Oxidative Medicine and Cellular Longevity

Role of Antioxidants in the Protection from Aging-Related Diseases

Lead Guest Editor: Daria M. Monti
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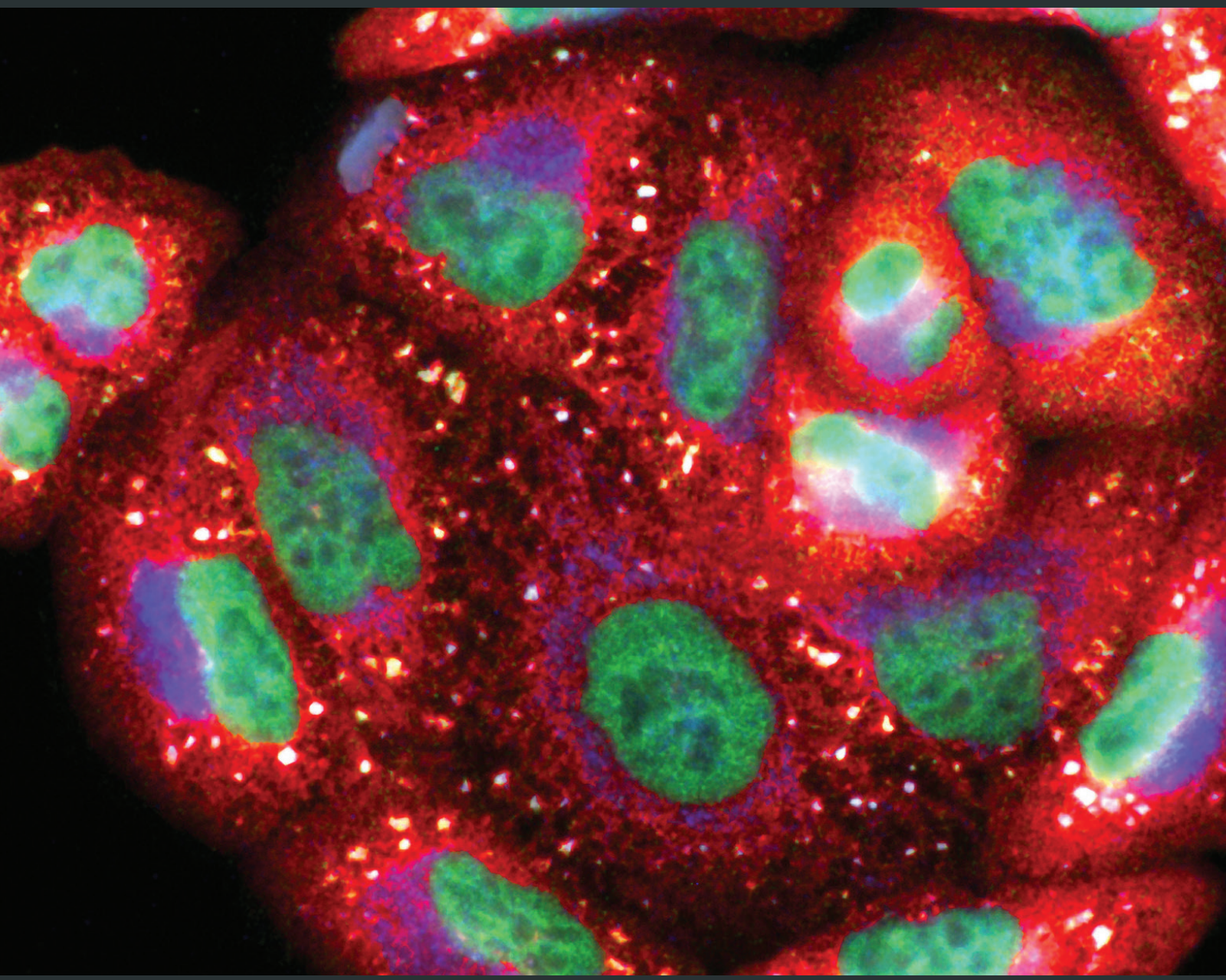

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Volume 2019, Article ID 7450693, 2 pages
https://doi.org/10.1155/2019/7450693

Editorial

Role of Antioxidants in the Protection from Aging-Related Diseases

Daria Maria Monti,[1] **Maria Manuela Rigano**,[2] **Simona Maria Monti**,[3] **and Herbenya Silva Peixoto**[4]

[1]*Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, via Cinthia 4, 80126 Naples, Italy*
[2]*Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055 Portici (Naples), Italy*
[3]*Institute of Biostructures and Bioimaging, National Research Council, Naples 80134, Italy*
[4]*Faculty of Pharmacy, Universidade Paulista UNIP, Campus Manaus BR, Av. Mário Ypiranga, 4390-Parque 10 de Novembro, 69050-030 Manaus, AM, Brazil*

Correspondence should be addressed to Daria Maria Monti; mdmonti@unina.it

Received 25 November 2018; Accepted 26 November 2018; Published 8 January 2019

In recent years, oxidative stress has been considered an important factor in the pathogenesis and development of lifestyle-related diseases. In particular, the increase in reactive oxygen species (ROS) has been related to the onset and progression of human aging. ROS can be generated by normal metabolic activity as well as by lifestyle factors. Although many epidemiological studies have shown that the adoption of a healthy diet is mandatory, the molecular mechanisms by which nutrients exert antioxidant effects remain poorly understood. Concurrently, oxidation damage can also depend on inherited or acquired defects in enzymes involved in the redox-mediated signaling pathways. The resulting redox-unbalance leads to increased susceptibility towards oxidative aging-related pathologies. For these reasons, the investigation of the role of cellular antioxidant scavenger enzymes, active towards oxidative stress, is gaining increased attention.

In this special issue, we invited researchers to shed light on the molecular mechanisms involved in fighting aging-related diseases by antioxidant molecules which are able to promote healthy aging and counteract oxidative stress.

In the review from B.A.Q. Gomes et al., they analyzed the possibility to counteract Alzheimer's disease, a severe neurodegenerative disorder, whose insurgence is related to age and presents high levels of oxidative stress. The authors reported that resveratrol is able to inhibit Aβ peptide aggregation. Resveratrol is a well-known phenol endowed with antioxidant and anti-inflammatory activity, and it plays also an important role in neuronal differentiation through the activation of silent information regulator-1; thus, it can be considered as a promising tool in disease prevention.

Moreover, in the context of neurodegeneration, the anti-inflammatory mechanisms of several phytochemicals, such as curcumin, resveratrol, propolis, polyunsaturated fatty acids (PUFAs), and ginsenosides, have been extensively studied in the review from J. Wang et al. In particular, the authors reported that these phytochemicals are able to modulate and suppress neuroinflammation of the brain by different mechanisms of action. Therefore, some phytochemicals can represent a useful tool to counteract systemic inflammation and oxidative stress related to neurodegenerative diseases.

Still in the context of neurodegeneration, L.A. Ramos-Chávez et al. reported a study, on 77 women over the age of 50, on the relation between the changes in Trp catabolism and cognitive impairment associated with age. The authors hypothesize using circulating Trp levels as a new biomarker for cognitive impairment.

In the review from A. Perrelli et al., the role of avenanthramides (from *Avena sativa*) in fighting age-related diseases was deeply analyzed. Interestingly, avenanthramides have been identified as therapeutic candidates for the treatment of a cerebrovascular disorder. Finally, by using proteomic approaches, the authors found distinctive molecular pathways and redox protein modifications associated with avenanthramide activity.

The paper from Y. Ma et al. was on the protective effect and mechanism of action of an ethanol extract from the leaves of *Diospyros kaki*. The extract was used on mice treated with D-galactose, known to mimic aging in animal models. The authors found that the extract, rich in flavonoids, decreased oxidative stress levels and inflammatory mediators, restored memory impairment, and ameliorated the impairment of synaptic-related proteins.

The role and molecular mechanism of action of proteins containing reactive sulfhydryl groups involved in the response to oxidative stress has been clearly described by A. Di Fiore et al., with a special focus on carbonic anhydrases III and VII. These enzymes have been found to play an important role in cells in which oxidative stress is activated. Interestingly, both proteins are mainly localized in tissues characterized by a high rate of oxygen consumption and contain, on their molecular surface, two reactive cysteine residues eventually undergoing S-glutathionylation.

As for plant secondary metabolites, their role has been deeply analyzed in the review by G. Petruk et al., with a special focus on skin photoaging. Phenolic compounds, polyphenols, and carotenoids have been analyzed for their antiaging properties related to the reduction of oxidative stress pathways.

Finally, L. Di Renzo et al. analyzed the role of the Mediterranean diet in preventing noncommunicable diseases and, in particular, the role of wine. They found that the association of low/moderate intake of alcohol beverages, with nutraceutical-proven effectiveness and ethanol, in association with a Mediterranean diet, could determine a reduction of atherosclerosis risk onset through a positive modulation of antioxidant gene expression helping in the prevention of inflammatory and oxidative damages.

Thus, we hope that readers will find these contributions interesting and stimulating for the next coming research. We believe that these reviews will add knowledge in the correlation between oxidative stress and antioxidants in age-related pathologies.

Conflicts of Interest

None of the authors has any conflict of interest.

Daria Maria Monti
Maria Manuela Rigano
Simona Maria Monti
Herbenya Silva Peixoto

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 8938207, 12 pages
https://doi.org/10.1155/2018/8938207

Research Article

Flavonoid-Rich Ethanol Extract from the Leaves of *Diospyros kaki* Attenuates D-Galactose-Induced Oxidative Stress and Neuroinflammation-Mediated Brain Aging in Mice

Yingjuan Ma,[1] Bin Ma,[2] Yuying Shang,[1,3] Qingqing Yin,[1] Dejie Wang,[4] Song Xu,[1] Yan Hong,[1] Xunyao Hou,[1] and Xueping Liu [1,5,6]

[1]*Department of Senile Neurology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, 250021 Shandong, China*
[2]*School of Pharmaceutical Sciences of Shandong University, Jinan, 250012 Shandong, China*
[3]*Department of Critical Care Medicine, Jining No. 1 People's Hospital, Jining 272011, China*
[4]*Corporate Health Management Center, Shandong Electric Powder Central Hospital, Jinan, 250001 Shandong, China*
[5]*Department of Anti-Aging, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, 250021 Shandong, China*
[6]*Anti-Aging Monitoring Laboratory, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, 250021 Shandong, China*

Correspondence should be addressed to Xueping Liu; liuxueping62@163.com

Received 2 May 2018; Revised 4 July 2018; Accepted 30 July 2018; Published 23 December 2018

Academic Editor: Maria Manuela Rigano

Aging is a major factor that contributes to neurological impairment and neuropathological changes, such as inflammation, oxidative stress, neuronal apoptosis, and synaptic dysfunction. Flavonoids act as protective antioxidant and anti-inflammatory agents against various age-related neurodegenerative diseases. Here, we investigated the protective effect and mechanisms of the flavonoid-rich ethanol extract from the leaves of *Diospyros kaki* (FELDK) in the cortex and hippocampus of D-galactose- (gal-) aged mice. Our results showed that FELDK treatment restored memory impairment in mice as determined by the Y-maze and Morris water maze tests. FELDK decreased oxidative stress levels via inhibiting reactive oxygen species (ROS) and malondialdehyde (MDA) production and elevating antioxidative enzymes. FELDK also alleviated D-gal-induced neuroinflammation via suppressing the expression of advanced glycation end products (AGEs) and receptor for AGEs (RAGE) and activating microgliosis and astrocytosis, nuclear factor kappa B (NF-κB) nuclear translocation, and downstream inflammatory mediators. Moreover, FELDK inhibited the phosphatidylinositol 3-kinase (PI3K)/Akt and C-jun N-terminal kinase (JNK) apoptotic signaling pathways and ameliorated the impairment of synapse-related proteins. Hence, these results indicate that FELDK exerts neuroprotective effects on D-gal-induced brain aging. Thus, FELDK may be a potential therapeutic strategy for preventing and treating age-related neurodegenerative diseases such as Alzheimer's disease.

1. Introduction

Aging is a major factor underlying brain dysfunction, which is marked by memory loss, cognitive impairment, and many age-related neurodegenerative disorders, such as Alzheimer's disease (AD) [1]. Multidimensional pathological features, including oxidative stress, neuroinflammation, and cell metabolic imbalance, are associated with aging of the brain [2, 3], which consequently result in neuronal death and synaptic dysfunction. Oxidative stress and reactive oxygen species (ROS) are known contributors to age-related neurodegenerative disorders [4, 5]. The accumulation of excessive oxidative stress and ROS in the brain can trigger neuroinflammation and induce dangerous modifications of cellular proteins, lipids, and DNA, which subsequently impair cellular activity and destabilize neuronal homeostasis, ultimately leading to neuronal loss [1, 5, 6].

Chronic inflammation is a potential risk factor in age-associated diseases [7]. D-Galactose (D-gal) can mimic aging in animal models by inducing oxidative stress and

inflammation, and these models have been widely used in antiaging research [8–11]. Accumulation of D-gal triggers ROS generation, which leads to oxidative stress and formation of advanced glycation end products (AGEs) via reacting with amino peptides and proteins [12]. In addition, binding of AGEs to the receptor for advanced glycation end products (RAGE) is common in many age-related degenerative disorders. This interaction leads to downstream activation of nuclear factor kappa B (NF-κB), followed by release of ROS and proinflammatory mediators [13]. Evidence indicates that ROS and AGEs result in neuroinflammation, activation of microglia and astrocytes, loss of neuronal cells, and reduction of synapse-related proteins, which can aggravate the deficits of learning and memory [6, 14, 15]. Thus, we used D-gal-induced aging mice in this study to study the aging brain.

Extracts from persimmon leaves, of which flavonoid is the main component, have been widely used in China to treat apoplexy and its sequela [16]. In addition to its well-known antioxidant and neuroprotective effects, this leaf extract also provides a number of other beneficial aspects. Studies reported that a standardized flavonoid extract of persimmon leaves (FLDK) exerted protective effects *in vivo* in two different brain ischemia and reperfusion injury rat models, as well as protective effects *in vitro* in hippocampal and primary cortical neurons following glutamate or hypoxia injury [16]. FLDK also protected NG108-15 cells from oxidative injury induced by hydrogen peroxide, possibly by improving the cellular redox state and upregulating Bcl-2 levels [17]. Ethyl acetate extract from persimmon leaves showed a potential protective effect on $A\beta_{1\text{-}42}$-induced cognitive dysfunction in rats, probably via improving the antioxidative defense system and attenuating mitochondrial-mediated neuronal apoptosis. Flavonoids and triterpenoids are likely the major active components in the ethyl acetate extract [18]. However, it is unclear if the flavonoid-rich ethanol extract from the leaves of *Diospyros kaki* (FELDK) could delay aging. Therefore, the present study is aimed at investigating the neuroprotective effects of FELDK on D-gal-induced learning and memory impairment, neuroinflammation, oxidative stress, apoptosis, and synaptic function.

2. Materials and Methods

2.1. Reagents. FELDK was provided by Professor Bin Ma (School of Pharmaceutical Sciences of Shandong University). FELDK was preparated as we previously described [19]. High-performance lipid chromatography (HPLC) analyses were carried out using an Agilent 1200 series including a binary pump, a column oven, a 4-flow channel degasser (Agilent Technologies, Palo Alto, CA, USA), and an autosampler (NASCA 5100, Shiseido Co. Ltd., Tokyo, Japan). FELDK were separated on a Phenomenex Luna C_{18} column (5 μm particle size, 250 mm × 4.6 mm i.d. with a 4 mm × 3 mm precolumn (Security Guard C18 cartridge, Phenomenex Inc., USA)). The mobile phases were composed of acetonitrile (A) and water with 0.1% formic acid (B) using a multistep gradient elution of 15% A for 0–10 min, 35% A for 10–20 min, 95% A for 20–45 min, 95% A for 45–55 min, 15% A for 55–55.1 min, and 15% A at 55.10–60.0 min with the flow rate kept at 1.0 mL/min. The injected sample volume was set at 5 μL, and the column oven temperature was 20°C.

After separation, the HPLC flow was directed to the mass spectrometer (MS) inlet after 1 : 10 splitting. FELDK analyses were performed on an API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) with an electrospray ionization source (ESI) in negative mode. Nitrogen was used as the carrier, heater, and collision gases. The Applied Biosystems Analyst version 1.5.2 software was used to control for data acquisition and analysis [20].

HPLC-MS verified that the ethanol extract from the leaves of *Diospyros kaki* contained the following flavonoids: myricetin, quercetin, kaempferol, hyperoside, astragalin, and vitexin [18, 21]. After being hydrolyzed to aglycone, the extract was contained more than 79.35% total flavonoids as determined by UV spectrophotometry as previously described [22].

Enzyme-linked immunosorbent assay (ELISA) kits for mouse AGEs were from Cell Biolabs (San Diego, CA, USA). The RAGE antibody was from Santa Cruz Biotechnology (CA, USA), NF-κB p65 antibody was from Millipore (Temecula, CA, USA), and tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) antibodies were purchased from ImmunoWay Biotechnology (Newark, DE, USA). The phosphatidylinositol 3-kinase (PI3K), phosphorylated- (p-) Akt, total- (t-) Akt, p-C-jun N-terminal kinase (JNK), and t-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor molecule (Iba1), Bcl-2, Bax, synaptophysin, synaptotagmin, p-cAMP response element-binding protein (CREB), t-CREB, and p-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) antibodies were from Abcam (Burlingame, CA, USA). The Iba1 antibody used for immunohistochemical staining was from Wako Pure Chemical Industries (Osaka, Japan). The histone H3, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) antibodies were from Proteintech Group (Chicago, IL, USA). Glyceraldehyde phosphate dehydronase (GAPDH) and secondary antibodies were from Zhongshan Jinqiao Biotechnology (Beijing, China). The BCA Protein Assay Kit was from Biocolor BioScience & Technology Company (Shanghai, China).

2.2. Animals and Treatment. Thirty-six 10-week-old Kunming male mice (purchased from the Experimental Animal Center of Shandong University, Jinan, China) were housed in a constant environment (12 h/12 h light-dark schedule; 19–23°C; 45–65% humidity) with free access to water and food. After feeding adaptation for one week, the mice were randomly divided into four groups ($n = 9$ per group). The aging model group (D-gal group) received subcutaneous injections of D-galactose at 100 mg/(kg d) for 10 weeks to mimic aging and was orally gavaged with vehicle. The normal control group (NC group) were injected with normal saline and received the same volume of vehicle by oral gavage. The flavonoids F1 and F2 intervention groups were fed 40 and 80 mg/(kg d) FELDK, respectively, by oral gavage after subcutaneous injection of D-gal. The NC group

and D-gal group were given the same volume of normal saline. Animal breeding and all experiments complied with the Provisions and General Recommendation of the Chinese Experimental Animals Administration Legislation, and the experiments were approved by the Animal Care Committee of the Provincial Hospital Council, Shandong University, China.

2.3. Behavioral Tests. After 10 weeks of oral FELDK treatment, the Y-maze test and the Morris water maze (MWM) test were performed for behavioral analysis. The two tests were separated by one day.

2.3.1. Y-Maze Test. The Y-maze test was performed as previously described to determine working memory performance [6, 9]. After being acclimated in the environment, each mouse was placed at the end of one arm of the maze. The series of arm entries were recorded during three 8 min sessions. Spontaneous alteration behavior was defined as the number of entries into three arms. Alteration behavior (%) was calculated as follows: $(\text{alterations/total entries} - 2) \times 100$.

2.3.2. MWM. Two days after the Y-maze test, the MWM test was used to assess learning and memory in the mice. The MWM task consisted of a five-day training period followed by a one-day probe test. The MWM device was a circular tank (120 cm in diameter and 50 cm in height) with a platform (10 cm in diameter) situated in the center of one of the four equally sized quadrants and submerged ~1 cm under water ($22 \pm 1^{\circ}$C) during the training period. The platform was removed on the last day of the probe test. On training days, mice were placed into one of the four quadrants. Escape latency (EL) was measured as the time that mice spent discovering the platform. Mice that failed to find the platform were assisted to the platform and kept on the platform for 15 sec. For these mice, EL was recorded as 60 sec. In the probe trial, mice were allowed to swim freely for 60 sec. EL, the number of times mice crossed the platform area, and the time spent in the target quadrant were counted. All data were recorded by a computerized video recording system (HVS Image, UK) with a video camera located above the center of the pool.

2.4. Preparation of Tissue Samples. During the experiment, three mice (one in the D-gal group, one in the F1 group, and one the in F2 group) died and were excluded from data analysis. After the MWM test, all mice were anesthetized and perfused transcardially with 30 mL normal saline. The left hemisphere of the mice brains was soaked in 4% paraformaldehyde for 48 h, followed by postfixation in 70% ethanol for 24 h, and then dehydrated and embedded in molten paraffin. The brains were sliced coronally into 5 μm thick serial sections for immunohistochemistry. The right cortex and hippocampus of hemispheres were dissected and stored in liquid nitrogen; parts were used for biochemical detection, and the others were used for western blot analysis.

2.5. Tissue Homogenates. For biochemical assays, tissues were homogenized in 1/10 (w/v) normal saline containing a protease inhibitor and phosphatase inhibitor (Sigma-Aldrich) and then centrifuged at 12000 g at 4°C for 10 min. The supernatants were collected and stored at −80°C. For western blot analysis, brains were homogenized in RIPA lysis buffer (1 : 4, $w : v$) containing 1% protease inhibitors and 1% phosphatase inhibitors (Sigma-Aldrich) and then centrifuged at 12000 g at 4°C for 30 min. Nuclear protein was extracted to measure NF-κB p65 expression according to the manufacturer's instructions (Thermo Scientific, Waltham, MA). Protein concentration was quantified using the BCA Protein Assay Kit.

2.6. Biochemical Analysis

2.6.1. ELISA for AGEs. Brain AGEs levels were detected using the OxiSelect™ AGEs ELISA kit according to the manufacturer's instructions. Absorbance at 450 nm was measured, and content was expressed as ng/mg protein.

2.6.2. Measurement of Oxidative Stress. ROS and malondialdehyde (MDA) levels, as well as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activity, were detected as previously published [23]. ROS was measured by determining the oxidation levels of 2′,7′-dichlorodihydrofluorescein-diacetate compared to 2′,7′-dichlorofluorescein-diacetate. MDA levels were estimated using the thiobarbituric acid reactive method. SOD activity was measured by the inhibition of nitro blue tetrazolium reduction. GSH-Px activity was determined by 5,5′-dithiobis-p-nitrobenzoic acid. CAT activity was assayed as the rate of reduction in absorbance of H_2O_2 at 240 nM/min/mg protein in the presence of CAT.

2.7. Western Blotting. Equal amounts of protein were separated on SDS-PAGE gels and then transferred to PVDF membranes. Membranes were incubated with antibodies against RAGE (1 : 500), GFAP (1 : 1000), Iba1 (1 : 1000), NF-κB (1 : 1000), TNF-α (1 : 1000), IL-1β (1 : 1000), COX-2 (1 : 1000), iNOS (1 : 1000), PI3K (1 : 1000), p-Akt (1 : 2000), t-Akt (1 : 1000), p-JNK (1 : 1000), t-JNK (1 : 1000), Bcl-2 (1 : 500), Bax (1 : 1000), synaptophysin (1 : 20000), synaptotagmin (1 : 1000), p-CREB (1 : 5000), t-CREB (1 : 1000), p-CaMKII (1 : 1000), GAPDH (1 : 1000), and histone H3 (1 : 1000), followed by the appropriate corresponding secondary antibody (1 : 5000) for 1 h at room temperature. Proteins were visualized with enhanced chemiluminescence reagents using an image analyzer (Alpha Innotech, San Leandro, CA, USA). Protein bands were quantified using ImageJ (NIH, USA).

2.8. Immunohistochemistry. Brain sections were dewaxed, rehydrated, and boiled in citrate buffer (10 mM, pH 6.0) for antigen retrieval, followed by incubation with 0.3% H_2O_2 and then goat serum. Primary rabbit anti-Iba1 (1 : 200) and rabbit anti-GFAP (1 : 200) antibodies were incubated overnight at 4°C. After repeated washing, the slices were then incubated with biotinylated goat anti-rabbit IgG followed by incubation with horseradish perioxidase (HRP) conjugated streptavidin. Finally, the slices were incubated with DAB and counterstained with hematoxylin.

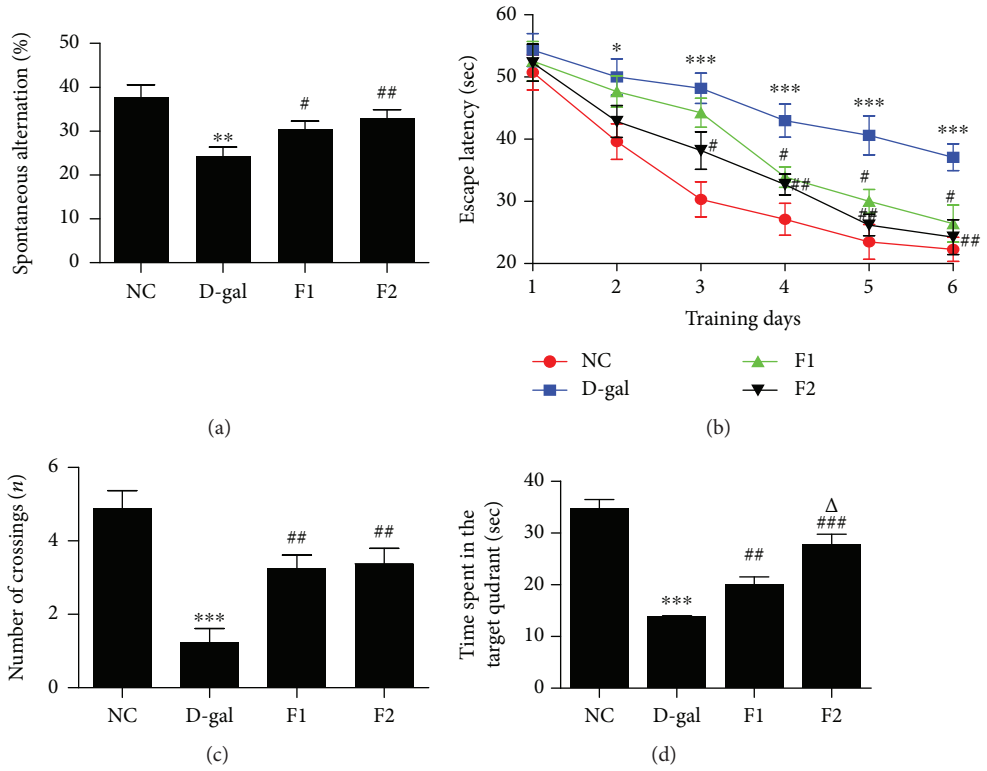

Figure 1: Effects of FELDK on memory impairment in D-gal-treated mice. (a) The percentage of spontaneous alteration in the Y-maze test. (b) Comparison of EL during 6 days in the MWM. (c) The number of crossings and (d) the time spent in the target quadrant where the platform had been previously situated during the spatial probe trial on day 6. Data are presented as mean value ± SEM for $n = 9, 8, 8, 8$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the D-gal group.

2.9. Statistical Analysis. Statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad Software Inc., La Jolla, CA, USA). Significant differences between treatment groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's test. Repeated measures ANOVA followed by Tukey's test was used for comparison of escape latency in the MWM test. Data are expressed as means ± standard error of the mean (SEM). P values < 0.05 were considered statistically significant.

3. Results

3.1. Effects of FELDK on Memory Impairment in D-gal-Aged Mice. The Y-maze was used to assay spatial working memory in mice. The D-gal group showed a significant decrease in the percentage of spontaneous alteration compared to the NC group ($P < 0.01$) (Figure 1(a)), indicating decreased working memory in the D-gal-aged mice. The percentage of spontaneous alteration was significantly increased in FELDK-treated mice at both 40 and 80 mg/kg ($P < 0.05$ and $P < 0.01$, respectively), indicating that FELDK improves working memory in D-gal-aged mice.

The MWM test was used to measure the effects of FELDK on spatial learning and memory. As shown in Figure 1(b), EL increased in the D-gal group on the second day compared to the NC group ($P < 0.05$). Administration of oral FELDK (40 and 80 mg/kg) significantly reduced EL (both $P < 0.05$). In the probe test (Figures 1(c) and 1(d)), the searching frequency (the number of times that the mice crossed the site where the platform had been placed) and the time spent in target quadrant (the swimming time in the site where the platform had been placed) decreased in the D-gal group compared to the NC group (both $P < 0.001$). FELDK at both dosages (40 and 80 mg/kg) alleviated the decreased number of crossings and the time spent in target quadrant ($P < 0.01$). In addition, the high dose of FELDK (80 mg/kg) showed better effects than the low dose of FELDK (40 mg/kg) on the time spent in the quadrant ($P < 0.05$). These results suggest that FELDK alleviates spatial learning and memory impairment in D-gal-aged mice.

3.2. Effects of FELDK on AGEs and RAGE Levels in D-gal-Aged Mice. As shown in Figure 2(a), D-gal significantly ($P < 0.01$) increased AGEs levels in the hippocampus and cortex regions, which was attenuated by FELDK ($P < 0.05$). RAGE is a cell surface protein that is a member of the immunoglobulin superfamily. Our results showed that the interaction of D-gal with its receptor, RAGE, significantly increased RAGE expression in the hippocampus and cortex regions compared to the NC group ($P < 0.01$). However, FELDK markedly reversed this upregulation of RAGE compared to the D-gal group ($P < 0.05$) (Figures 2(b) and 2(c)).

3.3. FELDK Suppressed Microglial and Astrocytes in D-gal-Aged Mice. Glial cells have been considered the major pathological contributor to neurodegeneration [6]. Iba1 and GFAP

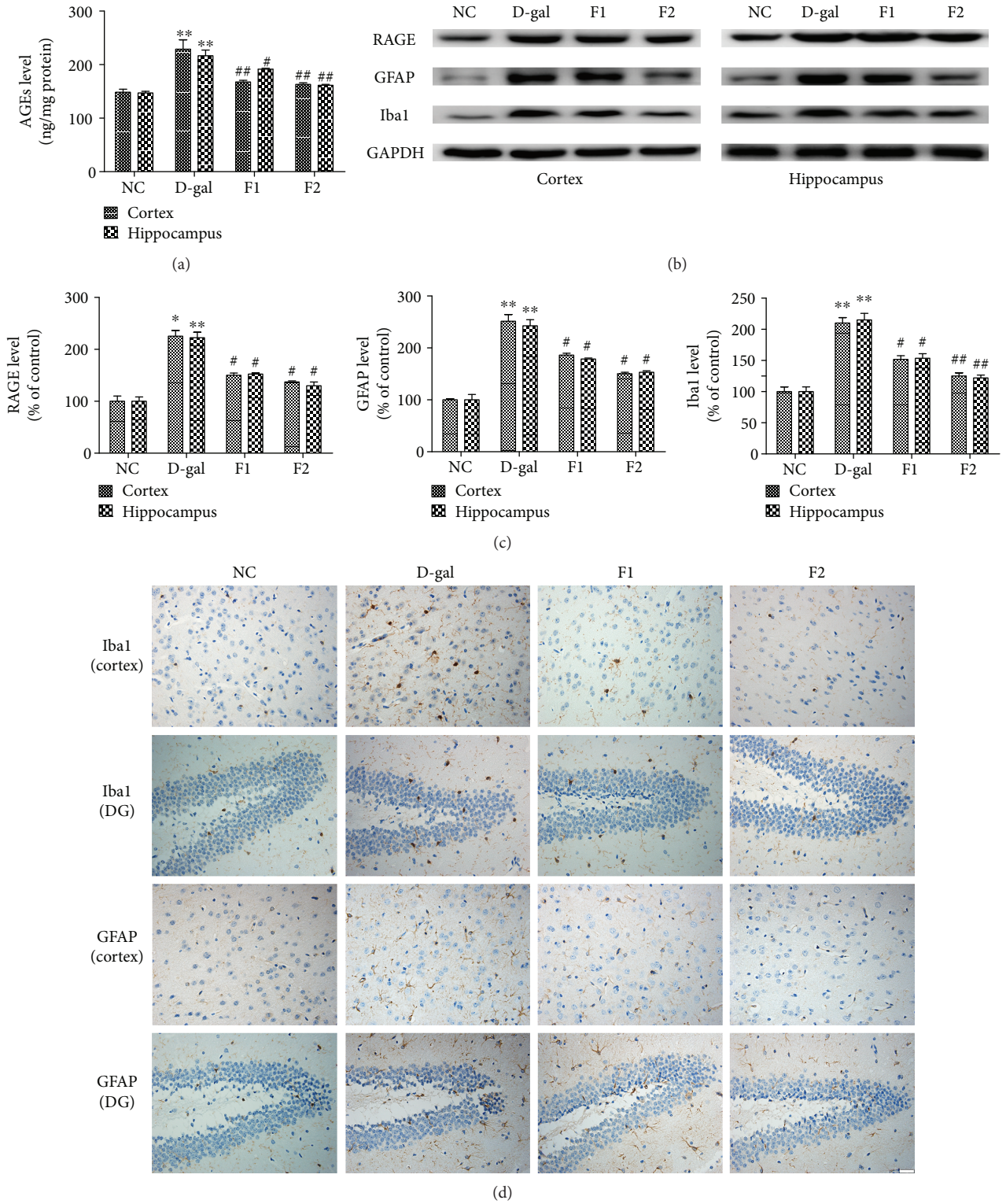

Figure 2: Effects of FELDK on AGEs, RAGE, GFAP, and Iba1 expression in the cortex and hippocampus of D-gal-aged mice. (a) AGEs levels were determined by ELISA ($n = 9, 8, 8, 8$). (b) Representative western blots showing protein expression of RAGE, GFAP, and Iba1 ($n = 3$). (c) Quantification of western blot analysis of RAGE, GFAP, and Iba1 protein expressed as percent of control. (d) The immunohistochemical images of active microglia and astrocytes in the cortex and hippocampal dentate gyrus (DG) regions of mice ($n = 9, 8, 8, 8$). Scale bars = 50 μm. All data are presented as mean values ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the D-gal group.

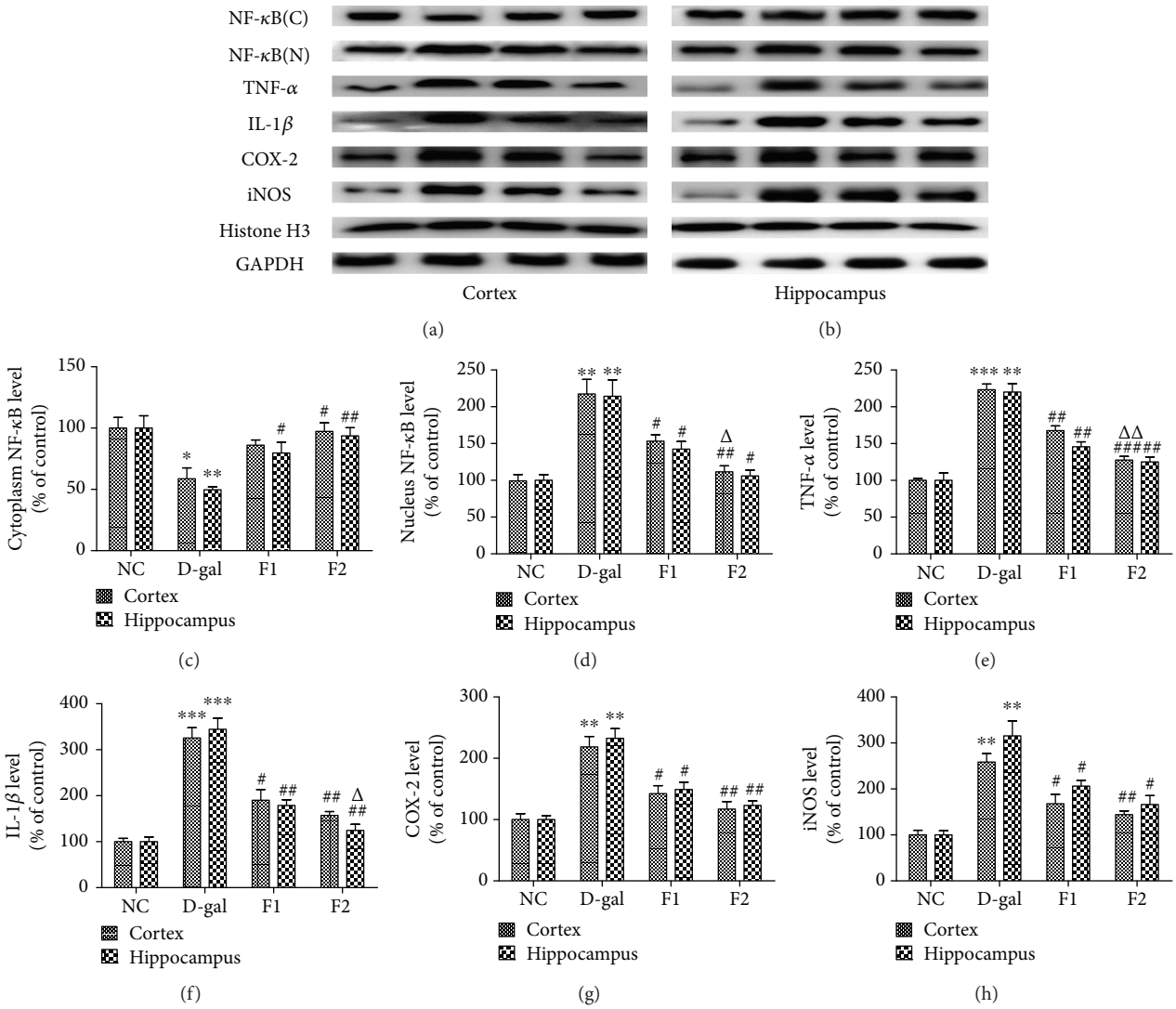

Figure 3: Effects of FELDK on NF-κB nuclear translocation and expression of inflammatory markers in the cortex and hippocampus of D-gal-aged mice. (a) Western blot images showing the expression of cytoplasmic and nuclear NF-κB, TNF-α, IL-1β, COX-2, and iNOS protein levels. (b) Quantification of the western blot analysis of cytoplasmic and nuclear NF-κB, TNF-α, IL-1β, COX-2, and iNOS protein expressed as percent of control. All data are presented as mean values ± SEM for $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the D-gal group; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ versus the F1 group.

are specific markers of microglia and astrocyte activation, respectively. A previous study demonstrated that D-gal activates glial cells [6]. As shown in Figures 2(b) and 2(c), western blots showed significant increases in GFAP and Iba1 protein expression in the D-gal group compared to the NC group ($P < 0.01$), and these expression levels were attenuated by low and high doses of FELDK ($P < 0.05$). Similar to the western blotting results, immunohistochemistry showed an increase in activated Iba1^{+} and GFAP^{+} cells in the cortex and hippocampal dentate gyrus (DG) regions of the D-gal vehicle group compared to the vehicle NC group (Figure 2(d)). FELDK treatment decreased the number of activated microglia and astrocytes in the cortex and hippocampal dentate gyrus regions of D-gal-FELDK-cotreated mice.

3.4. FELDK Reduced Nuclear NF-κB Translocation and Various Inflammatory Markers in D-gal-Aged Mice. AGEs promote proinflammatory cytokines through RAGE/NF-κB signaling [15]. To confirm this finding, we measured nuclear and cytoplasmic NF-κB levels. As shown in Figure 3, subcutaneous injection of D-gal significantly increased nuclear translocation of NF-κB (cytoplasm: $P < 0.05$; nucleus: $P < 0.01$) compared to the NC group. FELDK administration attenuated nuclear translocation (nucleus $P < 0.05$; cytoplasm $P < 0.05$). Nuclear translocation of NF-κB increased the expression of proinflammatory cytokines. In this study, the D-gal group mice showed a significant increase in inflammatory biomarkers, including TNF-α, IL-1β, COX-2, and iNOS ($P < 0.01$, $P < 0.001$, $P < 0.01$, and $P < 0.01$, respectively). Cotreatment with FELDK at both low and

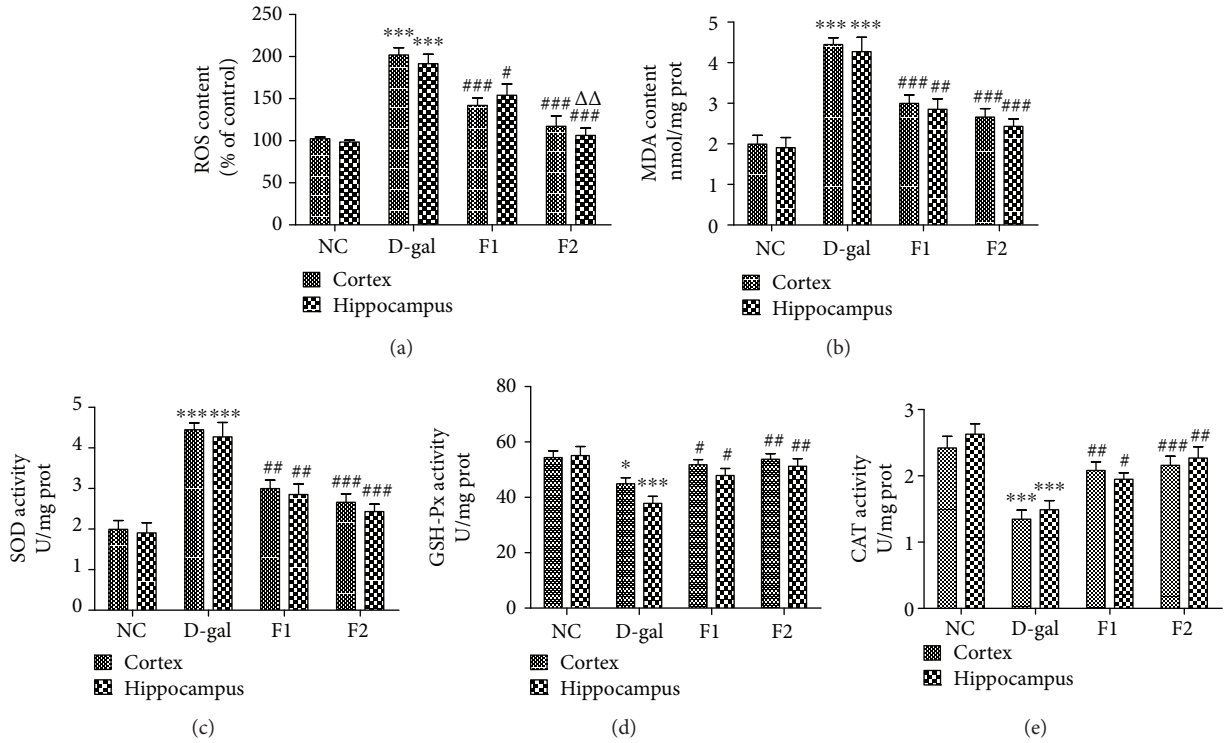

Figure 4: Effects of FELDK on ROS and MDA production and SOD, GSH-Px, and CAT activity in the cortex and hippocampus of D-gal-aged mice. (a) ROS levels. (b) MDA levels. (c) SOD activity. (d) GSH-Px activity. (e) CAT activity. All data are presented as mean values ± SEM for $n = 9, 8, 8, 8$. $^{*}P < 0.05$, $^{***}P < 0.001$ versus the NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the D-gal group, $^{\triangle\triangle}P < 0.01$ versus the F1 group.

high doses attenuated neuroinflammatory cytokine production (TNF-α, IL-1β, COX-2, and iNOS) compared to the D-gal group ($P < 0.05$).

3.5. FELDK Attenuates Oxidative Stress in D-gal-Aged Mice. Oxidative stress is initiated early in neurodegenerative diseases, such as AD. Therefore, we measured ROS production to determine the level of oxidative stress in the aged mice brains. D-gal markedly increased ROS production compared to the NC group ($P < 0.001$), while cotreatment of D-gal and FELDK significantly alleviated ROS production ($P < 0.05$) (Figure 4(a)). MDA is a marker of lipid peroxidation. As shown in Figure 4(b), there was a marked increase in MDA in the D-gal-aged mice ($P < 0.001$), while FELDK treatment significantly reduced MDA levels in the F1 and F2 groups ($P < 0.01$). Additionally, SOD, GSH-Px, and CAT activity were measured to determine the antioxidant capacity of the aged brains (Figures 4(c)–4(e)). All of the above markers were reduced after D-gal administration ($P < 0.001$, $P < 0.05$, and $P < 0.001$, respectively), while FELDK significantly improved SOD, GSH-Px, and CAT activity ($P < 0.05$).

3.6. FELDK Alleviates Neuronal Apoptosis in the Brains of D-gal-Aged Mice. D-gal has been reported to cause neuronal apoptosis [14]. The PI3K/Akt signaling axis is a major signaling pathway that promotes cell survival. Thus, we used western blot analysis to measure the effects of FELDK on D-gal-induced neuronal apoptosis. The results showed that PI3K and p-Akt decreased markedly in the cortex and hippocampus of D-gal-aged mice compared to the NC mice ($P < 0.05$). However, the reduction of PI3K and p-Akt was largely suppressed in the mice treated with FELDK after D-gal injection ($P < 0.05$). We did not observe any significant changes in total Akt (Figure 5).

Elevated p-JNK was reported in D-gal-treated mice [6]. Our results suggest that the activation of p-JNK in the D-gal group was comparable to the NC group ($P < 0.05$). FELDK suppressed p-JNK activation in the F1 and F2 groups compared to the D-gal group ($P < 0.05$) (Figure 5).

The results also indicate that D-gal reduced antiapoptotic Bcl-2 and increased proapoptotic Bax compared to the NC group ($P < 0.01$ for both). In contrast, treatment with low and high doses of FELDK reversed the effects on Bcl-2 and Bax protein expression in the cortex and hippocampus of D-gal-aged mice ($P < 0.05$), suggesting that FELDK has antiapoptotic properties (Figure 5).

3.7. FELDK Rescues D-gal-Induced Reduction of Synaptic Proteins in the Brain. Synaptic protein expression was significantly reduced following subcutaneous injection of D-gal [24]. We used western blot analysis to investigate the effect of FELDK on D-gal-induced alterations of synapse-related proteins. The results indicate that synaptic proteins, including synaptophysin, synaptotagmin, p-CREB, and p-CaMKII, were significantly reduced in the D-gal group compared to the NC group ($P < 0.05$). Additionally, FELDK reversed the reduction of synaptic proteins in the F1 and F2 groups compared to the D-gal group ($P < 0.05$) (Figure 6).

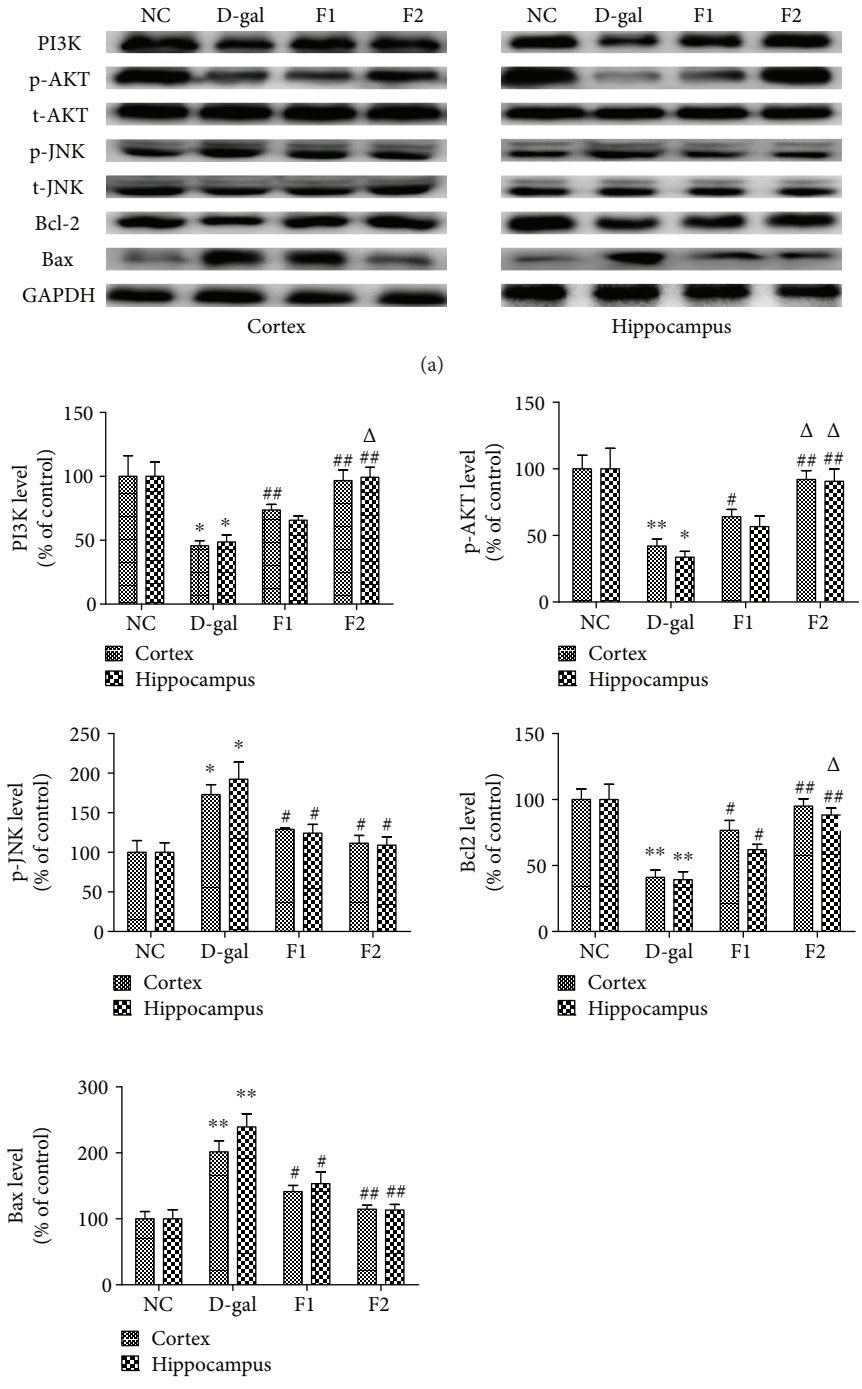

Figure 5: Effects of FELDK on the PI3K-Akt pathway, JNK activation, and Bcl-2 and Bax expression in the cortex and hippocampus of D-gal-aged mice. Western blots and related quantification of PI3K, p-Akt (Ser473), p-JNK, Bcl-2, and Bax expressed as percent of control. All data are presented as mean values ± SEM for $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the D-gal group; $^{\triangle}P < 0.05$ versus the F1 group.

4. Discussion

Very little is known about the potential antiaging effects of FELDK *in vivo*. Therefore, we investigated the effects and underlying mechanisms of FELDK using the D-gal-aged mouse model. Data obtained from the present study clearly demonstrated the following. First, consecutive subcutaneous injections of D-gal (100 mg/kg/d) daily for 10 weeks resulted in learning and memory deficits, microglia and astrocyte activation, increased AGEs/RAGE and NF-κB nuclear translocation, release of inflammatory mediators, elevated oxidative stress, neuronal cell apoptosis, and dysfunction of

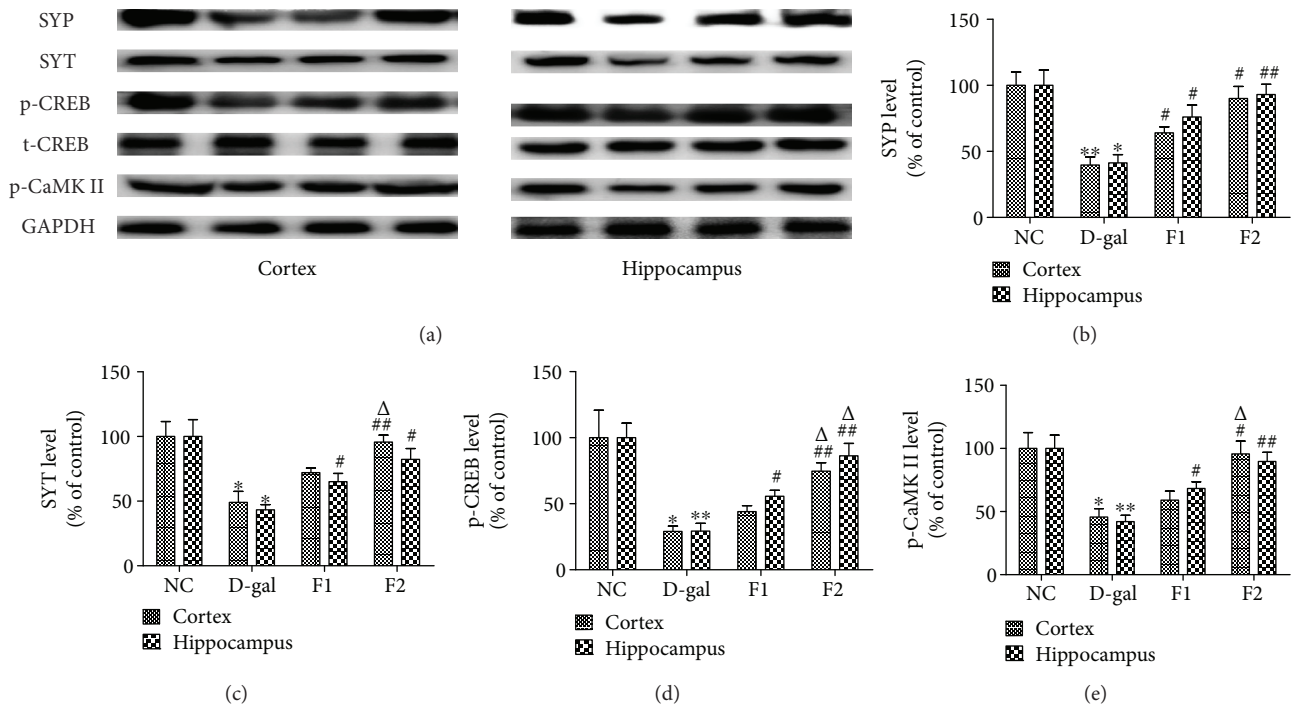

Figure 6: Effects of FELDK on synaptic protein expression, including synaptophysin, synaptotagmin, p-CREB, and p-CaMKII, in the cortex and hippocampus of D-gal-aged mice. Western blot and related quantification of synaptophysin, synaptotagmin, p-CREB, and p-CaMKII protein expression expressed as percent of control. All data are presented as mean values ± SEM for $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the D-gal group; $^{\triangle}P < 0.05$ versus the F1 group.

synaptic proteins in the brain. Second, oral administration of FELDK (40 or 80 mg/kg/d) significantly attenuated the above-mentioned changes induced by D-gal.

A low dose of D-gal is harmless, as D-gal can be converted into glucose by galactose-1-phosphate uridyltransferase and galactokinase [25]. However, galactose oxidase can increase D-gal and generate ROS [26]. D-gal also reacts readily with amino acids in peptides and proteins to form AGEs, which bind to and upregulate the expression of their receptor, RAGE [9]. ROS and AGEs are both involved in the pathological processes of aging and age-related diseases [27–29]. Studies have demonstrated that chronic subcutaneous administration of D-gal induces ROS and AGEs, which ultimately results in oxidative stress, leading to the activation of glial cells accompanied by neuroinflammation, neuronal cell apoptosis, reduction of synaptic proteins, and memory impairment [6, 14, 15].

ROS and binding of AGEs to RAGE both stimulate NF-κB, which can in turn lead to glial activation and gene transcription of many inflammatory mediators under resting conditions. NF-κB is maintained in the cytoplasm through interaction with the inhibitory subunit IκB. After activation, IκB becomes phosphorylated, followed by polyubiquitination and destruction. As a result, NF-κB is activated and easily translocates from the cytoplasm into the nucleus [30]. Consistent with the previous study, we found that D-gal elevated AGEs, RAGE, Iba1, and GFAP expression, while FELDK reversed AGEs/RAGE signaling and microglia and astrocyte activation. In addition, we demonstrated that D-gal induced nuclear translocation of NF-κB and enhanced the expression of downstream inflammatory markers, including TNF-α, IL-1β, COX-2, and iNOS. Nevertheless, FELDK attenuated the translocation of NF-κB and release of downstream inflammatory mediators.

Oxidative stress, which is increased in many neurodegenerative diseases, is an important pathological change associated with D-gal-induced aging. Oxidative stress also generates ROS, leading to many other pathological changes that ultimately accelerate the aging process [26]. The degree of oxidative damage can be determined by the production of antioxidant enzymes and markers of oxidative damage products, such as SOD, GSH-Px, CAT, and MDA. SOD combats oxidative stress by converting superoxide anions into H_2O_2, a more stable compound [31]. GSH-Px and CAT can further transform H_2O_2 molecules into H_2O [31]. MDA is a pivotal indicator of toxic lipid peroxidation induced by oxidative damage, which indirectly reflects ROS production. It has been reported that FLDK attenuates H_2O_2-induced injury in NG108-15 cells by increasing the expression and activity of GSH, CAT, and GSH-Px and reducing MDA levels [17]. In the present study, D-gal-aged mice showed elevated levels of ROS and MDA and decreased activity of SOD, GSH-Px, and CAT compared to the NC mice. However, FELDK markedly reduced ROS and MDA production and upregulated the expression of all measured antioxidative enzymes.

Evidence suggests that apoptosis contributes to the pathological process of D-gal-induced brain injury [32]. PI3K/Akt signaling inhibits cell survival, while JNK activation induces apoptosis through regulating Bcl-2 family gene

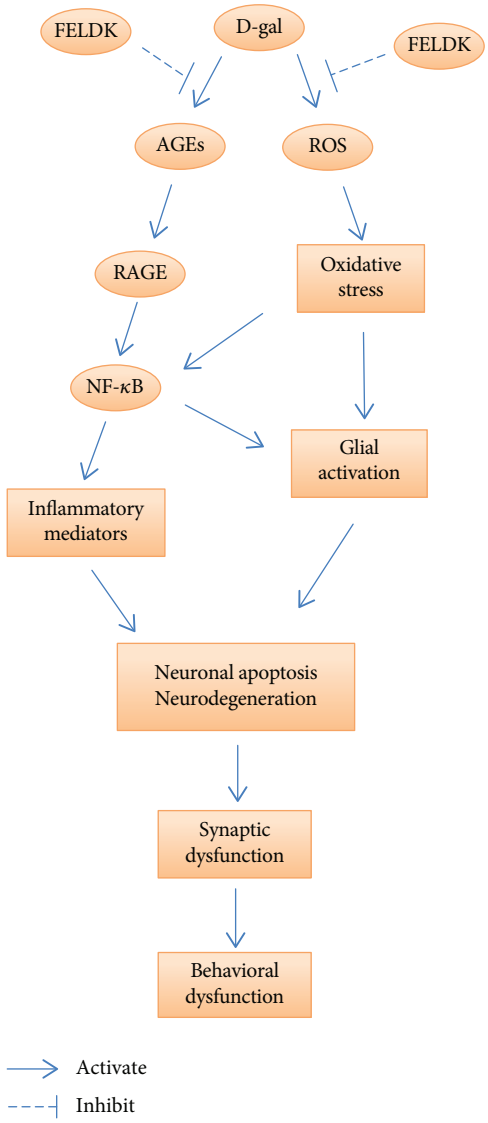

Figure 7: Schematic diagram of the protective effects of FELDK on D-gal-induced brain aging.

transcription [33]. In addition, the activation of PI3K/Akt signaling can inhibit the phosphorylation and activation of JNK [34, 35]. Bcl-2 and Bax are members of the Bcl-2 family and function as proapoptotic and antiapoptotic proteins, respectively. Researches have demonstrated that D-gal-induced ROS activates apoptotic signaling via stimulation of JNK in aging animal models [6, 14]. Our findings are consistent with these studies, such that D-gal increased neuronal apoptosis by inhibiting the PI3K/Akt pathway and activating JNK, which ultimately decreased the expression of Bcl-2 and increased the expression of Bax. Previous studies have also shown that FLDK-P70 upregulates Bcl-2 expression in H_2O_2-induced NG108-14 cells and protects against hypoxia- and reoxygenation-induced apoptosis *in vitro* [16, 17]. Ethyl acetate extract from the leaves of persimmon significantly attenuated $A\beta_{1\text{-}42}$-mediated hippocampus apoptosis in rats by modulating JNK/caspase-3 and the ratio of Bax/Bcl-2 [18]. In the present study, FELDK rescued neuronal apoptosis by upregulating PI3K/Akt and suppressing JNK activation, suggesting that FELDK might possess antiapoptotic activity in the aging mouse brain.

Synaptic proteins correlate closely with cognitive decline and increased oxidative stress [36], providing a good predictor for severity of neurodegeneration [37]. The previous study demonstrated that D-gal reduced the expression of presynaptic and postsynaptic protein markers [9, 24]. Synaptophysin, an important protein that forms small synaptic vesicle membranes, regulates short-term and long-term synaptic plasticity and synapse formation [38, 39]. Synaptotagmin plays a regulatory role in membrane interaction during trafficking of synaptic vesicles at the active zone of the synapse and is involved in exocytosis and endocytosis [40–42]. Autophosphorylation of CaMKII at Thr286 results in its persistent activation and is considered to be pivotal for long-term potentiation and information storage [43]. Phosphorylation of CREB at Ser133 is an important transcriptional process for memory function and synapse formation [8]. Consistent with the previous studies, our data showed that synaptophysin and synaptotagmin, postsynaptic protein p-CaMKII, and p-CREB were all reduced in D-gal-aged mice and oral administration of FELDK markedly enhanced the expression of synaptic proteins, which might be related to improved learning and memory in the aging mice.

In conclusion, FELDK administration alleviated oxidative stress induced ROS production and lipid peroxidation and sustained activity of endogenous antioxidant enzymes. Moreover, FELDK reduced microglia and astrocyte activation, decreased AGEs/RAGE expression, downregulated NF-κB nuclear translocation, inhibited the release of inflammatory mediators, attenuated neuronal apoptosis, and alleviated synaptic dysfunction. As summarized in Figure 7, these effects partially explain the mechanisms by which FELDK attenuates learning and memory impairment in D-galactose-aged senescent mice.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have declared that there is no conflict of interest.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (Grant no. 81371225) and the Science and Technology Development Planning Project of Shandong Province (Grant no. 2014GSF118056).

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 8604718, 10 pages
https://doi.org/10.1155/2018/8604718

Research Article

Low Serum Tryptophan Levels as an Indicator of Global Cognitive Performance in Nondemented Women over 50 Years of Age

L. A. Ramos-Chávez,[1,2] G. Roldán-Roldán,[1] B. García-Juárez,[3] D. González-Esquivel,[2] G. Pérez de la Cruz,[4] B. Pineda,[5] D. Ramírez-Ortega,[2] I. García Muñoz,[6] B. Jiménez Herrera,[6] C. Ríos,[2] S. Gómez-Manzo,[7] J. Marcial-Quino,[8] L. Sánchez Chapul,[9] P. Carrillo Mora,[3] and V. Pérez de la Cruz[2]

[1]*Laboratorio de Neurobiología de la Conducta, Departamento de Fisiología, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico*
[2]*Departamento de Neuroquímica, Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, S.S.A., México, DF 14269, Mexico*
[3]*División de Neurociencias/Subdivisión de Neurobiología, Instituto Nacional de Rehabilitación S.S.A., México, DF 14389, Mexico*
[4]*Instituto Nacional de Estadística y Geografía (INEGI), México, DF 03730, Mexico*
[5]*Laboratorio de Neuroinmunología, Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, S.S.A., México, DF 14269, Mexico*
[6]*División de Rehabilitación Geriátrica, Instituto Nacional de Rehabilitación, Ciudad de México, S.S.A. 14389, Mexico*
[7]*Laboratorio de Bioquímica Genética, Instituto Nacional de Pediatría, Secretaría de Salud, México City 04530, Mexico*
[8]*CONACYT-Instituto Nacional de Pediatría, Secretaría de Salud, Ciudad de México 04530, Mexico*
[9]*Laboratorio de Enfermedades Neuromusculares, Instituto Nacional de Rehabilitación, Ciudad de Mexico, S.S.A. 14389, Mexico*

Correspondence should be addressed to P. Carrillo Mora; neuropolaco@yahoo.com.mx
and V. Pérez de la Cruz; veped@yahoo.com.mx

Received 11 April 2018; Revised 17 July 2018; Accepted 12 August 2018; Published 21 November 2018

Academic Editor: Simona M. Monti

Aging is a physiological decline process. The number of older adults is growing around the world; therefore, the incidence of cognitive impairment, dementia, and other diseases related to aging increases. The main cellular factors that converge in the aging process are mitochondrial dysfunction, antioxidant impairment, inflammation, and immune response decline, among others. In this context, these cellular changes have an influence on the kynurenine pathway (KP), the main route of tryptophan (Trp) catabolism. KP metabolites have been involved in the aging process and neurodegenerative diseases. Although there are changes in the metabolite levels with age, at this time, there is no study that has evaluated cognitive decline as a consequence of Trp catabolism fluctuation in aging. The aim of this study was to evaluate the relation between the changes in Trp catabolism and cognitive impairment associated with age through KP metabolites level alterations in women over 50 years of age. Seventy-seven nondemented women over 50 years old were examined with a standardized cognitive screening evaluation in Spanish language (Neuropsi), Beck anxiety inventory (BAI), and the geriatric depression scale (GDS). Also, serum levels of Trp, kynurenine (Kyn), kynurenic acid (KYNA), and 3-hydroykynurenine (3-HK) and the glutathione ratio (GSH/GSSG) were measured. Results showed a negative correlation between age and Trp levels and a positive correlation between age and KYNA/Trp and 3-HK/Trp ratios. The level of cognitive impairment showed a significant positive association with age and with kynurenine pathway activation and a significant negative correlation with Trp levels. The GSH/GSSG ratio correlated positively with Trp levels and negatively with Kyn/Trp and 3-HK/Trp ratios. The depression score correlated negatively with Trp and positively with the 3-HK/Trp ratio. We concluded that KP activation increases with age and it is strongly associated with the level of cognition performance in nondemented women over 50 years of age.

1. Introduction

Aging is a time-dependent physiological process that is characterized by a progressive loss of physiological integrity, leading to impairment of functions, and an increased vulnerability to death, affecting all higher organisms [1]. The rising life expectancy leads to higher risk in development of age-related diseases, such as cancer and neurodegenerative diseases [2]. Factors that converge during aging are mitochondrial dysfunction, oxidative stress, decline in antioxidant defense, cellular senescence, stem cell exhaustion, alterations of intercellular communication, genomic instability, epigenetic alterations, deregulated nutrient sensing, and chronic low-grade inflammatory state, among others [3–6].

Some of these factors, such as inflammation and redox state alteration, directly influence the Trp catabolism, which also changes with age. Trp is an essential amino acid, which is metabolized mainly through the kynurenine pathway (KP) (~95%) [7]. Trp can be catabolized by Trp 2,3-dioxygenase (TDO) in the liver and by indoleamine 2,3-dioxygenase (IDO) elsewhere to produce kynurenine (Kyn). Kyn can be a substrate for three enzymes: (1) kynurenine aminotransferases to produce kynurenic acid (KYNA), (2) kynureninase to form anthranilic acid (AA), and (3) kynurenine-3-monooxygenase (KMO) to produce 3-hydroxykynurenine (3-HK), which is further hydrolyzed by kynureninase to 3-hydroxyanthranilic acid (3-HANA). 3-HANA is catabolized as a substrate of 3-hydroxyanthranilate 3,4-dioxygenase which produces an unstable intermediate that rapidly can be converted to quinolinic acid (QUIN) by nonenzymatic cyclization or to produce picolinic acid by the 2-amino-3-carboxymuconate semialdehyde decarboxylase. Finally, quinolinate phosphoribosyl transferase catabolizes QUIN to produce NAD+. KP is controlled mainly by TDO and IDO, which are modulated in different ways. TDO is inducible by glucocorticoids, while IDO is activated by proinflammatory cytokines and superoxide [8–10]. The clinical importance of the KP is due to the fact that metabolites with redox and neuroactive properties as 3-HK, 3-HANA, KYNA, and QUIN are formed through it. QUIN is an agonist of NMDAr, while KYNA is an antagonist of NMDAr and can also inhibit noncompetitively α7-nicotinic receptors [11, 12]. It has been observed that brain KYNA level fluctuations impact the cognition [13–21].

There are a few human studies that relate aging with kynurenine pathway components. Pertovaara and coworkers [22] found that the Kyn/Trp ratio is higher in older people than in healthy and younger controls and was able to predict mortality in nonagenarian population. In another study, age was positively associated with kynurenine levels in human serum [23]. It has been shown that picolinic acid concentration in CSF correlates positively with age [24]. Also, KYNA in CSF increased during aging and correlated with high titers of IgG and β_2-microglobulin (markers of immune system activation) [25]. Even with this evidence which suggests that activation of KP occurs during aging and knowing that aging is associated with impairment in cognitive information processing, decline in attention, memory, and other cognitive functions, until now, there have been no studies correlating cognitive performance and KP metabolite levels during aging. The purpose of this study was to determine whether cognitive decline associated with age is related to Trp catabolism fluctuations in nondemented women over 50 years of age.

2. Materials and Methods

2.1. Chemicals. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and J.T.Baker® (Center Valley, PA, USA), unless otherwise mentioned in the text.

2.2. Ethical Approval. The protocol, previously approved by the institutional committees (reference no. 114/15), was in agreement with the Declaration of Helsinki and local regulations regarding research on human subjects (Reglamento de la Ley General de Salud en Materia de Investigación para la Salud en México). Written informed consents were obtained from all recruited subjects.

2.3. Subject Recruitment Inclusion. The individuals included in the study were 77 adult women, over 50 years old, without evident cognitive impairment, that is, individuals with complete functionality and independent in their basic activities of daily life. No history of neurodegenerative, psychiatric, chronic inflammatory, or autoimmune diseases was present. Also, inclusion criteria required no history of cerebrovascular disease in the previous 6 months and no current use of immunosuppressive or immunomodulatory drugs. Subjects with severe visual or auditory deficit that could affect the cognitive evaluation were excluded. Subjects who fulfilled all the inclusion criteria gave their informed consent to participate in the study.

2.4. Cognitive and Emotional Evaluations. For cognitive status evaluation in subjects, a standardized and validated neuropsychological test battery, in adult population, was used, which also allowed to weight the effect of scholarship and age of subjects (Neuropsychological Assessment in Spanish language, *Neuropsi*) [26]. This evaluation has reference standards made in the Mexican population being able to identify individuals with normal performance or with different levels of cognitive impairment as mild, moderate, and severe. Likewise, the Beck anxiety inventory (BAI) and the geriatric depression scale (GDS) were applied, in order to rule out that the cognitive alterations were caused by some emotional state disturbance. All evaluations were performed immediately before the peripheral blood collection.

2.5. Sample Blood Collection. Blood samples (5 ml) were collected by vein puncture and allowed to clot, and serum was obtained by centrifugation at 2500 rpm for 20 minutes and stored at −70°C until analysis.

2.6. Serum GSH and GSSG Determinations. Serum reduced glutathione (GSH) and oxidized GSH concentrations were determined using a fluorometric method reported by Senft et al. [27] and adapted by Ramos-Chavez et al. [28]. The method is based in the GSH reaction with o-phthaldialdehyde (OPA) to form a highly stable and

fluorescent isoindole derivative. Briefly, 50 μl of serum sample was treated with 150 μl of 5% (w/v) metaphosphoric acid and vigorously mixed. Then, tubes were placed on ice for 15 minutes and centrifuged at 14,000 rpm for 20 minutes at 4°C. 5 μl of supernatant was used for GSH and 30 μl for GSSG determination. For GSSG determination, the first step was to inhibit GSH isoindole derivation using N-ethylmaleimide; subsequently, GSSG is reduced to GSH by dithionite treatment and then derivation with OPA to obtain the isoindole. The fluorescence was measured at 370 nm excitation and 420 nm of emission (*FLx800* Multimode Lector BioTex, Houston, Texas, USA). Calibration curves were built for GSH and GSSG, and the concentrations were obtained by interpolation in the standard curve. The results are expressed as μmol/l.

2.7. Kynurenines Determination. Kynurenines were measured by an HPLC method with fluorescence detection for KYNA, Trp, and Kyn, while 3-HK was determined using electrochemical detection [29–32]. The equipment used was a PerkinElmer chromatograph (PerkinElmer, Waltham, MA, USA) coupled to a variable wavelength UV detector (PDA Plus Detector Flexar), a fluorescence detector (model S200), an electrochemical detector (CC-5E LC-4C Amperometric Detector), an automatic delivery pump (Flexar binary LC pump), and an autosampler injector (Flexar LC autosampler). 200 μl of the serum sample was treated with 200 μl of 6% perchloric acid, centrifugated at 14,000 rpm and 4°C. The supernatant was stored at −70°C until analysis.

2.7.1. KYNA and Kyn Determinations. 20 μl of the serum supernatant sample or standard solution was injected onto an Eclipse XDB-C18 reverse phase column (5 μm, 4.6 × 150 mm, Agilent, Santa Clara, CA, USA) and isocratically eluted with a mobile phase consisting of 50 mM of sodium acetate, 250 mM of zinc acetate, and 3% of acetonitrile, pH adjusted to 6.2 with glacial acetic acid, at a flow rate of 1 ml/min; Kyn was eluted with the same mobile phase but without acetonitrile. Both metabolites were detected by fluorescence, KYNA at excitation wavelength of 344 nm and emission wavelength of 398 nm and Kyn at excitation wavelength of 368 nm and emission wavelength of 480 nm. The retention time for KYNA was ~7 min and for Kyn was ~10 min.

2.7.2. Trp Analysis. Trp levels were determined using a ZORBAX Eclipse AAA column (3.5 μm, 4.6 × 150 mm, Agilent, Santa Clara, CA, USA) and isocratically eluted with a mobile phase containing 100 mM of zinc acetate and 3% of acetonitrile (pH adjusted to 4.2 with glacial acetic acid) at a flow rate of 1 ml/min. 20 μl of biological sample or standard solutions was injected for Trp determination. Trp was detected by fluorescence (excitation wavelength: 254 nm and emission wavelength: 404 nm). The retention time of Trp was ~5 min.

2.7.3. 3HK Measurement. The 3HK was determined using an electrochemical method described by Heyes and Quearry et al. [29]. Briefly, 3HK was eluted at a constant flow rate of 0.6 ml/min with a mobile phase containing 9% of triethylamine, 0.59% phosphoric acid, 0.27 mM EDTA, and 8.9 mM heptane sulfonic acid; 40 μl of the sample or standard was injected onto an Adsorbosphere Catecholamine C18 reverse phase column (3 μm, 4.6 mm × 100 mm, Fisher Scientific, Hampton, Nuevo Hampshire, USA). The retention time was ~11 min.

2.8. Statistical Analysis. Correlation between each pair of the following variables was assessed with Spearman's rho coefficient: age, cognition, Trp, Kyn/Trp, KYNA/Trp, 3-HK/Trp, depression score, anxiety, and GSH/GSSG. The Mann-Whitney test and T-test were used to compare distributions associated with normal (undetected) and detected cognitive impairment groups; the T-test was obtained using the logarithmic scale. Finally, a logistic regression model was adjusted considering age and Trp levels in the logarithmic scale as covariables, where the dependent variable corresponds to the binary variable associated with cognitive impairment (1 = normal and 0 = CI). The logistic regression model obtained was the following:

$$\log\left(\frac{p}{1-p}\right) = -31.9 + 7.7^{*} \times \log(\text{age}) - 0.83^{**} \times \log(\text{Trp}). \quad (1)$$

3. Results

Demographic and clinical characteristics of the participants are shown in Table 1. The average age was 71.9 years (SD: 11.8), the average number of years of education was 8.4 (SD: 5.0), and only 26% of them had education over 9 years. 77% of them had between 1 and 3 comorbidities (e.g., hypertension, diabetes, dyslipidemia, osteoarthritis, and obesity). Regarding their cognitive performance, 70% of them obtained a normal evaluation, and 30% ($n = 23$) presented some degree of cognitive impairment (CI). 33% found significant symptoms of depression and 53% with some symptoms of anxiety. Descriptive statistics of analytes is shown in Table 2.

Pairwise correlations among age, cognition level, Trp levels, and the Kyn/Trp, KYNA/Trp, and 3-HK/Trp ratios as well as depression, anxiety, and GSH/GSSG ratio are showed in Figure 1. As expected, age correlated positively with the cognitive impairment level (four ordinal categories were used: normal, mild, moderate, and severe). Trp levels (pmoles/μl) correlated negatively with age, while Kyn (pmoles/μl)/Trp, KYNA (fmoles/μl)/Trp, and 3-HK (pmoles/μl)/Trp ratios correlated positively with age. Also, the cognitive impairment level was associated negatively with Trp levels and positively with KYNA/Trp and 3-HK/Trp ratios. As it has been described by other groups [23], the depression score correlates positively with anxiety and negatively with the levels of Trp, as was observed in this study. We also found a positive correlation between the depression score and 3-HK/Trp ratio. The GSH/GSSG ratio was determined as a redox status marker and correlated

Table 1: Clinical and demographic features of all individuals ($n = 77$).

Age (years)	Mean ± SD	71.92 ± 11.84
	Min, Max	51.00–97.00
Education	Mean ± SD	8.48 ± 5.02
	0–4 years	11 (14%)
	5–9 years	46 (60%)
	10–24 years	20 (26%)
Civil status	Married	30 (39%)
	Single	19 (25%)
	Widow	28 (36%)
Occupation	Paid employment	47 (61%)
	Vacant/retired	30 (39%)
Comorbidities	0–3	59 (77%)
	4–6	16 (20%)
	7–9	2 (3%)
Cognition profile	Mean ± SD	85.86 ± 20.24
	Min, Max	23.00 ± 119.00
	Normal	54 (70%)
	Mild impairment	10 (13%)
	Moderate impairment	11 (14%)
	Severe impairment	2 (3%)
GDS (depression), $n = 63$	Mean ± SD	3.44 ± 2.60
	Min, Max	0.0 ± 10.00
	Without depression symptoms	42 (67%)
	With depression symptoms	21 (33%)
BAI (anxiety)	Mean ± SD	12.52 ± 10.98
	Min, Max	0.0–42.00
	Minimal	36 (47%)
	Mild	21 (27%)
	Moderate	14 (18%)
	Severe	6 (8%)

GDS: geriatric depression scale; BAI: Beck anxiety inventory.

positively with Trp and negatively with Kyn/Trp and 3-HK/Trp ratios.

To extend the analysis on the association between Trp catabolism and cognitive impairment, only two groups of subjects were considered. The first one consisted of those subjects who did not present with cognitive impairment, the second, those that presented some level of cognitive impairment. The results of the statistical tests comparing the distributions of these two groups for the serum levels of Trp, Kyn/Trp, KYNA/Trp, and 3-HK/Trp are shown in Table 3. It was found that the levels of Trp were significantly different in women with cognitive impairment in comparison with those without any cognitive impairment; the median of the Trp levels in women with cognitive impairment was around half the median value found of those in women without cognitive impairment (Table 3). Also, Kyn/Trp, KYNA/Trp, and 3-HK/Trp serum ratios were significantly different between women with cognitive impairment and those without any cognitive impairment.

Moreover, to study the correlation between the presence of cognitive impairment and Trp levels, a logistic regression model including age as a covariable was adjusted; as was mentioned before, there was a significant correlation between age and cognitive impairment. The results show that both covariables, age and Trp levels, are significant in the model, and for a given age, it means that at lower Trp levels, there is a greater probability of observing cognitive impairment. Figure 2 shows the decision boundary for the adjusted logistic regression.

4. Discussion

According to our knowledge, this is the first time that Trp metabolites have been correlated with cognitive performance during normal aging in women. Our results suggest an overactivation of the KP during aging since we found a negative correlation between age and Trp levels and a positive influence of age on Kyn/Trp, KYNA/Trp, and 3-HK/Trp ratios; which is supported by the fact that the serum Kyn/Trp ratio is a measure of the beginning of KP activity [33, 34]. These data are consistent with previous studies in which low plasma, serum, and CSF Trp levels and high values of the Kyn/Trp ratio were also observed in elderly people [22, 23, 33, 35–39]. These alterations on the Kyn/Trp ratio may be due to enhanced activity of IDO and/or TDO. Although the major site of Trp conversion into kynurenine is the liver via TDO, it has been shown that liver TDO activity, both holoenzyme and apoenzyme, decreases significantly with age in rats [40], so that if we consider that during aging there is an increased concentration of inflammatory markers, the Kyn/Trp ratio could reflect mainly IDO activity [41]. Supporting this idea, it has been previously described that IL-6 correlates positively with Kyn and the Kyn/Trp ratio in serum of older people [4], suggesting that IDO activity increases with aging [37]. In addition, it has been found that the Kyn/Trp ratio and neopterin concentration (marker associated with inflammation and oxidative stress) were significantly correlated with increased age, while Trp correlated negatively with age in CSF samples from women [33].

Additionally, our results indicated a strong association between KP activation and redox status. The ratio of GSH and GSSG (GSH/GSSG) has been noted as an index of oxidative stress. Specifically, in this study, the GSH/GSSG ratio correlated negatively with KP activation, and as was mentioned before, some metabolites produced through KP have neuroactive and redox properties [10]. Kyn and KYNA have shown antioxidant properties [32, 42], while 3-HK and 3-HANA can also be scavengers in a concentration-dependent way and after their interaction with ROS leads to more toxic compounds inducing cellular death [43–45]. An important point is that GSH levels can be reduced because they can produce an adduct with the 3-HK glucoside, which, at the same time, is a product of 3-HK deamination [46, 47]. Keeping this in mind, the low GSH/GSSG

Table 2: Descriptive statistics of GSH, GSSG, and KP metabolites for each group (normal and detected cognitive impairment (CI)).

	Min	Percentile 25	Median	Mean	Percentile 75	Max	n
GSH (μmol/l)							
Total	53.760	151.030	196.860	209.920	236.740	766.000	79
Normal	53.760	148.710	187.210	206.580	220.590	766.000	48
CI	129.700	165.100	218.800	213.900	253.300	340.400	18
GSSG (μmol/l)							
Total	0.090	12.650	40.180	39.700	62.800	118.780	79
Normal	0.090	14.480	41.440	41.750	63.470	118.780	48
CI	7.410	19.430	40.720	43.260	67.950	85.270	18
Trp (pmoles/μl)							
Total	2.380	11.450	22.740	26.360	37.290	87.600	82
Normal	3.401	15.802	23.370	28.208	35.173	87.599	48
CI	2.380	5.017	11.472	15.384	17.506	47.816	21
KYNA (fmoles/μl)							
Total	0.832	2.708	4.525	10.534	6.662	209.894	79
Normal	0.832	2.514	4.465	13.325	7.191	209.894	47
CI	1.694	2.768	3.731	4.595	4.908	15.561	19
3-HK (pmoles/μl)							
Total	0.000	0.025	0.036	0.046	0.053	0.376	82
Normal	0.000	0.025	0.041	0.050	0.057	0.376	48
CI	0.006	0.026	0.036	0.045	0.052	0.155	21
L-Kyn (pmoles/μl)							
Total	0.000	3.478	12.513	20.305	22.417	283.361	80
Normal	0.015	5.636	14.697	20.158	22.820	224.934	47
CI	2.174	6.396	12.513	14.127	17.542	39.094	20

ratio found in this study could be related to the high levels of redox kynurenines.

In this study, the Kyn/Trp, KYNA/Trp, and 3-HK/Trp ratios that are associated with age reflect a greater amount of KP metabolites in the circulation. However, just Trp, Kyn, and 3-HK can cross the blood-brain barrier, either through simple diffusion across the vascular membranes or as a result of active transport via the large neutral amino acid transporter [48–51]. Recently, Hestad and coworkers showed that serum Kyn levels correlated highly with CSF Kyn levels [23]. The alterations of blood KP metabolites can produce significant secondary changes in the levels of kynurenine metabolites in the CNS and consequently impact processes such as cognition, by altering the degree of activation or blockade of NMDAr as well as the α-7 nicotinic receptor [52, 53].

In this context, the 3-HK/Trp ratio is consistent with the increase in downstream metabolites such as 3-HANA, PIC, and QUIN found in human CSF, with age. In rats the increase of QUIN with age has also been described [24, 33, 54]. Moreover, Trp and Kyn that really cross the blood-brain barrier, can produce KYNA by KATs, considered the canonical way. However, there are other mechanisms by which KYNA is produced and that involve the interaction of D- and L-isomers of Trp and Kyn with reactive oxygen species (ROS) [32, 55, 56], which as we know, is an important factor during aging. Fluctuation in KYNA levels leads to behavioral and cognitive changes during aging [25]. According to data obtained in this study, Trp catabolism through KP and the level of cognitive impairment are associated with aging in women over 50 years of age. Experimental studies have shown that increased levels of Kyn are linked with deficits of spatial working memory [17, 57]. The deletion of the major KAT isoenzyme, KAT-II, results in a substantial decrease in the extracellular concentration of KYNA, improving cognitive performance in a range of behavioral tasks which include exploration, object recognition, and passive avoidance learning [20]. Also, it has been demonstrated that Trp depletion affects a variety of cognitive processes in healthy individuals, such as memory and learning skills and long-term memory consolidation, which can be associated with the bioactive kynurenines such as KYNA and QUIN [58, 59].

A strong relationship between KP activation and cognitive impairment is also observed in Alzheimer's disease, which is an age-related neurodegenerative disease. Widner and coworkers found decreased serum Trp levels and increased serum kynurenine levels, and these changes correlated with the level of cognitive decline in Alzheimer's disease patients [60, 61]. Also, plasma Trp concentrations were found to be lower in HIV+ compared with HIV− individuals, and a higher plasma Kyn/Trp ratio was associated with cognitive impairment and major depressive disorder in the overall HIV+ group [62]. Another study found positive

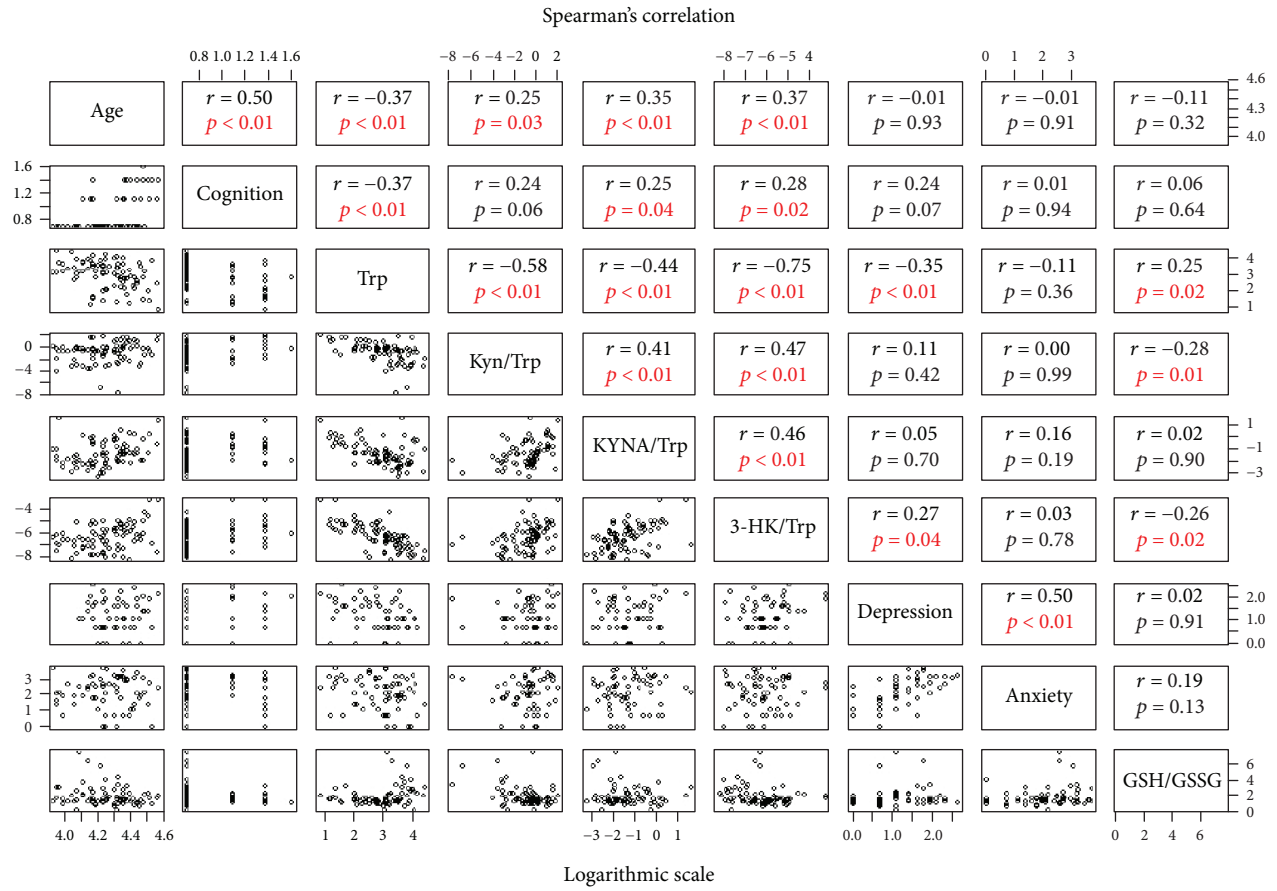

Figure 1: The lower triangular matrix contains the scatterplot for each pair of variables in the logarithmic scale. The upper triangular matrix contains Spearman's rank correlation coefficient and its associated p value. In this case, the variable cognition corresponds to an ordinal variable with four categories depending on the level of cognitive impairment: normal, mild, moderate, and severe.

Table 3: Descriptive statistics of Trp levels and Kyn/Trp, KYNA/Trp, and 3-HK/Trp ratios for each group (normal and detected cognitive impairment (CI)).

	Trp		Kyn/Trp		KYNA/Trp		3-HK/Trp	
	Normal	CI	Normal	CI	Normal	CI	Normal	CI
Descriptive statistics								
Percentile 25	15.802	5.017	0.198	0.359	0.117	0.236	0.001	0.001
Median	23.370	11.472	0.579	0.856	0.191	0.459	0.002	0.003
Percentile 75	35.173	17.506	1.045	3.094	0.352	0.686	0.003	0.005
Mean	28.208	15.384	0.915	2.120	0.454	0.661	0.003	0.007
n	48	21	47	18	46	19	47	21
p values								
Mann-Whitney test	0.0015		0.0700		0.0280		0.0262	
T-test	0.0017		0.0350		0.0306		0.0189	

correlations between cognitive function tests and lower plasma KYNA levels, and inverse correlations between these tests and increased QUIN levels in Alzheimer's patients [63]. Plasma and the CSF Kyn/Trp ratio were correlated with risk of dementia in Alzheimer's patients [63–65]. Interestingly, it has also been observed that increased KP activation and changes in the levels of kynurenine metabolites correlate negatively with cognitive performance in patients undergoing cardiac surgery, which suggest that Trp catabolism can be a biomarker of non-age-related cognitive impairment [66].

Noteworthy to mention is that in this study, Trp levels correlated negatively with the depression score and positively with the 3-HK/Trp ratio and anxiety in women over 50 years of age. These results suggest the KP activation in depression,

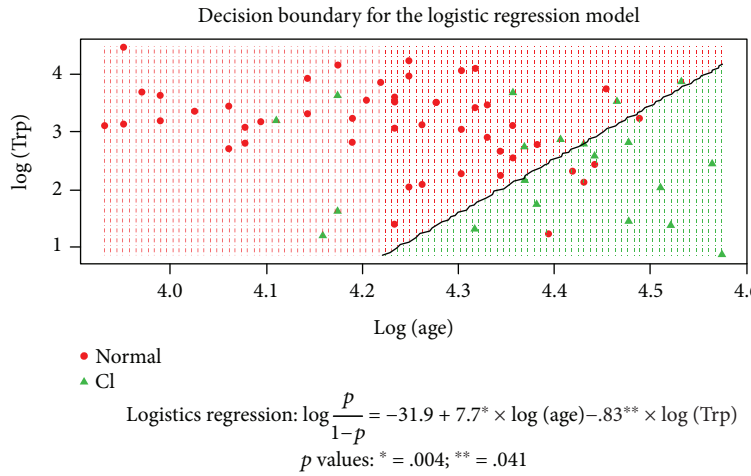

Figure 2: Scatterplot for age and Trp in the logarithmic scale distinguishing between the status of cognitive impairment: detected and normal. The adjusted logistic regression considering these two variables where the dependent variable corresponds to the binary variable associated with cognitive impairment (1: detected and 0: normal) is also shown together with the corresponding p values for the coefficients in the logistic regression model.

which may induce a transient reduction in serotonin synthesis, which may also be associated with depression [67–71]. In our study, we did not take into account serotonin production, considering that the serotonin synthesized in the periphery is unable to cross the blood-brain barrier, whereas the production of brain serotonin is dependent on the amount of circulating Trp [72]. Low Trp intake has been assumed to cause lower brain serotonin levels and to be an important risk factor involved in the onset and course of a variety of affective disorders, including depression [73]. In this context, Suga and coworkers showed an inverse association between Trp intake and depressive symptoms in young women participants (mean age around 18 years old), which suggests that the adequate intake is necessary to prevent depression [74]. A recent study observed that the increased Trp catabolism related to peripheral inflammation is accompanied by marked elevation in brain kynurenine and QUIN levels, and these alterations correlated with depressive symptoms in patients with hepatitis C [75].

Interestingly, our analysis suggests elements to establish that circulating Trp levels are a predictive biomarker and could be used to distinguish women over 50 years of age with some degree of cognitive impairment. However, it is important to take into account that this study was performed in women over 50 years of age; it is therefore necessary to confirm these results in a wider age range and to determine whether this effect is also present in men, in order to establish whether Trp levels are predictive for cognitive impairment in the general population.

5. Conclusion

The identification of novel biomarkers associated with the cognitive impairment that occurs during aging could provide key biological insights to identify an adequate intervention. This study confirms a close relationship between age, the Trp catabolism through KP activation, and cognitive impairment. However, based on our logistic regression model, given the age, Trp levels were the only significant predictor among the KP metabolites. Then, Trp levels can be considered as a useful indicator of cognitive impairment in women over 50 years of age, and these results afford a basis for further investigation in order to design future intervention strategies focused on prevention and treatment of cognitive impairment.

Data Availability

The cognitive and biochemistry data used to support the findings of this study are available with the corresponding author upon request.

Conflicts of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

This work was supported by CONACYT Grant 262010. LARC received a postdoctoral fellowship from DGAPA-UNAM.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 8152373, 15 pages
https://doi.org/10.1155/2018/8152373

Review Article

Neuroprotective Mechanisms of Resveratrol in Alzheimer's Disease: Role of SIRT1

Bruno Alexandre Quadros Gomes[1], **João Paulo Bastos Silva**[1], **Camila Fernanda Rodrigues Romeiro**[2], **Sávio Monteiro dos Santos**[3], **Caroline Azulay Rodrigues**[3], **Pricila Rodrigues Gonçalves**[2], **Joni Tetsuo Sakai**[3], **Paulo Fernando Santos Mendes**[3], **Everton Luiz Pompeu Varela**[3], **and Marta Chagas Monteiro**[1,2,3]

[1]*Neuroscience and Cell Biology Graduate Program, Institute of Biological Sciences, Federal University of Pará, Belém, Pará, Brazil*
[2]*Faculty of Pharmacy, Institute of Health Sciences, Federal University of Pará, Belém, Pará, Brazil*
[3]*Pharmaceutical Sciences Graduate Program, Institute of Health Sciences, Federal University of Pará, Belém, Pará, Brazil*

Correspondence should be addressed to Marta Chagas Monteiro; martachagas2@yahoo.com.br

Received 5 March 2018; Revised 21 July 2018; Accepted 15 August 2018; Published 30 October 2018

Academic Editor: Herbenya S. Peixoto

Alzheimer's disease (AD) is a progressive and neurodegenerative disorder of the cortex and hippocampus, which eventually leads to cognitive impairment. Although the etiology of AD remains unclear, the presence of β-amyloid (Aβ) peptides in these learning and memory regions is a hallmark of AD. Therefore, the inhibition of Aβ peptide aggregation has been considered the primary therapeutic strategy for AD treatment. Many studies have shown that resveratrol has antioxidant, anti-inflammatory, and neuroprotective properties and can decrease the toxicity and aggregation of Aβ peptides in the hippocampus of AD patients, promote neurogenesis, and prevent hippocampal damage. In addition, the antioxidant activity of resveratrol plays an important role in neuronal differentiation through the activation of silent information regulator-1 (SIRT1). SIRT1 plays a vital role in the growth and differentiation of neurons and prevents the apoptotic death of these neurons by deacetylating and repressing p53 activity; however, the exact mechanisms remain unclear. Resveratrol also has anti-inflammatory effects as it suppresses M1 microglia activation, which is involved in the initiation of neurodegeneration, and promotes Th2 responses by increasing anti-inflammatory cytokines and SIRT1 expression. This review will focus on the antioxidant and anti-inflammatory neuroprotective effects of resveratrol, specifically on its role in SIRT1 and the association with AD pathophysiology.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative pathology that causes impaired cognitive functioning and memory [1, 2]. Despite the disease being identified over 100 years ago [3], efforts are currently being expended to discover new chemical products (i.e., natural antioxidants) that act at determined points to block the progression of the disease [4, 5]. Resveratrol has been considered as a protector compound for the treatment of neurodegenerative diseases (i.e., AD, Parkinson disease, and amyotrophic lateral sclerosis) that have high levels of oxidative damage due to its antioxidant and anti-inflammatory properties [6]. Moreover, this compound can also modulate different molecular pathways dependent on silent information regulator-1 (SIRT1) in neurodegenerative diseases [6]. However, recent reviews also report other multipathways that are involved in the neuroprotective mechanisms of resveratrol such as inhibition of

nuclear factor-κappa B (NF-κB) expression and alteration in the signaling pathways of mitogen-activated protein kinases (P38-MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3K)/Akt, activation of autophagy, among others [7–10].

Interest in resveratrol has grown recently due to its beneficial effects in several neurological and autoimmune disorders [11, 12]. Resveratrol is a phytoalexin that mainly occurs in grapevine species (*Vitis* sp.) and other fruits, and attention has been drawn to it due to its versatile biological properties, including its antioxidant, anti-inflammatory, and neuroprotective activities [13–15]. In this sense, resveratrol could indirectly activate SIRT1 expression [16] and lead to neuroprotection in AD cases [17]. SIRT1 regulates the activity of several substrates, including p53 and peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) [18], which decrease the accumulation of β-amyloid (Aβ) and improve mitochondrial dysfunction [19].

Some studies have shown that resveratrol improves the impaired learning and memory in neurodegenerative disease and protects the memory decline in AD through its antioxidant activity [20]. Resveratrol is also effective at preventing blood-brain barrier (BBB) impairment and inhibiting Aβ1–42 from crossing the BBB and accumulating in the hippocampus [21, 22]. The hippocampus is a critical brain component for cognitive and memory functions, is a region that displays ongoing neurogenesis in adulthood, and is a very sensitive area in AD [23–25]. However, a significant reduction in hippocampal neurodegeneration was observed after intracerebroventricular injection of resveratrol in an animal model, which was associated with a decrease in SIRT1 acetylation [26, 27].

Karuppagounder et al. [28] showed that mice treated with resveratrol for 45 days had reduced Aβ toxicity. This suggests that the onset of neurodegeneration may be delayed by dietary chemopreventive agents (i.e., resveratrol) that protect against Aβ formation and oxidative stress [28]. Wang et al. [29] recently showed that resveratrol protected neurons against Aβ1–42-induced disruption of spatial learning, memory, and synaptic plasticity and rescued the reduction of SIRT1 expression in hippocampal rats. Thus, resveratrol is effective at reducing central nervous system (CNS) damage and decreasing the ischemia and toxicity induced by Aβ peptide, showing its potential therapeutic use in neurodegenerative diseases [30].

One of the major neuroprotective mechanisms of resveratrol is the activation of SIRT1 that is expressed in the adult mammalian brain, predominantly in neurons [31]. Activation of SIRT1 by resveratrol prevents Aβ-induced microglial death and contributes to improved cognitive function [32]. Although the major mechanisms of resveratrol are associated with the overexpression of SIRT1, its subsequent neuroprotective effect remains unknown. However, the overexpression of SIRT1 plays an important role in neuronal protection as it regulates reactive oxygen species (ROS), nitric oxide (NO), proinflammatory cytokine production, and Aβ expression in the brains of AD patients [33–36]. This review discusses the neuroprotective effects of resveratrol that are dependent on its action on SIRT1 and its implications in AD.

2. Resveratrol Plant Biosynthesis and Pharmacokinetics

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a polyphenol plant secondary metabolite that has a phytoalexin role in high plant species. This metabolite is commonly found in grapevines (*Vitis vinifera*), grape juice, and wine [37, 38]. Others food sources, including peanuts, pomegranate, spinach, and bananas, also contain high concentrations of resveratrol [39–43]. Table 1 shows the concentration of resveratrol in some food sources.

Resveratrol is synthesized in high plant species using the phenylpropanoid pathway under biotic and abiotic stress conditions (i.e., ultraviolet (UV) light radiation and tissue disruption) and in response to fungal infections (i.e., *V. vinifera* leaves infected by *Plasmopara viticola*) [44–46]. The biosynthesis of resveratrol begins with the generation of 4-coumaroyl-CoA units in the phenylpropanoid pathway [47]. At this point, stilbene synthase (STS) and chalcone synthase (CHS) enzymes promote the chain extension of 4-coumaroyl-CoA via the addition of three malonyl-CoA molecules to generate a polyketide compound (Figure 1). Despite both enzymes using the same substrate, STS possesses substantially more amino acids than CHS (the key enzyme in flavonoid biosynthesis), which explains the difference in the end products formed [48, 49].

The polyketide peptide suffers a fold that promotes the generation of aromatic rings in a Claisen-like reaction catalyzed by STS, which produces an unstable intermediate metabolite called stilbene-2-carboxylic acid [50, 51]. The final steps involve the stepwise reactions that promote the decarboxylation, dehydration, and enolization of stilbene-2-carboxylic acid to yield the resveratrol molecule [52]. Resveratrol can undergo other biochemical reactions to produce new stilbenes, including ε-viniferin, t-piceid, t-piceatannol, and t-pterostilbene [53].

Resveratrol is well absorbed but is quickly excreted, mainly by the urinary system [54]. Calliari et al. [55] reported that the pharmacokinetics of resveratrol have been studied in several organs and that its therapeutic effect is mainly dose dependent. After oral consumption, resveratrol is primarily metabolized by phase II enzymes, especially glucuronides and sulfatases, and absorbed in the small gut, predominantly in its glucuronidated form [12, 56]. In addition to the glucuronide metabolite, sulfated products of resveratrol are also commonly found in biological samples [57]; however, only trace amounts of free resveratrol can be detected in plasma [58]. In this regard, Sergides et al. [59] demonstrated higher plasma concentrations of glucuronidated (4083.9 ± 1704.4 ng/ml) and sulfated (1516.0 ± 639.0 ng/ml) resveratrol than its unmetabolized form (71.2 ± 42.4 ng/ml) following the consumption of a single resveratrol (500 mg) tablet in healthy volunteers. Resveratrol is mainly attained by dietary intake; however, there are some concerns regarding its low concentration in food sources and its poor oral bioavailability. This has highlighted the need for strategies that allow biologically active concentrations of resveratrol to reach its target tissues, including the brain [60]. In this regard,

Table 1: Resveratrol concentration in food sources.

Food source	Family	Resveratrol content	Reference
Banana peel (*Musa* sp.)	Musaceae	38.8 ± 0.1 mg/100 g	[41]
Caper bush (*Capparis spinosa*)	Capparidaceae	235.31 mg/100 g	[42]
Whole grapes (*V. vinifera*)	Vitaceae	8.4 ± 0.2 mg/100 g	[41]
White wine (*V. vinifera* cv. Chardonnay)	Vitaceae	0.04 ± 0.01 mg/l	[43]
Red wine (*V. vinifera* cv. Shiraz)	Vitaceae	0.53 ± 0.06 mg/l	[43]
Mulberry wine (*Morus rubra*)	Moraceae	145.31 ± 8.89 mg/l	[43]
Whole Mentha (*Mentha arvensis*)	Lamiaceae	9.4 ± 0.0 mg/100 g	[41]
Boiled peanuts (*Arachis hypogaea*)	Fabaceae	5.1 ± 2.8 μg/g	[40]
Peanut butter (*A. hypogaea*)	Fabaceae	0.3 ± 0.1 μg/g	[40]
Pomegranate pulp (*Punica granatum*)	Punicaceae	19.9 ± 0.2 mg/100 g	[41]
Whole spinach (*Spinacia oleracea*)	Amaranthaceae	19.3 ± 0.1 mg/100 g	[41]

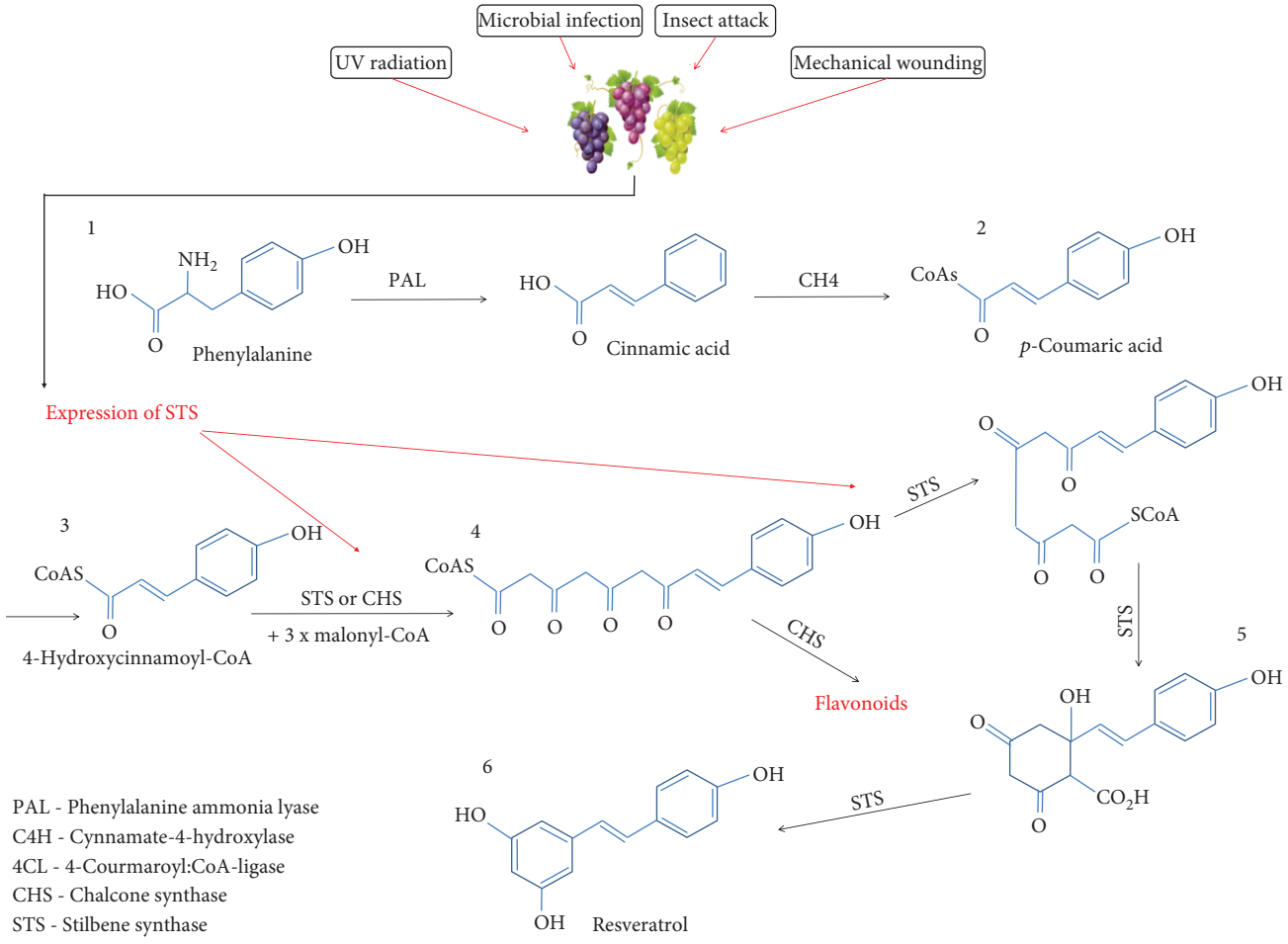

Figure 1: Resveratrol biosynthesis route in high plants.

Oliveira et al. [12] reported that the major problem of resveratrol treatment was its low bioavailability, with some human studies reporting that even high-dose resveratrol treatment (500 mg/day) produced low plasma concentrations (10–71.2 ng/ml) of this antioxidant.

The description of resveratrol concentrations in the brain is a challenge that remains to be overcome. Frozza et al. [61] reported that intravenous administration of resveratrol reached satisfactory target brain regions, while oral resveratrol treatment was not well absorbed and resulted in reduced

stability, increased photosensitivity, and accelerated metabolism, thus making it difficult to reach the brain. Turner et al. [62] showed that resveratrol and its metabolites crossed the human BBB, and these authors detected resveratrol in both the plasma and cerebrospinal fluid, thus showing its effects on the CNS. Preclinical data suggest that the main metabolite found in the rat brain after resveratrol consumption is resveratrol-3-glucuronic acid, which is also the main metabolite found in plasma [63]. To try to overcome the low oral bioavailability, several researchers focused on the microencapsulation technique or on the creation of prodrugs that, after metabolization, will give rise to resveratrol molecules [12, 64, 65]. Studies with new conjugated particles that improve the pharmacokinetics of resveratrol in the brain are of great importance, as the biologically active concentrations observed in *in vitro* experiments are much higher than those achieved after oral consumption are. Frozza et al. [61, 66] demonstrated that resveratrol nanoparticles reached the brain at higher concentrations than free resveratrol, resulting in increased bioavailability and possible neuroprotective effects. Resveratrol is considered a low-toxic substance, as humans have used several resveratrol-containing foods for a long time without related toxic effects. Data also confirm the safety of resveratrol on the basis of preclinical tests and clinical trials [67, 68].

Some studies have reported that resveratrol is an activator of SIRT1 [27, 69], although further evidence shows that resveratrol is not a direct activator of SIRT1 [70], and that its role may be related to the activation of substrates of SIRT1 [71]. The overexpression of SIRT1 results in neuroprotection in AD [17]. SIRT1 inhibits NF-κB signaling by decreasing Aβ-induced toxicity in primary mouse neuronal cultures [32]. SIRT1 may be capable of determining Aβ production by modulating β-secretase 1 expression through NF-κB signaling [32].

3. Role of SIRT1 in the Pathophysiology of AD

Oxidative stress and the overproduction of ROS are associated with the pathophysiology of neurodegenerative disorders, including AD, and lead to neural membrane injury and memory impairment [72–75]. Brain tissue is more susceptible to oxidative stress due to its high oxygen consumption rate, low regenerative capability, high polyunsaturated fatty acid content, and low concentration of antioxidants [76, 77]. ROS are major neurotoxic factors released by activated microglia and include superoxide radicals ($O_2^{\cdot}$), hydroxyl radicals ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2). These molecules are highly reactive, and their excessive production can induce lipid peroxidation, (deoxyribonucleic acid) DNA fragmentation, and protein oxidation and result in further cell dysfunction and cell death [78]. Therefore, mitochondria that are damaged during oxidative stress can produce ROS that damage proteins, nucleic acids, and polyunsaturated fatty acid membranes and cause lipid peroxidation, a loss of membrane integrity, and increased calcium (Ca^{2+}) permeability. ROS also increase the production of Aβ peptides, which induce oxidative stress both *in vitro* and *in vivo* [79]. Thus, a vicious cycle between ROS and Aβ accumulation may accelerate the progression of AD [80]. Studies *in vitro* and *in vivo* have shown that ROS increases Aβ production and induces oxidative stress, thus leading to neuronal apoptosis and accelerating the progression of AD [80–82].

AD is a progressive neurodegenerative disorder of the cortex and hippocampus that eventually leads to cognitive impairment. Although the etiology of AD remains unclear, multiple cellular changes have been implicated, including the production and accumulation of Aβ peptides, tau phosphorylation, oxidative stress, mitochondrial dysfunction, synaptic damage, and biometal dyshomeostasis. The neuroinflammatory response via microglial activation and acetylcholine deficits are also considered to play significant roles in the pathophysiology of AD [83, 84]. The main pathogenic event in AD is the cerebral aggregation of Aβ peptides [85]. Aβ is the major constituent of plaques and is generated from amyloid precursor protein (APP) by the action of β and γ-secretases [86]. The accumulation of Aβ could initiate a series of downstream neurotoxic events that result in neuronal dysfunction in AD patients [87, 88]. However, oxidative stress is also an important event in the pathogenesis of AD [89], as the generation and accumulation of ROS and reactive nitrogen species can accelerate fibrillization, increase the toxicity of Aβ, and promote neuronal death and neurodegeneration [90–93].

Decreased sirtuin levels, mainly SIRT1 expression levels, were recently correlated with elevated Aβ production and deposition in AD patients [94]. SIRT1 may regulate Aβ metabolism through the modulation of APP processing, and loss of SIRT1 is closely associated with exacerbated Aβ production [95]. However, SIRT1 overexpression decreases Aβ production [95, 96], which may represent an interesting therapeutic approach to block the neurodegeneration and cognitive impairments caused by the disease. SIRT1 is a member of a sirtuin family that utilizes nicotinamide (NAD^+) as a substrate to catalyze the deacetylation of various substrates [97]. SIRT1 plays an essential role in regulating cellular homeostasis by influencing neuron survival, insulin sensitivity, glucose metabolism, and mitochondrial biogenesis [98, 99]. In the adult brain, SIRT1 was shown to be essential for synaptic plasticity, cognitive functions [100], and the modulation of learning and memory function [101].

During normal aging, SIRT1 is responsible for the maintenance of neural systems and behavior, including the modulation of synaptic plasticity and memory processes [102]. The absence of SIRT1 expression in hippocampal neurons is correlated with impaired cognitive abilities, including immediate memory, classical conditioning, and spatial learning [100]. SIRT1 can also increase PGC-1α activity, which leads to the inhibition of Aβ production and improved mitochondrial dysfunction [19]. SIRT1 can also deacetylate a large number of other substrates, including p53, NF-κB, and Forkhead box O (FOXO), and prevent neuronal apoptosis [103, 104]. Therefore, the pharmacological activation of SIRT1 may represent a promising approach to preventing Aβ deposition and neurodegeneration in AD [105]. Thus inhibiting ROS production may be an important tool for protecting neuronal cells from oxidative damage and a therapeutic

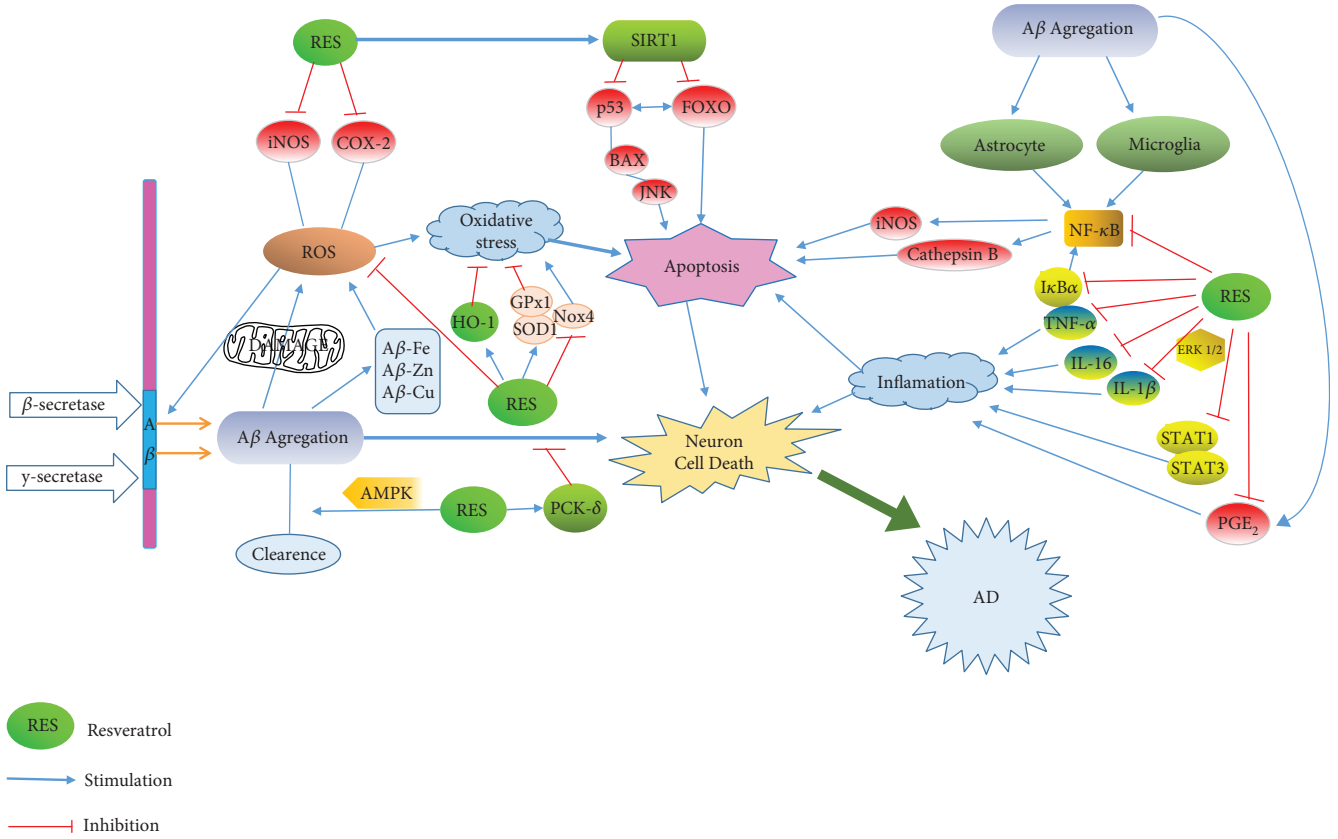

Figure 2: Main cellular routes proposed for the mechanisms of resveratrol in Alzheimer's disease. Modified from Ma et al. [72].

strategy in the treatment of neurological disorders [106]. Figure 2 summarizes the pathways by which resveratrol acts on SIRT1 in the pathology of Alzheimer's disease.

3.1. Antioxidant Mechanisms of Resveratrol in AD: Role of SIRT1. Oxidative stress induces neuronal damage, modulates intracellular signaling, and leads to neuronal death by apoptosis or necrosis. Therefore, antioxidant products (i.e., resveratrol) are used to protect against neuronal damage in neurodegenerative disorders (i.e., AD) [80]. The antioxidant properties of resveratrol were reported in several studies, which demonstrated that chronic resveratrol treatment reduced the production of malondialdehyde and nitrite and restored glutathione (GSH) levels [107, 108]. Additional antioxidant mechanisms of resveratrol were also described and include SIRT1 activation, Aβ aggregation and toxicity inhibition, metal chelation, and ROS scavenging [106, 108, 109]. These results demonstrate that this compound is an effective therapeutic strategy for AD therapy. Therefore, resveratrol not only plays a role in ROS protection but it can also modulate important glial functions, including glutamate uptake activity, GSH, improved functional recovery, and decreased DNA fragmentation and apoptosis [110–112].

3.1.1. In Vitro Studies. Resveratrol can dysregulate the metal ion balance (i.e., copper, zinc, and iron) and play a key role in neurodegeneration, which is related to cellular function changes and neuronal survival dysfunction [27]. These metal ions are able to bind Aβ and neurofibrillary tangles and promote their aggregation [106, 109], enhance the production of ROS, and contribute to AD pathogenesis. Hou et al. [113] demonstrated the interaction between resveratrol and SIRT1 using molecular dynamics simulation. The authors proposed that resveratrol was responsible for enhancing the binding affinity between SIRT1 and the substrate, thus functioning as a binding stabilizer. Nevertheless, Dasgupta and Milbrandt show that resveratrol is a potent activator of AMP-activated protein kinase (AMPK) function, and resveratrol-mediated AMPK activation was independent of SIRT1 [114]. In addition, in cell lines, resveratrol presented a decrease in the acetylation of PGC-1α, possibly due to the activation of AMPK [115]. Thus, showing a dose-dependent effect, resveratrol was able to activate AMPK independently of SIRT1 [116]. However, SIRT1 plays a key role in protecting neurons from the oxidative effects of ROS, NO, and Aβ peptides in the brains of AD subjects [117].

3.1.2. Animal Studies. One neuroprotective property attributed to resveratrol is the suppression of ROS formation through the inhibition of prooxidative genes (i.e., nicotinamide adenine dinucleotide phosphate oxidase) [118]. Huang et al. [119] showed that the neuroprotective activity of resveratrol included the suppression of inducible nitric oxide synthase (iNOS) production, which is involved in

Aβ-induced lipid peroxidation and heme oxygenase-1 downregulation, thereby protecting the rats from Aβ-induced neurotoxicity [120]. Moreover, resveratrol induced the expression of various antioxidant enzymes, such as superoxide dismutase (SOD), catalase, thioredoxin, and glutathione peroxidase (GPx) [121, 122]. However, Lee et al. [123] showed that resveratrol possesses chelator-metal ion properties to attenuate the metal imbalance and ROS production [124]. Furthermore, the oral administered of resveratrol in mice lowered the Aβ accumulation in the cortex due to the activation of AMPK signaling by enhancing cytosolic Ca^{2+} levels in neuronal cultures [120, 125].

Other studies also showed the neuroprotective action of resveratrol in animal models; for example, Simão et al. [126] evaluated the response to a 7-day resveratrol treatment (30 mg/kg) on postinduced ischemia in rodent models. Cerebral immunohistochemistry showed reduced activation of astrocytes and microglia in the hippocampus and suppression of the inflammatory response mediated by NF-κB, cyclooxygenase 2 (COX-2), and nitric oxide synthetase (NOS) in hippocampal cells, thus suggesting the anti-inflammatory potential of resveratrol in brain damage. Moreover, Wang et al. [127] suggested that resveratrol (200 mg/kg/day for 8 weeks) could act as an AD-adjuvant therapy after human umbilical cord stem cell transplantation. This occurred due to the increased expression of brain-derived neurotrophic factor precursor (BDNF), neuronal growth factor (NGF), and neurotrophin 3 (NT-3), which are associated with neurogenesis, survival, learning, and memory. Thus, resveratrol positively stimulated these cell-protected factors [128]. The overexpression of these neurotrophic factors is related to the ability of resveratrol to increase the activity of SIRT1 [13]. Similarly, resveratrol also induced an increase of SIRT1 in a mice model [129]. Another study also reported the preventive action of resveratrol in decrease the formation of insoluble Aβ plaques in the hippocampus of rats [21], as the etiology of the disease is associated with an imbalance in Aβ homeostasis. Resveratrol effectively reduced the cleavage activation of APP and promoted peptide clearance [10]; therefore, the authors suggested that resveratrol was efficient at reducing the formation of protein aggregates.

3.1.3. Human Studies. There are currently studies evaluating the effectiveness of resveratrol in AD; for example, a randomized double-blind placebo-controlled study evaluated the effects of resveratrol in 64 AD patients with a mild form of the disease. A resveratrol dose of 500–1000 mg was administered orally to these patients. However, the results demonstrate that resveratrol and its major metabolites able to cross the BBB and cause weight loss and reactions such as nausea and diarrhea. In addition, brain volume loss was greater in the group receiving resveratrol. Conversely, Imamura et al. [130] demonstrated the antioxidant effect of resveratrol on arterial stiffness in patients with type 2 diabetes mellitus (T2DM). In this randomized double-blind placebo-controlled clinical trial, 50 patients were selected: 25 received resveratrol (100 mg/day) and 25 received a placebo for 12 weeks. Supplementation with resveratrol improved several parameters in the T2DM patients and decreased oxidative stress, which was evaluated through metabolites of reactive oxygen. Mansur et al. [131] also conducted a study to evaluate the effects of resveratrol in humans. Slightly overweight elderly individuals were randomly divided into two groups: group one received 250 mg of resveratrol orally twice daily, while group two received a caloric restriction diet (1000 cal/day). SIRT1 concentrations were determined in both groups at the end of the 30-day treatment period. The serum concentration of SIRT1 was increased in both groups; however, this finding was not correlated with a better profile of metabolic markers for atherosclerotic processes.

3.2. SIRT1 and Anti-Inflammatory Mechanisms of Resveratrol. Neuroinflammation is an important contributor to the pathogenesis of AD [132]. Various reports show that inflammatory responses occur in the CNS, including the activation of microglia, astrocytes, lymphocytes, and macrophages that trigger numerous proinflammatory mediators and neurotransmitters [133]. However, the hallmark of brain neuroinflammation is microglia activation, which releases highly proinflammatory cytokines, ROS, and NO and leads to protein oxidation, lipid peroxidation, DNA fragmentation, neuronal inflammation, and cell death [78, 134]. Microglial cells are the resident macrophage-like population within the CNS and are a prime component of the brain immune system. In physiological conditions, microglia actively survey the microenvironment and ensure normal CNS activity by secreting neurotrophic factors (i.e., NGF). Although microglial activation plays an important role in the phagocytosis of dead cells in the CNS, overactivated microglia cause inflammatory responses that lead to neuronal and axonal degeneration and disruption of the immature BBB [135].

Inflammatory mediators such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and NO are produced by activated microglia and have recently been linked to the pathogenesis of neurological disorders [136]. Therefore, pharmacological interference with the overactivation of microglia may have a therapeutic benefit in the treatment of inflammation-mediated neurological disorders [137]. The activities of resveratrol against neuroinflammation appear to target activated microglia and result in the reduction of proinflammatory factors (i.e., TNF-α, IL-β, prostaglandin E2, cyclooxygenases, and iNOS through the modulation of signal transduction pathways) [138].

Gocmez et al. [139] showed that aging increased the levels of TNF-α and led to chronic neuroinflammation in the hippocampus and impaired spatial learning and memory. However, chronic administration of resveratrol reversed the cognitive deficits and inhibited the production of inflammatory cytokines. In addition, resveratrol also inhibited the activation of signal transducer and activator of transcription (STAT1 and STAT3) and prevented the proinflammatory effect of Aβ and Aβ-triggered microglial activation [140]. However, the role of resveratrol in microglia activation and the molecular mechanisms involved are not fully elucidated. The major pathway seems to involve SIRT1 activation, which promotes Th2 responses by increasing

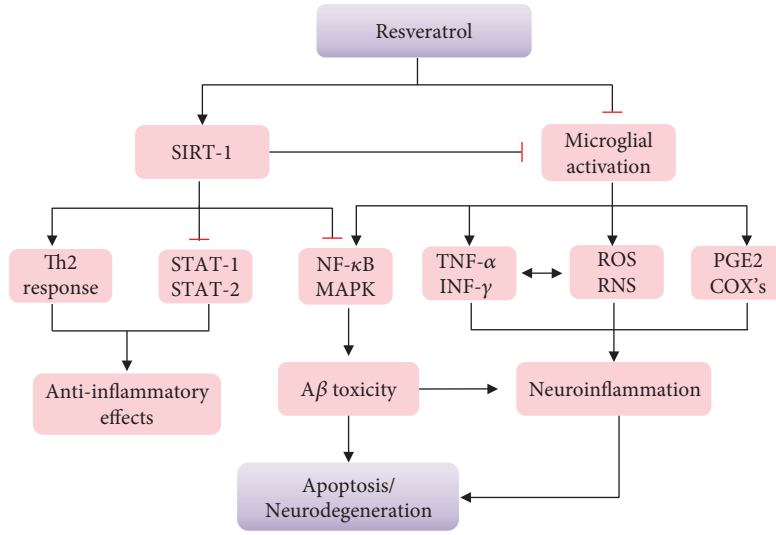

Figure 3: Anti-inflammatory effects of resveratrol and the role of SIRT1 in AD.

anti-inflammatory cytokine expression and upregulating PGC-1α (Figure 3) [141, 142].

3.2.1. In Vitro Studies. Resveratrol has numerous functions in neuroinflammation, as it induces mitophagy [143, 144]. Wang et al. [80] used a differentiated lineage of cell lymphomas from rat pheochromocytoma as a cellular model of AD treated with Aβ peptide Aβ1–42 (Aβ1–42). Resveratrol decreased the mitophagy-mediated mitochondrial damage and attenuated the oxidative stress caused by Aβ1–42 [141]. Neuroinflammation may also be related to the degradation of the BBB [145]. The BBB is constituted of structural and functional elements such as brain endothelial cells [146, 147]. Thus, Annabi et al. [145] demonstrated that human brain microvascular endothelial cells treated with a carcinogen can signal through NF-κB, allowing release of inflammatory markers such as matrix metalloproteinase 9 (MMP-9) and COX-2. However, resveratrol decreased secretion of MMP-9 and expression of COX-2 [145]. It also activated the expression of SIRT1, which regulated inflammation, inhibited NF-κB signaling, and prevented Aβ-induced degeneration [148].

3.2.2. Animal Studies. Several studies suggest that pharmacological activation of SIRT1 may represent a promising approach to prevent amyloid deposition and neurodegeneration in AD [99, 149]. The relationship between SIRT1 and AD is paramount, as a study of the SIRT1 serum concentration in healthy subjects and AD patients showed a reduced serum SIRT1 concentration that correlated with the increasing age of an individual. The decline was much more pronounced in patients with AD [93].

SIRT1 also exhibited therapeutic activity in a transgenic mouse model of AD [150]. Wang et al. [127] assessed an alternative therapy for AD that used mesenchymal stem cells derived from the umbilical cord combined with resveratrol in a mouse model of AD. Resveratrol also favored the formation of neurons and regulated SIRT1 expression in the hippocampus of AD rats [127]. Resveratrol has anti-inflammatory functions and can inhibit Aβ-induced NF-κB signaling in microglia and astrocytes [151]. Another study showed that mice overexpressing SIRT1 exhibited reduced brain inflammation (due to its action in tau phosphorylation) and reduced cognitive defects that were specific to the APP transgenic mouse [149, 150].

3.2.3. Human Studies. Some neurodegenerative diseases, such as AD, are associated with oxidative stress and neuroinflammation, and proteins that are closely related to this neurological disorder (i.e., AMPK, SIRT1, and PGC-1α) can be modulated by resveratrol [152]; however, there are few clinical studies on resveratrol in AD patients. Moussa et al. [153] reported that patients treated with resveratrol (1 g/day) for 52 weeks demonstrated reduced MMP-9 levels (an inflammatory marker related to AD) compared to a placebo group. In addition, patients treated with resveratrol had less cerebrospinal fluid decline, which resulted in less Aβ accumulation in the brain. Resveratrol probably strengthened the CNS, hampered the penetration of MMP-9, and reduced the activity of this inflammatory agent [154].

The anti-inflammatory effects of resveratrol are mediated, at least in part, by suppressing the activation of NF-κB, extracellular signal-regulated kinase-1 and kinase-2, and mitogen-activated protein kinase (MAPK) signaling pathways, which are all important upstream modulators of the production of proinflammatory mediators [137]. Resveratrol-mediated overexpression of SIRT1 markedly reduced NF-κB signaling and Aβ-mediated microglial activation and had strong neuroprotective effects [68, 155]. The polymerization of Aβ peptides was markedly inhibited by resveratrol, which stimulated the proteasomal degradation of Aβ peptides [30, 75].

Studies strongly suggest that resveratrol-induced SIRT1 inhibits NF-κB signaling in microglia and astrocytes and protects AD neurons against Aβ-induced toxicity. This

NF-κB signaling controls the expression of iNOS, which mediates apoptosis and neurodegeneration [32]. Resveratrol also effectively suppresses the apoptotic activities of both p53 and FOXO via SIRT1 overexpression and confers neuronal protection in AD [152, 156].

Therefore, the potential anti-inflammatory mechanisms for resveratrol-mediated neuroprotection involve (i) reduction of proinflammatory cytokine expression, (ii) suppression of MAPK signal transduction pathways, and (iii) activation of the SIRT1 pathway, which in turn suppresses the activation of the NF-κB signaling pathway and protects neurons against microglia-dependent Aβ toxicity [134].

In this context, the neuroprotective effects of resveratrol can involve the scavenging of ROS, decreased NO levels, improved antioxidant capacity, NF-κB inhibition, inhibition of inflammatory mediators, promotion of neuronal survival via SIRT1 activation [157, 158], the prevention of DNA lesions, and the prevention of lipid peroxidation in cell membranes [85]. Animal models also indicate that resveratrol improves the spatial memory by decreasing the accumulation of Aβ peptides and lipid peroxidation in the hippocampus, thus protecting against neuronal apoptosis [159].

Therefore, it is also important to emphasize that these neuroprotective effects can also be mediated by other action mechanisms of resveratrol. Another neuroprotective mechanisms of resveratrol include the following: (i) inhibits the tauopathy by interfering with the MID1-PP2A (midline 1-protein phosphatase 2A) complex or by altering or partially inhibiting of the glycogen synthase kinase 3 beta (GSK3β) and p53 interaction [6, 110]; (ii) improves learning and long-term memory formation through the microRNA (microribonucleic acid)-CREB (cAMP response element-binding protein)-BDNF pathway [20]; (iii) protects against Aβ-mediated neuronal impairment (inflammation and oxidative stress) by activation of AMP-activated protein kinase-(AMPK-) dependent signaling and inhibition of NF-κB expression and iNOS levels [160]; (iv) antioxidative activity by reduction in levels of ROS enhances the expression of various antioxidant defensive enzymes (heme oxygenase 1, catalase, glutathione peroxidase, and superoxide dismutase), downregulation of prooxidative stress proteins (i.e., plaque-induced glycogen synthase kinase-3β (GSK-3β), and AMPK [8, 10]; (v) improves cognitive impairment due to inhibition of cholinesterase activity [161]; (vi) inhibits the Aβ plaque synthesis by restoration of normal cellular autophagy via the TyrRS-PARP1 (auto-poly-ADP-ribosylation of poly (ADP-ribose) polymerase 1)-SIRT1 signaling pathway and enhancement of transthyretin (transporter protein) binding to Aβ oligomers [162]; (vii) inhibits mammalian target of rapamycin (mTOR) signaling and induces AMPK, thereby stimulating the clearance of Aβ aggregates [110]; (viii) prevents the neuronal cell death by attenuating apoptosis via Akt/p38 MAPK signaling and inhibits caspase-3 and B cell lymphoma-2 (Bcl-2)/Bcl-2-associated X protein signaling [163, 164]; (ix) increases intracellular calcium levels, promoting the activation of calcium/calmodulin-dependent protein kinase kinase β-CamKKβ-AMPK pathway, which alters mitochondrial function and leads to a decrease in ROS generation [165]; (x) attenuated injury and promoted proliferation of the neural stem cells, at least in part, by upregulating the expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), HO-1, and NAD(P)H:quinone oxidoreductase 1 (NQO1) [166]; and (xi) inhibits the neuronal electrical activity by mechanisms associated with large conductance of Ca^{2+} potassium channels and attenuates Aβ-induced early hippocampal neuron excitability impairment [167]. Therefore, resveratrol may be an important tool to protect neuronal cells from oxidative damage and a promising strategy in the treatment of AD.

4. Conclusions

Resveratrol is a potential compound for the treatment of AD due to its antioxidant and anti-inflammatory properties. The key neuroprotective mechanism of resveratrol in AD seems to be linked with SIRT1 activation. Although the mechanisms that link resveratrol to the overexpression of SIRT1 and neuroprotection are unknown, this expression may play an important role in neuronal protection from ROS, NF-κB signaling in activated microglia, prevent Aβ toxicity, and contribute to improved learning and memory function. Resveratrol can also effectively suppress the apoptotic activities of both p53 and FOXO via SIRT1 overexpression and confer neuronal protection in AD. Although this review focuses on the importance of SIRT1 activation for the neuroprotective role of resveratrol, it is also important to clarify that these mechanisms are still unclear and fully elucidated. In addition, resveratrol may act on CNS by inhibiting neuroinflammatory and prooxidant mechanisms by multiple action mechanisms that are independent of SIRT-1. These mechanisms are quite complex and involve stimulation or inhibition of multiple signaling pathways or alteration of potassium channels eading to inhibition of neuronal electrical activity. In summary, the major mechanisms that may be associated with the neuroprotective effect of resveratrol, in addition to SIRT1, include stimulation of regulation by microRNA-CREB-BDNF pathway, inhibition of mTOR and AMPK-dependent signaling pathways, inhibition of enzymes (cholinesterase activity), transcription factor (NF-κB) and apoptotic pathways, and stimulation of cellular autophagy and expression of Nrf2, HO-1, NQO1, among others. Therefore, we critically analyze and suggest that SIRT1 is one of the main mechanisms related to the beneficial effects of resveratrol; however, this compound can change multiple pathways simultaneously, and then, there is a need for crosstalk between signaling and regulatory functions to provide improvements in the development and progression of AD. In addition, caution is required in therapies with natural products, since intrinsic aspects of the patient, environmental factors, and characteristics of the compound studied are important for efficacy and therapeutic success.

Despite the neuroprotective potential of resveratrol demonstrated in several in vitro studies, the major limitation currently facing is the lack of information from clinical studies that correlates the SIRT1 activation and the inflammatory and oxidative status reduction associated with improvement in the development and progression

of AD. Overall, evidence from clinical trials is weak and largely inconclusive. Most human studies establish a link between consumption of foods rich in resveratrol and reducing the incidence or prevalence of AD, as well as improvement in learning, memory, visual and spatial orientation, and social behavior. However, these observed effects may be the result of complex direct and indirect interactions of the various constituents present in the diet, not only of resveratrol. In addition, other difficulties in clinical trials are the following: (i) the studies are mainly conducted with volunteers, not reflecting the target population, (ii) the participants' age is quite broad between 18 and over 80 years of age, and (iii) sample size is rarely calculated and the slow progression of AD is not investigated because it requires longer clinical time in the trials. Another important issue is the poor bioavailability of resveratrol, which makes it difficult to link with the optimal concentrations achieved in in vitro experiments. Although preclinical studies also indicate that resveratrol is able to cross the blood-brain barrier, low concentrations of this molecule have been detected in the brain, and only higher concentrations of resveratrol and its metabolites have been found in the blood. In addition, it is emphasized that the neuroprotective effects of resveratrol are mainly short term, varying according to dose, dosage form, duration of treatment, pharmacokinetic and pharmacogenetic parameters, food and drug interactions, among others. Thus, we conclude that, to date, evidence based on clinical studies is still insufficient, contradictory, and inconclusive, so we recommend that further clinical trials be conducted to substantiate the neuroprotective effects of resveratrol and its likely mechanisms of action in the body. However, we emphasize that resveratrol is promising in health promotion, not only for its antioxidant activities but also for its anti-inflammatory and neuroprotective properties. Thereby, further studies assessing other routes of administration or pharmaceutical formulations (i.e., nanoencapsulation) are required to improve the tissue-targeting concentration and allow resveratrol to exert its biological activities in AD.

Conflicts of Interest

The authors declare no conflict of interests.

Authors' Contributions

All authors participated in the design of the study and drafted the manuscript.

Acknowledgments

The authors were supported by the Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Amazônia Paraense de Amparo à Pesquisa (FAPESPA), and Federal University of Pará, and MCM thanks the fellowship from CNPq.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 1972714, 16 pages
https://doi.org/10.1155/2018/1972714

Review Article

Connection between Systemic Inflammation and Neuroinflammation Underlies Neuroprotective Mechanism of Several Phytochemicals in Neurodegenerative Diseases

Jintang Wang,[1] Yuetao Song,[1] Zheng Chen,[1] and Sean X. Leng[2]

[1]*Institute for Geriatrics and Rehabilitation, Beijing Geriatric Hospital, Beijing 100095, China*
[2]*Division of Geriatric Medicine and Gerontology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA*

Correspondence should be addressed to Sean X. Leng; sleng1@jhmi.edu

Received 15 April 2018; Revised 5 September 2018; Accepted 10 September 2018; Published 8 October 2018

Guest Editor: Daria Maria Monti

Oxidative damage, mitochondrial dysfunction, and neuroinflammation are strongly implicated in the pathogenesis of neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD), and a substantial portion of elderly population at risk of these diseases requires nutritional intervention to benefit health due to lack of clinically relevant drugs. To this end, anti-inflammatory mechanisms of several phytochemicals such as curcumin, resveratrol, propolis, polyunsaturated fatty acids (PUFAs), and ginsenosides have been extensively studied. However, correlation of the phytochemicals with neuroinflammation or brain nutrition is not fully considered, especially in their therapeutic mechanism for neuronal damage or dysfunction. In this article, we review the advance in antioxidative and anti-inflammatory effects of phytochemicals and discuss the potential communication with brain microenvironment by improved gastrointestinal function, enhanced systemic immunity, and neuroprotective outcomes. These data show that phytochemicals may modulate and suppress neuroinflammation of the brain by several approaches: (1) reducing systemic inflammation and infiltration via the blood-brain barrier (BBB), (2) direct permeation into the brain parenchyma leading to neuroprotection, (3) enhancing integrity of disrupted BBB, and (4) vagal reflex-mediated nutrition and protection by gastrointestinal function signaling to the brain. Therefore, many phytochemicals have multiple potential neuroprotective approaches contributing to therapeutic benefit for pathogenesis of neurodegenerative diseases, and development of strategies for preventing these diseases represents a considerable public health concern and socioeconomic burden.

1. Introduction

With rapid population aging, advanced age is a major risk factor leading to an increased prevalence of neurodegenerative diseases including Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and multiple sclerosis (MS). The common characteristic of these diseases is progressive neuronal loss and impaired neuronal function, and one of their critical backstage manipulators is extracellular neurotoxic microenvironment linked to oxidative stress, chronic inflammation, and mitochondrial dysfunctions [1]. These three situations not only possess their own signaling mechanism in neuronal loss, detrimental or beneficial, but also interact or crosstalk to promote neurodegenerative damage in a manner of vicious circle. Therefore, a better understanding of this pathological mechanism will help to develop therapeutic strategies for preventing or delaying disease processes. Currently, application of natural products in the prevention of these diseases is comparatively a new area, and supplementation of dietary phytochemicals is proven to be a promising nutritional intervention approach due to their neuroprotective properties such as antioxidation and anti-inflammation. Research shows that a wide variety of dietary phytochemicals, such as myricitrin, mulberry, and green tea, are endowed with antioxidative and anti-inflammatory features, and individual

dietary habit determines availability of phytochemical types for health and therapeutic purposes, especially for the age-related diseases. The phytochemicals can consequentially increase overall physical quality and reduce neurodegenerative pathologies through at least three therapeutic attributions: gastrointestinal function improvement, immunity enhancement, and neuroprotective outcomes [2]. These attributions act independently or crosstalk to influence neural cell activities through immune network or neural network of peripheral and central nervous system (CNS), including vagal reflex pathway responsible for gastrointestinal signaling to the brain [3], inflammatory infiltration pathway through the blood-brain barrier (BBB) and gateway reflex [4], and direct neuroprotective regulations on glial cells and neurons within the brain [5–7].

Traditionally, parenchymal cells (i.e., neurons, astrocytes, microglia, and oligodendrocytes) of the CNS are separated from the rest of body by BBB to form an immune-privileged organ, and peripheral immune cells and nutrients such as resveratrol and curcumin are restricted into the brain. However, in recent years, substantial evidence shows that the brain itself is fully immune competent due to the participation of microglia and astrocytes in immune response [2]. Especially under pathological stimulation, peripheral immune cells such as monocytes and T and B lymphocytes can readily infiltrate into the brain through disrupted BBB or gateway reflex [8] and activate innate and adaptive immunity [2]. In addition, inflammatory reflex (i.e., vagal reflex or gut-brain axis) is another connection approach, by which systemic inflammation occurring in gastrointestinal tract or peripheral immune system can be sensed to form peripheral inflammatory signals that transmit into the brain to exacerbate chronic neurodegeneration [3, 7]. Likewise, dietary intake of these phytochemicals can form nutritional signals to reverse neuroinflammatory microenvironment by vagal reflex [4, 9]. As nutritional neuroscience is quickly growing, phytochemicals or nutraceuticals are proven to be important regulators of brain health and diseases, which can decrease production of proinflammatory cytokines and oxidative damage [10–12], and exert significant neuroprotective effect in neurodegenerative diseases [2]. Therefore, dietary nutritional intervention is beneficial for the elderly to remain both physically and cognitively healthy into older age or to prevent from neurodegenerative diseases, which represents a considerable public health concern and a potential solution to socioeconomic burden associated with rapid population aging.

In this article, based on the availability in daily life and reported evidence, we chose five phytochemicals, i.e., curcumin, resveratrol, propolis, polyunsaturated fatty acids (PUFAs), and ginsenosides, as representatives of various phytochemicals and discuss their neuroprotective mechanism and therapeutic implication for neurodegenerative diseases, including their anti-inflammatory and antioxidative effects on gastrointestinal dysfunction, peripheral immune system, and brain innate immunity, as well as potential communication of their nutritional signals between the brain and the periphery. The data about phytochemicals curcumin, resveratrol, propolis, PUFAs, and ginsenosides are collected from PubMed database and addressed in terms of antioxidative or anti-inflammatory mechanism and nutritional or protective effects on the gastrointestinal tract, systemic immunity, and neuroimmunity.

2. Oxidative and Inflammatory Mechanisms Underlying Neurodegenerative Diseases

Oxidative stress associated with mitochondrial dysfunction and neuroinflammation is a common characteristic of neurodegenerative diseases, mainly due to metabolic features of the CNS: high oxygen consumption even under basal conditions and high production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) from specific neurochemical reactions, as well as increased metabolites such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) with aging [13, 14]. These three situations can interact with a causal relationship, as shown in Figure 1, to form a persistent stimulation to neuronal apoptosis and glial cell neurotoxicity, leading to neuronal dysfunction and damage or progressive loss [15].

Oxidative stress is defined as an imbalance between production of oxidants such as ROS and ability to detoxify reactive oxygen intermediates, causing cellular damage by free radicals. Normally, the brain function is highly sensitive to oxygen metabolic activity or production of ROS such as hydrogen peroxide (H_2O_2), hydroxyl free radical (•OH), superoxide anion ($O2^{-\bullet}$), and peroxynitrite (ONO_2^-), and approximately 98% of ROS is formed in mitochondria as by-products of cellular respiration [15]. Pathologically, various harmful factors, such as environmental factors (e.g., chemicals, UV light, and infectious organisms) and endogenous factors (e.g., dysfunctional mitochondria, abnormal enzyme activity, and aging factors), are accumulated to result in an imbalance between prooxidative and antioxidative reactions and subsequent oxidative damage (e.g., genetic mutations or epigenetic changes) to biomolecules (e.g., lipids, proteins, and DNA). These molecule alterations are proven to be the initiators of apoptotic mechanism linked to neuronal degeneration, involving apoptotic signaling pathway such as p53, caspase 3, caspase 9, Bcl2/Bax, Nrf2, and hemeoxygenase-1 (HO-1) and PI3K/Akt signaling pathway [16]. On the other hand, these molecules are also the activators of microglia and astrocytes to promote innate immunity and release of various proinflammatory cytokines such as tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1β, interferon- (IFN-) γ, and IL-6 and form a neurotoxic microenvironment [7].

Chronic inflammation, another aging contributor, plays a critical role in neurodegenerative pathogenesis from initiation and progression to outcome of diseases, as a consequence of persistent stimuli of chronic stress antigens such as PAMPs, DAMPs, and senescence-associated secretory phenotype (SASP) [7, 17]. In the inflammatory process, these chronic stress factors stimulate microglia (resident immune cells) and astrocytes to activate canonical inflammatory pathway such as toll-like receptors (TLRs), nuclear factor- (NF-) κB, and inflammatory cytokines IL-6, TNF-α, IL-1β, and IL-10 [18]. On the other hand, activated microglia is also

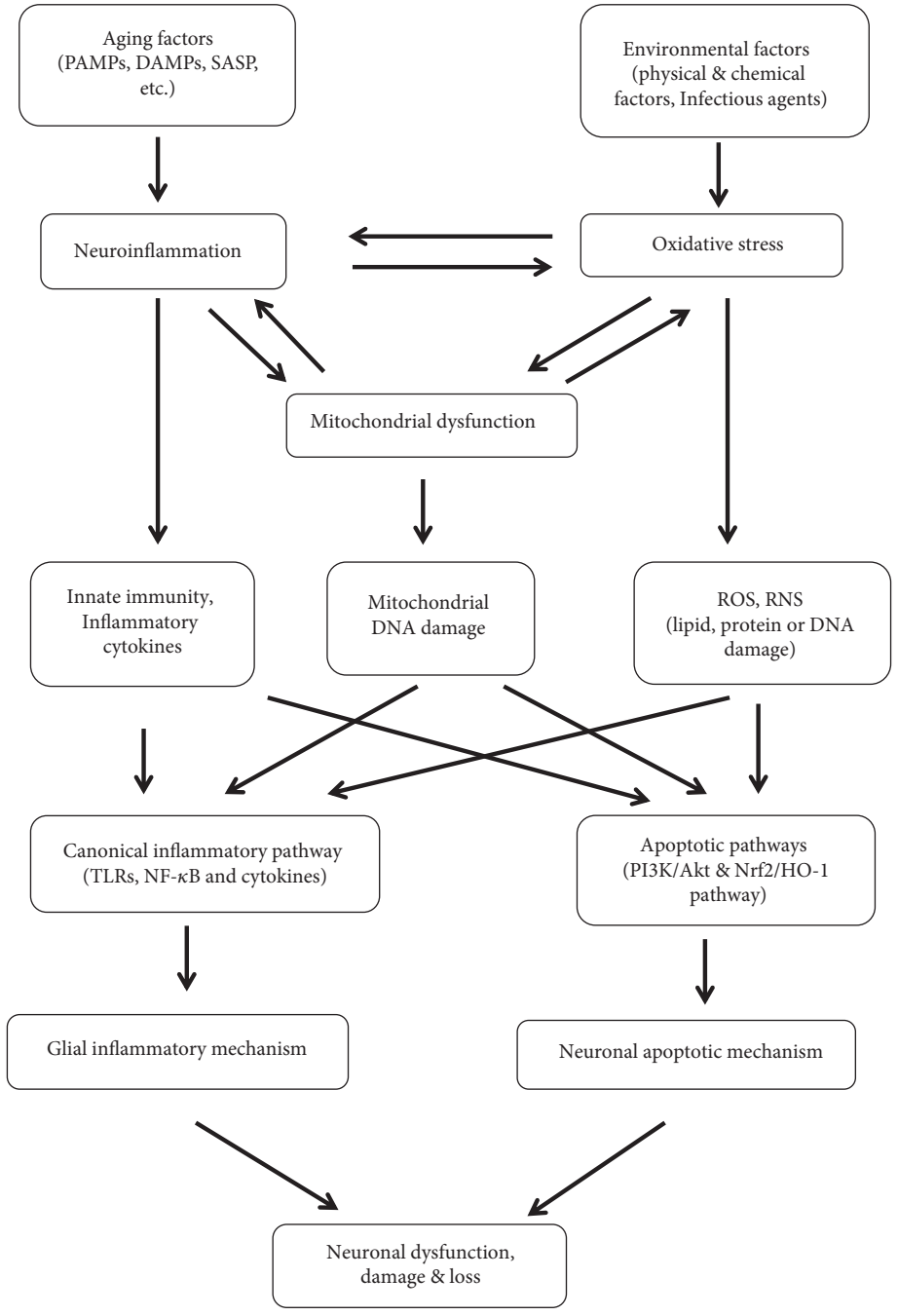

Figure 1: Correlation between oxidative stress, mitochondrial dysfunction, and neuroinflammation. Various aging factors and environmental factors stimulate glial cells to induce inflammatory response, oxidative stress, and mitochondrial dysfunction, which orchestrate to impact on neuronal apoptotic mechanism and glial inflammatory mechanism, leading to neuronal dysfunction or loss in neurodegenerative diseases.

an abundant source of free radicals in the brain to release excessive harmful ROS and RNS, etc., which in turn stimulate glial activation and innate immunity. Therefore, oxidative stress and neuroinflammation are two concomitant processes in aging and neurodegenerative diseases.

Mitochondrial dysfunction is a major source of ROS due to high energy demand and high dependence of brain activities on efficient mitochondria. It is readily affected by various environmental factors to occur early as a primary event in the aging process (e.g., mitochondrial DNA damage) and then is potentiated by microglia-mediated oxidative stress and neuroinflammation to fuel the pathogenesis of neurodegenerative disorders [14, 19]. For example, persistent oxidative stress and hypoperfusion in the brain can stimulate expression of nitric oxide synthase (NOS) to further drive formation of ROS and RNS and collectively contribute to BBB dysfunction and damage to brain parenchymal cells, and contrarily suppression of microglial

activation can produce neuronal cell survival [1]. Taken together, oxidative stress, neuroinflammation, and mitochondrial dysfunction are orchestrated to form a neurotoxic microenvironment responsible for neuronal damage, and the underlying neuronal apoptotic mechanism and glial inflammatory mechanism will provide potential therapeutic targets for nutrients and phytochemicals to reverse pathogenesis of neurodegenerative diseases.

3. Gastrointestinal Health by Phytochemicals and Its Connection with Brain Innate Immunity via Inflammatory Reflex

Gastrointestinal function acts on gut microbiota, mucus integrity, gut immunity, and intramural neural plexus, and these functional components are readily influenced or damaged by gut inflammation and oxidative stress linked to various adverse factors. Especially, the gut damage, as a peripheral inflammatory stimulation signal, can be sensed by the brain through vagal reflex and exacerbate brain inflammatory response in the onset and progression of neurodegenerative diseases [7, 20]. Likewise, the improved gut function by phytochemicals is also sensed by vagal reflex to ameliorate neuroinflammatory response occurring in the brain. Therefore, neuroinflammation or neuroprotection may initiate from the periphery, and peripheral conditions powerfully influence various brain pathologies through vagal reflex or disrupted BBB [21].

Inflammatory reflex or vagal reflex, a bidirectional neuroimmune communication pathway between the gut and brain, also called gut-brain axis, consists of an afferent arm that senses peripheral inflammation and an efferent arm that sends out the signals integrated in the brain to inhibit gut inflammation and innate immune response. In detail, afferent signals in the vagal nerve, while reaching the solitary tract nucleus and brainstem, activate central neurons that project to the hypothalamus and other CNS nuclei responsible for inflammatory response control [22]. Namely, the brain perceives peripheral inflammation such as gut condition or gut microbiota dysbiosis through vagal afferent. For example, increased cytokine levels in the periphery and exogenous administration of proinflammatory cytokines such as IL-1β, IL-6, and TNFs can elicit sickness behavior of the brain, indicating that vagal afferent inflammatory signals mediate inflammatory response and relevant receptor activation within the brain [7]. Then, the integrated signals in the brain send a response to suppress immune system and regulate gastrointestinal function through vagal efferent [23, 24]. In other words, gut nutritional or microbial stimuli such as dietary intake of phytochemicals can activate the vagal nerve and send nutritional signals or nature of gut function to the brain to change neurochemistry of the brain and its behavior [25, 26]. Therefore, understanding reversal of gut inflammation and dysfunction by dietary phytochemicals and concurrent transmission alteration of these signals in vagal neural reflex may have an important implication for developing anti-inflammatory intervention and microbe- or nutrition-based therapeutic strategies for neurodegenerative diseases.

3.1. Curcumin. Curcumin is an oil-soluble polyphenolic phytochemical from *Curcuma longa* or turmeric and has an inhibitory effect on gut inflammation and gut permeability as evidenced in several preclinical and clinical studies. For example, curcumin protects gut function and metabolism by reducing chaperone BiP expression to modulate endoplasmic reticulum (ER) stress and inflammatory response in human intestinal epithelial cell lines, T84 and Caco-2 [27], or by increasing signals of neurotransmitters such as brain-derived neurotrophic factor (BDNF), serotonin, and cAMP response element binding (CREB) in the hippocampus and peripheral intestinal system [28]. In animal models, curcumin inhibits visceral nociception via antagonizing transient receptor potential vanilloid-1 (TRPV1) receptor, indicating a treatment implication for functional gastrointestinal disease [29]. In mice with TNF-α-induced ulcerative colitis, curcumin suppresses neutrophil priming and inducible NOS to counteract oxidative bowel damages from imbalanced gut immune response [30]. Meanwhile in the intestine, curcumin modulates dendritic cells to express aldehyde dehydrogenase 1a and IL-10 and induces differentiation of naive CD4+ T cells into regulatory T cells (Treg) to inhibit antigen-specific T-cell activation *in vitro* and reduce colitis *in vivo* [31].

3.2. Resveratrol. Resveratrol, a nonflavonoid plant polyphenol mainly found in red grapes and wine, possesses an anti-inflammatory effect to benefit gut health as evidenced in various inflammation models. In H_2O_2-induced Caco-2 cells, resveratrol increases epithelial expression of occludin and zonula occluden to protect gut barrier function and reduces intracellular ROS accumulation, along with increased expression of superoxide dismutase (SOD) and HO-1, to prevent oxidative damage [32]. In mice with ileitis, resveratrol downregulates T helper immune responses and increases Treg numbers and intestinal epithelial cell proliferation to maintain gut barrier function and prevent bacterial translocation [33]. In rats with colitis, resveratrol increases lactobacilli and bifidobacteria and inhibits enterobacteria to improve colonic mucosa architecture and reduce levels of colonic mucosa prostaglandin E2 (PGE-2), cycloxygenase-2 (Cox-2), PGE synthase, nitric oxide (NO), and systemic inflammation markers, indicating that it is a gut beneficial compound and exerts antioxidative and anti-inflammatory effect [34].

3.3. Propolis. Propolis or bee glue is a resinous substance that bees collect from various living plants for construction and adaptation of their nests, consisting of three major components: flavonoids, phenolic compounds, and caffeic acid phenethyl ester (CAPE). As a functional food, it has a range of biological activities such as anti-inflammatory, antibiotic, antioxidative, anticancer, antifungal, anesthetic, and cytostatic effects [35]. In Caco-2 cells and in rats, propolis protects intestinal barrier function by increasing expression of tight junction loci occludin and zonula occluden and activating AMP-activated protein kinase and extracellular regulated protein kinase signaling [36]. In intestinal epithelial cells, CAPE, as an active constituent of honeybee propolis, inhibits nuclear factor- (NF-) κB signaling and TNF-induced and IFN-induced protein- (IP-) 10 expression, independently

of p38 mitogen-activated protein kinase (MAPK) and HO-1 signaling pathways, revealing its anti-inflammatory effect [37, 38].

3.4. PUFAs. PUFAs, a group of unsaturated fatty acids enriched in vegetable and fish oil, functionally act as important signaling molecules regulating diverse physiological processes. They are divided into two families: omega- (ω-) 3 fatty acids such as alpha-linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) and ω-6 fatty acids such as linoleic acid and arachidonic acid (AA). ω-3 PUFAs, as an anti-inflammatory agent, can decrease colonic damage and inflammation and exert a beneficial protective effect in gut dysfunction. For example, in inflammatory bowel disease, ω-3 PUFAs significantly inhibit expression or production of NF-κB, Cox-2, PGE-2, and leukotriene B4; induce expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) in the colon; revert gut microbiota composition; and increase production of anti-inflammatory compounds like short-chain fatty acids, indicating that they are a potent anti-inflammatory agent and can maintain intestinal wall integrity and interaction with host immune cells [39, 40]. PUFAs can regulate gastrointestinal function by activating transient receptor potential ankyrin 1 (TRPA1), which is a cation channel in sensory neurons and gut tissues to function as a sensor of PUFAs to excite enteroendocrine cells and primary sensory neurons [41]. In the rat intestine, ω-3 PUFAs inhibit IL-15 expression or reduce IL-4-mediated permeability of intestinal mucosa to decrease proportion of TCR$\alpha\beta$+CD8α+CD8β cells and expression of TNF-α, IFN-γ, IL-4, and IL-10, indicating a protective role in gut immune barrier function or gut barrier integrity [42, 43]. In addition, ω-3 PUFAs (EPA and DHA) in fish oil have been proven to inhibit AA metabolism into inflammatory eicosanoids, displaying their anti-inflammatory profile or less inflammatory attribution, and ω-6 PUFA AA is, however, known as precursor of inflammatory eicosanoids like PGE-2 and leukotriene B-4 to benefit inflammatory process [44].

3.5. Ginsenosides. Ginsenosides or panaxosides, a class of natural steroid glycosides and triterpene saponins in the plant *Panax ginseng*, are broadly divided into two families based on carbon skeletons: four-ring dammarane (major component of ginsenosides), including protopanaxadiols (Rb1, Rb2, Rg3, Rh2, and Rh3) and protopanaxatriols (Rg1, Rg2, and Rh1), and oleanane. These ginsenosides have various biological effects such as anti-inflammation, antistress, anxiolytic, and antitumor, as well as protective effect on gastrointestinal dysfunction and gut barrier function. In rats with postoperative ileus, expression of TNF-α, IL-1β, IL-6, and IL-10 is reduced by Rb1 in ileum tissue, along with gastrointestinal transit increased, indicating a potent anti-inflammatory effect [45]. In jejunal stimulation, ginsenosides display a bidirectional regulation: jejunal contractility is increased in low contractile states caused by cholinergic activation, whereas the contractility is decreased in high contractile states caused by adrenergic activation and NO [46]. Ginsenoside compound K from Rb1,2 enhances glucose uptake of intestinal epithelial cells by upregulating cAMP response element-binding protein (CREB) and glucose transporter 1, as well as epidermal growth factor receptor, indicating its functional regulation and anti-inflammatory effect in gut [47]. In mice with colitis, their oral administration inhibits expression of TNF-α, IL-1β, and IL-17 and restores Th17/Treg imbalance, indicating their anti-inflammatory effect in inflammatory gut diseases [48].

Taken together, the phytochemicals can counteract inflammatory and oxidative stresses by their intrinsic ability to scavenge free radicals and maintain homeostasis of gut microbiota, gut barrier integrity, and immune barrier function. The improved gastrointestinal function can activate vagal neural reflex or gut-brain axis or microbiome-gut-brain axis by gut nutritional signals to change brain neurochemistry and behavior and to reverse neuroinflammatory pathogenesis in the brain [9, 25, 26]. Therefore, the gut nutritional mechanism of phytochemicals may have an important implication for developing microbe- or nutrition-based therapeutic strategies for neurodegenerative diseases [23].

4. Systemic Immunity Regulation by Phytochemicals and Its Connection with Brain Innate Immunity across the Blood-Brain Barrier

Immunosenescence or age-based immunity decline is a gradual deterioration of the immune system with natural age advancement, involving decline in production of new naive lymphocytes and functional competence of memory cell populations. This phenomenon entails increased risk and severity of diseases such as infections, chronic inflammation, autoimmunity, and cancer, especially obscure presentation of nonspecific signs and symptoms, leading to increase in prevalence of neurodegenerative diseases [49]. To acquire a therapeutic procedure, dietary intake of phytochemicals is increasingly used to enhance systemic immunity function and reverse intrinsic inflammatory pathologies in the diseased brain. The underlying mechanism is attributed to the immune system-brain communication, involving mononuclear phagocyte/immune factor infiltration into the brain or inflammatory factor release by microglia/astrocytes, i.e., a crosstalk between systemic inflammation and brain inflammatory microenvironment [49]. This communication process contains at least three approaches: (1) vagal afferent, i.e., sensory arm of inflammatory reflex; (2) disrupted BBB, or gateway reflex-compromised BBB [4, 8], or circumventricular organs, responsible for inflammatory infiltration into the brain parenchyma to activate microglia and astrocytes, or vice versa [5, 6, 21]; and (3) healthy BBB-lacking choroid plexus, allowing systemic inflammatory mediators or microbial products to directly interact with brain endothelium or microglia and exaggerate brain inflammation and sickness behaviors in neurodegenerative diseases [50].

4.1. Curcumin. Curcumin, an antioxidative and anti-inflammatory agent, can enhance systemic immunity by influencing expression or release of proinflammatory

cytokines, ROS/RNS, TLRs, β-amyloid (Aβ), etc. Meta-analysis shows that curcumin supplementation significantly reduces concentration of circulating IL-6, a key regulator of the immune system, with more evident effect in patients with higher degree of systemic inflammation [51], indicating that curcumin can effectively inhibit inflammation propagation. In human dendritic cells (DCs), curcumin treatment limits DC maturation, reduces proinflammatory cytokine production, and prevents induction of allospecific T cell responses [52]. In the mononuclear cells from AD patients, curcuminoid compound reverses defective phagocytosis of Aβ and expression depression of TLRs such as TLRs 2–4, reflecting its ability to correct immune defects [53, 54]. Curcumin significantly decreases elevated production of IFN-γ and IL-17, upregulates secretion of IL-10 and PPAR-γ, and increases CD4(+)CD25(±) Foxp3(+) Treg cells in the CNS and lymphoid organs, showing its obvious immune enhancement effect [55]. In addition, curcumin inhibits intracellular inflammatory pathways to control lipopolysaccharide- (LPS-) induced expression of IL-6, TNF-α, PGE2, and Cox-2 and reverse inhibited expression of suppressor of cytokine signaling- (SOCS-) 1 and 3 and p38 MAPK in macrophages, showing its anti-inflammatory properties in chronic inflammatory diseases [56]. In patients with migraine, combination of ω-3 fatty acids and nanocurcumin significantly downregulates TNF-α mRNA expression, along with a greater decrease in the serum level than in the gene level, but no significant differences were observed in both single groups [57]. Taken together, curcumin is an effective immune enhancer to modulate systemic inflammation and brain pathologies through multiple communication mechanisms and is hopefully a particularly promising natural agent in fighting the ravages of aging and neurodegenerative diseases.

4.2. Resveratrol. Resveratrol, a well-known anti-inflammatory, antioxidant, immunomodulatory, and anticarcinogenic agent, can promote immune surveillance and reduce immunosenescence in rodents and humans. Evidence shows that resveratrol modulates transcription factors AP-1, NF-κB, and gene Cox-2 to reduce secretion of proinflammatory cytokines (e.g., IL-6, IL-8, and TNF-α) and expression of adhesion proteins (e.g., intercellular adhesion molecule-1, ICAM-1) and leukocyte chemoattractants (e.g., monocyte chemotactic protein-1, MCP-1), whereas it increases production of anti-inflammatory cytokines such as IL-10 [58]. In human macrophages, *trans*- and *cis*-resveratrol can inhibit production and secretion of IL-1β, purinergic receptor (P2X-7R), ER stress marker (Glc-regulated protein 78), ROS, Cox-2, and PGE-2 [59]. In peripheral blood mononuclear cells (PBMCs) stimulated with LPS, resveratrol significantly inhibits production of cytokines (TNF-α, IL-1α, IL-1β, IFN-γ, IL-10, and IL-1Ra), chemokines (C-C motif ligand 2 (CCL2), CCL5) and differentiation factors such as colony-stimulating factors (CSFs), indicating its potent anti-inflammatory, antioxidant, and immunomodulatory properties [60].

4.3. Propolis. Propolis has a wide spectrum of pharmacological activities such as anti-inflammation and antioxidation in systemic immunity, which are principally attributed to presence of flavonoids, phenolic compounds, and CAPE [61]. Studies show that propolis increases phagocytosis and expression of IL-1β, IL-6, TLR2, and TLR4 in peritoneal macrophages and cytotoxicity of natural killer (NK) cells, reflecting that it potentiates cellular immunity and activates initial steps of immune response [62–64]. Brazilian red propolis downregulates TLR2 and TLR4 signaling and attenuates production of proinflammatory mediators IL-12, GM-CSF, IFN-γ, and IL-1β in LPS-induced macrophages, along with slight upregulation of TNF-α and IL-6 and decrease of IL-4, IL-10, and transforming growth factor- (TGF-) β, indicating its anti-inflammatory role [65]. CAPE, as an active component of propolis, can decrease plasma concentration of proinflammatory cytokines (e.g., TNF-α, IL-1α, IL-1β, IL-6, IL-4, and ICAM-1), increase anti-inflammatory cytokines (e.g., IL-10) in LPS-induced systemic inflammatory response [66], and inhibit NO production, MAPK, and NF-κB signaling in mast cells and macrophages [67, 68]. In a word, comprehension of relationship between propolis and immune system has progressed in recent years, but its applicability to human health and action mechanism are not completely understood.

4.4. PUFAs. PUFAs, a group of immunomodulatory agents, function differently based on their families and cell contexts. In ω-3 PUFAs, EPA and DHA, as well as their bioactive derivatives (e.g., resolvins, protectins, and maresins), possess powerful anti-inflammatory and antioxidative effect in various inflammatory diseases, cardiovascular diseases, and cancer. AA in ω-6 PUFAs is generally known to be proinflammatory because it can be metabolized into PGE-2 and leukotriene B-4 by Cox and lipoxygenase enzymes, leading to production of proinflammatory eicosanoids and cytokines, whereas it is required for cell membrane fluidity and flexibility in the immune system and nervous system [69]. Moreover, PGE-2 also displays its anti-inflammatory profile by binding to one of its receptors, PGE receptor 4 (EP4), to suppress release of cytokines and chemokines in macrophages and T cells, and participates in innate and adaptive immunity and tissue repair [69]. Interestingly, in the spleen of transgenic mice overexpressing ω-3 fatty acid desaturase that converts ω-6 to ω-3 fatty acids, expression of IL-17, IL-6, and IL-23 is decreased, and Treg cells are expanded, along with Treg cell differentiation significantly higher, indicating an anti-inflammatory effect of ω-3 fatty acids [70]. Therefore, PGE-2 or AA is a dual regulator of inflammatory response due to binding to their different target receptors [69], and ω-3 PUFAs are well-established as potent immune nutritional agents, leading to positive clinical results and promising health promotion [71], and hopefully as an alternative therapeutic approach to impact systemic innate immunity and to control inflammatory process.

4.5. Ginsenosides. Ginsenosides play a critical role in regulating immune responses in inflammatory and immune-related diseases. In LPS-stimulated macrophages, ginsenoside Rg1 and its metabolites inhibit NF-κB activation, expression of TNF-α and IL-1β, and phosphorylation of TGF-β-activated

kinase 1 and IL-1 receptor-associated kinase, along with PI3K/Akt/mTOR pathways activated [48, 72], indicating their anti-inflammatory and enteric nutritional effects. In mice with cecal sepsis, Rg1 enhances the innate immunity by suppressing proinflammatory response and promoting bacterial clearance [73]. In a rat with postoperative ileus, ginsenoside Rb1 reduces serum concentration of TNF-α, IL-1β, IL-6, and IL-10, indicating its anti-inflammatory effect [45]. A ginsenoside derivative, 20S-dihydroprotopanaxadiol, upregulates functional activities of macrophages/monocytes in the innate immunity by promoting phagocytic uptake capability and generation of ROS [74].

Taken together, better understanding of immune enhancement mechanisms of phytochemicals and their relevant communication routes between the periphery and the brain, is essential to develop preventive strategies to counteract impact of systemic inflammation on brain activities in older adults, especially those with preclinical neurodegenerative diseases. The phytochemicals have a direct beneficial effect on the peripheral immune system, and so their dietary administration is an optimal choice to ameliorate systemic inflammation and then reverse pathogenesis of neurodegenerative diseases [50].

5. Brain Innate Immunity Regulation by Phytochemicals and Its Neuroprotective Implication

Neurodegenerative diseases are characterized by progressive neuronal loss and impaired neuronal function, along with activation of microglia and astrocytes and increased release of a range of proinflammatory cytokines such as TNF-α, IL-6, IL-1β, and IFN-γ and oxidative factors such as ROS, NOS, and PGE2 [5, 15, 18]. Targeting the pathogenesis of these diseases, dietary administration of phytobioactive compounds is an effective nutritional intervention choice due to their considerable antioxidant and anti-inflammatory properties [75]. It is also noted that in the striatum of PD brain, dietary administration only improves recovery and regeneration of dopamine terminals, rather than prevents their initial damage [76]. Also, the data shown in the following are largely established from *in vitro* studies, and their limited bioavailability and inability to detect them (e.g., curcumin) in the circulation or target tissues have hindered development of their therapeutic role in humans to some degree.

5.1. Curcumin. Curcumin, as a food additive with antioxidative and anti-inflammatory properties, has a neuroprotective effect in neurodegenerative diseases. Accumulated evidence shows that curcumin can prevent α-synuclein aggregation in PD, attenuate ROS-induced Cox-2 expression in ALS, ameliorate symptoms of MS and other brain injuries, and also suppress overexpression of inflammatory mediators in neuroinflammation. For example, in transgenic mice with AD, curcumin effectively counteracts p25-mediated glial activation and proinflammatory chemokine/cytokine production [10], reduces oxidative damage such as amyloid plaque burden and preformed Aβ fibrils, and reverses progression of tau/amyloid pathology, along with amelioration of cognitive impairments [77]. In prooxidant conditions of neuron culture, curcumin exerts a strong neuroprotective effect and prevents neurotoxicity of oxidative agents H_2O_2 and Fe^{+3} by slowing down tau aggregation and disassembling tau pathological oligomeric structures [78]. In an LPS-induced PD model, curcumin inhibits astrocyte activation and NADPH oxidase complex activation by downregulating NF-κB activity, intrinsic apoptotic pathway (Bax, Bcl-2, caspase 3, and caspase 9), proinflammatory cytokines (TNF-α, IL-1β, and IL-1α), and inducible NOS [79]. In primary neuron culture, curcumin effectively inhibits TNF-α-induced neuroinflammation (IL-6 and Cox-2), protects neurons from excessive ROS production and cellular apoptosis, and promotes expression of antioxidative enzymes HO-1, catalase, and SOD-2 [80]. Recently, conjugated curcumins such as nanocurcumin or curcumin-like analogs are developed to increase their bioavailability and potential neuroprotective efficacy in PD. Although curcumin therapeutic effect for neurodegenerative diseases has been increasingly studied, its *in vivo* metabolic evidence is still not fully reported, including its pharmacokinetics, metabolism, safety, tolerance, bioavailability, and even its entry across the BBB.

5.2. Resveratrol. Resveratrol, as a neuroprotective agent, can suppress overexpression of inflammatory mediators in activated microglia and astrocytes. In LPS-induced cortical neurotoxicity, resveratrol significantly protects cortical neurons against neuroinflammation by inhibiting microglia activation and subsequent production of proinflammatory and cytotoxic factors such as TNF-α, NO, and IL-1β [81]. In mice with intracerebral hemorrhage, resveratrol treatment attenuates acute neurological deficits, neurodegeneration, and cerebral edema with concomitant reduction in IL-1β expression [82]. In LPS-induced neuroinflammation *in vivo* and *in vitro*, resveratrol suppresses microglia activation by promoting microglia polarization from proinflammatory M1 toward anti-inflammatory M2 phenotype via PGC-1α and reduces inflammatory damage and sickness behavior of mice [83]. Interestingly, in BBB-disrupted mice, dietary supplementation of resveratrol markedly reduces BBB-crossing lymphocytes, protein IL-17A, and matrix metalloproteinases (a tight junction degradation protein); enhances tight junction proteins; and improves BBB integrity [84]. In primary mouse astrocytes, resveratrol inhibits LPS-induced production of NO, TNF-α, IL-1β, IL-6, and MCP-1, as well as production of IL-12p40 and IL-23 (a phenotype of T cells) and C-reactive protein, indicating its anti-inflammatory and antioxidative roles in brain innate or adaptive immunity in chronic inflammatory disorders [11]. Whole-genome microarray analysis shows that in rhesus monkeys with high-fat/high-sugar (HFS) stress, dietary administration (2 years) of resveratrol differentially modulates a number of genes and pathways linked to vascular health and inflammation in cerebral cortices and ameliorates neuroinflammatory process such as oxidative stress and NF-κB activation, indicating that long-term resveratrol intake elicits neuroprotective effects [85]. A robust epidemiological study indicates that moderate intake of red wine rich in resveratrol can counteract oxidative stress and metal ion deregulation produced by amyloid and

metal dysmetabolism in the AD brain [86]. Collectively, resveratrol is an active scavenger of free radicals and a modulator of prosurvival or proinflammatory signaling pathways, with a greater potential for therapeutic success in counteracting neurodegenerative diseases.

5.3. Propolis. Propolis has been confirmed to have neuroprotective effects. In the microglia treated by hypoxia, propolis significantly inhibits expression of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6; generation of ROS from mitochondria; and activation of NF-κB [12]. Likewise, CAPE also inhibits expression of NOS and Cox-2 and production of NO and increases expression of HO-1 and erythropoietin (EPO) in microglia, revealing a potent anti-neuroinflammatory effect [87]. In kainic acid-induced excitotoxicity, propolis supplementation significantly prevents increase of NOS, NO, TNF-α, and caspase-3 in the rat brain, reflecting its neuroprotective role in neurodegenerative disorders [61]. In rats with PD, CAPE improves scavenging of ROS and metal chelation and inhibits mitochondrial permeability transition (MPT), a mediator of neuronal death that triggers cytochrome c release and caspase-3 activation, but has no effect on brain mitochondrial function, suggesting that CAPE is a potential compound to protect dopaminergic neuronal loss in neurodegenerative diseases [88, 89]. Also, chrysin, another component of propolis, significantly inhibits expression of NF-κB, NO, inducible NOS, and Cox-2 and release of proinflammatory cytokines (TNF-α and IL-1β) [90]. Intriguingly, intragastric administration of propolis significantly inhibits acetylcholinesterase (AChE) activity in the hippocampus of scopolamine-treated mice, indicating that propolis may protect brain function through vagal reflex [91].

5.4. PUFAs. PUFAs, the structural component of cellular membrane in the brain, are a source of bioactive lipid mediators enzymatically derived from DHA of ω-3 PUFAs or AA of ω-6 PUFAs. DHA and AA are highly susceptible to free radical attack on microglia and astrocytes, and brain lipid metabolism relies on complex integration of diet, peripheral metabolism such as in the liver and blood, and entry of PUFAs into the brain, all of which are readily affected by genetics, sex, and aging [92, 93]. Therefore, habitual supplementation of PUFAs is an effective approach to maintain brain lipid homeostasis or counteract lipid metabolic disturbance in brain aging and neurodegenerative diseases. Evidence shows that PUFAs can be converted into essential membrane phospholipids and second messengers to modulate inflammatory response, oxidative stress, and neuronal function [94] and also regulate molecular signaling of brain innate immunity, especially in neuroinflammation and behavior disorders [92]. ω-3 PUFAs are important for regulating metabolic and inflammatory pathways or pleiotropic pathological activities of the brain, and DHA is especially found in high concentration in the brain (about 40% of neural phospholipids in plasma membrane). In IL-1β-injected rats, dietary supplementation of EPA improves ω-3/ω-6 PUFA imbalance, inhibits glial activation, reduces expression of amyloid precursor protein (APP) and TNF-α, and upregulates expression of BDNF and its receptor, indicating that EPA is an important candidate for anti-inflammatory therapy of neurodegenerative diseases [93]. In LPS-stimulated microglia, EPA and DHA suppress production of proinflammatory cytokines TNF-α and IL-6 and NF-κB expression by activating Sirt1 pathways [95]. In both healthy aged adults and AD patients, cognitive function declines along with DHA decrease, and higher dietary intake of DHA and higher concentration of plasma DHA can reduce the risk of cognitive impairment or AD [96]. In healthy elderly population, DHA administration can enhance learning and memory function but cannot benefit the patients with AD progression diagnosed already [97]. In AD rat models, DHA deficiency activates caspases and exacerbates decline of glutamatergic transmission in learning and memory function [98], and this phenomenon can be reversed by DHA supplementation, along with accumulation of neuronal Aβ and tau protein reduced [99]. By contrast, AA, an ω-6 PUFA, can be metabolized by Cox and lipoxygenase enzymes into proinflammatory eicosanoids and PGE-2 to stimulate cytokine production and activation of microglia and astrocytes, contributing to α-synuclein-mediated neurotoxicity through EP-2. In a word, brain lipid metabolism and lipid signaling pathways may be viable targets for regulating microglia/astrocyte phenotype or function and developing novel therapeutic approaches for neurologic disorders.

5.5. Ginsenosides. Ginsenosides, the major bioactive components of ginseng, possess multiple immunoregulatory effects, involving inhibition of neuroinflammation and oxidative stress, maintenance of neurotransmitter balance, and antiapoptosis and mitochondrial stabilization in the brain. In mice with LPS-induced depression, ginsenoside Rg3 significantly reduces plasma levels of IL-6 and TNF-α [100]. In mice with cognitive dysfunction, Rb1 mitigates expression of ROS, TNF-α, and IL-6 in the hippocampus [101]. In an ALS mouse model, Re reduces microglia activation and inhibits TLR4 signaling and proinflammatory proteins such as CD14 and TNFs [102]. In LPS-induced activation of microglia *in vitro*, Rh2 inhibits inflammatory response to LPS and prevents LPS neurotoxicity, including decreased generation of NO, TNF-α, IL-6, IL-1β, Cox-2, and inducible NOS [103]. Furthermore, in a PD mouse model, oral administration of Rg1 significantly attenuates behavior defects, loss of dopamine neurons, and abnormal ultrastructure changes and regulates activation of astrocytes and microglia by decreasing release of cytokines such as TNF-α and IL-1β in substantia nigra [104]. These data indicate that ginsenosides exhibit a potent neuroprotective effect against neuroinflammation and oxidative stress and are a promising therapeutics for PD. Taken together, beneficial or therapeutic effects of ginsenosides have been widely demonstrated by preclinical and clinical studies, with most evidence obtained from AD and PD, and better understanding of their neuroprotective properties may promote their dietary application in the patients with neurodegenerative disorders and the healthy elderly in communities in order to benefit the society.

Table 1: Anti-inflammatory or antioxidative mechanisms of several phytochemicals in gastrointestinal health, systemic immunity, and neuroimmunity.

Phytochemicals	Approaches	Action mechanisms	Major outcomes	References
Curcumin	Gastrointestinal health	BiP ↓ and IL-8 ↓, in IECs	Anti-inflammation ↑ and ER stress ↓	[27, 28, 29, 30, 31]
		Serotonin ↓, BDNF ↓, and pCREB ↓, in gut	Gut function ↑	
		Mesenteric afferent nerve response by colorectal distension or capsaicin ↓	Gut nociception ↓	
		NO ↓, lipid peroxides ↓, neutrophils infiltration ↓, and cell apoptosis ↓, in TNF-α-colitis	Antioxidation ↑	
	Systemic immunity	Naïve CD4(+) T cells differentiation ↑, Treg ↑, and IL-10-producing Tr1 cells ↑, in intestine	Intestinal lamina propria immunity ↑	[51, 52, 53, 54, 55, 56]
		Circulating IL-6 ↓, DC maturation ↓, proinflammatory cytokine ↓, and allospecific T cell response ↓	Systemic inflammation ↓	
		Monocyte phagocytosis of Aβ ↑ and TLRs 2–4 ↑, in AD	Systemic immunity ↑	
		IL-6 ↓, TNF-α ↓, IFN-γ ↓, IL-17 ↓, Cox-2↓, IL-10 ↑, and Treg cells ↑, in lymphoid organs or macrophages.	Anti-inflammation ↑ and innate immunity ↑	
	Neuroimmunity	Glial activation ↓, NF-κB↓, TNF-α ↓, IL-1β ↓, IL-1α ↓, IL-6 ↓; inducible NOS ↓, Cox-2 ↓; Bax↓, Bcl-2 ↓, caspase 3↓, and caspase 9↓, in AD and PD models	Anti-inflammation ↑, antioxidation ↑, and antiapoptosis ↑	[10, 77, 78, 79, 80]
		Tau aggregation ↓ and neurotoxicity ↓, in neurons	Neuroprotective effect ↑	
Resveratrol	Gastrointestinal health	Occludin ↑ and zonula occluden (ZO-1) ↑, in IECs	Intestinal mucus integrity ↑	[32, 33, 34]
		ROS accumulation ↓, SOD ↑, and HO-1 ↑	Antioxidation ↑	
		T helper cells ↓, Treg cells ↑, and IEC proliferation ↑, in ileitis	Gut barrier function ↑ and microbiota dysbiosis ↓	
		Lactobacilli ↑, bifidobacteria ↑, and enterobacteria ↓	Colonic mucosa architecture ↑	
		PGE-2 ↓, Cox-2 ↓, PGE synthase ↓, and NO ↓, in colonic mucosa	Antioxidation ↑ and anti-inflammation ↑	
	Systemic immunity	Cytokines (TNF-α, IL-1α, IL-1β, IFN-γ, IL-6, IL-8, and IL-10) ↓, chemokines (C-C motif ligand 2 (CCL2), CCL5) ↓, ROS ↓, Cox-2 ↓, PGE-2 ↓, ICAM-1 ↓, and CSFs ↓, in monocytes and macrophage	Antioxidation ↑ and anti-inflammation ↑	[58, 59, 60]
	Neuroimmunity	Glial activation ↓, NF-κB ↓, and cytotoxic factors (TNF-α, NO, IL-1β, IL-6, and C-reactive protein) ↓	Neuroprotective effect ↑, on cortical neurons	[11, 81, 84, 85, 86]
		Lymphocyte infiltration ↓, protein IL-17A ↓, matrix metalloproteinases, ↓, and tight junction proteins ↑, in BBB-disrupted mice	BBB integrity ↑	

Table 1: Continued.

Phytochemicals	Approaches	Action mechanisms	Major outcomes	References
Propolis (flavonoids, CAPE, or chrysin)	Gastrointestinal health	Occludin ↑, ZO-1 ↑ and colon fibrosis ↓, in IECs	Epithelial barrier function ↑	[35, 36, 37, 38]
		NF-κB↓, proinflammatory cytokines ↓, and IP-10 ↓	Antioxidation ↑ and anti-inflammation ↑	
		Phagocytosis↑ and cytotoxicity (IL-1β, IL-6, TLR-2, and TLR-4) ↑, in peritoneal macrophages	Cellular immunity ↑	
	Systemic immunity	Circulating proinflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6, IL-4, and ICAM-1) ↓ and anti-inflammatory cytokines (IL-10) ↑, in LPS-induced systemic inflammation	Systemic inflammation ↓	[62, 64, 65, 66, 67, 68]
		NO ↓, MAPK ↓, and NF-κB ↓, in mast cells and macrophages	Antioxidation ↑ and anti-inflammation ↑	
	Neuroimmunity	NF-κB ↓, TNF-α ↓, IL-1β ↓, IL-6 ↓, NOS ↓, NO ↓, ROS ↓, Cox-2 ↓, and caspase-3 ↓, in microglia or PD mice	Antioxidation ↑, and anti-inflammation ↑, for neurons	[12, 61, 87, 88, 89, 90, 91]
PUFAs (ω-3 PUFAs)	Gastrointestinal health	NF-κB ↓, Cox-2 ↓, PGE-2 ↓, and leukotriene B4 ↓	Anti-inflammation ↑, in gut	[39, 40, 41, 42, 43]
		TRPA1 activation ↑	Gastrointestinal function ↑	
		Intestinal mucosa permeability ↓, gut microbiota ↑, IL-15 ↓, TNF-α ↓, IFN-γ ↓, IL-4 ↓, and IL-10 ↓	Gut immune barrier function ↑	
	Systemic immunity	IL-17 ↓, IL-6 ↓, IL-23 ↓, and Treg cells ↑, in spleen	Anti-inflammation ↑ and immune function ↑	[69, 70, 71]
	Neuroimmunity	Glial activation ↓, ω-3/ω-6 PUFA balance ↑, amyloid precursor protein (APP) ↓, NF-κB ↓, IL-6 ↓, TNF-α ↓, BDNF, and its receptor ↑	Neuroprotection ↑, anti-inflammation ↑, and brain innate immunity ↑	[93, 94, 95, 96, 97, 98]
Ginsenosides (Rb1, Rb2, Rg3, Rh2, Rh3, Rg1, Rg2, and Rh1)	Gastrointestinal health	TNF-α ↓, IL-1β ↓, IL-6 ↓, IL-17 ↓, IL-10 ↓, CREB ↑, glucose transporter 1 ↑, and gut contractility ↑	Anti-inflammation ↑ and gastrointestinal function ↑	[45, 46, 47, 48]
	Systemic immunity	NF-κB ↓, TNF-α ↓, IL-1β ↓, and PI3K/Akt/mTOR pathways ↑	Anti-inflammation ↑ and enteric nutrition ↑	[45, 48, 72, 73, 74]
		Phagocytic uptake ↑ and ROS generation ↑	Innate immunity ↑	
	Neuroimmunity	Glial activation ↓, ROSs ↓, TNF-α ↓, and IL-6 ↓, in the hippocampus	Anti-inflammation ↑ and antioxidation ↑	[100, 101, 102, 103, 104]
		CD14 ↓, NO ↓, TNF-α ↓, IL-6 ↓, IL-1β ↓, Cox-2 ↓, and inducible NOS ↓, in microglia		

Notes: ↑: increased; ↓: decreased; IECs: intestine epithelial cells; abbreviations are shown in the text.

6. Conclusions

Collectively, neuroinflammation, oxidative stress, and mitochondrial dysfunction are three major situations in pathogenesis of neurodegenerative diseases (Figure 1). Nutritionally, these situations can be regulated by administration of many phytochemicals such as curcumin, resveratrol, propolis, PUFAs, and ginsenosides to mitigate inflammatory microenvironment and improve immune competency of the brain, as shown in Table 1. The phytochemicals may be an optimal therapeutic option to counteract pathogenesis of neurodegenerative diseases, mainly through four approaches (Figure 2): (1) reducing systemic inflammation by scavenging free radicals, NOS, and proinflammatory cytokines in the periphery to ease brain inflammation via the BBB; (2) reducing expression of ICAM-1 in endothelial cells to enhance integrity of disrupted BBB and inhibit inflammatory infiltration linked to gateway reflex; (3) permeating into the brain parenchyma

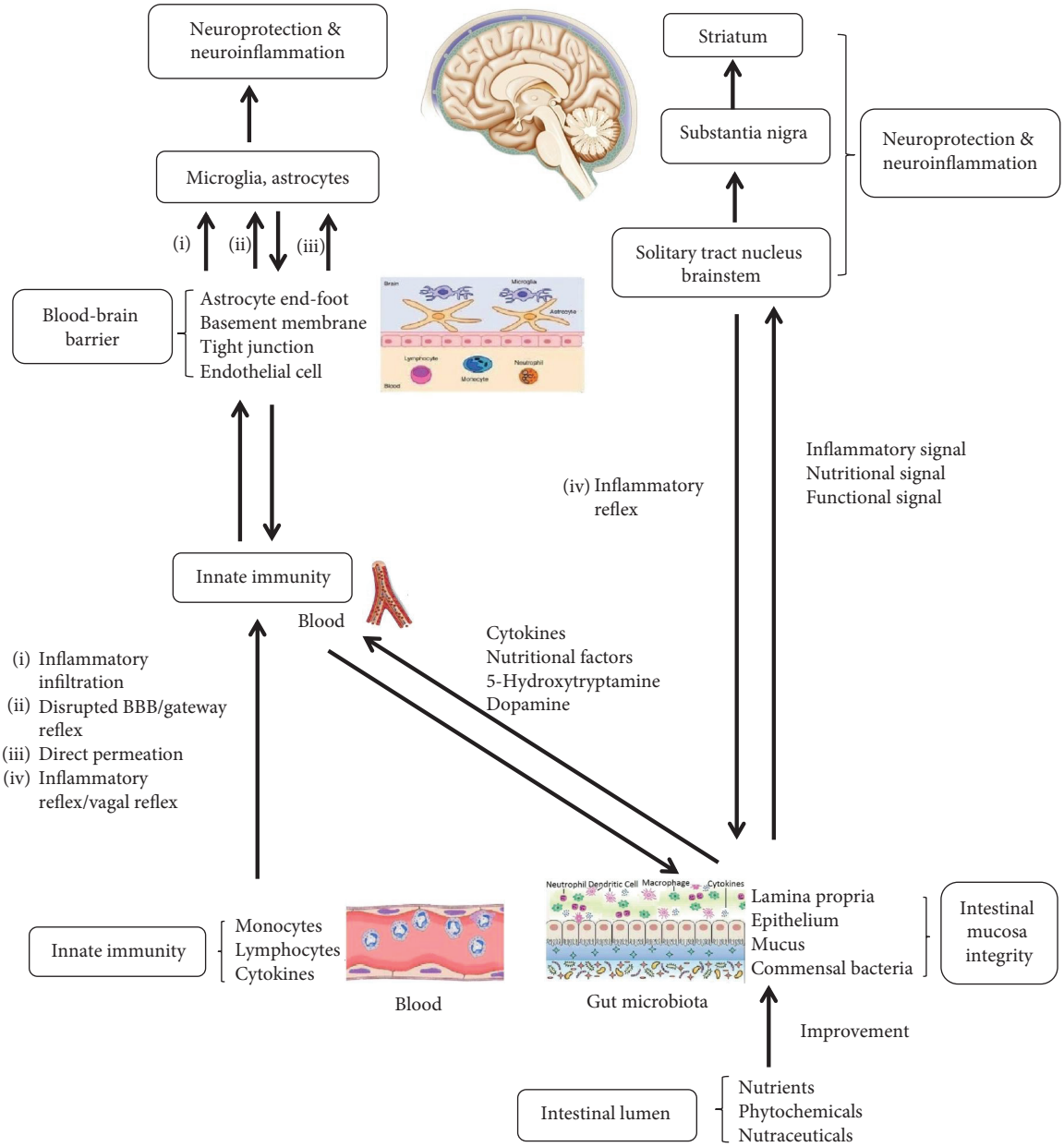

Figure 2: Communication between gut function, systematic immunity, and neuroinflammation. Gastrointestinal function improvement by many phytochemicals can stimulate vagal reflex to affect brain neuroinflammatory response, modulate gut functional secretion of hormones and cytokines, and facilitate systemic innate immunity, leading to neuronal functional improvement or damage reversal. There are at least four approaches connecting gut function, systematic immunity, and neuroinflammation, determining neuroinflammatory or neuroprotective outcomes through dietary intervention of the phytochemicals.

(e.g., PUFAs) to directly act on glial cells and decrease inflammatory response of the brain; and (4) providing protective effect on gastrointestinal function and sending nutritional signals to indirectly improve brain function through gut-brain axis or vagal reflex. In addition, combination application of phytochemicals has a synergistic effect, but their efficacy, bioavailability, metabolism, and safety remain to be fully clarified. Therefore, clinical development of phytochemicals may be a novel therapeutic strategy that only alteration of lifestyle and dietary intake habit becomes an effective alternative of routine drugs to reverse systemic inflammation, oxidative stress, and neuropathologies of neurodegenerative diseases.

Abbreviations

AA: Arachidonic acid
AD: Alzheimer's disease
ALA: Alpha-linolenic acid
ALS: Amyotrophic lateral sclerosis
BBB: Blood-brain barrier
BDNF: Brain-derived neurotrophic factor

CAPE: Caffeic acid phenethyl ester
CCL: C-C motif ligand
CNS: Central nervous system
Cox-2: Cycloxygenase-2
DAMPs: Damage-associated molecular patterns
DHA: Docosahexaenoic acid
EPA: Eicosapentaenoic acid
ERK: Extracellular regulated protein kinase
HD: Huntington's disease
HO-1: Heme oxygenase-1
ICAM-1: Intercellular adhesion molecule-1
IL: Interleukin
IP-10: Interferon-induced protein-10
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MCP-1: Monocyte chemotactic protein-1
MS: Multiple sclerosis
NF-κB: Nuclear factor-κB
NO: Nitric oxide
NOS: Nitric oxide synthase
PAMPs: Pathogen-associated molecular patterns
PBMCs: Peripheral blood mononuclear cells
PD: Parkinson's disease
PGE-2: Prostaglandin E2
PUFAs: Polyunsaturated fatty acids
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SASP: Senescence-associated secretory phenotype
SOD: Superoxide dismutase
TGF-β: Transforming growth factor β
TLR: Toll-like receptors
TNF: Tumor necrosis factor
Treg: Regulatory T cells.

Conflicts of Interest

All authors declare no conflicts of interest.

Authors' Contributions

JTW designed, organized, and wrote the review manuscript; YTS and ZC provided research facilities and conditions; SL helped design and review this paper; and all authors approved the final draft of the manuscript.

Acknowledgments

This work was supported by the funding (JT Wang) from the Milstein Medical Asian American Partnership (MMAAP) Foundation (http://www.mmaapf.org) and in part by the grants (SX Leng: R21-AG-043874, R01AI108907) from the National Institutes of Health.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 6015351, 17 pages
https://doi.org/10.1155/2018/6015351

Review Article

Biological Activities, Health Benefits, and Therapeutic Properties of Avenanthramides: From Skin Protection to Prevention and Treatment of Cerebrovascular Diseases

Andrea Perrelli,[1,2] Luca Goitre,[1,2] Anna Maria Salzano,[3] Andrea Moglia,[2,4] Andrea Scaloni,[3] and Saverio Francesco Retta[1,2]

[1]*Department of Clinical and Biological Sciences, University of Torino, Orbassano, Torino, Italy*
[2]*CCM Italia, Torino, Italy*
[3]*Proteomics & Mass Spectrometry Laboratory, ISPAAM, National Research Council, Napoli, Italy*
[4]*Plant Genetics and Breeding, Department of Agriculture, Forest and Food Sciences, University of Torino, Grugliasco, Torino, Italy*

Correspondence should be addressed to Saverio Francesco Retta; francesco.retta@unito.it

Received 27 April 2018; Accepted 24 July 2018; Published 23 August 2018

Academic Editor: Daria M. Monti

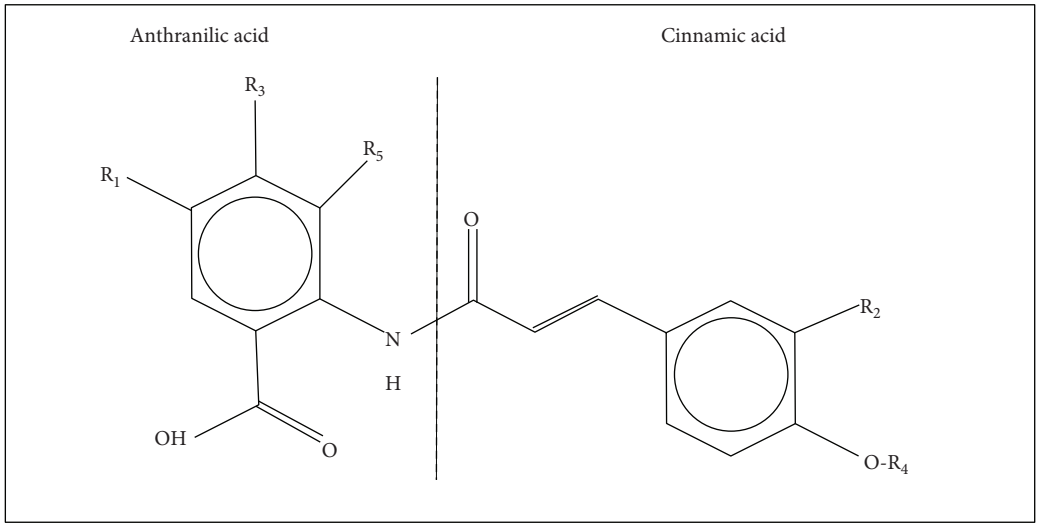
Oat (*Avena sativa*) is a cereal known since antiquity as a useful grain with abundant nutritional and health benefits. It contains distinct molecular components with high antioxidant activity, such as tocopherols, tocotrienols, and flavanoids. In addition, it is a unique source of avenanthramides, phenolic amides containing anthranilic acid and hydroxycinnamic acid moieties, and endowed with major beneficial health properties because of their antioxidant, anti-inflammatory, and antiproliferative effects. In this review, we report on the biological activities of avenanthramides and their derivatives, including analogs produced in recombinant yeast, with a major focus on the therapeutic potential of these secondary metabolites in the treatment of aging-related human diseases. Moreover, we also present recent advances pointing to avenanthramides as interesting therapeutic candidates for the treatment of cerebral cavernous malformation (CCM) disease, a major cerebrovascular disorder affecting up to 0.5% of the human population. Finally, we highlight the potential of foodomics and redox proteomics approaches in outlining distinctive molecular pathways and redox protein modifications associated with avenanthramide bioactivities in promoting human health and contrasting the onset and progression of various pathologies.

The paper is dedicated to the memory of Adelia Frison

1. Introduction

Oats are cereal grain crops belonging to the family of Poaceae (or Gramineae) [1]. Two main species of oat grow naturally, namely, *Avena sativa* and *Avena nuda*. The former, known as common oat, is the most widely cultivated, especially in the cool and moist regions of Northern Europe and North America [2]. Among the common cereal grains, oats are consumed at lower rates than wheat and rice all over the world. However, dietary fiber content, nutritional value, and health benefits of oats are high. Indeed, the increasing interest of consumers towards whole grain oats is mainly driven by its advantageous composition in macronutrients: (i) lipids with a high degree of unsaturation, including oleic and linoleic acids (about 40% and 36% of total fatty acids, resp.), (ii) proteins with a favorable composition of essential amino acids, and (iii) dietary fibers with a high content of β-glucan (2–8.5% w/w of oat seed). In particular, the high levels of β-glucan present in oats have been shown to contribute in reducing total plasma concentration of cholesterol and low-density lipoprotein (LDL) cholesterol, the main risk factors for coronary heart disease (CHD).

A growing body of evidence suggests that oats contain other important bioactive compounds, such as phenolic



Name	R_1	R_2	R_3	R_4	R_5	Structure
Avn-A	OH	H	H	H		N-(4′-hydroxycinnamoyl)-5-droxyanthranilic acid
Avn-B	OH	OCH_3	H	H		N-(4′-hydroxy-3′-methoxicinnamoyl)-5-hydroxyanthranilic acid
Avn-C	OH	OH	H	H		N-(3′-4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid
Avn-D	H	H	H	H		N-(4′-hydroxycinnamoyl)-hydroxyanthranilic acid
Avn-E	H	OCH_3	H	H		N-(4′-hydroxy-3′-methoxycinnamoyl)-hydroxyanthralinic acid
Tranilast	H	OCH_3	H	CH_3		N-(3′,4′-dimethoxycinnamoyl)-anthranilic acid
Yavn I	H	H	H	H	OH	N-(4′-hydroxycinnamoyl)-3-hydroxyanthranilic acid
Yavn II	H	OH	H	H	OH	N-(3′-4′-dihydroxycinnamoyl)-3-hydroxyanthranilic acid

Figure 1: Chemical structure and names of some natural (Avn), synthetic (Tranilast), and recombinant (YAvn) avenanthramides. Avns are low molecular weight phenolic compounds consisting of an anthranilic acid linked to a hydroxycinnamic acid with an amide bond. Different forms of Avns have been either extracted from oats, produced by chemical synthesis, or generated by recombinant DNA techniques in yeast cells.

compounds, which exert protective effects against the development of various pathologies, including cardiovascular diseases (CVDs), diabetes, inflammatory bowel disease (IBD), cancer, obesity, and celiac disease, acting synergistically with dietary fibers [3]. Phenolic compounds are major secondary products of plant metabolism, consisting of at least one aromatic ring bearing one or more hydroxyl groups (phenolic unit). Their chemical structure may range from that of a simple phenolic molecule (phenolic acids) to that of a complex high molecular weight polymer (polyphenols). Depending on the number and type of phenolic units, they can be divided into at least 10 different molecular classes, including simple phenols (phenolic acids), and intermediate (e.g., flavonoids and anthocyanins) and high (e.g., stilbenes, coumarins, and tannins) molecular weight polyphenols. From the biological point of view, phenolic compounds have been show to possess numerous activities, the most important being the antioxidant activity, which prevents lipid peroxidation and cellular oxidative damage mediated by harmful free radicals [4, 5]. This property is related to the ability of phenolic compounds to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations, which is dictated mainly by the number and position of the hydroxyl groups and the nature of substitutions on the aromatic rings, as demonstrated by structure-activity relationship analyses. More in general, phenolic compounds have been involved in cellular defense against various stressful events [2, 6, 7] and shown to possess several health-promoting properties [8], including prophylactic activity against arteriosclerosis, CVDs, inflammatory processes, and certain forms of cancer [9, 10].

The type and concentration of phenolic compounds in whole-grain cereals are influenced by the plant variety and grain nature. In particular, besides containing high levels of phenolic acids, tocopherols, and alk(en)ylresorcinol derivatives, oats are a unique source of avenanthramides (Avns; also known as N-cinnamoylanthranilate alkaloids or anthranilic acid amides), which are not present in other cereals [11]. Avns are low molecular weight phenolic amides consisting of an anthranilic acid linked to a hydroxycinnamic acid with an amide bond (Figure 1). They were originally identified as phytoalexins produced by the plant in response to exposure to pathogens, such as fungi [12, 13]. Oats contain a unique group of approximately 40 different types of Avns, which are present in both oat grains and leaves [14–16]. The most abundant

are Avn-A (N-(4′-hydroxycinnamoyl)-5-hydroxyanthranilic acid), Avn-B (*N*-(4′-hydroxy-3′-methoxycinnamoyl)-5-hydroxyanthranilic acid), and Avn-C (*N*-(3′-4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid) (Figure 1), which are amides of 5-hydroxyanthranilic acid with *p*-coumaric, ferulic, and caffeic hydroxycinnamic acids, respectively [13, 17, 18]. These Avns are constitutively expressed in the kernel, reaching the highest concentration in the bran, and appear in almost all milling fractions [17]. A number of studies demonstrate that these natural products have strong antioxidant activity both *in vitro* and *in vivo*, as well as anti-inflammatory, anti-itching, anti-irritant, anti-atherogenic, and antiproliferative activities, which may prevent or limit cellular oxidative dysfunctions and the development of oxidative stress-related diseases, such as neurodegenerative and cardiovascular diseases, and provide additional protection against skin irritation, aging, CHD, and cancer [16, 17, 19–32].

Apart from natural compounds isolated from oats, avenanthramide analogs endowed with important biological properties have been artificially produced by organic synthesis methodologies, including the pharmaceutical drug Tranilast™ (N-[3′,4′-dimethoxycinnamoyl]-anthranilic acid; Rizaban, Kissei Pharmaceutical Co., Japan), which is currently used in Japan and South Korea as an antihistamine to treat bronchial asthma, atopic dermatitis, keloids and hypertrophic scars, allergic conjunctivitis, allergic rhinitis, and other allergic disorders [33–35]. Notably, whereas several years of clinical use have established that Tranilast has very low adverse effects and good toleration by patients, the beneficial effects of this drug have also been seen in a variety of other pathologies, such as scleroderma and other skin diseases related to excessive fibrosis, cancer, diabetes, and autoimmune, cardiovascular, and renal diseases [36]. Tranilast efficacy has been mainly attributed to its capacity to inhibit the release of proinflammatory factors from leukocytes, including mast cells and macrophages, and suppress collagen deposition, and has been associated mainly with the inhibition of the TGF-β pathway, although this drug affects other pathways as well [37, 38].

Besides natural and synthetic Avns, novel Avn analogs have been produced in recombinant yeast, including N-(4′-hydroxycinnamoyl)-3-hydroxyanthranilic acid (YAvn I) and N-(3′-4′-dihydroxycinnamoyl)-3-hydroxyanthranilic acid (YAvn II), which were generated by engineering a *Saccharomyces cerevisiae* strain with two plant genes (*4cl-2* from tobacco and *hct* from globe artichoke) encoding key proteins involved in the biosynthesis of phenolic esters [39]. Remarkably, YAvn I and YAvn II share structural similarity with Avn-A and Avn-C, respectively (Figure 1), and were shown to possess bioactive properties relevant to biomedical applications, including potent antioxidant, anti-inflammatory, and antiproliferative properties. Indeed, they were effective in rescuing major pathological phenotypes in both cellular and animal models of Cerebral Cavernous Malformation (CCM) disease, a human cerebrovascular disorder of genetic origin implicating oxidative stress and inflammation as main pathogenetic events [40, 41].

2. Radical-Scavenging and Antioxidant Activity of Avenanthramides

The antioxidant activity of oat components was initially suggested by the evidence that oat flour could be used as a food preservative from oxidative deterioration due to its ability in retarding the initial peroxide formation and rancidity [42, 43]. Subsequently, Lingnert and coworkers originally determined the antioxidative capacity of *N*-(4′-hydroxy-3′-methoxy-(*E*)-cynnamoil)-5-hydroxyanthranilic acid and *N*-(4′-hydroxy-3′-methoxy-(*E*)-cynnamoil)-5-hydroxy-4-methoxyanthranilic acid in oxygen consumption experiments with a linoleic acid-based system [44]. The antioxidant properties of oat extracts and their components, including avenanthramides, were eventually demonstrated directly by assaying purified compounds from different oat cultivars [45, 46]. In particular, Emmons and colleagues examined oat milling fractions to determine their potential as dietary antioxidants, showing that three avenanthramide isoforms (Avn-A, Avn-C, and Avn-K) were among the most important oat metabolites endowed with antioxidant activity [46]. Then, Peterson and coworkers synthesized the three major oat avenanthramides (Avn-A, Avn-B, and Avn-C) and tested their antioxidant activities using two *in vitro* assays, such as the inhibition of beta-carotene bleaching and the reaction with the free radical 2,2-diphenyl-1-picrylhy-drazyl (DPPH), demonstrating that Avn-C has greater antioxidant activity than Avn-B and Avn-A [47].

The antioxidant properties of Avns were also investigated in *in vivo* models. In particular, Avn-C supplementation in the diet of rats at a concentration of 0.1 g/kg was effective in reducing reactive oxygen species (ROS) levels in the soleus muscle. Moreover, Avn-C-fed rats had higher superoxide dismutase activity in the vastus lateralis muscle (DVL), liver, and kidney, and higher glutathione peroxidase activity in the heart and DVL, compared to control rats. In addition, Avn-C supplementation attenuated the increased ROS production in the soleus and lipid peroxidation in the heart induced by exercise [19].

The bioavailability of Avns was examined by Chen and coworkers in hamsters [22], where it was observed that plasma concentration of Avns and phenolic acids peak at 40 min after the animals were gavage with saline containing 0.25 g oat bran phenol-rich powder. While *p*-coumaric acid was the most bioavailable among oat phenolics, Avn bioavailability appeared very low, probably due to metabolite distribution in other tissues and the corresponding biotransformation rate. The same authors also investigated the bioavailability and antioxidant action of major Avns, including Avn-A, Avn-B, and Avn-C, in humans [21]. At doses of 0.5 and 1.0 g of an Avn-enriched mixture (AEM), Avns reached the maximum peak in plasma at 1.5 and 2.3 h, respectively. Avn-A and Avn-B bioavailability was 18- and 5-fold higher in humans than in hamsters, respectively. Interestingly, consumption of Avn-enriched oat extracts significantly increased the plasma concentration of reduced glutathione (GSH), the body's master antioxidant. Specifically, after consumption of 0.1 g of AEM, plasma GSH levels increased

Table 1: Antioxidant activity of natural, synthetic, and recombinant avenanthramides.

Year	Compound	Effects	Ref.
1937	Oat flour	Food preservation from oxidative deterioration	[42, 43]
1987	Tranilast	Reduction of intracellular levels of ROS, including hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^•$)	[51]
2003	Avns	Antioxidant activity demonstrated by using DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant potential), and linoleic acid assays	[20, 24, 28]
2003	Avn-C	Upregulation of superoxide dismutase and glutathione peroxidase activities and attenuation of exercise-induced ROS production and lipid peroxidation in the heart and skeletal muscles of rats	[19]
2004	Supplementation of Avn-enriched extract of oats	Interaction with vitamin C to enhance hamster and human LDL resistance to oxidation	[22]
2007	Consumption of Avn-enriched extract of oats	Antioxidant activity in humans: increase of the plasma-reduced glutathione level after consumption	[21]
2010	Avn-rich extract from oat	Effective against D-galactose-induced oxidative stress	[48]
2010	YAvns	Reduction of intracellular ROS levels in a cellular model of CCM disease	[39]
2015	YAvns	Upregulation of FOXO1 and SOD2 expressions in a cellular model of CCM disease	[40]
2015	Avns	Upregulation of heme oxygenase-1 (HO-1) expression in both a dose- and time-dependent manner mediated by Nrf2 translocation	[50]
2017	YAvns	Antioxidant effects in a mouse model of CCM disease	[41]
2018	Natural and synthetic Avns	Antioxidant effects on CaCo-2 and Hep3B cancer cells	[83]

Avns: avenanthramides; FOXO1: forkhead box protein O1; ROS: reactive oxygen species; SOD2: superoxide dismutase 2; YAvns: yeast avenanthramides.

21% from baseline at 15 min, without apparent adverse side effects [21]. Moreover, Avn-rich extract from oat was also reported to possess an effective antioxidant activity against D-galactose-induced oxidative stress [48]. Furthermore, it was demonstrated that Avns, including Avn-A, significantly increased heme oxygenase-1 (HO-1) expression in HK-2 cells in both dose- and time-dependent manners, showing that this effect involved ROS production and Nrf2 nuclear translocation [49, 50].

The Avn analog Tranilast was also reported to be effective in reducing the generation of ROS, including hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^•$), suggesting potential clinical applications [51]. However, the mechanisms of its antioxidant activity are yet to be clarified.

On the other hand, Avn analogs produced in recombinant yeast, including YAvn I and YAvn II, were originally shown to have strong antioxidant activity when tested in an $ABTS^{•+}$ radical quenching assay, as well as the capacity to reduce intracellular ROS levels in a cellular model of CCM disease, as evaluated with a cellular antioxidant assay [39]. Subsequent *in vitro* studies demonstrated that YAvn I and YAvn II positively regulate cell antioxidant defense mechanisms through the upregulation of forkhead box protein O1 (FOXO1) and superoxide dismutase 2 (SOD2) expression levels [40]. In addition, recent studies in an animal model of CCM disease have extended these findings, demonstrating the effectiveness of YAvns in major oxidative stress-related disease phenotypes [41] (Table 1).

3. Anti-Inflammatory Activity of Avenanthramides

The ancient literature already described the anti-inflammatory and anti-itching properties of oat extracts. In fact, Greek and Latin literatures report the use of oatmeal as topical therapy for a variety of dermatological conditions [29]. Since 1945, several studies showed the benefits of colloidal oatmeal bath as soothing treatment as well as nonirritating, cleansing formulation for inflamed, itchy skin associated with various xerotic dermatitis [16, 29]. Despite widespread use for skin irritation, the phytochemicals present in oat and responsible for the anti-inflammatory activity were not defined until 2004. Liu and colleagues first reported the potential anti-inflammatory and antiatherogenic properties of Avn-enriched extracts of oats, which inhibited the IL-1β-stimulated endothelial cell secretion of proinflammatory cytokines (IL-6) and chemokines (IL-8 and MCP-1), as well as expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and adhesion of monocytes to endothelial cell monolayer [29]. Similarly, CH_3-Avn-C, a synthetically prepared methyl ester derivative of Avn-C, significantly and dose-dependently decreased mRNA expression and secretion of IL-6, IL-8, and MCP-1 in endothelial cells and inhibited IL-1β- and TNFα-stimulated NF-κB activation by preventing the phosphorylation of IκB kinase and IκB [26]. Moreover, Sur and colleagues found that keratinocites treated with Avns displayed a significant inhibition of TNF-induced NF-κB activity and subsequent reduction of IL-8 release, suggesting that oat Avns may have putative anti-itching activity [16].

Table 2: Anti-inflammatory activity of natural, synthetic, and recombinant avenanthramides.

Year	Compound	Effects	Ref.
1997	Tranilast	Inhibition of COX-2 and iNOS expression	[56]
2002	Tranilast	Inhibition of cytokine-induced NF-κB activation	[16]
2004	Avn-enriched extract of oats	Inhibition of IL-6, IL-8, and MCP-1 secretion and ICAM-1, VCAM-1, and E-selectin expression	[29]
2008	CH_3-Avn-C	Reduction of mRNA expression and secretion of IL-6, IL-8, and MCP-1 and inhibition of IL-1β- and TNFα-stimulated NF-κB activation in endothelial cells	[26]
2008	Avns	Inhibition of TNF-induced NF-κB activity and reduction of IL-8 release keratinocites. Putative anti-itching activity	[29]
2008	Avns	Inhibition of tumor necrosis factor alpha (TNF-alpha) induced NF-κB luciferase activity and subsequent reduction of interleukin-8 (IL-8)	[16]
2014	Avn-based diet supplementation	Attenuation of exercise-induced inflammation	[53]
2015	Avn-enriched oat bran	Modulation of specific biomarkers of inflammation in older, overweight, or obese adults	[82]
2017	YAvns	Inhibition of NF-κB and rescue of inflammatory phenotypes in cellular and mouse models of CCM disease	[41]
2017	DH Avn-D	Interaction with the neurokinin-1 receptor (NK1R), inhibition of mast cell degranulation, and reduction of the secretion of the cytokine interleukin-6 (IL-6)	[52]
2018	Natural and synthetic Avns	Anti-inflammatory effects on CaCo-2 and Hep3B cancer cells	[83]

Avns: avenanthramides; CCM: cerebral cavernous malformation; CH_3-Avn-C: methyl ester of Avn-C; COX-2: cyclooxygenase-2; DH Avn-D: dihydro-avenanthramide D; ICAM-1: intercellular adhesion molecule 1; iNOS: inducible nitric oxide synthase; MCP-1: monocytic chemotactic protein-1; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; TNFα: tumor necrosis factor alpha; VCAM-1: vascular cell adhesion molecule 1; YAvns: yeast avenanthramides.

Furthermore, dihydro-avenanthramide D (DH Avn-D), a synthetic analog of avenanthramide, was shown to inhibit mast cell degranulation and exhibit anti-inflammatory effects through the activation of the neurokinin-1 receptor [52]. In addition, avenanthramide supplementation was able to attenuate exercise-induced inflammation in postmenopausal women by reducing neutrophil respiratory burst activity, plasma C-reactive protein and IL-1β levels, and NF-κB activation in peripheral blood mononuclear cells [53]. Notably, differences in the ability to inhibit NF-κB among Avns have been ascribed to molecular structural variations [54].

On the other hand, the anti-inflammatory properties of the Avn analog Tranilast have been attributed mainly to its capacity to inhibit the release of chemical mediators from mast cells and basophils [34, 55], suppress COX-2 and iNOS expression [56], and limit TNFα-induced secretion of IL-6 and surface expression of vascular adhesion molecules, including VCAM-1, ICAM-1 and E-selectin, in endothelial cells by inhibiting NF-κB-dependent gene transcription [38]. Furthermore, Tranilast anti-inflammatory properties were shown to be triggered by the induction of HO-1 expression via ERK1/2 activation [55]. Finally, recombinant YAvns were demonstrated to downregulate NF-κB and rescue inflammatory phenotypes in cellular and animal models of CCM disease [41] (Table 2).

4. Antiproliferative Activity of Avenanthramides

Clear evidence demonstrates that Avns, including Avn-C and its methylated derivative (CH_3-Avn-C), can significantly inhibit proliferation of distinct cell lines, such as human colon and breast cancer cells, and vascular smooth muscle cells (VSMC) [25, 31, 57]. Indeed, it has been reported that Avns induce cell cycle arrest at the G1 phase by upregulating the p53-p21cip1 pathway and inhibiting phosphorylation of the retinoblastoma protein (pRB) [25], and may activate apoptosis [57]. Moreover, methylated Avn-C was shown to inhibit proteasome activity and increase the levels of ubiquitin-conjugated proteins in endothelial cells, suggesting that inhibition of proteasome activity and consequent stabilization of the p53 protein are a plausible mechanism underlying the inhibitory effect of Avns on the cell cycle [25]. In particular, by testing the antiproliferative effect of Avns on distinct cancer cell lines, it was found that Avn-enriched oat extracts, Avn-C, and the methyl-ester derivative of Avn-C were more effective on colon cancer cell lines, including CaCo-2, HT29, LS174T, and HCT116 cells, than on prostate or breast cancer cell lines [25]. Furthermore, the synthetic DH Avn-D was shown to inhibit human breast cancer cell invasion through inhibition of MAPK/NF-κB and MAPK/AP-1 pathways and suppression of MMP-9 expression [32]. In addition, the Avn analog Tranilast was shown to exert inhibitory effects on proliferation, epithelial-mesenchymal transition (EMT), and invasion of cancer cells [58]. Moreover, it was reported to inhibit proliferation, chemotaxis, and tube formation of human microvascular endothelial cells *in vitro* and angiogenesis *in vivo* [59], as well as vascular endothelial growth factor (VEGF)-induced vascular permeability [60], suggesting that it might ameliorate angiogenesis-related diseases, such as tumor metaplasia, rheumatoid arthritis, diabetic retinopathy, and age-related

Table 3: Antiproliferative activity of natural, synthetic, and recombinant avenanthramides.

Year	Compound	Effects	Ref.
1994–1996	Tranilast	Blockage of PDGF-induced cell-cycle progression at the G1/S checkpoint, inhibition of VSMC proliferation, and suppression of intimal hyperplasia after photochemically induced endothelial injury in the rat	[31]
1994–1997	Tranilast	Proposed as a putative therapeutic agent for prevention and treatment of diseases associated with neovascularization, such as diabetic retinopathy, senile discoid macular degeneration, neovascular glaucoma, and rheumatoid arthritis	[59–62]
2001	Tranilast	Inhibition of migration and invasiveness of human malignant glioma cells	[37]
2002	Tranilast	Inhibition of pancreatic cancer cell proliferation and tumor angiogenesis	[58]
2003	Tranilast	Inhibition of oral squamous cell carcinoma growth and invasion	[76]
2006	Avn-C and CH_3-Avn-C	Inhibition of VSMC proliferation	[31]
2006	Avn-C	Inhibition of SMC proliferation by upregulating the p53-p21cip1 pathway and inhibiting pRB phosphorylation	[30, 31]
2009	Tranilast	Inhibition of human prostate adenocarcinoma cell proliferation	[74]
2009	Tranilast	Inhibition of neurofibroma cell growth	[81]
2010	Tranilast	Effectiveness in the treatment of desmoid tumor of the chest wall and inhibition of breast cancer stem cells	[73]
2010	Tranilast	Inhibition of murine and human breast cancer cell proliferation and migration	[79, 80]
2010	Avn-enriched extracts of oats, Avn-C, and CH_3-Avn-C	Antiproliferative effects on distinct colon cancer cell lines	[25]
2011	DH Avn-D	Inhibition of human breast cancer cell invasion through downregulation of MAPK/NF-κB and MAPK/AP-1 pathways and suppression of MMP-9 expression	[32]
2015	YAvns	Stronger antiproliferative properties than natural Avns, including Avn-B, due to enhanced capacity of reducing intracellular ROS levels and cyclin D1 expression	[40]
2017	Avns	Antiproliferative effect on breast cancer cells through an antiapoptotic mechanism as revealed by annexin V and caspase activities	[57]
2018	Natural and synthetic Avns	Cytotoxic and proapoptotic effects on CaCo-2 and Hep3B cancer cells	[83]

Avns: avenanthramides; CH_3-Avn-C: methyl ester of Avn-C; DH Avn-D: dihydro-avenanthramide D; PDGF: platelet-derived growth factor; ROS: reactive oxygen species; VSMC: vascular smooth muscle cells; YAvns: yeast avenanthramides.

macular degeneration, acting as a novel angiogenesis inhibitor [59, 61, 62]. Finally, recent evidence demonstrates that recombinant YAvn I and YAvn II are endowed with stronger antiproliferative properties than natural Avns, including Avn-B, due to their enhanced capacity of reducing intracellular ROS levels and cyclin D1 expression [40] (Table 3).

5. Therapeutic Benefits of Avenanthramides

There is compelling evidence that oxidative stress plays a major role in the pathogenesis and progression of major human diseases, including atherosclerosis, diabetes, inflammatory diseases, cardiovascular diseases, cancer, and neurological disorders, such as amyotrophic lateral sclerosis, Alzheimer's (AD) and Parkinson's (PD) diseases [63], and is also implicated in aging [64].

Oxidative stress occurs either when excess ROS are produced in cells, which could overwhelm the normal antioxidant capacity, or upon impairment of antioxidant defense mechanisms. ROS toxicity contributes to protein, lipid and DNA damage, inflammation, cell and tissue injury, and apoptosis. Nevertheless, ROS also play important physiological functions, whereas emerging evidence demonstrates that the biological impact of ROS depends not only on their intracellular levels and rate of formation and decay but also on their chemical nature and subcellular localization [65, 66]. Thus, inappropriate removal of ROS by antioxidants may cause paradoxical reductive stress and thereby induce or promote disease [63, 67, 68].

Due to their capacity to scavenge ROS and prevent oxidative stress, antioxidants (including natural and synthetic phenolic compounds) have long been credited with helping to live longer and stay healthier, and looked upon as effective therapeutic options for prevention and treatment of various oxidative stress-related diseases. Natural antioxidants are primarily phenolics that may occur in all parts of

plants [69]. Specifically, beneficial effects on human health of phenolic compounds with high antioxidant properties obtained from oats have been reported in many studies and shown to protect cells against oxidative damage [23, 70]. Furthermore, several compositions containing oat Avns or derivatives have been described in pharmaceutical patents for use in cosmetic, nutraceutical, and therapeutic preparations due to their antioxidant, anti-inflammatory, antiitching, antiallergic, antihistaminic, antiasthmatic, and antiaging activities. In particular, the synthetic drug Tranilast has been approved since 1982 in Japan and South Korea and, as mentioned above, is currently used as an antihistamine to treat bronchial asthma, atopic dermatitis, allergic conjunctivitis, allergic rhinitis, and other allergic disorders, with indications for keloids and hypertrophic scars, scleroderma, and other skin disease related to excessive fibrosis [36]. In addition, it was proposed for treatment of autoimmune diseases, such as arthritis and multiple sclerosis, and as an inhibitor of angiogenesis [37, 71]. Moreover, the high potential of Tranilast in inhibiting pathological cellular growth processes, such as tumor-related ones, was investigated with promising results [37, 58, 72–81].

On the other hand, a randomized, placebo-controlled, double-blind pilot study, led to determine whether the Avn-enriched bran reduces biomarkers of inflammation, demonstrated that consuming Avns in a whole food form, that is, Avn-enriched oat bran, may affect specific biomarkers of inflammation in older, overweight, or obese adults [82]. Considering the anti-inflammatory properties of Avns and their capacity to inhibit smooth muscle cell proliferation and increase NO production, these compounds were proposed for prevention or therapy of atherosclerosis and associated cardiovascular diseases. Data also pointed to the potential benefit of including oats and oat bran in daily meals over the long term [26]. Interestingly, recent evidence highlighted the combined antioxidant, anti-inflammatory, and anticancer effects of individual synthetized Avns and a mixture of natural Avns on CaCo-2 and Hep3B cancer cells, showing that both natural and synthetic Avns activate caspases 2, 8, and 3 and downregulate hTERT, MDR1, and COX-2 genes, and suggesting that oat-based foods fortified with Avns could be an alternative to produce functional foods with major health benefits [83]. Furthermore and importantly, recent findings demonstrated that both Tranilast and YAvns were effective in rescuing prooxidant and proinflammatory phenotypes associated with CCM disease, a cerebrovascular disorder associated with altered redox homeostasis and signaling and enhanced susceptibility to oxidative stress and inflammatory insults, thus widening the therapeutic potential of these compounds [41].

6. Avenanthramides as Potential Therapeutics for Cerebral Cavernous Malformation Disease

CCM, also known as cavernous angioma or cavernoma, is a major cerebrovascular disease characterized by clusters of abnormally dilated and leaky capillaries occurring in brain, spinal cord, and retina, with a prevalence of 0.3–0.5% in the general population. These vascular anomalies, referred to as CCM lesions, can be single or multiple (up to hundreds), as detected by magnetic resonance imaging, and may result in severe clinical symptoms at any age, including recurrent headaches, focal neurological deficits, seizures, stroke, and intracerebral hemorrhage (ICH) [84]. CCM disease has proven genetic origin (OMIM 116860), being caused by loss-of-function mutations in three genes, *KRIT1* (*CCM1*), *CCM2*, and *PDCD10* (*CCM3*). It may arise sporadically or is inherited as autosomal dominant condition with incomplete penetrance and highly variable expressivity even among members of the same family, including wide differences in lesion number, size, and susceptibility to ICH [84–86]. Despite significant recent advances in our understanding of the pathophysiology of CCM disease, no direct therapeutic approaches are available so far, besides the surgical removal of accessible lesions [84, 87].

Accumulated evidence demonstrates that loss-of-function mutations of CCM genes have pleiotropic effects on several redox-sensitive molecules and mechanisms that control cellular homeostasis and defenses against oxidative stress and inflammation, thereby sensitizing cells to local oxidative stress and inflammatory events [84, 86, 88–95]. In particular, KRIT1 loss-of-function has been shown to affect major antioxidant pathways and mechanisms, including the FOXO1-SOD2 axis and the Nrf2 antioxidant pathway [89, 94], and the autophagic degradation of dysfunctional, ROS-generating mitochondria [89, 91]. On the other hand, there is emerging evidence that Avns, including YAvns, can enhance cellular defenses against oxidative stress by inhibiting the activity of prooxidant and proinflammatory proteins, such as NADPH oxidase and NF-κB [41], and stimulating the upregulation of antioxidant molecules, such as GSH and SOD2 [21, 40]. Indeed, treatment of KRIT1-knockout and KRIT1-silenced cellular models with YAvns was effective in reverting molecular phenotypes caused by KRIT1 loss-of-function, including the downregulation of FOXO1 and SOD2 and the upregulation of cyclin D1 [40]. Furthermore, both YAvns and Tranilast were able to induce a rescue of major phenotypic signatures in a mouse model of CCM disease, including altered redox homeostasis and signaling, destabilized endothelial cell-cell junctions and blood-brain barrier, enhanced vascular permeability, and reduced susceptibility to oxidative stress and inflammatory insults, suggesting potential therapeutic benefits for CCM disease [41]. Further studies aimed at a comprehensive characterization of the pleiotropic effects and mechanisms of action of natural and recombinant Avns will provide useful insights into these and other promising therapeutic benefits.

7. Avenanthramide and Aging Processes: A New Elixir of Youth?

Oatmeal has been used for centuries as a soothing agent to relieve itch and irritation associated with various xerotic dermatoses. Today, it is available in various dosage forms from powders for the bath to shampoos, shaving gels, and

moisturizing creams, and has been approved as a skin protectant by the US Food and Drug Administration (FDA) [27].

Among oat constituents, Avns are known to suppress histamine release at very low doses, helping to plump up the skin, reduce wrinkles, and restore the skin natural barrier. Indeed, oat Avns have been shown to represent the main group of active polyphenolic antioxidants responsible for oatmeal anti-inflammatory, antierythema (antiredness), antipruritic (anti-itching), and antihistaminic properties. Consistently, several studies have demonstrated their benefits in reducing eczema and other inflammatory skin conditions [16]. Another health and antiaging benefit of oat Avns is their antigenotoxic activity, which can protect the DNA of epidermal cells against environmental insults, including UV irradiation [21]. In hair care, oat Avns have been shown to prevent lipid peroxidation in human hair follicles and alleviate scalp itchiness and tenderness, indicating Avns as an ideal active ingredient for scalp care formulations [25]. Furthermore, Avns have been shown to prevent oxidation of LDL cholesterol and inhibit the first stages of atherosclerosis, gaining the reputation of being able to protect the aging cardiovascular system. In addition, as also described in this review, several studies in the past few years have suggested that oat Avns may be beneficial in the treatment of various aging-related human diseases associated with chronic oxidative stress and inflammation [96]. Notably, Avns exert their strong antioxidant and anti-inflammatory properties even at very low doses.

Taken together, the established beneficial effects of Avns in skin protection and treatment of dermatological diseases, and their emerging potentiality to prevent and treat chronic oxidative stress and inflammation associated with onset, progression and severity of aging-related diseases, including metabolic, cardiovascular, cerebrovascular and neurodegenerative diseases, point to these compounds as promising new elixir of youth with both cosmetic and pharmaceutical applications.

8. Foodomics for Elucidating Molecular Pathways Underlying Biological Effects of Avenanthramides in Chronic Diseases

Nutrition research has traditionally explored the functional importance of diverse food categories through a careful evaluation of various physiological phenomena and molecular markers characterizing a group of individuals fed with a defined diet. Bioactive food constituents may have significant beneficial effects for health promotion and disease prevention, with various compounds active in reducing the sustained oxidative stress and inflammation accompanying chronic diseases, for example, CVDs and/or metabolic syndromes. Unfortunately, chronic disorders are often complex, multifactorial pathogenetic processes; they are the result of combined genomic variant peculiarities interacting with environmental/behavioral factors. Hence, not only genetic factors but also homeostatic alterations related to the environment may be crucial in disease onset, progression, and severity.

In the last few years, nutrition research has moved from classical physiology and epidemiology to chemical biology, molecular biology, and genetics [97]. It has evolved similarly to pharmacological research, where the topic effect of a specific drug is evaluated on a defined cellular/organism model subjected to controlled perturbative events (such as drug treatment at a specific concentration and for a defined time), which are then assayed according to a holistic perspective through combined molecular approaches [98, 99]. In this context, foodomics has emerged as a novel and multidisciplinary research field in nutrition science, which aims at elucidating how diet can influence organism health [100, 101]. It is well known that bioactive compounds present in foods, when assimilated, can affect gene expression profiles in organism tissues/organs, and corresponding protein levels and metabolite representations, thus contributing to modulating the incidence of several chronic diseases. The study of these complex interactions requires the integration of different analytical approaches generating various dataset, which then are interpreted according to a system biology perspective by dedicated bioinformatic methods [102]. Thus, in a foodomics experiment, (i) genomics takes advantage of DNA microarray technologies to detect mRNA expression changes in response to diet; (ii) proteomics uses quantitative LC and MS methods combined with isotopic labelling procedures (TMT, iTRAQ, or SILAC) to define protein profile variations in dietary interventions; (iii) metabolomics uses the same separation and measure techniques to define the bioavailability of bioactive molecules in food and their molecular changes after ingestion, as well as organism plasma/urine metabolite profiles in response to diet; (iv) genetics defines common genetic variants involved in the individual response to diet through whole genome sequencing techniques. Integration of all information according to a multiomic elaboration allows simultaneously deciphering gene expression pathways, protein levels, and metabolite concentrations that are affected in healthy individuals experiencing a certain diet; the same information can be obtained for subjects suffering a certain pathological condition. Thus, it is possible to formulate dietary recommendations based on a system biology perspective to ensure a healthy condition or to prevent and treat chronic diseases, such as CVDs, obesity, and cancer [103]. In this context, we particularly underline the importance of foodomics studies that over time have been performed on human, animals, and animal models of human diseases administered with (i) rosemary extracts rich in polyphenols [104–106] and corresponding isolated metabolite carnosol and carnosic acid [107, 108]; (ii) red-to-blue fruit extracts rich in anthocyanins [109]; (iii) vegetable extracts rich in flavonoids [110] and isoflavones or isolated genistein, daidzein [111], and flavone [112]; (iv) green tea extracts rich in polyphenols [113]; (v) olive oil extracts rich in polyphenols [114]; (vi) fish oil extracts rich in polyunsaturated fatty acids [115]; (vii) resveratrol-containing foods [116]; (viii) inulin-containing prebiotics and isolated inulin; (ix) increased dietary protein [117, 118]; (x) nutrients lacking normal Zn supplement [119]; (xi) augmented folate [120] and multivitamin/mineral supplement [121]. Due to the complexity of these studies,

their results were often published in different articles. In most cases, experiments were performed on individuals fed with a food matrix containing various bioactive compounds; this is similar to other "omic" investigations, where traditional pharmacological remedies were tested through holistic approaches [122–124]. In the next future, it is desirable that advanced foodomics studies, analogous to those reported above for other foods, will also be performed on organisms or animal models of human diseases fed with oat compounds, including isolated Avns and their recombinant derivatives, to unveil the molecular mechanisms underlying the corresponding biological effects and therapeutic benefits reported above. To this regard, particular attention should be paid to the effects of Avns on the intestinal microbiome, as this has been recognized as a fundamental player in human health and disease, affecting a variety of conditions such as host energy balance and immune responses [125], and has been recently implicated also in the pathogenesis of CCM disease, suggesting that manipulation of the bacterial microbiome may indeed be an effective therapeutic approach [126]. It is therefore important that future foodomics investigations will also include information from gut-residing bacteria and consequent modulation of the gut-brain axis.

9. Redox Proteomics for Detailing Chemical Modifications Hampered by Avenanthramides in Chronic Diseases

Oxidative and nitrosative stresses, due to an imbalance between the generation of ROS and reactive nitrogen species (RNS), and the antioxidant defense capacity of the organism, are important pathophysiological events contributing to the onset and progression of several human pathologies, including cardiovascular diseases and metabolic syndromes [127, 128]. ROS include superoxide anion ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$) and peroxyl ($RO_2^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals, as well as nonradical compounds, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and organic peroxides, which can be produced from either endogenous (e.g., mitochondrial electron transport chain, cytochrome P450 monooxygenases, and NADPH oxidases) or exogenous sources (e.g., pollutants, drugs, xenobiotics, and radiation). On the other hand, RNS are reactive compounds derived from nitric oxide ($NO^{\bullet}$) following the activity of inducible nitric oxide synthases, and include peroxynitrite ($ONOO^-$), alkyl peroxynitrite (ROONO), nitrogen dioxide ($NO_2^{\bullet}$), and other molecules [129].

ROS and RNS affect major cellular components, including lipids, DNA and proteins, modifying their structure. In particular, hundreds of adducts of distinct nature have been identified in proteins as a result of the reaction of ROS and RNS with chemical groups present in amino acid side chain [130]. Through modulation of protein structure/function, ROS and RNS can influence a number of enzymatic activities and protein functions, thus affecting intracellular signal transduction pathways and gene expression profiles. While several enzymatic and nonenzymatic markers of chronic oxidative and nitrosative stresses are well known in different organs and body tissues/fluids, early protein targets of oxidative and nitrosative injuries are now becoming to be defined. The identification of putative oxidative biomarkers takes advantage of redox proteomics [131], which is indeed a branch of proteomics specifically designed to identify oxidized and nitrosized proteins and determine nature, extent, and location of oxidative/nitrosative posttranslational modifications in the proteomes of interest. *Gel-based* and *gel-free* redox proteomics techniques often use liquid chromatography coupled to mass spectrometry as the major platform to achieve the goal of identifying and fully characterizing oxidized and nitrosized target proteomes. In this context, dedicated redox proteomics methods have been developed to quali-quantitatively investigate (i) Cys oxidation to sulfenic, sulfinic, and sulfonic acid; (ii) Cys conversion to intra- and intermolecular cystine derivatives; (iii) Cys conversion into S-nitrosyl-cysteine; (iv) Met sulfoxidation to sulfone/sulfoxide derivatives; (v) Trp oxidation and nitrosation to (di)hydroxytryptophan, N-formylkynurenine, hydroxykynurenine, kynurenine, and nitrotryptophan; (vi) His oxidation to oxindolylalanine, 2-oxo-histidine, and 5-hydroxy-2-oxo-histidine; (vii) Tyr oxidation, nitrosation, and halogenation to di- and tri-hydroxyphenylalanine, 3,3′-dityrosine, 3-nitrotyrosine, and 3,(5)-(di)halotyrosine, respectively; (viii) Pro, Arg, Lys, and Thr direct oxidation to 2-pyrrolidone, glutamic acid semialdehyde, aminoadipic semialdehyde, and 2-amino-3-ketobutyric acid, respectively; (ix) Lys and Arg glyco-oxidation to generate more than fifty distinct derivatives; (x) Cys, His, and Lys reactions with α,β-unsaturated aldehydes deriving from lipid peroxidation to generate more than thirty distinct derivatives; (xi) Cys modification with electrophic prostaglandins and isoprostanes deriving from arachidonic acid oxidation. Once identified, oxidized and nitrosized proteins can be placed in specific molecular pathways to provide insights into affected molecular and cellular functions associated with human diseases.

Conventional and early detection of above-mentioned oxidized and nitrosized protein markers in various diseases and metabolic syndromes has thus enabled to hypothesize a relationship between pathological hallmarks of such disorders and protein structural/functional modifications. This is the case of distinctive identifications of (i) carbonylation, Tyr chlorination, and Met sulfoxidation of target proteins in plasma and atherosclerotic lesions from subjects affected by coronary artery diseases [132, 133]; (ii) glycated, carbonylated, Met-sulfoxidized, Tyr-nitrated, and *S*-nitrosylated proteins in biological fluids and tissues of diabetic patients, or tissues of related animal models [134–144]; (iii) carbonylation, Tyr nitration, and *S*-glutathionylation of target proteins in brain tissues of AD and mild cognitive impairment patients, or animal models of AD, PD, HD, and ALS [145–150]; (iv) oxidized, carbonylated, and Tyr-nitrated and chlorinated proteins in body tissues of patients and animal models experiencing various acute inflammatory syndromes [151, 152]; (v) carbonylated proteins in bronchoalveolar lavage of patients with sarcoidosis and pulmonary fibrosis [153]; (vi) carbonylated proteins in the diaphragm and muscle tissues of severe chronic obstructive pulmonary disease

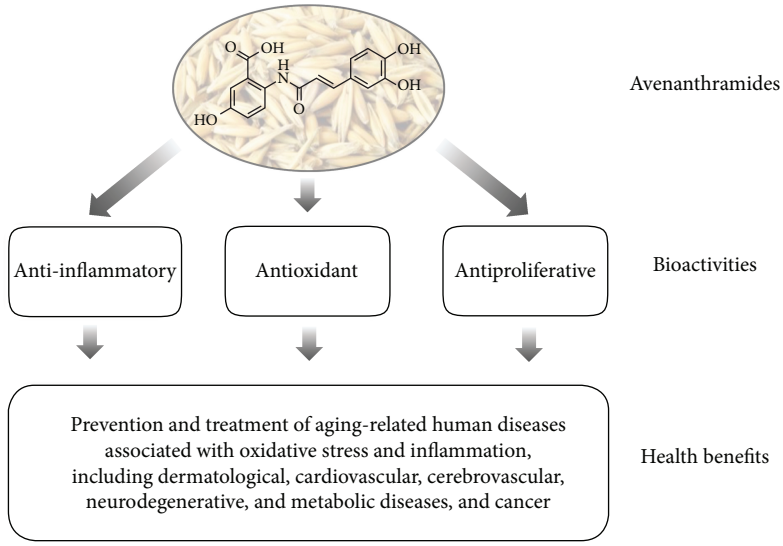


Figure 2: Bioactivities and potential health benefits of avenanthramides. Both natural, synthetic, and recombinant avenanthramides have been shown to exhibit strong antioxidant, anti-inflammatory, and antiproliferative activities, which may provide protection against various cellular dysfunctions and human pathologies, including aging-related diseases.

patients and related animal models [154, 155]; (vii) glycoxidized and carbonylated proteins in urine and the kidney of patients with dialysis-related amyloidosis [156–158]; (viii) oxidized proteins in human liver tissues after ischemia/reperfusion [159]; (ix) redox modified proteins in various tissues as a result of aging [160–168]; (x) carbonylated, Tyr-nitrated, and S-sulphenylated proteins in hypertensive kidney disease [169–171]; (xi) oxidized proteins in the amniotic fluid of preeclamptic women [172]. Whenever inserted in perturbative experiment pipelines, redox proteomics approaches are now allowing a monitoring of the degree of corresponding body tissue damage and the response to pharmacological therapies.

At the same time, they will provide a rationale to the positive/negative effects of a diet on healthy individuals and/or on patients suffering pathological conditions. In this context, pioneering experiments have been performed to evaluate the impact of (i) excessive caloric intake on oxidized and carbonylated proteins from adipose tissues of healthy men [173]; (ii) fasting on Cys-oxidized proteins from healthy animals [174]; (iii) high-fat and high-sucrose diet on carbonylated proteins from tissues and body fluids of healthy animals [175]; (iv) the assumption of glutathione derivatives on Tyr-nitrated proteins from brain-injured animal models [176]; (v) antioxidant-fortified diet on carbonylated and Tyr-nitrated proteins from brain tissues of animal models of AD [177]; (vi) high-fat and alcohol diet on carbonylated and Cys-oxidized proteins from tissues and body fluids of fatty liver disease patients and related animal models [178–184]. In the close future, it is hypothesized that redox proteomics studies will also be performed on organisms or animal models of human diseases fed with oat compounds and derivatives, including isolated natural and recombinant Avns, in order to evaluate the capacity of such nutraceuticals to modulate oxidized and nitrosized proteomes in target tissues and body fluids.

10. Concluding Remarks

It is generally accepted that antioxidants exert health-promoting effects by scavenging intracellular ROS; thus, their consumption as food additives and nutraceuticals has been greatly encouraged. Nonetheless, to date, there is little clinical evidence for the long-term benefits of most antioxidants, while there are even alarms of health risks consequent to supplementation of lipophilic antioxidants [68]. Accordingly, the existence of a physiological role of specific ROS concentrations can explain the negative results from clinical trials, where large doses of exogenously-administered antioxidants or hyperactivation of antioxidant pathways with electrophilic therapeutics failed to improve outcomes of oxidative stress-related diseases or resulted even deleterious [63, 67, 185, 186]. Indeed, it is now well established that redox reactions bear the Janus faceted feature of promoting both physiological signaling responses and pathological cues in all biological systems, as well as that endogenous antioxidant molecules and mechanisms participate in both scenarios [63, 67]. Consistently, emerging evidence demonstrates that only intermediate levels of major regulators of antioxidant responses are beneficial, although both the low and high concentration thresholds for physiological versus pathological effects may vary largely depending on genetic and environmental factors and the cellular context [185]. Thus, given that most of the emerging therapeutic compounds with antioxidant properties influence redox-sensitive mechanisms, both their low and high concentration thresholds for physiological versus pathological effects have to be carefully considered.

In this light, further studies are necessary to fully address the beneficial effects of Avns in human health, including antioxidant, antiproliferative, anti-inflammatory, antiaging, and anticancer activities (Figure 2). In particular, useful insights could be derived from foodomics and redox proteomics

studies aimed at a comprehensive characterization of molecules and mechanisms that mediate the pleiotropic effects of Avns in cellular and animal models of human diseases, including oxidative posttranslational modifications of structural and regulatory proteins. Moreover, novel therapeutic approaches, including combinatorial therapy and nanotechnology-based targeted drug delivery, are encouraged in order to allow site-directed application, appropriate dosing regimens, pharmacological repair of oxidized biomolecules, and triggering of endogenous antioxidant response systems, which could also be guided by the identification of predictive biomarkers.

Abbreviations

Avn: Avenanthramide
CCM: Cerebral cavernous malformations
CHD: Coronary heart disease
COX-2: Cyclooxygenase-2
FOXO1: Forkhead box protein O1
HO-1: Heme oxygenase-1
ICAM-1: Intercellular adhesion molecule 1
IκB: Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor
LDL: Low-density lipoprotein
MMP: Matrix metalloproteinase
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
VCAM-1: Vascular cell adhesion molecule 1
ROS: Reactive oxygen species
SOD2: Superoxide dismutase 2
TGF-β: Transforming growth factor-β
TNFα: Tumor necrosis factor *alpha*
VSMC: Vascular smooth muscle cells
YAvn: Yeast avenanthramide.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Andrea Perrelli and Luca Goitre contributed equally to this work.

Acknowledgments

The authors are grateful to Eliana Trapani, Valerio Benedetti, Giulia Costantino, Federica Geddo, Alessia Zotta, Giovanna Bratti, and Gaudenzio Inverso for critically reading the manuscript and suggesting improvements, and to Santina Barbaro for helpful discussion. They also acknowledge the Italian Research Network for Cerebral Cavernous Malformation (CCM Italia, http://www.ccmitalia.unito.it/) and the Associazione Italiana Angiomi Cavernosi (AIAC, http://www.ccmitalia.unito.it/aiac/) for fundamental collaboration and support. Figure 2 is an artwork of Federica Geddo. This work was supported by grants from Telethon (GGP15219/coordinator) and the University of Torino (Local Research Funding 2014-17) to Saverio Francesco Retta.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 2018306, 9 pages
https://doi.org/10.1155/2018/2018306

Review Article

Protective Role of Carbonic Anhydrases III and VII in Cellular Defense Mechanisms upon Redox Unbalance

Anna Di Fiore,[1] **Daria M. Monti**,[2] **Andrea Scaloni**,[3] **Giuseppina De Simone**,[1] **and Simona M. Monti**[1]

[1]*Institute of Biostructures and Bioimaging, National Research Council, 80134 Naples, Italy*
[2]*Department of Chemical Sciences, University of Naples Federico II, 80126 Naples, Italy*
[3]*Proteomics and Mass Spectrometry Laboratory, ISPAAM, National Research Council, 80147 Naples, Italy*

Correspondence should be addressed to Giuseppina De Simone; gdesimon@unina.it and Simona M. Monti; marmonti@unina.it

Received 28 May 2018; Accepted 24 July 2018; Published 5 August 2018

Academic Editor: Reiko Matsui

Under oxidative stress conditions, several constitutive cellular defense systems are activated, which involve both enzymatic systems and molecules with antioxidant properties such as glutathione and vitamins. In addition, proteins containing reactive sulfhydryl groups may eventually undergo reversible redox modifications whose products act as protective shields able to avoid further permanent molecular oxidative damage either in stressful conditions or under pathological circumstances. After the recovery of normal redox conditions, the reduced state of protein sulfhydryl groups is restored. In this context, carbonic anhydrases (CAs) III and VII, which are human metalloenzymes catalyzing the reversible hydration of carbon dioxide to bicarbonate and proton, have been identified to play an antioxidant role in cells where oxidative damage occurs. Both proteins are mainly localized in tissues characterized by a high rate of oxygen consumption, and contain on their molecular surface two reactive cysteine residues eventually undergoing S-glutathionylation. Here, we will provide an overview on the molecular and functional features of these proteins highlighting their implications into molecular processes occurring during oxidative stress conditions.

1. Introduction

In physiological conditions, reactive oxygen species (ROS) are generated intracellularly as a result of metabolism in peroxisomes, mitochondria, and by several cytosolic enzymes [1]. However, their generation is also triggered by exogenous sources, such as UV-light, chemotherapeutics, inflammatory cytokines, and ionizing radiations [1, 2]. Normally, the levels of ROS in cells are tightly regulated by sophisticated enzymatic and nonenzymatic antioxidant defense systems, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione (GSH), and vitamins (vitamin A, C, and E). Generated ROS, within certain boundaries, are fundamental to preserve cell homeostasis and serve as important regulatory mechanisms for many cellular activities [1]. When ROS levels are unbalanced, detrimental effects on the physiological functions of the cell may occur. This can eventually lead to accelerated aging, age-related diseases, and, ultimately, to cell death [1]. ROS, which include oxygen-centered radical species, as hydroxyl radical ($^{\cdot}OH$), superoxide anion ($O_2^{\cdot-}$), and peroxyl radical ($R\text{-}O_2^{\cdot}$); and nonradical compounds, as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and ozone (O_3), are known to modify proteins, nucleic acids, and lipids through a number of oxidative pathways [3–6].

In proteins, oxidative posttranslational modifications can either be permanent, as result of irreversible molecular oxidative damage, or temporary, which prevent lasting oxidative damage under stressful cellular conditions [7]. Cysteine is the most susceptible residue toward oxidative modifications. This is a nonabundant amino acid, being less than 3% compared to the other residues of the mammalian proteome [8]. It is nucleophilic, redox sensitive, and can undergo reversible and irreversible modifications in response to an altered localized redox environment [9]. Cysteine thiols have a pKa value of about 8.5 [10]. For this reason, they are not

reactive at physiological pH values. However, within the protein three-dimensional structure, cysteines can be affected by the presence of specific residues altering their reactivity. In particular, the spatial proximity of acidic residues will raise the pKa value of thiol group, leaving cysteine uncharged and making it less prone to modification [11]. On the contrary, an alkaline environment given by the vicinal presence of basic residues can lower the corresponding pKa value, facilitating thiol deprotonation [11]. Cys modification is also affected by accessibility, since steric factors may prevent its alteration, even if the pKa of the thiol residue is relatively low [12]. For these reasons, oxidation of reactive cysteines is a highly selective process [13–15].

The thiolate anion renders proteins susceptible targets for a variety of oxidative modifications, generating cysteine derivatives containing intramolecular and intermolecular disulfide linkages, adducted species bearing disulfide bonds with low-molecular mass nonprotein thiols (mainly with GSH and to a lesser extent with free cysteine, forming PS-SG and PS-SCys, resp.) and sulfenic acid (P-SOH) (Figure 1). Sulfenic acid-containing proteins can undergo glutathionylation or be further oxidized leading to the irreversible formation of sulfinic ($P\text{-}SO_2H$) and/or sulfonic acid ($P\text{-}SO_3H$) derivatives [9]. The latter oxidative modifications often can irreversibly alter the structure and function of the involved protein. On the contrary, protein S-glutathionylation is a reversible reaction that can be efficiently reverted primarily by glutaredoxin (GRx) [16] and also by thioredoxin (TRx) [17], sulfiredoxin [18], or spontaneously in the presence of a high ratio of GSH/GSSG [19] (Figure 1). In oxidative stress conditions, protein thiolate anion may also undergo S-nitrosylation with reactive nitrosative species (RNS). Resulting protein S-nitrosothiol has a relatively short half-life since it reacts with physiological GSH forming the S-glutathionylated protein [9, 20, 21] (Figure 1).

Reversible S-modification can affect protein structure and function; thus, it has been reported as a molecular switch able to reversibly activate or deactivate regulatory processes, such as serving to transduce redox signals, control of gene expression, cell proliferation, and apoptosis [15, 22]. Besides its role in redox signal transduction, S-glutathionylation has been suggested also to be a cellular mechanism by which cells preserve enzyme functionality from further irreversible oxidation [22, 23].

Among the different antioxidant systems that cells have developed, it has been reported that proteins containing reactive cysteines could play a protective role against oxidative insult occurring either in stressful conditions or under pathological circumstances. These proteins participate in the cellular defense system by means of reversible thiol modification of their cysteine residues. In this view, we here report on the antioxidant role of two human metalloenzymes, namely, carbonic anhydrases (CAs) III and VII, whose function in mediating the oxidative insult and aging has been recently reported [24].

CAs are ubiquitous enzymes [25], encoded by seven genetically distinct gene families: α-, β-, γ-, δ-, ζ-, η-, and θ-CAs [25–30]. Human CAs (hCAs) belong to the α-class with fifteen isoforms being so far identified that differ in tissue distribution, catalytic activity, response to inhibitors, and cellular localization. Indeed, five isoforms are cytosolic (CAs I–III, VII, and XIII), four are membrane-associated (CAs IV, IX, XII, and XIV), two are mitochondrial (CAs VA and VB), and one is secreted in milk and saliva (CA VI) [25, 31, 32]. CAs catalyze a simple physiological reaction, the reversible hydration of carbon dioxide to bicarbonate and proton, following a two-step mechanism [33, 34]. Several studies reported that CAs are involved in a variety of physiological processes, such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, and ureagenesis. Consequently, abnormal levels or activities of these enzymes have been often associated with different human pathological conditions [31, 35].

Among the cytosolic CA isoforms, CA III and CA VII are enzymes having properties that deserve further attention [24]. In particular, recent studies showed that these proteins are mainly present in tissues characterized by a high oxygen consumption rate, such as skeletal muscle, liver, and brain, where they could participate in cell defense processes counteracting oxidative damage [24, 36]. In this review, by examining CA III and CA VII functional and structural features, we will provide insights into their newly proposed protective role against oxidative stress.

1.1. Tissue Distribution, Catalytic Activity, and Molecular Features of CA III and CA VII. The distribution pattern of CA III has been investigated using different techniques, such as Western blotting and immunohistochemistry experiments, indicating that this isoform is highly expressed in liver and skeletal muscle [37] and at lower levels in other tissues [37–45]. CA VII was thought initially to have a more limited tissue distribution, being detected only in some brain regions including the cortex, hippocampus, and thalamus where it plays a role as a molecular switch for GABAergic excitation [46–48]. Subsequently, CA VII has been found also in other human tissues such as colon, muscle, and liver [49], revealing a strong similarity with CA III.

CA III and CA VII present also high similarity in their primary structures with 49% sequence identity (Figure 2). Notably, they contain a higher number of cysteine residues (5 and 4, resp.) with respect to other cytosolic CAs having only a single cysteine (Figure 2) [24].

It has been reported that both enzymes have two highly reactive cysteines on the protein surface, namely, Cys183 and Cys188 for CA III and Cys183 and Cys217 for CA VII (numbering refers to hCA I isoform [50]). These cysteines have been reported to be S-glutathionylated both *in vitro* [51] and *in vivo* [52, 53], without affecting enzyme catalytic activity [51, 53]. Thus, the localization of CA III and CA VII in organs and tissues characterized by a high propensity for oxidative stress, combined with the presence of reactive sulfhydryls in their primary structure, provided the first evidence that these enzymes may act as oxyradical scavengers involved in cell protection from oxidative damage.

Despite the above-described similarities, CA III and CA VII present a very different catalytic efficiencies for hydration of CO_2, with CA VII being one of the most efficient catalysts among mammalian isoforms [51, 54] and CA III being the

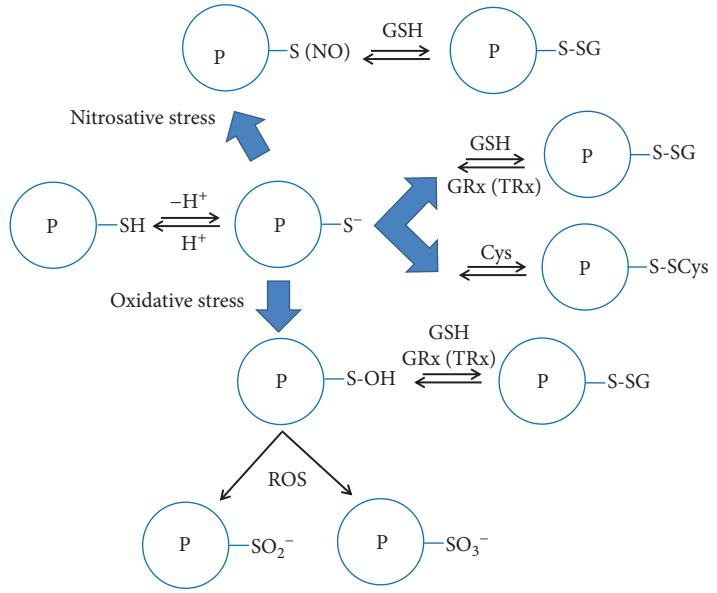

Figure 1: Schematic representation of protein oxidative modifications involving its reactive cysteine and nonprotein thiols with low molecular weight mass.

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hCA  III   2   -AKEWGYASHNGPDHWHELFPNAKGENQSPVELHTKDIRHDPSLQPWSVSYDGGSAKTILNNGKTCRVVF
hCA  VII   0  TGHHGWGYGQDDGPSHWHKLYPIAQGDRQSPINIISSQAVYSPSLQPLELSYEACMSLSITNNGHSVQVDF
hCA  I     1  -ASPDWGYDDKNGPEQWSKLYPIANGNNQSPVDIKTSETKHDTSLKPISVSYNPATAKEIINVGHSFHVNF
hCA  II    2  --SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEF
hCA  XIII  1  -SRLSWGYREHNGPIHWKEFFPIADGDQQSPIEIKTKEVKYDSSLRPLSIKYDPSSAKIISNSGHSFNVDF

hCA  III   71  DDTYDRSMLRGGPLPGPYRLRQFHLHWGSSDDHGSEHTVDGVKYAAELHLVHWN-PKYNTFKEALKQRDG
hCA  VII   71  NDSDDRTVVTGGPLEGPYRLKQFHFHWGKKHDVGSEHTVDGKSFPSELHLVHWNAKKYSTFGEAASAPDG
hCA  I     71  EDNDNRSVLKGGPFSDSYRLFQFHFHWGSTNEHGSEHTVDGVKYSAELHVAHWNSAKYSSLAEAASKADG
hCA  II    71  DDSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWN-TKYGDFGKAVQQPDG
hCA  XIII  71  DDTENKSVLRGGPLTGSYRLRQVHLHWGSADDHGSEHIVDGVSYAAELHVVHWNSDKYPSFVEAAHEPDG

hCA  III   141  IAVIGIFLKIGHENGEFQIFLDALDKIKTKGKEAPFTKFDPSCLFPACRDYWTYQGSFTTPPCEECIVW
hCA  VII   141  LAVVGVFLETGDEHPSMNRLTDALYMVRFKGTKAQFSCFNPKCLLPASRHYWTYPGSLTTPPLSESVTW
hCA  I     141  LAVIGVLMKVGEANPKLQKVLDALQAIKTKGKRAPFTNFDPSTLLPSSLDFWTYPGSLTHPPLYESVTW
hCA  II    141  LAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTW
hCA  XIII  141  LAVLGVFLQIGEPNSQLQKITDTLDSIKEKGKQTRFTNFDLLSLLPPSWDYWTYPGSLTVPPLLESVTW

hCA  III   210  LLLKEPMTVSSDQMAKLRSLLSSAENEPPVPLVSNWRPPQPINNRVVRASFK-
hCA  VII   210  IVLREPICISERQMGKFRSLLFTSEDDERIHMVNNFRPPQPLKGRVVKASFRA
hCA  I     210  IICKESISVSSEQLAQFRSLLSNVEGDNAVPMQHNNRPTQPLKGRTVRASF--
hCA  II    210  IVLKEPISVSSEQVLKFRKLNFNGEGEPEELMVDNWRPAQPLKNRQIKASFK-
hCA  XIII  210  IVLKQPINISSQQLAKFRSLLCTAEGEAAAFLVSNHRPPQPLKGRKVRASFH-
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Figure 2: Sequence alignment of cytosolic hCAs. Reactive cysteines of CA III and VII are highlighted in green and reduced ones in yellow, whereas CA VII cysteines involved in the formation of an intramolecular disulfide bond are in pink.

worst one (k_{cat}/K_M of $0.3 \times 10^6\,M^{-1}\,s^{-1}$ for CA III and of $7.2 \times 10^7\,M^{-1}\,s^{-1}$ for CA VII) [55].

Structural studies on hCA III [56] and on a mutated form of hCA VII, named dmCA VII [54, 57], showed that both enzymes adopt a three-dimensional arrangement similar to that of other cytosolic CAs [50, 58]. Both proteins are monomeric, and their fold presents a central ten-stranded β-sheet surrounded by additional β-strands and several α- and 3_{10}-helices. Their active site is located in a large conical cavity, containing the catalytic zinc ion at the base, which is coordinated by three conserved histidine residues (His94, His96, and His119). The X-ray structures of bovine and rat CA III have also been solved [53, 59].

Remarkably, the structural analysis of rat CA III provided very interesting data clarifying the molecular determinants responsible for the above-mentioned high redox reactivity of Cys183 and Cys188. Indeed, these two residues, which are located on the molecular surface of the protein, were

found S-glutathionylated in the crystal structure [53]. Since analysis of the binding sites gave no evidence for a specific recognition of GSH, it was suggested that S-glutathionylation was due to the high reactivity of Cys183 and Cys188 and to the great abundance of GSH in cell, which reaches *in vivo* millimolar concentration [2, 23, 60]. The disulfide linkage between the cysteine residue and the GSH molecule does not alter the overall structure of the protein, nor the conformation of residues located close to Cys183 and Cys188, in agreement with the observation that S-glutathionylation does not have effect on the catalytic activity of the enzyme [53]. Moreover, the electron density maps of the two Cys-GSH adducts indicated a conformational flexibility of the glutathionyl moieties, with the disulfide bridge involving Cys183 adopting two different orientations, and that involving Cys188 only one.

Among the two cysteines, Cys188 is located in an environment characterized by a lower negative charge, thus explaining its greater propensity to react [61]. On the contrary, Cys183 is located in a depression of the surface showing a greater negative charge, making this residue less reactive. Two positively charged residues surrounding Cys188, namely, Lys213 and Arg189, were hypothesized to act as main modulators of thiol reactivity, lowering its pKa value. Interestingly, mutagenesis studies showed that only Lys213 is responsible for the lowering of the pKa value of Cys188, whereas Arg189 seems not to affect it. In addition, the acid residues Asp190 and Glu214 were also found to interact with the thiol of Cys188, decreasing its reactivity and partially counteracting the presence of the above-mentioned basic residues (Figures 3(a) and 3(b)) [61]. The crystallographic structure of S-glutathionylated CA III provided also information on the different reactivity of adducted protein thiols towards the process of deglutathionylation, with the Cys188-glutathione adduct being more accessible to nucleophilic attack compared to the corresponding Cys183-adduct, due to its greater steric accessibility [53].

In the case of CA VII, Cys54 and Cys178 were proved to be involved in an intramolecular disulfide bond, whereas the two remaining cysteines, Cys183 and Cys217, are exposed on the molecular surface [54, 57]. At present, no crystallographic information is available for S-glutathionylated Cys183 and Cys217. Thus, starting from the crystallographic structure of dmCA VII, we have built a model of hCA VII and used it to investigate the chemical environment potentially affecting Cys183 and Cys217 reactivity. Interestingly, we observed that Cys183 is located within a region devoid of acidic residues. The presence of a basic residue (His154) distant about 5 Å from the side chain of Cys183 could lower its pKa value, making it highly reactive (Figure 3(c)). His154 is located also in close proximity of Cys217; however, the presence of several acidic residues located nearby the thiol group may diminish its reactivity (Figure 3(d)). Further studies are necessary to clarify this matter.

1.2. Role of CA III as Antioxidant Agent. Important results elucidating the physiological role of CA III in aging and aging-related processes have been obtained from a study on the nucleus pulposus (NP) phenotype [62]. NP is part of the intervertebral disc, and its integrity is strictly related to intervertebral disc degeneration. This, in turn, is associated with low back and neck pain, leading to a very common disability in the United States [63, 64]. One of the major etiologic factors responsible for this pathological condition is aging disc [65], which is characterized by the time-dependent accumulation of cellular and molecular damage, predominantly caused by oxidative and inflammatory processes [65, 66]. Notably, NP is a hypoxic niche where the two hypoxia-inducible CAs IX and XII are robustly expressed and regulate intracellular pH level, which is essential for the maintenance of cellular functions [67]. mRNA and protein expression of CA III are hypoxia-sensitive, being upregulated in low oxygen tension. However, unlike CAs IX and XII, CA III expression is insensitive to HIF-1α, and this isoform does not play a role in the regulation of intracellular pH homeostasis [62], but it participates to the antioxidant defense mechanism of NP cells. Indeed, NP cells silenced for CA III expression showed high sensitivity to oxidative stress-dependent apoptosis through caspase-3 activation. Therefore, it has been suggested that mechanisms regulating CA III expression may represent novel therapeutic targets to reduce the negative effects of oxidative damage associated with aging in the degeneration of the intervertebral disc [62].

In agreement with this proposed protective role of CA III, it was demonstrated that this protein inhibited apoptosis in H_2O_2-stressed mature osteocytes [68] and in cotransfected NIH/3T3 cells [69]. In particular, CA III expression increased with osteoblast differentiation and was also involved in diminishing ROS production and in protecting cells from hypoxic stress [68]. Interesting data were also reported on the repression of CA III gene transcription in Rat1 cells expressing high level of Evi1 [70], a zinc finger protein involved into cancer progression [71]. In these cells, Evi1 overexpression corresponded to a decreased level of CA III and to an enhanced sensitivity to H_2O_2-induced apoptosis [70]. These results may be used to exploit novel therapies targeting oxidative stress.

Even though many papers describe the role of CA III in cellular defense processes from oxidative insult, very little information is available on the molecular mechanisms responsible for this behavior. It has been suggested that the antioxidant activity of this protein is related to the presence of the above-described reactive cysteines, which *in vivo* are S-glutathionylated [53]. To get more insights into this process, Mallis and coworkers investigated in detail the chemical modification of CA III sulfhydryl groups upon exposure to different oxidant agents, such as H_2O_2, HOCl, or peroxy radicals [72]. The authors revealed that the type of cysteine modification depends on cellular GSH content. Indeed, CA III was reversibly S-glutathionylated when GSH concentration was approximately equimolar to that of protein thiols, whereas irreversible oxidation of cysteines to sulfinic or sulfonic acid derivatives occurred at low GSH levels. In agreement with these data, CA III was found to be highly and irreversibly oxidized in old rats with respect to young animals, since reduced GSH levels are a hallmark of aging.

Further insights into the role and mechanism of action of CA III as antioxidant agent were obtained by Zimmerman

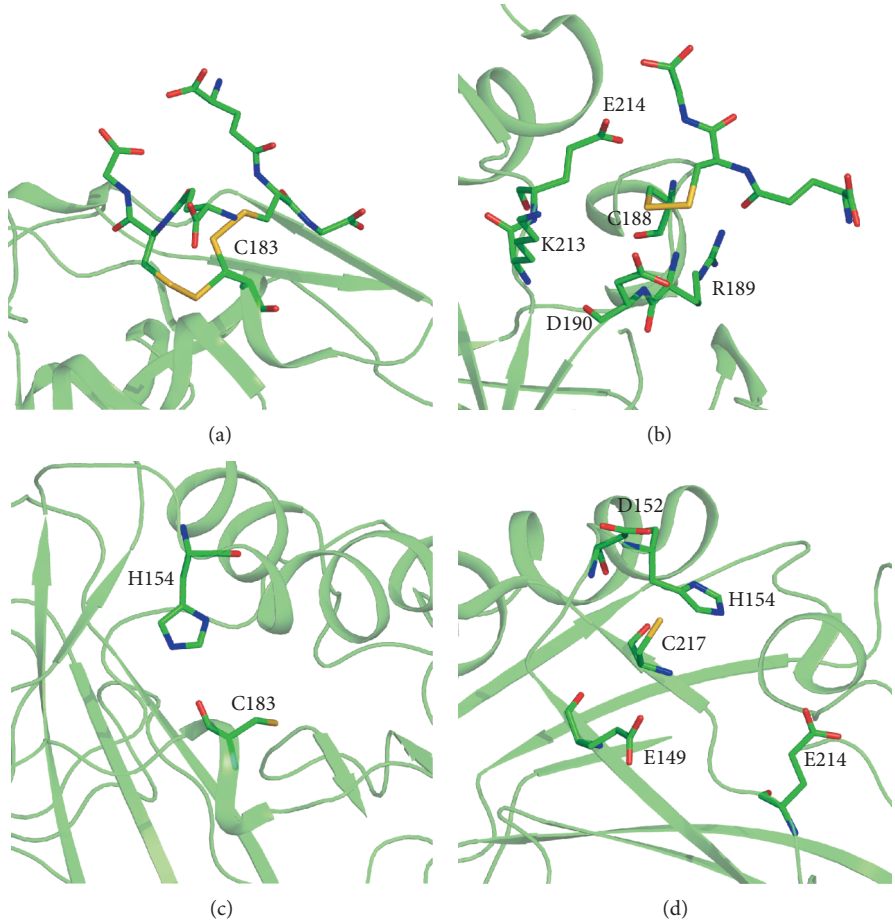

Figure 3: Chemical environment of CA III and VII reactive cysteines. (a) S-glutathionylated Cys183 of CA III showing two alternative conformations. (b) S-glutathionylated Cys188 of CA III surrounded by Arg189, Asp190, Lys213, and Glu214 which affect its pKa. (c) Model of Cys183 in CA VII structure adjacent to His154. (d) Model of Cys217 in CA VII structure showing the nearby acidic and basic residues.

et al. through the evaluation of S-glutathionylation and irreversible oxidation of the two protein reactive cysteines in the presence of increasing intensities of the oxidative stress insult [73]. These analyses indicated that Cys183 and Cys188 were differentially oxidized in skeletal muscle when different oxidative stress conditions were induced. Specifically, only Cys188 underwent reversible S-glutathionylation under a mild or brief redox stress, involving about 20% of total skeletal muscle protein, whereas a prolonged or harsh stress produced irreversible oxidation of both sulfhydryl groups. Thus, the high content of CA III in skeletal muscle could represent a storage of reactive sulfhydryl moieties able to repair acute and chronic oxidative insults. Moreover, in resting skeletal muscle, less than 10% of CA III was S-glutathionylated, suggesting that cysteine oxidation may represent a regulative physiological mechanism performed by the enzyme within the cell. A further proof of a possible involvement of CA III in the glutathione-mediated antioxidant processes was obtained by comparing the gene expression profile of CA3-knockout and wild type mice by microarray strategy [73]. Even though CA III-deficient mice had a normal development, fertility, and longevity, at least under experimental standard laboratory conditions, they showed a transcriptional alteration of more than 500 out of 12,000 genes analyzed; most of them were associated with the GSH-mediated antioxidative system [74].

1.3. Role of CA VII as Antioxidant Agent. Available data on reactive cysteines in CA VII [51], together with the observation that this enzyme is expressed in tissues with a high oxygen consumption rate (similarly to CA III), led us to hypothesize that this protein may have a functional role *in vivo*, that is of protecting cells from oxidative stress [52]. To test this hypothesis, human cancer (HeLa) cells, which usually do not express endogenous CA VII, were transiently transfected to express the wild-type protein, in the presence or in the absence of oxidative stress. Consistent with our hypothesis, cells expressing CA VII were less sensitive to apoptosis induced by oxidative stress. This was clearly demonstrated by measuring: (i) apoptotic protein levels and (ii) apoptotic cells. In particular, mock-transfected cells showed a significant alteration in procaspases (8 and 3), Bcl-2 and Bax levels after induction of oxidative stress, whereas corresponding protein levels were almost unaltered in CA VII

expressing cells. Moreover, in the presence of oxidative stress, an increase in CA VII expression was observed, suggesting an attempt by the cell to protect itself by overexpressing the protein [52]. A further confirmation of the importance of the cysteine residues was obtained by performing the same experiments in the presence of a mutated version of the protein, in which all cysteines were replaced by serines (TM-CA VII). When cells were transiently transfected with the vector encoding for the mutated protein and then stressed, no protection was observed. Indeed, a similar alteration in procaspase-3 and 8, Bax and Bcl-2 levels as well as apoptotic cells, was observed for mock and TM-CA VII transfected cells [52]. Thus, cysteine residues in CA VII exert a protective functional role. Notably, the lack of the protective effect was not related to a different catalytic activity of the mutated enzyme, since it showed the same kinetic parameters of native CA VII [52].

It is commonly accepted that increased levels of ROS are related to oxidative stress, and may lead to the development of many pathologies, including cancer [75–78]. Interestingly, a reduced CA VII expression is observed in colorectal carcinoma [79], thus allowing one to speculate that low protein levels may be related to a higher cellular sensitivity to oxidative stress and cancer progression.

2. Conclusions

In summary, cells have developed different sophisticated defense systems to counteract oxidative stress, and their ability to respond to ROS/RNS production is connected to aging, cancer, and other disease states. Among the several molecular systems, the contribution of CA III and CA VII as scavengers towards oxidative insult has been recently proposed, highlighting an unexpected functional role of these two CAs. Notably, these CAs are abundantly expressed in tissues such as brain, liver, and skeletal muscle, which present high oxygen consumption rates. By means of different approaches including *in vitro* and *in vivo* experiments, it was shown that CA III and CA VII exert their protective role due to the presence of reactive cysteines on their protein surface.

In particular, from a molecular point of view, the chemical environment generated by residues nearby the reactive sulfhydryls of CA III and CA VII assists the formation of a thiolate anion which may undergo S-glutathionylation. This reversible modification is thought to protect cellular proteins and preserves their functionality under oxidative stress conditions.

Significant findings on the peculiar role of these proteins were obtained by the observation that both CA III and CA VII protect cells from apoptosis induced by oxidative agents, thus participating in the cellular defense system. Further studies are necessary to better clarify their molecular and biological mechanism and a putative role as novel antiaging molecules.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

All authors drafted the manuscript and approved the final version of the manuscript.

Acknowledgments

The authors thank Luca De Luca, Maurizio Amendola, and Giosuè Sorrentino for their technical assistance. This work was supported by a grant from CNR-DSB Progetto Bandiera "InterOmics".

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 1454936, 11 pages
https://doi.org/10.1155/2018/1454936

Review Article

Antioxidants from Plants Protect against Skin Photoaging

Ganna Petruk,[1] Rita Del Giudice,[2] Maria Manuela Rigano,[3] and Daria Maria Monti[1,4]

[1]*Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, via Cinthia 4, 80126 Naples, Italy*
[2]*Department of Experimental Medical Science, Lund University, 221 84 Lund, Sweden*
[3]*Department of Agricultural Sciences, University of Naples Federico II, via Università 100, 80055 Portici, Naples, Italy*
[4]*Istituto Nazionale di Biostrutture e Biosistemi (INBB), Rome, Italy*

Correspondence should be addressed to Daria Maria Monti; mdmonti@unina.it

Received 26 April 2018; Revised 5 July 2018; Accepted 11 July 2018; Published 2 August 2018

Academic Editor: Laura Bravo

Exposure to UV light triggers the rapid generation and accumulation of reactive oxygen species (ROS) in skin cells, with consequent increase in oxidative stress and thus in photoaging. Exogenous supplementation with dietary antioxidants and/or skin pretreatment with antioxidant-based lotions before sun exposure might be a winning strategy against age-related skin pathologies. In this context, plants produce many secondary metabolites to protect themselves from UV radiations and these compounds can also protect the skin from photoaging. Phenolic compounds, ascorbic acid and carotenoids, derived from different plant species, are able to protect the skin by preventing UV penetration, reducing inflammation and oxidative stress, and influencing several survival signalling pathways. In this review, we focus our attention on the double role of oxidants in cell metabolism and on environmental and xenobiotic agents involved in skin photoaging. Moreover, we discuss the protective role of dietary antioxidants from fruits and vegetables and report their antiaging properties related to the reduction of oxidative stress pathways.

1. Introduction

Reactive oxygen species (ROS) are normally produced in cell metabolism, but, when the balance between free radicals and antioxidants favours the former, they can also take part in a pathological process known as oxidative stress. Oxidative stress may result in cell damage, thus leading to the development of many types of diseases, as well as aging [1]. With aging, a decreased performance of cell endogenous antioxidant system occurs; thus, elderly people are more susceptible to oxidative stress [2, 3]. Several secondary plant metabolites are endowed with antioxidant activity and have been studied to prevent, retard, and control the development of age-related pathologies [4]. The skin is considered the largest organ with a protective role against external noxious sources, such as UV radiations. In particular, exposure to UV light triggers the rapid generation and accumulation of ROS in skin cells, which may result in photoaging. In this review, we focus our attention on the role of oxidants in their physiological context and in pathological conditions, with a special attention on skin photoaging. Then, the protective role of antioxidants from fruits and vegetables is discussed. Their antiaging properties, related to the activity of intracellular oxidative stress pathways, are reported.

2. Physiological Role of Oxidants

All life processes are governed by redox signalling; thus, the maintenance of a physiological level of oxidants is mandatory for proper cellular functioning. This can be obtained by switching on/off some regulation pathways or programmed cell death. Oxidants are responsible for a well-known process, senescence, as they are involved in telomere shortening. Different authors demonstrated that cells grown in the presence of strong oxidative environments have a shorter life span compared with cells grown in low oxygen tension [5–7]. Indeed, oxygen is one of the most abundant oxidants. This chemical element is necessary for all aerobic organisms and acts as terminal oxidant in the mitochondrial respiratory

chain, which is the main source of energy for the cell [8]. In eukaryotic cells, oxygen can be partially reduced by several enzymatic and nonenzymatic reactions, thus inducing the production of reactive intermediates, such as superoxide radical ($O_2^{\bullet-}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), and hydroxyl ($HO^{\bullet}$), better known as reactive oxygen species (ROS). All these molecules need to be stabilized by reacting with other molecules, such as nitric oxide ($NO^{\bullet}$), and forming reactive nitrogen species (RNS). This constitutes the basis for the formation of a multitude of additional oxidative signalling elements, including the highly reactive and potentially damaging peroxynitrite ($ONOO^{-}$) [9, 10]. Both ROS and RNS may target cysteine thiols, leading to oxidative modifications and to the formation of reactive sulphur species (RSS) [11].

Despite this, a small, nontoxic increase in ROS levels plays a key role in the prevention of the insurgence of different diseases by assisting the immune system, mediating cell signalling, and playing an essential role in apoptosis [12]. Indeed, ROS can alter the mitochondrial membrane potential and induce the release of cytochrome c, which induces caspase activation [13]. Cellular oxidants are mainly by-products of endogenous processes: (1) mitochondrial ATP production, (2) phagocytosis, (3) β-oxidation of long-chain fatty acids (>C20), and (4) other metabolic pathways, such as inflammation [14, 15]. Normally, damages caused by free radicals are repaired by a class of molecules named antioxidants. However, when antioxidant defences are not adequate, that is, when excessive amounts of free radicals are generated, the cell undergoes oxidative stress. In this condition, several damages may occur at protein, enzyme, lipid, and nucleic acid levels. In the latter, the production of reactive singlet oxygen can react with all DNA bases. More in detail, when the single oxygen reacts with guanine, the process generates 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) [16]. While guanine normally pairs with cytosine, 8-oxo-dG pairs with adenine; thus, the resulting point mutation will be translated in a mutated protein.

Generally, cell damages may alter downstream cell signalling and cause a variety of diseases, such as cardiovascular diseases, neurodegenerative disorders, cancers, and also aging, including skin aging [17–25].

3. Environmental and Xenobiotic Agents Involved in Skin Aging

Aging is a complex biological process, as it induces progressive deterioration of anatomical structures and of the physiological functions of the organs [26]. The skin is the outermost barrier of the body and the biggest organ, and its changes are among the most visible signs of aging. Indeed, with aging, the skin loses some of its properties, such as elasticity, thickness, and colour [27]. The normal cellular oxidative metabolism can generate different by-products responsible for molecular damage, thus contributing to skin aging (intrinsic aging). However, it has been reported that up to 80–90% of skin aging is due to environmental and xenobiotic agents (extrinsic aging) [26, 28].

Several external factors may represent a cause of free radical production and consequently induce skin aging. Among them, it is worth mentioning air and water contaminants, tobacco smoke, different organic solvents, several drugs (such as bleomycin and gentamycin), kitchen scraps (i.e., used oil and fat), and heavy or transition metals (such as lead, cadmium, mercury, and iron) ([18] and references therein). In particular, air pollution includes biological and gaseous contaminants, as well as particulate. Pollution has been reported to exert deleterious effects on the skin in different ways [29]: (a) ultrafine particles can penetrate tissues and localize in mitochondria, thus inducing ROS generation [30]; (b) diesel exhaust particles induce activation of the inflammatory cascade in keratinocytes [31]; (c) pollutants are among the activators of the aryl hydrocarbon receptor (AhR), a cytosolic ligand-activated transcription factor that regulates cellular proliferation, inflammation, and melanogenesis [32]; and (d) pollutants can alter skin microflora [33, 34]. Another external stress factor is arsenic. This chemical element is widely present in food, water, air, and soil and is mostly found in its trivalent (As^{3+}, such as sodium arsenite and arsenic trioxide) or pentavalent (As^{5+}) inorganic form. There are many pieces of evidence demonstrating that the deleterious effects of trioxide arsenic are mostly due to its inorganic state, rather than to the organic form [35–37], since it induces the generation of free radicals in cells and, consequently, leads to oxidative stress, resulting in oxidative DNA damage and finally into apoptosis [38–42].

Tobacco smoking has been underestimated as stress factor for a long time and its damages have resulted more evident with the increase of life expectancy [43, 44]. Smoking dates back to as early as 5000 BC in the Americas in shamanistic rituals [45], but the first report that described a link between smoking and cancer was published in 1928 [46]. To date, in PubMed, there are more than 53,000 research entries on this theme. Despite that, millions of people continue to smoke and the risk of cancer, as well as premature skin aging, is ignored [47, 48].

Nowadays, sunlight is among the most harmful exogenous factors able to induce ROS formation. The spectrum of sunlight includes infrared energy (above 760 nm), visible light (400–760 nm), and ultraviolet (UV) light (below 400 nm) [49]. UV radiations can be further divided in UVA (400–315 nm), UVB (315–280 nm), and UVC (280–100 nm). Photobiological responses are mostly generated by exposure to UVB and UVA radiations. UV radiations are the major cause of stem cell DNA damage; they can contribute to depletion of stem cells and damage of stem cell niche, eventually leading to photoinduced skin aging [50]. In particular, in the UVB range, direct light absorption by DNA mainly results in dimerization reactions between adjacent pyrimidine bases. Nevertheless, UVA radiations are considered more dangerous than UVB as, although they are weakly absorbed by DNA, they can excite endogenous chromophores, leading to DNA damage. Moreover, several endogenous and exogenous molecules, once exposed to photoexcitation, can lead to ROS formation [51].

4. Skin Photoaging as a Consequence of Oxidative Stress

Exposure to UV irradiation induces photochronical generation of ROS that activates cell surface growth factors,

Table 1: Defence levels and mechanism of action of antioxidants.

The first line of defence		The second line of defence		The third line of defence	
Antioxidant	Mechanism of action	Antioxidant	Mechanism of action	De novo enzymes	Mechanism of action
Superoxide dismutase	$O_2^{\bullet -} \rightarrow H_2O_2$	Ascorbic acid	Chain breaking: donate an electron to the free radical	Polymerases	DNA repair
Catalase	$2H_2O_2 \rightarrow O_2 + H_2O$	Uric acid		Glycosylases	
Glutathione peroxidase	$H_2O_2 + GSH \rightarrow GSSG + H_2O$	Glutathione		Nucleases	
Transferrin	Metal chelators or sequesters	α-Tocopherol		Proteinases	Protein proteolysis
Caeruloplasmin		Ubiquinol		Proteases	
		β-Carotene	Incorporation of free radical	Peptidases	
		Lycopene			

cytokine receptors, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [52]. This induces signal propagation within the cell through phosphorylation of tyrosine residues on the receptors and their associated adaptor proteins [53]. In particular, two nuclear transcription factors, activator protein 1 (AP-1) and nuclear factor-κB (NF-κB), involved in transcription of genes for matrix-degrading enzymes and proinflammatory cytokines, respectively, are activated [54]. These transcription factors are associated with skin dryness, pigmentation, laxity, deep wrinkling [55, 56], and apoptosis activation [57, 58].

However, photoaging is a cumulative process, as many factors contribute to it, such as the degree of sun exposure and skin pigment. Indeed, it has been demonstrated that people with lighter skin colour, living in sunny countries, developed more easily skin cancers, such as basal cell cancer, squamous cell carcinoma, and melanoma [54].

To hide the effects of aging, several people undergo plastic surgery. However, in most cases, this practice is expensive, very invasive, and could potentially lead to complications. In this context, development of new antiaging therapies is gaining more importance.

During the last decade, different studies demonstrated that chronological aging and photoaging activate the same oxidative stress-related pathways. However, photoaging accounts for about 80% of the aging-related adverse effects. In this context, antioxidants are known to inhibit damages induced by oxidation, caused by ROS, as described in this review in paragraph 5 [54, 59, 60].

5. Antioxidants as Antagonists of ROS in Skin Disorders

The aerobic world is characterized by high levels of toxic oxygen by-products. To survive in this adverse environment, the organism has evolved antioxidant systems to protect itself. Khlebnikov et al. defined the antioxidants as "any substance that directly scavenges ROS or indirectly acts to upregulate antioxidant defences or inhibit ROS production" [61]. However, antioxidants are also characterized by the ability to form a new, more stable radical, through intramolecular hydrogen bonding and further oxidation [62]. In addition, antioxidants can regulate gene expression inducing the translocation of the nuclear factor-erythroid 2-related factor 2 (Nrf-2) from the cytosol to the nucleus, upon dissociation from its inhibitor, Kelch-like erythroid cell-derived protein 1 (Keap-1). Once in the nucleus, Nrf-2, can bind antioxidant response elements (ARE) and induces the transcription of stress response genes, such as glutathione S-transferase (GST), heme oxygenase-1 (HO-1), and NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1) [14, 63, 64]. The defence system of the cell from oxidative stress consists of an interacting network of different antioxidants that acts at different levels and with different mechanisms, which are summarized in Table 1.

Antioxidants belonging to the first line of defence suppress the formation of free radicals, whereas those of the second line counteract the chain initiation and/or break the chain propagation reactions of free radicals. Following oxidative stress, the cell is able to induce the transcription and translation of de novo enzymes, involved in repair processes. If the cell is able to counteract the negative effects of stress injury, it will undergo adaptation and restore the physiological antioxidant levels. On the other hand, in case of prolonged or excessive stress, the cell will undergo programmed cell death, as schematically represented in Figure 1.

In general, antioxidants can be grouped as endogenous, that is, produced by the body, and exogenous, that is, obtained from the diet. The first class can be divided in enzymatic and nonenzymatic defences. The first group includes superoxide dismutase (SOD) [65], catalase [65], and glutathione peroxidase [65], whereas the nonenzymatic defenders include iron- and copper-binding extracellular proteins (e.g., albumin, transferrin, lactoferrin, haptoglobin, and ceruloplasmin) [66] as well as other cellular compounds (e.g., quinones, glutathione, uric acid, and bilirubin) [66].

Enzymatic and nonenzymatic defenders are complementary to each other, since they act against different oxidative species in different cellular compartments. Moreover, they may act in a synergistic way with the exogenous antioxidant systems. This last family of antioxidants can be divided into synthetic and natural antioxidants. Some examples for each class are reported in Table 2, with a focus on antioxidants able to protect the skin from photoaging.

6. Fruits and Vegetables as Powerful Sources of Antiaging Antioxidants

Exogenous supplementation with dietary antioxidants and/or skin pretreatment with antioxidant-based lotions before

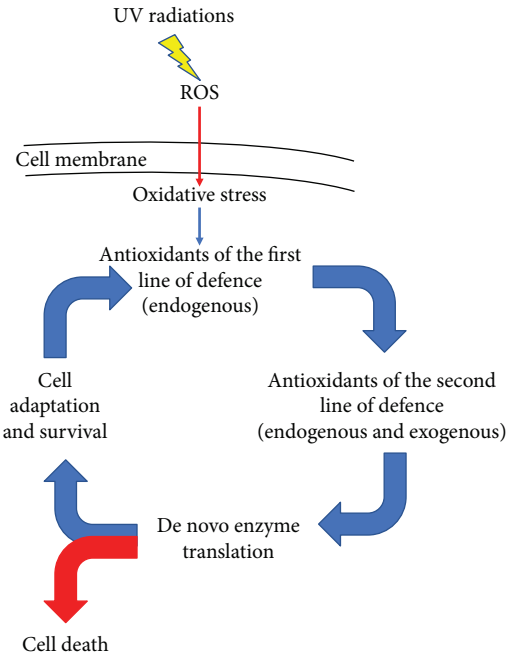

Figure 1: Schematic representation of the cell antioxidant response following oxidative stress injury. Upon UV radiations, ROS levels increase and oxidative stress is induced. Endogenous antioxidants suppress ROS formation and exogenous and endogenous antioxidants cooperate to suppress propagation reactions. Cell damages are repaired by de novo enzymes. Finally, if the cooperation among these antioxidant-related networks is able to counteract oxidative stress injury, the cell will survive after an adaptation process; otherwise, in case of prolonged or excessive stress, the cell will undergo cell death.

sun exposure might be a winning strategy against age-related skin oxidative damage [86]. Indeed, a regular intake of vitamins, polyunsaturated fatty acids, and polyphenols from plant sources has been shown to contribute to the prevention of age-related diseases. The search for effective natural compounds able to protect against the deleterious effects of photoaging has been intensified in recent years. Indeed, the list of molecules with antiaging potential extracted from different parts of a number of plant species is continuously growing [87, 88].

In this context, plants produce many secondary metabolites to protect themselves from UV radiations and these molecules can be used as natural antioxidants able to protect the skin from photoaging. These active compounds can protect the skin by (i) absorbing UV radiations, (ii) inhibiting free radical reactions induced by UV in cells, and (iii) modulating endogenous antioxidant and inflammatory systems [4, 89].

In the following part of the review, we will describe some of these natural antioxidants, the plants from which these compounds are normally extracted and their role in photoaging.

6.1. Phenolic Compounds. Natural compounds used against photoaging comprise phenolic compounds, including flavonoids (catechins, isoflavones, proanthocyanidins, and anthocyanins), phenolic acids (benzoic, gallic, and cinnamic acids), and stilbenes derived from plants such as tea, grape, bergamot, fernblock, rooibos, grapefruit, and red orange [4, 88, 90]. All these compounds can prevent penetration of radiations into the skin and, in addition, they can reduce inflammation, oxidative stress, and influence several signalling pathways in order to protect the skin against UV damage [4]. We recently demonstrated that two phenolic compounds, malvidin and cyanidin, extracted from fruits of the açai tree (*Euterpe oleracea* Mart.), a South American palm, were able to counteract UVA-induced oxidative stress in immortalized fibroblasts [74]. Indeed, the preincubation of UVA-irradiated BALB/3T3 cells with açai phenolic compounds interfered with ROS production and kept GSH levels and lipid peroxidation comparable to normal cellular levels [74]. In another paper, we showed the beneficial effects of water extracts from *Opuntia ficus-indica* L. cladodes on human keratinocytes [91]. In particular, the phenolic compounds eucomic and piscidic acids were found to be the main active molecules responsible for the protection of keratinocytes against the UVA-induced oxidative stress and apoptosis [91].

Several studies have demonstrated the health-promoting effect of grape (*Vitis vinifera*) against age-related diseases. This is due to the high content of phenolic compounds present in this plant. Indeed, grape seeds and peels constitute a rich source of polyphenols including quercetin, catechin, epicatechin, gallic acid, and oligomeric proanthocyanidins [4, 92]. Recently, it has been found that also grape extracts from the stems, a part of the grape tree rich in phenolic compounds, are able to reduce UVB-induced oxidative damage [93]. Indeed, the topical application of stem's grape extracts on mice skin before UVB treatment was able to prevent epidermal thickness, erythema, pigmentation, mast cell and inflammatory neutrophil infiltrations, collagen degradation, and the expression of COX-2, Nrf-2, and HO-1 genes [93].

Grape seeds are also rich in phytoalexin resveratrol (trans-3, 4′,5-trihydroxy-stilbene), a polyphenolic antioxidant with strong anti-inflammatory and antiproliferative activity [4, 87]. A single application of resveratrol on hairless mice's skin before exposure to UVB radiation led to the inhibition of skin edema, cyclooxygenase, and ornithine decarboxylase induction and lipid peroxidation in the skin [4, 76]. Interestingly, the human skin has been shown to have specific binding sites for resveratrol. As recently reviewed by Davinelli and colleagues [94], the interaction of resveratrol with the specific binding partner is able to block apoptotic events and mitochondrial dysfunctions in keratinocytes, delaying skin aging. In human keratinocytes, resveratrol can also modulate cytokine (IL-6, IL-8, and TNF-α) levels and stimulate the expression of HSP70, a factor important for cell repair and also for cytoprotection ([75] and references therein). However, one should keep in mind that resveratrol has very low solubility and high sensitivity to oxidation, thus making this molecule very unstable ([94] and references therein).

Human intervention studies have also been carried out, and most of them have focused on the supplementation of epigallocatechin gallate (EGCG) from green tea. In one of

Table 2: Antioxidants involved in protection from photoaging.

Antioxidants	Class	Bioactive compound	Skin protection from photoaging	Ref
Synthetic	Nitroxides (mimetics of SOD)	Tempol	Protection from UVA- and UVB-induced damage *in vitro* and *in vivo* Inhibition of extracellular matrix degradation and preservation of collagen production *in vitro*	[67–70]
	Coenzyme Q analogues	Idebenone	Protection from oxidative stress damage in living skin Suppression of sunburn cell formation	[71]
Natural	Flavonoids	Quercetin	Inhibition of UV-induced inflammation in primary human keratinocytes Protection of mice skin from UV radiation-induced damage	[72, 73]
		Malvidin and Cyanidin derivatives	Protection of murine fibroblast from UVA damages	[74]
	Polyphenols	Resveratrol	Protection of HaCaT cells from UVB irradiation through attenuation of the caspase pathway Counteraction of UVB damages in hairless mice Reduction of skin wrinkling and skin oxidative stress	[75–77]
	Carotenoids	β-Carotene Lycopene Luthein	Prevention and repair from photoaging Protection of human skin against UV radiation in human clinical studies	[78–80]
	Vitamins	Vitamin C	Protection of HaCaT cells from UVA irradiation through attenuation of inflammation and activation of apoptosis Antioxidant, photoprotection, antiaging, antipigmentary effects on the skin	[81–84]
		Vitamin E	Skin photoprotection against UV-induced oxidative stress	[84, 85]

these studies, human subjects received 800 mg of EGCG, in one dose or divided in two doses, and this treatment consistently reduced the erythema size caused by the exposure to UV radiation [95]. Attention should be paid to the local EGCG concentration as, when tested in nM concentrations, it acts as antioxidant, whereas when tested in the μM concentration range, EGCG acts as a prooxidant [96]. In another study on human subjects, high dose of flavanols from cocoa powder alleviated the erythema size upon UV radiation exposure [97].

6.2. Vitamin C. Vitamin C (ascorbic acid) is an essential cofactor in several enzymatic reactions but, since it cannot be synthesized by the human body, it has to be introduced in the organism by diet [98]. The antioxidant activity of ascorbic acid, which is found in fruits such as acerola, orange, lemon, tangerine, and tomato, makes it a good candidate as a protective compound against UV irradiation [99, 100]. Tomato (*Solanum lycopersicum*) fruits are a good source of ascorbic acid. Notably, we recently demonstrated the ability of an ascorbic acid-enriched tomato genotype to fight the oxidative stress induced by UVA in normal human keratinocytes [83]. In particular, pretreatment of cells with ascorbic acid or with tomato extracts before UVA exposure was able to maintain ROS, GSH, and lipid peroxidation levels at the basal levels and there was no evidence of apoptosis or inflammation [83]. These findings have been corroborated by Pullar et al., who demonstrated that ascorbic acid prevents lipid peroxidation and protects keratinocyte exposed to UV radiation from apoptosis [100]. In humans, it has been found that ascorbic acid acts as a photoprotectant at doses above the minimal erythema dose and that stimulates collagen synthesis, protects against damage from UVA/B radiation, and mitigates inflammation in the skin [86, 99, 100]. Finally, it has been demonstrated that topical application of an antioxidant mixture containing grape seed extract, vitamin E, ubiquinone, and ascorbic acid was able to protect the human skin against infrared A radiation-induced MMP-1 upregulation [101]. Unfortunately, many factors influence vitamin C stability, such as its concentration, temperature, and the pH used for aqueous formulations (which should be used at a pH lower than its pKa) [102].

6.3. Carotenoids. Carotenoids are dietary antioxidants that have demonstrated photoprotective activity. In plants, these compounds are components of the photosynthetic machinery where they act as accessory light-harvesting pigments and protect from photooxidative damage [103]. The photoprotective effects of several carotenoids have been investigated by intervention studies in humans, in which a carotenoid-rich diet has been investigated for its ability to decrease the erythema size upon UV radiation exposure [104–106], even though a long period (at least ten weeks) is necessary for successful intervention [106].

Among carotenoids, β-carotene, lycopene, canthaxanthin, and lutein that are derived from different plant sources such as tomato, carrots, and algae are the most abundant [107]. Carotenoids capsanthin and capsorubin from red pepper (*Capsicum annuum* L.) fruits have also been found to have protective properties against UVB-induced DNA damage in human dermal fibroblasts [88, 108]. Indeed, cell

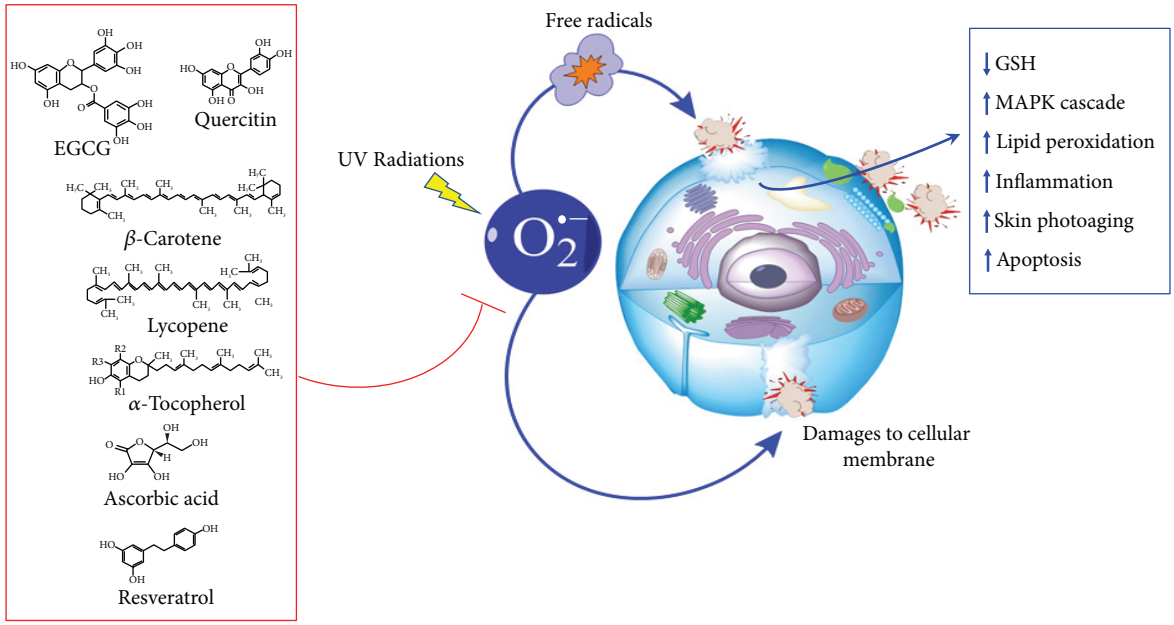

Figure 2: Cartoon representing cellular responses to oxidative stress in the presence (red lines) or in the absence (blue lines) of antioxidants. After oxidative stress induction by UV radiations, there is an increase in free radicals, which, in turn, induces different responses in the cell, such as depletion in GSH, activation of MAPK cascade, increase in lipid peroxidation, inflammation, skin photoaging, and apoptosis. All these processes can be inhibited or counteracted by antioxidant's activity (reported in the red box).

pretreatment with these carotenoids decreased the formation of UVB-induced DNA strand breaks and counteracted caspase-3 activation [108]. Studies on β-carotene protective effects go back to early '70s as this molecule showed very promising results. In 1996, a trial was reported on 12 years of supplementation with β-carotene (50 mg on alternate days) on healthy men but did not show any protective effect from melanoma insurgence [109].

Moreover, safety concerns have been recently raised with regard to its supplementation for over long periods of time [79]. Although the photoprotective effects of beta-carotene are thought to originate from its antioxidant properties, some studies documented prooxidant effects of β-carotene. For this reason, recent studies are focused on carotenoids other than β-carotene, such as lycopene, the primary carotenoid in tomatoes. We recently demonstrated that tomato extracts, rich in lycopene, are effective in counteracting the detrimental effects induced by oxidative stress caused by treatments with sodium arsenite on different human cell lines [80]. In particular, we found that carotenoids extracted from both fresh and processed tomato fruits showed cytoprotective activity, were able to mitigate ROS production induced by oxidative stress, and prevented GSH depletion and lipid peroxidation [80].

It has been demonstrated that lycopene protects against various skin alterations induced by UV radiation [90]. As an example, lycopene has been shown to have a role in the prevention of skin cancer. Indeed, lycopene preexposure on UVB-irradiated human keratinocytes was found to play a diversified role in UVB-irradiated keratinocytes, depending on the level of damage, correcting the injury in mild photodamaged cells and acting as a cytotoxic agent in preneoplastic cells [110]. In particular, in irradiated keratinocytes, lycopene pretreatment resulted in the overexpression of BAX gene, a cell cycle delay at S-phase transition, and in a consequent decrease of cells in G0/G1 phase [110].

In a placebo-controlled, double-blinded, randomized study, oral supplementation with lycopene-rich tomato nutrient complex (TNC) and lutein has been shown to be able to protect the human skin against UVA and UVA/UVB radiations [79]. In particular, oral supplementation with TNC inhibited UV-induced upregulation of the genes heme oxygenase-1, intercellular adhesion molecule 1, and matrix metallopeptidase 1, indicators of oxidative stress, photodermatoses and photoaging [79].

Several studies suggested that the protection from UV radiation is more effective upon treatment with combined tomato antioxidant compounds, compared to the effects of lycopene treatment alone, and this is probably due to a synergistic effect of the different tomato phytonutrients [79, 111]. This could be related to the fact that the interaction between structurally different molecules, endowed with different antioxidant properties, may provide a more comprehensive protection against oxidative injury [84].

Accordingly, consumption of tomato paste has been shown, in a randomized controlled study, to protect against acute and long-term photodamage [112]. In particular, supplementation of tomato paste before UV exposure was able to dampen skin erythema and to reduce mitochondrial DNA damage [112, 113]. Moreover, it has been recently showed that, in hairless and immunocompetent mice, tomato consumption was able to protect against the development of UVB-induced keratinocyte carcinoma [113].

7. Conclusions

Skin photoaging is a consequence of the oxidative stress generated upon exposure to UV radiation. However, the skin is normally protected from the negative effects of oxidative stress by endogenous antioxidant systems, which, unfortunately, undergo a progressive decline during aging. Several lines of evidence support the hypothesis that secondary metabolites from plants act as natural antioxidants able to decrease or retard the development and progression of life style-related diseases.

The intake of dietary antioxidants plays a fundamental role in the protection against oxidative injury; therefore, a correct diet is crucial to extend lifespan. Accordingly, several *in vitro*, *in vivo*, and human intervention studies demonstrated that antioxidants deriving from natural products, most of them assumed with the Mediterranean diet, are particularly effective in the protection of skin from photoaging, as schematically reported in Figure 2.

However, the use of natural antioxidants not only is restricted to oral diet but also includes a potential topical use against UV radiations. This is possible as some natural compounds show UV absorption properties and act as antioxidants, thus reducing the damaging effects of UV radiation exposure. Thus, increasing the antioxidant capacity of skin cells by using exogenous antioxidants could be a valuable strategy for preventing UV-induced skin damage.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

All authors contributed to write the review article and have read and approved the final manuscript.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 5461436, 13 pages
https://doi.org/10.1155/2018/5461436

Research Article

Alcoholic Beverage and Meal Choices for the Prevention of Noncommunicable Diseases: A Randomized Nutrigenomic Trial

Laura Di Renzo,[1] **Giorgia Cioccoloni**,[2] **Paola Sinibaldi Salimei**,[1] **Ida Ceravolo**,[3] **Antonino De Lorenzo**,[1] **and Santo Gratteri**[4]

[1]*Clinical Nutrition and Nutrigenomic Section, Department of Biomedicine and Prevention, University of Rome Tor Vergata, Via Montpellier 1, 00136 Rome, Italy*
[2]*PhD School of Applied Medical-Surgical Sciences, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy*
[3]*Department of Experimental Biomedicine and Clinical Neuroscience, Ophthalmology Section, University of Palermo, Piazza Marina 61, 90133 Palermo, Italy*
[4]*Department of Surgery and Medical Science, Magna Græcia University, 88100 Catanzaro, Germaneto, Italy*

Correspondence should be addressed to Laura Di Renzo; laura.di.renzo@uniroma2.it

Received 29 March 2018; Accepted 23 May 2018; Published 27 June 2018

Academic Editor: Maria M. Rigano

Background. Noncommunicable diseases (NCDs) are the first cause of death worldwide. Mediterranean diet may play a crucial role in the prevention of NCDs, and the presence of wine in this diet could play a positive role on health. *Methods*. 54 healthy volunteers consumed one of the following beverages: red (RW) or white wine (WW), vodka (VDK), and/or Mediterranean meal (MeDM) and high-fat meal (HFM). *Results*. OxLDL-C changed significantly between baseline versus HFM, MeDM versus HFM, and HFM versus HFM + RW ($p < 0.05$). Significant upregulation of catalase (CAT) was observed only after RW. Conversely, WW, VDK, RW + MeDM, HF + WW, and HF + VDK determined a significant downregulation of CAT gene. Superoxide dismutase 2 (SOD2) gene expression was upregulated in WW, MeDM + VDK, and RW. Contrariwise, HFM + VDK determined a downregulation of its expression. RW, RW + MeDM, and RW + HFM caused the upregulation of glutathione peroxidase-1 (GPX1). *Conclusions*. Our results suggest that the association of low/moderate intake of alcohol beverages, with nutraceutical-proven effectiveness, and ethanol, in association with a Mediterranean diet, could determine a reduction of atherosclerosis risk onset through a positive modulation of antioxidant gene expression helping in the prevention of inflammatory and oxidative damages.

1. Introduction

Noncommunicable diseases (NCDs) are the first cause of death worldwide. In 2011, the United Nations assembly recognized the social-economical-medical importance and the inevitability of preventive politics of NCDs in order to reduce the mortality indexes [1, 2], and in 2013, the World Health Organization (WHO) has laid down a policy paper for the institution and promotion of prevention politics in order to reduce the damages due to these pathologies. Cardiovascular diseases (CVDs) represent the 48% of NCDs, followed by cancer (21%), respiratory chronic diseases (12%), and diabetes (3.5%) [3, 4].

NCDs are the result of the individual predisposition, that is, genetic component, lifestyle habits, and pathological changes that lead to an untreatable full-blown chronic degenerative illness [5]. According to WHO, three of the most important behavioral risk factors for NCDs are harmful use of alcohol, unhealthy diets, which promote the progression and pathogenesis of polygenic diet-related diseases, and sedentary behavior [3].

The effect of some alcoholic beverages and dietary compounds on metabolic pathways related to several NCDs is currently under investigation and is leading the traditional methods of nutritional counseling towards a more complex approach based on the modulation of gene expression by food.

Wine is only the fourth (8.0%) most consumed among alcoholic beverage worldwide, and its consumption is higher in European regions (25.7%). In fact, globally, individuals

above 15 years of age drink 13.5 g/day of pure alcohol, mainly in the form of spirits (50.1%) and beer (34.8%) [6]. It has been shown a relationship to "curved J-shaped" among the consumption of alcohol and the mortality, in which harmful effects are reached after 89 g of average intake for day and a maximum protective effect to 20 g of pure average alcohol intake per day [7].

If on one side, the toxic role of the consumption of alcoholic beverages is broadly shown, associated with an increased overall mortality, cardiomyopathy, hypertension, acute cerebrovascular events, liver diseases, and cancer, and on the other side, several epidemiologic studies have encountered an inverse association between risk of cardiovascular mortality and moderate alcohol consumption [8]. It is likely that the cardioprotective effect of alcohol is due to its ability to increase high-density lipoprotein-cholesterol (HDL-C) levels and its antithrombotic properties.

Moreover, wine represents an important component of the Mediterranean diet (MeD), contributing to the reduced incidence of NCDs of this dietary habit [9]. Important evidences about a likely positive relation between nutrition and cardiovascular disorders were showed thanks to epidemiological studies on Greek and southern Italian people, in which the incidence of CVDs was remarkably low compared with other regions of the world [9, 10]. In Italy, even today, wine is the favorite beverage among adults [11].

Among alcoholic beverages, wines, especially red ones, could play a positive role against CVDs, as observed in PREDIMED study [12]. For instance, this beverage, thanks to the resveratrol and polyphenolic compounds, may decrease blood viscosity, antagonize the development of endothelial dysfunction increasing nitric oxide (NO) bioavailability, and reduce atherosclerosis by inhibiting lipoprotein oxidation and thrombosis [13].

Considering the remarkable influence of red wine on CVDs, in further researches, it has been analyzed the possibility that other alcoholic beverages could have the same influence. For the time being, some interesting results were showed for white wine. This beverage, which contains phenols like red wine and olive oil, seems to have a good effect in preventing inflammatory cytokine release and it may also play an interesting role in the cardioprotection [14]. On the contrary, for spirits, conflicting data are demonstrated. For instance, vodka may have good capability to prevent both hyperoxia-induced increase of arterial stiffness [15] and endothelial dysfunction, reducing oxidative stress in the myocardium [16]. In other studies data for this beverage are not encouraging [17].

Another important aspect for the prevention of NCDs is the type and quality of food. It is widely accepted that the consumption of fruits and vegetables prevents diseases related to the oxidative processes [9]. Conversely, one of the common causes of oxidative stress onset, and then of the vascular alterations of NCDs, is the huge consumption of salt, sugars, and processed meals and a low intake of vegetables and fruit [18].

Further researches demonstrated that MeD might play a crucial role in the prevention of NCDs, because of the high content of polyunsatured fatty acids (PUFA), polyphenols, and fiber and the low content of saturated fatty acids, cholesterol, and sodium [9, 19–21].

We have previously demonstrated that red wine in association with McDonald's® and a Mediterranean meal modulated the inflammatory status and could represent an essential component of a holistic approach to combatting chronic NCDs linked to inflammation [22].

Based on previous results, we hypothesized that 30 g of ethanol and polyphenol present in different types of alcoholic beverages, that is, red wine, white wine, and vodka, could amplify the effect of an antioxidant meal and lead to a change in oxidative status, contributing to a reduction of chronic NCDs linked to inflammation [23]. The first endpoint of this study was to examine the oxidative status of LDL; the second end point was the evaluation of gene expression of selected genes belonging to inflammatory and oxidative stress pathway, as catalase (CAT), superoxide dismutase 2 (SOD2), and glutathione peroxidase 1 (GPX1). Therefore, a controlled randomized clinical trial was performed on healthy volunteers in fasting status or in the postprandial time, after a Mediterranean or a high-fat meal, with or without alcoholic beverages intake.

2. Materials and Methods

2.1. Subjects and Study Design. For this study, 55 healthy volunteers were recruited at the Clinical Nutrition and Nutrigenomic Section at the University of Rome Tor Vergata. In order to be included in the study, subjects had to respect the following eligibility criteria: age between 18 and 65 years old and BMI between 18.5 and 35 kg/m^2. At the same time, exclusion criteria were the following: BMI > 35 kg/m^2, active tobacco smoking, past or active cardiovascular, hepatic, metabolic, autoimmune, and neoplastic diseases, and drug consumption. The randomized parallel group study was conducted as shown in Figure 1. Blood samples for oxLDL-C concentration and biochemical and genomic analyses were collected at baseline (B), and genomic analysis also after 2 h of intervention in order to evaluate nutritional, oxidative, and inflammation status. Nutritional status assessment and genomic analysis were performed at the Clinical Nutrition and Nutrigenomic Section, Department of Biomedicine and Prevention of University of Rome Tor Vergata. Lifestyle habits of healthy volunteers did not change during the study period. Clinicians assessed any adverse effects from the interventions by going through a checklist of symptoms, including bloating, fullness, or indigestion, altered bowel habit, dizziness, and other symptoms that were possibly associated with the interventions. No abnormality was presented during the study period. All patients completed the study. All participants, in accordance with principles of the Declaration of Helsinki, signed a statement of informed consent. This protocol has been registered with ClinicalTrials.gov NCT01890070.

2.2. Dietary Intervention. During the study, subjects randomly consumed one of the following beverages or meals: (a) fasting + 30 g of ethanol from red wine (RW), (b) fasting

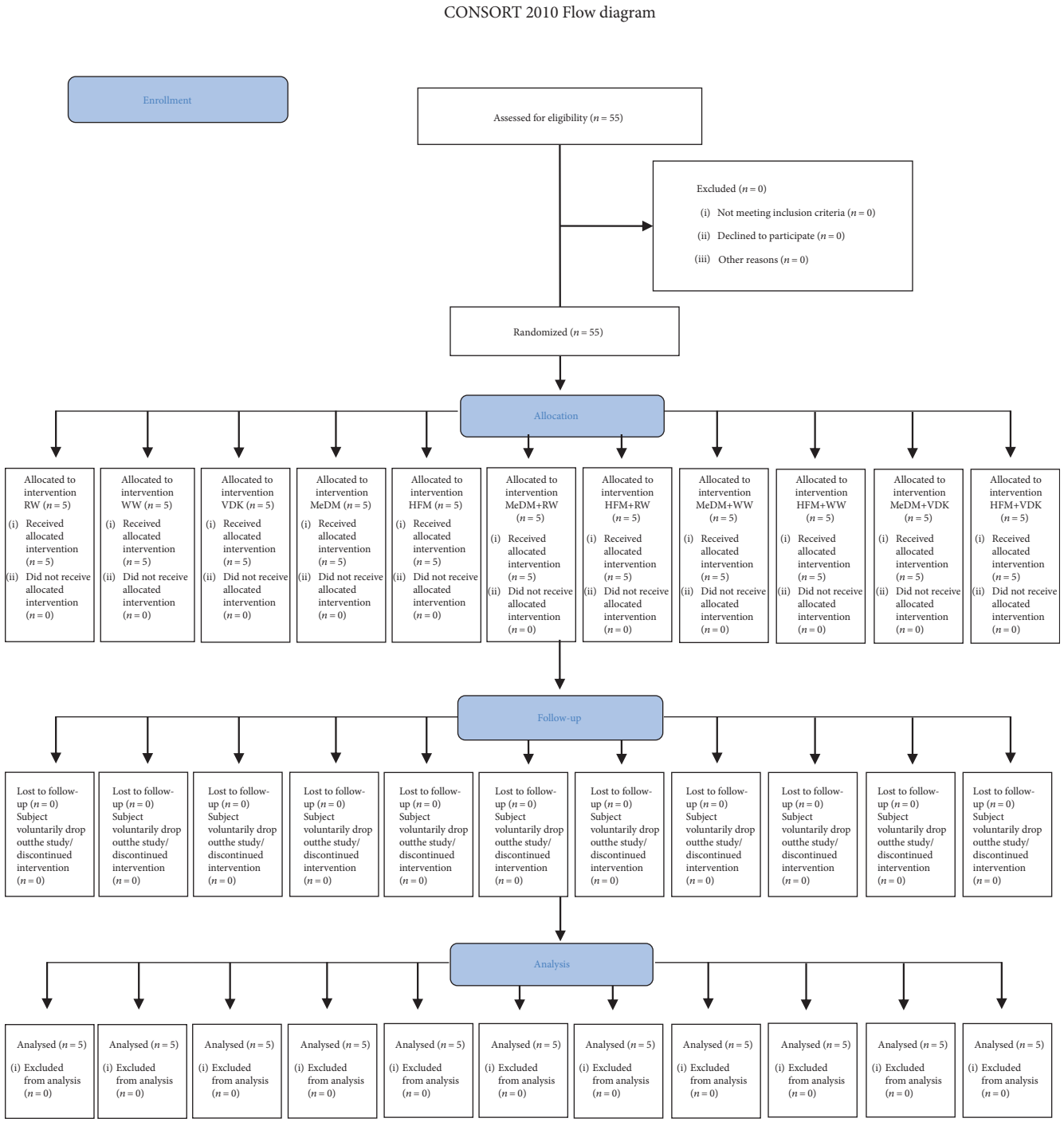

Figure 1: Study design. Clinical trial design. RW: fasting red wine; WW: fasting white wine; VDK: fasting vodka; MeDM: Mediterranean meal; HFM: high-fat meal; MeDM + RW: Mediterranean meal plus red wine; MeDM + WW: Mediterranean meal plus white wine; MeDM + VDK: Mediterranean meal plus vodka; HFM + RW: high-fat meal plus red wine; HFM + WW: high-fat meal plus white wine; HFM + VDK: high-fat meal plus vodka.

+ 30 g of ethanol from white wine (WW), (c) fasting + 30 g of ethanol from vodka (VDK), (d) Mediterranean meal (MeDM), (e) high-fat meal (HFM), (f) MeDM + 30 g of ethanol from RW, (g) MeDM + 30 g of ethanol from WW, (h) MeDM + 30 g of ethanol from VDK, (i) HFM + 30 g of ethanol from RW, (j) HFM + 30 g of ethanol from WW, and (k) HFM + 30 g of ethanol from VDK. MeMD was composed by 150 g of whole pasta, 300 g of eggplants, 150 g of tomatoes, 150 g of peppers, 100 g of rocket salad, 100 g of radicchio, 60 g of anchovies, 20 g of walnuts, and 20 g of capers. The HFM was bought in McDonald's restaurant and was represented by n.1 Big Tasty Bacon® and n.1 small French

fries package. The MeDM was prepared, distributed, and consumed as well as HFM, at the Clinical Nutrition and Nutrigenomic Section, Department of Biomedicine and Prevention, University of Rome Tor Vergata. Meal analyses were performed by the Dietosystem dietary software (DS Medica S.r.l., Milan, Italy).

RW, made from Merlot (75%), Tocai Rosso (10%), and Cabernet Sauvignon (15%) grapes, was used in the study. RW characteristics are as follows: unfiltered wine, no added sulfites, total alcohol: 14.52% volume, residual sugar: 0.7 g/l, total acidity: 5.9 g/l, dry extract: 30 g/l, volatile acidity: 0.59 g/l, and total sulfur dioxide: 2 mg/l. WW made from Garganega grapes (95%) and other variety (5%) was used in the study. WW characteristics are as follows: unfiltered wine, no added sulfites, total alcohol: 12.5% volume, residual sugar: 0.6 g/l, total acidity: 4.7 g/l, dry extract: 18 g/l, volatile acidity: 0.48 g/l, and total sulfur dioxide: 9 mg/l. The commercial vodka used in this study was a pure grain distilled with total alcohol volume of 38%.

Subjects were not blinded to the type of beverages or meal they consumed.

2.3. Anthropometric Measurements. All volunteers were subjected to anthropometric evaluation after overnight fasting. Body weight and height were measured according to standard procedures [20]. Body weight was evaluated with balance scale to the nearest 0.1 kg (Invernizzi, Rome, Italy). Height was measured with a stadiometer to the nearest 0.1 cm (Invernizzi, Rome, Italy). BMI was calculated using the formula: BMI = body weight (kg)/height (m^2).

2.4. Biochemical Analysis. Blood tests were carried out at the accredited Clinical Chemical Laboratories of the "Policlinic Tor Vergata (PTV)" of Rome, Italy. Analyses were performed only at baseline after a 12-hour overnight fast. Blood samples (10 ml) were collected into tubes with EDTA (Vacutainer®). Samples were directly placed on ice, and plasma was separated by centrifugation.

Laboratory analysis included complete blood count, fasting glucose, total cholesterol (TC), HDL-C, LDL-cholesterol (LDL-C), triglycerides (Tg), aspartate aminotransferase (AST/GOT), alanine transaminase (ALT/GPT), fibrinogen, albumin, creatinine, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and insulin. All clinical chemistry analyses, except fasting glucose, serum lipid, CRP, and Tg analyses, were carried out with an ADVIA®1800 Chemistry System (Siemens Healthcare). Plasma glucose concentrations were measured with glucose oxidase method (COBAS INTEGRA 400, Roche Diagnostics, Indianapolis, IN, USA); serum lipid profile components were determined by standard enzymatic colorimetric techniques (Roche143 Modular P800, Roche Diagnostics, Indianapolis, IN, USA). Serum Tg was measured by a coupled enzymatic method on the Beckman Synchron LX20 automated system. Serum CRP was measured by a high-sensitivity sandwich enzyme immunoassay (Immundiagnostik, Bensheim, Germany). In order to minimize variability, all tests were performed using the same lot of reagents or assay plates.

The assessment of insulin resistance was evaluated with the homeostasis model assessment of insulin resistance (HOMA-IR) corresponding to the following formula:

$$\text{HOMA-IR} = \frac{\text{fasting glucose(mg/dl)} \times \text{fasting insulin(uU/ml)}}{405}. \quad (1)$$

2.5. Sample Collection and RNA Extraction. Blood samples were collected in PAXgene Blood RNA Tubes (PreAnalytiX Qiagen, Hombrechtikon, Switzerland) and stored at −80°C until use. RNA from each sample was extracted with PAXgene Blood miRNA Kit (PreAnalytix Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. RNA quantification was performed through spectrophotometry (NanoDrop, Wilmington, USA).

2.6. Quantitative Real-Time PCR and Data Analysis. For the assessment of SOD2 (NCBI Reference Sequence: NC_000006.12), CAT (NCBI Reference Sequence: NC_000011.10), and GPx1 (NCBI Reference Sequence: NC_000003.12) genes, the human oxidative stress (PAHS-065ZA) and the inflammatory cytokine and receptor (PAHS-011Z) RT^2 Profiler PCR Arrays (Qiagen, Netherlands) were used. Each sample was analyzed in triplicate and repeated twice according to the manufacturer's instructions (Qiagen, Netherlands). We used β-actin (ACTB) (NM 001101) as a housekeeping gene. To determine gene expression level, comparative threshold (CT) cycle was used. CT value was normalized using the formula $\Delta CT = CT\ (gene) - CT$ (housekeeping gene). The relative gene expression levels were determined according to the following formula: $\Delta\Delta CT = \Delta CT\ \text{sample} - \Delta CT\ \text{calibrator}$. The value used to plot relative gene expression was determined using the expression fold change $(FC) = 2^{(-\Delta\Delta CT)}$.

2.7. Low-Density Lipoprotein Oxidative Status. Blood samples were collected, stabilized, and centrifuged in EDTA tubes (Vacutainer). Plasma was removed and stored at −80°C until use. Mercodia Oxidized LDL ELISA test (Mercodia AB, Sweden) was used to quantify oxLDL-C according to the manufacturer's protocol. All the analyses were performed in triplicate.

2.8. Statistical Analysis. Statistical analysis was carried out using IBM SPSS 21.0 for Windows (IBM Corp., Armonk, NY, USA). After the Shapiro-Wilk test, a paired t-test or a nonparametric Wilcoxon test was performed to evaluate differences before and after nutritional interventions. Parametric t-test or nonparametric Mann–Whitney U test was performed to evaluate differences between nutritional interventions. All tests were considered significant at $p \leq 0.05$. Percentage variations were calculated as the percentage difference between baseline and the subsequent treatment. For genomic analysis, the value used to plot relative gene expression was determined using the expression fold change $(FC) = 2^{(-\Delta\Delta CT)}$, using β-actin (ACTB) as housekeeping gene. Only genes with a $FC \geq 2$ were considered significant upregulated for differentially expressed genes. Conversely, genes

with a FC ≤ 0.5 were considered significant downregulated for differentially expressed genes.

3. Results

3.1. Meal Analysis. MeDM was composed of 55% of carbohydrates, 20% of proteins (>50% of vegetable derivation), <30% of lipids (on total kcal: saturated fat < 10%, 6–10% polyunsaturated fatty acids (PUFA), n-6 : n-3 PUFA ratio of 3 : 1, 15% of monounsaturated fatty acids (MUFA), and <1% trans-fatty acids), and 30 g of fiber. HM was composed by 24.3% of carbohydrates, 23% of proteins (>80% animal proteins), 52% of total fat (saturated fat 19.5% of total kcal), and 5.60 g of fiber. Furthermore, MeDM had the following nutritional index values: cholesterol-saturated fat index (CSI) of 8.80, thrombogenic index (TI) of 0.46, atherogenic index (AI) of 0.26, and potential renal acid load (PRAL) of −23.56. At the same time, HF meal had the following nutritional index values: CSI of 24.34, TI of 1.73, AI of 1.97, and PRAL of 29.57.

3.2. Subject Characteristics. Of the fifty-five subjects enrolled, one of them was excluded from the trial (subject declined to participate). Finally, fifty-four patients completed the study (Figure 1). No changes to trial outcomes after the trial commenced occurred. The average age of subjects was 32.47 ± 7.25 years, 58.8% females and 41.2% males; none of the subjects presented metabolic diseases (Table 1).

3.3. OxLDL-C Analysis. Comparing oxLDL-C levels at baseline and after the consumption of different beverage and/or meal treatments, significant changes were observed only between baseline and HFM treatment ($\Delta\% = 18.49$; $p < 0.05$) (Figure 2). Among treatments, we noticed significant differences in oxLDL-C levels between MeDM and HFM ($p < 0.05$) and HFM and HFM + RW ($p < 0.05$) (Table 2). Conversely, no statistical significant modifications between beverages treatments, MeDM meals associated with RW, WW, and VDK consumption as well as among HFM and WW and VDK administrations were determined.

3.4. Gene Expression Data. Significant upregulation of CAT, with a fold change exceeding the threshold set at 2, was observed only after RW ($2^{(-\Delta\Delta CT)} = 4.04$). Conversely, WW and VDK administration determined a significant downregulation of CAT gene expression ($2^{(-\Delta\Delta CT)} = 0.30$ and $2^{(-\Delta\Delta CT)} = 0.23$, resp.) as well as the combination of HFM with WW ($2^{(-\Delta\Delta CT)} = 0.48$) and VDK ($2^{(-\Delta\Delta CT)} = 0.23$) (Figure 3(a)). The expression of SOD2 gene was upregulated in WW, MeDM + VDK treatment, and especially in RW administration ($2^{(-\Delta\Delta CT)} = 3.32$, $2^{(-\Delta\Delta CT)} = 2.63$, and $2^{(-\Delta\Delta CT)} = 32.73$, resp.). On the other hand, HFM + VDK treatment determined a downregulation of its expression ($2^{(-\Delta\Delta CT)} = 0.49$) (Figure 3(b)). RW alone and its association with MeDM and HFM treatments caused the upregulation of GPX1 gene expression ($2^{(-\Delta\Delta CT)} = 9.12$, $2^{(-\Delta\Delta CT)} = 8.99$, and $2^{(-\Delta\Delta CT)} = 10.5$, resp.) (Figure 3(c)).

Table 1: Anthropometric and bioclinical baseline characteristic of study subjects.

Baseline characteristic of volunteers Parameters	Median ± SE	Min–Max
Age (y)	32.47 ± 7.25	25.00–52.00
Weight (kg)	65.11 ± 12.21	48.00–92.70
Height (cm)	168.06 ± 11.89	150.00–186.00
BMI (kg/m^2)	22.98 ± 3.15	18.75–32.00
WBC (K/μl)	6.51 ± 2.90	3.30–13.40
LYM (K/μl)	1.96 ± 0.55	1.30–3.30
MON (K/μl)	0.35 ± 0.13	0.20–0.60
GRN (K/μl)	4.12 ± 2.36	1.70–10.30
EOS (K/μl)	0.09 ± 0.08	0.00–0.20
BAS (K/μl)	0.02 ± 0.06	0.00–0.20
LYM (%)	28.83 ± 10.18	1.84–41.00
MON (%)	5.03 ± 1.92	0.37–8.00
GRN (%)	57.00 ± 17.92	1.96–77.00
EOS (%)	1.61 ± 1.45	0.00–5.00
BAS (%)	0.19 ± 0.38	0.00–1.00
RBC (M/μl)	4.62 ± 0.31	4.02–4.95
HGB (g/dl)	13.84 ± 1.26	11.10–16.50
HCT (%)	42.25 ± 2.98	36.90–48.20
MCV (fl)	91.51 ± 4.97	81.00–100.00
MCH (pg)	29.98 ± 2.55	24.30–34.70
MCHC (g/dl)	32.71 ± 1.38	30.20–17.90
RDW (%)	15.31 ± 1.63	13.00–17.90
PLT (K/μl)	225.54 ± 78.24	135.00–438.00
PCT (%)	0.15 ± 0.02	0.12–0.19
MPV (fl)	7.30 ± 0.60	6.10–8.10
PDW (%)	51.30 ± 3.88	44.40–55.80
TC (mg/dl)	174.43 ± 28.86	128.00–233.00
HDL-C (mg/dl)	50.77 ± 10.21	38.00–69.00
LDL-C (mg/dl)	99.00 ± 25.01	51.00–137.00
Tg (mg/dl)	67.85 ± 28.16	35.00–120.00
Glycemia (mg/dl)	84.57 ± 10.10	65.00–101.00
Fibrinogen (mg/dl)	328.82 ± 96.84	226.00–480.00
GOT (U/l)	25.15 ± 11.23	10.00–56.00
GPT (U/l)	22.61 ± 7.52	10.00–38.00
CPR (mg/dl)	0.51 ± 0.51	0.10–1.72
ESR (mm/h)	8.85 ± 4.45	3.00–17.00
Insulin (μM/ml)	6.82 ± 1.68	5.00–8.00
HOMA-IR	1.26 ± 0.31	0.83–1.63
Albumin (g/dl)	3.83 ± 0.33	3.50–4.31
Creatinine (mg/dl)	0.70 ± 1.14	0.92–0.16

Results are expressed in median ± standard error and minimum and maximum for each parameter. ALT/GPT: alanine transaminase; AST/GOT: aspartate aminotransferase; BAS: basophiles; CRP: C-reactive protein; EOS: eosinophils; ESR: erythrocyte sedimentation rate; GRN: granulocytes; HDL-C: high-density lipoprotein cholesterol; HCT: hematocrit; HGB: hemoglobin; HOMA-IR: homeostasis model assessment of insulin resistance; LDL-C: low-density lipoprotein cholesterol; LYM: lymphocytes; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; MPV: mean platelet volume; MON: monocytes; PDW: platelet distribution width; PLT: platelets; PCT: procalcitonin; RBC: red blood cells; RDW: red cell distribution width; TC: total cholesterol; Tg: triglycerides; WBC: white blood cells.

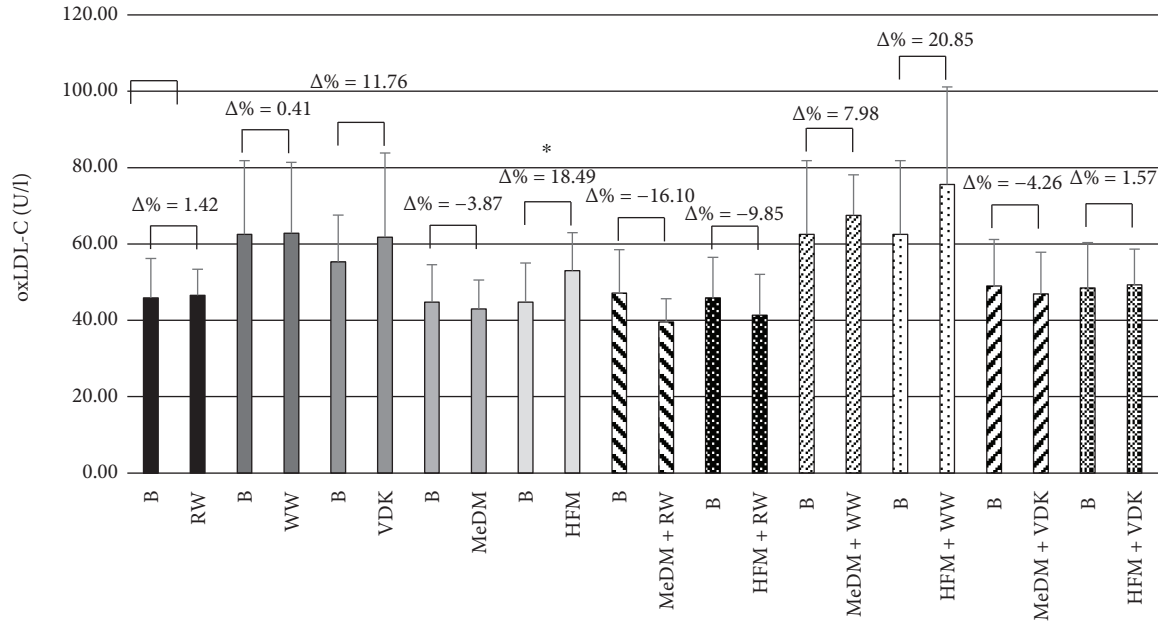

Figure 2: Variation of oxLDL-C levels between baseline and treatments. Comparative values of oxLDL-C levels for each treatment intervention. The significant values are expressed as B versus HFM ($^*p < 0.05$). B: baseline; RW: fasting red wine; WW: fasting white wine; VDK: fasting vodka; MeDM: Mediterranean meal; HFM: high-fat meal; MeDM + RW: Mediterranean meal plus red wine; MeDM + WW: Mediterranean meal plus white wine; MeDM + VDK: Mediterranean meal plus vodka; HFM + RW: high-fat meal plus red wine; HFM + WW: high-fat meal plus white wine; HFM + VDK: high-fat meal plus vodka.

Table 2: OxLDL-C percentage variation between baseline and dietary treatment.

OxLDL-C percentage variation		
	Mean ± SD	Median (Min–Max)
Δ% B-RW	6.99 ± 23.96	11.3 (−39–48)
Δ% B-WW	1.21 ± 8.50	4.08 (−13–10)
Δ% B-VDK	9.76 ± 19.96	9.05 (−11–32)
Δ% B-MeDM	−1.32 ± 20.43[a]	3.66 (−43–47)
Δ% B-HFM	21.29 ± 29.93[a,b]	20.71 (−22–69)
Δ% B-MeDM + RW	−12.08 ± 23.20	−8.55 (−50–34)
Δ% B-HFM + RW	−4.97 ± 33.18[b]	−2.05 (−57–81)
Δ% B-MeDM + WW	−7.36 ± 5.66	−9.765 (−11–1)
Δ% B-HFM + WW	−5.37 ± 7.09	−2.755 (−16–1)
Δ% B-MeDM + VDK	−3.37 ± 12.99	−9.765 (−16–27)
Δ% B-HFM + VDK	−2.60 ± 37.38	−2.755 (−101–75)

Results are expressed in mean value ± standard deviation and median, minimum, and maximum for each treatment. Significant values ($p \leq 0.05$) are expressed as [a]Δ% B-MeDM versus Δ% B-HFM and [b]Δ% B-HFM versus Δ% B-HFMRW. B: baseline; RW: fasting red wine; WW: fasting white wine; VDK: fasting vodka; MeDM: Mediterranean meal; HFM: high-fat meal; MeDM + RW: Mediterranean meal plus red wine; MeDM + WW: Mediterranean meal plus white wine; MeDM + VDK: Mediterranean meal plus vodka; HFM + RW: high-fat meal plus red wine; HFM + WW: high-fat meal plus white wine; HFM + VDK: high-fat meal plus vodka.

4. Discussion

NCDs share lifestyle as a risk factor. It seems particularly important to consider patient living conditions, before that NCDs lead to an untreatable full-blown chronic degenerative illness. In fact, lifestyle habits could make a difference in normal and pathological conditions. Primary prevention starts essentially from small lifestyle changes. Actually, in 2012, the Medical American Association invited health professionals to apply the "lifestyle medicine" clinical skills as a NCDs primary prevention [24].

One of the best known biochemical mechanisms underlying aging is the progressive loss by senescent cells of perfect replication of DNA in daughter cells. The "errors" accumulated in the transcription of cellular DNA after several replication processes end up by activating particular gene sequences which are self-replicating and which accumulate within the cell over time, causing its degeneration and death. The formation of these "cell scars" is closely related to aging. The possibility of reducing accumulated errors, modulating the inflammatory processes that underlie them, is the subject of numerous studies that focus on nutraceutical aspects and the concept of quality.

The glucose and lipid hematic concentrations in postprandial period, as the increased concentration of LDL-C converted in their oxidize form, the oxidized low-density lipoprotein-cholesterol (oxLDL-C), lipoxygenases, and the reactive oxygen species (ROS) [25], were related to the chronic process pathogenesis of NCDs [26].

ROS levels raise in postprandial state damaging cellular structures and contributing to the activation of some transcription factors that are able to regulate the expression of genes involved in immunity, inflammation, cell proliferation, growth, and apoptosis [27, 28]. OxLDL-C accumulates in the tunica intima where engulfed by macrophages, thereby

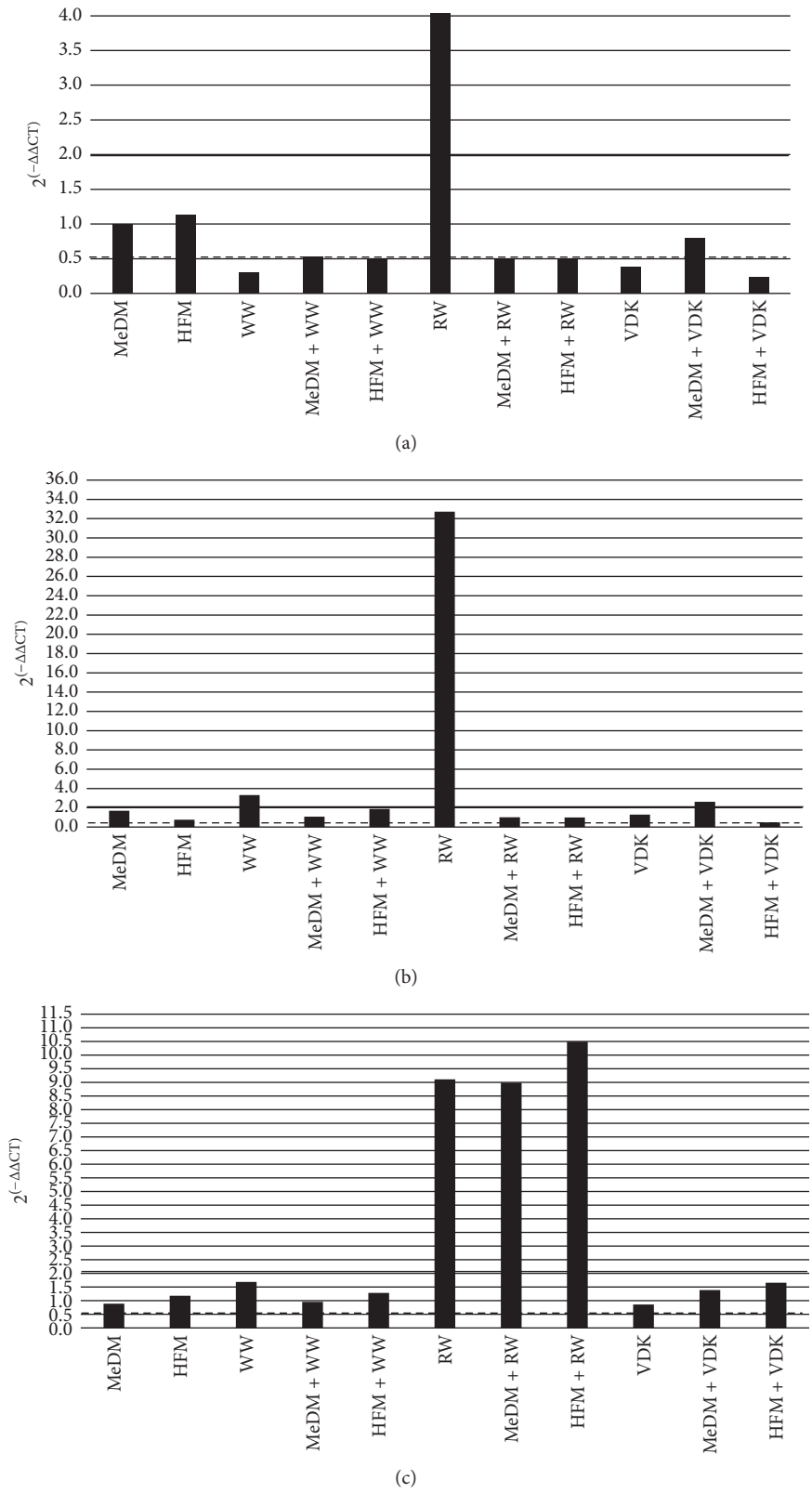

Figure 3: Gene expression after treatments. Different levels of fold change of genes analyzed: (a) CAT: catalase, (b) SOD2: superoxide dismutase 2, and (c) GPX1: glutathione peroxidase 1. RW: fasting red wine; WW: fasting white wine; VDK: fasting vodka; MeDM: Mediterranean meal; HFM: high-fat meal; MeDM + RW: Mediterranean meal plus red wine; MeDM + WW: Mediterranean meal plus white wine; MeDM + VDK: Mediterranean meal plus vodka; HFM + RW: high-fat meal plus red wine; HFM + WW: high-fat meal plus white wine; HFM + VDK: high-fat meal plus vodka.

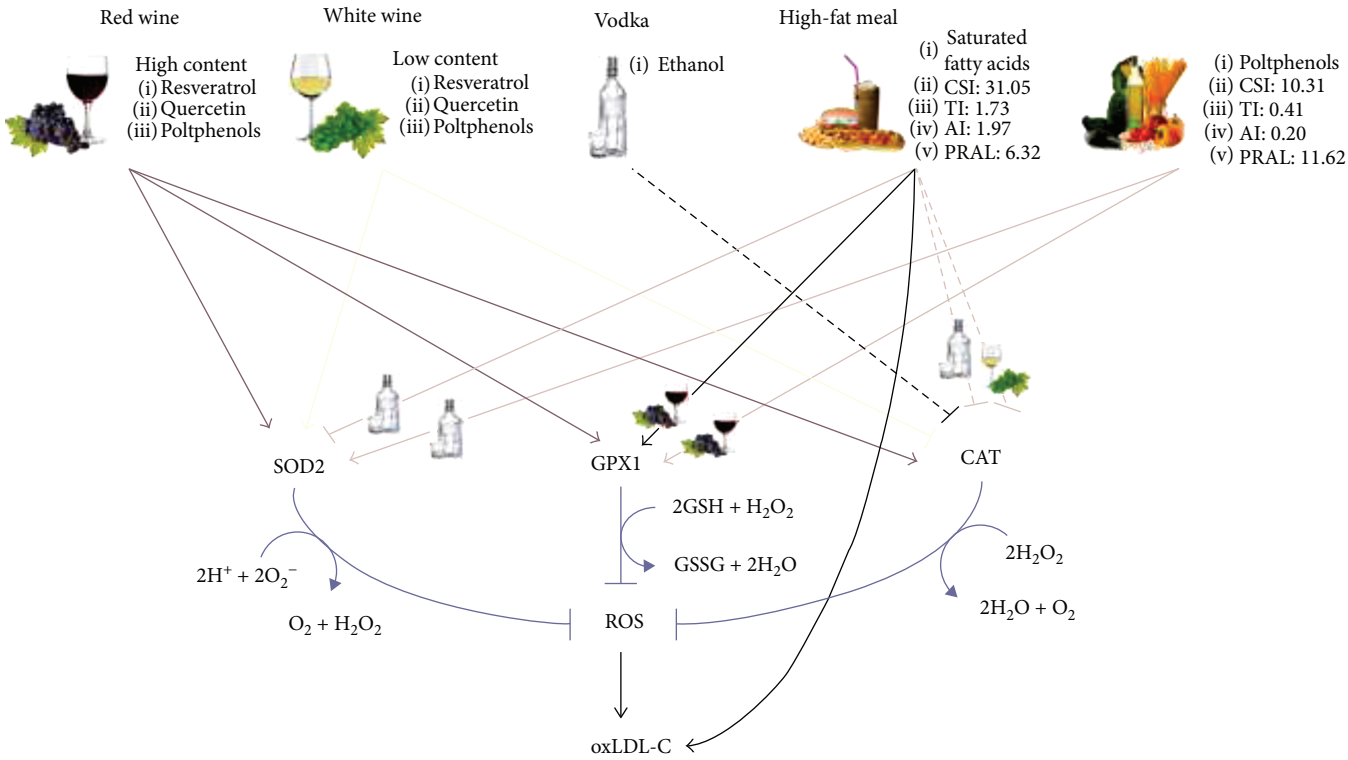

Figure 4: Effects of beverage and/or meal administration on oxLDL-C and gene expression. Effects of fasting red wine, fasting white wine, fasting vodka, Mediterranean meal, high-fat meal, Mediterranean meal plus red wine, Mediterranean meal plus white wine, Mediterranean meal plus vodka, high-fat meal plus red wine, high-fat meal plus white wine and high-fat meal plus vodka on catalase (CAT), superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPX1), and oxidized low-density lipoprotein-cholesterol (oxLDL-C).

formation of cholesterol laden foam cells, which is considered the initial event of atherosclerosis [16]. Furthermore, the inflammation induced by oxidative stress and oxLDL-C on vascular cells increases monocyte and macrophage adhesion and infiltration into the vessel wall causing the foam cell development [29, 30]. In this frame, antioxidant enzymes like SODs, GPX1, and CAT take on great importance in the reduction of circulating ROS as well as in LDL oxidation process. In fact, superoxide anion (O_2^-), which belongs to the free radical molecules of ROS family, can be converted naturally or enzymatically by SODs into hydrogen peroxide (H_2O_2), which, in turn, can be transformed into water by CAT or GPX1 [31]. SOD2, through the control of mitochondrial ROS and NO, regulates endothelial and vascular smooth muscle cells. Its deficiency seems to be involved in onset and development of atherosclerosis through the increase of O_2^- levels and mitochondrial dysfunction, which leads to mitochondrial DNA damage [32]. CAT, instead, can react with H_2O_2 through the four groups of porphyrin heme iron, speeding up its conversion into water. It was observed that reduced expression of CAT can induce atherosclerosis onset and progression [33]. GPX1 is one of the most represented enzymes of GPX family and its ability to reduce H_2O_2 into water makes it inversely associated with CVD risk [34]. In fact, GPX1 shortage determines increasing foam cell formation by oxLDL-C, enhancing atherosclerosis process [35].

For many years, the benefits of fruits and vegetables have long been considered due to their fibers, minerals, and vitamin contents, but studies made in the last few decades have highlighted the importance of phytochemicals in preventing disease and increasing life expectancy [36]. The life expectancy of humans can be prolonged by an appropriate diet, rich in vegetables and fruit, which contain antioxidants. Foodstuffs with a relevant antioxidant effect do contain molecules that can prevent damage to the cellular system incurred by ROS [37].

Novel dietary strategies provide a new window of opportunity in the efforts being made to reduce NCDs and numerous so-called functional foods being proposed for the promotion of health, with simple lifestyle changing. From an evolutionistic point of view, our genes are evolved for metabolizing fermented plant materials, so that the moderate consumption of wine became physiologically an integral part of our diet.

The preventive role of the moderate consumption of wine has broadly been shown and envoy in relationship to the content of phenolic substances [38], mainly resveratrol and proanthocyanidins and other polyphenols (mostly present in red wine) including flavonols, monomeric flavan-3-ols, and anthocyanin [39–41]. The polyphenols, particularly resveratrol and quercetin, contribute to prevent or delay the onset of chronic diseases such as diabetes, inflammation, Alzheimer's disease, and cardiovascular disease. Moreover,

resveratrol induces neuroprotection and inhibits proliferation of human cancer cell lines [42–46].

Both resveratrol and quercetin are able to reduce ROS concentrations in different tissues [44–46], and their dietary supplementation increases CAT, SOD, and GPX expression [47–49]. Thanks to its high concentrations of polyphenols, RW consumption reduces LDL oxidation and prevents endothelial dysfunction [23]. Conversely, WW exhibits lower antioxidant capacity reducing LDL oxidation when compared to RW [50]. Furthermore, WW in rat and human vascular smooth muscle cells has no effect on the development of atherosclerosis [51]. A possible explanation is the low polyphenolic content of WW compared to RW [52] due to the lack of expression of the enzyme flavonoid $3'$,$5'$-hydroxylase in white grapes, which restricts the presence of flavonol and anthocyanin contents [53]. These evidences support the common thought that polyphenols are the reason why wine has beneficial effect on CVD prevention, independently from ethanol content. In fact, dealcoholized RW but not WW greatly increased in vitro plasma antioxidant capacity, reduced oxLDL-C concentrations, improved flow-mediated vasodilation, and increased human endothelial NO synthase [54, 55]. However, data on the role of ethanol and spirts are controversial. Recently, Fawole and Opara [56], throughout an in vitro digestion model, demonstrated that the total phenolic concentration and total flavonoid concentration of a food rich in polyphenols are influenced by the extraction solvents used. In particular, the polyphenol bioavailability is higher in ethanol solvent.

VDK decreases protein oxidative stress in the myocardium but has no effect on normalizing endothelial dysfunction and platelet aggregation [16, 57, 58]. However, VDH seems to not exert protection against oxygen-induced oxidative stress in plasma lipid peroxides [59].

According to previous studies [55, 60], we did not observe significant changes in oxLDL-C concentrations following RW, WW, and VDK consumption as well as among beverage treatments. WW and VDK administrations determined a reduction of CAT expression ($2^{(-\Delta\Delta CT)} = 0.30$ and $2^{(-\Delta\Delta CT)} = 0.23$, resp.), suggesting a possible downstream reduction of antioxidant enzyme gene expression caused by alcohol with low or null polyphenolic content. On the other hand, in WW and RW treatments, but not after VDK consumption, we observed an upregulation of SOD2 ($2^{(-\Delta\Delta CT)} = 3.32$ and $2^{(-\Delta\Delta CT)} = 32.73$, resp.), suggesting a possible greater sensitivity of this gene to both high and low polyphenol concentrations, as reported also by Zhao et al. [60]. At the same time, RW consumption, beyond the increased levels of SOD2, determined an upregulation of CAT and GPx1 expression ($2^{(-\Delta\Delta CT)} = 4.04$ and $2^{(-\Delta\Delta CT)} = 9.12$, resp.) (Figure 4), demonstrating that there is an acute indirect antioxidant response to RW polyphenolic content. However, the fast antioxidant activity exerted by RW did not highlight any changes in oxLDL-C levels. This result is probably due to the little time of plasma exposure to the RW, as also observed by Caccetta et al. [61].

Like RW, Mediterranean diet has been associated with a reduction of coronary events and CVD risk and mortality, probably due to the high content of antioxidants which are contained in the most represented foods of this dietary pattern, that is, vegetables, fruits, legumes, grains, nuts and seeds, fish, and wine [62–65]. Previous studies demonstrated the inverse correlation between Mediterranean diet and plasma oxLDL-C levels [66] as well as the relationship between this diet and antioxidant genes [22]. Conversely, one of the most important risk factors for CVD onset is the regular consumption of a high-fat diet. High-fat meals usually determine increased levels of oxidative stress and endothelial impairing in postprandial period. This process is mainly due to the high presence of saturated fatty acids in meals, which determine a transient hypertriglyceridemia that can activate mitochondrial metabolism and consequently enhance ROS production, leading to oxidative stress and/or lower antioxidant defenses and vascular damage [66]. According to our previous study [22], MeDM administration, with or without beverage combinations, did not determine a significant reduction of oxLDL-C levels compared to baseline as well as between MeDM alone and its association with beverages. A significant reduction of oxLDL-C levels was predictably observed between HFM and MeDM ($p < 0.05$). This effect is probably due to the different polyphenolic and antioxidants intake among meals [22] as well as the increasing levels of oxLDL-C among HFM and baseline ($\Delta\% = +18.49$; $p < 0.05$), depending on the high presence of saturated fatty acids in the meal, which are able to enhance oxidative stress and reduce antioxidant defenses [66].

However, we noticed an upregulation of SOD2 expression ($2^{(-\Delta\Delta CT)} = 2.63$) after MeDM + VDK administration, increased GPX1 gene expression in MeDM + RW ($2^{(-\Delta\Delta CT)} = 8.99$) and HFM + RW group ($2^{(-\Delta\Delta CT)} = 10.5$), and the upregulation of all antioxidant genes observed after the ingestion of RW, acknowledging the role of the antioxidant molecules present in RW on oxidative stress. Conversely, HFM treatment in association with VDK reduced both CAT and SOD2 expressions ($2^{(-\Delta\Delta CT)} = 0.23$ and $2^{(-\Delta\Delta CT)} = 0.49$, resp.), as its combination with WW administration downregulated CAT ($2^{(-\Delta\Delta CT)} = 0.48$). According to Fawole and Opara [56], these results, together with the reduction of oxLDL-C levels observed after HFM + RW treatment compared to HFM ($p < 0.05$) (Figure 4), suggest a pivotal role of ethanol on the bioavailability of polyphenols during digestion.

5. Conclusions

In order to maintain a good health status, it is necessary to have good nutritional habits. Food provides substances that can affect internal homeostasis and then lead to an untreatable full-blown chronic degenerative illness. Our findings support, for the first time based on nutrigenomic approach, the evidence that moderate alcohol consumption has significant health benefits, justifying the promotion of longevity and reduction of the risks of most of the age-related diseases. However, our data should be confirmed on a larger number of subjects, with a prospective long-term trial.

In this work, we observed that genetic regulation due to red wine consumption occurs both with the beverage alone

and in combination with a meal, resulting as a protective food in postprandial state mainly because of its polyphenolic content, which is activated by alcohol. On the other hand, ethanol has a positive effect on gene oxidation pathway only if combined with an antioxidant meal, exerting a potential increase of polyphenols bioavailability during digestion and antioxidant genes expression, controlling LDL-C oxidation pathway.

Within a comprehensive vision of lifestyle medicine [67], according to other studies [68], our results suggest that the association of low/moderate intake of alcohol beverages with nutraceutical-proven effectiveness and ethanol in association with a Mediterranean diet could determine a reduction of atherosclerosis risk onset maintaining postprandial oxLDL-C levels steady and a positive modulation of antioxidant gene expression. Moreover, we highlighted the importance to choose healthy meals associated to alcoholic beverages for the prevention of inflammatory and oxidative damages.

In conclusion, we suggest that a good dietetic plan, finalized to the reduction of NCDs onset and progression, should contemplate a moderate consumption of alcoholic beverages.

Abbreviations

ALT/GPT: Alanine transaminase
AST/GOT: Aspartate aminotransferase
AI: Atherogenic index
BAS: Basophiles
CVDs: Cardiovascular diseases
CAT: Catalase
CSI: Cholesterol-saturated fat index
CT: Comparative threshold
CRP: C-reactive protein
EOS: Eosinophils
ESR: Erythrocyte sedimentation rate
FC: Fold change
GPX1: Glutathione peroxidase 1
GRN: Granulocytes
HDL-C: High-density lipoprotein cholesterol
HCT: Hematocrit
HGB: Hemoglobin
HFM: High-fat meal
HOMA-IR: Homeostasis model assessment of insulin resistance
LDL-C: Low-density lipoprotein cholesterol
LYM: Lymphocytes
MCH: Mean corpuscular hemoglobin
MCHC: Mean corpuscular hemoglobin concentration
MCV: Mean corpuscular volume
MPV: Mean platelet volume
MeDM: Mediterranean meal
MON: Monocytes
MUFA: Monounsaturated fatty acids
NO: Nitric oxide
NCDs: Noncommunicable diseases
oxLDL-C: Oxidized LDL-C
PDW: Platelet distribution width
PLT: Platelets
PUFA: Polyunsaturated fatty acids
PRAL: Potential renal acid load
PCT: Procalcitonin
RBC: Red blood cells
RDW: Red cell distribution width
ROS: Reactive oxygen species
RW: Red wine
SOD2: Superoxide dismutase 2
TI: Thrombogenic index
TC: Total cholesterol
Tg: Triglycerides
VDK: Vodka
WBC: White blood cells
WW: White wine
ACTB: β-Actin.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

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

Authors' Contributions

Laura Di Renzo and Giorgia Cioccoloni contributed equally to this work. Laura Di Renzo designed the research and wrote the paper; Giorgia Cioccoloni analyzed the data and wrote the paper; Paola Sinibaldi Salimei and Ida Ceravolo conducted the research; Santo Gratteri and Antonino De Lorenzo had primary responsibility for the final content. All authors read and approved the final manuscript.

Acknowledgments

The authors thank Doctor Simone Falco for technical research assistance and the "Master of Science Degree in Physical Activity and Health Promotion" for their support and the involvement of their students finalized to their master's dissertation production. This study was supported by the grants from the Ministry of Agriculture, Food and Forestry (DM 18829/7818/2009).

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