, 23]. Downstream signals then activate PI3-K/PKB (Akt) and two MAPK pathways [24]. VSMC dedifferentiation is determined by activation of Akt pathway, p42/44 MAPK, and p38 MAPK pathways. Ultimately, this results in VSMC dedifferentiation via the recruitment, and activation of specific signaling pathway may mediate the migration and proliferation of VSMCs in response to injury such as the development of atherosclerosis and hypertension. Several studies have revealed that PDGFR targeted by synthetic tyrosine kinase inhibitors and antisense treatment reduce neointima formation in injured arteries [25, 26].

EGCG has been shown to have protective effects on the cardiovascular system, including antiatherosclerotic, antihypercholesterolemic, and antirestenosis effects [27–29]. Also, several studies have stated that EGCG inhibited proliferation, migration, and invasion of barrier by inhibition

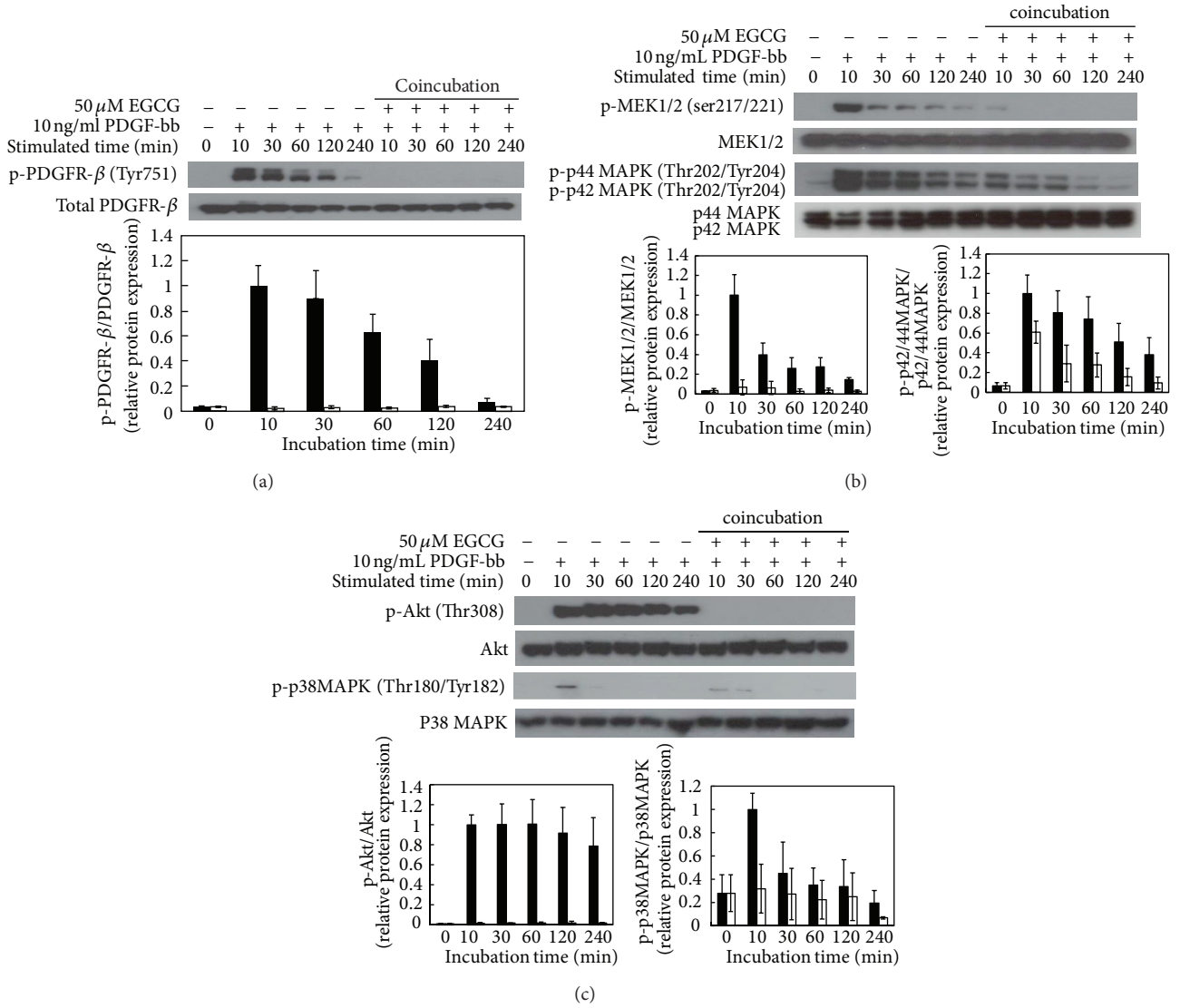


Figure 5: The effect of EGCG on modulation of PDGF-bb stimulatory signal pathways in RAOSMC. Serum-starved RAOSMC was stimulated with 10 ng/mL PDGF-bb and 50 μM EGCG for the desired time (10 m, 30 m, 1 h, 2 h, and 4 h, resp.), lysed, and lysates were immunoblotted with antibodies. After densitometric quantification using the imageJ program, data were each expressed as the mean ± SD from three independent experiments. The black bar indicates expression by PDGF-bb stimulation. The white bar indicates expression by PDGF-bb stimulation with EGCG. (a) The expression of phospho-PDGFR-β (Tyr751) in a time-dependent manner. The band intensity was normalized to total PDGFR-β expression. (b) The expression of phospho-MEK1/2 (Ser217/221) and phospho-p42/44 MAPK (Thr202/Tyr204) in a time-dependent manner. The band intensity was normalized to total MEK1/2 and p42/44 MAPK expression. (c) The expression of phospho-Akt (Thr308) and phospho-p38 MAPK (Thr180/Tyr182) in a time-dependent manner. The band intensity was normalized to total AKt and p38 MAPK expression.

via intracellular signaling transduction pathway signals on VSMC stimulated with growth factor, such as angiotensin II [30, 31] and basic fibroblast growth factor (bFGF) [32]. A previous study showed that EGCG induced apoptosis of VSMCs in a p53- and NF-κB-dependent manner [33, 34].

However, the dosage of polyphenols and flavonoids in cell culture studies may be much higher that than which occurs after oral administration in the body. The compounds may lose most of their functions after undergoing metabolism and circulation in vivo, but this may not be possible to evaluate in vitro. For that reason, concentration determined by in vitro experiment may be difficult to apply as a physiological dose to animals or humans [35–37]. Although not all cell culture findings are applicable for animal experiments, in vitro studies have provided important insights into the action mechanisms of flavonoids that would be physiologically achievable in human [37].

Polyphenolic catechins and flavonoids are generally safe and may possess beneficial properties for human health. Various clinical studies have revealed that they are effective at various organ sites [38]. However, unusually high dosage of natural products supplements may exhibit toxicity in vivo [36, 37]. Accordingly, numerous studies have been performed to improve the stability and enhance the physiological activity

of native compounds, with combination with other agents, synthetic modification, and adoption of analog and prodrug [39, 40].

Our results observed that RAOSMC stimulation by PDGF-bb induced proliferation and cell cycle progression through intracellular pathways: p42/44 MAPK, p38 MAPK, and Akt cascade, in addition to the activation of PDGFR-β. However, PDGF-bb did not induce proliferation and mitogenesis on RAOSMC preincubated with EGCG (Figure 1). Also, pretreated EGCG inhibited the gelatinolytic activity of MMP-9 and conversion from pro-MMP-2 to active MMP-2. Therefore, gelatinolytic activity of MMPs was inhibited dose-dependently in PDGF-bb stimulated RAOSMCs by EGCG (Figure 3). Previous studies reported that EGCG enhanced pro- and active MMP-2 binding to TIMPS and upregulated TIMP-2 expression as one of the major mechanisms for inhibition of SMC invasion [16, 41]. These results suggest that EGCG regulates the activation of MMPs and TIMPs for inhibition of invasion in dedifferentiated VSMCs.

These results suggest that EGCG may mediate the inhibition of PDGF-bb directly binding with PDGFR-β on the RAOSMC membrane of RAOSMC and thus deactivate the PDGF signal pathway related to mitogenesis (Figure 4). Some studies reported that EGCG is hijacked by the laminin receptor (LamR), a lipid raft protein, and alters membrane domain composition to prevent epidermal growth factor (EGF) from binding to its receptor (EGFR) [42, 43]. Also, EGCG has been shown to incorporate itself into the plasma membrane to lead to reversible binding of PDGF-bb to a nonreceptor target site, reducing PDGF binding to its receptors [44]. Thus, EGCG inhibits a surface-membrane linked mechanism [45].

In this study, we could also demonstrate on the direct interaction between EGCG and PDGF-bb when they are cotreated. As shown in Figure 2, low concentration of EGCG (10 μM) induces antiproliferation and cell cycle arrest, and cell stimulation occurred in the presence of EGCG. This effect is accompanied by the fact that EGCG inhibits PDGF-induced mitogenesis by disturbing PDGFR-β phosphorylation (Figure 5). Also, the inhibitory effect of EGCG was mediated by the blockage of PDGFR-β phosphorylation early in the experiment. Thus, EGCG may already have interacted with PDGF-bb in media and inhibited VSMC dedifferentiation by blocking the early signal transduction pathway. Other research groups showed that EGCG is able to interact with various biomolecules, especially proliferation-related proteins, each being proved by various cell line experiments [46–51]. Therefore, recent studies have revealed that EGCG binds with high affinity to residues located in the serum albumin under physiological conditions [52, 53].

Based on our findings, we suggest that EGCG inhibits RAOSMC mitogenesis by interruption of PDGF-bb signaling, probably by blockage of PDGF-bb binding and PDGFR-β phosphorylation, as well as the activation of p42/44 MAPK, p38 MAPK, and Akt, important downstream events of PDGFR-β. Therefore, EGCG may be a potential target for inhibiting PDGFR and may be of use in the prevention and treatment of vascular diseases.

Conflict of Interests

The authors declare that there is no conflict of interests with any financial organization regarding the commercial identities mentioned in the paper.

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Research Article

The Natural Stilbenoid Piceatannol Decreases Activity and Accelerates Apoptosis of Human Neutrophils: Involvement of Protein Kinase C

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Neutrophils are able to release cytotoxic substances and inflammatory mediators, which, along with their delayed apoptosis, have a potential to maintain permanent inflammation. Therefore, treatment of diseases associated with chronic inflammation should be focused on neutrophils; formation of reactive oxygen species and apoptosis of these cells represent two promising targets for pharmacological intervention. Piceatannol, a naturally occurring stilbenoid, has the ability to reduce the toxic action of neutrophils. This substance decreased the amount of oxidants produced by neutrophils both extra- and intracellularly. Radicals formed within neutrophils (fulfilling a regulatory role) were reduced to a lesser extent than extracellular oxidants, potentially dangerous for host tissues. Moreover, piceatannol did not affect the phosphorylation of $p40^{phox}$—a component of NADPH oxidase, responsible for the assembly of functional oxidase in intracellular (granular) membranes. The stilbenoid tested elevated the percentage of early apoptotic neutrophils, inhibited the activity of protein kinase C (PKC)—the main regulatory enzyme in neutrophils, and reduced phosphorylation of PKC isoforms α, βII, and δ on their catalytic region. The results indicated that piceatannol may be useful as a complementary medicine in states associated with persisting neutrophil activation and with oxidative damage of tissues.

1. Introduction

Piceatannol (*trans*-3,4,3′,5′-tetrahydroxystilbene, Figure 1) is a naturally occurring hydroxylated analogue of resveratrol, displaying remarkable antioxidative, anticancer, and anti-inflammatory properties. The latter activity has been attributed to the capacity of piceatannol to prevent activation of transcription factors, mRNA expression, and production of inflammatory mediators, for example, nitric oxide, prostaglandin E_2, interleukins, monocyte chemotactic protein MCP-1, cyclooxygenase-2, and tumour necrosis factor α [1–4]. Piceatannol, as a potent spleen tyrosine kinase (Syk) inhibitor, has a great potential to suppress allergic and autoimmune disorders by blocking immune receptor signalling in a variety of inflammatory cells, including neutrophils [5–9].

Neutrophils (neutrophilic polymorphonuclear leukocytes) represent the body's primary line of defense against invading pathogens. Nevertheless, these cells are able to release cytotoxic substances and inflammatory mediators, which, along with their delayed apoptosis, have a potential to maintain permanent inflammation [10, 11]. Therefore, treatment of diseases associated with chronic inflammation should be focused on neutrophil functions; formation of reactive oxygen species (ROS) and apoptosis of these cells represent two promising targets for pharmacological intervention.

Formation of ROS is initiated by the activation of phagocyte NADPH oxidase (NOX2/$gp91^{phox}$), the first identified and the best studied member of the NOX family. During activation, the cytosolic oxidase subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac2 translocate to the plasma membrane and associate with the membrane-bound cytochrome b_{558} complex. Cytochrome b_{558} is formed by two subunits—$gp91^{phox}$ (also known as NOX2) and by $p22^{phox}$, and this

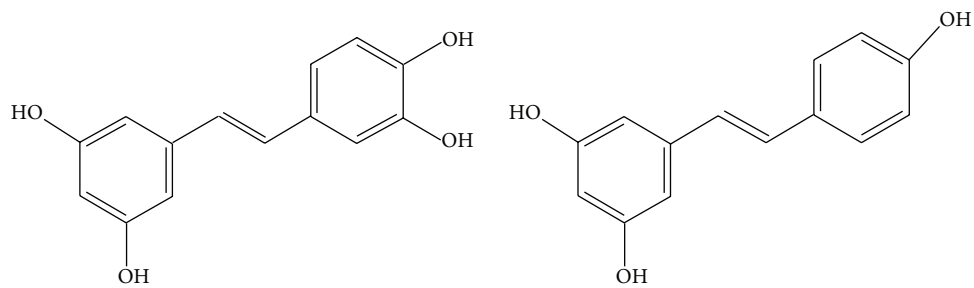

Figure 1: Piceatannol (*trans*-3,4,3′,5′-tetrahydroxystilbene) and its related compound *trans*-resveratrol (*trans*-4,3′,5′-trihydroxystilbene).

heterodimer transfers electrons from NADPH to molecular oxygen. Flavin adenine dinucleotide (FAD) and two heme groups serve as the redox pathway which enables the transfer of electrons across the membrane [12–14]. If the assembly of catalytically active oxidase occurs on the plasma membrane, the generated oxidants are liberated extracellularly or into phagosomes. These radicals are involved in the elimination of pathogens; however, their overproduction may result in tissue damage. ROS produced intracellularly—on membranes of specific granules, participate in the initiation of neutrophil apoptosis [15, 16] and they are considered key suppressors of inflammation [17, 18]. Since the optimum therapy is expected to minimise tissue damage without reduction of the physiological function of neutrophils, separate analysis of extra- and intracellular effects of antioxidants is of particular importance.

The production of ROS is limited by the programmed death of neutrophils. Apoptosis represents a precisely regulated process, which includes release of proapoptotic proteins into the cytosol, gradual activation of caspases, DNA fragmentation, chromatin condensation, loss of membrane asymmetry, and formation of apoptotic bodies. Alterations in plasma membrane (e.g., externalisation of phosphatidylserine) facilitate the recognition and clearance of apoptotic neutrophils by macrophages, resulting in safe removal of these cells from the site of inflammation. Since the delayed apoptosis and impaired clearance of neutrophils aggravate and prolong tissue injury, pharmacological intervention focused on neutrophil apoptosis is studied as an original approach for the design of new anti-inflammatory strategies [16, 19, 20].

Modulation of protein kinase C activity represents a prospective method to regulate neutrophil functions. Immunochemical studies have shown that human neutrophils express five PKC isoforms, α, βI, βII, δ, and ζ, which participate in NADPH oxidase activation as well as in proapoptotic and antiapoptotic signalling [21–25].

The present paper investigated the impact of piceatannol on the viability and oxidative burst of human neutrophils. We analysed separately the effects of this stilbenoid on the concentration of ROS produced by neutrophils extra- and intracellularly. Protein kinase C activity was examined as an assumed target of piceatannol action and the phosphorylation of PKCα, PKCβII, and PKCδ (the most abundant PKC isoforms in neutrophils) was assessed.

2. Material and Methods

2.1. Chemicals. Piceatannol was purchased from Acros Organics (Geel, Belgium). Luminol, isoluminol, PMA (4β-phorbol-12β-myristate-13α-acetate), Ca^{2+}-ionophore A23187, superoxide dismutase, dextran (average MW 464 000 kDa), zymosan (zymosan A from *Saccharomyces cerevisiae*), luciferase from *Photinus pyralis*, and D-luciferin sodium salt were from Sigma-Aldrich Chemie (Deisenhofen, Germany); HRP (horseradish peroxidase) and catalase were obtained from Merck (Darmstadt, Germany) and lymphoprep (density 1.077 g/mL) was purchased from Nycomed Pharma AS (Oslo, Norway). Propidium iodide and rh Annexin V-FITC (produced in *E. coli* and conjugated with fluorescein isothiocyanate—FITC) was received from eBioscience (Vienna, Austria) and PKC kinase activity kit was from Enzo Life Sciences AG (Lausen, Switzerland). Phosphospecific antibodies *versus* PKC isoforms and *versus* $p40^{phox}$ were obtained from Cell Signaling Technology (Danvers, MA, USA). Secondary anti-rabbit antibody and Lumigen Detection Reagent were supplied by GE Healthcare Life Sciences (Little Chalfont, UK).

This work was approved by the Local Ethic Committee, Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava.

2.2. Blood Collection and Isolation of Human Neutrophils. Fresh blood was obtained at the blood bank by venipuncture from healthy male donors (20–50 years) who had not received any medication for at least 7 days. The samples were mixed with 3.8% trisodium citrate, in the ratio of 9 mL of blood to 1 mL citrate. Erythrocytes were allowed to sediment in 1% dextran solution (1 ×g, 25 min, 22°C) and the suspension of leukocytes and platelets in plasma (buffy coat) was used for flow cytometric analyses or for neutrophil isolation. For neutrophil isolation, the buffy coat was centrifuged, cells were resuspeded in phosphate-buffered saline, and neutrophils were separated on Lymphoprep (500 ×g, 30 min, 22°C). The contaminating erythrocytes were removed with hypotonic cold haemolysis and neutrophils were washed with phosphate-buffered saline. Neutrophil count was assessed by Coulter Counter (Coulter Electronics, High Wycombe, England) and adjusted to a final concentration of 10^4 cells/1 μL. The final suspension of neutrophils contained more than 96%

of viable cells, as evaluated by trypan blue exclusion, and was used maximally for 2 h—as long as control chemiluminescence kept constant.

2.3. Formation of ROS in Neutrophils. Oxidative burst of neutrophils was recorded on the basis of enhanced chemiluminescence [26, 27], in a microtitre plate computer-driven luminometer LM-01T (Beckman Coulter, Prague, Czech Republic). Chemiluminescence of human whole blood (250x diluted) enhanced with luminol (250 μmol/L) was stimulated with phorbol myristate acetate (PMA, 0.05 μmol/L), opsonized zymosan (0.5 mg/mL), or Ca^{2+}-ionophore A23187 (1 μmol/L). Chemiluminescence of isolated human neutrophils (5×10^5/sample) was initiated by PMA (0.05 μmol/L). Oxidants released extracellularly were determined in the system containing isoluminol (5 μmol/L) and HRP (8 U/mL); HRP was added to the system, ensuring sufficient extracellular peroxidase concentration. Intracellular chemiluminescence was enhanced with luminol (5 μmol/L) in the presence of the extracellular scavengers superoxide dismutase (100 U/mL) and catalase (2 000 U/mL). Concentration of oxidants was evaluated on the basis of integral values of chemiluminescence over 1 800 s (isolated neutrophils and A23187 stimulated whole blood) and over 3 600 s (whole blood chemiluminescence initiated with PMA or zymosan).

2.4. Chemiluminescence of Cell-Free System. The antioxidative activity of piceatannol was measured in cell-free system containing piceatannol (0.01–100 μmol/L), HRP (2 U/mL), luminol (10 μmol/L), and hydrogen peroxide (100 μmol/L) in 50 μL aliquots. Chemiluminescence was determined for 10 min at 37°C [28].

2.5. Activity of Protein Kinase C. Isolated neutrophils (5×10^5 cells) were incubated with piceatannol (1–100 μmol/L) for 30 min at 37°C and stimulated with PMA (0.15 μmol/L) for 3 min. Stimulation was stopped by the addition of a tenfold volume of ice-cold phosphate buffer. After centrifugation, neutrophils were broken by lysing solution and by sonication, centrifuged, and the supernatant was used for the determination of protein kinase C activity using an enzyme-linked immunosorbent assay kit.

2.6. Phosphorylation of $p40^{phox}$ and PKCα, βII, δ. Western blot analysis was performed as previously described [29, 30]. Isolated human neutrophils (5×10^6 cells) were incubated at 37°C with piceatannol (10 or 100 μmol/L, 1 min), stimulated with PMA (0.15 μmol/L, 1 min) and lysed by sonication in a lysing solution containing protease, and phosphatase inhibitors. Debris from the lysed cells was pelleted by centrifugation and the supernatant was taken for blotting assay. Protein concentration was measured using Bradford Dye Reagent detection kit (Bio-Rad, Hercules, CA, USA). Proteins (20 μg per lane) were separated by SDS polyacrylamide gel electrophoresis and transferred to Immobilon-P Transfer Membrane. From each membrane, the area between 60 and 100 kDa was detected with primary anti-phospho-PKCα/βII (Thr638/641) antibody (1 : 5 000) and with the secondary antibody conjugated to HRP (1 : 5 000). Phosphorylated PKC isoforms α and βII were visualised with Lumigen Detection Reagent kit, scanned, and quantified densitometrically using ImageJ programme. The membrane was then stripped, using stripping buffer (Re-blot Plus Mild Solution, Millipore, Temecula, CA, USA), and reprobed with anti-phospho-PKCδ (Thr505) antibody (1 : 1 000). The membrane area between 30–60 kDa was detected with regard to the presence of the internal standard β-actin (β-actin antibody, 1 : 4 000) and the phosphorylated subunit of NADPH oxidase $p40^{phox}$ (anti-phospho- $p40^{phox}$ (Thr154) antibody, 1 : 5 000).

2.7. Neutrophil Integrity. Damaging effect of piceatannol on the integrity of plasma membranes was evaluated on the basis of ATP liberation, measured by the luciferin-luciferase chemiluminescence method [31]. Suspension of isolated neutrophils (3×10^4 cells) was incubated with piceatannol (1–100 μmol/L) for 15 min at 37°C. Then, the mixture of luciferin (1.6 μg) and luciferase (45 000 U) was added and chemiluminescence was recorded for 60 seconds. Chemiluminescence of ATP standards (1–500 nmol/L) was measured in each experiment and concentrations of ATP in samples were calculated from the calibration curve. Total ATP content was assessed immediately after sonication of neutrophils for 10 seconds.

2.8. Analysis of Apoptosis. Human plasma buffy coat (see Section 2.2) was incubated with piceatannol (1–100 μmol/L) for 10 min at 37°C. The cells were double-stained with annexin-V conjugated with FITC (in dark at 4°C for 10 min) and with propidium iodide and analysed by cytometer Cytomics FC 500 (Beckman Coulter, Inc., Brea, CA, USA). From the granulocyte area, 5 000 cells were gated and the percentage of apoptotic (annexin positive and propidium iodide negative), dead (double positive), and viable cells (double negative) was determined as described previously [32, 33].

2.9. Data Analysis. All values were given as the mean ± SEM and the statistical significance of differences between means was established by Student's t-test. P values below 0.05 were considered to be statistically significant and were indicated in the figures by $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

3. Results

Piceatannol reduced the oxidative burst of human neutrophils measured in whole blood (Table 1). It inhibited chemiluminescence initiated by the stimulation of protein kinase C, increased calcium concentration, and the activation of membrane receptors at the respective mean effective concentrations of 0.65 ± 0.07 μmol/L (PMA), 2.71 ± 0.41 μmol/L (A23187) and 9.43 ± 0.53 μmol/L (zymosan).

In isolated neutrophils stimulated with PMA, extra- and intracellular chemiluminescence was recorded separately (Table 2). Piceatannol decreased both the extracellular and intracellular chemiluminescence of neutrophils at the respective mean effective concentrations 1.87 ± 0.35 μmol/L and

Table 1: Dose-dependent inhibition of neutrophil oxidative burst in the presence of piceatannol. Chemiluminescence, measured in whole blood, was initiated with PMA (0.05 μmol/L), Ca^{2+}-ionophore A23187 (1 μmol/L), or opsonized zymosan (0.5 g/L). The incubation of neutrophils with piceatannol was 60 min (PMA, zymosan) or 30 min (A23187), depending on the kinetics of oxidative burst. Mean ± SEM, $n = 8$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ *versus* Control.

Piceatannol (μmol/L)	Inhibition of chemiluminescence (% of control)		
	PMA	A23187	Zymosan
0.01	93.21 ± 2.64*	96.74 ± 1.41	98.10 ± 1.10
0.1	84.45 ± 1.99**	91.64 ± 1.84**	98.63 ± 1.42
1	41.19 ± 2.19**	73.89 ± 4.39**	93.22 ± 1.98*
10	4.12 ± 0.21**	19.40 ± 3.30**	50.17 ± 1.37**
100	0.25 ± 0.04**	0.07 ± 0.07**	0.26 ± 0.07**

Table 2: Effect of piceatannol on extra- and intracellular chemiluminescence of isolated human neutrophils stimulated with PMA (0.05 μmol/L) and on the chemiluminescence produced by cell-free system. The exposure to piceatannol was 30 min (extra-, intracellular) or 10 min (cell-free system). Mean ± SEM, $n = 3$–8, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ *versus* Control.

Piceatannol (μmol/L)	Inhibition of chemiluminescence (% of control)		
	Extracellular	Intracellular	Cell-free system
0.01	96.89 ± 4.05	101.72 ± 4.34	99.43 ± 1.19
0.1	90.73 ± 5.80	102.40 ± 2.16	93.77 ± 0.55*
1	75.74 ± 5.55*	101.29 ± 1.96	33.60 ± 0.13**
10	5.31 ± 0.82**	59.95 ± 2.51**	0.72 ± 0.04**
100	0.01 ± 0.01**	0.65 ± 0.15**	0.71 ± 0.02**

12.59 ± 0.96 μmol/L; in cell-free system, the EC_{50} of piceatannol was 0.63 ± 0.01 μmol/L. The phosphorylation of $p40^{phox}$ (a component of NADPH oxidase essential for intracellular oxidant formation) was increased more than three times after PMA stimulation. This increase was not modified by the treatment of neutrophils with piceatannol (Figure 2). Considering the high efficiency of piceatannol in neutrophils stimulated with PMA and its recorded intracellular activity, in further experiments the effect of this phytochemical was evaluated on PKC activity (Figure 3). The stimulation of neutrophils with PMA increased protein kinase C activity by 50%; piceatannol dose-dependently reduced this rise until the values of activity were comparable with those produced by resting cells. The phosphorylation of protein kinases C α, βII, and δ (the most abundant PKC isoforms in neutrophils) was also decreased after piceatannol treatment (Figure 4). Phosphorylation of PKCα and βII was reduced in the presence of both concentrations used, whereas in the case of PKCδ phosphorylation, only 10 μmol/L piceatannol was effective. The observed inhibitory effects were not associated with neutrophil damage as in the presence of piceatannol no increase in extracellular ATP concentration was recorded (Figure 5). Spontaneous ATP liberation from isolated neutrophils was minimal, approximately 5% of the total ATP content. This amount remained unchanged or

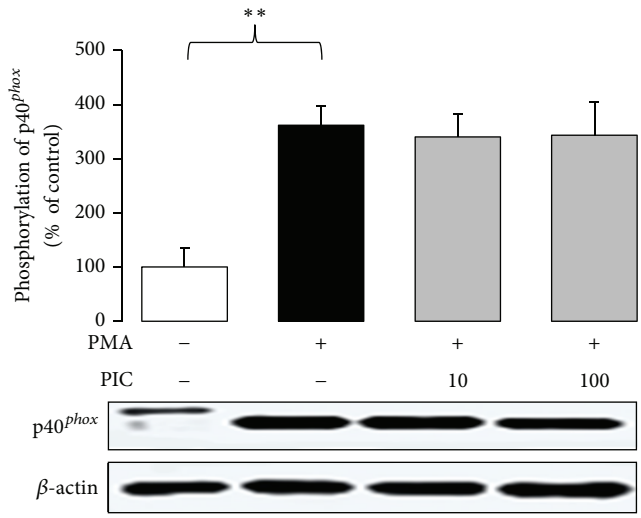

Figure 2: Phosphorylation of $p40^{phox}$ in PMA-stimulated neutrophils treated with 10 and 100 μmol/L piceatannol. Phosphorylated NADPH oxidase subunit was isolated by Western blotting and detected with anti-phospho- $p40^{phox}$ (Thr154) antibody. The values are presented as percentage of resting control. Control value, given as optical density of $p40^{phox}$ band corrected to β-actin content, was 38.13 ± 10.67. Mean ± SEM, $n = 6$, $^{**}P \leq 0.01$.

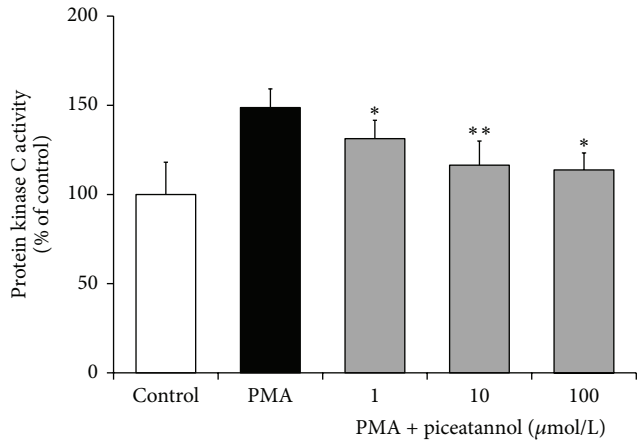

Figure 3: . Effect of piceatannol on PKC activity. Neutrophils were incubated with piceatannol (30 min) and stimulated with PMA (3 min). PKC activity was assessed by ELISA kit in the supernatant of cell lysate. The values are presented as percentage of resting control (PKC activity in absence of PMA). Control value given as kinase activity per 1 mg of protein was 13 376 ± 2 417. Mean ± SEM, $n = 5$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ *versus* PMA.

was slightly decreased after treatment of neutrophils with piceatannol. Spontaneous neutrophil apoptosis was accelerated by piceatannol as indicated by the significantly elevated number of apoptotic neutrophils (Figure 6). The percentage of dead (propidium iodide positive) neutrophils ranged from 0.1 to 0.2% and was not significantly increased in the presence of piceatannol.

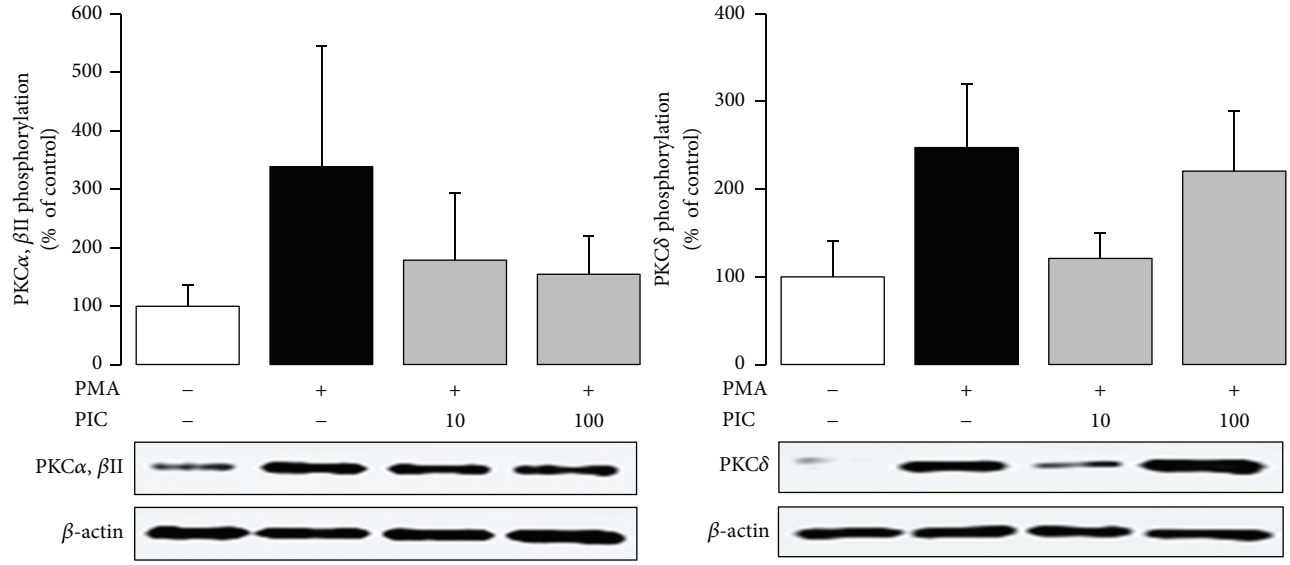

Figure 4: Phosphorylation of PKCα, PKCβII, and PKCδ in PMA-stimulated neutrophils treated with 10 and 100 μmol/L piceatannol (PIC). Phosphorylated PKC isoforms were isolated by Western blotting and detected with anti-phospho-PKCα/βII (Thr638/641) and anti-phospho-PKCδ (Thr505) antibodies. The values are presented as percentage of resting control. Control values, given as optical density of PKC bands corrected to β-actin content, were 78.07 ± 17.86 (PKCα, βII) and 84.84 ± 18.80 (PKCδ). The representative blot manifests elevated phosphorylation of PKC isoforms in neutrophils stimulated with PMA as well as the effect of piceatannol on this increase. Mean ± SEM, $n = 4$.

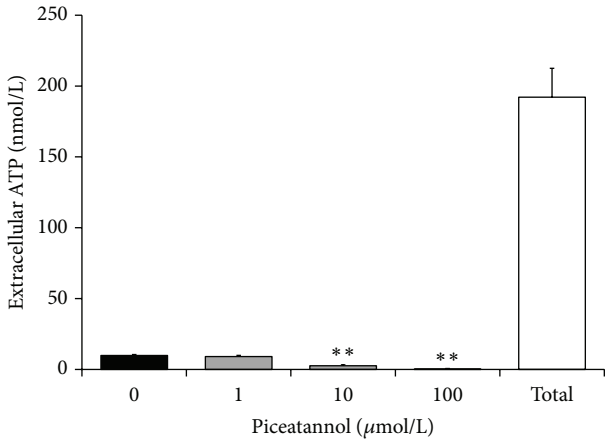

Figure 5: Integrity of neutrophils assessed on the basis of ATP liberation in the absence (0) and in the presence of piceatannol (1–100 μmol/L). Total—amount of ATP determined immediately after complete neutrophil destruction. The given values represent the extracellular ATP concentration in samples containing 30 000 neutrophils. Mean ± SEM, $n = 6$, $^{**}P \leq 0.01$ *versus* Control (0).

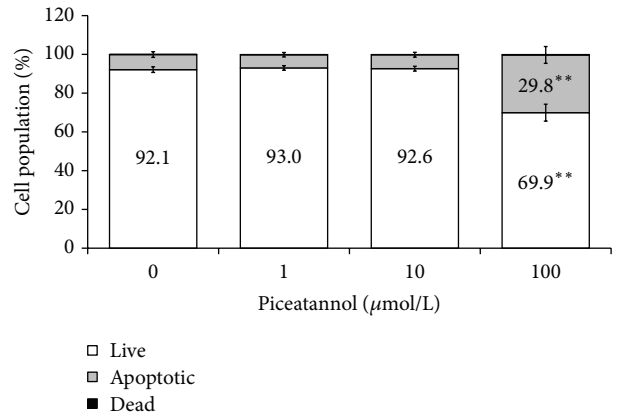

Figure 6: Effect of piceatannol on neutrophil apoptosis. The lifespan of human neutrophils was recorded by flow cytometry, using double-staining with annexin-V (AN) and propidium iodide (PI). Of the population of 5 000 granulocytes, the percentage of live (AN^-/PI^-), apoptotic (AN^+/PI^-), and dead cells (AN^+/PI^+) was calculated. The percentage of dead neutrophils (0.1-0.2%) was not significantly increased in the presence of piceatannol. Mean ± SEM, $n = 7$, $^{**}P \leq 0.01$ *versus* Control (0).

4. Discussion

Novel strategies of anti-inflammatory therapy are based upon pharmacological agents capable of enhancing the resolution—that is, the termination of the beneficial inflammation before it may turn into an adverse chronic stage [34, 35]. It is likely that several phytochemicals would act in this way, but this point has not been investigated. In this paper, the natural stilbenoid piceatannol was analysed, considering its ability to affect activity and apoptosis of neutrophils—two important inputs of resolution [19, 20, 36].

The incubation of human neutrophils with piceatannol resulted in decreased production of ROS. Several mechanisms—inhibition of Syk kinase, ROS scavenging, and the interference with neutrophil activation through suppressed PKC activation—could be involved in the reduction of the chemiluminescence signal. Piceatannol, widely used

as an inhibitor of Syk kinase [6, 8, 9], has the potential to block oxidative burst at the receptor signaling level. However, this mechanism might not be essential, with regard to the pronounced inhibition of chemiluminescence initiated with PMA or A23187, that is, by receptor bypassing stimuli. The marked inhibition of the chemiluminescence produced by cell-free system suggested participation of antioxidant activity, which is closely involved in various effects of other natural polyphenols [37–41]. Piceatannol was found to be a potent scavenger of hydroxyl radicals and superoxide anion [42], much more effective than resveratrol. This high effectiveness results from the presence of an additional hydroxyl group in stilbene rings, which makes the abstraction and transfer of electrons easier and increases the stability of the resulting piceatannol semiquinone radical [4]. Due to the fact that the inhibition of chemiluminescence occurred in the presence of each stimulus used, the interference of piceatannol with a process involved in all mechanisms of initiation has been suggested. One of the potential candidates could be the signalling enzyme protein kinase C. Piceatannol inhibited PKC activation initiated by PMA, which was reflected in the decreased activity of the enzyme as well as in the reduced phosphorylation of protein kinase isoenzymes α, βII, and δ on their catalytic region. Since PKC participates in the activation of neutrophil NADPH oxidase [13, 14, 22, 25], its inhibition may result in reduced oxidant formation and thus explain the decreased chemiluminescence of neutrophils treated with piceatannol. The precise mechanism of piceatannol-mediated PKC inhibition is still not completely clear. Similar to other natural polyphenols, it may involve the competition for phorbol ester or calcium binding to the regulatory domains of PKC [43, 44], inhibition of PKC translocation from cytosol to membrane fraction [45], oxidation of thiol groups present within the catalytic domain of PKC [46], or piceatannol-induced alterations in membrane ordering [47].

Since activated neutrophils form and liberate ROS both extra- and intracellularly [13, 17, 18], it was important to identify which part of the chemiluminescence signal was reduced in the presence of piceatannol. This stilbenoid was active in both compartments, however, at different mean effective concentrations –12.59 μmol/L (intracellular) and 1.87 μmol/L (extracellular). It means that the radicals formed within neutrophils (fulfilling a regulatory role) were reduced to a lesser extent than extracellular oxidants, potentially dangerous for host tissues. Moreover, piceatannol did not affect the phosphorylation of $p40^{phox}$—a component of NADPH oxidase, responsible for the assembly of functional oxidase in intracellular (granular) membranes [17, 48, 49]. Finally, the phosphorylation of PKCδ (responsible for intracellular oxidant production [50]) was affected by piceatannol to a lesser extent than the phosphorylation of PKCβ, which is involved in the extracellular formation of oxidants [16]. Yet the interference of piceatannol with PKCδ may not be a decisive mechanism involved in the inhibition of intracellular chemiluminescence, as in the presence of 100 μmol/L piceatannol, the chemiluminescence was strongly reduced, despite the fact that the inhibition of PKCδ phosphorylation was minimal at this concentration.

The more pronounced extracellular activity results from the structure of piceatannol. Compared to resveratrol, the additional hydroxyl group makes the molecule of piceatannol more hydrophilic and less able to pervade biological membranes [47]. Thus, piceatannol could minimise tissue damage with the minimal reduction of beneficial intracellular oxidants, involved in the suppression of neutrophil proinflammatory activity [17, 18] and in the initiation of neutrophil apoptosis [15, 16].

The observed reductions in chemiluminescence and in the activity of protein kinase C were not associated with neutrophil damage because in the presence of piceatannol no increase in extracellular ATP concentration was recorded. As confirmed by flow cytometry, this stilbene enhanced spontaneous apoptosis of neutrophils. This was indicated by an increased number of annexin-positive cells, that is, cells displaying a more pronounced externalisation of phosphatidylserine. The expression of phosphatidylserine on the external side of plasma membrane facilitates the recognition of apoptotic neutrophils by macrophages and their safe removal from the site of inflammation [15]. The ability of piceatannol to increase apoptosis has been extensively studied in cancer cells, where it involves the increased activities of caspases, activation of proapoptotic factors Bid, Bax, Bak, or the inhibition of the antiapoptotic factor Bcl-xL [4]. In neutrophils, all these mechanisms may be operative, along with the repressed activation of the antiapoptotic enzyme phosphoinositide-3-kinase [51, 52]. Moreover, the accelerated apoptosis, observed in the presence of piceatannol, may result from the inhibition of PKCα and PKCδ, as these PKC isoforms participate in antiapoptotic signalling in neutrophils [23, 53]. Increased neutrophil apoptosis was observed in the presence of 100 μmol/L piceatannol, that is, in a concentration several times higher than assumed piceatannol plasma levels obtained by dietary intake. Yet it should be taken into account that, in comparison to *in vivo* conditions, in these samples neutrophil count was substantially higher and the exposure of the cells to piceatannol lasted only 30 minutes. Similarly, the accelerated apoptosis of leukemic cells was detected after 24–48 h incubation with 10–60 μmol/L piceatannol [54, 55].

5. Conclusion

Piceatannol decreased the concentration of ROS produced by neutrophils and accelerated spontaneous apoptosis of these cells. The observed effects classified piceatannol as a potentially useful complementary medicine in states associated with persistent activation of neutrophils, oxidative damage of tissues, and persistent inflammation. However, the bioavailability and toxicity of this promising natural substance is a decisive question requiring further studies.

Conflict of Interests

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

Acknowledgments

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Review Article

Dietary Anthocyanins as Nutritional Therapy for Nonalcoholic Fatty Liver Disease

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Nonalcoholic fatty liver disease (NAFLD), defined by excessive lipid accumulation in the liver, is the hepatic manifestation of insulin resistance and the metabolic syndrome. Due to the epidemics of obesity, NAFLD is rapidly becoming the leading cause of altered liver enzymes in Western countries. NAFLD encompasses a wide spectrum of liver disease ranging from simple uncomplicated steatosis, to steatohepatitis, cirrhosis, and hepatocellular carcinoma. Diet may affect the development of NAFLD either by increasing risk or by providing protective factors. Therefore, it is important to investigate the role of foods and/or food bioactives on the metabolic processes involved in steatohepatitis for preventive strategies. It has been reported that anthocyanins (ACNs) decrease hepatic lipid accumulation and may counteract oxidative stress and hepatic inflammation, but their impact on NAFLD has yet to be fully determined. ACNs are water-soluble bioactive compounds of the polyphenol class present in many vegetable products. Here, we summarize the evidence evaluating the mechanisms of action of ACNs on hepatic lipid metabolism in different experimental setting: *in vitro*, *in vivo*, and in human trials. Finally, a working model depicting the possible mechanisms underpinning the beneficial effects of ACNs in NAFLD is proposed, based on the available literature.

1. Introduction

In the last decades, the pandemic of overweight and obesity related to sedentary lifestyle and excess intake of refined foods has led to a dramatic rise in the prevalence of the metabolic syndrome and associated conditions, such as type 2 diabetes and dyslipidemia, leading to accelerated atherosclerosis [1], but also to nonalcoholic fatty liver disease (NAFLD) [2, 3].

Lifestyle and dietary habits represent both major risk and protective factors in the development and progression of degenerative diseases [4].

Diets rich in fruits and vegetables are among the recommended lifestyle modifications to decrease the risk of degenerative diseases, such as cardiovascular disease but also to reduce the complications associated with metabolic disorders and advanced atherosclerosis. Diet is in fact affordable and available and usually does not include the side effects and the metabolic and physiologic burden that medications impose on body systems [5].

In this regard, many different dietary components are under study for their possible pharmacologic activity in several pathophysiological conditions at different levels (e.g., vascular, immune, hepatic, etc.).

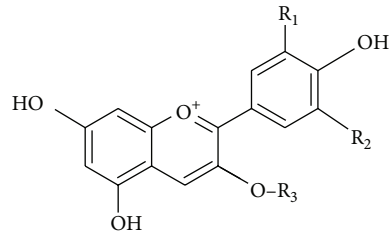
Most bioactive compounds have been documented in fruits and vegetables [6] and their mechanisms of action investigated both in *in vitro* and in *in vivo* models. In particular, great interest has been devoted to several classes of polyphenols and especially to a specific subset of molecules called anthocyanins (ACNs).

Table 1: Anthocyanin concentrations in selected food sources.

Food description	Cyanidin mg/100 g	Delphinidin mg/100 g	Malvidin mg/100 g	Pelargonidin mg/100 g	Peonidin mg/100 g	Petunidin mg/100 g
Berries						
Arctic bramble berries (*Rubus arcticus*)	88.3			0.7		
Bilberry (*Vaccinium myrtillus*)	85.3	97.6	39.2		20.4	42.7
Blackberries (*Rubus spp.*)	99.9	0	0	0.4	0.2	0
Blueberries (*Vaccinium spp.*)						
Cultivated	8.5	35.4	67.6	0	20.3	31.5
Wild	19.4	37.6	57.2	2.6	10	23.5
Chokeberry	344.1	0.6	1.2	1	0.1	2.8
Cranberries (*Vaccinium macrocarpon*)	46.4	7.7	0.4	0	49.2	0
Currants						
Black (*Ribes nigrum*)	61.3	87.9		1.2	0.6	3.9
Red	65.5	9.3			0.2	
Golden (*Ribes aureum*)	108.8	0.7			0.1	
Elderberries (*Sambucus spp.*)	485.3	0		0		0
Raspberries						
Black	669			16.7	1.1	
Raspberries (*Rubus spp.*)	45.8	1.3	0.1	1	0.1	0.3
Saskatoon berries (*Amelanchier canadensis*)	110.6	50.4	10.6	0	3	6.3
Strawberries (*Fragaria X ananassa*)	1.7	0.3	0	24.8	0	0.1
Other fruits						
Cherries, sweet	30.2	0	0	1.4	1.5	0
Grape						
Red	1.2	2.3	39	0	3.6	2
Concord (*Vitis vinifera*)	23.8	70.6	6		4.8	14.9
Pistachio nuts, raw (*Pistacia vera*)	7.3	0	0	0	0	0
Plums						
Black diamond (with peel)	56	0	0	0	0	0
Purple	17.9				5.2	
Plums (*Prunus spp.*)	5.63	0	0	0	0.3	0
Vegetables						
Black beans (*P. vulgaris*)		18.5	10.6			15.4
Cabbage red picked	11.8					
Eggplant raw (*Solanum melongena*)		85.7				
Onions red	3.2	4.3		0	2.1	
Radicchio (*Cichorium intybus*)	127	7.7				
Radishes (*Raphanus sativus*)	0	0	0	63.1	0	0
Sweet potato purple (cooked)	10.6	0.9		0		

2. Anthocyanins

ACNs are water-soluble bioactive compounds, which belong to the widespread group named flavonoids within the polyphenol class. Chemically, ACNs consist of two aromatic rings linked by three carbons in an oxygenated heterocycle. The chromophore of ACNs is the 7-hydroxyflavylium ion. In particular, ACNs consist of an aglycon base or flavylium ring (anthocyanidins), sugars, and possibly acylating groups (Figure 1) [7]. ACNs are responsible for the red, purple and blue colors of many flowers, cereal grains, fruit, and vegetable. They are generally found in the skins, and their content is usually proportional to color intensity. ACN content varies greatly depending on the different food sources considered (Table 1) [8]. More than 600 different ACNs have been identified in vegetables, derived from twenty-three different aglycones (anthocyanidins) classified according to the number and position of hydroxyl and methoxyl groups on the flavan nucleus. The six anthocyanidins commonly found in fruit and vegetables are pelargonidin, cyanidin, delphinidin,



Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1: General chemical structures of anthocyanins in the diet. R_3 = sugar (i.e., glucose, arabinose, galactose, as monomers, or dimers). Sugars can be present also on ring A; moreover acylation of sugars with aliphatic and/or aromatic acids can be found.

peonidin, petunidin, and malvidin which are combined with sugars (mostly glucose, galactose, and arabinose) (Figure 1) [8].

ACN intake has been estimated to range between 180 mg/day and 215 mg/day, but these values can be 10 times lower in industrialized countries [9–11]. ACN bioavailability is reported to be lower than that of other polyphenols, and less than 1% of consumed ACNs is generally absorbed, reaching plasma concentrations in the nanomolar order [12]. In addition, ACNs are rapidly metabolized and their presence in the circulation is limited to a few hours. Despite their low absorption and rapid metabolism, the regular intake of ACNs may result in beneficial effects on human health by reducing the risks of cardiovascular disease and cancer [13–15]. Indeed, they possess high antioxidant capacity and can play a key role in the prevention of oxidative stress by scavenging reactive oxygen species and free radicals and by modulating endogenous defense system, as demonstrated in several *in vitro* and *in vivo* studies [16–18]. ACNs have also been documented to ameliorate hyperglycemia, to modulate endothelial function, and to decrease inflammation [19–24]. Moreover recently ACNs have been studied for their role in the modulation of lipid metabolism and fat deposition [25–27] in different tissues, including the liver.

3. Nonalcoholic Fatty Liver Disease

NAFLD is characterized by liver fat deposition, that is, steatosis, related to systemic insulin resistance (IR) [28]. In susceptible individuals, steatosis may be associated with oxidative hepatocellular damage, inflammation, and activation of fibrogenesis, defining nonalcoholic steatohepatitis (NASH) [29, 30]. NASH, but not simple steatosis, is a potentially progressive liver disease leading to cirrhosis and hepatocellular carcinoma [31]. Following the epidemics of obesity and the metabolic syndrome, NAFLD is rapidly becoming the leading cause of altered liver enzymes in Western countries [2, 32, 33], and NASH will become the major cause of end-stage liver disease and hepatocellular carcinoma within the next 10–20 years.

Fatty liver, that is, hepatic fat accumulation exceeding 5% of total liver mass, results from an unbalance between triglyceride deposition and synthesis on one hand and oxidation and secretion by lipoproteins on the other hand [34] and initially represents a protective mechanism against the toxicity resulting from an increased flux of free fatty acids (FFAs) to the liver [35]. Most of excess hepatic lipid content derives from increased peripheral lipolysis [36], which is caused by adipose tissue insulin resistance [37], and is a typical feature of obesity. Other contributing factors are increased lipogenesis induced by hyperinsulinemia or directly by diet. Indeed, the major risk factor for NAFLD is systemic IR due to central obesity and the metabolic syndrome [28, 38]. Impaired ability to secrete lipoproteins [39] and changes in fattyacid oxidation also contribute to hepatic fat accumulation [40].

Development of NASH has classically been explained by the occurrence of a so-called second-hit, leading to the activation of inflammation, in the context of hepatic steatosis (the "first hit") [41]. This second insult likely represents a combination of insults related to (a) direct hepatic lipotoxicity, (b) hepatocellular oxidative stress secondary to free radicals produced during β- and ω-oxidation of FFAs, (c) inflammation triggered by endotoxins engaging Toll-like receptor-4 (TLR-4) in Kupffer cells (the hepatic macrophages) and hepatocytes due to increased intestinal permeability, bacterial overgrowth, and altered intestinal flora [42–44], (d) cytokine release, and (e) endoplasmic reticulum stress. These combine to produce inflammation, cellular damage, and activation of fibrogenesis. Genetic factors, and in particular the I148 M variant of Patatin-like phospholipase domain containing-3 (PNPLA3), play a major role in determining individual susceptibility to develop steatosis or NASH and progressive liver disease, interacting with dietary factors [45, 46].

4. Anthocyanins in NAFLD

Recent studies documented that ACNs can reduce hepatic lipid accumulation, but their impact on NAFLD has yet to be determined.

We have classified the available evidence according to the experimental setting: *in vitro*, *in vivo*, and in human trials. For the revision of the literature, the PubMed database was searched up to June 2013 (keywords: steatosis or nonalcoholic fatty liver disease or steatohepatitis plus anthocyanins or single anthocyanin names). No publication data restrictions

Table 2: Studies evaluating the effect of anthocyanins on hepatic lipid metabolism and hepatocellular lipotoxicity *in vitro*.

Paper	Anthocyanin	Food	Model	Effects	Mechanism
46	ACN-rich extract	Bilberry	Primary rat hepatocytes	⇓ tBH induced damage ⇓ MTT, LDH, TBARS	Antioxidant
47	ACN-rich fraction	Blueberry	HepG2 cells	⇓ OA induced TG accumulation at high doses	?
48	Anthocyanin factor	Sweet potato	HepG2 cells	⇑ pAMPK ⇓ Srepb1c, FAS	⇑ pAMPK
49	Cyanidin-3-O-β-glucoside	—	HepG2 cells	⇓ lipogenesis	⇑ pPKC ζ ⇓ MtGPAT1 translocation to OMM
50	Cyanidin chloride	Blackberry	HepG2 cells	⇑ antioxidants (SOD, catalase)	⇑ pMAPK, ⇑ Nrf2 and PPARα
51	Cyanidin-3-O-β-glucoside	—	HepG2 cells	⇓ ROS induced by glucose ⇑ antioxidants (GSH)	⇑ PKA and CREB
52	Cyanidin-3-O-β-glucoside	—	HepG2 cells	⇑ pAMPK and pACC, ⇑ CPT1 and FFAs oxidation	AMPK activation mediated by calmodulin kinase kinase
53	ACN-rich extract	Mulberry	HepG2 cells	⇑ pAMPK and pACC, ⇑ PPARα, CPT1 and FFAs oxidation ⇓ Srebp1c and lipogenesis	AMPK activation
54	Cyanidin	—	HepG2 cells	⇓ lipogenesis ⇑ lipolysis	PPAR$\alpha\beta/\delta$ agonist

AMPK: adenosine monophosphate protein kinase; Srebp1c: sterol regulated element binding protein 1c; ACC: acetyl-coenzyme A carboxylase; p: phospho; glycerol 3 phophate acyl transferase; PKC: protein kinase C; OMM: outer mitochondrial membrane; SOD: superoxide dismutase; MAPK: mitogen associated protein kinase; Nrf2: nuclear factor erythroid 2-related factor 2; PPARα: β/δ peroxisomes proliferator activated receptor α; ROS: reactive oxygen species; GSH: reduced glutathione; PKA: protein kinase A; CREB: cAMP-response element binding protein; CPT-1: carnitine-palmytoil-transferase-1; ACN: anthocyanins; OA: oleic acid; tBH: tert-butyl hydroperoxide; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; LDH: lactate dehydrogenase; TBARS: thiobarbituric acid reacting substances.

were applied. Papers were selected for inclusion in this review on the basis of their relevance, and additional papers were obtained from their reference lists.

4.1. In Vitro. Studies evaluating the effect of ACNs *in vitro* on lipid metabolism and oxidative stress in hepatocytes, typical of NAFLD and NASH, are presented in Table 2. Most studies were conducted in human hepatoma HepG2 cells [47–55], an established model of hepatic lipid metabolism. Both ACN-rich extracts of foods (berries and potatoes) and synthetic ACNs (cyanidin hydrochloride and cyanidin-3-O-β-glucoside) were employed. Unfortunately, interpretation of the overall evidence is hindered by differences in cellular models, experimental protocols, and the molecular pathways evaluated. However, most studies are concordant on the fact that ACNs reduce hepatocellular lipid accumulation [48–50, 53–55] by inhibiting lipogenesis [49] and possibly by promoting lipolysis [53–55], although the different aspects of lipid metabolism were not evaluated in all studies. Furthermore, ACNs also reduce cellular oxidative stress by promoting the antioxidant response [47, 51, 52]. Interestingly, three independent studies reported that activation of the adenosine monophosphate protein kinase (AMPK) pathway was implicated in mediating the effect of ACNs on hepatic lipid metabolism and antioxidant response [49, 51, 53, 54]. However, another study suggested that ACNs may act as direct agonist of PPAR receptors in hepatocytes [55].

4.2. In Vivo. Studies evaluating the effect of ACNs *in vivo* on hepatic lipid metabolism, steatosis, oxidative stress, and steatohepatitis are presented in Table 3. Also in this case, the interpretation of the overall evidence is difficult, due to the very different experimental models of NAFLD and metabolic syndrome employed and to the different outcomes for the evaluation of lipid metabolism, oxidative stress, and liver damage. In addition, in some studies, animals were exposed to synthetic ACNs (i.e., cyanidin-3-O-β-glucoside) [50, 52, 56, 57], whereas in others they were exposed to extracts of ACN-rich foods (e.g., sweet potato, berries, and oranges) [27, 49, 58–62]. Mirroring the results obtained *in vitro*, there is ample convergence supporting an effect of ACNs in reducing hepatic lipid accumulation, that is, steatosis [49, 50, 52, 56–58, 60–63]. In addition, the majority of studies also reported an improvement in hepatic and systemic IR and serum lipids, often related to reduced weight gain [57, 58, 60–62]. Again, increased activation of PPARα inducing lipolysis and reduced lipogenesis were postulated to be responsible for decreased hepatic fat content [27, 59–61]. Increased activity of

Table 3: Studies evaluating the effect of anthocyanins on hepatic steatosis and steatohepatitis *in vivo*.

Paper	Anthocyanin	Food	Model	Metabolic effects	Molecular effects
48	Anthocyanin factor	Sweet potato	Mice fed HFD	⇓ weight gain ⇓ steatosis	⇑ pAMPK and pACC ⇓ Srepb1c, FAS, ACC
49	Cyaniding-3-O-β-glucoside	—	KKAy mice	⇓ steatosis	⇓ GPAT1 translocation to OMM
51	Cyanidin-3-O-β-glucoside	—	db/db mice	⇑ antioxidants (GSH) ⇓ steatosis, ROS, and inflammation	⇑ PKA and CREB
55	Cyanidin-3-O-β-glucoside	Blackcurrant	Rats	⇓ steatosis ⇓ hepatic saturated FAs ⇑ antioxidants	?
56	Cyanidin-3-O-β-glucoside	—	C57Bl/6 on HFD and db/db	⇓ glucose and IR ⇓ cytokines and adipose tissue inflammation ⇓ steatosis	⇓ hepatic JNK ⇓ hepatic FOXO1 activity and gluconeogenesis
57	Several	Tart cherry	Dahl Salt-Sensitive rat	⇓ fasting glucose ⇓ hyperlipidemia ⇓ hyperinsulinemia ⇓ steatosis	⇑ PPARα ⇑ acyl-coenzyme A oxidase
58	—	Vitis coignetiae Pulliat leaves (yama-budo)	Rats on HFD choline deficient diet	⇓ liver enzymes and liver fibrosis ⇓ CYP2E1 ⇑ antioxidants	?
59	Several	Moro orange juice	C57Bl/6 mice on HFD	⇓ weight gain ⇓ IR, ⇓ TGs, ⇓ steatosis	⇓ LXR, FAS ⇑ PPARα, Srebp1c
27	Several	Wild blueberry (Vaccinium angustifolium)	Zucker rats	⇓ hyperlipidemia	⇑ PPARα ⇓ Srebp1c
60	—	Blueberry	Zucker rats on HFD	⇓ IR and lipids ⇓ adiposity ⇓ steatosis	⇑ PPARα
61	—	Mulberry	Hamsters on HFD	⇓ weight gain and visceral fat, ⇓ TGs, chol, FFAs ⇓ steatosis	⇓ HMG-CoA, FAS ⇑ PPARα, CPT-1
62	Several	Elderberry	Hamsters fed high fat fish oil	⇓ serum lipids ⇓ steatosis ⇓ lipoperoxidation	?
63	—	Mulberry	Rats on HFD	⇓ serum lipids ⇓ hepatic and serum lipoperoxidation	⇑ antioxidants

HFD: high fat diet; IR: insulin resistance; TGs: triglycerides; LXR: liver X receptor; FAS: fatty acid synthase; GAPT1: glycerol 3 phosphate acyl transferase; PPARα: peroxisomes proliferator activated receptor α; chol: cholesterol; FFAs: free fatty acids; CPT-1: carnitine-palmitoyl-transferase-1; HMG-CoA red: 3-hydroxymethyl-3-glutaryl-coenzyme A reductase; p: phospho; AMPK: adenosine monophoshopate protein kinase; Srebp1c: sterol regulated element binding protein 1c; ACC: acetyl-coenzyme A carboxylase; ROS: reactive oxygen species; JNK: c-Jun N-terminal kinase; FOXO1: forkhead box O1.

the AMPK pathway was confirmed *in vivo* in one study [49], and increased hepatic antioxidant activity after exposure to ACN was also widely confirmed in experimental models of NAFLD [52, 56, 59, 63, 64]. However, whether improved redox status was secondary to or independent of reduced hepatic lipids and improved metabolic status was not tested. In some studies, these effects of ACN exposure translated in an improvement in inflammation, that is, in reduced severity of steatohepatitis [53, 58, 60]. The involvement of AMPK activation in mediating the beneficial effect of ACN on insulin sensitivity is also supported by evidence that bilberry extract ameliorates insulin resistance and hepatic lipid metabolism via this pathway [65].

4.3. Clinical Studies. There is only one study evaluating the effect of ACN on NAFLD patients, which is summarized in Table 4 [66]. Suda and coworkers recruited 48 adult men with increased liver enzymes negative for viral hepatitis, thereby likely affected by NAFLD. During a eight-week intervention, about 200 mg of acylated ACNs or placebo was administered twice daily. Acylated ACN intake was associated with reduced levels of liver enzymes, in particular gamma-glutamyltransferases. However, liver damage was not directly assessed, fatty liver was not confirmed by direct imaging, and the effect of acylated ACNs was not compared to that of a control food or to the lack of intervention.

Table 4: Studies evaluating the effect of anthocyanins on hepatic steatosis and steatohepatitis in patients.

Paper	Anthocyanin	Food	Subjects	Metabolic effects	Mechanism
64	Acylated anthocyanins	Purple sweet potato beverage 8 wks	Healthy humans with borderline hepatitis	⇓ GGT (AST, ALT) ⇓ oxidative stress	⇓ oxidative stress

GGT: g-glutamyl transferase; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

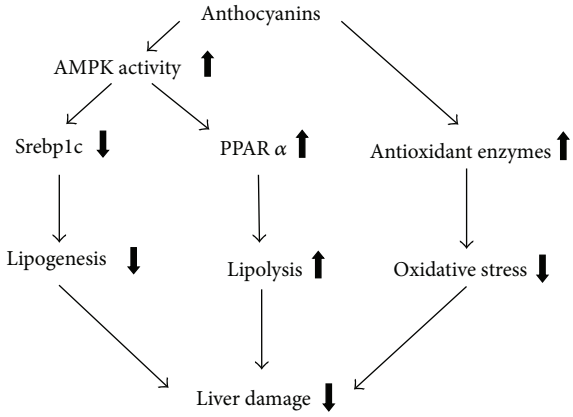


Figure 2: Possible mechanisms underpinning the beneficial effects of anthocyanins in NAFLD and NASH: a Srebp1c working model based on available studies. Anthocyanins may prevent the progression of liver damage related to NAFLD by three independent mechanisms: inhibition of lipogenesis by reducing Srebp1c, promotion of lipolysis by induction of PPARα activity, and reduction of oxidative stress by induction of anti-oxidant enzymes. The effects of anthocyanins on lipid metabolism seem to be dependent on the activation of the AMPK pathway in hepatocytes.

5. Conclusions

It is widely accepted that exploring the role of foods and more specifically the effect of bioactive compounds such as ACNs on the metabolic processes involved in *chronic diseases* is critical for preventive strategies. For instance, similar therapeutic activities have been shown for docosahexaenoic acid on steatosis severity in children with NAFLD [67]. The availability of data demonstrating cause-effect relationships and the specific mode of action of such compounds are of paramount importance in order to support any dietary recommendation or supplementation.

A working model depicting the possible mechanisms underpinning the effects of ACN in NAFLD, based on the available findings in the literature, is presented in Figure 2. ACNs may prevent the progression of liver damage related to NAFLD by three independent mechanisms: inhibition of lipogenesis by reducing Srebp1c, promotion of lipolysis by induction of PPARα activity, and reduction of oxidative stress.

On the basis of these data, it seems that ACN-rich foods can be promising for the prevention of NAFLD and its complications. Additional studies are required to clarify the molecular mechanisms and to test the specific effect of single compounds and food extracts *in vitro* and *in vivo*. Randomized controlled studies are warranted to test foods on histological damage or noninvasive biomarkers of liver damage progression in patients with NASH.

Conflict of Interests

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

Authors' Contribution

Luca Valenti and Patrizia Riso have contributed equally to this paper. They designed the study, independently reviewed the literature, and wrote the first paper draft. Anna Mazzocchi performed the literature search. Silvia Fargion, Marisa Porrini, and Carlo Agostoni critically reviewed the paper and supervised the study.

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Research Article

Anti-Inflammatory Effect of Procyanidins from Wild Grape (*Vitis amurensis*) Seeds in LPS-Induced RAW 264.7 Cells

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In the present study, the anti-inflammatory effect and underlying mechanisms of wild grape seeds procyanidins (WGP) were examined using lipopolysaccharide- (LPS-) stimulated RAW 264.7 cells. We used nitric oxide (NO) and prostaglandin E_2 (PGE_2) and reactive oxygen species (ROS) assays to examine inhibitory effect of WGP and further investigated the mechanisms of WGP suppressed LPS-mediated genes and upstream expression by Western blot and confocal microscopy analysis. Our data indicate that WGP significantly reduced NO, PGE_2, and ROS production and also inhibited the expression of proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expressions. Consistently, WGP significantly reduced LPS-stimulated expression of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin- (IL-) 1β. Moreover, WGP prevented nuclear translocation of nuclear factor-κB (NFκB) p65 subunit by reducing inhibitory κB-α (IκBα) and NFκB phosphorylation. Furthermore, we found that WGP inhibited LPS-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK). Taken together, our results demonstrated that WGP exerts potent anti-inflammatory activity through the inhibition of iNOS and COX-2 by regulating NFκB and p38 MAPK pathway.

1. Introduction

Vitis amurensis, a wild-growing grape species, is widely distributed in Republic of Korea, China, and Japan. The root and stem have been used as traditional medicines for treatment of cancer and various pains in Republic of Korea and Japan [1]. The fruit is not consumed fresh but is used primarily for production of juice and wine due to its strong stringency. Some studies have suggested that wild grape root and stem have antiangiogenic [2], antioxidant [3], anti-inflammatory [4], and neuroprotective effects [5]. In a recent study, our group addressed the separation and chemopreventive properties of procyanidins from wild grape seeds relating their induction of nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway in the human hepatoma HepG2 cell line [6]. Some procyanidins from other species have been found to display anti-inflammatory effect [7, 8], but their molecular mechanisms underlying the anti-inflammatory effects have not been elucidated.

Inflammation is involved in a variety of chronic diseases including cancer and heart disease. Pathogen- and host-derived molecules, such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ), stimulate macrophages to, in turn, upregulate inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and reactive oxygen species (ROS), as well as proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [9]. NO is overproduced endogenously by iNOS which is induced in response to proinflammatory cytokines and LPS [10]. COX-2 is also induced by several stimuli and is responsible for the production of large amounts of proinflammatory prostaglandins at the inflammatory sites [11]. Therefore, the inhibition of these inflammation mediators is an important target pathway in the treatment of disease with anti-inflammatory components [12, 13].

Multiple studies have shown that the expressions of several cytokines genes, including tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β), are associated with

activation of nuclear factor-κB (NFκB), which is a transcription factor that plays a major role in the regulation of genes associated with inflammation [14–16]. In non-stimulated cells, NFκB dimmers of p50 and p65 subunits remain inactive in the cytoplasm through interaction with an inhibitory protein, IκB. However, in response to cell stimulation, IκB kinase complex is rapidly degraded and phosphorylated. The transcription factor, NFκB, then translocates into the nucleus and binds to the DNA site that regulates transcription of inflammatory mediators [17]. Recently, many studies have demonstrated the role of phytochemicals in anti-inflammatory activity through downregulation of NFκB pathway [18–20].

The mitogen-activated protein kinases (MAPKs) such as extracellular signal regulated kinase (ERK), p38, and c-Jin NH2-terminal kinase (JNK) are a group of signaling molecules that play a critical role in the regulation of cell growth and differentiation, as well as in the control of cellular responses to cytokines and stresses [21, 22]. Phosphorylation of MAPKs is known to be a critical component in the production of NO and proinflammatory cytokines in activated macrophages [23, 24]. Also, it has been demonstrated that the specific MAPK inhibitors suppress the expression of the iNOS and COX-2 genes [6, 25]. Moreover, several studies have shown that MAPKs play a critical role in the activation of NFκB [23, 26]. In addition, it has been shown that the PI3K/Akt signaling pathway plays an important role in negatively regulating LPS-induced acute inflammatory responses *in vitro* and *in vivo* [27, 28]. Inhibition of the PI3K/Akt signaling pathway can enhance the activation of NFκB transcription factors and the expression of iNOS and COX-2 in RAW 264.7 cells [29].

In this present study, we examine the anti-inflammatory effects of wild grape seed procyanidins (WGP) in LPS-stimulated RAW 264.7 cells. The results show that WGP suppressed LPS-induced NO, PGE_2 and ROS production by inhibiting activation of NFκB pathway, as well as the p38 MAPK signaling pathway in LPS-stimulated RAW 264.7 cells.

2. Materials and Methods

2.1. Materials. Lipopolysaccharide (LPS, *Escherichia coli* O127:B8) was obtained from Sigma-Aldrich Co. (St. Louis, MO). 2′,7′-Dichlorodihydrofluorescein (DCF-DA) was purchased from Molecular Probes Inc. (Eugene, OR). Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Hyclone (Logan, UT). Antibodies against iNOS, COX-2, and β-actin, as well as horseradish peroxidase-conjugated anti-goat and anti-rabbit IgG, were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-phospho-JNK, phospho-ERK, phospho-p38, phospho-IκBα, phospho-p65, IκBα, p65, IL-1β, and TNF-α were purchased from Cell Signaling Technology Inc. (Beverly, MA). All other reagents used in this study were of the highest grade available.

2.2. Preparation of WGP. Seeds were collected from wild gape (*Vitis amurensis*) from a vineyard (Dooraemaeul, Inc., Hamyang, Republic of Korea), during transfer of the musts for wine fermentation. WGP were extracted from wild grape seeds and analysed as described previously [6]. Briefly, dried powder of wild grape seeds were extracted with 70% aqueous acetone. After the extraction, 70% acetone extract of wild grape seeds was partitioned with *n*-hexane to remove hydrophobic compounds and chromatographed over a Toyopearl HW-400F (Tosho, Tokyo, Japan) using an aqueous solution of 50% MeOH and 66% acetone and 100% acetone to yield a procyanidins fraction. The procyanidins fraction was analysed with various analytical techniques including the vanillin assay, butanol-HCl hydrolysis, and HPLC-MS analysis after depolymerization with phloroglucinol. The major procyanidins of WGP were determined as a mixture of prodelphinidins and procyanidins with the average polymerization degree of 6.22 and 4.65, respectively.

2.3. Cell Culture. RAW 264.7 cells were purchased from American Type Cell Culture (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL of penicillin/streptomycin and maintained at 37°C in a humidified CO_2 incubator.

2.4. Cell Viability Assay. Cell viability was determined by the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Briefly, cells were plated at a density of 2×10^4 cells in a 24-well plate, and WGP was added to each plate at the indicated concentrations. After 24 h incubation period, the absorbance was measured at 490 nm with a PowerWave XS microplate reader (BioTek Instruments, Inc., Winooski, VT). This assay was repeated three times with triplicate samples at each measurement.

2.5. Measurement of ROS Production. The level of intracellular ROS was quantified by fluorescence with DCF-DA. The cells (2×10^4 cells/well) were plated in 96 well plates and preincubated with the DCF-DA for 1 h at 37°C in darkness. After washing out the excess probe, the cells were treated with WGP or WGP and LPS for 24 h. The fluorescence was measured at 485/20 nm excitation and 528/20 nm emission in a fluorescence multidetection reader (Synergy HT Multidetection Microplate Reader; BioTek, VT).

2.6. Measurement of NO Production. The RAW 264.7 cells were plated at 1×10^5 cells/well in 24 well plates and then incubated with or without LPS (1 μg/mL) in the absence or presence of WGP for 24 h. Nitrite levels in culture media were determined using the Griess reaction assay and presumed to reflect NO levels. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and 0.1% (w/v) naphthyl ethylenediamine dihydrochloride), incubated at room temperature for 10 min and then the absorbance at 540 nm was measured in a microplate reader (PowerWave XS). The amount of nitrite in the samples was measured with the serial dilution standard curve of sodium nitrite.

2.7. Measurement of PGE_2 Production. The amount of PGE_2 produced from endogenous arachidonic acid was measured using a PGE_2 Parameter Assay Kit (R&D Systems, Minneapolis, MN). RAW 264.7 cells (1×10^5 cells/well) were treated with WGP for 1 h and stimulated with LPS for 24 hr, the conditioned media was collected to perform PGE_2 enzyme immune-metric assay according to the manufacturer's protocol.

2.8. Preparation of Whole Cell, Cytosolic, and Nuclear Extracts. The preparation of whole cell extract was previously described [6]. RAW 264.7 cells were seeded at 2×10^5 cells/well on 6-well plates and were treated with WGP and stimulated with LPS. After treatment, cells were collected by centrifugation and washed twice with ice-cold phosphate buffered saline. The cells pellets were resuspended in lysis buffer on ice for 1 h; and cell debris was removed by centrifugation. The cytosolic and nuclear proteins were extracted using a Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology Inc., Rockford, IL), and protein levels were determined by BCA protein assay (Pierce biotechnology).

2.9. Western Blot Analysis. Equal amounts of proteins (whole cell extracts: 30 μg/lane, cytosolic extracts: 30 μg/lane, nuclear extracts: 10 μg/lane) were loaded onto a 12% SDS-polyacrylamide gel electrophoresis unit and then transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). The membranes were incubated in a blocking buffer (5% w/v skim milk in PBST) for 1 h, and then incubated with overnight with primary antibody. After washing three times with 0.1% PBST buffer, the membranes were incubated with the anti-goat or anti-rabbit secondary antibodies conjugated with horseradish peroxidase and detected by the Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

2.10. Confocal Microscopy Analysis. Cells were plated at 2×10^5 cells/well on cover glasses bottom dishes and fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min at RT. Permeabilization was performed in PBS with 0.3% Triton X-100 for 10 min at RT. After blocking for 2 h with 3% bovine serum albumin, the cells were incubated with anti-p65 primary antibody at RT for 2 h. After washing with PBS, Alexa Fluor 555-confugated secondary antibody (Cell signaling) was added for 2 h in the dark. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI; Thermo Scientific, Rockford, IL), and the cells were visualized under LSM 510 laser confocal microscope (Zeiss, Jena, Germany).

2.11. Statistical Analysis. The data were expressed as means ± standard deviation (S.D.). Statistical analyses were performed using SigmaPlot 8.0 software (Systat Software Inc., Chicago, IL). Student's t-test and one-way ANOVA were used to determine the statistical significant difference between the LPS-treated and WGP plus LPS-treated cells. P value of <0.05 was considered statistically significant.

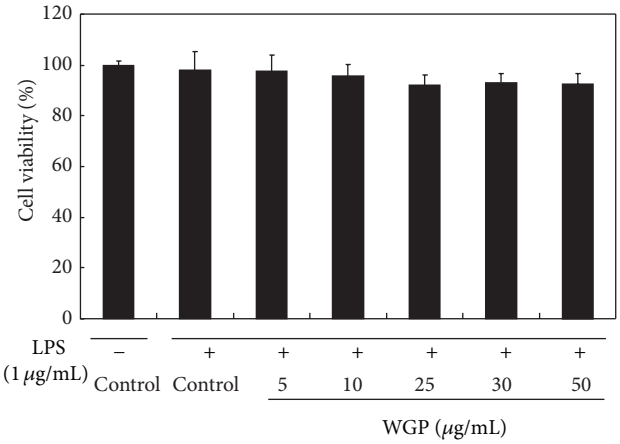

Figure 1: Effects of WGP on cell viability in RAW 264.7 cells. Cells were treated with the indicated concentration of WGP in combination with LPS for 24 h. Cell viabilities were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each value represents means ± SD of six independent experiments.

3. Results

3.1. Effects of WGP on Cell Viability in LPS-Induced RAW 264.7 Cells. The effects of WGP on the viability of RAW 264.7 cells were determined by a colorimetric MTS assay after 48 hr treatments. The data were expressed as percent cell viability compared to those of control (DMSO, 0.1%) (Figure 1). WGP did not cause any cytotoxicity at 50 μg/mL in RAW 264.7 cells. Therefore, subsequent experiments were performed with concentrations at or below 50 μg/mL.

3.2. Effects of WGP on the Production of ROS in LPS-Induced RAW 264.7 Cells. We examined the effects of WGP on LPS-induced ROS production in RAW 264.7 cells. Treatment of RAW 264.7 cells with LPS rapidly increased intracellular ROS level, as determined by using DCF-DA, which was effectively attenuated by pretreatment with WGP (Figure 2).

3.3. Effects of WGP on the Production of NO and PGE_2 in LPS-Induced RAW 264.7 Cells. The effects of WGP on the level of NO and PGE_2 in the culture media of RAW 264.7 cells were determined after 24 h treatment of 1 μg/mL LPS with WGP. NO production and iNOS protein expression by WGP were evaluated with the dose range of 1–50 μg/mL. Treatment with WGP at 35 μg/mL dramatically inhibited the NO production and iNOS protein expression in LPS-stimulated RAW 264.7 cells (Figure 3). Therefore, further experiments were performed with concentrations at 35 μg/mL. As illustrated in Figure 4(a), treatment of the cells with LPS resulted in increased NO production; however, WGP at 35 μg/mL significantly inhibited the production of NO. Treatment with WGP also significantly decreased LPS-induced PGE_2 production (Figure 4(b)).

3.4. Effects of WGP on the Protein Expression of iNOS and COX-2 in LPS-Induced RAW 264.7 Cells. To investigate

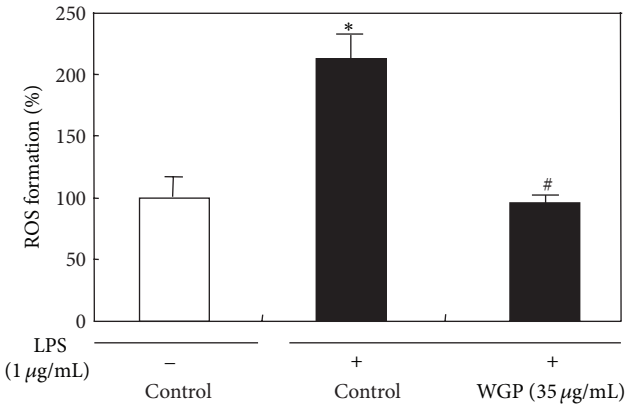

Figure 2: Effects of WGP on LPS-induced ROS production in RAW 264.7 cells. The level of intracellular ROS was measured with DCF-DA. The cells were pretreated with DCF-DA for 1 h, and then exposed to WGP at 35 μg/mL for 24 h. The formation of ROS in the cells was evaluated by the arbitrary fluorescence unit and described as fold induction test via vehicle (DMSO, 0.1%). Each value represents mean ± SD of six independent experiments. $^{*}P < 0.05$ indicates differences from the unstimulated control group. $^{\#}P < 0.05$ indicates differences from the LPS-treated group.

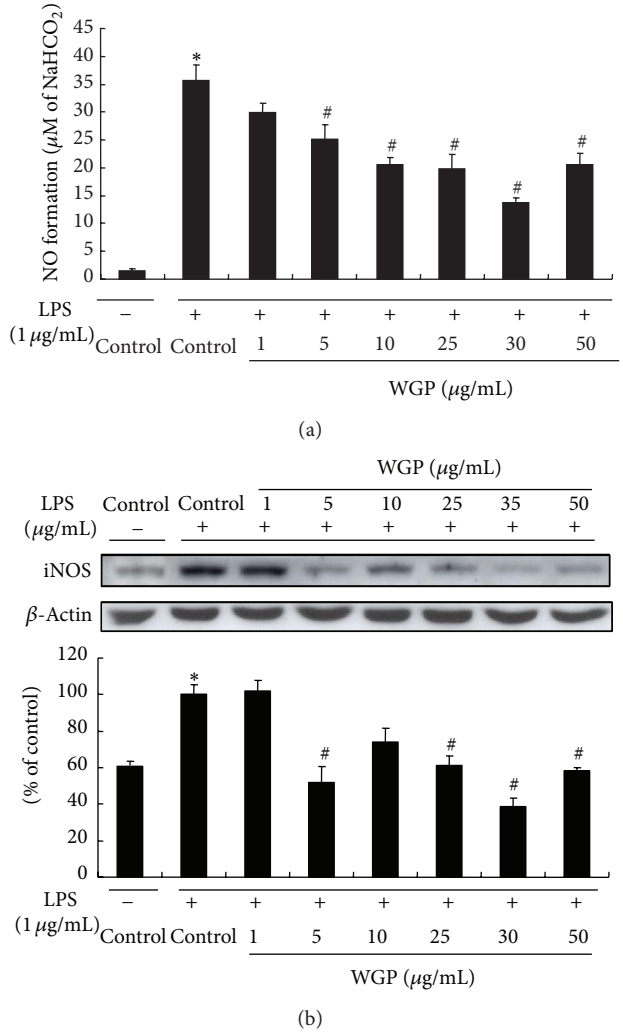

Figure 3: Effects of WGP on LPS-induced NO and iNOS protein expression in RAW 264.7 cells. (a) NO formation. The cells were treated with LPS only or with WGP ranging from 1 to 50 μg/mL for 24 h. The culture media were collected, and the nitric oxide concentration was measured by the Griess reaction. Each value represents mean ± S.D. of triplicate experiments. (b) The cells were treated with WGP ranging from 1 to 50 μg/mL for 1 h and then treated with LPS for 24 h. Equal amounts of total protein were subjected to Western blot analysis as described in Section 2. The ratio of immunointensity between the iNOS and the β-actin was calculated. The bar represents means ± S.D. from three independent experiments. $^{*}P < 0.05$ indicates differences from the unstimulated control group. $^{\#}P < 0.05$ indicates differences from the LPS-treated group.

whether the inhibitory effect of WGP on NO and PGE_2 production was via inhibition of corresponding gene expression, the protein expressions of iNOS and COX-2 were determined by Western blot. As displayed in Figure 4(c), the protein levels of iNOS and COX-2 were undetectable in RAW 264.7 cells without LPS stimulation. Treatment with LPS alone markedly increased iNOS and COX-2 protein levels, whereas cotreatment with WGP significantly suppressed the expression of iNOS and COX-2 proteins. The reduced expressions of iNOS and COX-2 protein were consistent with the reductions in total NO and PGE_2 in culture media.

3.5. Effects of WGP on the Protein Expression of TNF-α and IL-1β in LPS-Induced RAW 264.7 Cells. To examine the effects of WGP on the expression of proinflammatory cytokines following LPS treatment, Western blot analyses were performed. As shown in Figure 5, LPS significantly stimulated the expression of TNF-α and IL-1β. On the contrary, WGP significantly inhibited the LPS-stimulated TNF-α and IL-1β expression.

3.6. Effects of WGP on LPS-Induced Nuclear Translocation of NFκB and on the Phosphorylation of IκBα in LPS-Induced RAW 264.7 Cells. Because levels of iNOS and COX-2 protein were inhibited by WGP, we then examined the effects of WGP on the activation of NFκB. The translocation of NFκB was measured by extracts of nucleus and cytosol and subjected to analyses of Western blot and immunofluorescence. LPS stimulation for 1 h caused the translocation of p65, a subunit of NFκB, to the nucleus in the macrophage cells (Figure 6(a)). However, WGP treatment effectively blocked the LPS-induced nuclear translocation of p65 in the cells. These results were confirmed by NFκB and DAPI costaining in LPS-treated RAW 264.7 cells (Figure 6(b)). As illustrated in Figure 6(c), the treatment with WGP attenuated the LPS-stimulated phosphorylation of p65 as well as that of IκBα.

3.7. Effects of WGP on MAPKs and Akt Phosphorylation in LPS-Induced RAW 264.7 Cells. To investigate whether the inhibition of inflammatory response by WGP is mediated through the MAPK and PI3K/Akt pathways, we examined the effect of WGP on the LPS-stimulated phosphorylation of upstream kinases including ERK1/2, JNK, p38, and Akt in

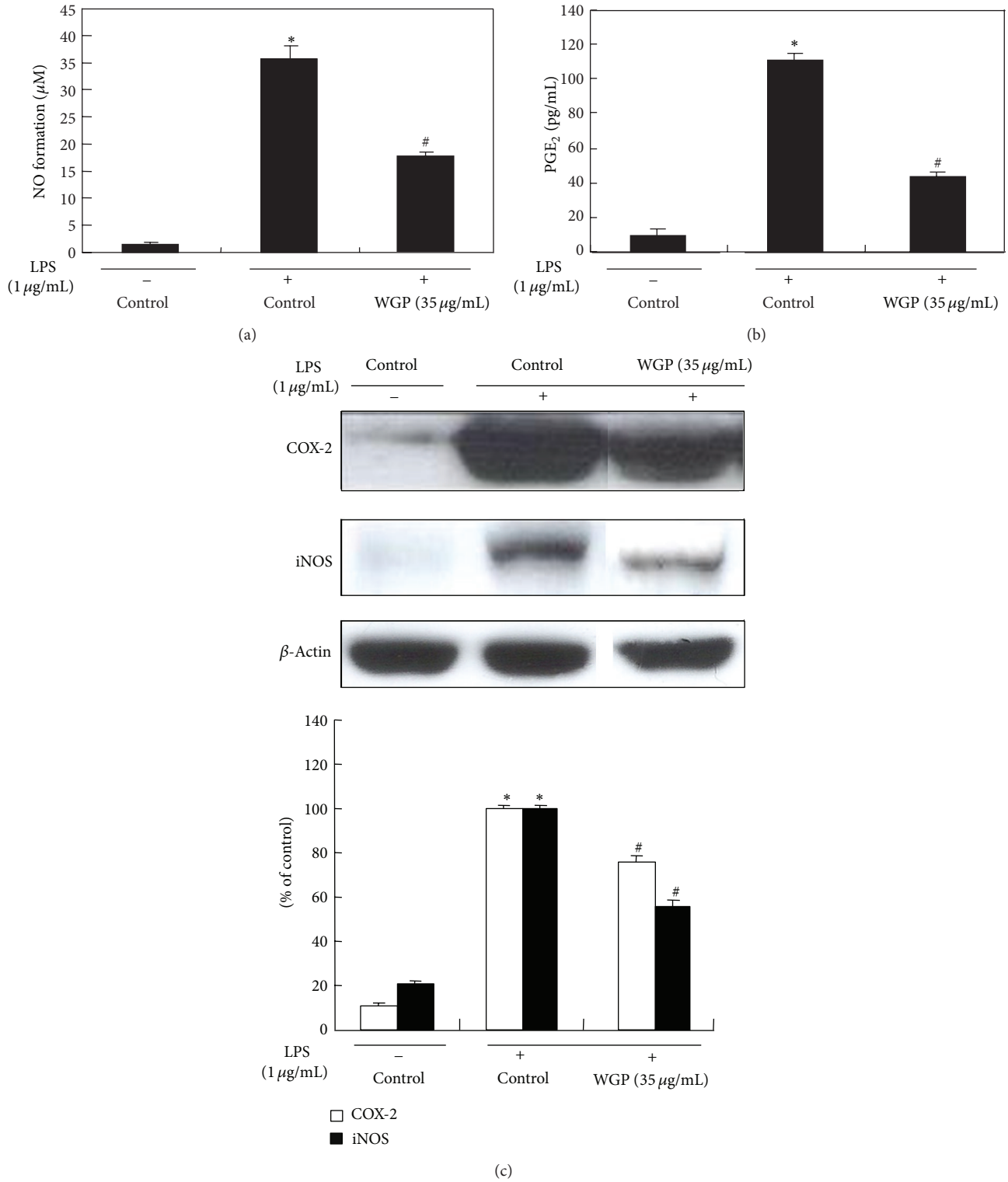

Figure 4: Effects of WGP on LPS-induced NO and PGE_2 production and iNOS and COX-2 protein expression in RAW 264.7 cells. (a) NO formation. The cells were treated with LPS only or with WGP at 35 μg/mL for 24 h. The culture media were collected, and the nitric oxide concentration was measured by the Griess reaction. (b) PGE_2 formation. Each culture supernatant was collected, and the amount of PGE_2 was measured using the PGE_2 parameter assay kit. Each value represents mean ± SD of triplicate experiments. (c) The cells were treated with WGP (35 μg/mL) for 1 h and then treated with LPS for 24 h. Equal amounts of total protein were subjected to Western blot analysis as described in Section 2. The ratio of immunointensity between the iNOS/COX-2 and the β-actin was calculated. Each bar (open bar, iNOS; closed bar, COX-2) represents means ± S.D. from three independent experiments. $^*P < 0.05$ indicates differences from the unstimulated control group. $^\#P < 0.05$ indicates differences from the LPS-treated group.

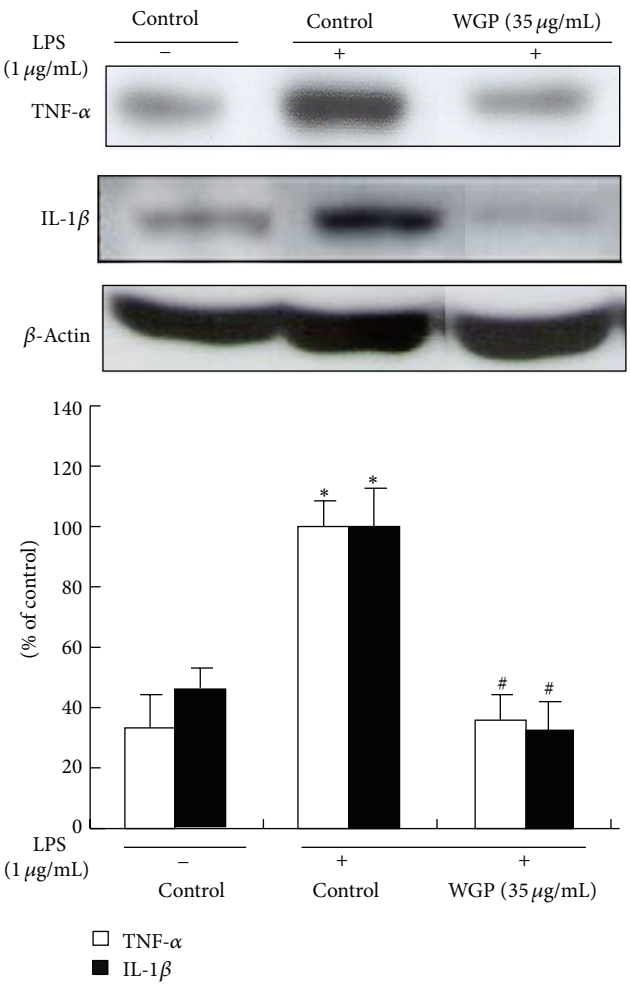

Figure 5: Effects of WGP on LPS-induced TNF-α and IL-1β protein expression in RAW 264.7 cells. The cells were treated with WGP (35 μg/mL) for 1 h and then treated with LPS for 24 h. Equal amounts of total protein were subjected to Western blot analysis, as described in Section 2. The ratio of immunointensity between the TNF-α/IL-1β and the β-actin was calculated. Each bar (open bar, TNF-α; closed bar, IL-1β) represents means ± SD from three independent experiments. $^{*}P < 0.05$ indicates differences from the unstimulated control group. $^{\#}P < 0.05$ indicates differences from the LPS-treated group.

RAW 264.7 cells. As displayed in Figure 7, WGP significantly inhibited the phosphorylation of p38 and its possible upstream kinase Akt, whereas phosphorylation of ERK and JNK was not affected. These results suggest that the anti-inflammatory effect of WGP might come from its modulation on p38/Akt signaling pathway.

4. Discussion

Procyanidins, plant polyphenols formed by the polymerization of flavan-3-ols, as cytoprotective agents have become an important source in human health research [30]. Epidemiological studies have indicated that populations that consume procyanidin-rich foods have lower incidences of inflammatory diseases [31]. Our previous study successfully demonstrated the separation and chemopreventive properties of procyanidins from wild grape seeds [6]. In this study, we investigated the anti-inflammatory activities of procyanidins, a main component isolated from wild grape (*Vitis amurensis*) seeds, in LPS-stimulated RAW 264.7 cells.

Macrophages are generally an important component in the immune defense mechanism. During the progress of inflammation, macrophages actively participate in inflammatory responses by releasing proinflammatory cytokines and mediators [32]. Furthermore, proinflammatory mediators such as ROS, NO, iNOS, and COX-2 play a key role in the pathogenesis of many acute and chronic inflammatory diseases [33]. ROS are well documented to function as signaling molecules, stimulating cellular activities ranging from cytokine secretion to cell proliferation, and at higher concentration, they can induce cell injury and death [34]. The iNOS and COX-2 pathway is known to play an important role in inducing ROS production [35, 36]. Since ROS is critical for LPS-induced inflammation through the activation of NFκB-related signaling [37], we first performed experiments to determine the effects of WGP on intracellular ROS accumulation. Pretreatment of cells with WGP significantly reduced the LPS-induced ROS production. Recent studies also demonstrated that lipid soluble extracts of red ginseng and *Salvia miltiorrhiza* possessed anti-inflammatory effects in LPS-induced RAW 264.7 cells by decreasing ROS production [38, 39]. Overproduction of NO produced by overexpression of iNOS has been implicated in the pathogenesis of septic shock, inflammation, and carcinogenesis [40]. COX-2 is another inducible enzyme that catalyzes biosynthesis of PGE_2, which contributes to pathogenesis of various inflammatory diseases, edema, angiogenesis, invasion, and growth of tumor [41]. Recently, mounting evidence both *in vitro* and *in vivo* has indicated an existing cross-talk between the release of NO and PGE_2 in the modulation of molecular mechanisms that regulate inflammation pathway [42, 43]. Thus, the anti-inflammatory agents that decrease NO and PGs production by simultaneously inhibiting the iNOS and COX-2 gene may have a potential therapeutic effect on the treatment of inflammatory and infectious diseases. According to our results, WGP strongly inhibits LPS-induced NO and PGE_2 production by attenuating the protein expression of iNOS and COX-2 without notable cytotoxicity. Our data implicate that WGP might inhibit NO and PGE_2 production by regulating the transcription molecules of iNOS and COX-2, which could be activated by LPS treatment. Several plant-derived components including curcumin, resveratrol, isoflavones, and red ginseng oil, have been reported to inhibit iNOS and COX-2 and exert anti-inflammatory activities in different types of cells [39, 44–46].

Inflammatory disorders are characterized among other events, by the production of significant amounts of free radicals and nitrogen reactive species as well as cytokines such as TNF-α, IL-1β, and IL-6 [47]. In particular, IL-1β is an important component in the initiation and enhancement of inflammatory response. TNF-α is also a pivotal proinflammatory cytokine and is regarded as an endogenous mediator of LPS-induced fever [48]. Our results showed that WGP could

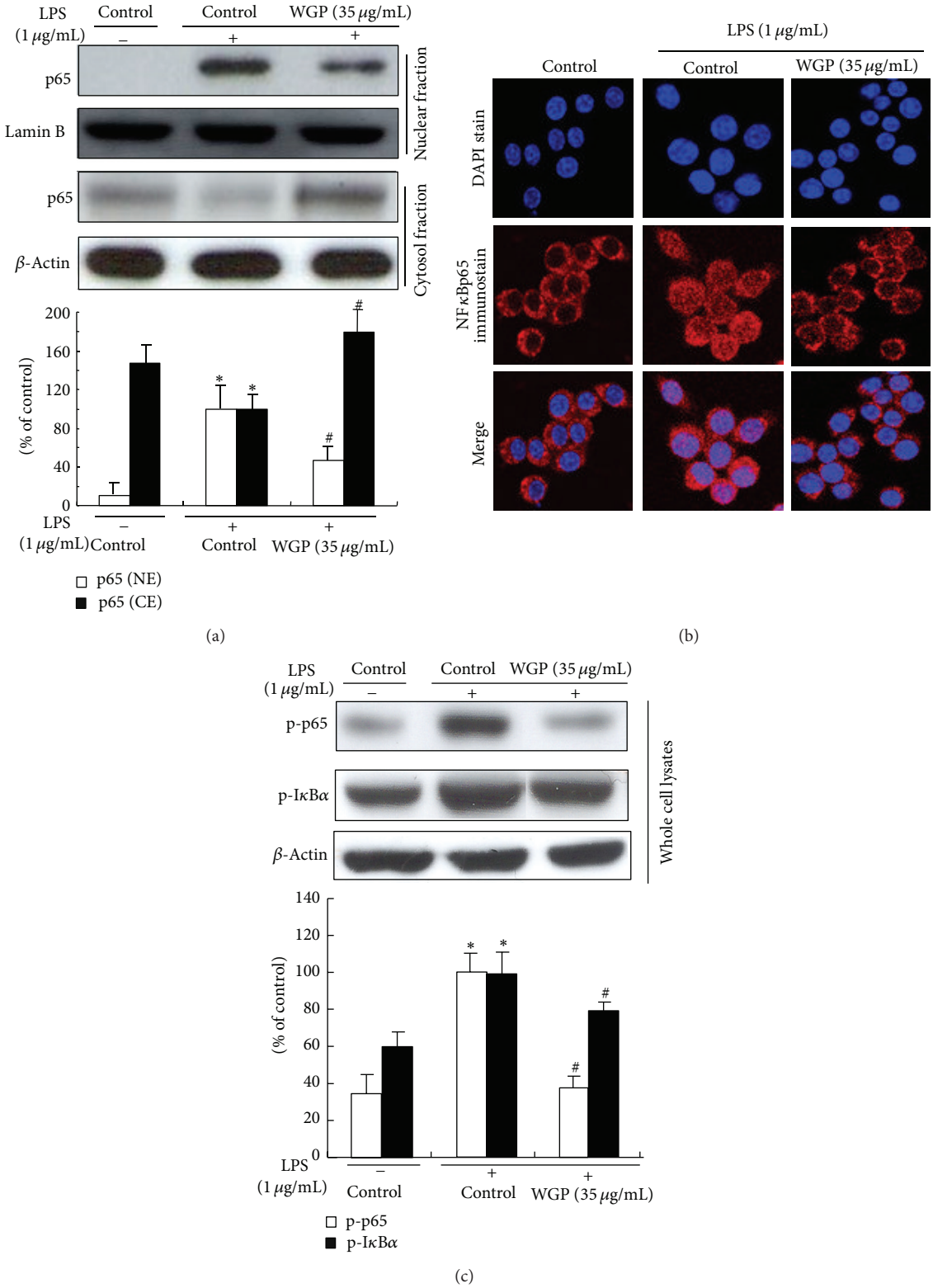

Figure 6: Effects of WGP on LPS-induced p65 nuclear translocation and p65 and IκBα phosphorylation in RAW 264.7 cells. (a) The cells were treated with 1 μg/mL LPS alone or with WGP (35 μg/mL) for 2 h. Cytosolic and nuclear fractions were prepared and analyzed by Western blotting. The ratio of immunointensity between the p65 (nuclear fraction: NE)/p65 (cytosolic fraction: CE) and the Lamin B and β-actin was calculated. Each bar (open bar, p65 (NE); closed bar, p65 (CE)) represents means ± S.D. from three independent experiments. (b) Cells were pretreated with WGP (35 μg/mL) for 1 h followed by stimulation with LPS for 2 h. Samples were stained by anti-p65 antibody and DAPI then prepared for confocal microscopy analysis. (c) The whole cells were treated with WGP at 35 μg/mL for 2 h and treated with LPS for 1 h. Equal amounts of total protein were subjected to Western blot analysis. The ratio of immunointensity between the p-p65/p-IκBα and the β-actin was calculated. Each bar (open bar, p-p65; closed bar, p-IκBα) represents means ± S.D. from three independent experiments. $^{*}P < 0.05$ indicates differences from the unstimulated control group. $^{\#}P < 0.05$ indicates differences from the LPS-treated group.

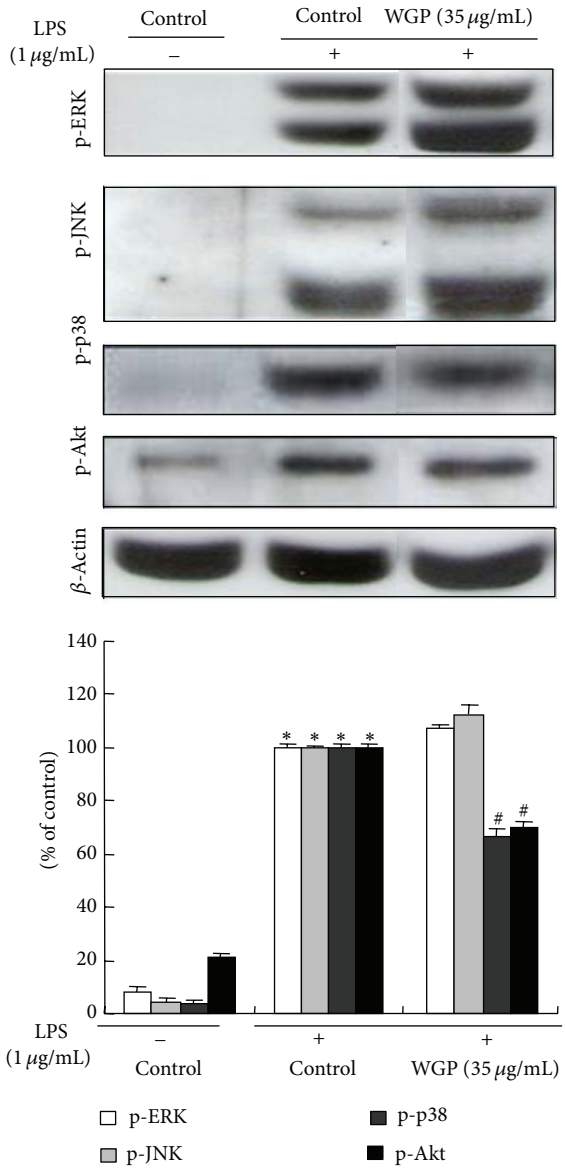

Figure 7: Effects of WGP on LPS-induced activation of MAP kinases and Akt in RAW 264.7 cells. The expression of phospho-ERK 1/2 (p-ERK), phospho-p38 (p-p38), phospho-JNK 1/2 (p-JNK), and phospho-Akt (p-Akt) was analyzed by Western blot. The cells were treated with WGP at 35 μg/mL for 1 h and treated with LPS (1 μg/mL) for 1 h. $^{*}P < 0.05$ indicates differences from the unstimulated control group. $^{\#}P < 0.05$ indicates differences from the LPS-treated group.

significantly suppress TNF-α and IL-1β expression. Excessive production of cytokines can be induced by inflammatory stimuli such as LPS treatment in macrophages and it will increase the immune response which in turn results in inflammation [49]. Therefore, the inhibition of the proinflammatory cytokines has been identified as a target for anti-inflammatory therapies, supporting our findings that WGP possesses an anti-inflammatory activity via the inhibition of TNF-α and IL-1β protein expression.

Much evidence suggests that transcription factor NFκB is involved in the regulation of LPS-induced inflammatory gene expression [17, 50]. Among the promoter regions of iNOS and COX-2, the transcription factor NFκB binding site is mainly responsible for the transcription activation of these genes by LPS stimulation [51]. Our results demonstrate that WGP has ability to inhibit LPS-induced phosphorylation of p65 and IκBα as well as the nuclear translocation of p65. This inhibitory effect might be through suppressing the phosphorylation and proteasome-mediated degradation of its inhibitor IκBα. In addition, phosphorylation of NFκB by upstream kinase has been reported to increase transcriptional potential of p65 subunit in LPS-stimulated macrophage [52]. The therapeutic potential of inhibiting NFκB pathway in chronic inflammatory diseases and inflammatory bowel disease has also been reported [53]. Moreover, recent studies have suggested that several natural products suppress inflammatory responses by regulating the NFκB pathway [12, 54]. These findings concur with our finding that the transcriptional inhibition of proinflammatory mediators by WGP is associated with the blockade of NFκB signaling pathway.

In addition to NFκB, LPS is a potent activator of MAPK and PI3K/Akt pathways. MAPKs not only play an important role in the LPS-mediated expression of iNOS and COX-2 but also regulate cytokine release in RAW 264.7 cells [26]. In this study, WGP treatment markedly suppressed LPS-stimulated phosphorylation of p38 and Akt, suggesting that suppression of p38 MAPK phosphorylation by WGP might be involved in the inhibition of LPS-induced production of proinflammatory substances in RAW 264.7 cells. Other studies have reported that NFκB-dependent gene expression is downregulated by p38 pathway or by dominant-negative p38 expression, but no significant difference was observed in NFκB translocation and DNA binding, which suggests that p38 could have an indirect influence on NFκB transcription [55, 56]. Additionally, the present result showed that WGP inhibits the LPS-induced phosphorylation of Akt, which is a critical step in PI3K activation. The PI3K/Akt pathway has also been shown to control a variety of cellular processes, including cell survival and proliferation [57]. Recently, studies have shown that the PI3K/Akt signaling pathway plays a crucial role in regulating LPS-induced acute inflammatory responses *in vitro* and *in vivo* [58–60]. However, the role of PI3K/Akt signaling cascades in the regulation of NFκB transactivation remains controversial. The present study agrees with previous investigations which demonstrate that PI3K/Akt pathway promotes the p65 inhibition [61]. However, other studies showed that the inhibition of the PI3K/Akt pathway augmented the p65 activation [59]. The reason for such inconsistency is not clear at this moment and further studies are needed to elucidate the exact molecular mechanisms involved in anti-inflammation by different agents. These results suggest that WGP may block LPS-induced NFκB translocation by inhibiting the phosphorylation MAPKs and PI3K/Akt, and subsequently decreasing the NO, PGE_2, and ROS production and the protein levels of iNOS, COX-2, and cytokines (Figure 8).

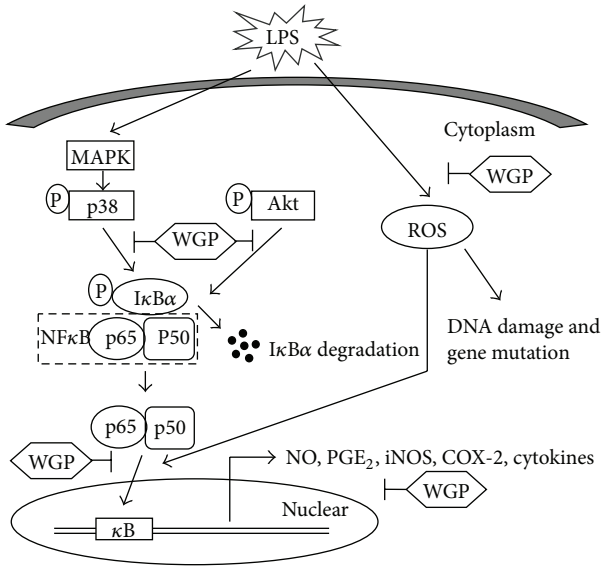

Figure 8: Schematic diagram illustrating the signaling pathways involved in WGP's anti-inflammatory effects in LPS-induced RAW 264.7 cells.

5. Conclusion

In conclusion, our findings indicate that WGP was shown to suppress many inflammatory events including production of NO, PGE_2, and ROS in RAW 264.7 cells stimulated with LPS. In addition, WGP plays a role in suppressing the protein expressions of iNOS and COX-2, two critical inducible enzymes responsible for the production of NO and PGE_2, as well as the expression of proinflammation cytokines such as TNF-α and IL-1β. These effects might be mediated through the inhibition of NFκB activity via downregulation of the p38 MAPK and Akt signaling pathways. Taken together, WGP may be used as a potent natural anti-inflammatory agent.

Abbreviation

WGP:	Wild grape seeds procyanidins
LPS:	Lipopolysaccharide
NO:	Nitric oxide
PGE_2:	Prostaglandin E_2
ROS:	Reactive oxygen species
iNOS:	Inducible nitric oxide synthase
COX-2:	Cyclooxygenase 2
TNF-α:	Tumor necrosis factor α
IκBα:	Inhibitory κB-α
NFκB:	Nuclear factor-κB
ERK:	Extracellular regulated kinase
JNK:	c-Jun N-terminal kinase
PI3K:	Phosphoinositide 3-kinase
MAPK:	Mitogen-activated protein kinase
DCF-DA:	2′,7′-Dichlorodihydrofluorescein.

Acknowledgments

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Research Article

Effects of Resveratrol on Vitrified Porcine Oocytes

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Vitrified MII porcine oocytes are characterized by reduced developmental competence, associated with the activation of the apoptotic pathway. Resveratrol (R), a polyphenolic compound present in several vegetal sources, has been reported to exert, among all its other biological effects, an antiapoptotic one. The aim of this study was to determine the effects of R (2 μM) on the apoptotic status of porcine oocytes vitrified by Cryotop method, evaluating phosphatidylserine (PS) exteriorization and caspases activation. R was added during IVM (A); 2 h postwarming incubation (B); vitrification/warming and 2 h postwarming incubation (C); all previous phases (D). Data on PS exteriorization showed, in each treated group, a significantly higher ($P < 0.05$) percentage of live nonapoptotic oocytes as compared with CTR; moreover, the percentage of live apoptotic oocytes was significantly ($P < 0.05$) lower in all R-treated groups relative to CTR. The results on caspase activation showed a tendency to an increase of viable oocytes with inactive caspases in B, C, and D, while a significant ($P < 0.05$) increase in A compared to CTR was recorded. These data demonstrate that R supplementation in various phases of IVM and vitrification/warming procedure can modulate the apoptotic process, improving the resistance of porcine oocytes to cryopreservation-induced damage.

1. Introduction

Vitrification of oocytes is the most recent cryopreservation methodology, used in different species such as human [1], bovine [2], goat, and swine [3].

Currently, several studies have been carried out to improve the efficacy of cryopreservation protocols, validating different cryoprotectant solutions, incubation times, oocytes containers, and other many conditions [4].

Recent progresses in the vitrification technique are attested by the high number of born after cryopreservation of human [5], mouse [6], cat [7], and bovine [8] oocytes, but no piglets have been obtained from cryopreserved swine oocytes so far.

Compared with other domestic species, the high intracellular lipid content [9] and the wide cell volume make porcine oocytes more susceptible to storage at low temperature, with a consequent decrease of oocytes survival rate and apoptotic progression after thawing [10, 11].

Moreover, the survival and development of unfertilized vitrified porcine oocytes are significantly lower than those of fertilized vitrified ones [12, 13]. During the vitrification/warming process many oocyte ultrastructures, such as mitochondria, smooth endoplasmatic reticulum, meiotic spindle, and plasma membrane, show considerable damages that contribute to reduce the developmental potential of oocytes after fertilization [14, 15].

In addition, oocytes that survive cryopreservation significantly reduce their glutathione (GSH) content and accumulate reactive oxygen species (ROS) [16].

ROS, such as superoxide anions (O^-_2), hydroxyl radicals (OH^-), and H_2O_2, are generated during intermediate steps of oxygen reduction; their heap, also associated with the glutathione efflux, is one of the main factors which are effective in inducing the apoptotic activation, characterized by biochemical events that result in specific morphological changes including cell shrinkage and progressive DNA and cell membrane damage, ultimately leading to cell death. Signals to death receptors (extrinsic apoptotic pathway) or to mitochondria (intrinsic apoptotic pathway) concur in the activation of caspases, a family of cysteine proteases with similar aminoacid sequences, structure, and specificity that promote morphological and biochemical cell changes, typical of apoptosis [17, 18]. Finally, phosphatidylserine (PS), that is normally confined to the inner plasma membrane leaflet,

after apoptotic stimuli is externalized and subsequently recognized by specific PS receptors of macrophages and other phagocytic cells, inducing apoptotic cells engulfment [19–21].

Therefore, one of the current challenges to reproductive cryobiologists is to prevent oocytes degeneration in order to maintain their developmental competences.

To preserve the antioxidant defence system in oocyte, specific substances that play antioxidant roles, such as ascorbate [22], epigallocatechin-3-gallate [23], β-mercaptoethanol [13], anthocyanin [24], and trans-ε-viniferin [25], were added during in vitro oocytes/embryo culture and storage.

Recently, treatment of porcine oocytes with 2 μM Resveratrol during IVM [26] reduces the intracellular level of ROS and increase GSH concentration in matured oocytes, resulting in increase of blastocyst development after parthenogenetic activation (PA) and in vitro fertilization (IVF).

Resveratrol, through its simultaneous activity on multiple molecular targets, is effective in modulating different cell pathways and, depending on its concentration, and its effect may be reversed [27]. Resveratrol has been reported to act as antioxidant because of its ability to decrease mitochondria ROS production, scavenge superoxide radicals, inhibit lipid peroxidation, and regulate the expression of antioxidant cofactors and enzymes [28].

On thise basis we decided to add 2 μM Resveratrol in maturation, vitrification, and postvitrification media in order to determine its effect on apoptotic process and oocytes viability.

For this purpose the externalization of phosphatidylserine using Annexin V (Annexin V/Hoechst 33342/PI) and the caspase activation through FITC-VAD-FMK staining (FITC-VAD-FMK/Hoechst 33342/PI) were evaluated.

2. Materials and Methods

Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (Milan, Italy). Cryotops (Kitazato, Fuji, Japan) were obtained from BioCare Europe (Roma, Italy).

2.1. In Vitro Maturation (IVM) of Cumulus-Oocyte Complexes (COCs). Ovaries were collected at a local abattoir and transported to the lab within 2 h in a thermos filled with physiological saline at 30–35°C. COCs from follicles 3–6 mm in diameter were aspirated using a 18-gauge needle attached to a 10 mL disposable syringe. Under a stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. Only COCs with complete and dense cumulus oophorus were used. After three washes in NCSU 37 [29] supplemented with 5.0 mg/mL insulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 50 μM β-mercaptoethanol, and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a 4-well multidish (Nunclon) containing 500 μL of the same medium per well and cultured at 39°C in a humidified atmosphere of 5% CO_2 and 7% O_2 in N_2. During the first 22 h of maturation, the IVM medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL eCG (Folligon, Intervet, Boxmeer, The Netherlands), and 10 IU/mL hCG (Corulon, Intervet). After culturing for 22 h, COCs were transferred to fresh maturation medium and cultured for a further 24 h period [29].

2.2. Oocyte Vitrification with Cryotops and Warming. The protocol of vitrification with Cryotop carrier and solution has been described by Kuwayama et al. [30, 31]. Briefly, denuded oocytes ($N = 5$) were transferred into equilibration solution (ES) consisting of 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) in Hepes-buffered Ham's F10 (HF10; Gibco, Invitrogen, Monza, Italy) and 20% fetal calf serum (FCS; Gibco) at 39°C for 5–15 min. Thereafter, oocytes were transferred into 20 μL drops of vitrification solution (VS) consisting of 15% EG, 15% DMSO, and 0.5 M sucrose dissolved in HF10 and 20% FCS. After incubation for 20–30 s, oocytes were loaded on Cryotop and plunged into liquid nitrogen (LN2). The entire process, from exposure in VS to plunging into LN2, was completed within 45–60 s. Vitrified oocytes were warmed by submerging vitrification devices directly into 39°C thawing solution (1.0 M sucrose dissolved in HF10 and 20% FCS) for 1 min, and then they were transferred to a dilution solution (0.5 M sucrose dissolved in HF10 and 20% FCS) for 3 min. Subsequently, oocytes were washed twice for 5 min in washing solutions (HF10 supplemented with 20% FCS) before being transferred in IVM medium for 2 h.

2.3. Annexin V Staining of Phosphatidylserine Residues. The Annexin V binding assay was employed to detect phosphatidlserine (PS) externalization on the plasma membrane. Annexin V, a member of the phospholipid-binding annexin family, binds most efficiently to PS, which is externalized on the outer plasma membrane of cells exposed to apoptotic stimuli.

Oocytes were washed three times with Dulbecco's phosphate-buffered saline (DPBS) and transferred to 100 μL of binding buffer (Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit, Molecular Probes, Eugene, USA) with 5 μL of Alexa Fluor Annexin V, 1 μL of propidium iodide (PI) (100 μg/mL), and 0.2 μL of 5 mg/mL Hoechst 33342 (Ho) for 20 min at 39°C in the dark. After incubation, the oocytes were washed three times in binding buffer and then mounted on glass slides which were examined with an Eclipse E 600 (Nikon Europe BV, Badhoevedorp, The Netherlands) epifluorescence microscope equipped with a digital camera.

Oocytes were classified as follows:

(i) live nonapoptotic oocytes with Ho-positive nuclei and no annexin staining (A−/PI−) (Figure 1(a)),

(ii) live apoptotic oocytes with Ho-positive nuclei and annexin-positive signal on the membrane (A+/PI−) (Figure 1(b)),

(iii) necrotic oocytes which showed PI-positive red nuclei, indicative of membrane damage with or without annexin staining on the membrane (PI+) (Figure 1(c)).

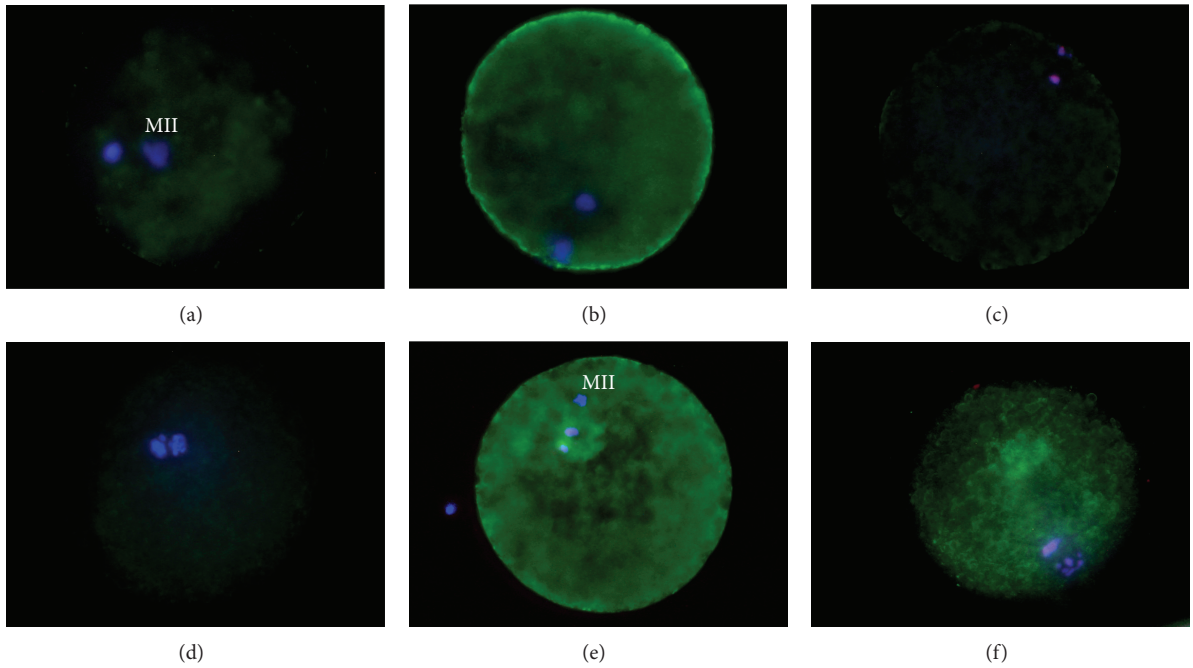

Figure 1: ((a)–(c)) Fluorescent micrographs of oocytes after combined staining with Annexin V/Hoechst 33342 (Ho)/PI. (a) Live non apoptotic oocytes with Ho positive nuclei and no annexin staining (A−/PI−); (b) Live apoptotic oocytes with Ho positive nuclei and annexin positive signal on the membrane (A+/PI−); (c) Dead oocyte which showed PI positive red nuclei (PI+). ((d)–(f)) Representative fluorescent micrographs of pig oocytes after FITC-VAD-FMK, Ho and PI staining. (d) Live non apoptotic oocytes with Ho positive nuclei and no FITC-VAD-FMK staining (VAD−/PI−); (e) Live apoptotic oocyte with the cytoplasm stained in green by FITC-VAD-FMK (VAD+/PI−); (f) Dead oocyte with the metaphase plate stained in red by PI (PI+).

2.4. Assessment of Activated Caspases. The activation of caspases was detected through FITC-VAD-FMK (Molecular Probes, Leiden, The Netherlands); VAD-FMK is a cell permeable caspase inhibitor that covalently binds activated caspases, conjugated to FITC. Oocytes were washed twice with DPBS and then incubated with 500 μL of DPBS containing 1 μM of FITC-VAD-FMK and 0.2 μL of 5 mg/mL Ho for 30 min at 39°C. During the final 5 min of VAD-FMK/oocyte incubation, 3 μL of a solution 1 mg/mL of propidium iodide were added to detect dead oocytes. Oocytes were then washed twice for 5 min with DPBS and mounted on glass slides. Samples were assessed by fluorescence microscopy. Stained oocytes were classified in three groups:

(i) viable oocytes without active caspases (VAD−/PI−)(Figure 1(d)),

(ii) viable oocytes with FITC-VAD-FMK positivity, indicative of caspase activation (VAD+/PI−) (Figure 1(e)),

(iii) dead oocytes (PI+) (Figure 1(f)).

2.5. Experimental Design. All oocytes were submitted to IVM, vitrification, and warming procedures and were evaluated after incubation for 2 h into maturation medium.

Oocytes were divided into the following 5 experimental groups:

(CRT) without Resveratrol addition;

(A) 2 μM Resveratrol supplementation during IVM;

(B) 2 μM Resveratrol supplementation during the postwarming incubation for 2 h;

(C) 2 μM Resveratrol supplementation during vitrification/warming and 2 h post-warming;

(D) 2 μM Resveratrol supplementation in all previous steps.

2.6. Statistical Analysis. Each experiment was repeated at least 3 times. All statistical analyses were performed using R version 2.15.2. [32]. Chi square test was performed and the level of significance was set at $P < 0.05$.

3. Results

3.1. Experiment 1: Detection of Apoptosis by Annexin V Labeling. The addition of R, in all treated groups, induced a percentage of live nonapoptotic oocytes (A−PI−) significantly higher ($P < 0.05$) than nontreated control (Table 1). Moreover, in all groups supplemented with R the percentage of live apoptotic oocytes (A+PI−) was significantly ($P < 0.05$) lower than in CTR group. Finally, R supplementation in B, C, and D groups significantly ($P < 0.05$) reduced the percentage of dead oocytes (PI+) compared to CTR.

3.2. Experiment 2: Assessment of Activated Caspases. The results on caspases activation showed a tendency to an increase of viable oocytes with inactive caspases (VAD−PI−)

Table 1: Effect of Resveratrol supplementation on exteriorization of phosphatidylserine, assayed by Annexin V/Hoechst 33342/PI staining, in vitrified oocytes. Data are presented as mean percentage.

Group	*N* oocytes	% viable oocytes (A− PI−)	% apoptotic oocytes (A+ PI−)	% dead oocytes (PI+)
CTR	310	66.1[a]	28.4[a]	5.5[a]
A	150	78.7[b]	15.3[b]	6[a]
B	124	83.1[b]	16.9[b]	0[b]
C	122	80.3[b]	18.9[b]	0.8[b]
D	114	81.6[b]	17.6[b]	0.9[b]

Different superscripts within the same column indicate significant differences ($P < 0.05$).
CTR: control group; (A) 2 μM R supplementation during IVM; (B) 2 μM R supplementation during the postwarming incubation for 2 h; (C) 2 μM R supplementation during vitrification/warming and 2 h after warming; (D) 2 μM supplementation in all previous steps.

Table 2: Effect of Resveratrol on caspase activation in vitrified oocytes, as assayed by FITC-VAD-FMK/Hoechst 33342/PI staining. Data are presented as mean percentage.

Group	*N* oocytes	% live oocytes (PI−)		% dead (PI+)
		VAD−	VAD+	
CTR	165	81,82[a]	10,30[a]	7,88[a]
A	84	91,67[b]	7,14[a]	1,19[b]
B	76	86,84[ab]	9,21[a]	3,95[ab]
C	75	84,00[ab]	13,33[a]	2,67[ab]
D	91	89,01[ab]	7,69[a]	3,30[ab]

Different superscripts within the same column indicate significant differences ($P < 0.05$).
CTR: control group; (A) 2 μM R supplementation during IVM; (B) 2 μM R supplementation during the postwarming incubation for 2 h; (C) 2 μM R supplementation during vitrification/warming and 2 h after warming; (D) 2 μM supplementation in all previous steps.

in B, C, and D groups, while a significant ($P < 0.05$) increase in A group compared to CTR was recorded.

No significant variations were observed in the percentage of live oocytes with active caspases (VAD+/PI−), while a significant reduction of dead oocytes (PI+) was detected in A group compared to CTR (Table 2).

4. Discussion

While ascorbate [22] and β-mercaptoethanol [13] have been already assayed in vitrification-warming solutions, in mouse embryos and porcine oocytes, respectively, this study, for the first time, tested Resveratrol in the improvement of oocyte cryopreservation. Ascorbate (0.1 mmol/l) has been demonstrated to reduce the levels of hydrogen peroxide in mouse embryos, increasing the inner cell mass when added in slow-freezing or vitrification solutions; β-mercaptoethanol (50 μmol/l) decreased ROS activity but did not improve viability and fertilization ability of vitrified-warmed MII oocyte, while significantly increased blastocyst formation ability of porcine oocytes vitrified after in vitro fertilization.

Our results demonstrate that 2 μM R in IVM and vitrification-warming phases increases oocytes viability, modulating the apoptotic process. Annexin V labeling showed a significant increase in live nonapoptotic oocytes and a parallel reduction of live apoptotic oocytes in all groups added with R as compared with CTR groups.

FITC-VAD-FMK staining evidenced a tendency to an increase of viable oocytes with inactive caspases in all R-treated groups compared with CTR; a significant difference was recorded only between A and CTR groups. However, the percentage of viable oocytes with active caspases was not affected by R treatment.

Several recent studies demonstrated that PS-mediated phagocytosis can occur without caspases activation [33–35]. Thus, we can hypothesize that the different trends in viability and apoptosis observed in annexin V and FITC-VAD-FMK assays may depend on an involvement of caspases and PS externalization in the whole apoptotic process, with a prevalent action of R in one of these two events.

Our results show that R mainly influences PS exteriorization, rather than caspases activation. This R effect can be related to GSH increase, observed by Kwak et al. [26] after the addition of the same R concentration to IVM solution. This hypothesis is supported also by He et al. [36], who demonstrated that inhibition of GSH efflux had no effect on the activation of caspases 3, 8, and 9, but decreased the translocation of PS. Cellular GSH homeostasis plays a crucial role in radical scavenging activity [18] and in cytoplasmatic maturation of porcine oocyte [37–39]. Somfai et al. [16] reported that low intracellular GSH levels and high H_2O_2 concentration in vitrified porcine oocytes, besides other ultrastructural cryodamages, increase the sensitivity to oxidative stress at the beginning of embryo culture, reducing porcine oocytes development and subsequent male pronucleus formation.

In addition, several studies demonstrated the ability of R to scavenge ROS [40, 41] and to modulate intracellular GSH depletion or synthesis, in relation to its concentration [42–44]

Therefore we can suppose that R could be able to maintain GSH homeostasis, with a subsequent inhibition of PS externalization.

Our results on R effect on caspase activation, which is not as evident as PS externalization in vitrified oocytes assayed after 2 h incubation after thawing, agree well with those by Vallorani et al. [11], who observed that caspase activation seems to be a reversible phenomenon. That work showed a significant reduction of vitrified live oocytes presenting a faint FITC-VAD-FMK staining after 2 h of postwarming incubation, compared to those observed immediately after warming; moreover, no significant differences were detected, in the same time lapse, in the number of apoptotic oocytes (A+P−) as assayed by Annexin V staining. Taken together, our and those results seem to suggest that 2 h of postwarming incubation may be beneficial in modulating the caspase cascade that can be further reduced by R addition, in a significant manner when added in IVM solutions.

A previous study [45] demonstrated that in neuronal cells the externalization of PS occurs during the late phase of apoptosis while caspase activation begins in the early one.

Therefore, we can hypothesize that while caspase activation can be arrested after 2 h post warming incubation, PS externalization may be a tardive and irreversible apoptotic event that could be avoided by R addition in one or more steps of the IVM and vitrification procedure.

In our study we did not observe any algebraic sum of positive effects of Resveratrol when added during vitrification/warming and 2 h of culture after warming (C group) or in the whole IVM-vitrified-warming-postwarming process (D group). Resveratrol could be immediately oxidized, modulating the GSH redox balance and thus increasing the oocyte-reducing power. Therefore, it seems that it might be employed either in IVM, in vitrified-warming, or 2 h post warming solutions, in order to prevent, inhibit, or repair cryoinjury damages.

5. Conclusions

In conclusion, the present results confirm the occurrence of vitrification-induced oocyte injuries, as previously reported, and suggest to improve vitrification protocols by Resveratrol addition.

Supplementation with 2 μM Resveratrol in IVM, vitrification-warming, or 2 h postwarming solutions could improve and optimize the quality and the resistance of IVM porcine oocytes to cryopreservation, modulating cell apoptotic process.

Other polyphenolic compounds could help in minimizing cryodamage and in optimizing current Cryotop vitrification method, improving the success of applications in female gamete preservation.

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Research Article

Neuroprotective Effects of a Variety of Pomegranate Juice Extracts against MPTP-Induced Cytotoxicity and Oxidative Stress in Human Primary Neurons

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an environmental toxin which selectively induces oxidative damage and mitochondrial and proteasomal dysfunctions to dopaminergic neurons in the substantia nigra leading to Parkinsonian syndrome in animal models and humans. MPTP is one of the most widely used *in vitro* models to investigate the pathophysiology of Parkinson's disease (PD) and, screen for novel therapeutic compounds that can slow down or ameliorate this progressive degenerative disease. We investigated the therapeutic effect of pomegranate juice extracts (PJE), Helow, Malasi, Qusum, and Hamadh against MPTP-induced neurotoxicity in primary human neurons by examining extracellular LDH activity, intracellular NAD^+ and ATP levels, and endogenous antioxidant levels including lipid peroxidation products, catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and reduced glutathione (GSH) levels. MPTP induced a reduction in SOD and GPx activities and intracellular NAD^+, ATP, and GSH levels parallel to an increase in extracellular LDH and CAT activities, although lipid peroxidation was not altered. We report that helow and malasi can ameliorate MPTP-induced neurotoxicity by attenuating the observed changes in redox function to a greater extent than qusum and hamedh. Selected PJE varieties may exhibit properties which may be of therapeutic value to slow down age-related degeneration and neurodegeneration in particular.

1. Introduction

As the ageing population continues to grow at an alarming rate, the likelihood of people developing debilitating neurodegenerative deficits such as Parkinson's disease (PD) is growing rapidly. PD represents the second most common neurological disorder after Alzheimer's disease (AD), and it affects 2% of the population over the age of 60. PD is characterised by the chronic and progressive loss of dopaminergic neurons in the substantia nigra [1]. Although the etiology of PD is not yet known, current studies have suggested that oxidative stress may be a major player [2]. An imbalance between the formation of free radicals and reactive oxygen species (ROS) and the body's endogenous antioxidant defense mechanisms has also been implicated in the pathogenesis of other neurodegenerative diseases such as AD, Huntington's disease (HD), Pick's disease, amyotrophic lateral sclerosis (ALS), epilepsy, schizophrenia, and hypoxic-ischemic brain injury. ROS can induce oxidative damage to

lipids, nucleic acids, and proteins, promote abnormal aggregation of cytoskeletal proteins, inactivate major metabolic enzymes, and facilitate mitochondrial dysfunction and the formation of reactive nitrogen species (RNS) and advanced glycation end products formation leading to further oxidative stress formation [3–20]. Therefore, an increased total antioxidant capacity has been associated with protection against neurodegeneration [12].

The environmental toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) can induce neurotoxicity to humans [21], subhuman primates, and mice [22–24] by eliciting damage to dopaminergic neurons and subsequently resulting in Parkinsonian-like syndrome in animals and humans. MPP^+ (1-methyl-4-phenylpyridinium ion) represents the neurotoxic form of MPTP which is formed by 4-e oxidation of MPTP in brain mitochondria [25]. It remains unclear whether or not MPP^+ is the main neurotoxic product of MPTP, and why other organs apart from the brain are not vulnerable to MPTP-induced cytotoxicity. The "mitochondrial hypothesis" suggests that MPP^+ can inhibit mitochondrial respiration similar to the synthetic pesticide, rotenone. On the other hand, the "oxidative stress" hypothesis assumes that the nigrostriatal cell death observed in PD is due to the MPTP-mediated formation of hydroxyl and superoxide radicals. Nevertheless, the neuronal lesions and neurological symptoms induced by MPTP and its congeners are similar to those reported in idiopathic parkinsonism and provides additional evidence to suggest that environmental toxins of related structure may play a causal role in human PD [26, 27].

The pomegranate (*Punica granatum* L.) is a polyphenolic rich fruit that has been extensively referenced in medical folklore [28]. In several countries of the Arabian Peninsula and notably Yemen, pomegranates are widely used for the treatment of common ailments, including diarrhea, stomachache, healing wounds, acidosis, dysentery, microbial infections, haemorrhage, and various infectious and noninfectious respiratory pathologies [29]. Phytochemicals such as polyphenols (including the phenolic acids and flavonoids which are concentrated in pomegranates) have demonstrated antioxidant properties and can inhibit inflammation and other deleterious processes involved in degenerative diseases [30]. Pomegranate pericarp is also highly rich in tannins (gallic acid, ellagic acid), which are potent antioxidants [31]. These polyphenols have been shown to inhibit carcinogenesis [32] and display various anticancer properties [33]. Tannins which are present in high levels in commercially processed pomegranate juice from pressing the whole fruit and the peels also augment the juices antioxidant power [34].

The antinflammatory and antibacterial potentials of pomegranate have been previously reported [35–43]. Further research has demonstrated that polyphenols possess powerful antioxidant properties which represent the most likely mechanism responsible for pomegranate's protective benefits [41]. Although pomegranate juice extract has been previously shown to reduce amyloid load and improve cognitive behavioural deficits in mouse models for AD, little is known about the potential beneficial effects of pomegranates in PD. Therefore, we investigated whether various pomegranate extracts could protect against MPTP-induced oxidative stress in primary human neurons *in vitro*.

2. Materials and Methods

2.1. Pomegranate Juice Extracts (PJE) Preparation. Fresh pomegranate (Helow, Malasi, Qusum, and Hamedh) varieties were obtained from Al-Jabal Al-Akdhar farms, Oman. The seeds were isolated and ground to obtain juice for all varieties separately. The juices (PJ) were air-dried at 40°C and concentrated under reduced pressure to obtain PJ extract (PJE). Appropriate preparation of PJE is vital to prevent some undesirable reactions, such as enzymatic and nonenzymatic degradation, fat oxidation, vitamin degradation, and protein denaturation prior to experimentation. Drying is the most commonly used method for the dehydration of food products. Various fruits and vegetables such as onions, red pepper, garlic cloves, and apricots have been dried, although this led to a reduction in size and loss of colour, texture and nutritional-functional properties (reviewed in [44]). Our PJE were dried as previously described by Bchir et al. (2012) with the lowest impact on the gallic acid equivalent fresh matter (FM) of total phenolics, FM of anthocyanins, antioxidant activity, and texture [45] of the extract used in the study.

2.2. Measurement of Total Phenolics in Pomegranate Juice Extracts. Total phenolics of PJE were measured by the modified Folin-Ciocalteu assay as previously described [46]. Briefly, 250 μL Folin-Ciocalteu reagent was mixed with 10 μL of PJE. After a short incubation of 5 mins, 750 μL of sodium carbonate (1.9 M) was added and incubated for 2 h at 25°C. The absorbance at 765 nm was measured and compared with that from gallic acid (GA) standards. The concentration of phenolics in pomegranate juice extracts was expressed as gallic acid equivalents (GAE). All the measurements were taken in triplicate, and the mean values were calculated.

2.3. Human Primary Neuronal Cell Cultures. Human foetal brains were obtained from 16- to 19 week-old foetuses collected following therapeutic termination with informed consent. Mixed brain cultures were prepared and maintained using a protocol previously described by Guillemin et al. [47]. Neurons were prepared from the same mixed brain cell cultures as previously described [48]. Briefly, cells were plated in 24-well culture plates coated with Matrigel (1/20 in Neurobasal) and maintained in Neurobasal medium supplemented with 1% B-27 supplement, 1% Glutamax, 1% antibiotic/antifungal, 0.5% HEPES buffer, and 0.5% glucose.

2.4. Cell Culture Treatments. Human neurons were divided into four groups: (1) control group: the cells were treated with 0.1% DMSO solution (control) alone; DMSO at the concentrations used had no effect on cell viability; (2) MPTP-treated group: the cells were treated with pathophysiological concentrations of MPTP (0.05 mM) for 24 hours; (3) PJE-treated group: the cells were treated with varieties of PJE at 1, 10, 50, and 100 μM for 24 hours; (4) PJE/MPTP-treated group: neurons were pretreated with PJE at 1, 10, 50, and 100 μM for

1 hour and then 0.05 mM MPTP (final concentration) was added for 24 hours into cultured neurons in 24-well plates containing supplemented Neurobasal medium as described above. Experiments were performed in quadruplicates using cultures derived from three different human foetal brains.

2.5. NAD(H) Microcycling Assay for the Measurement of Intracellular NAD^+ Concentrations. Intracellular NAD^+ concentration was measured spectrophotometrically using the thiazolyl blue microcycling assay established by Bernofsky and Swan [49] and adapted for 96-well plate format by Grant and Kapoor [50].

2.6. Extracellular LDH Activity as a Measurement for Cytotoxicity. The release of lactate dehydrogenase (LDH) into culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate measure of cellular toxicity. LDH activity was assayed using a standard spectrophotometric technique described by Koh and Choi [51].

2.7. Measurement of Intracellular ATP Levels. Human neuronal cell lysates were collected by centrifugation, and intracellular ATP was measured with a luminometer using an ATP Bioluminescence Assay Kit HS II (Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly 50 μL of reaction mixture was added to 50 μL of cell homogenate and the count was measured by luminometer (BD Biosciences). The level of ATP was determined from the standard curve. The standard was prepared for each day of measurements.

2.8. Malondialdehyde-Thiobarbituric Acid (MDA) as a Marker for Lipid Peroxidation. The level of lipid peroxidation was quantified by measuring the amount of malondialdehyde-thiobarbituric acid (MDA-TBA) adduct formed by the reaction of MDA and TBA at 100°C in neuronal cell lysates. MDA levels were measured using a standardised commercial assay kit (Caymen Chemical Co. Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, 50 μL of sample was added to 50 μL SDS solution and 1 mL TBA and incubated at 100°C for 1 hr. Afterwards, the samples were placed on ice for 10 minutes to terminate the reaction and centrifuged at 1600 g for 10 min to remove debris. The absorbance for the newly formed product was read at 540 nm using the Model 680XR microplate reader (BioRad, Hercules, USA).

2.9. Superoxide Dismutase Activity Assay. Superoxide dismutase (SOD) activity was assayed using a colorimetric assay kit (Cayman, MI, USA). The kit measures all three types of SOD (Cu/Zn-, Mn-, and Fe-SOD). The kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The absorbance was read at 450 nm using the Model 680XR microplate reader (BioRad, Hercules).

2.10. Catalase Activity Assay. Catalase (CAT) activity was assayed by the decomposition of hydrogen peroxide using a colorimetric assay kit (Cayman, MI, USA). The method is based on a two-stage reaction. The rate of dismutation of H_2O_2 to water and molecular oxygen correlates with catalase activity. A known amount of H_2O_2 was added to the cell homogenate and incubated for exactly 1 minute. The reaction was stopped using sodium azide. The amount of H_2O_2 remaining in the reaction mixture was then determined by the oxidative coupling reaction of 4-aminophenazone (AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) catalyzed by horseradish peroxidase. The resulting quinone imine dye was measured at 520 nm using the Model 680XR microplate reader (BioRad, Hercules).

2.11. Glutathione Peroxidase Activity Assay. The activity of glutathione peroxidase (GPx) was assayed using a colorimetric assay kit (Cayman, MI, USA). This assay is based on the principle that oxidized glutathione (GSSG) produced upon reduction of an organic peroxide by GPx is immediately converted to its reduced form (GSH) with concomitant oxidation of NADPH to $NADP^+$. The oxidation of NADPH was monitored spectrophotometrically using the Model 680XR microplate reader (BioRad, Hercules) as a decrease in absorbance at 340 nm.

2.12. Estimation of Glutathione. The total glutathione (GSH) content was measured using the reduced glutathione assay kit (Cayman, MI, USA). In this assay, o-phthalaldehyde (OPA) reacts with GSH present in the sample, and the fluorescence intensity (ex. 340 nm, em. 420 nm) was measured every 30 sec for a total of 60 min using Fluostar Optima Fluorometer (NY, USA).

2.13. Bradford Protein Assay for the Quantification of Total Protein. All assays were normalised for variations in cell number using the Bradford protein assay described by Bradford [52].

2.14. Data Analysis. Results obtained are presented as the means ± the standard error of measurement (SEM). One way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison tests were used to determine statistical significance between treatment groups. Differences between treatment groups were considered significant if P was less than 0.05 ($P < 0.05$).

3. Results

3.1. Total Phenolic Content of Various Pomegranate Juice Extracts. Our analysis of polyphenolic derivatives in PJE indicates the presence of gallic acid equivalents (GAE) in the Helow, Malasi, Qusum and Hamedh varieties (Table 1). Helow and Malasi showed the highest GAE compared to Qusum and Hamadh, the latter showed the least GAE.

3.2. Neuroprotective Effects of Pomegranate Juice Extracts on MPTP-Induced Neurotoxicity. Extracellular LDH activity and intracellular NAD^+ levels were used as measurements

Table 1: Gallic acid equivalent content in selected varieties of pomegranate juice extracts from Oman.

Name of pomegranate varieties	GAE (mg/100 g)
Helow	572.739 ± 0.261
Malasi	544.155 ± 0.506
Qusum	314.452 ± 0.086
Hamadh	281.671 ± 0.101

GAE: gallic acid equivalent.

of neurotoxicity in primary human foetal neurons. Our data shows that treatment with MPTP (0.05 mM) alone for 24 hours not only increased extracellular LDH activity, but also reduced intracellular NAD^+ levels significantly. In contrast, pretreatment with all PJE varieties significantly reduced extracellular LDH activity (Figure 1(b)) and ameliorated the MPTP-mediated decline in intracellular NAD^+ levels in a dose-dependent manner (Figure 1(a)). The Helow and Malasi varieties showed the greatest neuroprotective effect compared to Qusum and Hamadh. As well, MPTP treatment leads to a significant depletion of intracellular ATP levels after 24 hours (Figure 1(c)). Pretreatment with PJE increased ATP levels significantly, compared to MPTP treatment alone, with Helow and Malasi showing the greatest effect compared to that of Qusum and Hamadh. No significant changes in extracellular LDH activity (Figure 1(d)) and intracellular NAD^+ levels (Figure 1(e)) were detected in neuronal cells treated with PJE alone. Thus, it was possible to conclude that PJE varieties were effective for the protection of human neurons against MPTP-mediated toxicity *in vitro*.

3.3. Effect of PJE Varieties on MPTP-Induced ROS Formation and Alterations to Antioxidant Enzyme Activity. Since free radicals are thought to play a major role in the mechanism(s) of MPTP-induced neurotoxicity, we investigated whether MPTP administration can increase formation of lipid peroxidation and alter the activity of the endogenous antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and glutathione (GSH) levels *in vitro*. Interestingly, MPTP treatment alone (0.05 mM) did not significantly increase the levels of malondialdehyde (MDA), and no significant effect in MDA levels was observed in PJE-pretreated cells (Figure 2(a)). However, a significant reduction in GPx (Figure 2(b)) activities and GSH levels (Figure 2(c)) was observed following MPTP treatment, which was attenuated when the cells were pretreated with PJE in a dose-dependent manner. In contrast, total SOD (Figure 2(d)) and CAT activities (Figure 2(e)) increased by one- and two-fold, respectively, in neuronal cells treated with MPTP, and the effect was reduced following pretreatment of MPTP-treated cells with PJE in a dose-dependent manner. Again, Helow and Malasi showed the greatest antioxidant effect compared to Qusum and Hamadh.

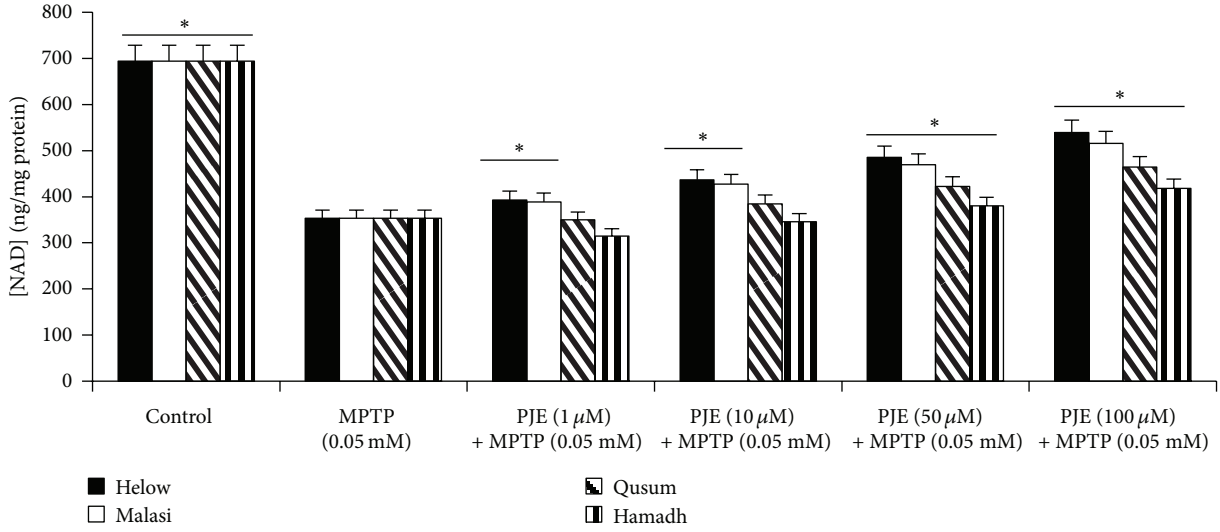
4. Discussion

Although several new therapies have emerged to treat PD, these treatment strategies only provide symptomatic relief and do not affect the progression of the disease [53]. Moreover, long-term use of these drugs can induce adverse side effects which may not be tolerated by patients with PD. Therefore, newer, more effective drugs that specifically target PD development are needed. Recent studies have focused on the benefits of naturally occurring phytochemicals that exhibit potent antioxidant effects as potential neuroprotective agents [54, 55]. Pomegranate has antioxidant function that may help protect neurons against MPTP-induced neurotoxicity. The neuroprotective effects of PJE have been previously demonstrated using an *in vivo* transgenic animal model of AD [56]. However, the effects of PJE on MPTP-induced neurotoxicity in primary human neuronal cells have not been investigated previously.

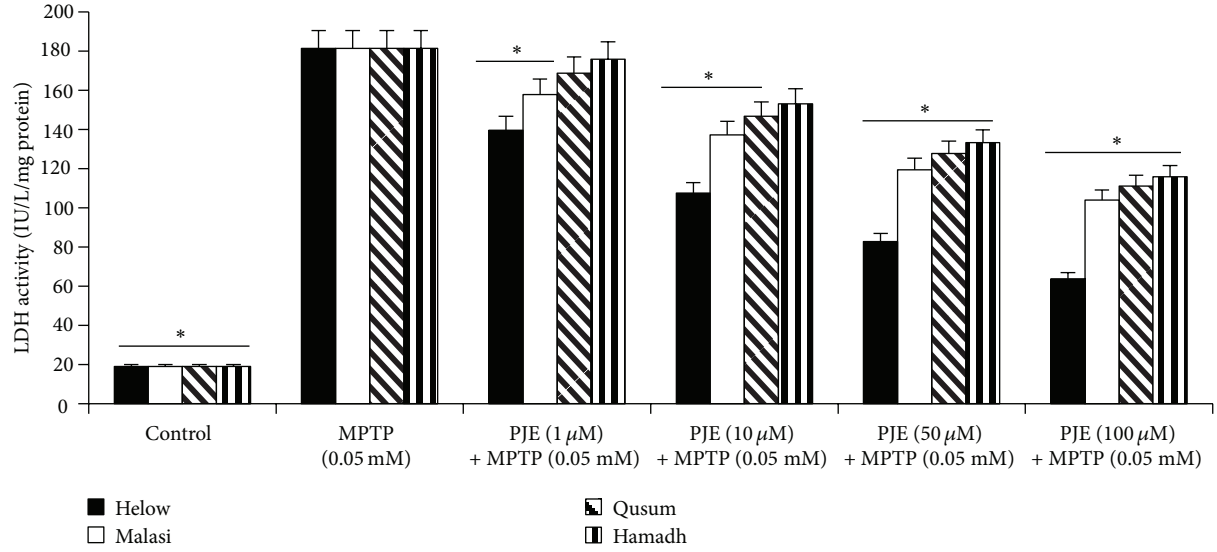
The biotransformation of MPTP into MPP^+, which is catalyzed by the mitochondrial enzyme monoamine oxidase B, represents the major route for MPTP-mediated neurotoxicity [57]. The conversion of MPTP to MPP^+ has been suggested to induce the formation of ROS. This notion is supported by previous studies which showed increased superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) levels during the biotransformation of MPTP (reviewed in [58]). While the damage induced by $O_2^{\bullet-}$ is limited, it can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$) which readily forms the more reactive $^{\bullet}OH$ radical. Other studies have shown that MPTP induces toxicity through ATP depletion and mitochondrial dysfunction. Moreover, Kutty et al. (1991) showed that ATP depletion plays a major role in MPTP-induced neuronal cell death [59].

In this paper, we showed that selected PJE can protect against MPTP-induced neurotoxicity in primary human neurons in a dose-dependent manner by attenuating MPTP-induced increase in extracellular LDH activity. However, we did not observe a significant increase in lipid peroxidation, an established measure for oxidative stress. MDA is widely used to assess lipid peroxidation both *in vitro* and *in vivo* [60]. However, it is likely that MDA can form complexes with other biological components such as protein, lipids, and nucleic acids which can contribute to an underestimation of endogenous lipid peroxidation [61]. On the contrary to our lipid peroxidation data, we also show that MPTP can lead to distinct alterations in endogenous antioxidant defense mechanisms. MPTP treatment has been previously shown to significantly increase Mn-SOD and CuZn-SOD activities in the striatum of C57BL/6 mice, which is suggestive of acute oxidative stress insult [62]. SOD is upregulated in cells when $O_2^{\bullet-}$ is produced in excessive levels [63]. This observation suggests that SOD may play a role in the toxicity observed following acute treatment of MPTP, although ROS formation may not play a major role in MPTP-induced toxicity. We also observed a significant increase in CAT after a 24-hour treatment with MPTP. CAT is an enzyme that is involved in the detoxification of ROS and the elimination of hydrogen peroxide (H_2O_2) in particular [64]. The increase in both intracellular SOD and CAT activities may therefore represent an adaptive response due to the leakage of free radicals during impaired mitochondrial respiration.

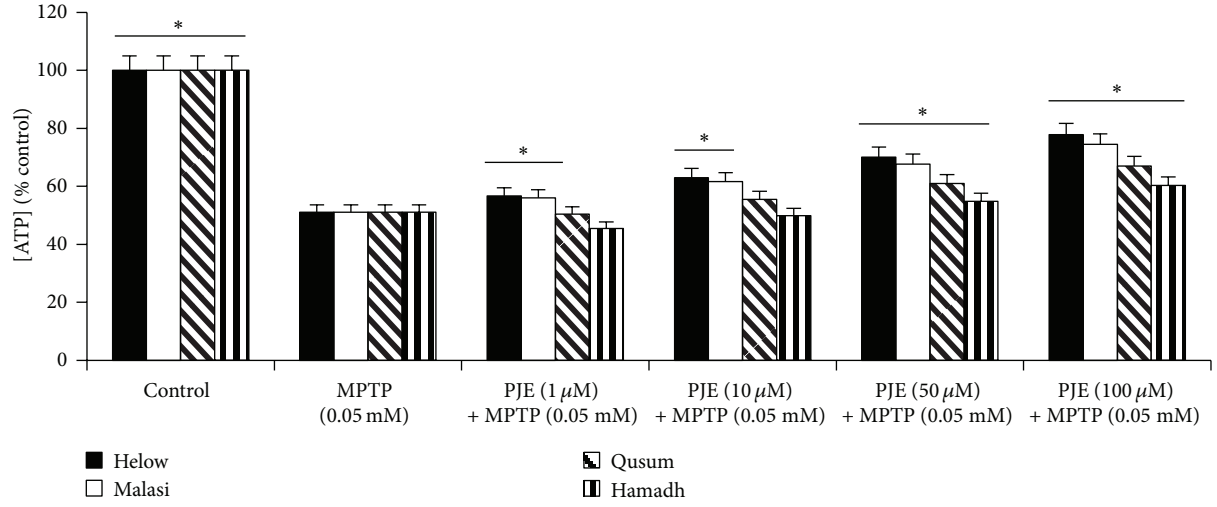
Treatment with MPTP also leads to reduced activity of GPx and decreased levels of the essential pyridine nucleotide



(a)



(b)



(c)

Figure 1: Continued.

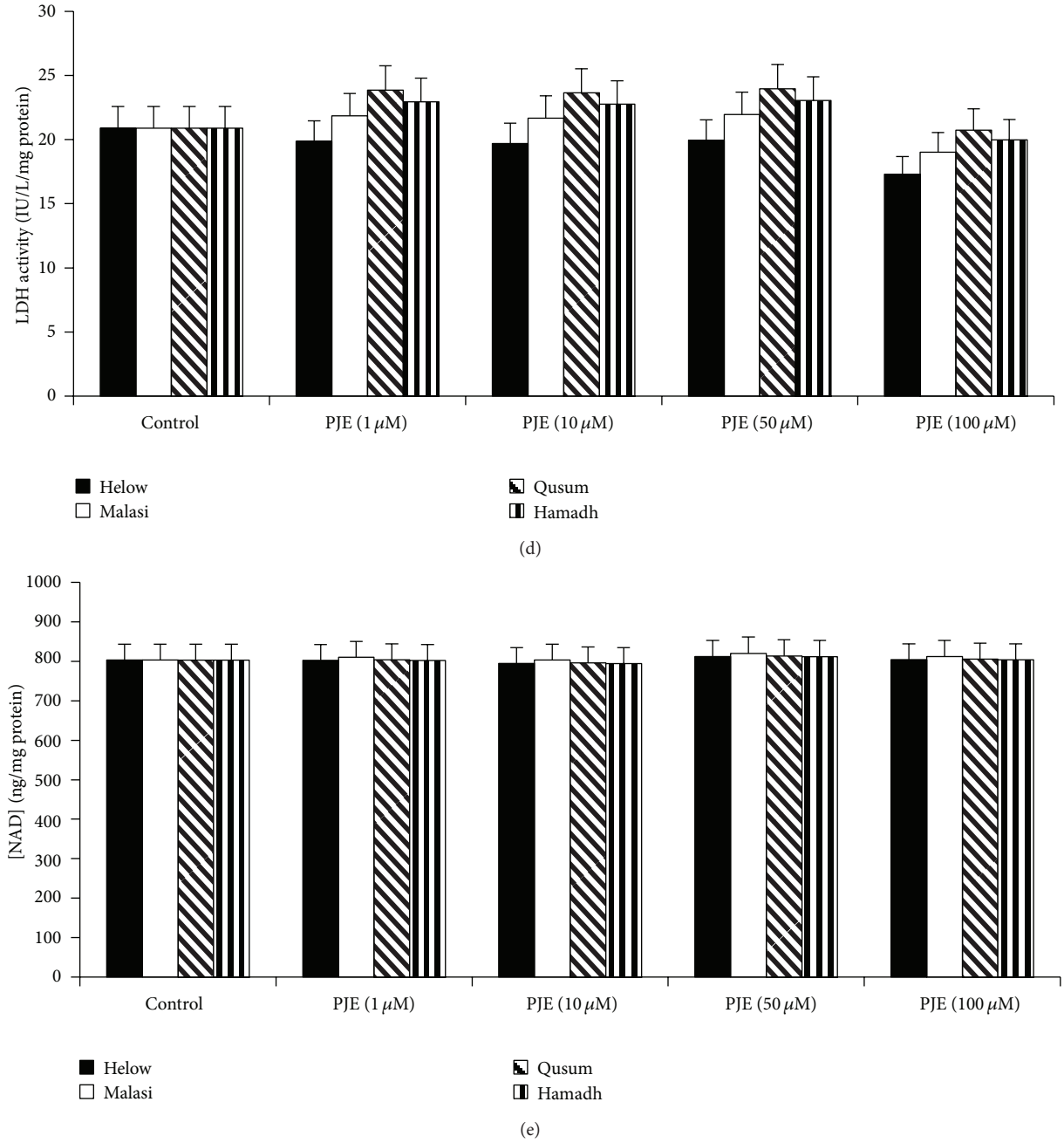
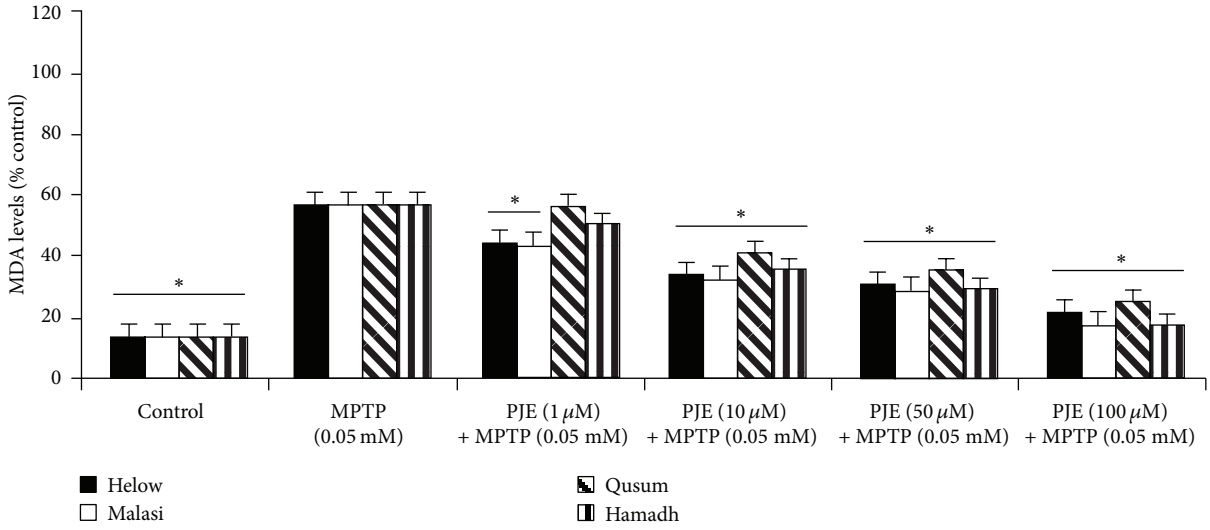
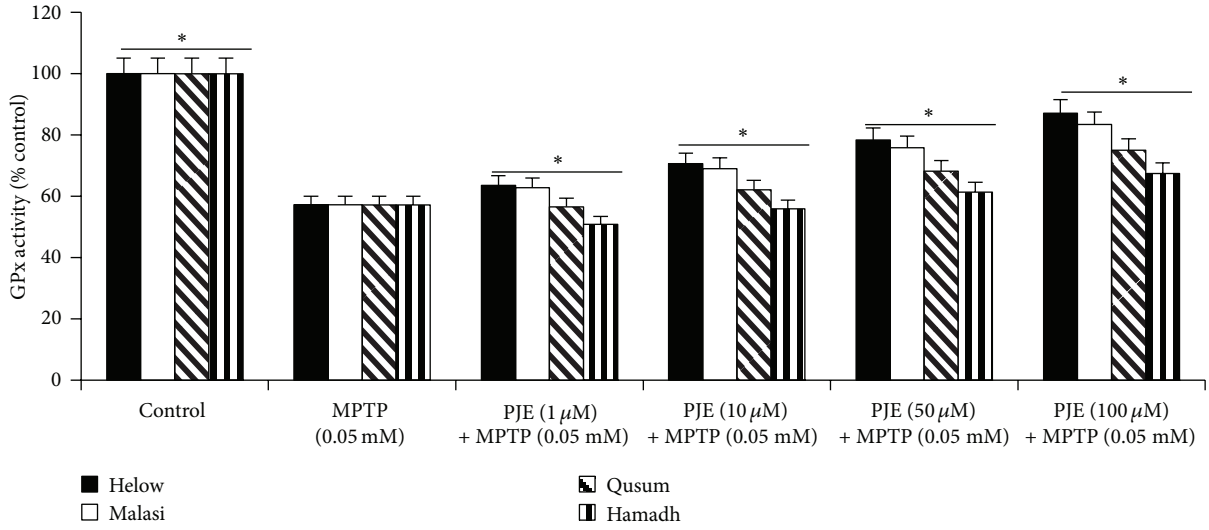


Figure 1: Effect of PJE on MPTP-induced NAD^+ and ATP depletions and cell viability in human neurons. Effect of (a) Helow, Malasi, Qusum, and Hamadh varieties on NAD^+ depletion in the presence of MPTP (0.05 mM) for 24 hours ($^*P < 0.05$ compared with 0.05 mM MPTP alone); (b) Helow, Malasi, Qusum, and Hamadh varieties on extracellular LDH activity in the presence of MPTP (0.05 mM) ($^*P < 0.05$ compared with 0.05 mM MPTP alone), $n = 4$ for each treatment group; (c) Helow, Malasi, Qusum, and Hamadh varieties on intracellular ATP levels in the presence of MPTP (0.05 mM) ($^*P < 0.05$ compared with 0.05 mM MPTP alone), $n = 4$ for each treatment group; (d) Helow, Malasi, Qusum, and Hamadh varieties alone on extracellular LDH activity ($^*P < 0.05$ compared with control), $n = 4$ for each treatment group; (e) Helow, Malasi, Qusum, and Hamadh varieties alone on intracellular NAD^+ levels ($^*P < 0.05$ compared with control), $n = 4$ for each treatment group.

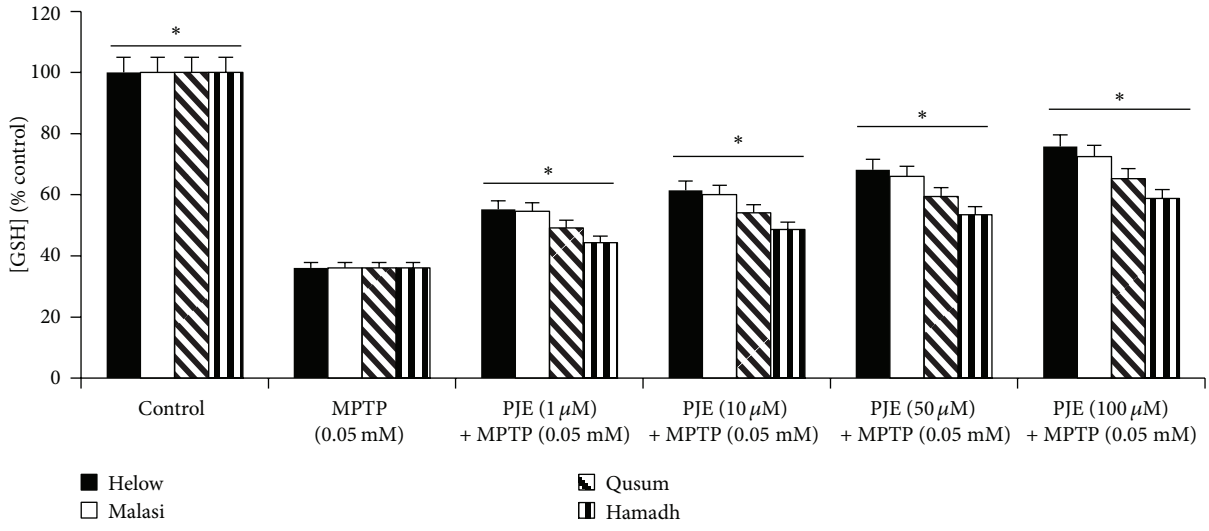
NAD^+, ATP, and GSH in primary human neurons after a 24-hour exposure. The maintenance of GPx activity appears crucial for the maintenance of cell viability during oxidative insult [65–68]. One study showed that increased GPx expression could protect against H_2O_2-mediated oxidative stress due to methamphetamine as measured by extracellular LDH activity [69]. Moreover, previous studies have shown that MPP^+, the metabolite of MPTP induces GSH depletion without increasing the levels of oxidized glutathione disulfide (GSSG) [70]. Reduced GSH levels may occur due to that MPP^+ induced decline in intracellular NAD^+ and ATP stores which are necessary for GSH anabolism, release, and



(a)



(b)



(c)

Figure 2: Continued.

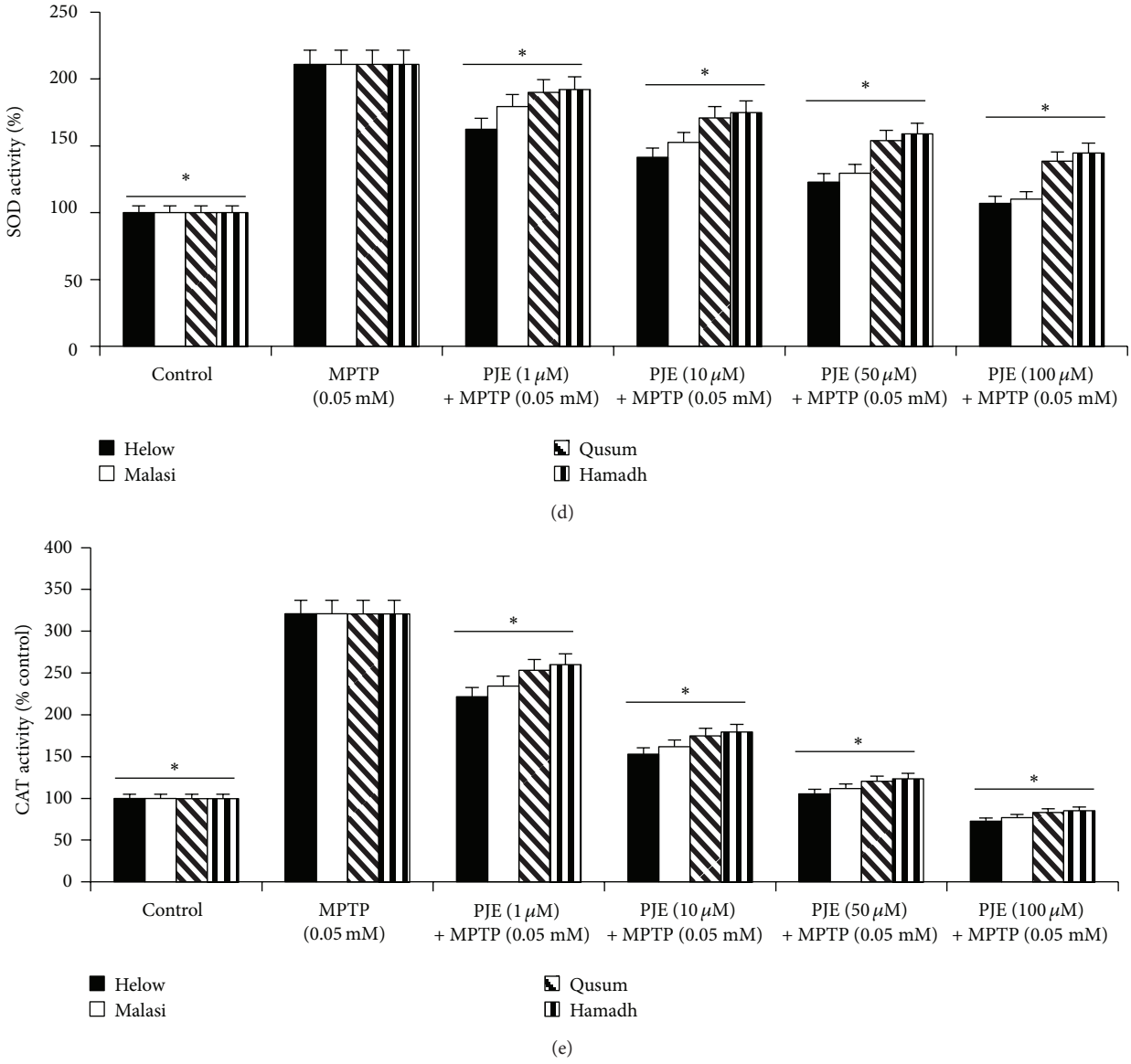


Figure 2: Effect of PJE on MPTP-induced increase in lipid peroxidation and MPTP-mediated changes to endogenous antioxidant activities in human neurons. Effect of (a) Helow, Malasi, Qusum, and Hamadh varieties on MDA levels in the presence of MPTP (0.05 mM) for 24 hours ($^*P < 0.05$ compared with 0.05 mM MPTP alone); (b) Helow, Malasi, Qusum, and Hamadh varieties on GPx activity in the presence of MPTP (0.05 mM) for 24 hours ($^*P < 0.05$ compared with 0.05 mM MPTP alone); (c) Helow, Malasi, Qusum, and Hamadh varieties on intracellular GSH levels in the presence of MPTP (0.05 mM) for 24 hours ($^*P < 0.05$ compared with 0.05 mM MPTP alone); (d) Helow, Malasi, Qusum, and Hamadh varieties on SOD activity in the presence of MPTP (0.05 mM) for 24 hours ($^*P < 0.05$ compared with 0.05 mM MPTP alone); (e) Helow, Malasi, Qusum, and Hamadh varieties on CAT activity in the presence of MPTP (0.05 mM) for 24 hours ($^*P < 0.05$ compared with 0.05 mM MPTP alone).

consequent hydrolysis. Taken together, our data suggests that MPTP exposure can limit the endogenous antioxidant defense, subsequently increasing the vulnerability of neuronal cells to additional oxidative stress. An imbalance in the function of endogenous antioxidant defense mechanisms can lead to the accumulation of free radicals and ROS and increased susceptibility to oxidative stress, which contributes to the pathogenesis of PD.

Different brain cell types are used to study the effects of oxidative stress in culture. Although our data relate to fetal neuronal cultures, it is likely that our results also reflect what is happening in adult astrocytes and neurons. Human cell culture models have demonstrated a neuroprotective role of both astrocytes and microglial cells against ROS mediated neuronal cell death. However, at the same time, evidence exist, linking neurotoxicity to oxidative stress mediated astrocyte/microglial activation [71, 72]. We have previously shown that the inflammatory profile is conserved between human and simian adult and foetal astrocytes and neurons [73, 74]. Therefore, human foetal brain cells are

a relevant model to study neurodegenerative diseases and MPTP-induced toxicity in particular.

PJE are known to exhibit antioxidant and anti-inflammatory properties. PJE have been shown to have a variety of protective effects in several disease models, including reduced low-density lipoprotein (LDL) aggregation, oxidative stress, amyloid load, and improved cognitive behaviour in AD mice [35–43]. Nevertheless, the effect of PJE against the MPTP toxicity in human neurons has not been previously investigated. Our data shows that PJE can reverse the effect of MPTP on the activities of antioxidant enzymes and attenuate MPTP-induced toxicity in a dose-dependent manner. Helow and Malasi varieties showed a more potent effect against MPTP toxicity compared to Qusum and Hamadh. An assessment of total phenolic compounds present in these varieties suggests that the latter have the lowest concentration of phenolics compared to Helow and Malasi varieties and can explain the lower protective effects observed by Qusum and Hamadh. The neuroprotective effects of polyphenols have been associated with their antioxidant and free-radical scavenging, iron/metal chelating ability, as well as their anti-inflammatory properties [75–78].

Our findings show that PJE at the stated concentrations have no toxic effect on human neurons and may therefore be therapeutically safe. However, little information is available in the literature regarding the absorption, bioavailability, biodistribution, and metabolism of important bioactive constituents found in PJE, such as phenolic acids, flavonoids, and tannins [79]. An *in vitro* study of the digestion of pomegranate phenolic compounds showed that these molecules are present during digestion in relatively large amounts (29%). However, anthocyanins are metabolised or degraded (97%). Seeram et al. (2008) investigated the bioavailability of polyphenols derived from PJE in liquid and lypophilised form. Plasma bioavailability, determined by examining GAE levels 6 hours after consumption, was not statistically different between the 2 interventions. The time of maximum concentration was delayed in the polyphenol powder extract (2.58 ± 0.42 h) compared with that of pomegranate juice (0.65 ± 0.23 h) and polyphenol liquid extract (0.94 ± 0.06 h) [80]. It is likely that the bioavailability of pomegranate polyphenols may be affected by several factors, including individual variability, differential processing of pomegranate juice, and the analytical techniques employed to detect low postprandial concentrations of these metabolites [81].

In conclusion, PJE provide protection against the neurotoxic effects of MPTP in human neurons, and the mechanisms of protection may be related to their antioxidant activity and botanical phenolic constituents. The potential neuroprotective effects of PJE warrant further investigation.

Conflict of Interests

The authors of the paper do not have direct financial relationship with the commercial identities mentioned in this paper that might lead to a conflict of interests.

Authors' Contribution

Nady Braidy and Gilles J. Guillemin wrote this paper. Nady Braidy, Subash Selvaraju, and Musthafa Mohamed Essa performed the experiments presented in this paper. Nady Braidy, Gilles J. Guillemin, Musthafa Mohamed Essa, Samir Al-Adawi, Abdullah Al-Asmi, Hamed Al-Senawi, Ammar Abd Alrahman Alobaidy, and Ritu Lakhtakia contributed to the revision of this paper. The work was performed in the laboratory of Gilles J. Guillemin.

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Research Article

Neuroprotective Effects of Hesperidin, a Plant Flavanone, on Rotenone-Induced Oxidative Stress and Apoptosis in a Cellular Model for Parkinson's Disease

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Rotenone a widely used pesticide that inhibits mitochondrial complex I has been used to investigate the pathobiology of PD both *in vitro* and *in vivo*. Studies have shown that the neurotoxicity of rotenone may be related to its ability to generate reactive oxygen species (ROS), leading to neuronal apoptosis. The current study was carried out to investigate the neuroprotective effects of hesperidin, a citrus fruit flavanol, against rotenone-induced apoptosis in human neuroblastoma SK-N-SH cells. We assessed cell death, mitochondrial membrane potential, ROS generation, ATP levels, thiobarbituric acid reactive substances, reduced glutathione (GSH) levels, and the activity of catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) using well established assays. Apoptosis was determined in normal, rotenone, and hesperidin treated cells, by measuring the protein expression of cytochrome c (cyt c), caspases 3 and 9, Bax, and Bcl-2 using the standard western blotting technique. The apoptosis in rotenone-induced SK-N-SH cells was accompanied by the loss of mitochondrial membrane potential, increased ROS generation, the depletion of GSH, enhanced activities of enzymatic antioxidants, upregulation of Bax, cyt c, and caspases 3 and 9, and downregulation of Bcl-2, which were attenuated in the presence of hesperidin. Our data suggests that hesperidin exerts its neuroprotective effect against rotenone due to its antioxidant, maintenance of mitochondrial function, and antiapoptotic properties in a neuroblastoma cell line.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. It is characterised by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta and subsequent depletion of dopamine in the striatum, the main projection area of the substantia nigra. Numerous studies using postmortem human tissues, animal models, and neuronal cell lines have reported the involvement of several pathological mechanisms responsible for the loss of dopaminergic neurons in PD, including elevated levels of iron, ubiquitin-proteasome system (UPS) dysfunction and impairment, altered calcium homeostasis, excitotoxicity, inflammation, oxidative stress, and release of apoptotic factors [1, 2].

Rotenone is a naturally occurring lipophilic compound exhibiting insecticide-like properties and is obtained from the roots of certain plants species (*Derris* and *Lonchocarpus*)

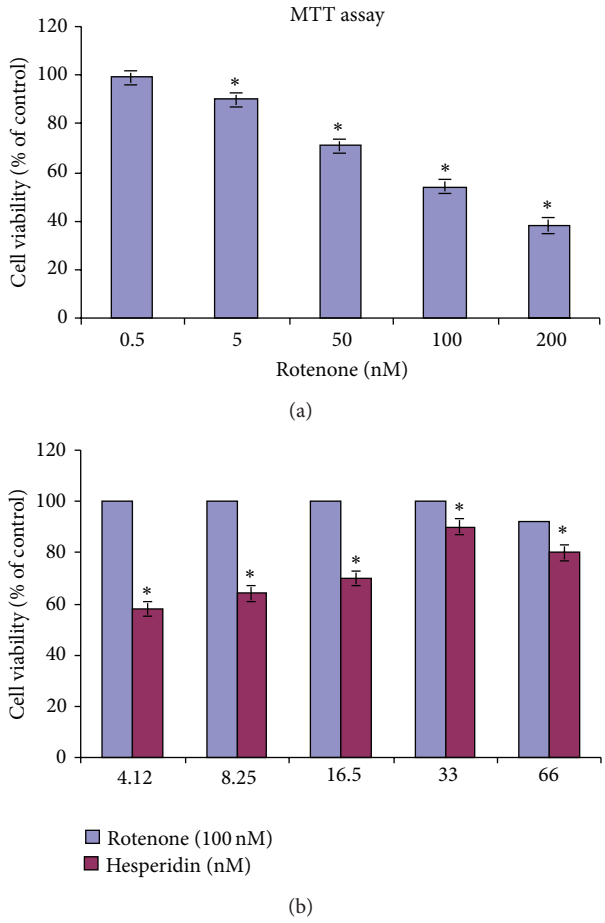

FIGURE 1: Effect of hesperidin on rotenone-induced reduction in cell proliferation in SK-N-SH neuroblastoma cells. (a) The dose-dependent effect of rotenone (0.5, 5, 50, 100, and 200 nM) on cell proliferation after 24 h. Values are presented as mean ± SD of four experiments in each group. 50% inhibition concentration value (IC_{50}) was found to be 100 nM. ∗ indicates significance compared to nontreated cells. (b) The dose-dependent effect of hesperidin (2.5, 5, 10, 20, and 40 μg) alone and against rotenone-induced changes on cell proliferation. Values are presented as mean ± SD of four experiments in each group. Treatment with hesperidin alone (blue column) (2.5, 5, 10, 20, and 40 μg) did not affect cell proliferation. Hesperidin (2.5, 5, 10, and 20 μg) pretreatment dose dependently enhanced cell proliferation against rotenone toxicity.

[3]. It is one of the common neurotoxic agents used to examine the development of PD in animal models [4] and induces similar toxicity in primary dopaminergic cultures derived from embryonic mouse mesencephalon [5], PC12 cells [6], and human neuroblastoma SH-SY5Y cells [7]. It inhibits mitochondrial electron transfer chain (ETC) complex I, which enhances the formation of ROS and leads to modest depletion of ATP and mitochondrial dysfunction culminating in apoptotic cell death [8].

Current pharmacological therapies for PD are inadequate, and alternative strategies such as stem cell therapy, neurotransplantation, and deep brain stimulation are still in infant stage. There has been considerable interest in the development of neuroprotective drugs from natural origins as a therapeutic strategy for PD [9]. Citrus fruits and their products are important sources of health-promoting constituents and are widely consumed around the world [10]. Hesperidin is a naturally occurring flavanone that exists in citrus and other plants and can be isolated in large amounts from the peels of *Citrus aurantium* (bitter orange), *Citrus sinensis* (sweet orange), and *Citrus unshiu* (satsuma mandarin) [11]. Hesperidin is reported to exert a wide range of pharmacological effects such as antioxidant, anti-inflammatory, antihypercholesterolemic, and anticarcinogenic properties [12]. It has also been demonstrated that hesperidin can protect neurons against various types of insults associated with many neurodegenerative diseases [13]. In this study, we investigated the neuroprotective effect of hesperidin on rotenone-induced cellular model for PD by analysing its effect on rotenone-mediated oxidative stress generation, mitochondrial dysfunction, and apoptosis in human neuroblastoma SK-N-SH cells.

2. Materials and Methods

2.1. Chemicals. Rotenone, hesperidin, thiobarbituric acid (TBA), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-diacetyl dichlorofluorescein (DCFH-DA), rhodamine 123 (Rh-123), heat-inactivated fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), glutamine, penicillin-streptomycin, EDTA, and trypsin were purchased from Sigma Chemicals Co., St. Louis, USA. Anti-Bcl-2, anti-Bax, caspase 3, caspase 9, and cytochrome c antibodies were obtained from Cell Signalling (USA) and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc., USA. ATP Bioluminescence Assay Kit HS II was purchased from Roche Molecular Biochemicals. Anti-mouse and anti-rabbit secondary antibodies were purchased from Genei, Bangalore, India.

2.2. Cell Culture. The SK-N-SH neuroblastoma cell line was obtained from the National Centre for Cell Science (NCCS) Pune, India. Cells were grown in (DMEM) 12 (1 : 1), supplemented with 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 U/mL), gentamicin (100 μg/mL), and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Sigma Chemicals Co, St Louis, USA), and the medium was changed every two days. Cells were maintained at 37°C in CO_2 incubator in a saturated humidity atmosphere containing 95% air and 5% CO_2. Rotenone and hesperidin were dissolved in fresh DMSO (0.05%) prior to each experiment. Hesperidin was added 4 h prior to rotenone treatment.

In Experiment I, cells were incubated with different concentrations of rotenone (2.5, 5, 50, 100, and 200 nM) for 24 h, and MTT assay was performed to detect IC_{50} value of rotenone. In Experiment II, cells were pretreated with different concentrations of hesperidin (2.5, 5, 10, 20, and 40 μg) for 4 h and then incubated with rotenone (effective dose) for 24 h. The effective dose of hesperidin was used to identify potential neuroprotective effects against rotenone toxicity.

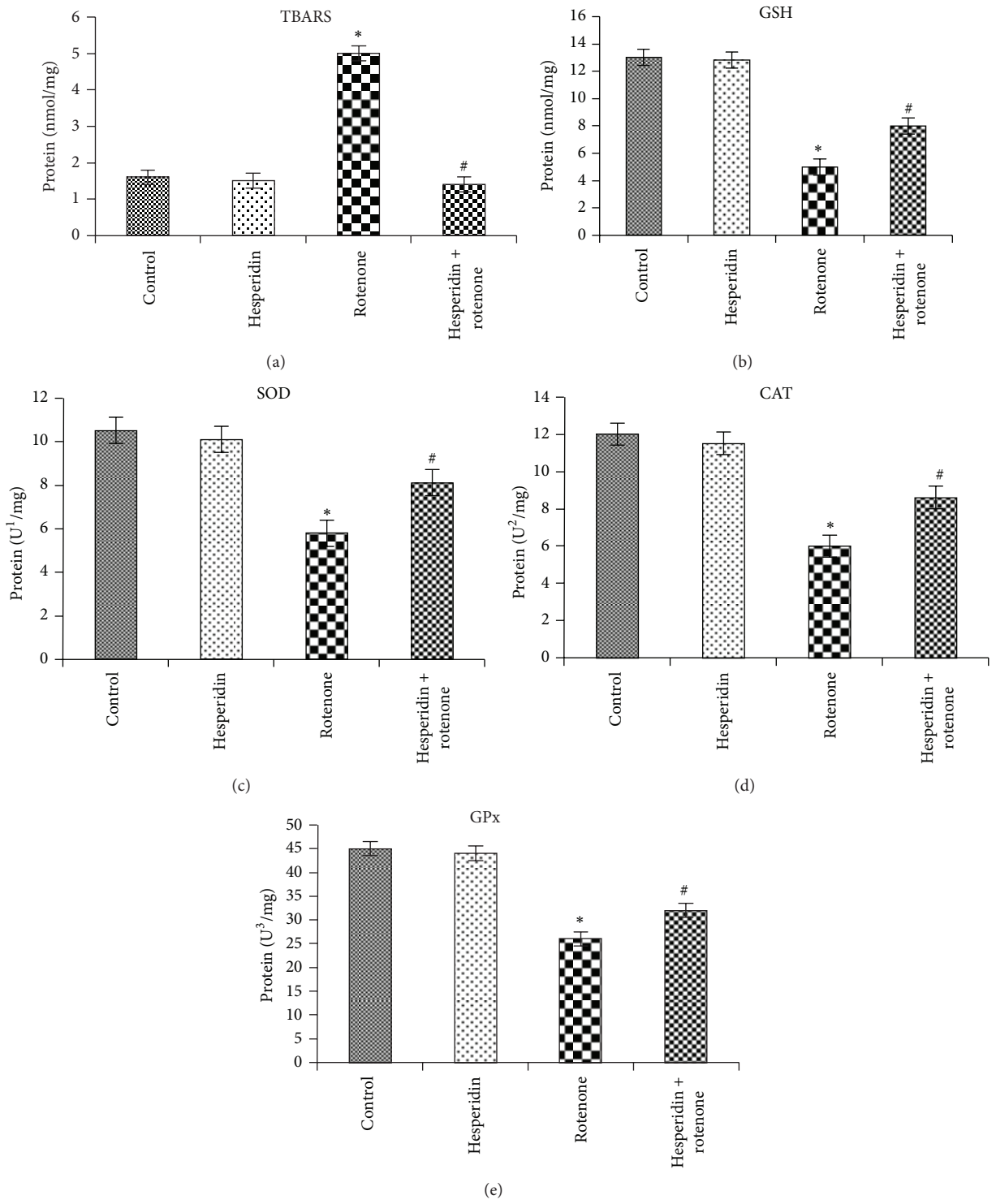

FIGURE 2: Effect of hesperidin (20 μg) on rotenone (100 nM)-induced oxidative and antioxidative indices. Rotenone treatment significantly increased and decreased the levels of TBARS and GSH, respectively, as compared to control cells, while hesperidin pretreatment significantly decreased and enhanced the levels of TBARS and GSH as compared to rotenone alone treated cells (Figures 2(a) and 2(b)). Values are presented as mean ± SD of four experiments in each group. $^{*}P < 0.05$ compared to control, and $^{\#}P < 0.05$ compared to rotenone group (DMRT). Rotenone treatment enhanced the activities of SOD, CAT, and GPx as compared to untreated cells, while hesperidin pretreatment significantly downregulated the activities of enzymatic antioxidants as compared to rotenone alone treated cells ((c), (d), and (e)). Values are given as mean ± SD of four experiments in each group. [1]Enzyme concentration required for 50% inhibition of nitroblue tetrazolium reduction in 1 min. [2]Micromoles of hydrogen peroxide consumed per minute. [3]Micrograms of glutathione consumed per minute.

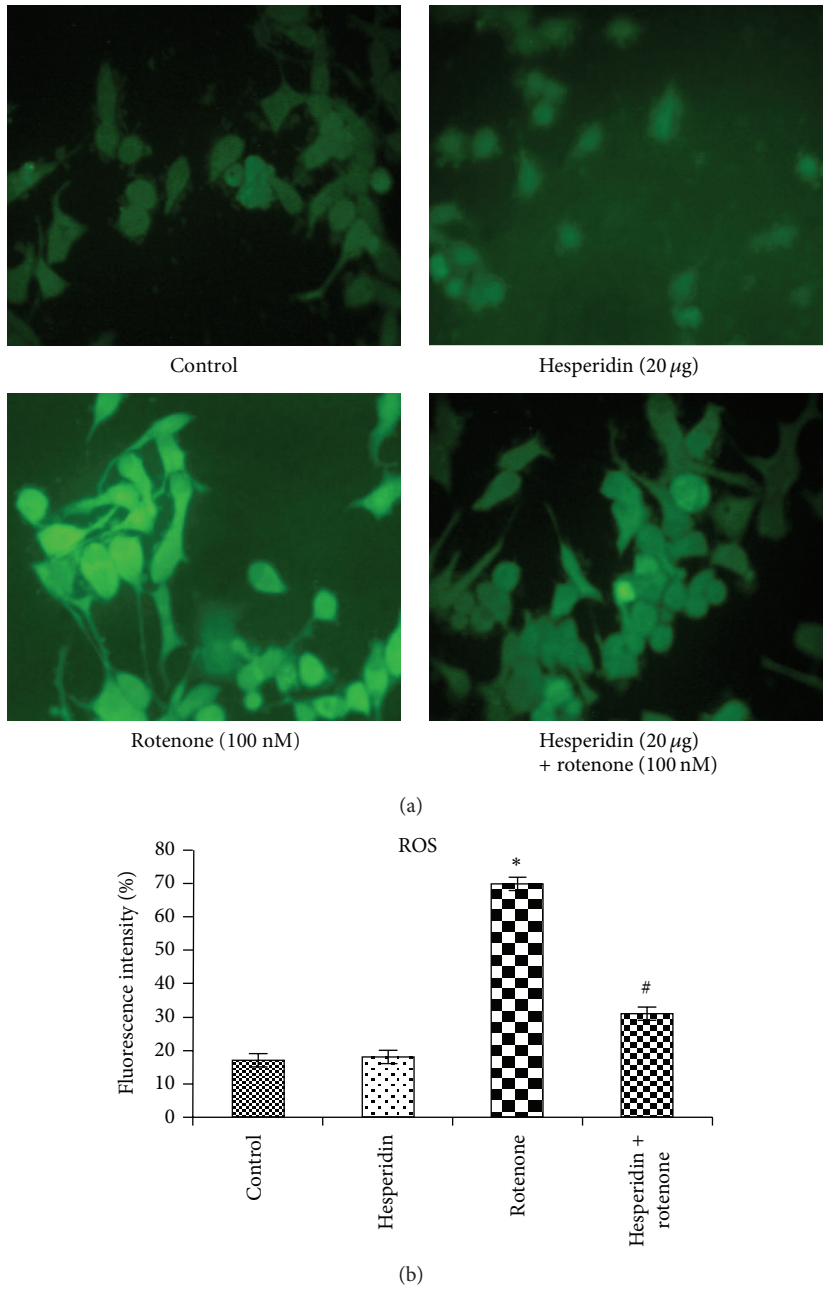

Figure 3: Effect of hesperidin on rotenone-induced ROS generation in SK-N-SH cells. (a) Microscopic images showing the preventive effect of hesperidin against rotenone-induced ROS generation by DCFDA staining. (b) Rotenone (100 nM) treatment significantly increased the levels of ROS as compared to control cells, while hesperidin (20 μg) pretreatment significantly decreased the levels of ROS as compared to rotenone alone treated cells. Values are given as mean ± S.D. of four experiments in each group. $^{*}P < 0.05$ compared to control, and $^{\#}P < 0.05$ compared to rotenone group (DMRT).

2.3. MTT Assay. The proliferation of cells treated with various concentrations of rotenone and hesperidin was determined by the MTT assay based on the detection of mitochondrial dehydrogenase activity in living cells [14, 15]. MTT was added to each well, and the plates were incubated at 37°C for 4 h. Afterwards, the cells were centrifuged for 10 min, and the supernatant was removed, 200 μL of DMSO was added into each well, and absorbance was measured in a microplate reader (Molecular Devices, CA, USA) at 595 nm.

2.4. Determination of Intracellular ROS Generation. The formation of ROS was measured by using a nonfluorescent probe, 2,7-diacetyl dichlorofluorescein (DCFH-DA) that can penetrate into the intracellular matrix of cells, where it is oxidized by ROS to form fluorescent dichlorofluorescein (DCF) [16]. The percentage of ROS was estimated in the control, hesperidin, and rotenone-treated SK-N-SH neuroblastoma cells. Briefly, an aliquot of the isolated cells 8×10^6 cells/mL was made up to a final volume of 2 mL in

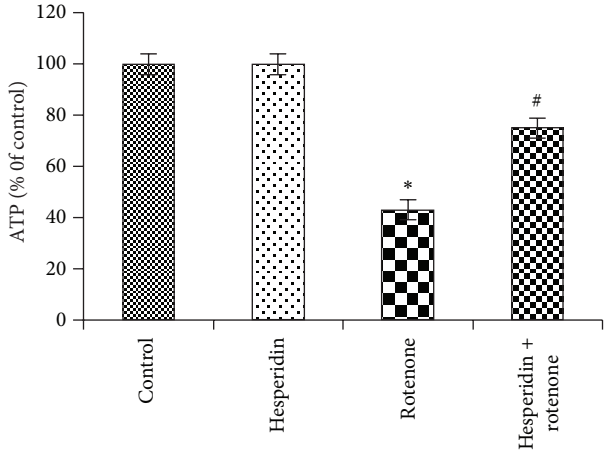

Figure 4: Measurement of intracellular ATP levels. Rotenone (100 nM) treatment significantly reduced the levels of ATP as compared to control cells, while hesperidin (20 μg) pretreatment significantly enhanced the levels of ATP as compared to rotenone alone treated cells. Values are given as mean ± S.D. of four experiments in each group. $^{*}P < 0.05$ compared to control, and $^{\#}P < 0.05$ compared to rotenone group (DMRT).

normal PBS (pH 7.4). Then, 1 mL aliquot of cells was taken to which 100 μL DCFH-DA (10 μM) was added and incubated at 37°C for 30 min. Fluorescent measurements were made with excitation and emission filters were set at 485 ± 10 nm, and 530 ± 12.5 nm respectively (Shimadzu RF-5301 PC spectrofluorometer). All initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were expressed as percentage; increase in fluorescence was calculated using the formula $[(F_{t_{30}} - F_{t_0})/(F_{t_0} \times 100)]$, and the fluorescence intensities at 0 and 30 min were measured.

2.5. Measurement of Intracellular ATP Levels. Cells were collected by centrifugation, and intracellular ATP was measured with a luminometer using an ATP Bioluminescence Assay Kit HS II (Roche Molecular Biochemicals) according to the manufacturer's instructions.

2.6. TBARS Assay. SK-N-SH neuroblastoma cells were suspended in 130 mM KCl and 50 mM PBS containing 0.1 mL of 0.1 M dithiothreitol (DTT) and centrifuged at 20,000 g for 15 min (4°C). The supernatant was taken for biochemical estimation. The level of lipid peroxidation was determined by analysing TBARS as previously described [17]. The pink-coloured chromogen formed by the reaction of 2-TBA with breakdown products of lipid peroxidation was measured.

2.7. SOD Activity Assay. Superoxide dismutase (SOD) activity was assayed by the method based on the inhibition of the formation of (NADH-PMS-NBT) complex as previously described [18].

2.8. Catalase Activity Assay. Catalase (CAT) activity was assayed by the decomposition of hydrogen peroxide as previously described [19]. A decrease in absorbance due to H_2O_2 degradation was monitored at 240 nm for 1 min.

2.9. Glutathione Peroxidase Activity Assay. The activity of glutathione peroxidase (GPx) was assayed spectrophotometrically as previously described [20]. Briefly, a known amount of enzyme preparation was allowed to react with hydrogen peroxides (H_2O_2) and GSH for a specified time period. The GSH content remaining after the reaction was measured.

2.10. Estimation of Glutathione. The total GSH content was measured by the method as previously described [21]. This method was based on the development of a yellow colour, when 5,5-dithiobis (2-nitrobenzoic acid) was added to compound containing sulfhydryl groups.

2.11. Changes in Mitochondrial Transmembrane Potential ($\Delta\psi m$). The change in $\Delta\psi m$ in different treatment groups was observed microscopically and determined fluorometrically using the fluorescent dye rhodamine 123 (Rh-123) as previously described [22]. Briefly, after incubation with treatment compounds for 24 h, 1 μL of fluorescent dye Rh-123 (5 m mol/l) was added to the cells and returned to the incubator for 15 min [22]. The cells were then washed with PBS, observed under fluorescence microscope, and estimated by using blue filter (450–490 nm) (Olympus BX60 fluorescence microscope). Polarized mitochondria emit orange-red fluorescence, and depolarized mitochondria emit green fluorescence. The fluorescence intensity was measured at 535 nm using FLUOstar OPTIMA fluorometer (Durham, NC, USA).

2.12. Western Blotting. Briefly, cells seeded in 6-well plates were harvested and washed with PBS. Cells were lysed in 100 μL lysis buffer containing 20 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 30 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride and subjected to 12.5% polyacrylamide gel electrophoresis. A total volume of 30 μg of protein was loaded per lane. The separated proteins were blotted onto a PVDF membrane by semidry transfer (Bio-Rad). After blocking with 5% nonfat milk in TBS, the membranes were then incubated with various antibodies: Bcl-2, Bax, caspases 3 and 9, cytochrome c, and β-actin. The following dilutions were used for Bcl-2 and Bax (1 : 500), cytochrome c, caspases 3 and 9 (1 : 1000), and β-actin (1 : 2000). After primary antibody incubation, the membranes were incubated with secondary antibody at a concentration of 1 : 2000. Then, the membranes were washed with Tris-buffered saline and 0.05% Tween 20 thrice for 10 min interval; after extensive washes in TBST, the bands were visualized by treating the membranes with 3,3′-diaminobenzidine tetrahydrochloride (western blot detection reagent, Sigma, USA). Densitometry was performed using "Image J" analysis software.

2.13. Data Analysis. Statistical analysis was performed using one-way analysis of variance followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 12.0. Results were expressed as mean ± SD for four experiments in each group. P values < 0.05 were considered significant.

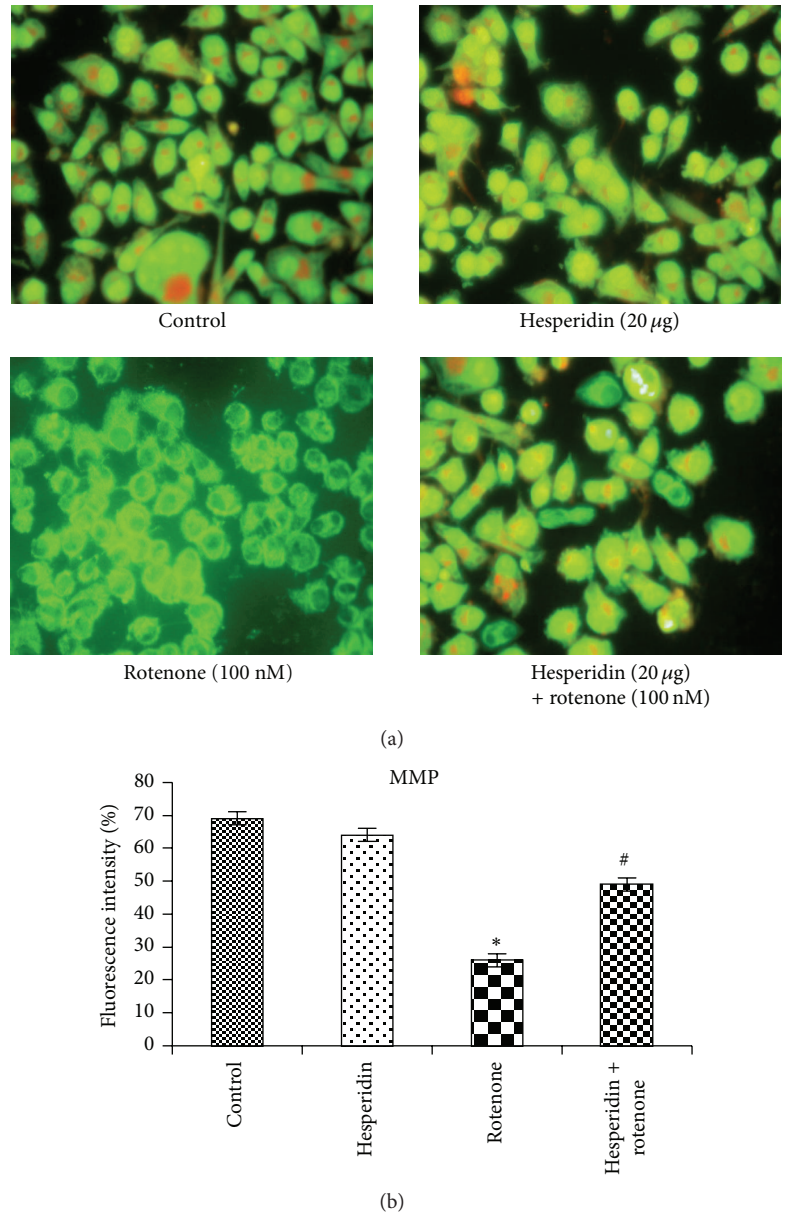

Figure 5: Alteration in mitochondrial membrane potential of control, hesperidin, and rotenone-treated SK-N-SH cells. Rotenone (100 nM) significantly decreased mitochondrial membrane potential, while hesperidin (20 μg) pretreatment significantly increased MMP in rotenone-treated SK-N-SH cells ((a) and (b)). Values are given as mean ± S.D of four experiments in each group. $^{*}P < 0.05$ compared to control, and $^{\#}P < 0.05$ compared to rotenone groups (DMRT).

3. Results

3.1. Effect of Hesperidin on Rotenone-Induced Cell Death. Rotenone treatment (2.5, 5, 50, 100, and 200 nM for 24 h) of SK-N-SH cells induced a dose-dependent reduction in cell proliferation, with approximately 50% proliferation observed at 100 nM (Figure 1(a)). Hesperidin dose dependently (0.5, 5, 10, 20, and 40 μg) attenuated the changes in cell proliferation induced by 100 nM rotenone (Figure 1(b)), with approximately 85% protection following treatment with 20 μg hesperidin after 24 h.

3.2. Effect of Hesperidin on Rotenone-Induced ROS Formation. Figures 2(a) and 2(b) indicate the levels of TBARS and GSH in rotenone-treated SK-N-SH cells incubated with and without hesperidin. Rotenone treatment (100 nM) significantly increased the levels of TBARS parallel to decreased levels of GSH in SK-N-SH cells compared with nontreated cells. Pretreatment with hesperidin (20 μg) to rotenone-treated cells significantly decreased the levels of TBARS and increased GSH levels significantly, compared to cells treated with rotenone alone. Figures 2(c), 2(d), and 2(e) elucidate the activities of SOD, catalase and GPx in rotenone-treated

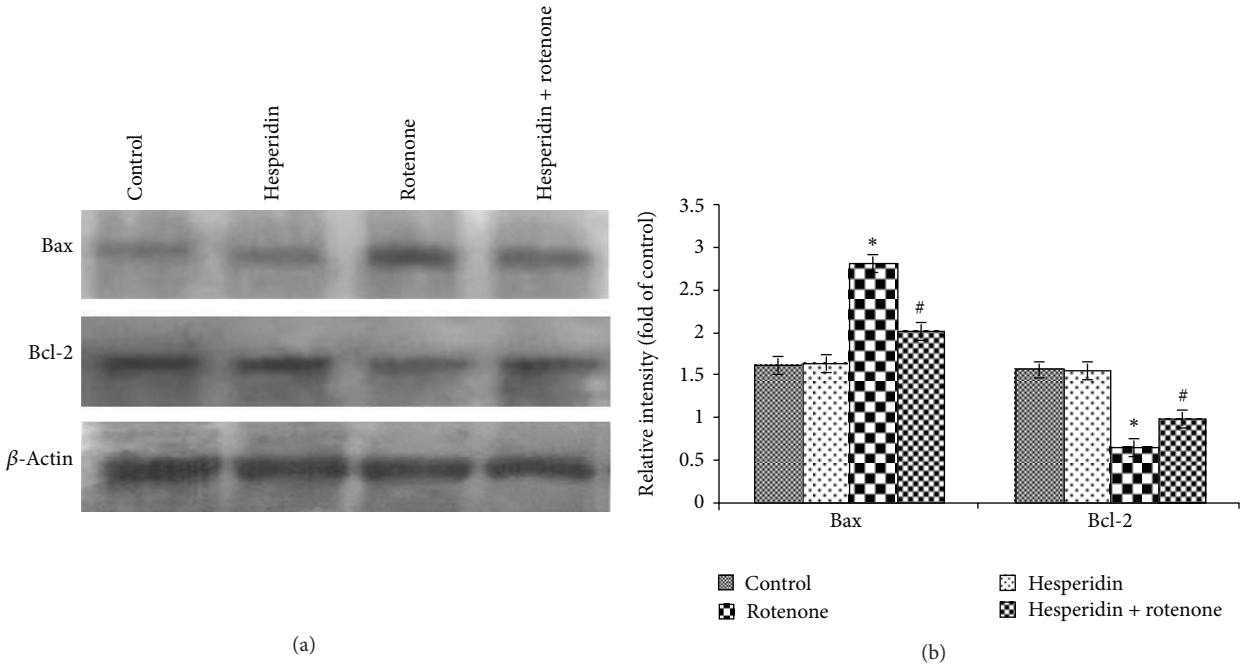

Figure 6: Effect of hesperidin on rotenone-induced Bax and Bcl-2 expressions in SK-N-SH cells. Rotenone (100 nM) significantly enhanced the expression of Bax and diminished the expression of Bcl-2, while hesperidin (20 μg) pretreatment significantly diminished the expression of Bax and elevated the expression of Bcl-2 in rotenone-treated SK-N-SH cells ((a) and (b)). Western blot data are quantified by using β-actin as an internal control, and the values are expressed as arbitrary units and given as mean ± SD of four experiments in each group. $^{*}P < 0.05$ compared to control, and $^{\#}P < 0.05$ compared to rotenone alone treated group.

SK-N-SH cells incubated with and without hesperidin. Compared with untreated cells, rotenone (100 nM) treatment increased SOD, catalase and GPx activities in SK-N-SH cells (Figures 2(c), 2(d), and 2(e)). Pretreatment with hesperidin significantly decreased the activities of SOD, catalase, and GPx, compared to cells treated with rotenone alone.

3.3. Effect of Hesperidin on Intracellular ROS Generation. The formation of intracellular ROS was measured in terms of fluorescence by DCF (Figures 3(a) and 3(b)). Addition of rotenone (100 nM) to cells caused a significant increase (~411%) in DCF fluorescence. Pretreatment of the cells with hesperidin (20 μg) lowered rotenone-induced free radical release as compared to rotenone-treated cells alone. No significant changes in ROS formation were detected in SK-N-SH cells treated with hesperidin alone.

3.4. Effect of Hesperidin on Rotenone-Induced ATP Depletion. Rotenone treatment (24 hr) depleted cellular ATP levels (57 ± 4.8% at 100 nM) (Figure 4). Pretreatment with hesperidin increased ATP levels significantly compared to rotenone only treated cells. No significant changes in ATP levels were detected in SK-N-SH cells treated with hesperidin alone.

3.5. Effect of Hesperidin on Mitochondrial Membrane Potential ($\Delta\psi m$). Figures 5(a) and 5(b) show the mitochondrial membrane potential ($\Delta\psi m$) measured by determining the red/green fluorescence ratio in the presence of Rh-123. Cells treated with 100 nM rotenone resulted in significant dissipation of $\Delta\psi m$. The average green fluorescence ratio was increased as a result of rotenone treatment as compared to untreated controls. Pretreatment with hesperidin to rotenone-treated cells displayed much higher red/green fluorescence, indicating a polarized state of mitochondrial membrane as compared to rotenone treatment alone.

3.6. Effect of Hesperidin on Apoptotic Markers. Rotenone treatment (100 nM) decreased the protein expression levels of antiapoptotic B-cell CLL/lymphoma 2 (Bcl-2) and increased the protein expression levels of proapoptotic Bcl-2-associated X protein (Bax) (Figures 5(a) and 5(b)). Hesperidin pretreatment (20 μg) attenuated the rotenone-induced reduction in Bcl-2 expression and increased expression of Bax (Figures 6(a) and 6(b)). We also examined the effect of hesperidin on the protein expression of cyt c and caspases 3 and 9. Treatment with rotenone (100 nM) for 24 h increased the expression of cyt c, caspases 3 and 9 at the protein level (Figures 7(a) and 7(b)). Hesperidin pretreatment significantly ameliorated the rotenone-mediated increase in cyt c, caspases 3 and 9 protein expression levels (Figures 7(a) and 7(b)).

4. Discussion

Our results show that rotenone is cytotoxic to SK-N-SH neuroblastoma cells in line with previous studies. However, our group is the first to show that hesperidin can attenuate the toxic cascade in rotenone-treated cells. Reduction of the tetrazolium salt MTT to a blue formazan product is widely

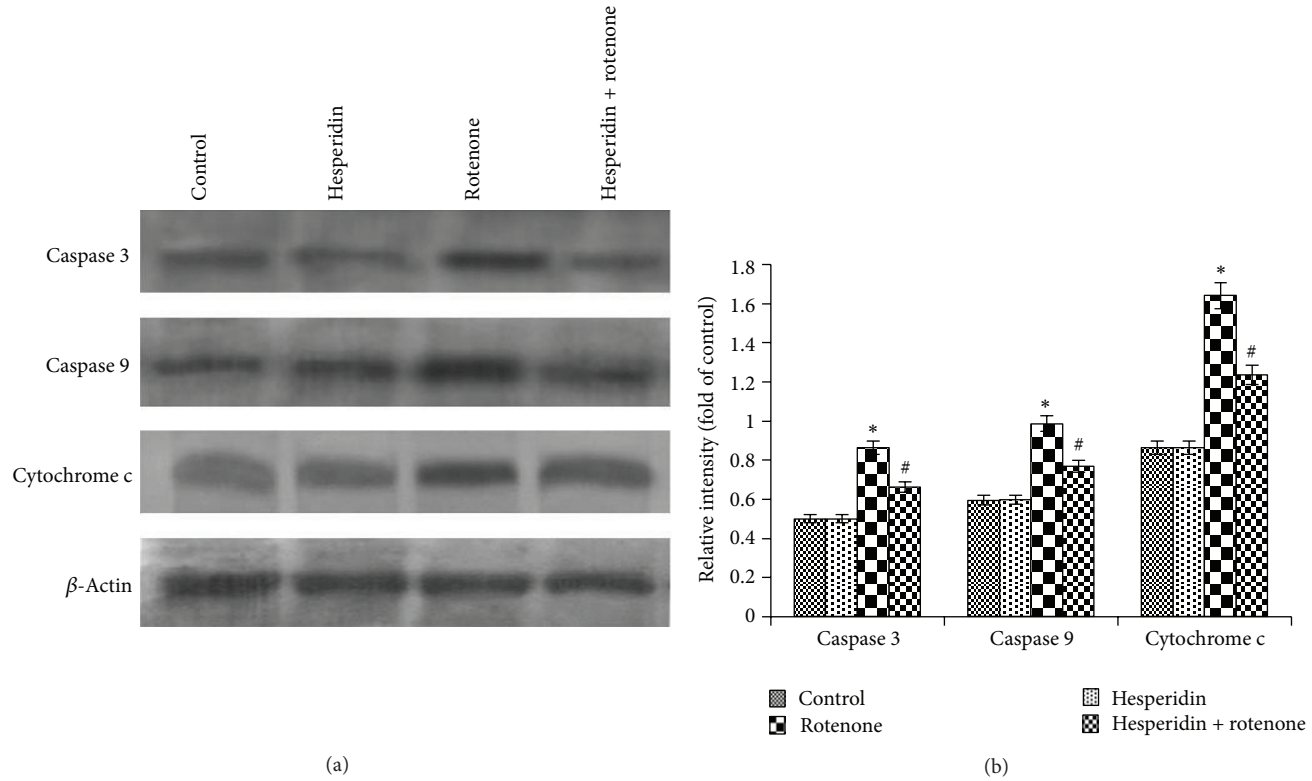

FIGURE 7: Effect of hesperidin on rotenone-induced cyt c and caspases 3 and 9 expressions in SK-N-SH cells. Rotenone (100 nM) significantly enhanced the expressions of cyt c, caspases 3 and 9, while hesperidin (20 μg) pretreatment significantly diminished the expressions of cyt c, caspases 3 and 9 in rotenone-treated SK-N-SH cells ((a), (b), and (c)). Western blot data are quantified by using β-actin as an internal control, and the values are expressed as arbitrary units and given as mean ± SD of four experiments in each group. $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to rotenone groups.

used for assessing cellular proliferation. The reduction is mainly catalyzed by dehydrogenases localized in the mitochondria of proliferating cells [23]. Optimal mitochondrial function is a determinant of the proliferation and hence overall viability of these cells by playing a central role in regulating apoptotic cell death signalling by controlling cellular energy metabolism, contribution of reactive oxygen species (ROS) formation, and release of apoptotic factors into the cytosol [24]. Data obtained from the MTT assay in the present study suggest a direct neuroprotective action of hesperidin against rotenone-mediated mitochondrial dysfunction.

Our data also demonstrate that rotenone treatment can stimulate the generation of intracellular ROS in SK-N-SH cells, as corroborated with previous studies [25, 26]. Inhibition of respiratory complex I by rotenone causes electrons to accumulate within respiratory chain components. These electrons can be added directly to oxygen molecules to produce O_2^- [27, 28]. The mitochondria are the principle intracellular sources of ROS and also the major targets of oxidative stress [29]. Hesperidin treatment significantly reduced ROS generation in rotenone-treated cells, which might be due to ROS scavenging property of hesperidin [30] and transition metal ions chelating properties [31]. Additionally, other potential mechanisms such as the ability of hesperidin to enhance glutathione content could be involved in its protective effect on rotenone-induced oxidative stress generation.

When cells were incubated with rotenone, we found increased levels of TBARS, indicating overproduction of free radicals along with glutathione depletion, which is corroborated with previous studies [7]. Our results show that the increase in the enzymatic activities of SOD, GPx, and CAT in neuroblastoma cells incubated with rotenone alone is likely due to a response towards increased ROS generation following rotenone treatment. Cassarino et al. [32] reported that complex I inhibition induces SOD activity in the brain tissue. Increment in GPx activity in rotenone alone treated cells indicated that there was an increase in the concentration of lipid peroxides and/or H_2O_2. Reduced GSH levels may also account for the increase in GPx activity [7].

In the present study, ATP levels were significantly reduced in rotenone-treated cells alone as compared to nontreated controls, which corroborates with previous studies [26, 33]. Since ATP is necessary for downstream events in the apoptotic cascade [34], the depletion of the intracellular ATP supply may not be expected to induce apoptosis. One study showed that both rotenone and 2-deoxyglucose caused similar ATP depletion. However, only rotenone exposure was toxic, suggesting that rotenone toxicity resulted from additional mechanisms such as oxidative damage [26, 35]. Similarly, mitochondrial ETC inhibitors such as rotenone deplete ATP and induce apoptosis, while oxidative phosphorylation inhibitors, which inhibit ATP synthesis without inhibiting the ETC, fail to induce apoptosis despite depleting

ATP to the same extent as ETC inhibitors in dopaminergic neuronal cells. Therefore, intracellular ATP depletion *per se* is not sufficient for apoptosis induction. Rather, ROS production plays an essential role in apoptosis induced by mitochondrial ETC inhibition. Pretreatment with hesperidin partially attenuated rotenone-induced ATP deficiency.

It was reported by several groups that complex I inhibition by rotenone may result in the opening of mitochondrial permeability transition pores (PTP), which induces a specific conformational change of complex I and massive production of ROS [36–38]. Increased levels of ROS within the mitochondria are known to cause further mitochondrial membrane depolarization and further release of ROS. Due to rotenone treatment, mitochondrial membrane potential is reduced, leading to increased mitochondrial permeability and results in the enhanced release of cyt c from the mitochondria, which triggers activation of caspases 3 and 9 culminating with cell death [39]. cyt c is not only an important electron carrier in the mitochondrial respiration chain, but also a death messenger in the cytosol, which forms apoptosome complexes with Apaf-1, dATP, and caspases 9 and 3 [40].

The efflux of the cyt c from the mitochondria is also regulated by BAD protein [41]. In an apoptotic cell, BAD protein displaces Bax and binds to the antiapoptotic members Bcl-2 and Bcl-xL [41]. Bax and Bcl-2 are involved in the regulation of caspase 3 mediated apoptosis [42]. Numerous studies have shown that Bcl-2, as a negative regulator of cell death in the Bcl-2 family members, protects cells against apoptosis induced by various stimuli in a wide variety of cell types [43], whereas Bax is a positive regulator of cell death which promotes or accelerates cell death. It was reported that rotenone induces Bax expression in dopaminergic neuronal cell line MND9 [44]. Elevations in proapoptotic proteins, such as Bax, are believed to stimulate mitochondrial generation of ROS and contribute to neuronal cell death in neurodegenerative diseases. Moreover, overexpression of Bcl-2 disrupts the proapoptotic proteins of Bax and prevents the mitochondrial release of cyt c, thereby inhibiting the activation of caspases and ultimately apoptosis [45, 46]. The ratio of Bcl-2 to Bax determines the survival or death of neurons following an apoptotic stimulus. Administration of hesperidin prevents the loss of $\Delta\psi m$, increases the mitochondrial permeability, and prevents the release of cyt c from the mitochondria, thereby inhibiting caspases 3 and 9, thus restoring the imbalance in the expression profiles of Bax and Bcl-2, and preventing cell death. Moreover, overexpression of Bcl-2 disrupts the proapoptotic proteins of Bax and prevents the mitochondrial release of cyt c, thereby inhibiting the activation of caspases, and apoptosis [45, 46].

Although the exact neuroprotective mechanism of action of hesperidin remains unclear, it is likely to demonstrate both antioxidant and cell signalling properties. Our data is in line with previous studies showing that hesperidin therapy could significantly attenuate ROS formation by reducing the levels of TBARS and restored antioxidant enzyme activity and GSH to physiological levels in the brain [47] and cultured PC12 cells [48]. Another study showed that hesperidin treatment could reduce stress-induced anxiety, impaired locomotor activity, and mitochondrial dysfunction in mice by modulating the nitrergic pathway [49]. Hesperidin also protected cortical neurons from ROS-mediated injury by activation of the prosurvival Akt and ERK1/2 signalling pathways [50]. These pathways are involved in the inhibition of the release of proapoptotic proteins such as apoptosis signal-regulating kinase 1 (ASK1), BAD, and caspases 3 and 9, suggesting that the neuroprotective effects of hesperidin may be due to its effect on a yet unidentified receptor [50]. Moreover, hesperidin can also protect against amyloid-beta- (Aβ-) associated neurotoxicity, and glutamate-induced excitotoxicity [13]. A more recent study showed that hesperidin treatment could reduce cerebral damage due to induced stroke in the rat brain due to the reduction of free radicals and associated neuroinflammation [51]. The results of our present study suggest that hesperidin attenuates neuronal damage induced by rotenone by reducing oxidative stress, mitochondrial dysfunction, and ameliorating apoptosis. These findings may have important implications in the use of hesperidin for the prevention of PD. However, further research involving various animal models and clinical trials is needed to validate hesperidin as a new therapeutic agent.

Disclosure

Kuppusamy Tamilselvam and Nady Braidy are the co-first authors.

Conflict of Interests

The authors of the paper do not have a direct financial relationship with the commercial identities mentioned in this paper that might lead to a conflict of interests.

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Research Article

Protective Effects of Extracts from *Fructus rhodomyrti* against Oxidative DNA Damage *In Vitro* and *In Vivo*

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Objective. To evaluate the potential protective effects of extracts from *Fructus rhodomyrti* (FR) against oxidative DNA damage using a cellular system and the antioxidant ability on *potassium* bromate- ($KBrO_3$-) mediated oxidative stress in rats. *Methods*. The effects of FR on DNA damage induced by hydrogen peroxide (H_2O_2) were evaluated by comet assay in primary spleen lymphocytes cultures. The effects of FR on the activities of SOD, CAT, and GPx and the levels of GSH, hydroperoxides, and 8-OHdG were determined in the plasma and tissues of rats treated with $KBrO_3$. *Results*. FR was shown to effectively protect against DNA damage induced by H_2O_2 *in vitro*, and the maximum protective effect was observed when FR was diluted 20 times. Endogenous antioxidant status, namely, the activities of SOD, CAT, and GPx and the levels of GSH were significantly decreased in the plasma, the liver, and the kidney of the $KBrO_3$-treated rats, while the pretreatment of FR prevented the decreases of these parameters. In addition, the pretreatment of FR was also able to prevent $KBrO_3$-induced increases in the levels of hydroperoxides and 8-OHdG in the plasma, the liver, and the kidney in rats. *Conclusions*. Our findings suggested that FR might act as a chemopreventive agent with antioxidant properties offering effective protection against oxidative DNA damage in a concentration-dependent manner *in vitro* and *in vivo*.

1. Introduction

Plants have played significant roles in maintaining human health and improving the quality of human life for thousands of years [1, 2]. *Fructus rhodomyrti* (FR) is the fruit of the *Rhodomyrtus tomentosa* growing on knolls in wilderness and widely distributed in Guangdong, Guangxi, Yunnan, Fujian, and Taiwan. FR has been used for the production of drinks and wine. FR is a traditional Chinese medicine material with antihepatitis property [3].

Cells that live in an oxygen-rich environment are inundated with various endogenous and exogenous sources of reactive oxygen species (ROS) [4]. The most important target for ROS in the carcinogenesis process is DNA [5, 6]. Irreparable DNA damage is involved in carcinogenesis, aging, and other degenerative diseases [4, 7]. However, enzymatic and nonenzymatic systems, which preserve the oxidant/antioxidant status, are disrupted during oxidative stress, a metabolic derangement due to an imbalance caused by excessive generation of ROS or a diminished capacity of the antioxidant defense system. Dietary factors and natural antioxidants that reduce the impact of ROS can protect DNA damage and thus reduce the risk of cancers [8, 9].

Hydrogen peroxide (H_2O_2) and potassium bromate ($KBrO_3$) are commonly used to induce oxidative damage [10–12]. $KBrO_3$ is a widely used food additive, a water disinfection by-product, and a known nephrotoxic agent. Cellular proliferation was enhanced in the kidney due to oxidative stress generated by $KBrO_3$. It has also been reported that $KBrO_3$ increased the levels of 8-hydroxydeoxyguanosine (8-OHdG), an oxidative DNA adduct, suggesting that it can indirectly induce DNA modifications by oxygen radicals that are involved in carcinogenesis [9, 13].

In a previous study, FR has been confirmed abundant in flavonoid glycosides and phenols, and the flavonoids extracts of FR displayed an antiradical action and antioxidant effects

in serum of rats [3]. However, the biological effects of FR remained poorly understood so far. It is unclear whether or not FR has any protective effects against oxidative DNA damage and antioxidant effects in target organs induced by xenobiotics. In the present study, the potential protective effects of extracts from FR against oxidative DNA damage *in vitro* and oxidative stress induced by $KBrO_3$ *in vivo* were explored.

2. Materials and Methods

2.1. Materials and Equipments. Low melting point agarose, Triton-X100, and sodium lauroylsarcosine were purchased from Sigma Company (USA). The normal melting point agarose (NMPA), RPMI1640 medium, and Tris were purchased from Promega Company. Neonatal calf serum was purchased from Beijing Bangding Company, and H_2O_2 (analytically pure) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products. The antibody against 8-OHdG and 8-OHdG enzyme-linked immunosorbent assay (ELISA) kits was purchased from JaICA (Japan). The confocal laser scanning microscope was purchased from Leica (Germany), and PAC200 transfer electrophoresis instrument was the product of BIO-RAD Company (USA). VE-186 transfer electrophoresis tank was purchased from Shanghai Jinpeng Analytical Instrument Company.

2.2. Preparation of FR Extract. FR originated in Shaoguan, Guangdong, was provided by Traditional Chinese Medicine University of Guangzhou. One kilogram of FR was ground and extracted with 2 L of distilled water at 4°C. After 24 h, the extracts were centrifuged at 15,000 ×g three times. The freeze dry sample was reextracted with 1 L of distilled water. The reextracted sample was extracted with 100% ethanol (1 : 4, *v*/*v*), and then the ethanol-insoluble fraction was collected and freeze-dried. The dried fraction was extracted with distilled water for further experiments. Stock solution (1 g/mL) was prepared with phosphate buffer solution (PBS) before use, boiled for 30 min, and then kept at 4°C. Appropriate concentrations were adjusted before use. The experimental concentration was 1 g/mL. Saline (0.85% NaCl) was used as a control.

2.3. Animals. Male albino rats (130–150 g) of Wistar strain were obtained from the Medical Experimental Animal Center of Sun Yat-Sen University, Guangzhou, China. The rats were housed in polypropylene cages in groups of six rats per cage in a room maintaining of 25 ± 2°C and a relative humidity of 40–70% with a 12 h light/dark cycle. The rats were allowed acclimatizing for one week before the experiments and had free access to standard laboratory feed and water ad libitum. The rats were sacrificed according to the guidelines of the current laws of Ethical Committee for the purpose of control and supervision of experiments on animals in China.

2.4. Culture of Spleen Lymphocytes. Sterile isolation of the intact spleen was immersed in PBS solution at 37°C. The spleen capsule and fat composition were removed. One mm^3 of spleen tissue was cut off, digested in pancreatin (1-2 min), and then placed in the metal filter net with PBS (pore size of 200 meshes). The spleen was triturated with disposable syringe needle so that the cells can be filtered out from the metal filters. The cell suspension was centrifuged at 1000 r/min (centrifugal radius 10 cm) for 3–5 min, and the supernatant was removed. The cells were suspended in the RPMI 1640 medium containing 10% fetal calf serum. Trypan blue staining indicated that the viable cell count was over 95%. The spleen cells density was adjusted to 5×10^6–10^7/mL, and the cells were incubated at 37°C in 5% CO_2 incubator.

2.5. DNA Damage Assay. Primary spleen lymphocyte cells were cultured for 24 h and centrifuged for 3–5 min (1000 r/min centrifugal radius 10 cm). The cells were resuspended in the RPMI 1640 medium with no fetal calf serum. Trypan blue staining shows that the viable cell count was over 95%, and cell density was adjusted to 5×10^6/mL. One ml of spleen cell suspension was added into each of 12 sterilization centrifuge tubes, which were randomly divided into 4 groups of 3 tubes. Twenty-five μL H_2O_2 solution of different concentrations (25, 50, and 125 μmol/L) was added into tubes of the 3 H_2O_2 groups, and equal volume of PBS was added into the blank control group and all tubes were incubated at 4°C for 20 min. Then, single-cell gel electrophoresis (SCGE) was performed.

2.6. Assay for FR Pretreatment In Vitro. A density of 1×10^7/mL of primary cells was prepared. One mL of lymphocyte suspension was added into each of the 18 centrifuged tubes, which were randomly divided into control group, H_2O_2 treatment group, and the FR treatment groups I, II, III, and IV, with 3 tubes in each group. The cells in the FR groups received the treatment of different concentrations of FR extracts (1 mL) and incubated at 37°C in 5% CO_2 for 60 min, followed by the treatment of 25 μL H_2O_2 solution (50 μmol/L) at 4°C for 20 min, while the cells in the H_2O_2 treatment group only received the treatment of 25 μL H_2O_2 solution. The control group cells received PBS treatment. After the treatment, the cells were harvested for SCGE.

2.7. Single-Cell Gel Electrophoresis. Improved methods were used in the study such as Singh, cell preparation in alkaline conditions, alkali treatment, electrophoresis, neutralization, EB staining, reviewing, and analyzing. A laser confocal microscope was used to analyze the morphology of the cells at a wavelength of 488 nm (10 ∗ 20 times magnification). One hundred randomly selected cells were used to calculate the DNA migration rate (tailing rate), and the total length (maximum length of the direction of comet) was considered as the tail length.

2.8. Animal Treatments. Different groups of animals were used to explore the effects of FR on $KBrO_3$-induced oxidative stress and 8-OHdG induction in the renal tissue of rats. Thirty male Wistar rats were randomly divided into 5 groups

(6 rats in each group). Group I received saline injection intraperitoneally (0.85% NaCl) at a dose of 10 mL/kg body weight. Group II received a single intraperitoneal injection of $KBrO_3$ at a dose of 125 mg/kg body weight. Group III received pretreatment with FR by gavage once a day for 5 days at a dose of 150 mg/kg body weight, and groups IV and V received the pretreatment with FR by gavage once a day for 5 days at a dose of 300 mg/kg body weight. After the last treatment with FR, the rats of groups II, III, and IV received a single intraperitoneal injection of $KBrO_3$ at a dose level of 125 mg/kg body weight. Twenty-four hours later, the rats were sacrificed by cervical dislocation.

Blood samples were collected in heparinised tubes, and the plasma was separated by centrifugation at 2000 ×g for 10 min. The tissues (liver and kidney) were isolated and immediately transferred to ice-cold containers containing 0.9% sodium chloride for various analyses. A known amount of tissue was weighed and homogenized in appropriate buffer for the evaluation of various biochemical parameters.

2.9. Biochemical Assays. The antioxidant status was evaluated by measuring the levels of reduced glutathione (GSH) by Khan's method [14] with minor modifications. The activities of glutathione peroxidase (GPx) and SOD were measured as described by Rotruck et al. [15] and Chen et al. [16], respectively. The content of H_2O_2 was assayed by the H_2O_2-mediated horseradish peroxidase-dependent oxidation of phenol red by the method of Pick and Keisari [17]. The activity of catalase (CAT) was measured by the method as described by Sinha [18] with minor modifications. The levels of 8-OHdG in plasma and tissues were measured according to the instruction provided in the assay kit for 8-OHdG.

2.10. Statistical Analysis. Statistical analysis was performed using SPSS15.0. One-way ANOVA ($P < 0.05$) and q test (Student-Newman-Keuls) were used to compare the means among the groups with measurement data after homogeneity testing for homogeneity of variance, and we analyzed enumeration data with chi-square statistics. The level of significance was set at $P < 0.05$.

3. Results

The results of the effects of different concentrations of H_2O_2 on DNA damage *in vitro* were summarized in Table 1. After treatment with 25 μmol/L H_2O_2 for 20 min, the number of comet tail cells (88%) was significantly increased as compared with the result (15%) of the control group. When the concentration of H_2O_2 reached 50 and 125 μmol/L, the numbers of comet cells were 100%. The comet tail length indicated the severity of DNA damage. The tail lengths of comet cells treated with H_2O_2 were significantly longer than those of the control cells (Table 1).

The effects of pretreatment of lymphocytes with FR were shown in Table 2. The data showed that FR of various concentrations could significantly reduce DNA damage induced by H_2O_2 in spleen lymphocytes. The protective effects of FR were significantly increased with increased FR concentrations from 20 to 80 dilution fold as compared with the positive control group ($P < 0.01$), and the maximum protective effect was observed when FR was diluted 20 times. However, when the concentration of FR was 10 dilution folds, the results of comet cell tailing rate and total comet length were significantly increased as compared with the positive control group ($P < 0.01$) (Figure 1, Table 2).

Table 1: DNA damage of lymphocytes induced by H_2O_2 ($n = 100$).

Treatment groups	Number of comet cells (%)	Distance of comet tail (μm) $\overline{x} \pm S$
H_2O_2 25 μmol/L	88.0**	42.89 ± 9.25**
H_2O_2 50 μmol/L	100.0**	50.45 ± 8.64**
H_2O_2 125 μmol/L	100.0**	53.28 ± 9.58**
Control	15.0	28.12 ± 6.75
	$\chi^2 = 298.5$	$F = 259.2$

** $P < 0.01$ compared with the control.

Table 2: The effects of FR on lymphocyte DNA damage induced by H_2O_2 ($n = 100$).

Treatment groups	Number of comet cells (%)	Distance of comet tail (μm) (mean ± SD)
Negative (A)	16.0	21.35 ± 2.54
Positive (B)	98.0	49.23 ± 7.27
Dilution ratio of FR (1 g/mL)		
80 times (C)	82.0*	40.51 ± 8.33*
40 times (D)	62.0**	35.29 ± 7.81**
20 times (E)	28.0**	25.45 ± 4.65**
10 times (F)	45.0**#	34.36 ± 8.12**
	$\chi^2 = 213.7$	$F = 163.5$

* $P < 0.05$, ** $P < 0.01$, compared with the positive control. # $P < 0.05$, compared with E group.

The effects of FR on $KBrO_3$-mediated oxidative stress and antioxidant ability in rats were shown in Tables 3, 4, 5, 6, 7, and 8. The treatment of $KBrO_3$ alone significantly reduced the activities of SOD, CAT, and GPx and the levels of GSH compared with those of the saline-treated control group, and the pretreatment of FR at 150 mg/kg body weight and 300 mg/kg body weight partially recovered the activities of SOD, CAT, and GPx and the levels of GSH in a concentration-dependent manner. However, treatment of FR alone did not produce any effects on the activities of the antioxidant enzymes.

The levels of hydroperoxides and 8-OHdG were significantly elevated in plasma and tissues of the rats treated with $KBrO_3$ as compared with the control rats. Pretreatment of FR significantly decreased the levels of hydroperoxides as compared with $KBrO_3$-treated rats.

4. Discussion

This study demonstrated that FR protected against oxidative DNA damage and to varying degrees reversed the damages

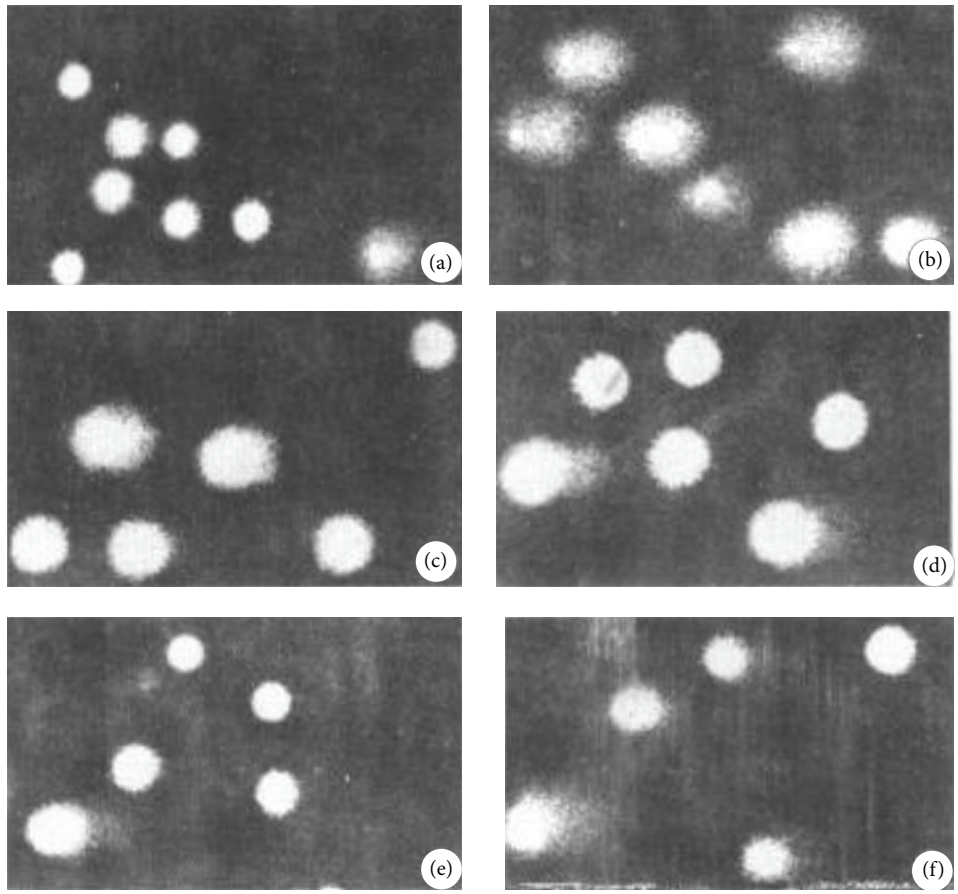

Figure 1: The effects of FR on lymphocyte DNA damage induced by H_2O_2 ($n = 100$). (a) Negative control; (b) positive control; (c) a dilution of 80 times of FR (1 g/mL); (d) a dilution of 40 times of FR (1 g/mL); (e) a dilution of 20 times of FR (1 g/mL); (f) a dilution of 10 times of FR (1 g/mL).

Table 3: The levels of hydroperoxides in plasma and tissues.

Treatment groups	Plasma ($\times 10^{-5}$ mM)	Kidney (mM/100 g tissue)	Liver (mM/100 g tissue)
Saline (control)	10.26 ± 0.71	38.59 ± 2.43	43.27 ± 2.83
$KBrO_3$ (125 mg/kg body weight)	$20.38 \pm 1.29^{\triangle\triangle}$	$73.50 \pm 5.28^{\triangle\triangle}$	$63.26 \pm 4.29^{\triangle\triangle}$
FR (150 mg/kg body weight) + $KBrO_3$	$12.86 \pm 0.83^{**}$	$59.32 \pm 4.22^{*}$	$49.55 \pm 3.36^{*}$
FR (300 mg/kg body weight) + $KBrO_3$	$11.60 \pm 0.78^{**}$	$53.78 \pm 3.73^{**}$	$46.27 \pm 3.69^{*}$
FR (300 mg/kg body weight) alone	10.52 ± 0.73	36.92 ± 2.79	42.65 ± 3.30

Values were expressed as mean ± SD, $n = 6$. The dose of $KBrO_3$ was 125 mg/kg body weight in each group.
$^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$ compared with the control.
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with $KBrO_3$-treated rats.

Table 4: The activities of SOD in hemolysate and tissues.

Treatment groups	Hemolysate U^A	Kidney U^B	Liver U^B
Saline (control)	4.82 ± 0.35	8.64 ± 0.65	9.28 ± 0.62
$KBrO_3$ (125 mg/kg body weight)	$2.65 \pm 0.17^{\triangle\triangle}$	$5.14 \pm 0.35^{\triangle\triangle}$	$6.50 \pm 0.45^{\triangle\triangle}$
FR (150 mg/kg body weight) + $KBrO_3$	$3.48 \pm 0.24^{*}$	$6.82 \pm 0.44^{*}$	$7.22 \pm 0.52^{*}$
FR (300 mg/kg body weight) + $KBrO_3$	$3.85 \pm 0.27^{**}$	$7.37 \pm 0.48^{**}$	$8.35 \pm 0.56^{**}$
FR (300 mg/kg body weight) alone	4.26 ± 0.32	8.43 ± 0.57	8.95 ± 0.59

Values were expressed as mean ± SD, $n = 6$. The dose of $KBrO_3$ was 125 mg/kg body weight in each group.
U^A: enzymes required for 50% inhibition of NBT reduction min/mg Hb; U^B: enzymes required for 50% inhibition of NBT reduction min/mg protein.
$^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$ compared with the control group.
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with $KBrO_3$-treated rats.

Table 5: The activities of CAT in hemolysate and tissues.

Treatment groups	Hemolysate U^A	Kidney U^B	Liver U^B
Saline (control)	3.65 ± 0.27	22.71 ± 1.25	68.26 ± 3.15
$KBrO_3$ (125 mg/kg body weight)	$1.59 \pm 0.13^{\triangle\triangle}$	$13.23 \pm 0.79^{\triangle\triangle}$	49.75 ± 2.62
FR (150 mg/kg body weight) + $KBrO_3$	$2.86 \pm 0.20^{*}$	14.82 ± 0.82	$60.35 \pm 2.79^{*}$
FR (300 mg/kg body weight) + $KBrO_3$	$3.19 \pm 0.25^{**}$	15.37 ± 0.85	$62.85 \pm 2.80^{*}$
FR (300 mg/kg body weight) alone	3.48 ± 0.31	21.28 ± 1.24	66.27 ± 3.02

Values were expressed as mean ± SD, $n = 6$. The dose of $KBrO_3$ was 125 mg/kg body weight in each group.
U^A: μ moles of H_2O_2 utilized/min/mg Hb; U^B: μ moles of H_2O_2 utilized/min/mg protein.
$^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$ compared with the control group.
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with $KBrO_3$-treated rats.

Table 6: The activities of GPx in hemolysate and tissues.

Treatment groups	Hemolysate U^A	Kidney U^B	Liver U^B
Saline (control)	26.28 ± 1.52	10.06 ± 0.61	11.12 ± 0.74
$KBrO_3$ (125 mg/kg body weight)	$13.75 \pm 0.94^{\triangle\triangle}$	$6.15 \pm 0.39^{\triangle\triangle}$	$7.68 \pm 0.46^{\triangle\triangle}$
FR (150 mg/kg body weight) + $KBrO_3$	$17.29 \pm 1.12^{*}$	7.67 ± 0.45	$9.10 \pm 0.65^{*}$
FR (300 mg/kg body weight) + $KBrO_3$	$23.82 \pm 1.50^{**}$	$8.74 \pm 0.56^{*}$	$10.06 \pm 0.63^{*}$
FR (300 mg/kg body weight) alone	26.65 ± 1.63	9.82 ± 0.59	10.55 ± 0.70

Values were expressed as mean ± SD, $n = 6$. The dose of $KBrO_3$ was 125 mg/kg body weight in each group.
U^A: μ moles of GSH utilized/min/mg Hb; U^B: μ moles of GSH utilized/min/mg protein.
$^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$ compared with the control group.
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with $KBrO_3$-treated rats.

caused by oxidative stress via its antioxidant activities. These findings support the hypothesis that FR exerted a protective effect *in vivo* as well as *in vitro*.

We evaluated the protective effects of FR against oxidative DNA damage by H_2O_2 with spleen lymphocytes being based on the following considerations. Firstly, oxidative DNA damage is closely related to aging, and immune senescence plays an important role in aging. During the process of aging, there will be varying degrees of degradation in the immune organs such as spleen and thymus, so it is of great significance to protect the immune cells to avoid oxidative DNA damage caused by chemicals [19]. Secondly, spleen is the largest immune organ in the body, and the cell model is easy to build. Thirdly, spleen lymphocyte models are widely used in experiment to study the protective effects of traditional Chinese medicines.

FR has been reported to contain flavonoid glycosides, phenols, amino acids, organic acids, and carbohydrates [3]. The major antioxidant active constituents of FR are flavonoid glycosides and phenols, which display free radical-scavenging activity and antioxidant properties [20–22]. The observed chemopreventive activity of FR in this study suggested that the protective effects of FR may be attributed to the action of these compounds in FR [23, 24].

In evaluating the effects of FR on lymphocyte DNA damage induced by H_2O_2, we have tried different dilutions of FR and found 10 times dilution working in a different way as compared with 20 times dilution. The difference between their values was statistically significant in terms of the number of comet cells but not statistically significant in terms of the distance of comet tail (Table 2). This is indeed a very interesting phenomenon and might provide evidence to the double-edged sword theory of antioxidants and reductive stress.

Antioxidants may promote oxidation at high concentrations. Skibola CF and Smith MT have found the potentially toxic effects of excessive flavonoid intake. At high doses, flavonoids may act as mutagens, prooxidants that generate free radicals, and as inhibitors of key enzymes involved in hormone metabolism. Thus, at high doses, the adverse effects of flavonoids may outweigh their beneficial ones. The unborn fetus may be especially at risk, since flavonoids readily cross the placenta [25, 26].

Redox imbalance in cells can lead either to oxidative or to reductive stress. Oxidative stress has been extensively studied for many years, and its possible clinical ramifications have been explored in considerable depth. Reductive stress, by contrast, has not been widely recognised. Yet reductive stress is probably both common and of clinical importance: indeed, reductive stress plus oxygen rather than oxidative stress may be the most common mechanism leading to the generation of reactive oxygen species (ROS). One possible link between the two may be the reduction of Fe^{3+} and its liberation from ferritin. The reduced metal could catalyse ROS generation [27].

Our results also showed decreased activities of enzymatic antioxidants SOD, CAT and GPx, and the levels of non-enzymatic antioxidant GSH in circulation, liver and kidney of $KBrO_3$-treated rats. ROS generated in tissues are normally scavenged by enzymatic and non-enzymatic antioxidants [28, 29]. Antioxidant defense system protects the aerobic organism from the deleterious effects of reactive oxygen metabolites [30].

Glutathione is a crucial component of the antioxidant defense mechanism and it functions as a direct reactive free

Table 7: The levels of GSH in plasma and tissues.

Treatment groups	Plasma (mg/dL)	Kidney (mg/100 g tissue)	Liver (mg/100 g tissue)
Saline (control)	35.28 ± 2.57	92.28 ± 5.74	125.72 ± 8.29
$KBrO_3$ (125 mg/kg body weight)	$20.85 \pm 1.62^{\triangle\triangle}$	$58.20 \pm 4.12^{\triangle\triangle}$	$78.27 \pm 5.71^{\triangle\triangle}$
FR (150 mg/kg body weight) + $KBrO_3$	$28.24 \pm 1.72^{**}$	$71.39 \pm 4.81^{*}$	$107.22 \pm 6.26^{**}$
FR (300 mg/kg body weight) + $KBrO_3$	$31.39 \pm 1.83^{**}$	$79.55 \pm 5.88^{**}$	$118.82 \pm 7.20^{**}$
FR (300 mg/kg body weight) alone	34.20 ± 1.96	89.26 ± 6.26	126.18 ± 8.75

Values were expressed as mean ± SD, $n = 6$. The dose of $KBrO_3$ was 125 mg/kg body weight in each group.
$^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$ compared with the control group.
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with $KBrO_3$-treated rats.

Table 8: The levels of 8-OHdG in plasma and tissues.

Treatment groups	Plasma (ng/mL)	Kidney (ng/mL)	Liver (ng/mL)
Saline (control)	0.69 ± 0.08	1.12 ± 0.15	0.92 ± 0.12
$KBrO_3$ (125 mg/kg body weight)	$9.65 \pm 1.26^{\triangle\triangle}$	$21.65 \pm 2.94^{\triangle\triangle}$	$13.73 \pm 2.13^{\triangle\triangle}$
FR (150 mg/kg body weight) + $KBrO_3$	$5.27 \pm 0.68^{**}$	$12.45 \pm 1.57^{*}$	$7.82 \pm 1.28^{**}$
FR (300 mg/kg body weight) + $KBrO_3$	$2.59 \pm 0.23^{**}$	$3.38 \pm 0.45^{**}$	$2.73 \pm 0.37^{**}$
FR (300 mg/kg body weight) alone	0.75 ± 0.10	1.05 ± 0.18	0.89 ± 0.15

Values were expressed as mean ± SD, $n = 6$. The dose of $KBrO_3$ was 125 mg/kg body weight in each group.
$^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$ compared with the control group.
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with $KBrO_3$-treated rats.

radical scavenger [31, 32]. The liver is the major organ with the highest content of GSH, which is transferred to kidney by distinct GSH transport system [33, 34]. The decreased levels of GSH in circulation and tissues in $KBrO_3$-treated rats may due to enhanced utilization during detoxification of $KBrO_3$. GPx and CAT, which act as preventive antioxidants and SOD, a chain-breaking antioxidant, play important roles in protection against the deleterious effects of lipid peroxidation [35]. Decreases in the activities of SOD, CAT and GPx in plasma, liver and kidney of $KBrO_3$-treated rats may due to the decreased synthesis of enzymes or oxidative inactivation of the enzyme proteins. In the present study, increased lipid peroxidation associated with decreased antioxidant status in $KBrO_3$-treated rats could therefore give rise to insufficient antioxidant potential.

The antioxidant effects of FR may be due to different causes: firstly, flavonoid's high diffusion into the membranes [36] allowed it to scavenge oxyradicals at several sites throughout the lipid bilayer; secondly, its pentahydroxyflavone structure allowed it to chelate metal ions via the orthodihydroxy phenolic structure, thereby scavenging lipid alkoxyl and peroxyl radicals [37, 38]. In spite of the free radical scavenging activities, flavanoid glycosides and phenols in FR might be also involved in the indirect induction of detoxifying genes [39], which might promote detoxification of $KBrO_3$ and decrease their toxicity. *In vivo* studies have shown that flavanoid glycosides inhibited Fe^{2+}-induced lipid peroxidation in the rat liver [40]. It is suggested that the lipid peroxidative indices were probably attenuated by the chain-breaking action of flavanoid in the free radical process of the oxidation of membrane lipids.

Our results also showed that the kidney was the major organ with the higher content of 8-OHdG after $KBrO_3$ treatment, suggesting that the kidney is the main target organ of $KBrO_3$-induced DNA oxidation. DNA damage was significantly decreased after the treatment of FR, which may be attributed to the antioxidant property of phenols in FR, as phenols are known to bind DNA at sites that would normally react with the active metabolites of carcinogen during carcinogen-DNA binding, a crucial step for initiation of carcinogenesis [41–43]. Alternatively, when the phenols bind to DNA, their molecules might be positioned in such a way so as to effectively scavenge reactive intermediates that approach the critical sites on DNA, or phenols may directly interact with the ultimate reactive metabolites of carcinogen by donating their electrons and rendering it inactive [44]. Dok-Go et al. demonstrated phenolsact in many cell-free experimental systems to scavenge reactive oxygen radicals and reduce oxidative DNA damage [45].

In summary, our data demonstrated that FR protected against $KBrO_3$ toxicity by decreasing oxidant status and DNA damage and increasing the antioxidant status, indicating that FR possesses a spectrum of antioxidant and DNA-protective properties. However, further investigations are necessary to elucidate the precise mechanisms of protection of FR against $KBrO_3$ toxicity, and the potential effects of FR against other carcinogens should be explored prior to evaluating as a chemopreventive agent against carcinogenesis.

Abbreviations

FR: *Fructus rhodomyrti*
SCGE: Single-cell gel electrophoresis
ROS: Reactive oxygen species
SOD: Superoxide dismutase
CAT: Catalase
GSH: Reduced glutathione
GPx: Glutathione peroxidase
8-OHdG: 8-Hydroxy-2′-deoxyguanosine.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Review Article

Nutrition and Healthy Ageing: Calorie Restriction or Polyphenol-Rich "MediterrAsian" Diet?

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Diet plays an important role in mammalian health and the prevention of chronic diseases such as cardiovascular disease (CVD). Incidence of CVD is low in many parts of Asia (e.g., Japan) and the Mediterranean area (e.g., Italy, Spain, Greece, and Turkey). The Asian and the Mediterranean diets are rich in fruit and vegetables, thereby providing high amounts of plant bioactives including polyphenols, glucosinolates, and antioxidant vitamins. Furthermore, oily fish which is rich in omega-3 fatty acids is an important part of the Asian (e.g., Japanese) and also of the Mediterranean diets. There are specific plant bioactives which predominantly occur in the Mediterranean (e.g., resveratrol from red wine, hydroxytyrosol, and oleuropein from olive oil) and in the Asian diets (e.g., isoflavones from soybean and epigallocatechin gallate from green tea). Interestingly, when compared to calorie restriction which has been repeatedly shown to increase healthspan, these polyphenols activate similar molecular targets such as Sirt1. We suggest that a so-called "MediterrAsian" diet combining sirtuin-activating foods (= sirtfoods) of the Asian as well as Mediterranean diet may be a promising dietary strategy in preventing chronic diseases, thereby ensuring health and healthy ageing. Future (human) studies are needed which take the concept suggested here of the MediterrAsian diet into account.

1. Introduction

It has been well established that diet plays a central role in maintaining health throughout life. In the Western world diet has significantly changed in recent years. Diets that are rich in saturated fats and cholesterol, low in fibre, and high in sugar and salt may increase the CVD risk [1–3]. We are currently facing an increase in the incidence of obesity associated diseases in general and specifically coronary heart disease in many parts of the world. Contrarily, a reduction of food and/or calorie intake without malnutrition may be associated with the prolongation of life- and healthspan. The National Academies Keck Futures Initiative; the Future of Human Healthspan, defined healthspan as the time an organism was of good health with health being the "the ability of a system to maintain or return to homeostasis in response to challenges" [4]. In mammals including primates, calorie restriction (CR) repeatedly delayed disease onset, thereby prolonging healthspan [5–7]. As diet composition also seems to influence health [8, 9], it has been hypothesized that CR and certain dietary factors can prevent age-related diseases like atherosclerosis and cardiovascular disease (CVD), type 2 diabetes mellitus, neurodegenerative diseases, and cancer. Dietary factors that decrease CVD risk may be the consumption of red wine and olive oil in the Mediterranean area [10] or seaweed in coastal Asia [11]. Interestingly, polyphenol consumption and calorie restriction (CR) seem to have similar effects on metabolism in humans [12, 13], and supplementation with polyphenols may attenuate negative effects from a high fat diet in mice [14]. Therefore, polyphenols are also referred to as CR mimetics. An important mechanism by which polyphenols induce CR-like signalling pathways seems to function through sirtuin activation [15]. Sirtuins are NAD-dependent deacetylases that were shown to be involved in the regulation of lifespan and metabolism in different organisms [15, 16]. Further evidence exists supporting a role of polyphenols in gene regulation via the gamma coactivator 1-alpha (PGC-1α) and transcription factors such as nuclear factor-erythroid 2 (Nrf2), forkhead box O (FoxO), and nuclear factor kappa B (NFκB) [17], all of which are also implicated in CR-mediated effects. In this review we will discuss the literature on CR and foods that induce CR-like

effects with a special focus on sirtuin activators that are found in the Asian and Mediterranean diets and their potential to promote healthy ageing. Because of their sirtuin-activating properties we refer to these foodstuffs as "sirtfoods".

2. Signalling Pathways That Are Induced by Calorie Restriction

CR induces body weight loss and has beneficial effects on plasma levels of triglycerides and cholesterol as well as on blood pressure, thereby preventing or delaying the onset of age-related diseases [18]. Additionally, in response to a reduction of body fat, adipose tissue-derived hormones including leptin and adiponectin are modulated. These hormones are central in the regulation of satiety and appetite in the hypothalamus [19]. As an important regulator of body weight [20], circulating leptin levels are directly proportional to the total amount of visceral adipose tissue. While CR decreases leptin levels, it increases adiponectin levels. In contrast to leptin, adiponectin exhibits antiatherogenic, anti-inflammatory, and insulin-sensitizing properties and is assumed to be cardioprotective [21].

2.1. Insulin-Like Growth Factor and Mammalian Target of Rapamycin Signalling. One of the main impacts of dietary restriction is the modulation of insulin and insulin-like growth factor-1 (IGF-1) signalling [22]. Interestingly, in mice impaired IGF-1 signalling leads to a similarly prolonged lifespan as compared to CR [23] and in *Caenorhabditis elegans* inactivation of the IGF/PI3K/Akt pathway promoted longevity [24].

Upon binding to their receptors, insulin and IGF-1 induce a number of signalling pathways and kinases such as the phosphatidylinositol 3-kinases class I (PI3KI), the serine/threonine kinase Akt, and the mammalian target of rapamycin (mTOR). mTOR promotes protein synthesis and cellular growth [25]. Besides downregulating mTOR via lowering IGF/PI3K/Akt signalling, CR also inhibits mTOR through activation of the 5′ adenosine monophosphate-activated protein kinase (AMPK) [26]. Inhibition of mTOR leads to prolongation of lifespan [27] which is in part mediated by autophagy, a lysosomal degradation process that degrades no-longer-needed proteins and organelles and also functions as a starvation response [28]. Consistent with CR attenuating age-related changes in metabolism and cellular functions, insulin sensitivity and autophagic activity decrease with age while being increased by CR [29].

2.2. Reactive Oxygen Species and the Hormesis Effect. Ageing is also characterized by accumulating oxidative damage. There are different mechanisms that lead to this build-up of damaged proteins, lipids, and organelles. Damaged macromolecules may accumulate because of reduced degradation, reduced antioxidant capacity, or increased reactive oxygen species production [30, 31].

It was shown that CR can decrease the mitochondrial release of reactive oxygen species (ROS) which contribute to this type of damage in ageing organisms [32]. However, recent studies have indicated that the metabolic rate increases with CR [33], and it has been hypothesized that deleterious substances including ROS may have beneficial effects at low concentrations. This concept of a biphasic dose-response with low and high doses of a substance mediating opposite effects is referred to as hormesis [34]. According to this concept, low doses of a certain substance benefit the organism by inducing adaptive effects. In line with the concept of hormesis, ROS may function as essential signalling molecules, for example, by regulating redox-sensitive transcription factors [35]. Because of a lack of energy supply under CR, mitochondrial activity and, as a consequence, oxidative phosphorylation in the respiratory chain could be enhanced, leading to increased ROS production [36].

2.3. The Redox-Regulated Transcription Factors Nrf2 and NFκB. One of the targets of increased ROS production is the nuclear factor-erythroid 2- (NFE2-) related factor (Nrf2). This Cap'n'Collar basic leucine zipper transcription factor controls the expression of a large number of antioxidant and phase II detoxifying enzymes such as the NAD(P)H dehydrogenase (quinone) 1 (NQO1), the heme oxygenase 1 (HO-1), glutathione *S*-transferase (GST), the glutathione peroxidase (GPx), and the γ-glutamylcysteine synthetase (γGCS) [37–39]. In CR rodents, upregulation and increased activity of these gene products have been described repeatedly [40, 41].

The nuclear factor kappa B (NFκB) is also a redox-sensitive transcription factor that induces the expression of genes involved in inflammation and cellular proliferation [42]. Although NFκB is activated by ROS, various studies have reported downregulation of NFκB by CR [43, 44]. It has been suggested that CR increases cytoplasmic levels of IκB which impedes NFκB translocation into the nucleus. Additionally, CR may influence NFκB translocation by decreasing nucleophosmin (NPM) expression. NPM is a nuclear phosphoprotein that shuttles between the nucleus and the cytoplasm and promotes NFκB activity [45]. Moreover, CR may inhibit the transcription of the NFκB subunit RelA/p65 through Sirt1 activation [46]. Considering that at a higher age NFκB activity and as a consequence inflammation seem to increase [47], the down-regulation of NFκB activity by CR may contribute to the ageing-related health benefits of CR.

2.4. Forkhead Box O Transcription Factors. The forkhead box O (FoxO) transcription factor family activates or represses gene expression of a wide variety of genes implicated in apoptosis, cell cycle and differentiation, DNA repair, and stress response [48, 49] and is also activated by CR [50]. Of the four human forkhead genes that have been identified so far (FoxO1, FoxO3, FoxO4 and FoxO6), FoxO3 has been identified as a longevity-associated gene in centenarians [51]. It has been suggested that FoxO transcription factors may be involved in longevity because of their ability to detoxify ROS and repair DNA damage.

FoxOs can be regulated by various mechanisms such as phosphorylation, acetylation, and proteasomal degradation. While under nutrient rich conditions, insulin/IGF-1 signalling leads to translocation of FoxO out of the nucleus

Table 1: FoxO regulation by Sirt1.

Cell culture	Treatment	Outcome	Reference
HEK 293T	Stress conditions (H_2O_2)	↑ Interaction between Sirt1 and FoxO3	[53]
MEF Sirt1$^{-/-}$	Treatment with LY 294002 (PI3K inhibitor): wild type Sirt1$^{-/-}$	↑ Acetylation of FoxO3 ↑ GADD45 (stress resistance) ↓ GADD45	
HepG2	Serum starvation => FoxO translocation into the nucleus	↑ Deacetylation of FoxO1 by Sirt1	[55]
HeLa	Sirt1 overexpression	↓ FoxO3 activity (↓ FoxO3 target genes Bim/p27)	[54]
HEK 293T	Inhibition of Sirt1	↓ FoxO4 activity ↓ MnSOD ↓ p27	[58]

Bim: Bcl−2 interacting mediator of cell death, proapoptotic protein; H_2O_2: hydrogen peroxide; HEK-293T: human embryonic kidney 293 cells containing the T antigen from simian virus; HepG2: human liver carcinoma cell line; MEF: mouse embryonic fibroblasts; MnSOD: manganese superoxide dismutase, part of antioxidative defence; p27: cyclin-dependent kinase inhibitor, controls cell cycle progression; PI3K: phosphatidylinositol 3-kinase.

Table 2: Cellular localization, activity, and biological function of sirtuins 1–7 according to [63].

Sirtuin	Localization	Activity	Biological function
Sirt1	Nucleus/cytosol	Deacetylase	Cell survival/metabolism
Sirt2	Cytosol	Deacetylase	Cell cycle
Sirt3	Mitochondria	Deacetylase	Thermogenesis/metabolism
Sirt4	Mitochondria	ADP-ribosyltransferase	Insulin secretion/metabolism
Sirt5	Mitochondria	Deacetylase	Unknown
Sirt6	Nucleus	ADP-ribosyltransferase	DNA repair
Sirt7	Nucleolus	Unknown	rDNA transcription

ADP: adenosine diphosphate; rDNA: ribosomal desoxyribonucleic acid; Sirt: sirtuin.

and subsequent degradation, and CR leads to activation of FoxO-mediated transcription [50]. An important mechanism for FoxO activation under conditions of limited nutrient supply is its deacetylation by Sirt1 [52] (see Table 1). It has been shown that Sirt1 leads to a type of FoxO3 activation that counteracts oxidative stress while suppressing FoxO3-induced apoptosis rather than leading to FoxO3-induced cell death [53]. The finding that Sirt1 can inhibit the transcription of one set of genes while switching on the transcription of another type of target genes could explain the observation made by Motta and colleagues [54] who stated that Sirt1-mediated deacetylation of FOXO3 led to its inhibition. However, in the case of FoxO1, Daitoku and coworkers [55] reported an upregulation via Sirt-mediated deacetylation. Interestingly, FoxO and the tumour suppressor p53 appear to be functionally linked as they can alter each other's functions [56]. Both FoxO and p53 are deacetylated by Sirt1, which thereby controls their activities. In addition, FoxO induces gene expression of Sirt1 [57]. Therefore, it seems plausible that CR, at least in part, leads to lifespan extension through regulating the insulin/IGF1, FoxO, and Sirt1 network.

3. Sirtuins, the Mammalian Homologues of the Yeast Longevity Gene SIR2

Sirtuins (Sirt1–7) are NAD-dependent histone deacetylases [59]. All sirtuins contain a catalytic core domain but differ in the protein sequences surrounding this domain and their cellular localization [60]. They act as protein deacetylases and/or ADP-ribosyltransferases. As they require NAD, it has been suggested that their activity depends on the metabolic state of the cell. It has been hypothesized that sirtuins may link energy intake to lifespan [61]. An inhibitory mutation of the Sirt1 ortholog SIR2 in yeast shortened lifespan, whereas overexpression of SIR2 extended lifespan [62]. Their cellular localizations, activities, and biological functions are listed in Table 2.

3.1. Sirt1 and the Control of Metabolism. Sirt1 is the best studied sirtuin to date and is sometimes referred to as a guardian against cellular oxidative stress and DNA damage. Apart from deacetylating p53 and FoxO transcription factors, it was also shown to interact with NFκB, the peroxisome proliferator-activated receptor gamma (PPARγ), and the PPARγ coactivator and inducer of mitochondrial biogenesis PGC-1α [64, 65] (Figure 1). *In vivo* studies have shown that CR upregulates the mammalian Sirt1 protein levels in muscle, brain, fat, and kidney (see Table 3) [57, 66]. In white adipose tissue (WAT) Sirt1 was shown to deacetylate and inhibit PPARγ. This nuclear receptor which is induced by fatty acids activates fat synthesis and adipogenesis. Thus, inhibition of PPARγ by Sirt1 led to fat loss [64]. A decrease in adipose tissue generally lowers the leptin/adiponectin ratio, thereby favouring insulin sensitivity and healthy ageing. It has also

Table 3: Effects of calorie restriction or starvation on Sirt1 in different tissues in mice and rats.

Species	Number animals	Caloric restriction	Tissue	Outcome	Reference
Mice (C57BL6)	10	40%	Muscle, fat	Sirt1 ↑ (protein)	[66]
			liver	Sirt1 ↓ (protein)	
Mice (C57BL/6×C3H/He F1 hybrid)	4	15%	Brain, liver	No effect on Sirt1 (protein)	[13]
			heart, muscle	Sirt1 ↓ (protein)	
Mice (C57BL6)	—	24 h starvation	Liver	Sirt1 ↑ (protein) no effect on mRNA	[65]
Mice (C57BL)	—	24 h starvation	Brain, heart, muscle, white adipose tissue, and kidney	Sirt1 ↑ (protein) no effect on mRNA	[70]
Mice (C57BLK/6×SV127 F1 hybrid)	10	30–40%	White adipose tissue	Sirt1 ↑ (protein)	[36]
Mice (C57BL6)	—	40%	White adipose tissue, liver, kidney, and brain	Sirt1 ↑ (protein) not significant	[71]
Rats (Fisher 344)	—	40%	Brain, fat, kidney, and liver	Sirt1 ↑ (protein)	[57]

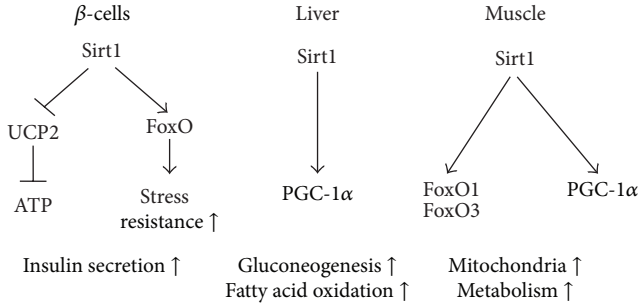


Figure 1: Sirt1-mediated regulation of metabolism in different tissues according to [92]. In pancreatic β-cells Sirt1 represses the expression of UCP2, thereby increasing insulin secretion, and Sirt 1 also regulates FoxOs, thereby protecting the β-cells against oxidative stress. In the liver Sirt1 regulates gluconeogenesis by activating PGC-1α. In muscle cells Sirt1 activates both PGC-1α and FoxO, thereby influencing mitochondrial biogenesis, respiration, and fatty acid oxidation. ATP: adenosine triphosphate; FoxO: forkhead box protein O; PGC-1α: peroxisome proliferator-activated receptor-γ coactivator 1 alpha; UCP: uncoupling protein 2.

been shown that Sirt1 activation shifts metabolisms away from using glucose as an energy source. While Sirt1 led to PPARγ inhibition, PPARα that transcribes genes involved in fatty acid oxidation was activated by Sirt1 localizing to PPAR response elements upon CR [67]. Additionally, deacetylation of PGC-1α by Sirt1 seems to lead to an activation of gluconeogenic gene transcription, while the expression of genes involved in glycolysis decreased [65]. With CR, PGC-1α is also induced on a transcriptional level and activated by AMPK-mediated phosphorylation [68]. Activated PGC-1α increases mitochondrial biogenesis by expressing several components of the respiratory chain. Moreover, PGC-1α contributes to the metabolic shift away from glycolysis under conditions of limited nutrient supply by promoting mitochondrial fatty acid oxidation and gluconeogenesis in the liver [69].

However, while various reports find increased Sirt1 protein levels in the muscle and WAT of CR animals, there is contradicting data as to whether liver Sirt1 changes upon CR (see Table 3). Chen and colleagues [66] even stated that hepatic Sirt1 levels were decreased by CR. This may be related to tissue-specific functions of Sirt1 (Figure 1). In the study by Chen and colleagues, the repression of hepatic Sirt1 by CR resulted in decreased hepatic fat synthesis and fat accumulation. Consistent with this finding, mice with a liver-specific knockout of Sirt1 fed a Western diet showed lower body weight gain than wild type mice. A possible explanation for this lowered fat accumulation in the liver may be a reduced coactivation of liver X receptor (LXR) resulting in decreased activation of the cholesterol transporter ABCA1 and the fat synthesis regulator SREBP1c [66]. However, in another study also using Sirt1 LKO contrary observations were made, and a weight gain in the LKO mice higher than in the wild type controls was reported [67]. As pointed out by Haigis and Sinclair [72], this may have resulted from the different types of diets used in these two studies, with the diet from the second trial containing more fat. Of interest, transgenic Sirt1 mice were protected against fatty liver when fed a high fat diet. Moreover, these mice showed lower NFκB activation and, consistently, lower levels of proinflammatory cytokines

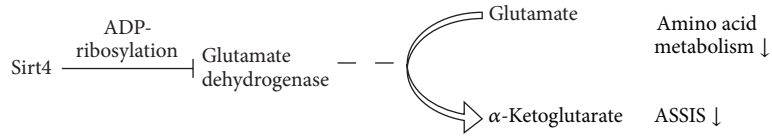


Figure 2: Sirt4 mediated amino acid metabolism according to [92]. ASSIS: amino acid-stimulated insulin secretion.

as well as lower glucose levels when compared to their wild type controls [73]. Thus, the authors hypothesize that Sirt1 protects from damage induced by a high fat diet.

3.2. Cardiovascular Disease and Sirt1. CVD is the primary cause of death in most countries and is characterized by elevated levels of low density lipoprotein cholesterol, oxidative damage to proteins and lipids and chronic inflammation. This environment promotes atherosclerotic plaque formation and a deteriorated endothelial function. Apart from genetic factors, a high fat diet and ageing are the main risk factors for developing this type of disease. Sirt1 induction by CR or by the "sirtfood"- constituent resveratrol has been shown to counteract elevated levels of inflammation and might lower cholesterol and triglyceride synthesis [74]. An important factor that contributes to the protective role of Sirt1 in CVD seems to be its function as a suppressor of the proinflammatory transcription factor NFκB. Sirt1 inhibits NFκB-mediated transcription by deacetylating its subunit RelA/p65 and as a consequence lowering its DNA-binding ability [46]. Various reports have shown that Sirt1 activation *in vitro* decreased the levels of proinflammatory mediators such as the tumour necrosis factor α, interleukins 1 and 6, intercellular adhesion molecule 1, and inducible nitric oxide synthase (NOS) [72]. In a mouse model for CVD, an apolipoprotein E double knockout, and endothelial Sirt1 overexpression lowered the formation of atherosclerotic plaques [75]. In mouse liver, it could be shown that Sirt1 interacts with the nuclear factor LXR and activates this transcription factor through its deacetylation. LXR induces reverse cholesterol transport by activating the transcription of the cholesterol efflux transporter ABCA1, thereby lowering peripheral cholesterol levels and promoting cholesterol excretion [76]. In contrast to inducible NOS which is found in macrophages and produces NO that harms microorganisms, endothelial NOS (eNOS) protects arteries. Interestingly, it was shown that Sirt1 activates eNOS and thus leads to endothelium-dependent vasodilatation.

3.3. Sirt1 Interaction with AMPK and Importance for Autophagy. Activation of the energy sensing kinase AMPK has shown similar effects on metabolism energy expenditure as compared to sirtuin signalling on various occasions [77]. This can be explained by the findings that Sirt1 activates AMPK [78] and vice versa [79]. Additionally, these reports point to the notion that both enzymes are needed to orchestrate the organism's response to limited nutrient supply or elevated energy demand during exercise. A part of the organism's response to such catabolic metabolic states is the lysosomal degradation pathway autophagy. Interestingly, autophagy was shown to be induced via Sirt1 and AMPK [80, 81]. In *Caenorhabditis elegans*, autophagy induction is necessary for and contributes to the lifespan prolonging effect of CR and its mimetic resveratrol [82]. In another experiment measuring lifespan extension, autophagy deficient worms did not benefit from caloric restriction to the same extent as wild type *C. elegans* [83]. Similarly to the activation of sirtuins, autophagy induction leads to lifespan extension in *Drosophila* and other organisms [84, 85]. In addition, sirtuins and autophagy are induced by CR [29]. Therefore, part of the ageing-related benefits from Sirt1 activation by CR or polyphenolic CR mimetics seems to be caused by autophagy [86]. In the case of the secondary plant metabolite resveratrol this connection could already be shown. By inducing autophagy via Sirt1 activation, resveratrol promoted longevity in worms and flies [87]. Interestingly, other polyphenolic Sirt1 inducers such as quercetin also promoted autophagic degradation [15, 88]. Considering that autophagy is a starvation and especially in higher eukaryotes a stress response, the activation of this lysosomal degradation pathway might be one the main mechanisms by which Sirt1 protects the organism from stressors (and thereby contributes to longevity).

3.4. The Mammalian Sirtuins 2–6. Apart from Sirt1, both Sirt3 and Sirt4 play important metabolic roles during food limitation. Additionally, in a study by Kawahara and coworkers [89], Sirt6-induced histone deacetylation led to prolongation of lifespan.

Sirt3 is mainly expressed in the liver, kidney, brain, and brown adipose tissue [90]. A high expression of Sirt3 in brown adipose tissue during CR induces the expression of PGC-1α and the uncoupling protein-1 (UCP-1) which is important for thermogenesis. Downregulation of Sirt4 by CR seems to affect amino acid metabolism and insulin secretion. Decreased Sirt4 levels lead to a higher conversion of glutamate to α-ketoglutarate by the glutamate dehydrogenase GDH [91], thereby promoting the use of amino acids as ATP source under CR. Contrarily, under nutrient-rich conditions Sirt4 ADP-ribosylates GDH which attenuates GDH activity [91]. Furthermore, CR-mediated Sirt4 decrease and GDH activity induce the amino acid-stimulated insulin secretion (AASIS) resulting in increased insulin secretion in response to CR [92] (Figure 2).

4. Potential Health Benefits of a MediterrAsian Diet

As in the Western world the prevalence of obesity and age-associated diseases such as type 2 diabetes mellitus, cancer, Alzheimer's, and atherosclerosis is increasing, and in order to

benefit from CR a lifelong restriction seems to be necessary [41], interest in dietary restriction mimetics has been rising (see Table 4).

However, although substances such as metformin can be used as an antidiabetic drug [97], most of the pharmacological CR mimetics have severe side effects. Therefore, the consumption of secondary plant bioactives such as polyphenols with the normal diet appears to be a safer strategy to benefit from potentially healthspan-improving CR mimetics.

Although further studies are needed to prove that these foods promote health in humans through their CR-mimicking secondary plant metabolite content, we hypothesize that the concept of combining foods from Asian and Mediterranean diets could possibly improve health status in an ageing population. Unfortunately, it can be difficult to study how dietary components influence health because of the low doses of bioactives found in diets when compared to the doses fed in some animal studies or the doses applied in most cell culture models. Additionally, bioactives are metabolised before and after adsorption from the gastrointestinal tract and could work synergistically or antagonistically with each other and other components in the diet. These factors make it complicated to extrapolate findings from *in vitro* and *in vivo* experiments to humans. Furthermore, when carrying out human studies, dietary effects tend to be smaller than in many pharmacological intervention studies and therefore require a higher number of participants to obtain measurable results. However, it seems that diets rich in saturated fats and cholesterol, low in fibre, and high in sugar and salt may increase CVD risk [1–3], and dietary factors such as olive oil consumption in the Mediterranean area [10] or seaweed in coastal Asia [11] may decrease CVD risk. Therefore, we believe that combining Asian and Mediterranean foods in a MediterrAsian diet could be a promising approach for improving human health.

4.1. Components of Mediterranean Diets. From a nutritional point of view there is a substantial overlap between the Asian and Mediterranean diets. These diets are rich in fruit and especially vegetables which are both important sources of dietary polyphenols, glucosinolates, and vitamin C (see Figure 3). Furthermore, for some parts of Asia and the Mediterranean a high consumption of oily fish rich in omega 3 fatty acids has been reported. Consistent with the low incidence of CVD in populations consuming such diets in Asia and the Mediterranean area, it has been suggested that foods with a high content of polyphenols, glucosinolates, and omega 3 fatty acids reduce the CVD risk.

In addition, there are also specific foods which are predominantly consumed in the Mediterranean area such as red wine. The same is true for olive oil which is also consumed to a higher extent in Southern as opposed to Northern Europe. Studies in model organisms (e.g., *C. elegans*, *D. melanogaster*), and laboratory rodents suggest that red wine constituents (e.g., resveratrol) may positively affect both health and lifespan [14, 16]. Our own data demonstrate that mice consuming diets rich in olive oil phenolics (e.g., hydroxytyrosol) exhibit decreased oxidative damage markers (e.g., lipid peroxides, protein carbonyls) and improved expression of Nrf2-dependent genes encoding antioxidant (γGCS, NQO1) and cardioprotective (paraoxonase) proteins. We have also shown that olive oil phenolics may induce proteasomal activity and Sirt1 signalling [98]. Recently, another group also observed Sirt1 induction by olive oil phenolics [99], and further evidence exists supporting a role of polyphenols in gene regulation via sirtuins and transcription factors such as Nrf2 and NFκB [17]. Resveratrol activated Nrf2 and attenuated oxidative stress, thereby protecting the endothelium in a mouse model [100] and inhibited inflammation in macrophages via downregulation of the proinflammatory NFκB [101]. Resveratrol has been repeatedly shown to induce Sirt1 [15, 82, 102], and therefore it seems possible that the endothelium protection and NFκB inhibition may also be connected to Sirt1 induction. The polyphenol quercetin found in onions could also induce Sirt1 [15]. In Table 5, we have listed polyphenols that were shown to induce sirtuins and the type of foods they are found in. Given the fact that Sirt1 activation appears to benefit health by mimicking CR, these "sirtfoods" may contribute to healthy ageing.

4.2. Components of Asian Diets. The Asian diet is rich in soy and turmeric. *Curcuma longa* is a significant source of curcumin [111], and soy contains considerable amounts of isoflavones [112, 113]. Similar to resveratrol and quercetin, curcumin has also been shown to induce Nrf2 [114] and other transcription factors [115] and inhibit NFκB-mediated inflammation [116, 117]. In contrast to the Asian diet, the Mediterranean diet is almost devoid of both isoflavones and curcumin. We and others have shown that soy isoflavones and curcumin mediates cardioprotective activity including reduction of LDL oxidation, inhibition of platelet aggregation, and improvement in vascular reactivity [118–120]. Interestingly, isoflavones such as daidzein were also shown to induce Sirt1 and PGC-1α [103]. Further constituents of the Asian diet that contain possible health-promoting bioactives are green tea and seaweed. Tea polyphenols seem to inhibit the proinflammatory transcription factor NFκB [121], while seaweed is a source of antioxidant vitamins and polyunsaturated omega 3 fatty acids [122] which may also prevent CVD [123, 124].

4.3. Human Intervention Studies on Secondary Plant Metabolites Found in the MediterrAsian Diet. Most of the *in vitro* and *in vivo* research and various human intervention studies with bioactives from the Mediterranean and Asian diets found promising results regarding possible health-promoting benefits [125, 126]. However, some studies reported controversial data. For example, red wine has been stated to decrease low density lipoprotein (LDL) peroxidation in humans which would be beneficial for CVD prevention [127]. In contrast, red or white wine with a reduced alcohol content did not decrease (LDL) peroxidation in human volunteers [128], and one group of researchers showed that although moderate red wine consumption could lower LDL oxidation, the other tested alcoholic beverages also did [129]. On the other hand, in one study only red wine as compared to white wine

Table 4: Dietary restriction mimetics: mechanisms and side effects.

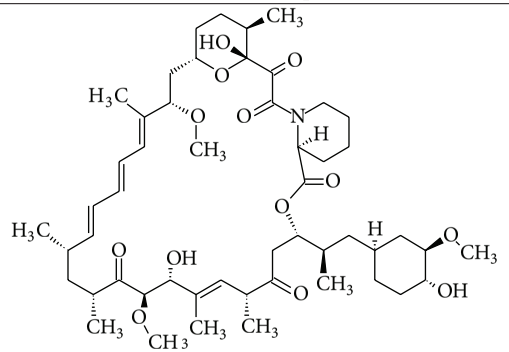
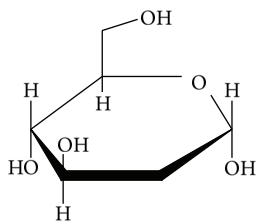
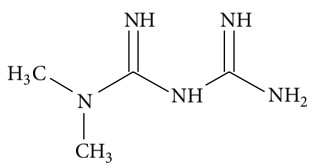
Name of compound	Structure of compound	Mechanism of action	Side effects	References
Rapamycin/sirolimus		Inhibition of mTor, induction of autophagy	Immunosuppression	[93]
2-Deoxy-D-glucose		Inhibition of glucose metabolism	Cardiotoxicity	[94]
Metformin		Inhibition of gluconeogenesis, stimulation of glucose uptake, inhibition of AMPK, and improvement of insulin sensitivity	Gastrointestinal complaints	[95–97]

Table 5: Sirt1-inducing plant bioactives and the "sirtfoods" they are found in.

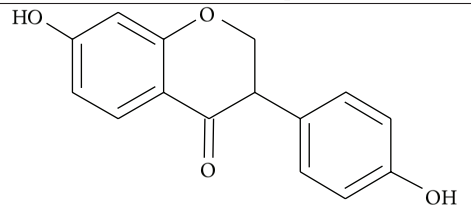
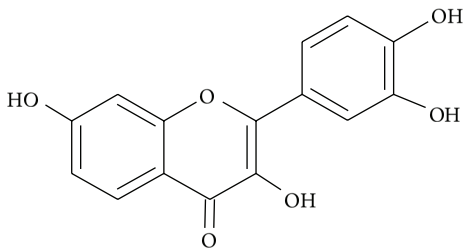
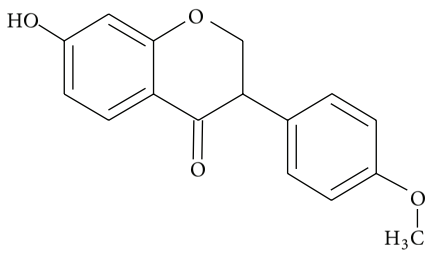
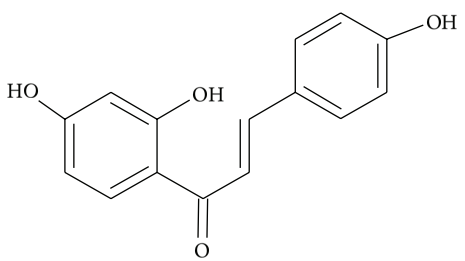
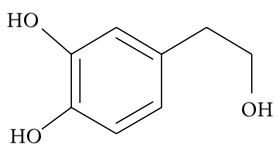
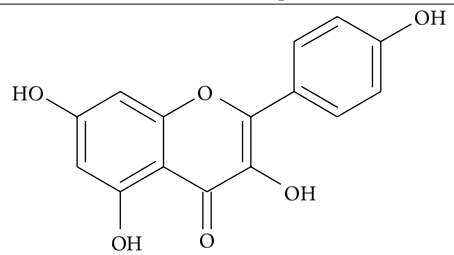
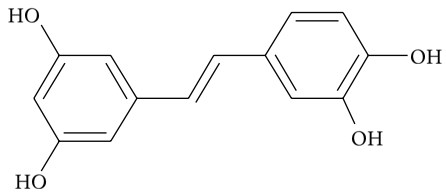
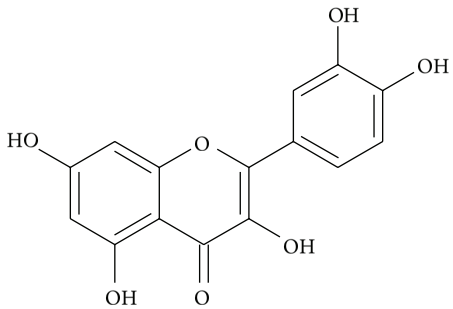
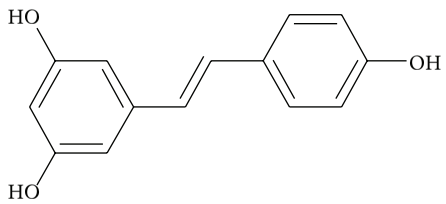
Name of compound	Structure of compound	"Sirtfoods" found in	References
Daidzein		Soybean, tofu, and other soy products	[103, 104]
Fisetin		Strawberries, apples, persimmons, grapes	[15, 105]
Formononetin		Soybean	[103]
Isoliquiritigenin		Soybean, shallots, and licorice	[15, 106, 107]
Hydroxytyrosol[◊]		Olive oil	[98, 99]

Table 5: Continued.

Name of compound	Structure of compound	"Sirtfoods" found in	References
Kaempferol		Cabbage, kale, parsley, different types of beans, *Corchorus olitorius*[*], and cruciferous vegetables	[105, 108]
Piceatannol		Red wine, grapes, and *Rhodomyrtus tomentosa*[#]	[15, 109, 110]
Quercetin		Onions, apples, white wine, capers, and *Corchorus olitorius*[*]	[15, 105]
Resveratrol		Red wine, red grapes	[15, 109]

[*] Consumed in Japan as "Molokheka"; [#] edible plant native to Asia; [◊] and possibly other phenolic compounds found in olive oil.

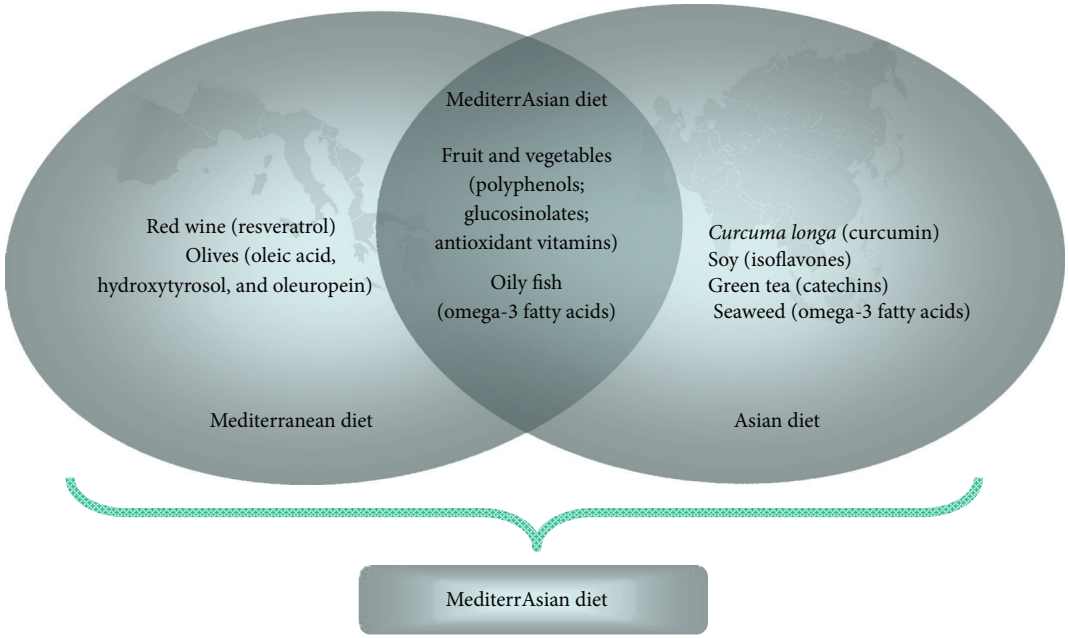


Figure 3: Important food items and their corresponding constituents in the Mediterranean, Asian, and so-called MediterrAsian diet.

lowered LDL oxidation in healthy humans, thereby pointing to the notion that certain components found in red but not white wine are responsible for this effect [130]. Similarly conflicting results were reported for the influence of fruit-derived polyphenols on blood pressure. While Aviram and colleagues observed that pomegranate juice reduced blood pressure in humans [131], in the study by Sumner and colleagues blood pressure did not change after administering pomegranate juice to the patients [132]. In the context of cancer prevention by soy-containing diets, a meta-analysis on the influence of isoflavones on a breast cancer biomarker found only a small positive effect that might or might not be clinically relevant [133]. In the case of curcumin, data from cell models and animal studies made this polyphenol appear an efficient treatment for dementia [134]. However, in clinical trials curcumin did not improve the symptoms of Alzheimer patients [135]. There are various factors that may contribute or explain these controversial findings including the differences in the health status of the patients, different doses and dosage forms, or different parameters measured (e.g., lipid peroxidation in plasma versus blood pressure for CVD). However, these conflicting results demonstrate that further research is needed to better understand the effect of dietary components on sirtuin activation and healthy ageing.

5. Conclusion

A high content of fruit, vegetables, and oily fish in the diets of Mediterranean and certain Asian populations is likely to cause the improved health status observed in the MediterrAsian area.

However, we suggest that plant bioactives, antioxidant vitamins, and omega-3 fatty acids do not work in isolation. Rather they may act synergistically, thereby preventing chronic diseases. Thus, it is possible that the complex mixture of diet-derived plant bioactives, antioxidatives, and omega-3 fatty acids cannot be substituted by a single purified compound [136].

Instead we propose combining healthy foodstuffs of the Asian and the Mediterranean diets especially rich in "sirtfoods" in order to prevent chronic diseases and ensure healthy ageing.

We would like to encourage future studies in cultured cells, model organisms, laboratory rodents, and ultimately humans to unravel and evaluate potential health benefits of the MediterrAsian diet from a molecular to the system biology level.

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Research Article

Red Chicory (*Cichorium intybus* L. cultivar) as a Potential Source of Antioxidant Anthocyanins for Intestinal Health

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Fruit- and vegetable-derived foods have become a very significant source of nutraceutical phytochemicals. Among vegetables, red chicory (*Cichorium Intybus* L. cultivar) has gained attention for its content of phenolic compounds, such as the anthocyanins. In this study, we evaluated the nutraceutical effects, in terms of antioxidant, cytoprotective, and antiproliferative activities, of extracts of the whole leaf or only the red part of the leaf of Treviso red chicory (a typical Italian red leafy plant) in various intestinal models, such as Caco-2 cells, differentiated in normal intestinal epithelia and undifferentiated Caco-2 cells. The results show that the whole leaf of red chicory can represent a good source of phytochemicals in terms of total phenolics and anthocyanins as well as the ability of these phytochemicals to exert antioxidant and cytoprotective effects in differentiated Caco-2 cells and antiproliferative effects in undifferentiated Caco-2 cells. Interestingly, compared to red chicory whole leaf extracts, the red part of leaf extracts had a significantly higher content of both total phenolics and anthocyanins. The same extracts effectively corresponded to an increase of antioxidant, cytoprotective, and antiproliferative activities. Taken together, these findings suggest that the red part of the leaf of Treviso red chicory with a high content of antioxidant anthocyanins could be interesting for development of new food supplements to improve intestinal health.

1. Introduction

Many degenerative diseases of the gastrointestinal tract, such as colorectal cancer or inflammatory bowel disease, are associated with a persistent state of oxidative stress which results from an imbalance in the production of reactive oxygen species (ROS) and cell antioxidant defenses at intestinal epithelial level [1, 2]. In particular, various exogenous and endogenous sources, including dietary oxidants and inflammatory processes, respectively, together with the disruption of the glutathione antioxidant defense system support oxidative stress within intestinal cells [3, 4]. Recent studies have shown that the induction of oxidative shift in the cellular redox status induces epigenetic events and cellular mitogenic or apoptotic responses which contribute to progression of colorectal cancer [5, 6].

In this context, there is increasing interest in food as a source of antioxidant phytochemicals which can provide an inexpensive readily applicable and easily accessible approach for cancer control and prevention [7, 8]. Several studies support this interest, showing that consumption of fruits and vegetables is associated with a decreased risk of several cancers, particularly colorectal cancer, possibly linked to their phytochemical content, which is of interest due to several proposed health benefits, including antioxidant and anticancer activities [9]. Preserving the redox status of intestinal cells as well as preventing the early events of cellular oxidative damage involved in the carcinogenesis processes by administrating dietary phytochemicals therefore provides an important strategy for colorectal cancer chemoprevention [10].

Among vegetables, chicory (*Cichorium intybus*), a typical vegetable indigenous to Europe and North and Western

America, has gained attention for its content of phytochemicals with potential nutraceutical effects, such as phenolic acids, flavonoids, and anthocyanins [11]. Using analytical methods, various studies demonstrate the ability of different chicory varieties to counteract various free radicals, as well as a linear correlation between phytochemical content and antioxidant capacity of this vegetable [12–14]. A more recent study shows an interesting antioxidant activity of the red chicory variety against the oxidative stress response in a eukaryotic model system suggesting its health activity at cellular level [15].

By contrast to other chicory varieties, red chicory is characterized by a high content of anthocyanin pigments [16]. The presence of anthocyanins in red chicory is of special interest because several studies have described many beneficial health or nutraceutical effects of anthocyanins on visual capacity, brain cognitive function, obesity, cardiovascular risk, and cancer prevention [7, 17–19]. The anticancer properties of dietary anthocyanins have been widely evaluated in *in vitro* studies and some animal gastrointestinal cancer models [7]. Although many studies have evaluated the anticancer effects, mainly as antiproliferative activity, of various berry fruit extracts or of anthocyanins from berries in human colon cancer cells, the ability of these dietary phytochemicals to also prevent oxidative events underlying colon cancer in appropriate intestinal models still remains unanswered. An integrated experimental approach with *in vitro* colon cancer and normal intestinal models is needed to characterize the potential nutraceutical effects, including both the prevention and the control of colon cancer, of food or part of a food.

The present study was planned to investigate the levels of phytochemicals, such as total phenolics and anthocyanins, and total antioxidant activity (TAA) as well as the *in vitro* bioactivity of red chicory of Treviso, a typical Italian red leafy vegetables which has been attributed to Protected Geographical Indication status according to European Union rules [20]. The *in vitro* bioactivity, in terms of antioxidant, cytoprotective, and antiproliferative activities, of red chicory extracts was studied using different intestinal models, including human colon carcinoma (Caco-2) cells differentiated in normal intestinal epithelia and undifferentiated Caco-2 cells. In particular, both antioxidant and cytoprotective activities were evaluated in differentiated Caco-2 cells, which provide a suitable model for assessment of the physiological response of intestinal epithelia to oxidative injury [21]. To mimic oxidative damage to intestinal epithelia, we used tert-butyl hydroperoxide (*t*-BuOOH) due to its ability to generate peroxyl and alkoxyl radicals, that catalyze the peroxidation of membrane lipids [22].

2. Materials and Methods

2.1. Chemicals. Gallic acid, tetrazolium salt (MTT), *t*-BuOOH, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2′-7′dichlorodihydrofluorescein diacetate (DCFH-DA), and Folin-Ciocalteu's phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade purity commercial available.

2.2. Sample Selection. Treviso red chicory (*Cichorium intybus* L. cultivar) from certified producers operating in Veneto (Italy) was purchased from a single retail outlet. Four different lots of red chicory at comparable ripening stages were analyzed. All red chicory samples were processed within 48–72 h of purchase.

2.3. Sample Extraction. Briefly, 100 g of weight of the whole leaf (WL) or only the red part of the leaf (RL), without the white side, of Treviso red chicory was mixed with cold methanol (HCl 0.1%) and homogenized using an Ultra Turrax homogenizer for 5 min. The mixture was filtered through a Whatman paper under vacuum, and the methanol in the filtrate was evaporated at 35°C. Subsequently, the residue was diluted to 10 mL with methanol and stored at −20°C until phytochemical content analysis. Subsequently, some samples of residue in methanol were dried completely and resuspended in appropriate culture mediums for the bioactivity determinations.

2.4. Total Phenolic Content Analysis. Total phenolic concentrations were measured using the Folin Ciocalteau assay [23]. Briefly, appropriate dilutions of extracts were treated with Folin Ciocalteau reagent, and the reaction was neutralized with 7% sodium carbonate. The absorbance of the resulting blue color was measured spectrophotometrically at 750 nm using a Beckman DU 7400 spectrophotometer. Gallic acid was used as standard and results expressed as milligrams of gallic acid equivalents (GAE) per 100 g of the fresh edible part of the red chicory.

2.5. Total Anthocyanin Content Analysis. Total anthocyanin content of the extracts of Treviso red radicchio was determined spectrophotometrically by the pH differential method of Rapisarda et al. [24].

2.6. Determination of TAA. The TAA of the extracts derived from Treviso red chicory was measured as reported by Re et al. [25]. This method is based on the ability of the antioxidant molecules in the vegetable extracts to reduce the radical cation of the ABTS, determined by the decolorization of $ABTS^{\bullet+}$ and measured as quenching of absorbance at 740 nm. Values obtained for each sample were compared with the concentration-response curve of a standard Trolox solution and expressed as micromoles of trolox equivalent antioxidant activity (TEAA)/100 g of fresh vegetable.

2.7. Cell Cultures of Human Colon Carcinoma Cells. Human colon carcinoma (Caco-2) cells were routinely grown at 37°C in a humidified incubator with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% (FCS), 2 mmol/L glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. To evaluate intracellular antioxidant and cytoprotective activities as well as antioxidant activity in membrane and cytosolic fractions of the WL and RL extracts,

Caco-2 cells were seeded at a density of 8×10^4 cells/cm^2 in multi-well dishes; once the cells were confluent, the medium was changed every 48 h using DMEM 20% FCS. Experiments were performed using completely differentiated cultures at 12–14 days after seeding. To evaluate the antiproliferative activity of the same extracts, undifferentiated Caco-2 cells were seeded in 96-well microliter plates at a density of 1.5×10^4 cells/cm^2. Experiments were performed after 24 h of incubation at 37°C in 5% CO_2.

2.8. Determination of Intracellular Antioxidant and Cytoprotective Activities. We evaluated the antioxidant and cytoprotective activities of WL and RL extracts of Treviso red chicory against both formation of intracellular ROS and cytotoxicity in differentiated Caco-2 cells after treatment with *t*-BuOOH. Formation of intracellular ROS was determined using a fluorescent probe, DCFH-DA, as described by Wang and Joseph [26]. Briefly, differentiated Caco-2 cells were incubated for 4 h with different concentrations of the extracts derived from Treviso red chicory samples corresponding to 5–30 mg vegetable/mL. Cells were washed with PBS and then incubated with 5 μM DCFH-DA in phosphate buffered saline (PBS) in 5% CO_2 at 37°C for 30 min. After removal of DCFH-DA and further washing, the cells were incubated with 0.5 mM *t*-BuOOH in PBS for 30 min. At the end of incubation, the fluorescence of the cells from each well was measured (wavelength: 485/535 nm) with a spectrofluorometer (Spectra Max Gemini, Molecular Devices, MN, USA). The values are expressed as percentage of increase of intracellular ROS evoked by exposure to *t*-BuOOH.

Cytotoxicity was monitored by trypan blue uptake as previously described [27]. Briefly, differentiated Caco-2 cells were incubated for 4 h with extracts of WL and RL (corresponding to 5–30 mg vegetable/mL), washed with PBS, and then incubated with 0.5 mM *t*-BuOOH in DMEM 20% FBS at 37°C in 5% CO_2. After 24 h of incubation, the cells were collected by gentle scraping in PBS and dispersed by repeated gentle pipetting. An aliquot of cell suspension was then diluted 1 : 1 with 0.5% trypan blue in 10 mM sodium phosphate buffer (pH 7.2) and placed on a hemocytometer with a cover slip. Percentages of viable cells were recorded on at least three separate counts.

2.9. Determination of Antioxidant Activity in Membrane and Cytosolic Fractions. Cytosolic and membrane-enriched fractions were separated as we previously reported [28]. Briefly, after 4 h of incubation with WL and RL extracts (corresponding to 5–30 mg vegetable/mL) at 37°C in 5% CO_2, differentiated Caco-2 cells were washed 3 times with cold PBS. Cells were subsequently collected in 1 mL of PBS and centrifuged for 10 min at 10,000 rpm at 4°C, after which the supernatant was removed and the cells were washed with 1 mL of PBS. This was repeated further 2 times, and the pellet was finally reconstituted in 600 μL of 0.05% Triton X-100. Cells were then homogenized and allowed to stand at 4°C for 30 min. Cytosolic and membrane fractions were subsequently separated by centrifugation at 14,000 rpm for 15 min at 4°C. Membrane and cytosolic fractions were stored at −20°C. Small amounts were removed for determination of the protein concentration using the Bradford method. TAA was then measured on cytosolic and membrane fractions using ABTS method as previously reported [25]. This experimental approach makes it possible to determine the cellular uptake of bioactive molecules and their ability to counteract the free radicals at different subcellular levels. Values obtained for each cellular fraction sample were expressed as μmol of Trolox equivalent antioxidant activity per mg of protein.

2.10. Determination of Antiproliferative Activity. The antiproliferative activity of WL and RL extracts of Treviso red chicory was determined in undifferentiated Caco-2 cells *in vitro* as we previously reported [21]. Briefly, after 96 h of incubation with WL and RL extracts (corresponding to 1–50 mg vegetable/mL), Caco-2 cells were washed with PBS and then incubated with MTT (5 mg/mL) in PBS for 4 h. After removal of MTT and further washing, the formazan crystals were dissolved with isopropanol. The amount of formazan was measured (405 nm) with a spectrophotometer (TECAN, Spectra model Classic, Salzburg, Austria). The cell viability was expressed as a percentage of control cells. At least three independent dose-response curves were plotted, and the concentration of red chicory extracts resulting in 50% inhibition of cell proliferation (IC_{50}) was calculated.

2.11. Statistical Analysis. Data are reported as mean ± SD of at least 3 independent experiments. Compositional data were statistically processed utilizing the Student's *t*-test. Statistical analysis of biological data was performed using one-way ANOVA with Dunnett's Post Hoc Test and Student's *t*-test, as appropriate, and Pearson's correlation coefficient for relations among variables. Differences were considered significant at $P < 0.05$. Analyses were performed using PRISM 3 software on a Windows platform.

3. Results and Discussion

We first determined the phytochemical contents such as total phenolics and anthocyanins in extracts obtained from edible samples of WL or RL of Treviso red chicory. As reported in Table 1, the extracts of RL had a significantly higher total phenolic and anthocyanin content than the corresponding extracts of WL (all $P < 0.05$). In parallel, the TAA of the same extracts was measured by ABTS radical cation decolorization assay and expressed as μmol of trolox equivalent antioxidant activity (TEAA)/100 g edible sample (Table 1). On the basis of weight, the TAA of the RL extracts was significantly higher than the activity of WL extracts ($P < 0.05$).

Taken together, the high levels of total phenolic and anthocyanin content found in WL extracts are in the range already reported in the literature for other varieties of red chicory [13, 14, 16, 29]. In this regard, we also confirmed the low levels of other antioxidant components such as total ascorbic acid in both WL and RL extracts of red chicory (data not shown). These findings are supported by a recent antioxidant characterization of *Cichorium intybus* that

Table 1: Total phenolics, total anthocyanins, and TAA of edible samples of WL or RL of Treviso red chicory[1].

Treviso red chicory	Total phenolics[2] mg of GAE/100 g samples	Total anthocyanins mg of anthocyanins/100 g samples	TAA[3] μmol TEAA/100 g samples
WL	311.6 ± 12.6	110.8 ± 8.2	506.7 ± 35.6
RL	370.4 ± 14.4*	142.6 ± 7.5*	655.2 ± 42.2*

[1]Values are means ± SD of at least four determinations (RL versus WL; $^{*}P < 0.05$ at Student's t-test).
[2]Values were expressed as gallic acid equivalents (GAE) in milligrams per 100 g of edible sample.
[3]Values were expressed as micromoles of trolox equivalent antioxidant activity (TEAA) per 100 g of edible sample.

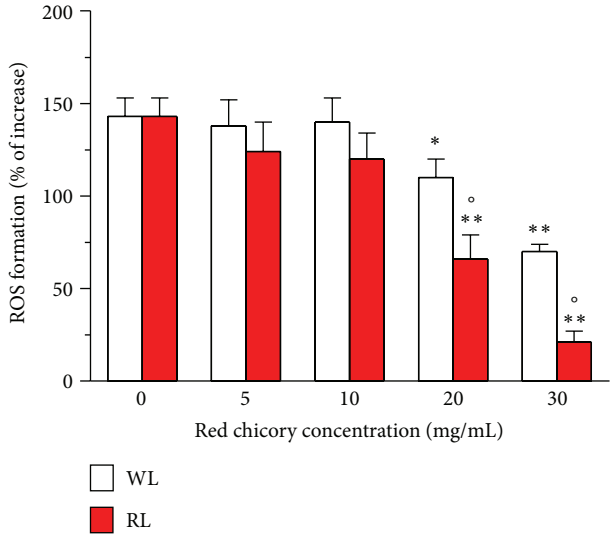

Figure 1: Antioxidant activity of WL and RL extracts of Treviso red chicory in Caco-2 cells differentiated in normal intestinal epithelia. The cells were treated with various concentrations of extracts for 4 h and then treated with t-BuOOH (0.5 mM) for 30 min. At the end of incubation, intracellular ROS formation was determined using a fluorescence probe, DCFH-DA, as described in the Materials and Methods section. The values are expressed as percentage of increase of intracellular ROS formation evoked by exposure to t-BuOOH. The values are shown as mean ± SD of four independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated cells, at ANOVA with Dunnett's Post Hoc Test; $^{\circ}P < 0.05$ versus cells treated with WL extracts at Student's t-test).

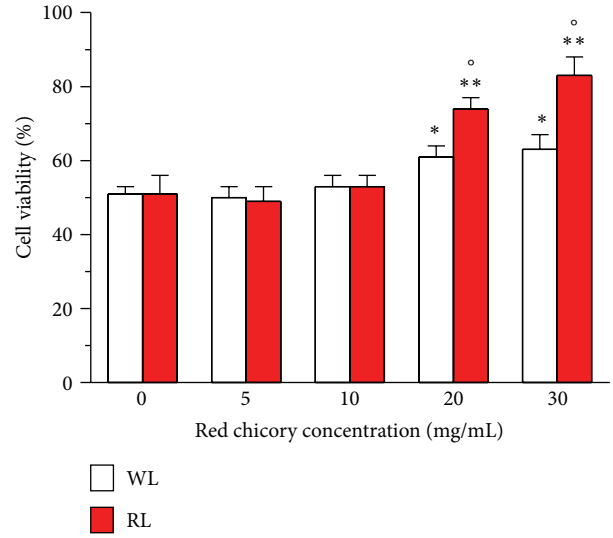

Figure 2: Cytoprotective activity of WL and RL extracts of Treviso red chicory in Caco-2 cells differentiated in normal intestinal epithelia. The cells were treated with various concentrations of extracts for 4 h and then treated with t-BuOOH (0.5 mM) for 24 h. At the end of incubation, cytotoxicity was determined using trypan blue assay as described in the Materials and Methods section. The values are expressed as percentage of cell viability after exposure to t-BuOOH. The values are shown as mean ± SD of four independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated cells, at ANOVA with Dunnett's Post Hoc Test; $^{\circ}P < 0.05$ versus cells treated with WL extracts at Student's t-test).

recorded the marginal role of total ascorbic acid in the total antioxidant activity of this vegetable [30].

Interestingly, the levels of the same phenolic compounds as well as TAA of RL extracts were significantly higher than WL extracts, supporting the hypothesis that phenolic compounds may be important antioxidant components to account for the observed antioxidant activity. In particular, recent studies on the phytochemical composition of red chicories recorded a large amount of hydroxybenzoic and hydroxycinnamic acids as well as of red anthocyanins which give red chicories an exceptionally high peroxyl radical scavenging activity, in terms of both capacity and efficiency [16]. Although we did not characterize the phenolic compound content, it is reasonable to suppose that the combination of these compounds, mainly the red anthocyanins, could be important due to the higher TAA observed in RL extracts and to confer a potential health value to this part of red chicory.

We then assessed the nutraceutical effects, in terms of the antioxidant and cytoprotective activities, of these WL and RL extracts of Treviso red chicory against both the intracellular ROS formation and cytotoxicity in differentiated Caco-2 cells after treatment with t-BuOOH. As shown in Figures 1 and 2, treatment of differentiated Caco-2 cells with both WL and RL extracts (5–30 mg/mL) showed a decrease, in a dose-dependent manner, of intracellular ROS formation and cytotoxicity elicited by t-BuOOH. The ability to counteract the ROS formation and cytotoxicity was significantly higher with RL than WL extracts for the concentrations 20 and 30 mg/mL (all $P < 0.05$).

To confirm the antioxidant and cytoprotective activities of WL and RL extracts at cellular level, we evaluated TAA (expressed as μmol TEAA/mg protein) at two different

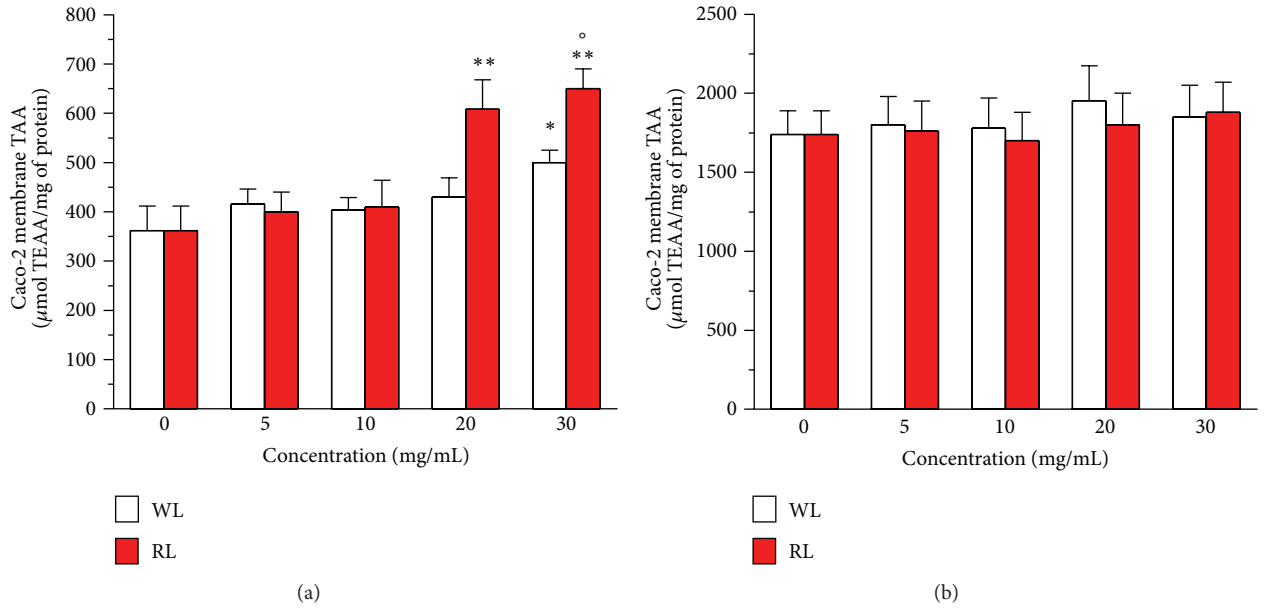

Figure 3: Effects of WL and RL extracts of Treviso red chicory on TAA of membrane (a) and cytosolic (b) fractions of Caco-2 cells differentiated in normal intestinal epithelia. The cellular fractions were submitted to the ABTS methods after 4 h of incubation with various concentrations of extracts as described in Section 2. The results obtained for each cellular fraction sample were expressed as μmol of TEAA per mg of protein. The values represent the mean ± SD of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated cells at ANOVA with Dunnett's Post Hoc Test; $^{\circ}P < 0.05$ versus cells treated with WL extracts at Student's t-test).

subcellular levels, membrane and cytosol. As depicted in Figure 3, the membrane and cytosolic fractions obtained from differentiated Caco-2 cells treated with WL (30 mg/mL) and RL (20 and 30 mg/mL) extracts for 4 h showed a significant increase of TAA in comparison with untreated cells. Remarkably, the recorded increase of membrane TAA was significantly higher in Caco-2 cells treated with RL than WL extracts at the concentrations of 30 mg/mL ($P < 0.05$). By contrast, both RL and WL extracts did not modify the basal levels of differentiated Caco-2 cytosolic fraction TAA. Interestingly, a highly significant linear correlation was found between Caco-2 cell membrane TAA and cytoprotective activity for both the WL ($r = -0.93$, $P < 0.001$ at Pearson's correlation coefficient) and RL ($r = -0.91$, $P < 0.001$) extracts.

Taken together, these results show that RL extracts led to greater increases in antioxidant and cytoprotective activities in the differentiated Caco-2 cells than WL extracts. In particular, the highest TAA observed at Caco-2 membrane level could be attributed to higher TAA levels of the anthocyanin fraction of the RL extracts. Our previous studies suggest an accumulation and antioxidant activity of these phenolic compounds present in anthocyanin-rich fruits, such as red orange and strawberry, in the membrane of various human epithelial cells, including intestinal cells [26, 27, 31]. There is evidence that the hydrophobic nature of the ring structure of anthocyanins determines their interactions with the hydrophobic component of the cell membrane, influencing the membrane fluidity and preventing lipid peroxidation and oxidative damage [32]. A recent study supports these scientific considerations, showing the ability of various *Cichorium intybus* extracts to prevent lipid peroxidation and intracellular ROS formation using neuron cell-based assays [33].

Last, we measured a nutraceutical effect as the antiproliferative activity of the WL and RL extracts in undifferentiated Caco-2 cells using MTT assay. To compare the antiproliferative activity of the extracts, we also used the IC_{50} (concentration of the extract resulting in 50% inhibition of colon cancer cell proliferation) extrapolated by a wide range of extract concentrations, from 1 to 50 mg/mL. As reported in Figure 4, treatment of Caco-2 cells with both WL and RL extracts induced a decrease of cell proliferation in a concentration-dependent manner. The inhibitory effects on Caco-2 cell proliferation were significantly higher with RL than WL extracts for the 5 and 10 mg/mL concentrations (all $P < 0.01$). At higher concentrations, the inhibitory effects were saturated for both extracts. Considering the concentration-inhibitor effect relationship, the IC_{50} was significantly lower with RL than WL extracts (14.85±0.26% versus 21.38±0.47%; $P < 0.05$ at Student's t-test).

These findings could be ascribed to a greater amount of bioactive molecules with anticarcinogenicity properties present in red chicory. In this context, it is interesting to note that the *Cichorium intybus* extracts at low concentrations (5 and 10 mg/mL) show antiproliferative effects in the absence of antioxidant and cytoprotective effects. There is an emerging view that phenolic compounds could exert anticarcinogenicity effects not only through their antioxidant potential but also through the modulation of signaling cascades, gene

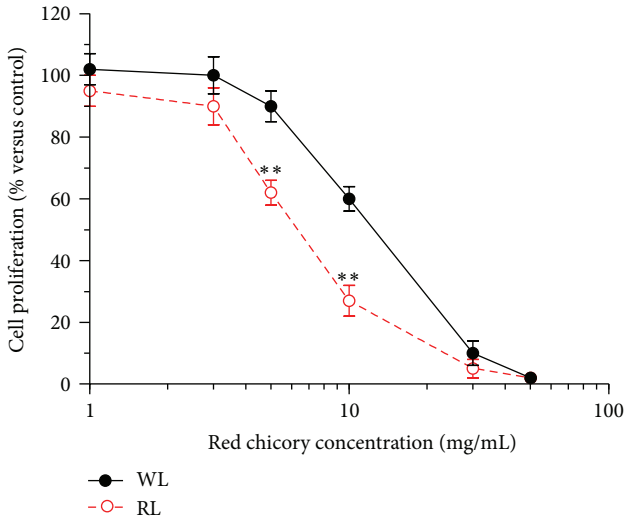

Figure 4: Effects of WL and RL extracts of Treviso red chicory on cell proliferation of undifferentiated Caco-2 cells. The cell proliferation was determined by the MTT assay after 96 h of incubation with various concentrations of extracts. The results were expressed as a percentage of control cells. The values represent the mean ± SD of three independent experiments ($^{**}P < 0.01$ versus cells treated with WL extracts at Student's t-test).

expression involved in the regulation of cell proliferation, differentiation, and apoptosis as well as the suppression of chronic inflammation, metastasis, and angiogenesis [34].

In particular, among phenolic compounds, it has been demonstrated that phenolic acids such as hydroxybenzoic and hydroxycinnamic acids can induce strong antiproliferative effects and apoptosis in human colon cancer cells [9]. Further, several studies also indicate that anthocyanins are able to inhibit the growth of different cancer cells, suggesting their possible role as chemopreventive agents [35, 36]. In particular, it has been suggested that the inhibitory effects of anthocyanin-rich fruit and vegetable extracts are based on the concentration rather than the composition of anthocyanins [37–39].

4. Conclusions

Our results showed that Treviso red chicory can represent a good source of nutraceutical phytochemicals for intestinal health. In particular, the high levels of antioxidant anthocyanins present in red chicory might exert a direct scavenging effect against ROS formation within the gastrointestinal tract. The anthocyanins and/or their metabolites could further contribute to intestinal health through their ability to spread out in internal intestinal tissue. Recent studies recorded the presence of glycoside, aglycone, and both methylated and glucuronide derivatives of anthocyanins in tissues including the stomach and small intestine of animal models fed either a single anthocyanin or berry extracts [7].

We also demonstrated that the red part of the leaf of red chicory could exert an interesting additional nutraceutical value, in terms of antioxidant and cytoprotective activities as well as antiproliferative activity, with respect to the whole leaf of red chicory. These findings, from an industrial point of view, indicate that the revaluation of some parts of red chicory with a high content of antioxidant anthocyanins could be interesting for the production of new commercial products, such as food supplements of high quality and low cost. However, further clinical studies are needed to confirm whether the whole and the red part of the leaf of Treviso red chicory are likely to improve intestinal health.

Abbreviations

ABTS:	2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt
Caco-2:	Human colon carcinoma cells
DCFH-DA:	2′-7′Dichlorodihydrofluorescein diacetate
GAE:	Gallic acid equivalents
MTT:	Tetrazolium salt
RL:	Red part of leaf
ROS:	Reactive oxygen species
TAA:	Total antioxidant activity
t-BuOOH:	*tert*-Butyl hydroperoxide
TEAA:	Trolox equivalent antioxidant activity
WL:	Whole leaf.

Authors' Contribution

Laura D'evoli and Fabiana Morroni contributed equally to this work.

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Research Article

Effects of Resveratrol on Daily Rhythms of Locomotor Activity and Body Temperature in Young and Aged Grey Mouse Lemurs

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In several species, resveratrol, a polyphenolic compound, activates sirtuin proteins implicated in the regulation of energy balance and biological clock processes. To demonstrate the effect of resveratrol on clock function in an aged primate, young and aged mouse lemurs *(Microcebus murinus)* were studied over a 4-week dietary supplementation with resveratrol. Spontaneous locomotor activity and daily variations in body temperature were continuously recorded. Reduction in locomotor activity onset and changes in body temperature rhythm in resveratrol-supplemented aged animals suggest an improved synchronisation on the light-dark cycle. Resveratrol could be a good candidate to restore the circadian rhythms in the elderly.

1. Introduction

Aging is associated with changes in circadian rhythmicity of endocrine, metabolic, and behavioral properties in several mammalian species [1]. It has important effect on thermoregulation processes [2] and on locomotor activity [3, 4].

Grey mouse lemur (*Microcebus murinus*, a Malagasy nonhuman primate) is a nocturnal species exhibiting high levels of locomotor activity during the dark period and a complete rest during the light period. Under exposure to short days, daily rhythm of body temperature (Tb) in mouse lemur is characterized by high values during the active period and high levels of locomotor activity (LA) and, before the onset of the light phase, a rapid and linear drop in Tb and LA, reaching minimal value after 3-4 hours. This hypothermia bout is followed by a spontaneous rewarming to normothermic Tb levels until the following dark phase. Tb, LA, and hypothermia are thus closely related and all driven by circadian regulation. In this species, age-related physiological changes have been identified (see [5] for review). Among these changes, an age-related decrease in amplitude of the seasonal rhythms of body mass, basal metabolic rate, and testosterone has been demonstrated [6]. More particularly, the daily rhythm of LA is fragmented and the amplitude of movement is reduced in aged animals compared to young [7, 8]. Thermoregulation processes are also impaired during aging in this species. During winter season, when ambient temperature is low, aged animals exhibit deeper hypothermia and increased levels of energy expenditure, impairing energy balance [9]. Aging is also known to shorten the free running period (*tau*) in male grey mouse lemurs [8].

Despite the importance of changes affecting the biological rhythms during aging, very few interventions are known to prevent such modifications. In humans, some studies using bright light therapy aimed to restore the circadian rhythms in elderly people. As an example, bright light therapy has been demonstrated as having beneficial effects on circadian rhythms in institutionalized elderly people [10]. Findings in animals allow us to propose nutritional interventions as another possible way to restore circadian rhythms during aging. Indeed, it was demonstrated that resveratrol (RSV), a natural compound found in grape and wine, regulates circadian clock genes in cultured Rat-1 fibroblast cells: a dose of 100 μM RSV increased the amplitude of oscillation of clock genes Per1, Per2, and Bmal1 [11]. This last result suggests that dietary RSV might act as a regulator of circadian clocks. RSV is known and tested as a potential mimetic of chronic calorie restriction, an intervention that may prolong lifespan in several species of invertebrates and mammals [12]. RSV is also

able to modify energy balance in several species from yeast to mice, by activating different proteins involved in energy regulation pathways, such as PGC1α [13] and SIRT1 [14], a nicotinamide adenosine dinucleotide-dependent deacetylase belonging to the sirtuins family. Interestingly, an important role of SIRT1 in biological clock processes has been identified [15, 16] so that the regulation of this protein by RSV could induce modifications of rhythm pattern of an organism's physiological parameters. In two recent studies on mouse lemurs, we demonstrated that RSV dietary supplementation was able to modify the endogenous period *tau* in young and aged animals [17] and was able to change the architecture of sleep-wake rhythms by lowering the amount of slow-wave sleep and increasing the proportion of activity in young animals [18].

Based on our previous findings, in the present study, we addressed the question whether RSV was able to restore the rhythms of daily locomotor activity and body temperature in aged mouse lemurs in comparison to young ones.

2. Experimental Procedures

2.1. Animals and Animal Care. We used eight young adult (mean age: 26 ± 11 months old) and five aged (mean age: 76 ± 10 months old) female grey mouse lemurs (*Microcebus murinus*, Cheirogaleidae, Primates) born in a laboratory breeding colony in Brunoy, France (Agreement no. 962773) from a population originally caught 40 years ago on the southwest coast of Madagascar. Conditions were constant with respect to ambient temperature (25°C), relative humidity (55%), and *ad libitum* water availability. Behavioral and physiological seasonal changes of mouse lemurs are dependent on the photoperiod and are reproduced in captivity by an artificial photoperiodic regimen. In the breeding colony, animals were exposed to an artificial photoperiodic regimen consisting of alternating 6-month periods of Malagasy winter-like short-day lengths (L : D 10 : 14) and of Malagasy summer-like long-day lengths (L : D 14 : 10). A greater plasticity of the body temperature adjustments was observed in animals under short-day photoperiod with higher torpor depth and duration, respectively, than in animals under long-day photoperiod [19]. These results are consolidated by the observation that grey mouse lemur in the field enters torpor spontaneously during the dry season but not during the rainy period [20]. This is the reason why the animals used in this study were in short-day photoperiod. More particular, the animals were in the middle of their short-day photoperiod, just after their fattening phase, to avoid any perturbation of their physiological processes due to a lack of food resource. Young animals presented a mean body mass of 128 ± 11 g at the beginning of the experiment and of 156 ± 14 at the end of the experiment. Aged animals presented a mean body mass of 137 ± 10 g at the beginning of the experiment and of 152 ± 13 at the end of the experiment. The animals were weighed during the control week, the second week and the fourth week of experiment. To minimize social influences, the animals were housed individually in cages (0.4 × 0.4 × 0.6 m), provided with nesting materials, and separated from each other by wooden partitions.

During the whole experiment, animals were fed with fresh fruits (banana and apple) and a mixture of cereals, milk, and eggs, providing them with a total of 120 kJ per day. The cereals are composed of 60% carbohydrates, 10% proteins, and 30% lipids. The cereals are primarily wheat flour (96%). After one control week to define basal levels of the different parameters analyzed in this study, the animals were fed, during the next 4 weeks (RSV1, RSV2, RSV3, and RSV4), with the same mixture as previous mentioned but 200 mg·kg^{-1} of RSV (Sequoia Research Products, United Kingdom) per day was added to the mixture. All the procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were done under personal licenses to experiment on mouse lemurs, delivered by the Ministry of Education and Science. Moreover, this study met the ethical standards of the journal [21].

2.2. Recording of Locomotor Activity and Body Temperature. Recording of locomotor activity (LA) and body temperature (Tb) was obtained by telemetry at a constant ambient temperature of 25°C. A small telemetric transmitter weighing 2.5 g (model TA10TA-F20, DataScience Co. Ltd, Minnesota, USA) was implanted into the visceral cavity under ketamine anesthesia (Imalgene, 100 mg/kg ip). After surgery, animals returned to their home cage and were allowed to recover for 15 days before start of experiment and continuous recordings of LA and Tb. Total recovery was checked by visual inspection of the complete healing of the surgical incision and by verification of a stable daily pattern of Tb variations. A receiver was positioned in the cage. Locomotor activity was continuously recorded by the receiver plate which detected vertical and horizontal movements (X-Y coordinate system, Dataquest Lab Pro v. 3.0, Data Science Co. Ltd, Minnesota, USA). LA data were summed in 5 min intervals and expressed in arbitrary unit (a.u.). The following parameters of LA have been defined: active phase LA (corresponding to the 8 most active hours of the dark period), LA onset (time between activity onset and the beginning of the dark period, expressed in min). LA onset was defined as the first 6 successive bins of 5 minutes in which activity was greater than the mean locomotor activity.

Tb was recorded every 10 min and allowed defining the following parameters: mean Tb during the dark phase (night Tb), mean Tb during the light phase (day Tb), and the minimal Tb reached during the hypothermia phase (Tb min). Entry into daily hypothermia was considered to start with the first value below 33°C, ending with the first value above 33°C (as defined by [19]). On this basis, the following hypothermia parameters were defined: hypothermia duration, reflecting the time during which Tb of an animal was under 33°C; hypothermia time drop (Hdrop), defined as the time from which mean Tb started to decrease (when 6 consecutive values were decreasing compared to the previous one); and time of minimal body temperature (Hmin), defined as the time at which Tb min was reached. For Hdrop and Hmin, the time of reference was the time of lights are on, with positive values before lights are on (phase advance) and negative values after lights on (phase delay). Tb was expressed in °C

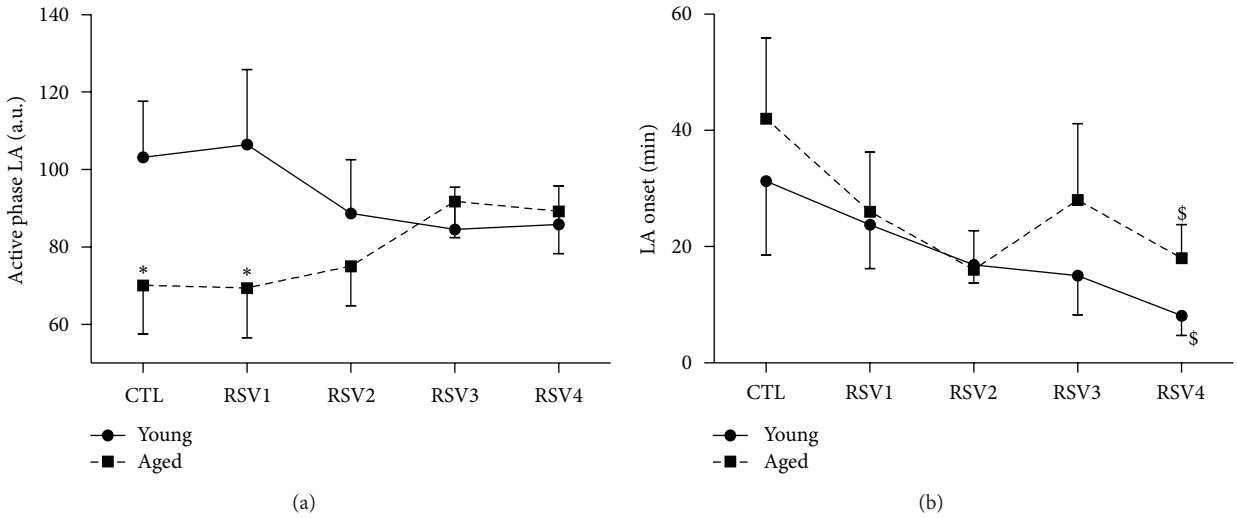

Figure 1: Locomotor activity (LA) parameters in young (solid lines) and aged (dotted lines) female mouse lemurs. (a) LA during the active phase is expressed in arbitrary units (a.u.). (b) LA onset is expressed in min. During the first week, animals received the control feeding (CTL), followed by 4 weeks of resveratrol (RSV) supplementation. Values are given as mean ± standard error of the mean. *represents a significant difference between young and aged animals ($P < 0.05$). $represents a significant effect of RSV supplementation compared to CTL condition ($P < 0.05$).

and hypothermia duration, Hdrop, and Hmin were expressed in min. LA and Tb data were averaged for each week of treatment (CTL, RSV1, RSV2, RSV3, and RSV4).

2.3. Statistics. All values are expressed as mean ± standard error to the mean. After checking for the normality of the distribution, ANOVA or repeated ANOVA for related samples was used to assert significant variations in locomotor activity, body temperature, and daily hypothermia parameters. Data from hypothermia duration were log-transformed in order to obtain data with a normalized distribution. Comparisons were considered to differ significantly when $P < 0.05$. All statistical computations were performed using SYSTAT for Windows (V9, SPSS Inc., USA).

3. Results

3.1. Locomotor Activity Parameters. Active phase LA was significantly higher in young animals compared to aged animals during the CTL and the first week of RSV supplementation ($P = 0.007$, Figure 1(a)). From RSV2 to RSV4 weeks, young and aged animals exhibited similar levels of active phase LA. LA onset was not significantly different between young and aged animals at the beginning of the study. Indeed, during CTL condition, a phase advance of 31 ± 13 min was observed for young animals and 42 ± 14 min for aged animals (Figure 1(b)). During the 4 weeks of RSV supplementation, young and aged animals then exhibited comparable reduction of the phase advance of LA onset to reach 8 ± 3 min and 18 ± 6 min respectively, at the end of RSV supplementation ($P = 0.03$, Figure 1(b)).

3.2. Body Temperature. Night Tb during CTL condition was similar in young and aged animals (36.1 ± 0.2°C and 35.9 ± 0.3°C, resp.) (Figure 2(a)). No significant change in night Tb occurred during the 4 weeks of treatment in animals of both age groups. Conversely, significant differences and variations in day Tb have been observed (Figure 2(b)). Under CTL condition, mean day Tb was 32.1 ± 0.6°C in young animals and 33.4 ± 0.7°C in aged animals, a difference that was statistically significant ($P = 0.004$). During RSV supplementation, day Tb significantly increased in both age groups, reaching 32.8 ± 1.0°C in young animals ($P = 0.04$) and 33.9 ± 0.3°C in aged animals ($P = 0.03$). Despite a transient and significant decrease of day Tb in aged animals during the third week of RSV supplementation, day Tb remained significantly higher in aged animals compared to young ones during the whole experiment ($P = 0.002$). Tb min, which was reached during the daily hypothermia, was comparable between young and aged animals at the start of the experiment (CTL condition) and then followed a similar pattern as day Tb (Figure 2(c)). Young animals exhibited an increase in Tb min from CTL condition (29.3 ± 0.7°C) to RSV4 (31.3 ± 1.0°C) ($P = 0.02$). Aged animals first exhibited an increase of Tb min from CTL condition (29.8 ± 1.4°C) to RSV2 condition ($P = 0.04$); then at RSV3, similarly to day Tb, they exhibited a transient decrease of Tb min. At RSV4, Tb min in aged animals reached similar values (30.7 ± 0.6°C) compared to those in young animals (31.3 ± 1.0°C).

3.3. Daily Hypothermia Parameters. Hypothermia duration was similar between young and aged animals and changed similarly during the experiment. Hypothermia duration during CTL condition was 510 ± 23 min in young animals and 562 ± 88 min in aged animals. It significantly decreased to 219 ± 73 min for young animals ($P = 0.02$) and 334 ± 67 min for aged animals during RSV4 condition ($P = 0.04$, Figure 3(a)). Time of Tb drop (Hdrop) was not significantly

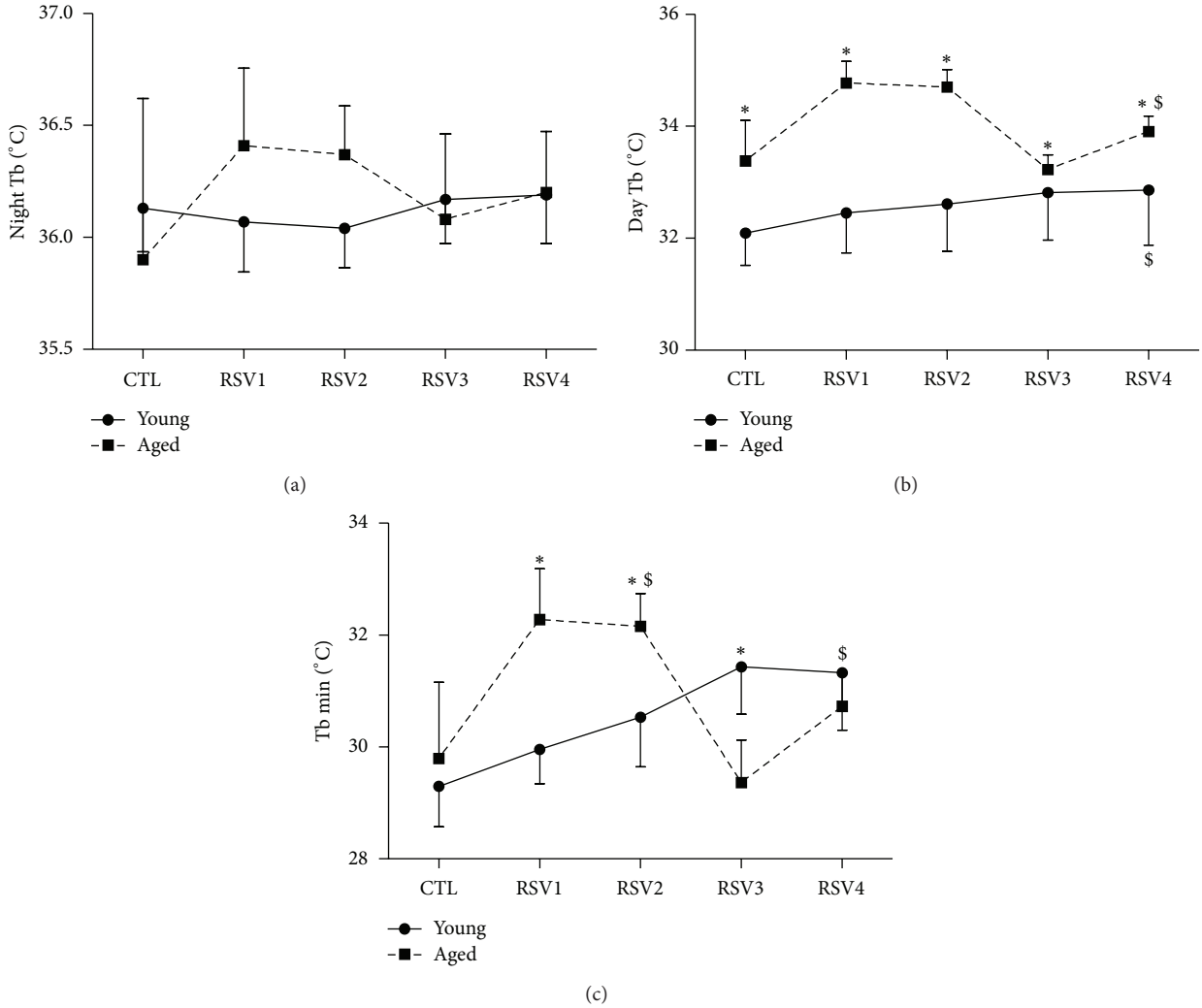

Figure 2: Body temperature (Tb) parameters in young (solid lines) and aged (dotted lines) female mouse lemurs. (a) Mean Tb during the dark phase. (b) Mean Tb during the light phase. (c) Lower Tb (Tb min) reached during hypothermia phase. During the first week, animals received the control feeding (CTL), followed by 4 weeks of resveratrol (RSV) supplementation. Tb was expressed in °C. Values are given as mean ± standard error of the mean. * represents a significant difference between young and aged animals ($P < 0.05$). $ represents a significant effect of RSV supplementation compared to CTL condition ($P < 0.05$).

different between young and aged animals ($P = 0.6$) and significantly decreased similarly in both age groups ($P = 0.04$ in both young and aged animals) (Figure 3(b)). Time at which Tb min was reached (Hmin) was not different between young and aged animals ($P = 0.9$) and did not change during the treatment ($P = 0.8$ and $P = 0.4$, resp., for young and aged animals) (Figure 3(c)).

4. Discussion

In the present study, we investigated the impact of RSV dietary supplementation on the rhythms of daily locomotor activity and body temperature in young and aged mouse lemurs.

RSV supplementation induced a reduction of LA onset suggesting a better synchronization with lights off. Young animals also exhibited a gradual increase in day Tb and Tb min under RSV supplementation. This increase in body temperature also led to a significant decrease in hypothermia duration after 4 weeks of RSV supplementation. In the same way, Hdrop was significantly delayed, leading to better synchronization with lights on and shortening of the torpor duration. These results are consistent with our previous observations in male grey mouse lemur in which a 4-week RSV supplementation inhibited the depth of daily torpor and significantly increased day Tb and Tb min [22]. Changes in LA onset and Hdrop confirm the specific impact of dietary RSV on circadian clock parameters previously observed by our group. Indeed, we observed that grey mouse lemurs in constant dark conditions (free-running experiments) exhibited a shortening of their endogenous period under RSV supplementation compared to controls [17].

In aged animals, active phase LA was lower in CTL condition compared to young animals. After 4 weeks of

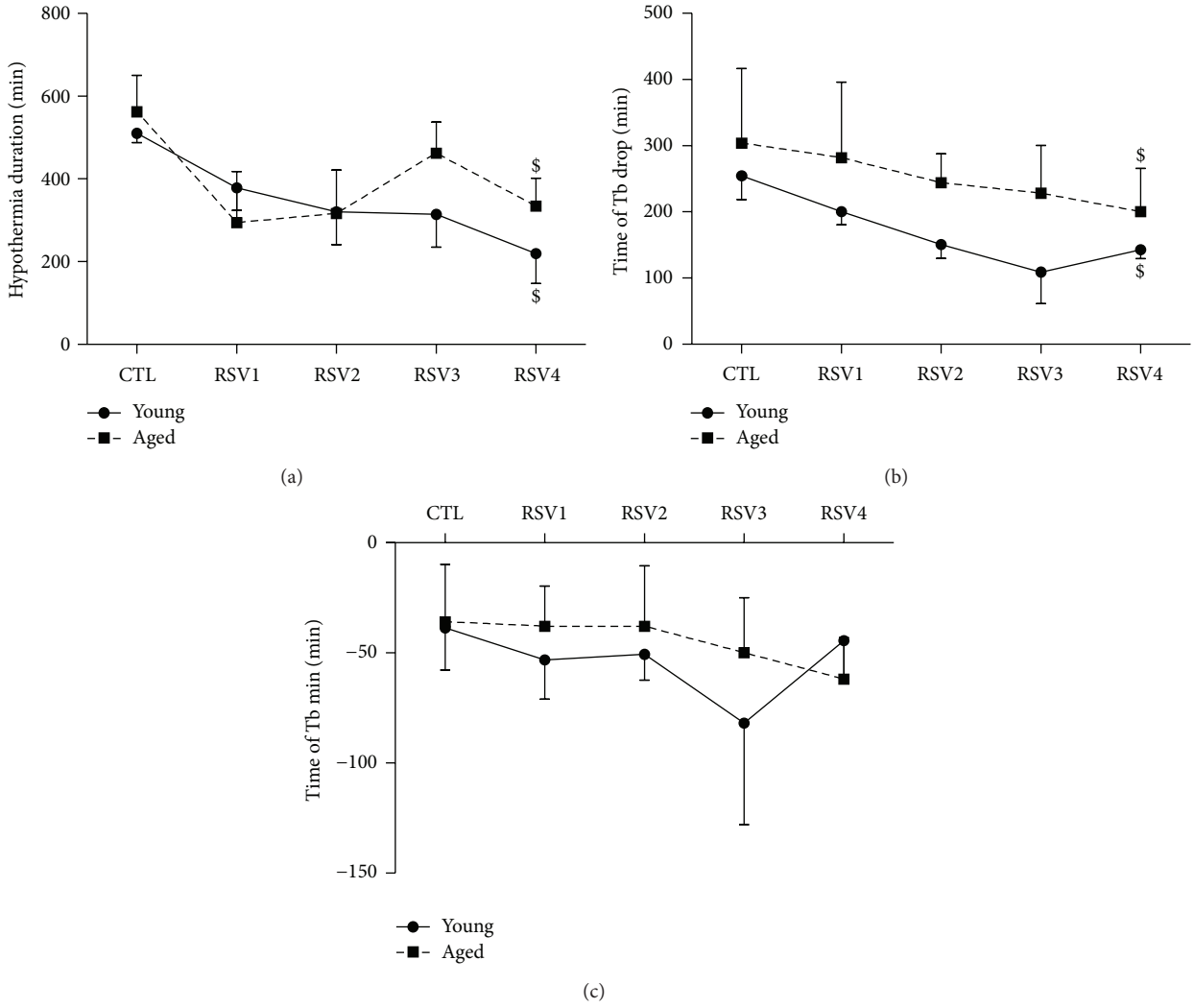

Figure 3: Daily hypothermia parameters in young (solid lines) and aged (dotted lines) female mouse lemurs. (a) Hypothermia duration (expressed in min). (b) Time from which the mean body temperature (Tb min) of the day decreased (Hdrop). (c) Time at which Tb min was reached (Hmin). During the first week animals received the control feeding (CTL), followed by 4 weeks of resveratrol (RSV) supplementation. Values are given as mean ± standard error of the mean. *represents a significant difference between young and aged animals ($P < 0.05$). $represents a significant effect of RSV supplementation compared to CTL condition ($P < 0.05$).

RSV supplementation, active phase LA reached similar values in aged and in young mouse lemurs. It is noteworthy that LA activity is affected differently by RSV supplementation in young and aged animals. It is widely described that aged animals [7, 8] and humans [10] exhibit a spontaneous impairment in their circadian rhythms that leads to increased resting phase LA and decreased active phase LA, showing the frailty of the aged individuals circadian clock. Even if no mechanism can be proposed at this time to explain the differential effect of RSV on young and aged animals, it is highly probable that this is due to the frailty of aged individual's circadian clock. Interestingly, under RSV conditions, we observed the same reduction of LA onset in aged animals as in young ones, suggesting a better synchronization with lights off in both age groups. Changes in locomotor activity parameters under RSV supplementation in aged animals suggest a restoration of some circadian rhythm parameters similar to those observed in young animals.

Day Tb remained significantly higher in aged animals compared to that in young ones during the course of the experiment ones and, similar to young animal, aged animals exhibited a gradual increase in day Tb with RSV supplementation. Tb min followed a similar pattern as day Tb with a final Tb min increase at the end of the 4 weeks of RSV supplementation. Similarly to young, hypothermia duration significantly decreased for aged animals during RSV4 condition and Hdrop was also significantly delayed. The shortening of torpor duration and higher day Tb suggest a specific impact of RSV on energy metabolism. We previously observed a similar difference in the impact of RSV on young or aged animals, with the endogenous period *tau* of aged animals being significantly more reduced by RSV

supplementation than the one of young animals [17]. These observations suggest that aged animals, known to exhibit impaired circadian rhythms [7, 8], may respond better to RSV positive effects, leading to an almost partial restoration of the rhythms, compared to young ones.

The results of the present study suggest that RSV might act as a potent regulator of circadian rhythms, more particularly during aging. Strong relations between metabolism and the regulation of the circadian clock have been recently evidenced [23]. Some studies suggest that relations between metabolism and circadian rhythms could be driven by changes in the expression of clock genes [24]. Caloric restriction, a regime known to prolong lifespan in various species [25], also affects circadian rhythms [26, 27] probably via activation of SIRT1. Since it has been demonstrated that RSV was able to activate the transcription of SIRT1 [14], which directly binds to the CLOCK/BMAL1 complex to regulate expression of clock genes [15, 16, 28, 29], the beneficial effects exerted by RSV could be mediated through resetting of the circadian clock, thus leading to better synchrony in metabolism and physiology [30]. However, it is noteworthy that the RSV-induced SIRT1 activation is now under debate [31]. Further studies focusing on the impact of RSV on clock and metabolism-related genes are now needed to address this hypothesis.

These results confirm the specific impact of RSV on rhythms parameters, with a more marked effect in aged animals (active phase LA and day Tb). The results of this study suggest that RSV might act as a potent regulator of both circadian clock and metabolism. RSV supplementation might thus represent a new and promising nonpharmacological treatment of circadian perturbations associated with normal or pathological aging such as Alzheimer's disease [32].

Conflict of Interests

The authors of the paper declare that they have no direct financial relationship with the commercial identities mentioned in the present paper that might lead to a conflict of interests.

Authors' Contribution

F. Pifferi and A. Dal-Pan contributed equally to this work.

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Review Article

Polyphenols in Exercise Performance and Prevention of Exercise-Induced Muscle Damage

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Although moderate physical exercise is considered an essential component of a healthy lifestyle that leads the organism to adapt itself to different stresses, exercise, especially when exhaustive, is also known to induce oxidative stress, inflammation, and muscle damage. Many efforts have been carried out to identify dietary strategies or micronutrients able to prevent or at least attenuate the exercise-induced muscle damage and stress. Unfortunately most studies have failed to show protection, and at the present time data supporting the protective effect of micronutrients, as antioxidant vitamins, are weak and trivial. This review focuses on those polyphenols, present in the plant kingdom, that have been recently suggested to exert some positive effects on exercise-induced muscle damage and oxidative stress. In the last decade flavonoids as quercetin, catechins, and other polyphenols as resveratrol have caught the scientists attention. However, at the present time drawing a clear and definitive conclusion seems to be untimely.

1. Introduction

Physical exercise, as well as nutritional behavior, is now widely considered to be an essential component of a healthy lifestyle. Moreover moderate exercise and an active lifestyle have been demonstrated to be useful in the primary and secondary prevention of cardiovascular diseases [1], type II diabetes [2], metabolic syndrome [3], and neurodegenerative diseases like Alzheimer's disease [4, 5]. It is now well known that regular and moderate exercise represents a mild source of stress able to induce an adaptive response. More importantly it seems that this adaptive response provides protection against other stressors [6], explaining how the practice of regular and moderate exercise plays a key role in the prevention of chronic and degenerative diseases.

The organism ability to adapt itself to stress is known as *hormesis*. This term refers to a J-shaped or inverted U-shaped dose-response curve produced by biological systems exposed to a stressor. Radak et al. [7] have firstly extended the *hormesis* theory to the exercise-induced effects. According to this theory, adaptation occurs only when the stressor dose, exercise bout, is within a specific range and followed by rest period. When the stressor is absent no adaptation can occur. On the other hand when exercise bouts are too heavy or not followed by rest periods (overtraining), pathological conditions as muscle damage, oxidative stress, and inflammation can occur as well. For a comprehensive review on this topic please refer to Radak et al. [8].

After many years of intensive research it is now well documented that exercise induces reactive oxygen species (ROS) production resulting in oxidative stress as clearly demonstrated by the induction of lipid peroxidation [9–12], superoxide anion generation through xanthine oxidase activation, and the increase in oxidized/reduced glutathione (GSSG/GSH) ratio [13, 14].

Many authors have investigated which metabolic pathways are influenced by exercise and if exercise may induce an adaptive response able to prevent, or at least delay, the onset of degenerative diseases. The potentially harmful condition of imbalance of the redox homeostasis plays a fundamental role in the organism adaptive response to exercise. In 2008, for the first time, Gomez-Cabrera et al. [15] defined moderate exercise as an antioxidant, explaining that the mild burst of ROS, generated by training, acts as a signal responsible for the activation of signaling pathways that lead to the induction of antioxidant enzymes in human tissue.

Other studies have investigated the pathways involved in this process, and it is now well documented that the major players are nuclear factor kB (NF-kB), the phosphoinositide 3-kinase/Akt (PI3 K/Akt), p53, heat shock proteins (HSPs), and mitogen-activated protein kinases (MAPKs) [16–19].

If it is commonly accepted that moderate exercise and training are key components of a healthy lifestyle and help to prevent or delay the onset of pathological conditions, it is now clear that these beneficial effects are lost when the exercise becomes exhaustive, indicating that the exercise intensity and duration are responsible for the beneficial or detrimental effects of physical activity.

A great body of literature has demonstrated that exhaustive exercise causes oxidative stress, inflammatory response, and structural damage to muscle cells, evidenced by an increase in the plasma activity of cytosolic enzymes, namely, lactic dehydrogenase (LDH) and creatine kinase (CK) [11, 20, 21]. So, many studies have investigated the possibility to prevent the exercise-induced oxidative stress and muscle damage through nutritional intervention, mainly using antioxidant vitamins and polyunsaturated fatty acids [22–29].

The effects of both Vitamins C and E have been investigated in a wide range of exercise conditions, using a variety of supplementation strategies, timing, and dosage. Even though antioxidant vitamin supplementation seems to be a reasonable strategy to reduce or prevent tissues damage in active muscles, there are only few and weak results able to support this thesis [22].

Thompson et al. [24] demonstrated that an acute supplementation (1000 mg) of Vitamin C 2 h prior to a 90 min intermittent shuttle running test does not affect the increases in serum CK level. Despite that some authors [23] found an increase in plasma total antioxidant capacity in subjects treated with 1000 mg Vitamin C for 2 weeks prior to 2.5 h cycling at 60% VO_2 max, others [30] found a reduction in IL-6 and malondialdehyde (MDA) plasma levels in subjects treated with 400 mg Vitamin C for 2 weeks prior to a 90 min intermittent shuttle running test. Similarly to Vitamin C, the effects of Vitamin E on exercise-induced muscles damage are still under debate, and the results of different studies are not in agreement. Cannon et al. [26, 31] found that the supplementation of 800 IU of α-tocopherol for 48 days does not prevent plasma CK release due to 45 min downhill running in young men, even though it reduces the secretion of IL-1β and IL-6. Two studies reported a positive effect of Vitamin E on exercise-induced muscles damage; Beaton et al. [25] and McBride et al. [32] found, respectively, that 30 days and 14 days supplementation of 1200 IU α-tocopherol reduces CK level in serum and plasma after different exercises. These results disagree with those published by Avery et al. [33] which found that 1200 IU α-tocopherol, 21 days before and 10 days after exercise, increases CK serum level when subjects undergo exercise bouts.

When Vitamins C and E are supplemented in combination some positive effect can be obtained, but, as recently reviewed [22], for each study providing positive effects [34] there are other studies that provide no effect [35, 36].

Only recently the attention has been shifted to the effects of nutraceutical bioactive compounds as polyphenols. Polyphenols are a class of organic chemical compounds, mainly found in plants, characterized by the presence of multiples of phenol structural units. Recently a great body of literature has underlined a potential relationship between bioactive compounds from plant foods and the prevention of cardiovascular and neurodegenerative diseases and other pathological conditions [37–41].

This review will summarize some of the actual knowledge on polyphenolic compounds that have been demonstrated both to exert a significant effect in exercise-induced muscle damage and to play a biological/physiological role in improving physical performance.

2. Flavonoids

Among nutraceutical compounds, flavonoids are the mainly studied ones for their positive effects on human health. Some of them have been proposed to be beneficial in exercise and exercise performance. Flavonoids are a family of plant bioactive compounds that share a common backbone. The flavonoid family includes many different subclasses: flavones, flavonols, flavanones, flavanones, isoflavones, and anthocyanidins; in Table 1 some examples are reported for each subclass.

On the left column flavonoid subclasses are reported; on the right some examples for each subclass are listed.

2.1. Flavonols. Flavonols are present in human nutrition as both glycosides and aglycone forms, and it has been estimated that the daily intake is within the range of 20–50 mg/die in Western population. Of these flavonols quercetin (Figure 1) accounts for about 13.82 mg/die [42], resulting in being one of the most abundant flavonols in Western diet.

Quercetin, mainly present as quercetin glycosides (rutin, spiraeoside, troxerutin quercitrin, isoquercitin, and hyperoside), is widely distributed in plant food; it is found in apples, berries, onions, grapes, tea, and tomatoes as well as in some medicinal plants as *Hypericum perforatum* and *Gingko biloba* [43–46].

Recent studies suggest that quercetin bioavailability is much higher than that originally thought [47]. Quercetin, assumed both as purified dietary supplement or as natural food source, has been clearly demonstrated to increase its plasma levels in humans, even though a high interindividual variability in plasma quercetin response has been reported [48–52]. Quercetin aglycone is a lipophilic molecule able to diffuse through the enterocyte membranes. Quercetin glucosides are easily hydrolyzed both in the mouth, during chewing, and in the gut, thanks to beta-glucosidase enzymes [53]. The overall result is that quercetin absorbed as aglycone is much higher than that expected from its food content [47].

Quercetin and other flavonoids have been reported to exert a variety of biological activities often related to their antioxidant nature. McAnulty et al. [54] have investigated the effects of quercetin supplementation in cycling athletes. Subjects were supplemented with 1000 mg/die quercetin or placebo for 6 weeks before and during 3 days in which they cycle for 3 h/die. In blood and plasma, F2-isoprostanes,

Table 1: Flavonoid classification.

Flavonoid subclasses	Representative compounds
Flavonols	*Quercetin, Kaempferol*
Flavones	*Apigenin, Luteolin*
Flavanols	*Epicatechin, Gallocatechin*
Flavanones	*Naringenin, Hesperidin*
Isoflavones	*Daidzein, Genistein*
Anthocyanidins	*Cyanidin*

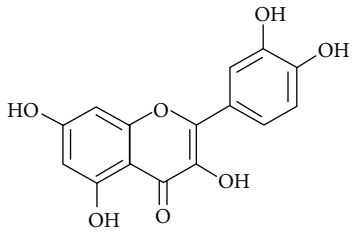

Figure 1: Quercetin structure. Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is a typical flavonol-type flavonoid and forms the backbone for many other flavonoids. Flavonoids are characterized by 2 benzene rings connected by an oxygen-containing pyrene ring.

nitrite, ferric-reducing ability, trolox equivalent antioxidant capacity, and C-reactive protein were analyzed before and after exercise. The authors concluded that cycling induces a strong increase in blood biomarkers of oxidative stress and inflammation, but, despite previous data demonstrating potent antioxidant actions of quercetin in *in vitro* and animal models [55–59], long-term quercetin supplementation was not able to exert any preventive effect on exercise-induced oxidative stress and inflammation biomarkers.

Similar conclusions have been reached when the effects of quercetin in association with catechin, isoquercetin, and PUFA were evaluated in an acute crossover study involving 20 endurance athletes supplemented with an oral dose providing 1000 mg quercetin [60]. Athletes were supplemented 15 min prior to a 2-hour run. Authors analyzed before, immediately after, and one hour after exercise plasma quercetin level, C-reactive protein, IL-6, and other cytokines and inflammatory biomarkers, confirming a good quercetin bioavailability but supporting that it does not prevent postexercise inflammation.

In another study [61] the effects of quercetin on inflammation and exercise-induced muscle damage in 39 trained cyclists were evaluated. In this study quercetin was provided in association with Vitamin C, niacinamide, and folic acid (Q) or in association with Vitamin C, niacinamide, folic acid, catechin, isoquercetin, and PUFA (Q-EGCG) for 2 weeks before, during, and 1 week after a 3 days period in which the subject cycled for 3 h/die. Q-EGCG association was specifically designed to improve quercetin bioavailability and extend its bioactive effects. Results confirm those previously published [54], showing that a long-term quercetin (Q) supplementation is not able to modulate serum C-reactive protein level and plasma IL-6 concentration and prevent CK relies. The Q-EGCG association decreased almost all biomarkers but not CK levels.

Recently Askari et al. [62] published a double-blind clinical trial on 60 male students with an athletic history of at least 3 years. They found that 500 mg quercetin plus 250 mg Vitamin C daily treatment lasting for 8 weeks was able to improve some markers such as lean body mass, basal metabolic rate, and total energy expenditure. In a previous study the same authors showed that quercetin plus Vitamin C treatment reduced CK plasma levels after treadmill exercise [63].

Beside the possibility to partially prevent exercise-induced muscle damage and inflammation, in the last decade a considerable effort has been performed to analyze the possibility that quercetin supplementation could improve aerobic exercise performance in human.

The rationale behind this hypothesis is given by the knowledge that some polyphenols as catechins, resveratrol, quercetin, and curcumin have been shown to activate sirtuins (SIRT1). SIRT1 activation modulates a variety of biological and physiological processes including skeletal muscle function and mitochondria biogenesis [64, 65].

Early human and animal studies reported a correlation between quercetin supplementation, endurance capacity, and mitochondrial biogenesis improvement. Davis et al. [66] published promising results showing that 7 days quercetin treatment (12.5 or 25 mg/kg b.w.) increases the expression of genes associated with mitochondrial biogenesis (PGC-1a and SIRT1), mitochondrial DNA content, and cytochrome-C concentration, both at muscle and brain levels in mice. Beside these biological data, quercetin-treated mice showed a significantly higher time to fatigue, in a treadmill running test, than their placebo-treated counterparts. A more recent study has evaluated quercetin ability to increase endurance capacity and VO_2 max in a cross-over protocol on healthy but untrained volunteers [67]. Data indicate that a 7 days 1000 mg/die quercetin supplementation is responsible for an improvement (13.2%) in time to fatigue, during a cycling test, and for a 3.9% increase in VO_2 max. Authors hypothesized a quercetin-induced increase in mitochondrial biogenesis that would lead to an increase in endurance capacity through a shift toward fat oxidation during exercise, but they did not investigate any mitochondrial biogenesis biomarkers.

Other studies have analyzed the effect of quercetin on exercise performance, some reporting positive effects [68, 69], while others do not [70–73], but to our knowledge an increase in mitochondrial biogenesis has not been reported in human even though Neiman et al. [68] have shown a modest and insignificant increase in relative mitochondrial DNA copy number following quercetin supplementation.

In a recent meta-analysis Kressler et al. [74] have demonstrated that quercetin can improve endurance capacity in humans, but they concluded that the benefit magnitude is only trivial to small. Because the "mitochondrial biogenesis" theory that could explain the quercetin-induced endurance capacity has not been demonstrated, some other mechanisms have been suggested. According to its ability to bind and act as antagonist at adenosine receptor level, quercetin may improve exercise performance in a caffeine-like manner

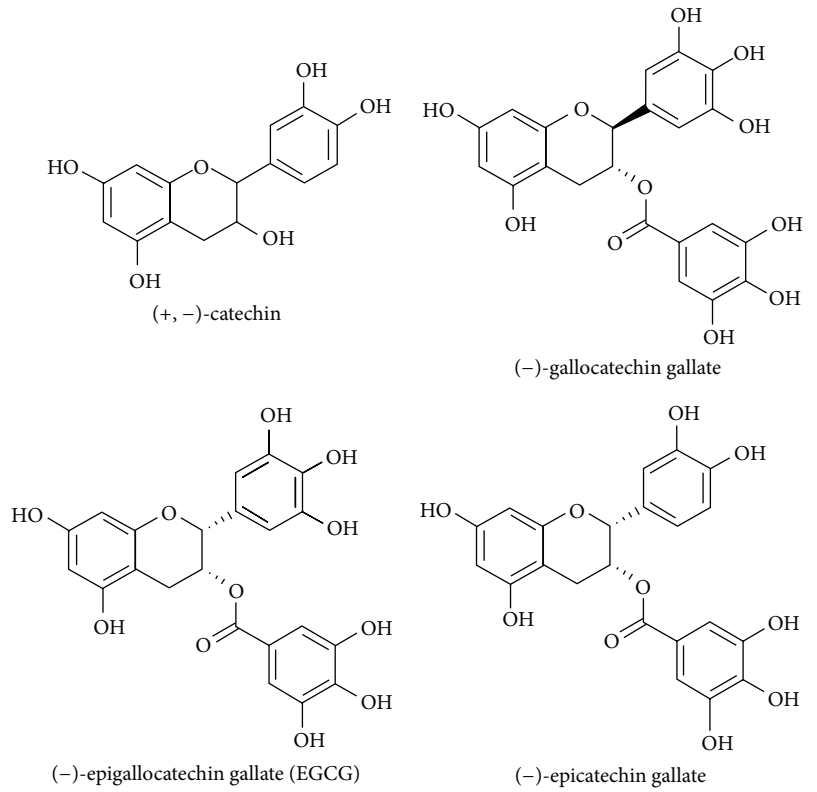

Figure 2: Chemical structure of the main catechins. Catechins are characterized by two benzene rings and a dihydropyran heterocycle with a hydroxyl group in position 3. Thanks to the presence of this hydroxyl group catechins are also called flavan-3-ol. Catechin gallates are gallic acid esters of the catechins as epigallocatechin gallate and gallocatechin gallate, which are typically found in tea.

[75, 76]. However, up to now, this hypothesis has still to be demonstrated, and the only study that has compared caffeine versus quercetin for their ability to improve exercise performance in the heat has failed to find any significant result [77].

2.2. Catechins. Although quercetin is the most studied flavonoid in relation to exercise, other molecules are under investigation for their ability to prevent exercise-induced muscle damage and to affect physical performance. Among them catechins have shown to exert some effects at least in animal models.

As all other flavonoids, catechins represent a family of plant secondary metabolites and belong to the flavanols subclass. Catechins (Figure 2) are easily found in edible foods and plants such as green tea and cacao. Catechin family consists mainly of epigallocatechin gallate (EGCG), gallocatechin gallate, and epicatechin gallate and have been demonstrated to be bioavailable and to possess various biological properties such as cardioprotective [78, 79], antiatherogenic [80], and anticarcinogenic effects [81, 82].

Some evidence on catechins ability to modulate exercise-induced muscle damage is rising. Haramizu et al. [83] have recently shown that 8 weeks catechins treatment is able to attenuate loss of muscle force, exercise-induced muscle damage, and oxidative stress biomarkers (CK, LDH, MDA) and to significantly sustain GSH/GSSG ratio after downhill running exercise in senescence-accelerated mice.

Catechins ability to modulate the exercise performance, both in animals and humans, has also been investigated. Murase et al. [84] have shown that green tea extract improves, in a dose-dependent manner, the time to exhaustion in BALB/c mice undergoing a swimming test after 7 weeks treatment. They have also demonstrated, through indirect calorimetry and biochemical analysis, that green tea extract induces a more efficient use of lipids as suggested by the increase in oxygen consumption, β-oxidation activity in muscle, and fat oxidation, suggesting that the green tea extract-induced lipid oxidation and mobilization are responsible for the increase in endurance capacity. These findings have been deepened, and recently it has been observed that EGCG treatment increases the expression of genes involved in mitochondrial fat oxidation at muscle level in high-fat fed mice [85]. Although most studies on green tea have been performed in animals, a considerable amount of data are now available in humans. Dulloo et al. [86] showed that a green tea extract rich in catechins and caffeine increases the daily energy expenditure in humans. More recently, an acute dose of green tea extract has been evaluated on healthy untrained men in a 30 min cycling test at 60% VO_2 max [87]. Results demonstrated that green tea extract improves fat oxidation and insulin sensitivity during moderate exercise.

Other recent findings report that short-term EGCG supplementation increases VO_2 max in adult humans [88], while Dean et al. [89] concluded that 6 days EGCG treatment does not significantly affect fat oxidation during a 60 min cycling exercise at 60% VO_2 max in moderately well-trained men. In a randomized, double-blind crossover study, 10 endurance-trained subjects exercised for 2 hours at 50% of their maximal power output before and after 3 weeks of green tea extract supplementation [90]. The treatment did not influence fat and energy metabolism biomarkers (oxygen uptake, respiratory exchange ratio, and energy expenditure), cytokines and inflammatory parameters (IL-6, C-reactive protein), and oxidative stress biomarkers (thiobarbituric acid-reactive substances, oxidized LDL). However,plasma CK level was significantly reduced. Recently Jowko at al. [91] published data obtained treating a group of 16 soccer players with a single dose of 640 mg green tea catechins. Athletes involved in the study performed a muscle-endurance test consisting in 3 bouts to exhaustion of bench press and back squat. Prior to and after exercise test plasma levels of thiobarbituric acid-reacting substances, uric acid, total catechins, total antioxidant status, and CK activity were analyzed.

None of the analyzed biomarkers was affected by the ingestion of green tea catechins, suggesting that the 640 mg dose was too low to attenuate exercise-induced oxidative stress and muscle damage. Green tea catechins-treated players were able to perform a higher number of lift repetitions during the test.

Taken together, data from available studies seem to suggest that catechins can improve physical performance particularly in term of endurance capacity and VO_2 max in untrained subjects, but the same results cannot be reached in physically active people and well-trained athletes.

3. Other Polyphenols

Resveratrol is a well-known bioactive compound able to induce a wide variety of biological responses. It has shown beneficial effects against most degenerative and cardiovascular diseases from atherosclerosis, hypertension, ischemia/reperfusion, heart failure, diabetes, obesity, aging and neurodegenerative diseases [40, 92]. Resveratrol, *3,5,4′-trihydroxystilbene* (Figure 3), is a natural phenol present in grape skin and seeds and in grape-derived products like red wine.

Only few studies have investigated resveratrol ability to modulate exercise performance and some evidence suggests that it could play a role improving endurance capacity. It has been demonstrated that after 12 weeks treatment resveratrol prevents the decline in running time to exhaustion, in oxygen consumption, and in lipid oxidation in a senescence-accelerated mice model (SAMP1). Data shown in this study demonstrate that the resveratrol induction of mitochondrial biogenesis (suggested by the mRNA levels of genes involved in mitochondrial biogenesis and energy metabolism, namely, peroxisome proliferator-activated receptor coactivator-1, cytochrome-C,

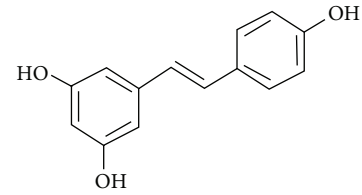

Figure 3: Resveratrol structure. Resveratrol (3,5,4′-trihydroxystilbene) belongs to the stilbene group. It exists both as *cis*- and *trans*-isomers.

and medium-chain acyl-CoA dehydrogenase) is responsible for the prevention of ageing-related performance impairment [93]. Accordingly, Dal-Ros et al. [94] have recently demonstrated that chronic red wine polyphenols intake prevents aging-induced performance decline in rats.

Recently some authors have focused their interest on the effect of caffeic acid, especially in its phenethyl ester form (CAPE) (Figure 4). Shen et al. [95] demonstrated that 10 mg/kg b.w. CAPE oral treatment protects rat muscle tissues from exercise-induced damages. In this study the protocol was represented by an intermittent downhill eccentric exercise able to induce both muscle damages and inflammation as demonstrated by the increase in CK serum level, NF-kB activation, iNOS and COX-2 expression, and IL-β level. Moreover, an *ex vivo* study by Chen et al. [96] shows that CAPE exhibits protective effect against hyperthermal stress, known to impair endurance capacity, in isolated peripheral blood mononuclear cells from competitive cyclists. Pretreatment of mononuclear cells with CAPE reduced hyperthermia-induced necrosis, superoxide production, glutathione depletion, and intracellular superoxide anion production in a dose-dependent manner.

Beside studies on single polyphenols, some researches have been focused on the effects of polyphenol mixtures obtained from fruits, plants, or algae. Hurst et al. [97] have investigated the effects of a blueberry extract on skeletal muscle cultured cells showing a dose-dependent protective effect on oxidative stress. Furthermore, Nakazato et al. [98] found that rats fed an apple polyphenol-enriched diet for 3 weeks showed a better oxidative stress biomarkers profile (thiobarbituric acid reactive substances and protein carbonyls), a lower force deficit, and earlier recovery after strenuous lengthening muscle contractions with respect to their control counterparts. Promising results have been published by Swamy et al. [99] on the effect of pomegranate peel extract supplementation in rats. In this study rats were supplemented for 28 days with 25 mg/die pomegranate peel extract, rich in polyphenols, and were forced to swim until exhaustion. Interestingly, they recorded a strong increase in time to exhaustion among rats fed pomegranate extract with respect to their control counterparts. Authors also found that pomegranate extract fed rats had a higher glycogen and ATP muscular content with respect to controls, and they hypothesized that pomegranate polyphenols are responsible for a better glucose utilization resulting in a longer swimming time to exhaustion. Moreover they found that LDH and

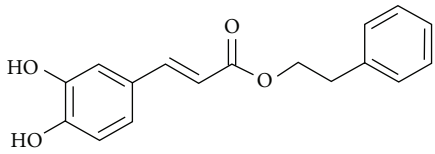

Figure 4: Caffeic acid phenethyl ester (CAPE) structure. CAPE is a polyphenolic compound primarily found in propolis.

CPK serum levels were reduced in pomegranate extract fed rats, suggesting a protective role of pomegranate polyphenols against exhaustive exercise-induced muscle damage.

Dark-chocolate polyphenols have been ascribed to be responsible for some positive effects of dark-chocolate consumption during exercise. A study investigated the effects of dark-chocolate (80 g/die for 2 weeks) consumption on oxidative stress biomarkers after prolonged exhaustive exercise. Plasma F2-isoprostanes were significantly lower both at exhaustion and after 1 hour recovery in dark-chocolate-supplemented subjects with respect to their control counterparts. Moreover dark-chocolate consumption was associated with lower oxidized low-density lipoprotein levels before and after exercise and with an increase in free fatty acids levels during exercise, even though the time to exhaustion was not significantly affected [100]. Similar conclusions were drawn when Davison et al. [101] investigated the acute effects of preexercise dark-chocolate consumption. In this study dark chocolate prevents the exercise-induced increase in plasma F2-isoprostane. These results suggest that dark-chocolate intake reduces exercise-induced oxidative stress biomarkers.

Recently it has been found that *Ecklonia cava* (a species of brown alga present in the ocean of Japan and Korea) polyphenols acute preexercise supplementation induces a slight but significant increase in time to exhaustion in healthy human subjects [102].

4. Conclusion

Overall, the available literature seems to suggest that nutraceutical bioactive compounds as polyphenols, known for their effects on degenerative and chronic diseases, can also provide protection against exercise-induced muscle damage and oxidative stress thanks to their antioxidant and anti-inflammatory properties. However the possibility to improve the exercise performances remains unclear. Even though this topic is extremely fascinating and has attracted a great research effort scientific data do not allow to draw a clear conclusion. On one hand, *in vitro* and *in vivo* animal studies suggest that polyphenols could really play a role in improving endurance performance; on the other hand, most human trials do not support this hypothesis and have failed to demonstrate that nutraceuticals as quercetin, catechins, or resveratrol can really affect exercise performance and VO_2 max. The reason for this discrepancy has still to be fully elucidated; however, some hypotheses are possible. Human studies often differ from each other in the training level of enrolled subjects; some studies have been carried on well-trained athletes while others on healthy but untrained subjects. Among recent studies those involving untrained subjects seem to have obtained better results on improving endurance capacity, although Kressler et al. [74], in their meta-analysis on quercetin, suggest that the variability among studies does not appear to be associated with the initial subject fitness level. Moreover while quercetin and catechins effects have been investigated both in animal and in human studies other polyphenols need to be further studied in human trials to reach a clear and unambiguous conclusion.

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Research Article

Dietary Flavonoids as Therapeutics for Preterm Birth: Luteolin and Kaempferol Suppress Inflammation in Human Gestational Tissues *In Vitro*

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Infection/inflammation is commonly associated with preterm birth (PTB), initiating uterine contractions and rupture of fetal membranes. Proinflammatory cytokines induce matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM) and prostaglandins which initiate uterine contractions. Nuclear factor-κB (NF-κB) and activator-protein- (AP-)1 have key roles in the formation of these prolabour mediators. In nongestational tissues, dietary flavonoids such as luteolin and kaempferol inhibit NF-κB, AP-1, and their downstream targets. The aim of this study was to determine if luteolin and kaempferol reduce infection-induced prolabour mediators in human gestational tissues. Fetal membranes were incubated with LPS, and primary amnion cells and myometrial cells were incubated with IL-1β in the absence or presence of luteolin or kaempferol. Luteolin and kaempferol significantly reduced LPS-induced secretion of proinflammatory cytokines (IL-6 and IL-8) and prostaglandins (PGE_2 and $PGF_{2\alpha}$) in fetal membranes, IL-1β-induced COX-2 gene expression and prostaglandin production in myometrium, and IL-1β-induced MMP-9 activity in amnion and myometrial cells. Luteolin and kaempferol decreased IL-1β-induced NF-κB p65 DNA binding activity and nuclear c-Jun expression. In conclusion, luteolin and kaempferol inhibit prolabour mediators in human gestational tissues. Given the central role of inflammation in provoking preterm labour, phytophenols may be a therapeutic approach to reduce the incidence of PTB.

1. Introduction

Preterm birth (PTB) is the single most important complication contributing to poor pregnancy and neonatal outcome, globally, being defined as childbirth occurring at less than 37-week gestation. According to the World Health Organisation (WHO), more than 1 in 10 babies is born preterm every year [1], and this incidence has been steadily rising since the 1980s despite obstetric intervention [2]. With long term health consequences, the care of the prematurely born infant is extremely expensive, and the emotional stress on the family is sizeable. The estimated cost associated with PTB in the United States alone, in terms of medical and educational expenditure and lost productivity in 2005, was more than US$26.2 billion [3].

Spontaneous PTB (sPTB) accounts for approximately 70% of all PTB with 60% due to idiopathic preterm labour and the remaining 40% due to preterm pre-labour rupture of the fetal membranes (PPROM) [4]. Infection is the biggest aetiological factor for the onset of sPTB [5]. Infection activates the maternal immune system, which causes production of the proinflammatory cytokines IL-1β and TNF-α. They bind to their respective receptors located on placenta, fetal membranes, and myometrium to induce the activity of the proinflammatory and prolabour transcription factors activator-protein- (AP-)1 and nuclear factor kappa B (NF-κB) [6–9]. Once activated, AP-1 and NF-κB upregulate transcription of proteases, prostaglandins, and cytokines which lead to delivery of the baby by causing cervical ripening, rupture of fetal membranes, and uterine contractions, three critical stages in the initiation of labour.

Epidemiological studies have revealed that a diet rich in plant-derived foods has a protective effect on human health [10, 11]. Luteolin is a citrus flavonoid found in high amounts in

parsley, thyme, peppermint, basil, herb, celery, and artichoke. Kaempferol is a flavonoid found in many edible plants including tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries, and grapes. Previous studies performed in nongestational tissues have demonstrated that luteolin and kaempferol are two phytophenols that have potent anti-inflammatory properties, exerting these actions via inhibition of NF-κB [12–15] and AP-1 [14, 16, 17]. The effect of luteolin and kaempferol as modulators of the inflammatory response associated with labour is, however, not yet known. Thus, the aim of this study is to determine whether luteolin and kaempferol will reduce infection-induced prolabour mediators in human gestational tissues. Experiments will be performed in the presence of LPS or IL-1β as these are likely to be factors stimulating proinflammatory cytokines, prostaglandins, and proteases in the context of infection-induced PTB. We analysed the effect of luteolin and kaempferol on (i) COX-2 and subsequent PGE_2 and $PGF_{2\alpha}$ production, (ii) proinflammatory cytokine (IL-6) and chemokine (IL-8) mRNA expression and release, and (iii) matrix-metalloproteinase- (MMP-)9 mRNA expression and release. The effect of luteolin and kaempferol on NF-κB (NF-κB p65 DNA binding activity and IκB-α expression) and AP-1 (nuclear c-Jun expression) was also examined.

2. Methods and Materials

2.1. Tissue Collection. Human placentae and attached fetal membranes were obtained (with Institutional Research and Ethics Committee approval) from nonobese women who delivered single, healthy infants at term gestational age (37–40-week gestation) undergoing elective Caesarean section, whether due to a medical or obstetrical reason or on patients request. Human myometrium was obtained from the upper margin of the incision made in the lower uterine segment at the time of term Caesarean section. Amnion and underlying choriodecidua were obtained 2 cm from the periplacental edge. None of the patients were in labour or had received uterotonics or tocolytics.

2.2. Fetal Membrane Explants. Fetal membranes (combined amnion and choriodecidua) were obtained within ten minutes of delivery, and dissected fragments were placed in ice-cold PBS. Tissue fragments were placed in Roswell Park Memorial Institute (RPMI) 1640 media at 37°C in a humidified atmosphere of 8% O_2 and 5% CO_2 for 1 h. Explants were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (200 mg wet weight/well). The explants were incubated, in duplicate, in 2 mL RPMI 1640 containing penicillin G (100 U/mL) and streptomycin (100 μg/mL). Explants were incubated, for 24 h, in the presence of 10 μg/mL LPS (to facilitate the production of proinflammatory mediators) in the absence (DMSO control) or presence of 20 μM luteolin or 100 μM kaempferol (Sigma, St. Louis, MO, USA) (n = 8 patients). A dose response was used to determine the concentrations of luteolin and kaempferol used for this study (data not shown), with the initial concentrations for the dose response determined from past studies [18–23]. After 24 h incubation, medium was collected and stored at −80°C until assayed for cytokine and prostaglandin concentrations as detailed below. Tissue was collected and stored at −80°C until assayed for gene expression by qRT-PCR. Experiments were performed in fetal membranes from eight patients.

2.3. Myometrial Cell Culture. Primary myometrial smooth muscle cells were used to investigate the effects of luteolin and kaempferol on the COX-prostaglandin pathway and MMP-9. Myometrial tissue was washed in PBS and finely dissected. Myometrium was minced and digested for 45 min in Dulbecco's Modified Eagle's Medium: nutrient mixture F12 (DMEM/F12) with 3 mg/mL type 1 collagenase (Worthington Biochemical, Freehold, NJ, USA) and 80 μg/mL DNase I (Roche Diagnostics Australia). Cells were centrifuged at 400× g for 10 min and grown in a 75 cm^2 flask in DMEM/F12 with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (37°C and 5% CO_2 and 21% O_2). Myometrial cells from passages 1–4 were trypsinised in TrypLE Express (Life technologies, Grand Island, NY, USA) and cultured in 12-well plates in DMEM/F12 with 10% heat inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin until they reached the required confluence (~90%). Cells were then incubated in 500 pg/mL IL-1β (to facilitate the production of proinflammatory mediators) and either in the absence (DMSO control) or presence of 20 μM luteolin or 100 μM kaempferol (n = 6 patients) for 24 h. For IκB-α studies, cells were pretreated with luteolin and kaempferol overnight, followed by a 30 min incubation with 500 pg/mL IL-1β. For c-Jun studies, cells were pretreated for 6 h with luteolin and kaempferol followed by an overnight incubation with 500 pg/mL IL-1β. The media were collected and stored at −80°C, until assayed for cytokine, prostaglandin, and MMP-9 concentrations as detailed below. Cell pellets were collected and stored at −80°C, before being analysed for IκB-α and c-Jun expression by Western blotting, gene expression by qRT-PCR, or NF-κB p65 DNA binding activity by transcription factor assay as detailed below. Experiments were performed in myometrium from six patients.

2.4. Primary Amnion Cell Culture. Primary amnion epithelial cultures were used to investigate the effects of luteolin and kaempferol on MMP-9 expression and enzyme activity. Cells were prepared as previously described [24]. Primary amnion cells (passage 1) at ~90% confluence were incubated in the absence or presence of 1 ng/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol (n = 6 patients). After 24 h incubation, medium was collected, and assessment of enzymes of ECM weakening and rupture (MMP-9) was performed as detailed below. Cells were also collected and MMP-9 gene expression analysed by qRT-PCR as detailed below. Experiments were performed in amnion from six patients.

2.5. Cytokine and Prostaglandin Assays. Conditioned medium from cell and tissue culture experiments was assessed for IL-6 and IL-8 concentrations using commercial ELISA

according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The release of PGE_2 and $PGF_{2\alpha}$ into the incubation medium was assayed using commercially available competitive enzyme immunoassay kits according to the manufacturer's specifications (Kookaburra Kits from Sapphire Bioscience, Redfern, NSW, Australia). All data were corrected for total protein and expressed as either ng or pg per mg protein. The protein content of tissue homogenates was determined using BCA protein assay (Pierce, Rockford, USA), using BSA as a reference standard, as previously described [25–27].

2.6. Gelatin Zymography. Assessment of enzymes of ECM weakening and rupture (MMP-9) was performed by gelatin zymography as previously described [27, 28]. Proteolytic activity was remodeled as clear zones of lysis on a blue background of undigested gelatin.

2.7. RNA Extraction and qRT-PCR. Total RNA from cells and tissues was extracted using TRI Reagent according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). RNA concentrations were quantified using a spectrophotometer (NanoDrop, Thermo Scientific). RNA quality and integrity were determined via the A_{260}/A_{280} ratio. Two hundred ng (fetal membranes) or 300 ng (amnion and myometrial cells) of RNA was converted to cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was diluted fiftyfold, and 4 μL of this was used to perform RT-PCR using Sensimix Plus SYBR green (Quantace, Alexandria, NSW) and 100 nM of primers: GAPDH (QT01192646), IL-6 (QT00083720), IL-8 (QT00000322), TNF-α (QT01079561), COX-2 (QT00040586), and MMP-9 (QT00040040) (Qiagen, Germantown, MD, USA). The specificity of the product was assessed from melting curve analysis. RNA without reverse transcriptase during cDNA synthesis as well as PCR reactions using water instead of template showed no amplification. Average gene C_T values were remodeled to the average GAPDH mRNA C_T values of the same cDNA sample. Fold differences were determined using the comparative C_T method and expressed relative to basal [29].

2.8. Western Blotting. Western blotting was performed as we have previously described [25, 30]. For IκB-α protein expression, cell lysates were prepared as detailed in [25, 27]. To assess c-Jun expression, nuclear protein was extracted as we have previously described [31]. Rabbit polyclonal anti-IκB-α and rabbit polyclonal anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at 1 μg/mL. Forty micrograms (IκB-α) or 5 μg (c-Jun) of protein was separated on polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to PVDF. Protein expression was identified by comparison with the mobility of protein standard. Membranes were viewed and analysed using the ChemiDoc system (Bio-Rad Laboratories, Hercules, CA, USA). For the IκB-α blot, the membranes were stripped and reprobed with β-actin (A5316; Sigma, St. Louis, MO, USA), used at 1.5 μg/mL to ensure even protein loading. For the c-Jun blot, the membrane was stained with Ponceau S to ensure even loading [32].

2.9. NF-κB p65 Transcription Factor Assay. Myometrial cells were pretreated with 20 μM luteolin and 100 μM kaempferol for 6 h, followed by 24 h treatment with 500 pg/mL IL-1β (n = 4 patients). Nuclear protein was extracted [31] and NF-κB p65 DNA binding in the nuclear protein assessed using a commercially available NF-κB p65 transcription factor ELISA according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA). A Bio-Rad xMark Microplate Spectrophotometer was used to read the sample absorbance at 450 nm, with data expressed as absorbance at 450 nm.

2.10. Statistical Analysis. All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data were first logarithmically transformed, before analysed by one-way ANOVA using Tukey multiple range tests. Statistical difference was indicated by a P value of less than 0.05. Data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Effect of Luteolin and Kaempferol on Proinflammatory Cytokines. In fetal membranes, qRT-PCR was performed to determine if luteolin and kaempferol reduced steady state levels of proinflammatory cytokines. As shown in Figure 1(a), LPS induced a significant increase in proinflammatory cytokine expression. Coincubation with luteolin or kaempferol significantly abrogated LPS-induced gene expression of TNF-α, IL-6, and IL-8. LPS induced a significantly higher concentration of both IL-6 and IL-8, when compared with basal concentration (Figure 1(a)). ELISA was then used to determine cytokine release. Coincubation with luteolin or kaempferol caused a significant reduction in LPS-induced IL-6 and IL-8 concentrations (Figure 1(b)). TNF-α concentration in the incubation media was below the limit of detection of the ELISA (data not shown).

Primary myometrial cells incubated with IL-1β were associated with significantly increased mRNA expression (Figure 1(c)) and release of IL-6 and IL-8 (Figure 1(d)). However, coincubation with luteolin or kaempferol had no effect on IL-1β-induced cytokine release or gene expression.

3.2. Effect of Luteolin and Kaempferol on the COX-Prostaglandin Pathway. Fetal membranes and myometrial cells were used to determine the effect of luteolin and kaempferol on COX-2 expression and prostaglandin levels. Experiments were performed as detailed above. When compared to basal, LPS-induced COX-2 mRNA levels (Figure 2(a)) and subsequent PGE_2 and $PGF_{2\alpha}$ (Figure 2(b)) concentrations were significantly greater in fetal membranes. Coincubation with luteolin and kaempferol significantly decreased LPS-induced PGE_2 concentration (Figure 2(b)). LPS-stimulated concentrations of COX-2 mRNA expression (Figure 2(a)) and

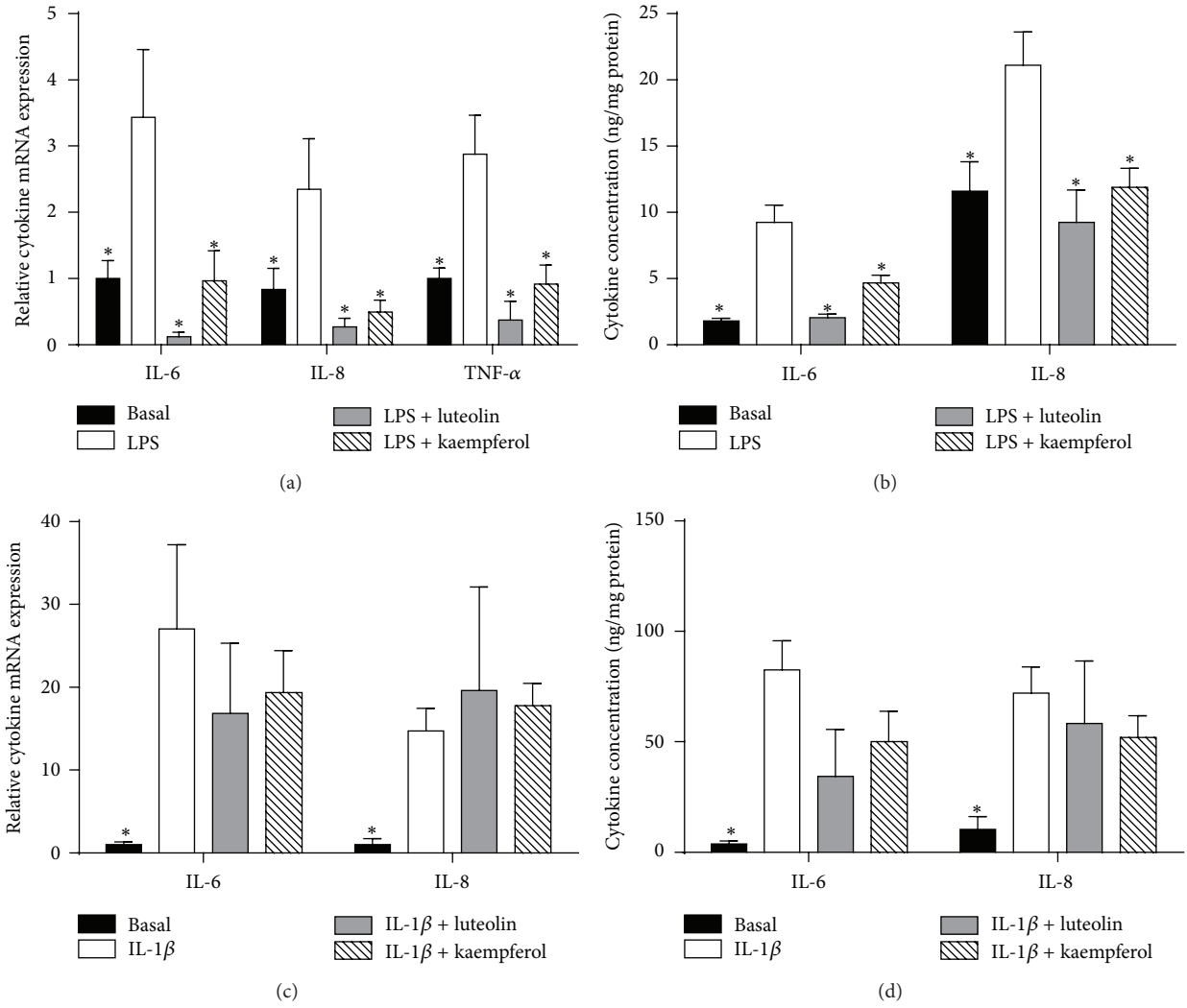

Figure 1: Effect of luteolin and kaempferol on proinflammatory cytokines. ((a), (b)) Fetal membranes were incubated with 10 μg/mL LPS in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 8$ patients). (a) TNF-α, IL-6, and IL-8 mRNA expression were quantified by qRT-PCR. (b) IL-6 and IL-8 concentrations in the conditioned media were assayed using ELISA. Each bar represents the mean ± SEM. $^*P < 0.05$ versus basal (one-way ANOVA). ((c), (d)) Primary myometrial cells were incubated with 500 pg/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 6$ patients). (c) IL-6 and IL-8 mRNA expressions were quantified by qRT-PCR. (d) IL-6 and IL-8 concentrations in the conditioned media were assayed using ELISA. Each bar represents the mean ± SEM. $^*P < 0.05$ versus basal (one-way ANOVA).

$PGF_{2\alpha}$ (Figure 2(b)) were statistically decreased by luteolin but not by kaempferol.

In myometrial cells incubated with IL-1β, COX-2 mRNA evels (Figure 2(c)) and subsequent PGE_2 and $PGF_{2\alpha}$ levels (Figure 2(d)) were significantly augmented. The addition of luteolin or kaempferol significantly attenuated IL-1β-induced PGE_2 and $PGF_{2\alpha}$ concentrations (Figure 2(d)). However, only kaempferol reduced COX-2 mRNA expression (Figure 2(c)).

3.3. Effect of Luteolin and Kaempferol on MMP-9 Expression and Activity. For amnion cells, IL-1β increased MMP-9 gene expression (Figure 3(a)) and pro-MMP-9 activity (Figure 3(b)). Coincubation with luteolin or kaempferol significantly attenuated both IL-1β-induced MMP-9 activity and expression. In myometrial cells, there was no change seen in the pro-MMP-9 bands; however there was increased active MMP-9 seen with the addition of IL-1β (Figure 3(d)). Both luteolin and kaempferol attenuated this IL-1β-induced increase in active MMP-9 activity. There was no change in MMP-9 mRNA expression with the addition of IL-1β in myometrial cells.

3.4. Effect of Luteolin and Kaempferol on NF-κB and AP-1 Transcriptional Pathways. In unstimulated cells, the NF-κB complex is made up of the p50/p65 subunits attached to IκB-α in the cytosol. While IκB-α is attached to the complex,

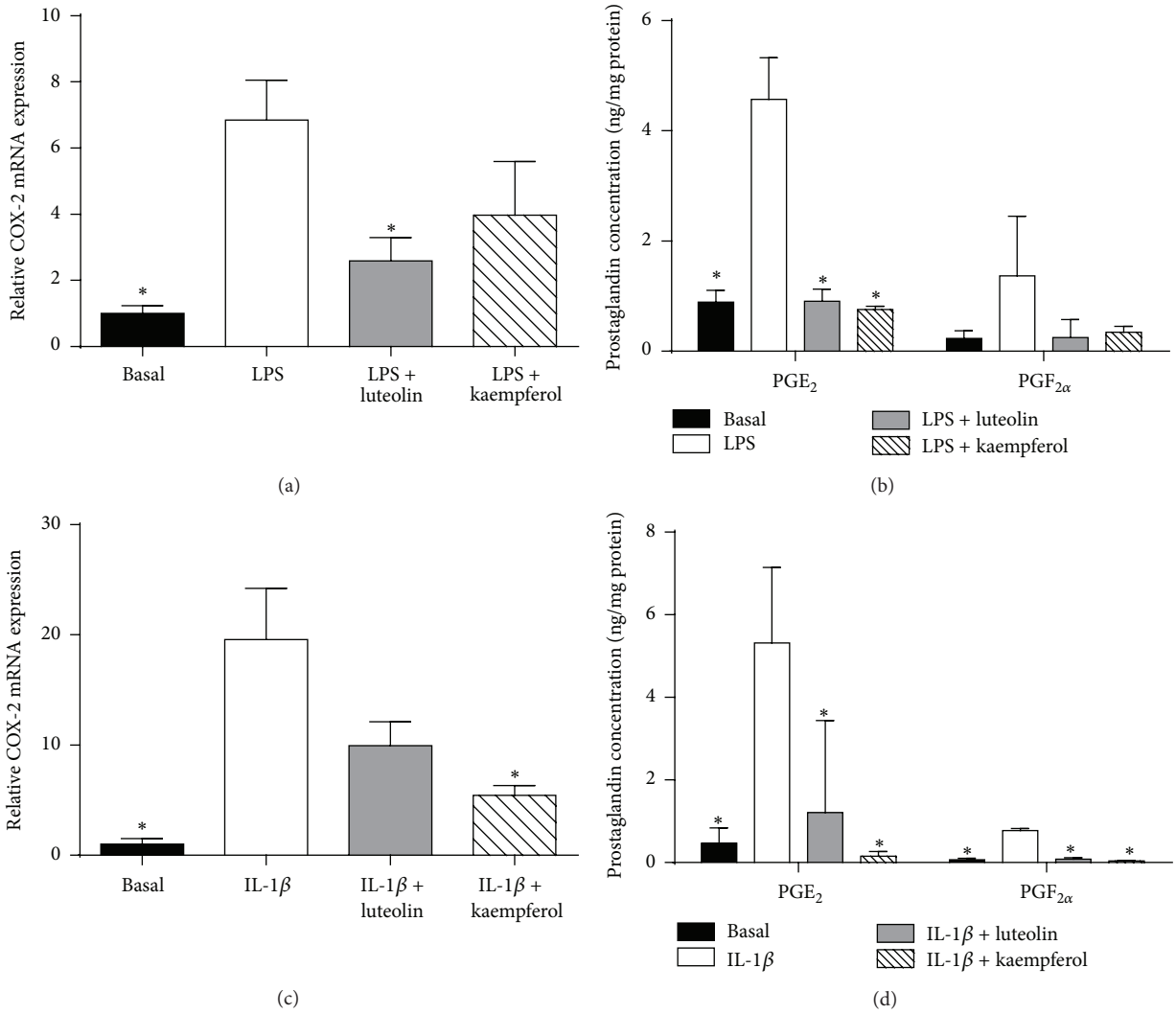

Figure 2: Effect of luteolin and kaempferol on the COX-prostaglandin pathway. ((a), (b)) Fetal membranes were incubated with 10 μg/mL LPS in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 6$ patients). (a) COX-2 mRNA expression was quantified by qRT-PCR. (b) PGE_2 and $PGF_{2\alpha}$ concentrations in the conditioned media were assayed using EIA. Each bar represents the mean ± SEM. $^*P < 0.05$ versus basal (one-way ANOVA). ((c), (d)) Primary myometrial cells were incubated with 0.5 ng/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 5$ patients). (c) COX-2 mRNA expression was quantified by qRT-PCR. (d) PGE_2 and $PGF_{2\alpha}$ concentrations in the conditioned media were assayed using EIA. Each bar represents the mean ± SEM. $^*P < 0.05$ versus basal (one-way ANOVA).

NF-κB is inactive. Activation by cytokines causes IκB-α to dissociate from the NF-κB complex. IκB-α is subsequently ubiquitinated and then degraded, allowing the NF-κB p50/p65 subunits to translocate to the nucleus [33]. The effect of luteolin and kaempferol treatment on IL-1β-induced IκB-α expression was determined in myometrial cells by Western blot analysis. Myometrial cells were pretreated with 20 μM luteolin and 100 μM kaempferol overnight, followed by 30 min 500 pg/mL IL-1β treatment ($n = 3$ patients). As expected, IL-1β induced a decrease in IκB-α expression (Figure 4(a)). However pre-treatment with either luteolin or kaempferol had no significant effect on attenuating this IL-1β-induced decrease in IκB-α expression (Figure 4(a)).

Once activated by cytokines, NF-κB enters the nucleus of cells where it binds to DNA to initiate gene transcription. There is potential for this binding to be inhibited by either blocking binding sites on the DNA itself or on NF-κB. NF-κB p65 DNA binding activity was assessed using a NF-κB p65 transcription factor assay. Nuclear protein was then extracted. IL-1β induced a significant increase in NF-κB DNA binding activity (Figure 4(b)). Co-treatment with both luteolin and kaempferol significantly attenuated IL-1β-induced NF-κB p65 DNA binding activity (Figure 4(b)).

To examine the effect of luteolin and kaempferol on AP-1, we examined c-Jun, as it forms part of the AP-1 early response transcription factor [34]. When compared to untreated cells (basal), IL-1β induced an increase in c-Jun expression (Figure 4(c)). Coincubation with luteolin and kaempferol attenuated the IL-1β-induced increase in c-Jun nuclear protein expression (Figure 4(c)).

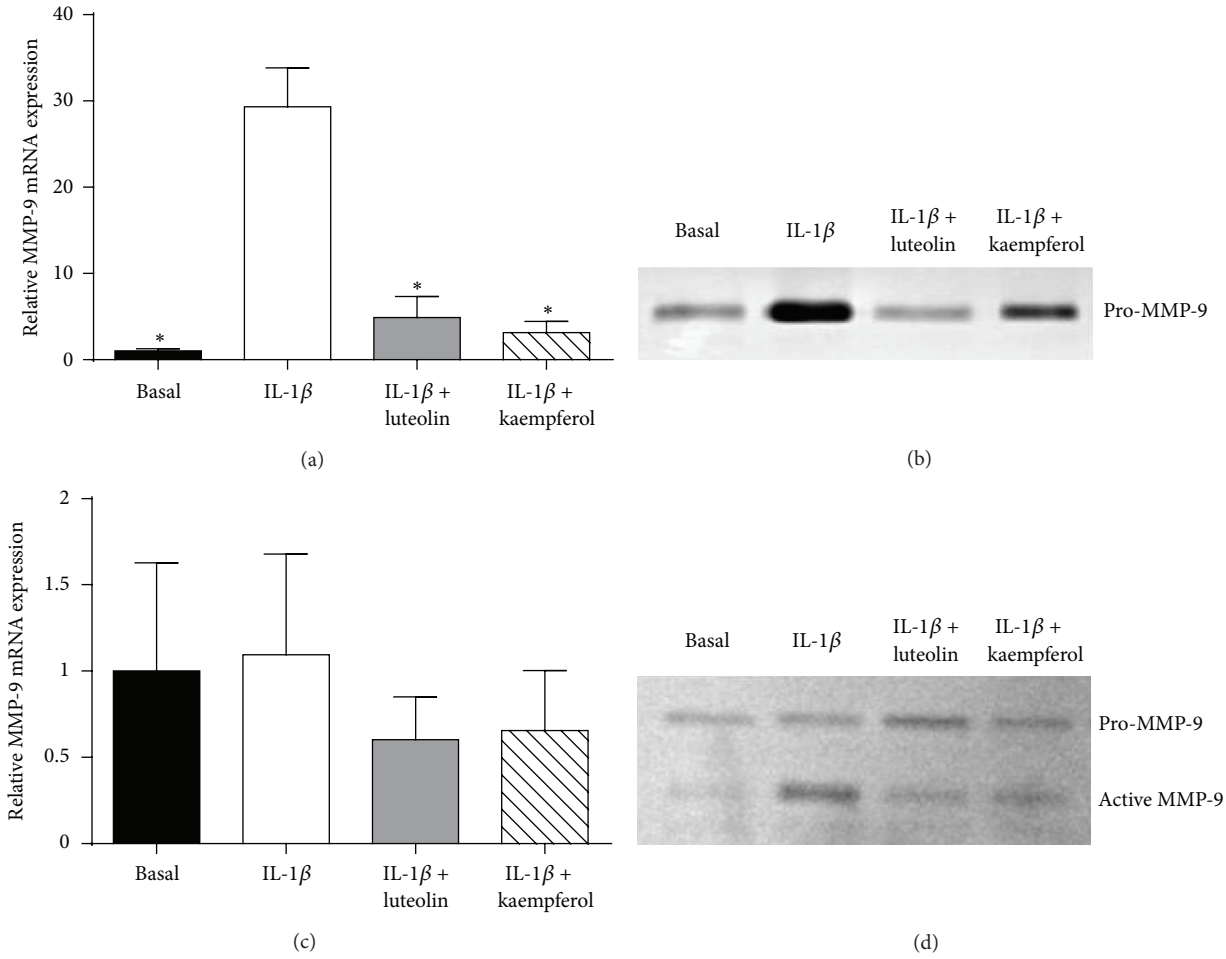

Figure 3: Effect of luteolin and kaempferol on MMP-9 expression and activity. ((a), (b)) Primary amnion cells were incubated with 1 ng/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 6$ patients). ((c), (d)) Primary myometrial cells were incubated with 500 pg/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 4$ patients). ((a), (c)) MMP-9 mRNA expression was quantified by qRT-PCR. ((b), (d)) The incubation medium was assayed for MMP-9 activity by gelatin zymography. Zymography from one patient is shown.

4. Discussion

The data presented in this study demonstrate that the two dietary phytophenols luteolin (from celery) and kaempferol (from grapefruit and tea) exert anti-inflammatory properties in gestational tissues. In human fetal membranes, luteolin and kaempferol treatment attenuated LPS, or IL-1β induced increases in mRNA expression and secretion of proinflammatory cytokines, COX-2 mRNA expression and subsequent prostaglandin release, and MMP-9 expression and secretion. In myometrium cells, luteolin and kaempferol significantly decreased COX-2 expression, prostaglandin release, and MMP-9 activity induced by IL-1β. There is, however, no effect of luteolin and kaempferol on proinflammatory cytokine expression or secretion in myometrial cells. Luteolin and kaempferol were found to act via the NF-κB and AP-1 pathways, inhibiting NF-κB p65 DNA binding activity and nuclear c-Jun expression in myometrial cells.

Proinflammatory cytokines play a key role as mediators of inflammation in preterm and term labour. In human gestational tissues and amniotic fluid, these cytokines are increased with the onset of human labour at term [35], and preterm [36] and more so in the presence of infection [37]. TNF-α and IL-1β exert proinflammatory actions such as the increase of prostaglandins and ECM degrading enzymes [38]. This leads to initiation of the three critical stages of human labour: rupture of fetal membranes, cervical ripening, and uterine contractions [35, 39, 40]. In this study, we used human fetal membranes and myometrial cells to determine the effect of luteolin and kaempferol on LPS or IL-1β-induced expression and release of the proinflammatory cytokines TNF-α, IL-6, and IL-8. We found that both luteolin and kaempferol significantly reduced the mRNA expression and secretion of proinflammatory cytokines in fetal membranes. Similarly, past studies performed in nongestational tissues have reported such anti-inflammatory actions for luteolin and kaempferol [12, 15–17]. However, it was found that luteolin and kaempferol had no effect on cytokine expression or secretion from pregnant myometrial cells. It is possible that longer

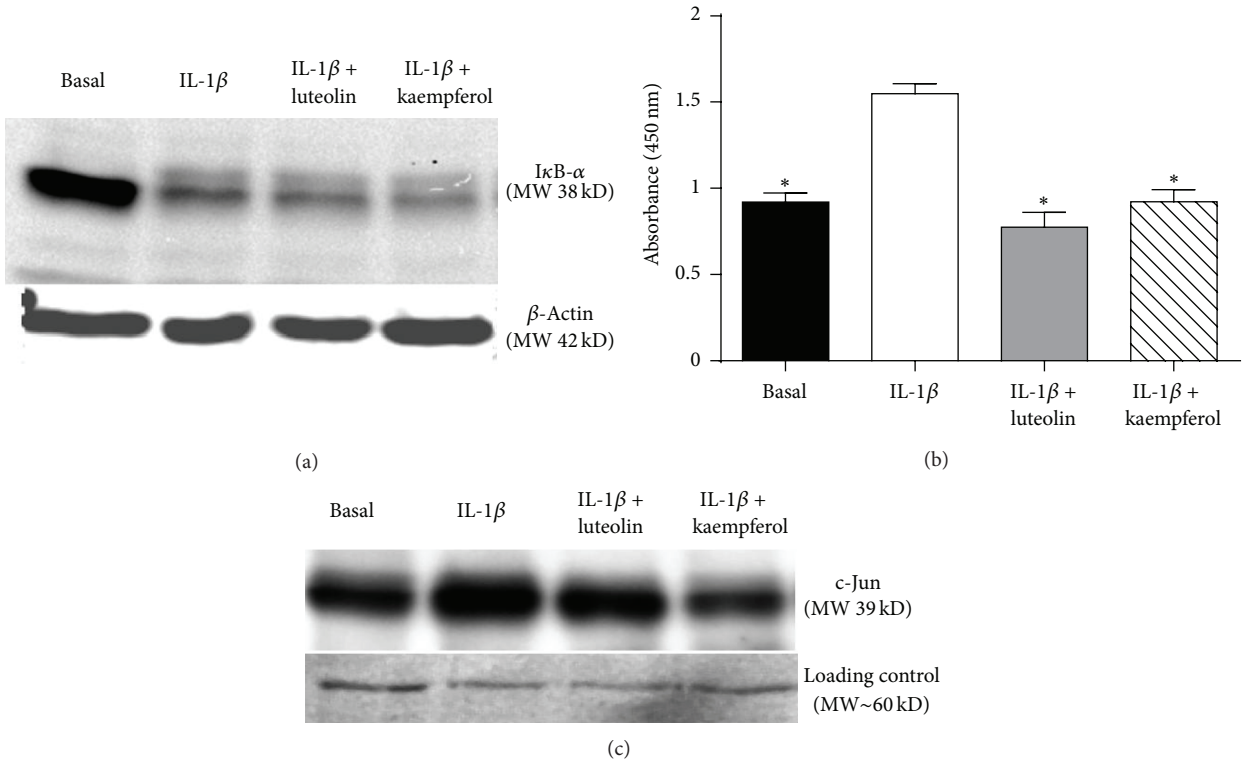

Figure 4: Effect of luteolin and kaempferol treatment on NF-κB DNA binding activity and c-Jun expression. ((a), (c)) Primary myometrial cells were pretreated with 20 μM luteolin or 100 μM kaempferol for 24 h, followed by 30 min incubation with 500 pg/mL IL-1β. (a) Representative IκB-α Western blot from one patient using β-actin as a loading control. Similar data were obtained for another two patients. (b) Primary myometrial cells were pretreated with 20 μM luteolin or 100 μM kaempferol for 6 h, followed by treatment with 500 pg/mL IL-1β for 24 h. NF-κB p65 DNA binding activity was assayed using a NF-κB p65 transcription factor assay ($n = 4$ patients). Data is displayed as absorbance at 450 nm. Each bar represents the mean ± SEM. $^*P < 0.05$ versus IL-1β-induced NF-κB DNA binding (one-way ANOVA). (c) Representative Western blot of nuclear c-Jun from one patient using Ponceau S as a loading control. Similar data were obtained for another four patients.

or shorter incubation times or higher concentrations of phytophenols are required to elicit a protective response in myometrial cells.

COX-2 stimulates production of prostaglandins, which are important in the initiation and maintenance of labour, by increasing uterine contraction and promoting cervical ripening and the decidual-fetal membrane activation [38, 41]. Luteolin and kaempferol have been demonstrated in nongestational tissues to cause inhibition of COX-2, hence causing inhibition of prostaglandins [42, 43]. In this study, we demonstrate that luteolin and kaempferol significantly decreased LPS, IL-1β-induced COX-2 gene expression, and subsequent PGE_2 and $PGF_{2\alpha}$ release in both fetal membranes and myometrial cells. This decrease in prostaglandin production could potentially detain the onset of uterine contraction and decrease the progression of cervical ripening, thus delaying the birth of the baby.

MMP-9 plays a role in the myometrium during parturition. During pregnancy the uterus is remodeled and enlarged by the addition of collagen to the myometrium, to accommodate the growing fetus, placenta, and amniotic fluid [44]. MMP-9 degrades this collagen during parturition, shrinking the uterus [44]. In addition, MMP-9 plays a major role in the degradation of the collagen matrix within fetal membranes, causing weakening, which, along with stretch forces, leads to membrane rupture [45]. Consistent with previous studies in nongestational tissues [46, 47], both luteolin and kaempferol attenuated IL-1β-induced MMP-9 activity in primary cells from amnion and myometrium. Of note, in myometrium, IL-1β did not increase MMP-9 gene expression, nor was there attenuation with luteolin or kaempferol. This may be explained by the fact that there are two different types of MMP-9 in myometrium, pro-MMP-9 and active MMP-9. The MMP-9 which is important in the context of parturition is active MMP-9, as this is the active form of the enzyme. qRT-PCR cannot distinguish between the two forms of MMP-9 and so hence gives the total MMP-9 expression, not just the active MMP-9 expression.

There is increasing evidence for the role of NF-κB and AP-1 in human term and preterm labour [6–9, 48]. Furthermore, mouse studies have been employed to demonstrate that by inhibiting NF-κB or AP-1, infection-induced PTB can be delayed [49, 50]. In nongestational tissues, luteolin and kaempferol are thought to exert their anti-inflammatory actions by inhibiting NF-κB [12–15] and AP-1 [14, 16, 17]. Similarly, in this study we show that luteolin and kaempferol

inhibited NF-κB DNA binding activity. In addition, both luteolin and kaempferol inhibited IL-1β-induced c-Jun expression, which is a nuclear protein that is part of the AP-1 transcription pathway. These findings suggest that, in human gestational tissues, luteolin and kaempferol may exert their inhibitory effects on proinflammatory cytokines, COX-2, prostaglandins, and MMP-9 via NF-κB. This is in agreement with previous studies, by our laboratory and others, that NF-κB and AP-1 regulate the transcription of prolabour mediators in human gestational tissues [6–9, 25].

There is emerging evidence for phytophenols as therapeutic agents for a number of pathological conditions including cancer [51]. They are readily available, inexpensive and have multitargeted potential [51]. However, their potential as therapeutics has also been heavily debated. They show low bioavailability and lose function due to metabolic processing when given via dietary supplementation [52]. If given at nutritionally relevant concentrations, extensive deglycosylation, glucuronidation, sulfation, and methylation reactions occur, mediated by a range of enzymes in the small intestine, liver, and colon. It has been shown that pharmacological doses that saturate metabolic pathways are required to obtain the free form of these phytophenols in the blood [52]. Whether luteolin or kaempferol can be used as therapeutics to prevent or delay PTB must first be addressed using experimental animal models of infection or inflammation-induced PTB. However, of promise is a recent study which has shown that kaempferol increases gestational length in a pregnant mouse model [53].

In addition to the anti-inflammatory actions, phytophenols possess a wide range of biological activities. For example, they have shown that both luteolin and kaempferol also possess antioxidant, antimicrobial, and anticancer activities [42, 43]. They have also been shown to have cardioprotective, antidiabetic, and neuroprotective effects. Luteolin has also shown antiallergic activity *in vitro* and *in vivo* [43].

In conclusion, in this study, we demonstrate that luteolin and kaempferol inhibit prolabour and proinflammatory mediators in human gestational tissues. Both luteolin and kaempferol have demonstrated anti-inflammatory properties in gestational tissues, by inhibiting NF-κB DNA binding activity, the AP-1 pathway, and their target genes. Given the central role of inflammation in provoking preterm labour, it is tempting to speculate that dietary phytophenols may be an effective, potential treatment or preventative for PTB. As a result of this research, further study is currently underway to determine effects of these phytophenols in an *in vivo* mouse model.

Conflict of Interest

There is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgments

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