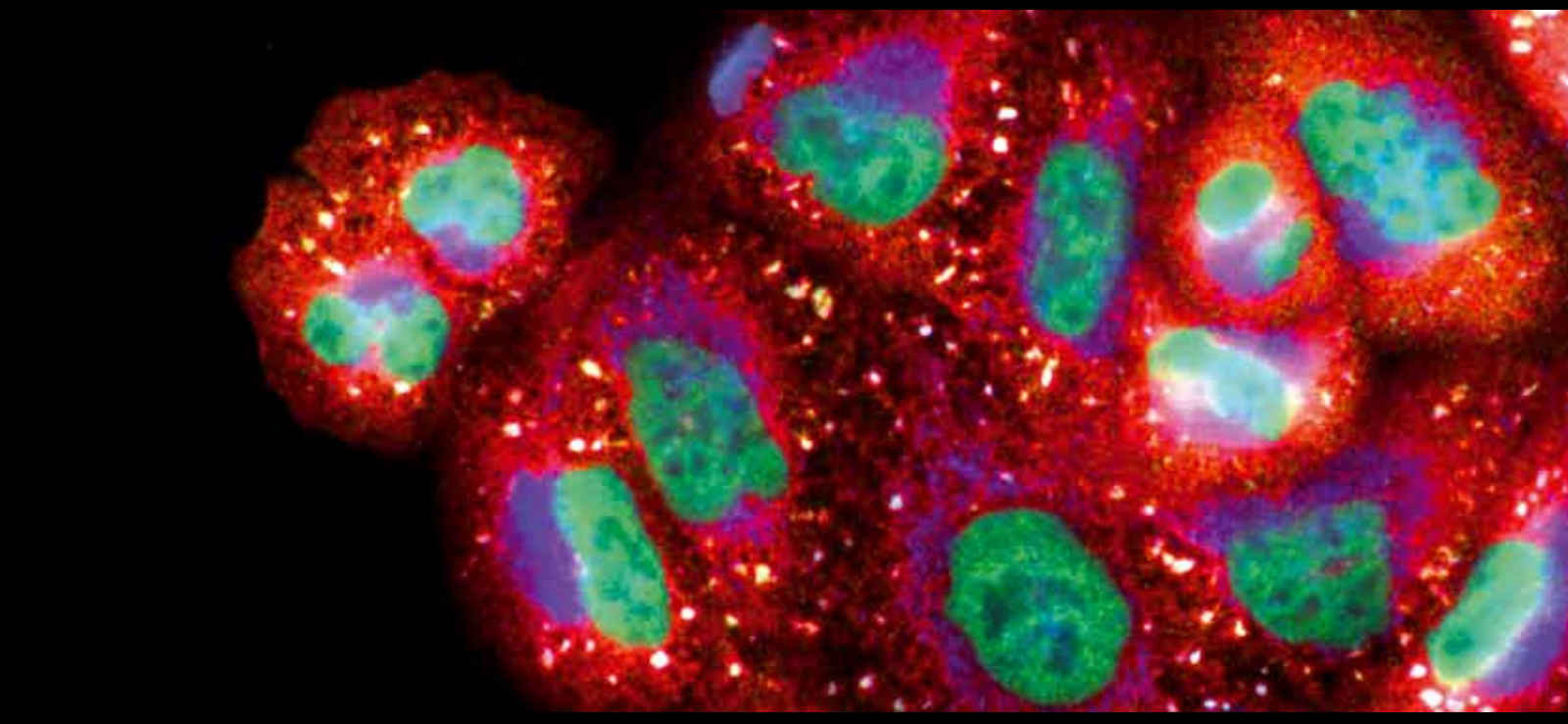
Oxidative Medicine and Cellular Longevity

Antioxidant Therapies for Neurodegenerative Diseases: Mechanisms, Current Trends, and Perspectives

Guest Editors: Daniel Pens Gelain, Guilherme Antonio Behr, Ramatis Birnfeld de Oliveira, and Madia Trujillo



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Editorial Board

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Editorial

Antioxidant Therapies for Neurodegenerative Diseases: Mechanisms, Current Trends, and Perspectives

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Over the last decades, the involvement of free radicals and oxidative stress in the molecular mechanisms related to ageing, cancer, atherosclerosis, neurodegenerative disorders, diabetes, and inflammatory diseases became increasingly clear. As a consequence, the interest on the potential therapeutic applications of antioxidants has also increased. Naturally occurring antioxidants as well as modified or synthetic compounds have been screened for biological activities that could be useful in the development of new therapies for diverse conditions. Research using *in vitro* (cell culture and chemical free radical-generating systems), *in vivo* (animal models), and *in silico* models as well as epidemiologic and different types of clinical studies have been conducted by a great number of researchers worldwide, in order to evaluate the possible therapeutic properties of compounds with antioxidant activity. However, despite the massive accumulation of evidence confirming the role of oxidants in several pathological conditions (especially cancer), the absolute majority of antioxidants developed for clinical use has been proven ineffective.

In fact, a considerable number of compounds intended to act as antioxidants in developing therapies were actually reported to induce acute or long-term toxic effects, including oxidative stress itself. One of the best documented examples is the case of vitamin A, which was widely regarded as an essential antioxidant component of the diet during the 1980 decade. A large clinical trial in the early 1990s The Beta-Carotene and Retinol Efficacy Trial (CARET) evaluated the effect of oral intake of beta-carotene and retinol in 18,314 men and women at high risk for lung cancer, but it had to be discontinued due to increased incidence of lung cancer, cardiovascular disease, and mortality. Following this study, several papers demonstrated that according the concentration, type of administration, and other factors, vitamin A may act as a strong pro-oxidant to living cells. Presently, some authors believe that even pro-oxidant agents or processes, which were earlier recommended to be avoided, may be important to maintain a healthy status, as low/mild exposure to free radicals would help cells to maintain an effective antioxidant defense system through physiological mechanisms, in a process that could be called "oxidative stress preconditioning." Thus, it is clear that antioxidants and antioxidant-based therapies must be cautiously addressed by researchers in the field of redox biology.

One of this special issue papers investigates the effect of the antioxidants trolox (hydrophilic analogue of alpha-tocopherol) and dithiothreitol (DTT) on mouse *locus coeruleus* neurons. This study demonstrates that pacemaker currents and firing rates were heterogeneously modulated in half of the neuronal population accessed, leading to the hypothesis that these neuronal populations could be the first ones affected in neurological disorders involving imbalance in the oxidant/antioxidant content. Another paper of this special issue presents an investigation on the potential of an

ancient traditional medicine used in China, the pine pollen, as a controller of cellular senescence through reduction of oxidative stress and free radical-related formation of advanced glycation endproducts (AGEs), which are relevant inducers of chronic inflammation and neurodegenerative processes. Another paper in this issue demonstrates that the redox impairment caused in hippocampus by the molecular constituent of amyloid plaques, the amyloid-beta peptide (Aβ), is a result of increased Aβ cellular internalization and deposition to mitochondria, and melatonin is an important endogenous protector in this context. Another article is a review on the possible mechanisms involved in the antioxidant action of S-allylcysteine and old garlic extract, which is enriched in S-allylcysteine, on different biological systems, evaluating the potential clinical and therapeutic applications of these products.

Another paper of this special issue reviews the clinical literature on oxidative and antioxidant effects associated with antidepressant agents and discusses their potential effects in the treatment of major depressive disorder. This interesting analysis suggests that augmentation of antioxidant defenses may be one of the mechanisms underlying the neuroprotective effects of antidepressants in major depressive disorder therapies. Another work published in this special issue addresses an innovative topic, reviewing the recent advances in therapies for neurodegenerative diseases that are based on glutathione (GSH) delivery systems. Glutathione is regarded as one of the major intracellular antioxidants, but its limited stability and bioavailability pose serious difficulties in the development of GSH-based therapies for clinical application. Another work in this issue addresses the redox and transcriptional effects of gases used in medicine (such as carbon monoxide, hydrogen sulphide and hydrogen) on antioxidant enzymes. Finally, another article of this special issue is an updated review on the current state of the use of antioxidants for therapies aimed to Alzheimer's disease. This comprehensive work summarizes many of the aspects that must be taken into account when dealing with antioxidants that exhibit a potential therapeutic application. Many compounds first appear to be promising in animal and in vitro models but fail to show relevant clinical results. Also, the relationship between endogenous antioxidants with disease prevention and/or prevalence revealed to be predictive in some epidemiological and transversal studies but were not observed in other similar works. The reasons accounting for such inconsistency are still not clear but certainly urge both caution and more extensive research on this subject.

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Review Article

Antioxidant Therapies for Alzheimer's Disease

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Alzheimer's disease (AD) is the most common neurodegenerative disease featuring progressive impairments in memory, cognition, and behavior and ultimately leads to death. The histopathological changes of Alzheimer's disease include neuronal and synaptic loss, formation of extracellular senile plaques and intracellular neurofibrillary tangles in brain. Multiple lines of evidence indicate that oxidative stress not only strongly participates in an early stage of Alzheimer's disease prior to cytopathology, but plays an important role in inducing and activating multiple cell signaling pathways that contribute to the lesion formations of toxic substances and then promotes the development of Alzheimer's disease. Many years of studies show that antioxidant therapies have enjoyed general success in preclinical studies. Therefore, this paper mainly focuses on the recent developments of common used antioxidant therapies for Alzheimer's disease and thus provides indications for future potential antioxidant therapeutic strategies of neurodegenerative diseases.

1. Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders that frequently cause dementia and affect the middle- to old-aged individuals, approximately one in four individuals over the age of 85. Dementia is characterized by a progressive cognitive decline leading to social or occupational disability. However, AD must be differentiated from other causes of dementia including vascular dementia, dementia with Lewy bodies, Parkinson's disease with dementia, frontotemporal dementia, and reversible dementias [1]. AD has multiple etiological factors including genetics, environmental factors, and general lifestyles [2], and its pathophysiological hallmarks include extracellular β-amyloid protein (Aβ) deposition in the forms of senile plaques and intracellular deposits of the microtubule-associated protein tau as neurofibrillary tangles (NFTs) in the AD brains. When the first case of a 51-year-old woman who presented with a relatively rapidly deteriorating memory along with psychiatric disturbances was diagnosed as AD by Alois Alzheimer in 1907, this disease was considered to be a relatively uncommon disorder with a variety of progressive and fatal neurological conditions including senile dementia and the early age at onset. Subsequent clinical and neuropathological studies identified senile plaques and NFTs as the most common causes of the disease in the elderly. Aβ is produced by sequential proteolytic processing of a larger Aβ protein precursor (APP) by β-secretase to generate a large secreted fragment sAβPP and a 99 aa cellular fragment CTFβ that includes Aβ, the transmembrane domain and the intracellular domain of APP [3]. AD is usually recognized as a syndrome, a common clinical-pathological entity with multiple causes. Specifically, the diagnosis of this disease is based not only on memory loss and impairment of at least one other cognitive domain, but also on a decline in global function, a deterioration in the ability to perform activities of daily living, and the appearance of disturbance in social or occupational function. What's more, characteristic idiopathic psychometric deficits upon clinical evaluation and further postmortem confirmations of the presence of the characteristic lesions mentioned above are quite necessary for its diagnosis.

Considerable variability in initial clinical presentation may be dependent on the brain regions affected. Over time, AD could be divided into two clinical phases depending on

the age of onset. A type of "presenile" dementia was usually defined for individuals younger than 65 years of age, whereas a similar dementia in the elderly, for example, in individuals over 65-year-old, was referred to as senile dementia of the Alzheimer type after the pioneering studies conducted by Roth et al. [4–6]. AD is now generally recognized as a single major cause of dementia with a prevalence that increases sharply after age 65. It has been reported that rare early-onset forms of AD are linked directly to highly penetrant autosomal dominant mutations in one of three different genes: amyloid precursor protein (APP) gene, presenilin (PS) 1 gene, or presenilin 2 gene. Early-onset forms of AD generally have extensive lesions including a tendency for white matter lesions and "cotton wool" plaques in some early onset cases associated with exon 9 presenilin-1 mutations [7]. Interestingly, in addition to classic AD pathology, Lewy body pathology has also been described in familial early onset cases [8]. However, late-onset AD (LOAD) which accounts for approximately 95% of all AD cases [9] likely results from the complex interplay of molecular, environmental, and genetic factors. It represents a significant and growing public health burden and is the third most costly medical conditions in the USA The causes of late-onset AD are not yet clarified, but aside from age, several other risk factors include family history of dementia, down syndrome, head trauma, being female, low education level, vascular disease, and environmental factors and genetic risk factors have been identified, therefore, high education, ingestion of estrogen, nonsteroidal anti-inflammatory drugs, and vitamin E may be protective for AD. AD pathology is found prematurely in down syndrome, which argues in favor of the amyloid cascade hypothesis, given that the β-amyloid protein precursor (AβPP) can be found on chromosome 21 and that down syndrome subjects have an extra copy of chromosome 21. Similar pathological presentations have been found in cases of down syndrome and most of these pathological features may also be present to a lesser extent in a large proportion of aged nondemented controls, lending support to the possibility that such individuals are in an extremely early preclinical stage of AD [10]. Previous studies have demonstrated that the most potent cause of late-onset AD is the ε4 allele of the apolipoprotein (apo) E gene (APOE) on chromosome 19 [11]. The effect appears to be dose dependent. It has been reported that the presence of a single ε4 allele increases the risk of AD by 2- to 4-fold while the double ε4 allele increases the risk of AD by 4- to 8-fold. However, possessing the ε4 allele is neither necessary nor sufficient for the development of AD. Therefore, though APOE genotyping may be useful for confirmation in some patients with dementia when a diagnosis of AD is unclear, it is not recommended by experts as a predictive test for AD in asymptomatic individuals, whereas APOE genotyping could be used in patients with a clinical diagnosis of AD and it may increase the specificity of the diagnosis.

2. Epidemiology

AD constitutes approximately 65% to 75% of all dementia cases. The prevalence of dementia in the United States in individuals aged 65 years or older is about 8%, incidence of AD increases with age, doubling every five to ten years from 1% to 2% at ages 65 to 70 years, to 30% and higher after the age of 85 years if those with milder forms of dementia or cognitive impairment are included. Rates of dementia are very much age dependent and increases exponentially with age. For persons between ages 65–69, 70–74, 75–79, 80–84, and 85 and older the incidence of AD has been estimated at 0.6%, 1.0%, 2.0%, 3.3%, and 8.4% [12]. Prevalence increases from 3% among individuals aged 65–74 to almost 50% among those 85 or older [13]. AD affects 25 million people worldwide and in the USA, prevalence was estimated at 5 million in 2007 and, by 2050, is projected to increase to 13 million [13]. Globally, the number of the elderly (aged 65 years and older) is projected to increase dramatically, more than doubling from 420 million in 2000 to 973 million in 2030 [1].

3. Pathophysiology

The exact mechanism of AD is still not clear. The most common and distinctive hallmark lesions present within the AD brains are the β-amyloid (Aβ-) containing senile plaques and the NFTs composed of hyperphosphorylated tau protein, which generate strong responses from the surrounding cellular environment and are responsible for much of the late-stage cognitive decline observed in AD patients. The neuritic plaque has been often highlighted as one of the major consensus criteria for the diagnosis of AD at autopsy. The other major hallmark NFTs are also occasionally called pretangles. Some studies have showed that the development of NFTs may protect against neuronal damage, since mutations in the Aβ precursor protein (APP) and the presence of NFT-containing neurons are associated with the reduced steady-state production of Aβ and reduced levels of oxidative stress. Alternative theories for the pathogenesis of AD suggest that such pathological formations of senile plaques and NFTs are primarily responsible for neurodegeneration. For example, when Aβ has aggregated sufficiently, this protein elicits a neuroinflammatory response via the activation of microglia and astrocytes [14, 15]. Following the initial neuroinflammatory response, the neurotoxic byproducts of inflammation cause additional oxidative damage to cells [16]. Similarly, the hyperphosphorylated tau fibrils create cytoskeletal stresses and promote neuronal dysfunction [17].

Neuronal and dendritic loss, neuropil threads, dystrophic neurites, granulovacuolar degeneration, Hirano bodies, and cerebrovascular amyloid are also typical of the AD brain. It is well recognized in recent years that [18] synapse loss is the most specific pathological feature of Alzheimer's disease, which is due at least in part to the recent studies to associate synapse loss with low-n soluble Aβ species. However, it should be pointed out that assessment of the synapse is not recommended in the diagnosis of AD at autopsy either by immunohistochemistry or electron microscopy [19].

The distinctive pathology between AD and aging is problematic, particularly in the elderly. Regarding the correlation between clinical diagnosis and pathological findings, it has

been reported that 76% of brains of cognitively intact elderly patients were interpreted as AD brains [20].

4. Oxidative Stress and Alzheimer's Disease

Accumulating evidence suggests that brain tissues in AD patients are exposed to oxidative stress during the development of the disease. Oxidative stress or damage such as protein oxidation, lipid oxidation, DNA oxidation, and glycoxidation [21] is closely associated with the development of Alzheimer's disease. AD is characterized by neuronal and synaptic loss, formation, and accumulation of extracellular Aβ plaques produced from APP processing and intracellular NFTs composed of aggregated hyperphosphorylated tau proteins in brain, proliferation of astrocytes, and activation of microglial. Oxidative stress is generally characterized by an imbalance in production of reactive oxygen species (ROS) and antioxidative defense system which are responsible for the removal of ROS [22], both systems are considered to have major roles in the process of age-related neurodegeneration and cognitive decline. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet$OH), singlet oxygen ($^1O_2$), alkoxyl radicals (RO$\bullet$), peroxyl radicals (ROO$\cdot$), and peroxynitrites ($ONOO^-$), contribute to pathogenesis of numerous human degenerative diseases [23]. Certain antioxidants including glutathione, α-tocopherol (vitamin E), carotenoids, ascorbic acid, antioxidant enzymes such as catalase and glutathione peroxidases are able to detoxify H_2O_2 by converting it to O_2 and H_2O under physiological conditions [24]. However, when ROS levels exceed the removal capacity of antioxidant system under pathological conditions or by aging or metabolic demand, oxidative stress occurs and causes biological dysfunction [24]. For example, high levels of protein oxidation, lipid oxidation, advanced DNA oxidation and glycoxidation end products, carbohydrates, formation of toxic substances such as peroxides, alcohols, aldehydes, free carbonyls, ketones, cholestenone, and oxidative modifications in nuclear and mitochondrial DNA [25] are the main manifestations of oxidative stress or damage occurred during the course of Alzheimer's disease. Elevated levels of those oxidated formations mentioned above have been described not only in brain, but in cerebrospinal fluid (CSF), blood, and urine of AD patients [25, 26].

Age-related memory impairments correlate with a decrease in brain and plasma antioxidants defense mechanism. An important aspect of the antioxidant defense system is glutathione (GSH) which is responsible for the endogenous redox potential in cells [27]. Its most important function is to donate electrons to ROS so as to scavenge them. Intracellular glutathione concentration decreases with age mammalian brain regions including hippocampus [28, 29] which may lead to a situation that the rate of ROS production exceeds that of removal thus induces oxidative stress. Therefore, the imbalance among the radical detoxifying enzymes is a cause for oxidative stress in AD.

Many studies have provided evidence for the deleterious consequences of oxidative stress products on certain cellular targets in AD. Protein oxidation and the oxidation of nuclear and mitochondrial DNA have been observed in both AD patients and in elderly patients without AD, but the oxidation of nuclear and mitochondrial DNA appears to be present in the parietal cortex [30], whereas protein oxidation appears to be more marked in AD patients in the regions presenting the most severe histopathologic alterations [31]. In addition, increased lipid peroxidation in the temporal lobe where histopathologic alterations are very noticeable in the AD brains has been observed. The APOE genotype and those with the ε4 allele seem to be more susceptible to peroxidation than those without this allele [32].

As mentioned earlier, several studies have identified many end products of peroxidation including malondialdehyde [33], peroxynitrite [14, 34], carbonyls [35], advanced glycosylation end products (AGEs) [33], superoxide dismutase-1 [36], and heme oxygenase-1 [37] which is a cellular enzyme that is upregulated in the brain and in other tissues in response to an oxidative stimuli in the brains of AD patients, particularly in the NFTs.

5. Lipid Oxidation in AD

The lipoperoxidation phenomena also exert important influences on the pathogenesis of AD. Aβ causes lipoperoxidation of membranes and induces lipid peroxidation products. Lipids are modified by ROS and the correlations among lipid peroxides, antioxidant enzymes, senile plaques, and NFTs in AD brains are very strong. Markesbery showed that lipid peroxidation is a major cause of depletion of membrane phospholipids in AD [38]. Several breakdown products of oxidative stress have been observed in AD brains compared to age-matched controls [39], including acrolein, malondialdehyde, F2-isoprostanes, and 4-hydroxy-2,3-nonenal (HNE) which is the most highly reactive and the major neuronal and hippocampal cytotoxic lipid peroxidation product in high concentrations in AD patients and it contributes to impairments of the function of membrane proteins such as the neuronal glucose transporter GLUT 3, inhibition of neuronal glucose and glutamate transporters, inhibition of Na^+/K^+ ATPases, activation of kinases, and dysregulation of ionic transfers and calcium homeostasis, thus the increased calcium concentration could itself cause a cascade of intracellular events, resulting in increased ROS and cellular death that ultimately induce an apoptotic cascade mechanism, and lead to neurodegeneration in AD. Some other supporting evidence shows that the glutamate-dependent flux of calcium is associated with the production of free radicals by the mitochondria. It is also worth mentioning that elevated level of cerebrospinal fluid concentrations of F2-isoprostane which is produced by free radical-catalyzed peroxidation of arachidonic acid has been observed in AD patients [40]. This discovery is significant not only because it confirms that lipid peroxidation is elevated in AD, but because it provides indications of the possible use of the quantification of cerebrospinal fluid F2-isoprostane

concentrations as a biomarker for AD diagnosis. In addition, Busciglio and Yankner showed that in down syndrome, which involves a similar neurodegenerative component to that of AD, neuronal death occurs according to a process of apoptosis that is related to an increase in lipoperoxidation and can be stopped by catalase and free radical scavengers [41].

6. DNA Oxidation in AD

DNA bases are vulnerable to oxidative stress damage and it has been observed in AD brains that ROS induces calcium influx via glutamate receptors and triggers excitotoxic responses leading to cell death. DNA and RNA oxidation is marked by increased levels of 8-hydroxy-2-deoxyguanosine (8OHdG) and 8-hydroxyguanosine (8OHD) [42] which mostly localized in Aβ plaques and NFTs. Increased levels of DNA strand breaks found in AD brains were first considered to be parts of apoptosis, but it is increasingly recognized that oxidative damage is responsible for DNA strand breaks, and this is consistent with the increased free carbonyls in the nuclei of neurons and glia in AD.

7. Glycoxidation in AD

Advanced glycation end products (AGEs), a diverse class of posttranslational modifications, are formed by a nonenzymatic reaction of a sugar ketone or aldehyde group with long-lived protein deposits and are also potent neurotoxins and proinflammatory molecules. Glycation of proteins starts as a nonenzymatic process with the spontaneous condensation of ketone or aldehyde groups of sugars with the free amino groups of a protein or aminoacid specifically lysine, arginine, and possibly histidine [43]. Accumulation of AGEs in the brain is a feature of aging and is also implicated in the pathophysiological development of AD. There is increasing evidence that the insolubility of Aβ plaques is caused by extensive covalent protein cross-linking and AGEs can be cross-linked to long-lived proteins [44]. Extracellular AGEs accumulation is caused by an accelerated oxidation of glycated proteins, also called glycoxidation and has been demonstrated in senile plaques in different cortical areas in both primitive and classic plaques. Immunohistochemical studies demonstrate that AGEs colocalize to a very high degree with APOE [45]. Some studies have shown the presence of AGEs in association with two major proteins of AD, Aβ and the microtubule-associated protein tau [33] which is the major component of the NFTs, and it has been shown to be subject to intracellular AGEs formation. Tau protein isolated from brains of AD patients can be glycated in the tubulin-binding region, giving rise to the formation of β-sheet fibrils [46], leading to its disability to bind to microtubules. Taken together, it can be concluded that AGEs are involved in the pathogenesis of AD, and free radicals are also involved in glycation processes and can promote the formation of Aβ cross-linking. Additionally, it has been reported that AGEs and β-amyloid activate specific receptors such as the receptor for advanced glycation end products (RAGE) and the class A scavenger-receptor to foster ROS production and regulate gene transcriptions of various factors participated in inflammation through NFκB activation [47].

8. Mitochondrial Dysfunction in Alzheimer's Disease

Mitochondria has been shown to be the center of ROS production. In AD, alterations in mitochondrial function and damaged mitochondria in the course of aging and neurodegeneration have been observed [48]. Consistent defects in mitochondria in AD include defects of the electron transport chain which are major factors contributing to the production of free radicals and the deficiencies in several key enzymes responsible for oxidative metabolism including α-ketoglutarate dehydrogenase complex (KGDHC) and pyruvate dehydrogenase complex (PDHC), which are two important enzymes involved in the rate-limiting step of tricarboxylic acid cycle and cytochrome oxidase (COX) which is the terminal enzyme in the mitochondrial respiratory chain that is responsible for reducing molecular oxygen [49]. It has been reported that postmortem cytochrome-c oxidase activity was lower than normal in the cerebral cortex (frontal, parietal, temporal, and occipital) [50], in the dentate gyrus and the CA4, CA3, and CA1 zones of the hippocampus and in the platelets of AD brains. A decline in cytochrome-c oxidase activity rather than amounts of cytochrome-c oxidase is associated with decreased expression of messenger RNA (mRNA) molecules, which is lower in the midtemporal region of the brains of AD patients [51]. The functional abnormalities in mitochondria promote the production of ROS. In addition, it has been found that damaged mitochondrial DNA (mtDNA) presents in vulnerable neurons in AD brains [52], and formation of mitochondrial-derived lysosomes and lipofuscin is evident in almost all of AD neurons [53]. Quantitative morphometric measurements of the percentage of different types of mitochondria (normal, partially damaged, and completely damaged) indicate that neurons in AD brains show an obvious lower percentage of normal mitochondria and a significantly higher percentage of the completely damaged mitochondria compared to the aged-matched control group [54]. The cybrid technique is very promising for the study of neurodegenerative diseases [54]. Some studies from cybrid cell lines with mitochondria DNA from AD patients showed abnormal mitochondrial morphology, membrane potential, and ROS production, which demonstrated that mutant mitochondrial DNA in AD contributed to its pathology. Low vascular blood flow, Aβ and β-amyloid protein precursor (AβPP) processing machinery have also been implicated in the development of AD and are associated with dysfunctional consequences for mitochondrial homeostasis [55]. AβPP is present in the mitochondrial import channel and potentially inhibits mitochondrial import [56], and it also impairs mitochondrial energy metabolism thus causes mitochondrial abnormalities. Furthermore, homocysteine is also a strong risk factor in the course of AD development and it inhibits several genes

encoding mitochondrial proteins and favors ROS production [57]. Apolipoprotein E4 (APOE4) is another factor that leads to mitochondrial dysfunction. Previous studies have shown that more APOE4 fragment in AD brains than in the age-matched controls, and it shows toxicity and impairs mitochondrial function and integrity [58].

The reason why mitochondrial defects exert an influence on the development of AD is that they can trigger two harmful events: the production of destructive free radicals and a reduction in energy resources. A reduction in the regional cerebral metabolic glucose concentration was observed in AD patients using positron emission tomography at rest. This was seen throughout the neocortex and was found to associate with the severity of dementia.

9. Therapeutic Strategies for Alzheimer's Disease

While no drug has been shown to completely protect neurons, there are two possible conceptual approaches to the treatment of AD. One approach is the treatment that prevents the onset of the disease by sequestering the primary progenitors or targets and reduces the secondary pathologies of the disease, slows disease progression or delay onset of disease, leads to the cessation or even the repair of neuronal damage after onset of disease, and eventually prevents the development of AD; another approach is the symptomatic treatment that treats the tertiary cognitive symptoms of the disease and protects from further cognitive decline. This approach reflects the current state of treatment and usually includes treating the cognitive impairment, decline in global function, deterioration in the ability to perform activities of daily living and behavioral disturbances. Notably, the appropriate treatment strategies depend on the severity of the disease and the specificity of each individual; however, currently available therapeutic agents are mainly targeted at specific symptoms of AD, agents such as cholinesterase/acetylcholinesterase inhibitors which are involved in the enhancement of cholinergic neurotransmission and inhibit the degradation of acetylcholine within the synapse are the major treatments for Alzheimer's disease. Other therapeutic agents and strategies including neurotrophins, antioxidants, statins, nonsteroidal anti-inflammatory drugs (NSAIDs), hormone replacement therapy, blocking of excitotoxicity, the Aβ vaccine trials, immunotherapy, and secretase effectors have also been studied, but their use remains controversial. Therefore, preventive and disease-modifying treatment strategies still need further investigations for the eradication of AD from the general population.

Our knowledge of the pathophysiology and the history of AD has been increased greatly over the past decade, yet the definitive causes remain unclear and the cures have been elusive. Antioxidant therapy, as one of the promising therapeutic strategies for AD, has been studied for years. It has been reported that antioxidants such as lipoic acid (also called thioctic acid), vitamin E, vitamin C and β-carotene may help break down intracellular and extracellular superoxide radicals and H_2O_2-cell-damaging compounds that are byproducts of normally functioning cells before these radicals damage cells or activate microglia through their action as intracellular second messengers [59, 60]. However, antioxidants may act as prooxidants under some defined circumstances. For example, vitamin E acts as a prooxidant in isolated lipoprotein suspensions such as parenteral nutrition solutions in clinical conditions [61]. The prooxidative activity of vitamin E at low concentrations of various oxidants has been widely documented in plasma lipoproteins [62–64]. Multiple lines of evidence suggest that the activity of vitamin E towards oxidation of plasma lipoproteins depends on oxidative conditions. Vitamin E consistently inhibits oxidation under strong oxidative conditions which is at high fluxes of free radicals as compared to the concentration of the vitamin [63], while under mild oxidative conditions, vitamin E behaves as a prooxidant and accelerates oxidation by a mechanism of tocopherol-mediated peroxidation [63]. In addition, in 2004, Kontush and Schekatolina reported that computer modeling of the influence of vitamin E on lipoprotein oxidation showed that the vitamin E developed antioxidative activity in CSF lipoproteins in the presence of physiologically relevant, low amounts of oxidants [65]. By contrast, under similar conditions, vitamin E behaved as a prooxidant in plasma lipoproteins, consistent with the model of tocopherol-mediated peroxidation established by Stocker in 1994 [62]. Vitamin A [66] and trace elements with antioxidant properties such as copper and selenium [67] may also become prooxidant both in vivo and in vitro under the right conditions. The prooxidant effects of selenium have been investigated in cultured vascular cells exposed to parenteral nutrition containing various forms and quantities of selenium [67]. In a recent study, Nakamura et al. [68] suggested that vitamin C may play an important role to prevent the prooxidant effect of vitamin E in LDL oxidation. Vitamin C combined with ferrous iron is a standard free radical generating system. The theory that the entire antioxidant systems normally form an important and complex network suggests that antioxidant intake from food is superior to vitamin supplements because high-dose supplementation with a single antioxidant vitamin in isolation could disrupt the balance of the network [69].

Therefore, taking considerations of the pathogenesis and oxidative damage-related mechanisms underlying AD, the network existing among the different antioxidants, and the relationship between prooxidant and antioxidant factors, this paper mainly focuses on the recent developments of common used antioxidants therapies for Alzheimer's disease and thus provides indications for future potential antioxidants therapeutic methods of neurodegenerative diseases. The chemical structure and potential function of the antioxidants are summarized in Table 1.

10. Antioxidant Therapies

10.1. Vitamins and Carotene. Vitamin E (α-tocopherol), vitamin C, and β-carotene are exogenous chain-breaking

Table 1: Chemical structure and potential function of the antioxidants.

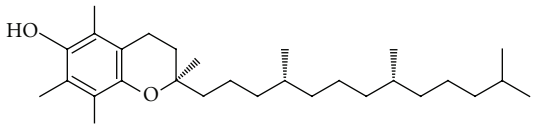
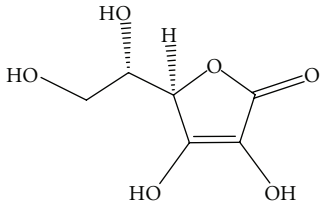
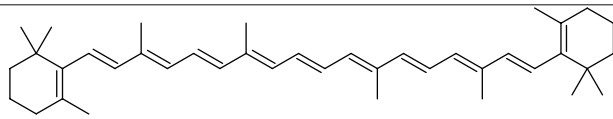
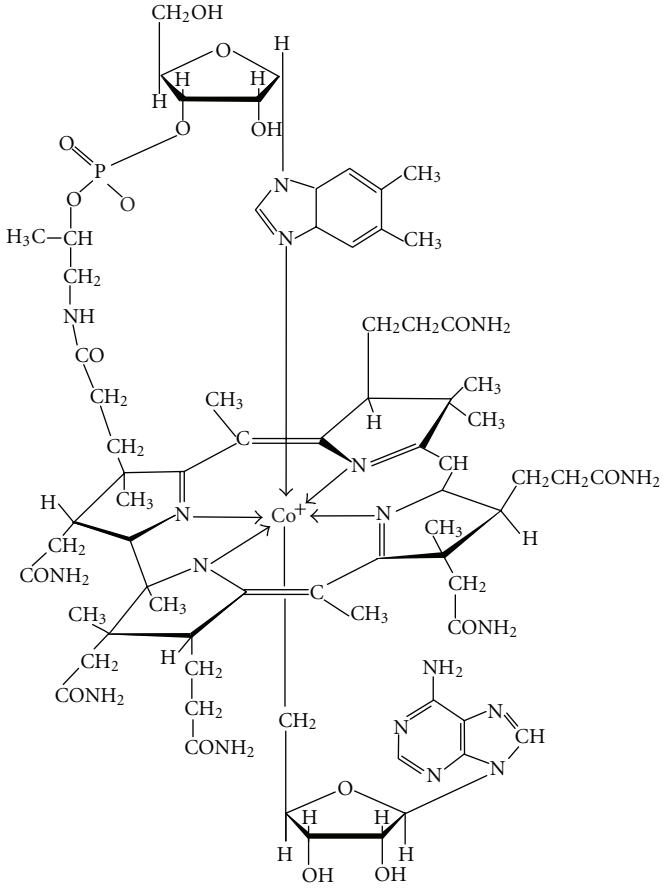
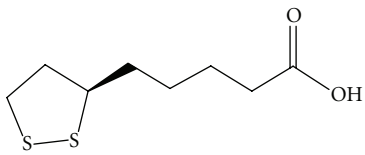
Antioxidant	Chemical structures	Potential functions
Vitamin E (α-tocopherol)		A powerful, lipid-soluble chain-breaking antioxidant found in lipid membranes, circulating lipoproteins and low-density lipoprotein (LDL) particles [70] and has been shown to decrease Free-radical-mediated damage caused by toxic chain reactions in neuronal cells and helps to inhibit dementia pathogenesis in mammalian cells.
Vitamin C		A water-soluble antioxidant, and an inhibitor of lipid peroxidation, acts as a major defense against free radicals in whole blood and plasma.
β-carotene		A lipid-soluble antioxidant which may reduce lipid peroxidation, improve antioxidant status [80], and quench singlet oxygen rapidly [81].
Vitamin B_{12}		An antioxidant that increases choline acetyltransferase activity in cholinergic neurons in cats [88] and improves cognitive functions in AD patients.
LA(α-lipoic acid)		A coenzyme of mitochondrial pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, can recycle other antioxidants such as vitamin C and E and glutathione and increase the production of acetylcholine or as a chelator of redox-active metals to combat the accumulation of lipid peroxidation products [94].

Table 1: Continued.

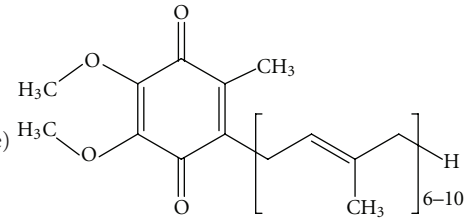
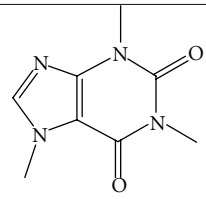
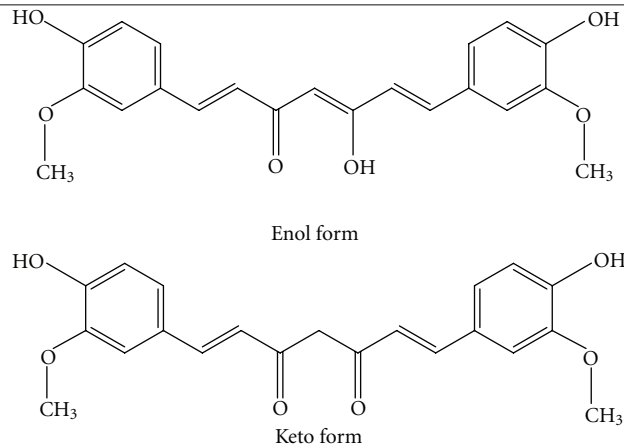
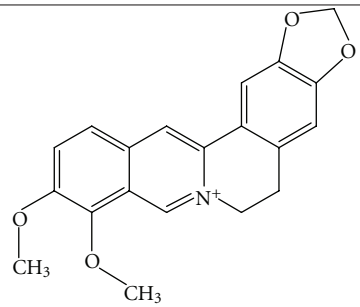
Antioxidant	Chemical structures	Potential functions
CoQ10 (ubiquinone)		A cofactor of the electron transport chain and it preserves mitochondrial membrane potential during oxidative stress and protects neuronal cells through attenuating Aβ overproduction and intracellular Aβ plaque deposits.
Selenium		A cofactor of glutathione peroxidase, and Se-glutathione peroxidase can also act as an antioxidant.
MitoQ		An antioxidant targeted to mitochondria and is produced by conjugation of the lipophilic triphenylphosphonium (TPP^+) cation to coenzyme Q [100].
Szeto Schiller (SS) peptide 31		A mitochondria-targeted antioxidants (MTAs) concentrate in the inner mitochondrial membrane and decrease mitochondrial toxicity [103], they can prevent the neurotoxin 1-methyl-4-phenylpyridium (MPP^+-) induced inhibition of oxygen consumption and ATP production and mitochondrial swelling [106].
Caffeine		An antioxidant that can inhibit amyloidosis and Aβ production and reduce brain Aβ levels in transgenic mouse models for early-onset familial AD.
Curcumin	 Enol form Keto form	An antioxidant that has antioxidant, antiinflammatory, and antiamyloid pathology activity in an Alzheimer transgenic mouse models, and it inhibits enzymes lipoxygenase and cyclooxygenase 2 that are responsible for the synthesis of the proinflammatory leukotrienes, prostaglandins, and thromboxanes [108].
Berberine		an antioxidant that exhibits anti-AD effects through both ChEs and Aβ pathways, and can also inhibit ROS and RNS [23].

Table 1: Continued.

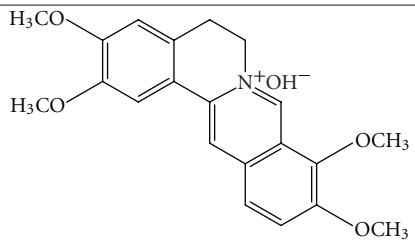
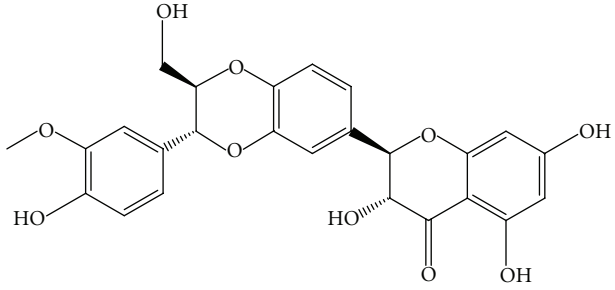
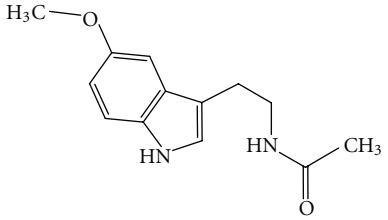
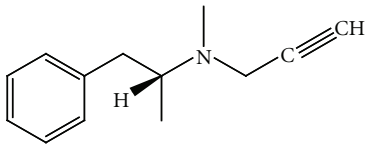
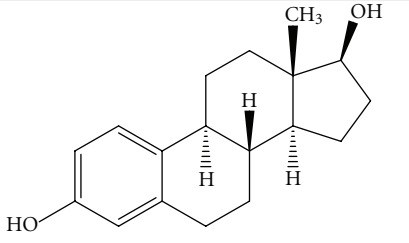
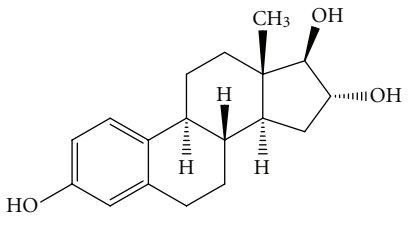
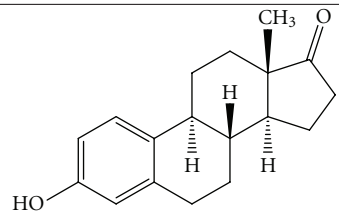
Antioxidant	Chemical structures	Potential functions
Palmatine		An antioxidant that exhibits anti-AD effects through both ChEs and Aβ pathways and can also inhibit ROS and RNS [23].
Silibinin (silybin)		A flavonoid derived from the herb milk thistle (Silybum marianum), it prevents memory impairment and oxidative damage induced by Aβ in mice.
Ginkgo biloba		A natural plant that contains a variety of compounds such as flavonoids and terpenoids which have free radical scavenging ability, and it can reduce amyloid precursor protein [112] and inhibit Aβ aggregation in vitro.
Melatonin		A mammalian hormone synthesized mainly in the pineal gland, and it scavenges oxygen and nitrogen-based reactants generated in mitochondria by stimulating the expression and activity of glutathione peroxidase, superoxide dismutase, and NO synthetase [116], and it also contributes to the reduction of oxidative damage in cells [79].
Selegiline (L-deprenyl)		A selective monoamine oxidase-B inhibitor which can generate potent vasodilator nitric oxide particularly in cerebral blood vessels rapidly [127] and protect the vascular endothelium from the toxic effects of Aβ peptide and enhance the function of nigral neurons or enhance their survival by inhibiting oxidative deamination [73, 115].
Estrogen	 Estradiol  Estriol	An antioxidant which protects neurons from the toxicity of Aβ.

Table 1: Continued.

Antioxidant	Chemical structures	Potential functions
Estrogen	 Estrone	An antioxidant which protects neurons from the tacitly of Aβ.

antioxidants, which decrease free-radical-mediated damage caused by toxic chain reactions in neuronal cells and help to inhibit dementia pathogenesis in mammalian cells. The most important lipid-phase antioxidant is α-tocopherol which is a powerful, lipid-soluble chain-breaking antioxidant found in lipid membranes, circulating lipoproteins and low-density lipoprotein (LDL) particles [70]. In experimental studies, vitamin E has been shown to attenuate toxic effects of β-amyloid and improve cognitive performance in rodents [71, 72]. In 1997, Sano et al. reported that in patients with moderately severe impairment from AD, treatment with α-tocopherol (2000 IU a day) reduces neuronal damage and slows the progression of AD [73], which indicates that the use of α-tocopherol may delay clinically important functional deterioration in AD patients. In 2004, Sung et al. reported that vitamin E suppresses brain lipid peroxidation and significantly reduces Aβ levels and senile plaque deposition in Tg2576 mice when it was administered early prior to the appearance of the pathology during the evolution of AD. However, if vitamin E supplementation was started at a later time point when amyloid plaques were already deposited, no significant effect is observed on the amyloidotic phenotype of these animals despite a reduction in brain oxidative stress [74]. In 2004, Nakashima et al. showed that treatment with α-tocopherol in transgenic (Tg) mice overexpressing human tau protein decreased carbonyls and 8-OHdG [75]. In 2007, Dias-Santagataet reported that administration of α-tocopherol significantly suppressed tau-induced neurotoxicity in Drosophila [76], and similar beneficial results were recently reported by others using transgenic mouse models of human tauopathies and AD [75], which underscored the therapeutic potential of vitamin E. In 2009, Pavlik et al. reported that patients whose regimens included vitamin E tended to survive longer than those taking no drug or a ChEI alone, and there was no evidence that treatment with high doses of vitamin E had an adverse effect on survival in an AD cohort followed-up for up to 15 years [77].

Vitamin C is a water-soluble antioxidant which is also a powerful inhibitor of lipid peroxidation and acts as a major defense against free radicals in whole blood and plasma. Bagi et al. have shown that chronic vitamin C treatment is able to decrease high levels of isoprostanes and oxidative stress in vivo, enhance NO bioavailability, restore the regulation of shear stress in arterioles, and normalize systemic blood pressure in methionine diet-induced hyperhomocysteinemia rats [78]. Besides, vitamin C reduces α-tocopheroxyl radicals rapidly in membranes and LDL to regenerate α-tocopherol and possibly inhibits α-tocopheroxyl radical-mediated propagation [79]. Carotenoid is another lipid-soluble antioxidant which may reduce lipid peroxidation and improve antioxidant status [80]. The most known and studied carotenoid is the β-carotene which is a potent antioxidant able to quench singlet oxygen rapidly [81]. Vitamin C, vitamin E, and carotenoids have shown to synergistically interact against lipid peroxidation [79, 82]. However, it is not completely defined whether nutrient therapy is an effective treatment of Alzheimer disease. Lloret et al.'s findings indicate that vitamin E does not lower plasma oxidative stress for half of the AD patients [83] and some other study results have suggested that either through the diet or by supplements, consuming carotenes or vitamins C and E did not decrease the risk of developing AD, which indicates that antioxidant therapies have only enjoyed general success in preclinical studies in animal models but little benefit in human preventive studies or clinical trials [84]. In 2002, Morris et al. reported that higher intake of foods rich in vitamin E may modestly reduce long-term risk of dementia and AD after adjustment for age, education, sex, race, APOEε4, and length of followup, and this association was observed only among individuals without the APOEε4 allele [85], while dietary intakes of vitamin C, β-carotene, and flavonoids were not associated with dementia risk. Thus, future trials are still needed with a special consideration and focus on individual monitoring of therapeutic potential to avoid toxicity and assess biomarkers of efficacy [86].

In addition to vitamin E and vitamin C, vitamin B_{12} may also have some roles in the treatment of AD. In most studies the serum levels of vitamin B_{12} in AD patients were significantly lower than that of control group, which may partly contribute to degeneration of neurons [87]. Several studies indicated that vitamin B_{12} supplementation increased choline acetyltransferase activity in cholinergic neurons in cats [88] and improved cognitive functions in AD patients [89]. Therefore, the inclusion of vitamin B_{12} in multiple antioxidant therapeutic strategies may be useful.

11. Antioxidant Enzymes

Preventive antioxidants such as metal chelators, glutathione peroxidases, and SOD enzymes including a cytoplasmic antioxidant enzyme-copper-zinc superoxide dismutase

(CuZnSOD) [90] and a prosurvival mitochondrial antioxidant enzyme-MnSOD, repair enzymes such as lipases, proteases, and DNA repair enzymes [21] have also been shown to be essential for neural survival and neuronal protection against oxidative damages [91] and can be used to treat cognitive and behavioral symptoms of Alzheimer's disease [21]. Recent studies suggest that MnSOD plays a protective role during AD development, and MnSOD deficiency increases Aβ levels and accelerates the onset of behavioral alteration in APP transgenic mice [92].

12. Mitochondria-Targeted Antioxidants (MTAs)

Besides well-characterized antioxidants such as vitamin A, carotenoids, vitamin C, and vitamin E mentioned above, other antioxidants such as α-lipoic acid (LA), coenzyme Q10, NADH, Mito Q, Szeto Schiller (SS) peptide, and glutathione which is effective in catabolizing H_2O_2 and anions have some potential therapeutic value in the treatment of certain neurodegenerative diseases. Mitochondrial dysfunction is involved in the pathogenesis of many neurodegenerative diseases including Alzheimer's disease, so developing new therapeutic strategies targeting mitochondrial may shed a new light to AD treatment. It is known that oxidative stress induces mitochondrial fragmentation and altered mitochondrial distribution in vitro, and it also causes both structurally and functionally damage of mitochondria during pathogenesis of Alzheimer's disease [93]. Since overproduction of ROS by mitochondria is one of the major factors that contribute to the course of AD, many drugs targeting mitochondria tested or in development belong to metabolic antioxidants. Those antioxidants including R-α-lipoic acid (LA) and coenzyme Q10 (CoQ10) that can easily penetrate not only the cell, but the mitochondria, may provide the greatest protection.

LA(α-lipoic acid), also called thioctic acid, as a powerful antioxidant therapeutic and the coenzyme of mitochondrial pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, can recycle other antioxidants such as vitamins C and E and glutathione and increase the production of acetylcholine or as a chelator of redox-active metals to combat the accumulation of lipid peroxidation products [94]. In this respect, LA used in conjunction with acetyl carnitine protects neuronal cells via cell signaling mechanisms including some extracellular kinase signaling pathways which are dysregulated in AD [95]. It has been reported that chronic administration of the antioxidant LA decreased the expression of lipid peroxidation markers of oxidative modification but not β-amyloid load within the brains of both control and AD mice models, and LA treatment improved Morris water maze performance in the Tg2576 mouse model but was ineffective at altering cognition in the Y-maze test or in the wild-type group for both tests [96]. Therefore, taken together results obtained from previous studies, we conclude the action of LA may be targeting mitochondria, the most affected organelle responsible for AD development.

CoQ10 (ubiquinone) is a potent antioxidant and also an important cofactor of the electron transport chain where it accepts electrons from complex I and II. It preserves mitochondrial membrane potential during oxidative stress and protects neuronal cells through attenuating Aβ overproduction and intracellular Aβ plaque deposits. It is well known that mitochondrial dysfunction is associated with AD, and coenzyme Q10 and nicotinamide adenine dinucleotide (NADH) are needed for the generation of ATP by mitochondria, so it is essential to use these antioxidants for AD prevention. A study has shown that coenzyme Q10 (ubiquinol) scavenges peroxy radicals faster than α-tocopherol [97] and, like vitamin C, can regenerate vitamin E in a redox cycle [98]. However, it is weaker to improve clinical symptoms in patients with mitochondrial encephalomyopathies [98]. NADH administration (10 mg/day before meal) has been shown beneficial in a pilot study of 17 AD patients [99]. What's more, it has been suggested that selenium is a cofactor of glutathione peroxidase, and Se-glutathione peroxidase can also act as an antioxidant. Therefore, Selenium supplementation together with other antioxidants may also contribute to therapeutic strategies of AD.

Mito Q is another promising therapeutic antioxidant that has been successfully targeted to mitochondria, and it is produced by conjugation of the lipophilic triphenylphosphonium (TPP^+) cation to coenzyme Q [100]. With help of TPP^+, coenzyme Q penetrates into the membrane core and reaches the mitochondrial matrix where it is reduced to its active form (the antioxidant ubiquinol) by complex II to decrease lipid peroxidation, resulting in reduced oxidative damage [101]. It has been reported that Mito Q also exerts protective effects on cells by reducing free radicals, decreasing oxidative damage, and maintaining mitochondrial functions [102]. Some studies report that Szeto Schiller (SS) peptide 31, another kind of mitochondria-targeted antioxidants (MTAs) concentrates in the inner mitochondrial membrane and decreases mitochondrial toxicity [103]. Intracellular concentrations of [3H] SS-31 were 6-fold higher than extracellular concentrations [104]. In 2010, Manczak et al. reported that MitoQ and SS31 prevent Aβ toxicity in mitochondria on neurons from a Tg2576 mouse model and on mouse neuroblastoma (N2a) cells incubated with the Aβ peptide, which suggested that MitoQ and SS31 are warranted to act as potential drugs to treat AD patients [105]. Studies with isolated mitochondria showed that both SS31 and SS20 prevented the neurotoxin 1-methyl-4-phenylpyridium (MPP^+-) induced inhibition of oxygen consumption and ATP production and mitochondrial swelling [106]. These findings provide strong evidence that neuroprotective peptides such as SS-31 which target both mitochondrial dysfunction and oxidative damage are promising for the treatment of Alzheimer's disease [104].

13. Dietary Supplements

Various dietary supplements have been also shown to provide treatment of AD such as omega-3 polyunsaturated fatty acid (docosahexaenoic acid) [21], caffeine, and curry spice curcumin. Caffeine (500 mg or 5-6 cups of coffee a day) [106], epigallocatechin-gallate esters from green tea [106]

and red wine (Cabernet Sauvignon) have been shown to inhibit amyloidosis and Aβ production in both cell culture and animal models. Caffeine has antioxidant properties and has been demonstrated to reduce brain Aβ levels in transgenic mouse models for early-onset familial AD. In 2010, Prasanthi et al. reported that in the cholesterol-fed rabbit model system for late-onset sporadic AD, caffeine decreases cholesterol-enriched diet-induced increase in Aβ production and accumulation, reduces cholesterol-induced increase in phosphorylation of tau, attenuates cholesterol-induced increase in ROS and 8-Iso-PGF2α levels, reduces glutathione depletion, and also protects against cholesterol-induced ER stress [107]. In addition, there is substantial in-vitro data indicating that curry spice curcumin is a promising agent in the treatment or prevention of AD. It has antioxidant, antiinflammatory, and anti-amyloid pathology activity in an Alzheimer transgenic mouse models since it inhibits enzymes lipoxygenase and cyclooxygenase 2 that are responsible for the synthesis of the proinflammatory leukotrienes, prostaglandins, and thromboxanes [108]. It has also reported that curry spice curcumin reduces carbonyls and facilitates disaggregation of Aβ and reduction in AD-associated neuropathology in APP Tg2576 mice [109]. Nonetheless, important information regarding curcumin bioavailability, safety, and tolerability, particularly in the elderly is lacking. Various other factors including life-style factors such as calorie restriction [110], high activity in environmental enrichment and voluntary exercise have been shown to synergistically interact with antioxidants in attenuating AD neuropathophysiology.

14. Traditional Herbal Antioxidants

Some traditional herbal antioxidants also exhibit potential for AD treatment. Jung et al. reported that three major alkaloids in Coptidis Rhizoma-groenlandicine, berberine, and palmatine can potentially exhibit anti-AD effects through both ChEs and Aβ pathways and antioxidant capacities to inhibit ROS and RNS [23]. In addition, silibinin (silybin), a flavonoid derived from the herb milk thistle (Silybum marianum), has been also shown to have antioxidative properties. Silibinin prevents memory impairment and oxidative damage induced by Aβ in mice and may be a potential therapeutic agent for Alzheimer's disease [111]. Ginkgo biloba is a natural plant that contains a variety of compounds such as flavonoids and terpenoids which have free radical scavenging ability. Previous studies have shown that Ginkgo biloba can reduce amyloid precursor protein and inhibit Aβ aggregation in vitro. However, Stackman et al. have reported that Tg2576 mice treated with Ginkgo biloba showed cognitive improvement without any effects on Aβ levels or senile plaque [112]. In 2010, Garcia-Alloza et al. reported that there was no significant effect on senile plaque size after treatment with Ginkgo biloba, and it did not show any effects on Aβ levels measured postmortem by ELISA [113]. In addition, there have been several reports of serious side effects associated with commercially available ginkgo, including coma, bleeding, and seizures [114]. At present, there is still little evidence to support the use of Ginkgo biloba for the treatment of AD [115].

15. Other Antioxidants

15.1. Melatonin. Melatonin is a mammalian hormone synthesized mainly in the pineal gland, and it scavenges oxygen and nitrogen-based reactants generated in mitochondria by stimulating the expression and activity of glutathione peroxidase, superoxide dismutase, and NO synthetase [116], and it also contributes to the reduction of oxidative damage in cells [79]. Currently conduced studies have shown that antioxidant melatonin could inhibit Aβ-induced toxicity [117] and mitigate tau hyperphosphorylation [118–123]. In vivo, melatonin improved learning and memory deficits in an APP695 transgenic mouse model and in vitro, melatonin attenuated Aβ-induced apoptosis in AD cell models such as mouse microglial BV2 cells, rat astroglioma C6 cells and PC12 cells [124]. Besides, studies in transgenic AD mice and cultured cells have suggested that administration of melatonin inhibited the Aβ-induced mitochondria-related bax increase and it may also initiate the survival signal pathways. Another experiment demonstrated that melatonin inhibited the phosphorylation of NADPH oxidase via a PI3K/Akt-dependent signaling pathway in microglia exposed to $A\beta_{1-42}$ [117]. Some studies also pointed that in APP Tg2576 mice models, melatonin decreased Aβ burden in young mice but showed no effects on F2-IsoPs or Aβ burden in older plaque-bearing mice [125, 126]. Taken together, the above evidence suggests that melatonin serves as a potential antioxidant therapeutic strategy for Alzheimer's disease but further clinical trials are still needed to determine the clinical value and efficacy of melatonin in the future.

15.2. Monoamine Oxidase-B Inhibitor. Selegiline (L-deprenyl) is a selective monoamine oxidase-B inhibitor with possible antioxidant properties and can be used in the treatment of neurodegenerative diseases. It can generate potent vasodilator nitric oxide particularly in cerebral blood vessels rapidly [127]. It may also protect the vascular endothelium from the toxic effects of Aβ peptide and enhance the function of nigral neurons or enhance their survival by inhibiting oxidative deamination [73, 115]. In 1997, Sano et al. reported that in patients with moderately severe impairment from AD, treatment with selegiline (10 mg a day) reduces neuronal damage and slows the progression AD [73]. These findings suggest that the use of selegiline may delay clinically important functional deterioration in patients with Alzheimer's disease. Though most of studies have shown that selegiline may bring improvements in cognition, behavior, and mood, little evidence shows a global benefit in cognition, functional ability, and behavior. In 2000, the authors of a meta-analysis of 15 clinical trials concluded that there was not enough evidence to recommend selegiline as a treatment for Alzheimer's disease.

15.3. Estrogen. Estrogen has been shown to act as an antioxidant to protect neurons from the toxicity of Aβ [115].

Table 2: Summary of the various antioxidants interventions in cells or animal models of AD and human trials studied for Alzheimer's disease.

Antioxidant intervention	Cells or animal models of AD	Human trials	Outcome	Reference
Vitamin E (α-tocopherol)	Aβ-induced AD rats model		Attenuated toxic effects of Aβ and improved cognitive performance.	[71]
		Treatment with α-tocopherol (2000 IU a day) in patients with moderately severe impairment from AD	Reduced neuronal damage and slowed progression of AD.	[73]
	APP Tg2576 mice		Suppressed brain lipid peroxidation, reduced Aβ levels and senile plaque deposition, and decreased F2-IsoPs levels.	[74]
	Transgenic mouse models of human tauopathies and AD		Suppressed tau-induced neurotoxicity, decreased carbonyls, and decreased 8OHdG.	[74, 75]
	Drosophila		Suppressed tau-induced neurotoxicity.	[76]
		AD patients whose regimens included vitamin E	Longer survival rate than those taking no drug or a ChEI alone.	[77]
Vitamin C	Methionine diet-induced hyperhomocysteinemia rats		Decreased oxidative stress in vivo, enhanced NO bioavailability, restored regulation of shear stress in arterioles, and normalized systemic blood pressure.	[78]
vitamin B_{12}	Cats		Increased choline acetyltransferase activity in cholinergic neurons.	[88]
		AD patients	Improved cognitive functions.	[89]
MnSOD	APP Tg2576 mice		Deficiency of MnSOD increases Aβ levels and accelerates the onset of behavioral alteration.	[92]
LA(α-lipoic acid)	APP Tg2576 mice		Decreased expression of lipid peroxidation markers of oxidative modification but not β-amyloid load within the brains; improved performance in Morris water maze but not effective at altering cognition in the Y-maze test.	[96]
MitoQ and Szeto Schiller (SS) peptide 31	APP Tg2576 mice and mouse neuroblastoma (N2a) cells incubated with the Aβ peptide		Prevented Aβ toxicity in mitochondria on neurons.	[105]
Caffeine	Cholesterol-fed rabbit model system for late onset sporadic AD		Decreased Aβ production and accumulation, reduced phosphorylation of tau, attenuated ROS and 8-Iso-PGF2α levels, and reduced glutathione depletion, and protection against cholesterol-induced ER stress.	[107]
Curcumin	APP Tg2576 mice		Reduced carbonyls and facilitated disaggregation of Aβ and reduction in AD associated neuropathology.	[109]
Silibinin	Aggregated A β_{25-35}-induced AD model mice		Prevented memory impairment and oxidative damage induced by Aβ.	[111]
Ginkgo biloba	APP Tg2576 mice		Improved cognitive functions but without any effects on Aβ levels or senile plaque.	[112]
		Postmortem brain ELISA measurement	No significant effects on senile plaque size or Aβ levels.	[113]

Table 2: Continued.

Antioxidant intervention	Cells or animal models of AD	Human trials	Outcome	Reference
Melatonin	APP695 transgenic mouse model		Improved learning and memory deficits.	[124]
	AD cell models such as mouse microglial BV2 cells, rat astroglioma C6 cells, and PC12 cells		Attenuated Aβ-induced apoptosis, inhibited Aβ-induced mitochondria-related bax increase.	[124]
	Microglia exposed to Aβ_{1-42}		Inhibited phosphorylation of NADPH oxidase via a PI3K/Akt-dependent signaling pathway.	[117]
	APP Tg2576 mice		Decreased Aβ burden in young mice; no effects on F2-IsoPs or Aβ burden in older plaque-bearing mice.	[125, 126]
Selegiline (L-deprenyl)		Treatment with selegiline (10 mg a day) in patients with moderately severe impairment from AD	Reduced neuronal damage and slowed progression of AD.	[73]

AD: Alzheimer's disease; 8OHdG: 8-hydroxy-2-deoxyguanosine; ChEI: cholinesterase inhibitors.

Although estrogen may have a neuroprotective effect [128], it does not appear to improve cognition or function in patients with Alzheimer's disease [129]. At present, there is no evidence that supports recommending the use of estrogen as an antioxidant to decrease the risk of AD or the progression of existing AD, therefore, further studies need to be conducted to determine if treatment with estrogen may prevent or delay the onset of AD or reduce its severity [115].

The various antioxidants interventions in cells or animal models of AD and human trials studied for Alzheimer's disease are summarized in Table 2.

16. Summary

Alzheimer's disease is recognized as a chronic neurodegenerative condition with a long asymptomatic period prior to recognizable clinical dementia. Multiple lines of evidence have shown strong implications that oxidative stress or damage plays essential roles in the pathogenesis of several neurodegenerative diseases such as AD through a number of mechanisms including oxidative damages caused by free radicals which may result in neuronal cell death. AD mice models show that increased oxidative damage is a relatively early event in the pathogenesis of AD that may be suppressed by antioxidants. Therefore, the development of approaches to prevent or reduce oxidative damages may provide therapeutic efficacy, and antioxidants have been identified as parts of these therapeutic strategies for Alzheimer's disease.

Metabolic antioxidants, mitochondria-directed antioxidants, and SS peptides have proved to be effective in AD mouse models and small clinical studies. It has also been shown that treatment with antioxidants vitamin E, vitamin C, selegiline, estrogen, Ginkgo biloba, and so forth may exert certain positive effects on the development of AD, but the efficacy of those antioxidants in clinical patients is still controversial and not so conclusive. Most antioxidant drugs show general success in animal models but less beneficial in human trials; clinical trials for AD prevention and treatment by antioxidants are still in their infancy. Therefore, further studies will be quite necessary to determine if antioxidants may decrease the risk or slow the progression of the disease for AD patients.

Acknowledgments

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Review Article

Therapeutic Approach to Neurodegenerative Diseases by Medical Gases: Focusing on Redox Signaling and Related Antioxidant Enzymes

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Oxidative stress in the central nervous system is strongly associated with neuronal cell death in the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. In order to overcome the oxidative damage, there are some protective signaling pathways related to transcriptional upregulation of antioxidant enzymes, such as heme oxygenase-1 (HO-1) and superoxide dismutase (SOD)-1/-2. Their expression is regulated by several transcription factors and/or cofactors like nuclear factor-erythroid 2 (NF-E2) related factor 2 (Nrf2) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α). These antioxidant enzymes are associated with, and in some cases, prevent neuronal death in animal models of neurodegenerative diseases. They are activated by endogenous mediators and phytochemicals, and also by several gases such as carbon monoxide (CO), hydrogen sulphide (H_2S), and hydrogen (H_2). These might thereby protect the brain from severe oxidative damage and resultant neurodegenerative diseases. In this paper, we discuss how the expression levels of these antioxidant enzymes are regulated. We also introduce recent advances in the therapeutic uses of medical gases against neurodegenerative diseases.

1. Introduction

The brain consumes 20 to 50% of total body oxygen (O_2) consumption, although it only accounts for 2% of the body weight, meaning that brain function is largely dependent on constitutive supply of O_2 [1]. Compared to the normal physiological condition, in which 2 to 5% of total oxygen consumed by cells is converted into reactive oxygen species (ROS) as a byproduct of mitochondrial respiration, excessive and unregulated production of ROS can occur in pathological conditions [2, 3]. Therefore, scavenging and regulating the amount of ROS in the brain is important to maintain normal brain activity.

Although aberrant production of ROS in the central nervous system (CNS) is critically linked to several neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), a set of antioxidant defense system can save the brain from severe injuries [4–8]. Oxidative stress activates a stress response, and adaptation against ROS-derived cellular injury maintains the redox balance and protects cells from lethal damage [9]. This adaptive response often requires upregulation of endogenous antioxidant enzymes, and their expression levels can be regulated by several transcription factors. To date, the importance of transcriptional regulation of antioxidant enzymes is recognized as a route to the discovery of neuroprotective strategies. In this paper, we highlight two major transcriptional regulation factors, nuclear factor-erythroid 2 (NF-E2) related factor 2 (Nrf2), and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α). Also, we focus on the role of heme oxygenase-1 (HOlinebreak and superoxide dismutase (SOD)

in neurodegenerative diseases, because these are the key components of antioxidant mechanism Figure 1. Finally, we would like to introduce recent research on several gases such as CO, H_2, and H_2S (now called medical gases), suggesting a new therapeutic approach against oxidative damage and resultant neurodegenerative diseases, most notably PD.

2. Nrf2: a Master of Redox Homeostasis

Nuclear factor-erythroid 2 (NF-E2) related factor 2 (Nrf2) is an important transcription factor and is recognized as a major contributor to the upregulation of multiple antioxidant defense system in response to oxidative stress. Nrf2 belongs to the cap'n'collar (CNC) family of basic region-leucine zipper (bZip)-type transcription factors [10]. NF-E2 is a heterodimeric protein which contains a large p45 and small p18 subunit [11]. Cloning of its cDNA revealed that p45 contains a cap'n'collar-(CNC-) type bZip domain [12]. The p45 subunit utilizes its CNC-bZip domain to form a heterodimer with p18; the latter has been identified as MafK, one of the small musculoaponeurotic fibrosarcoma oncogene (Maf) transcription factors [12, 13]. The heterodimer binds to an NF-E2 motif; the small Maf protein p18 confers DNA-binding activity to p45, while p45 activates transcription via its transactivation domain [13, 14]. Nrf2 binds to the antioxidant-responsive element (ARE) or the electrophile-responsive element (EpRE) [15, 16]. ARE has been detected in the promoter or upstream promoter regions of the genes encoding phase II antioxidant enzymes including glutathione S-transferase subunits (GST-Ya, GST-P, GST-M1/M3, etc.), glutamate-cysteine ligase catalytic (GCLC) and glutamate-cysteine ligase modifier (GCLM) subunits, the thioredoxin (TRX) and peroxiredoxin (PRX) families, and NAD(P)H:quinone oxidoreductase (NQO-1) [17–21]. Heme oxygenase-1 (HO-1) is also one of the Nrf2-ARE pathway-derived upregulated factors [22, 23], and its transcriptional upregulation is also mediated by some other transcription factors such as AP-1, CREB, and NF-κB [24]. In the CNS, genetic or pharmacological activation of Nrf2-ARE pathway can confer resistance against neurodegenerative disease insults such as AD, PD, HD, and ALS. A lentiviral vector encoding human Nrf2 reduced spatial learning deficits in aged APP/PS1 mice, a mouse model of AD [25]. Compared to neurons, astrocytes have a much greater ability to increase Nrf2-ARE pathway-derived gene expressions, as shown by a study using cortical neuronal cultures obtained from ARE-human placental alkaline phosphatase (hPAP) reporter mice [26], grown under condition where the mixed cortical cultures consist of 30% astrocytes and 70% neurons. Genetic-overexpression of Nrf2 in astrocytes using the glial fibrillary acidic protein (GFAP) promoter (GFAP-Nrf2) has shown protective effects against several animal models of neurodegeneration, for example, motor neuron degeneration produced by expressing mutant human superoxide dismutase 1 in ALS model mice [27], and dopaminergic neuronal loss by a neurotoxin (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP) in PD model mice [28]. Transplantation of astrocytes infected with adenovirus-Nrf2 protects striatal medium-spiny neuron degeneration by a mitochondrial complex II inhibitor (3-nitropropionic acid or malonate) in HD model mice [29].

HO-1, one of the antioxidant enzymes upregulated by Nrf2, is thought to be highly associated with AD pathology. In AD brain, HO-1 is expressed both in neurons and in astrocytes; 86% of GFAP-positive astrocytes in AD hippocampus exhibit HO-1 immunoreactivity, whereas those in age-matched normal tissues are in the range of only 6-7% [30]. HO-1 overexpression in astrocyte by transient transfection of *HMOX1* cDNA significantly decreased intracellular cholesterol concentrations and increased the level of at least four oxysterol species compared to untreated control cultures [30]. In mild cognitive impairment or early AD, enhanced HO-1 expression stimulated astrocyte cholesterol biosynthesis, oxysterol formation, and cholesterol efflux (to sites of neuronal repair and for egress across the blood-brain barrier). Glial cholesterol efflux exceeds biosynthesis, and total cholesterol levels in affected brains are normal or diminished. Regulation of sterol homeostasis is important in AD pathology because a massive increase in the free cholesterol pool saturates the sterol efflux mechanism, which results in an increase in brain cholesterol levels and exacerbates amyloid deposition and neurodegeneration in advanced AD. Upregulation of HO-1 has another therapeutic potential for clearance of tau protein by the ubiquitin-proteasome system (UPS) [30]. Proteasome activity is reduced in AD brain and amyloid beta (Aβ) inhibits the UPS in cultured cells [31, 32]. This influence on UPS, for which heme-derived iron and CO are responsible, promotes intracellular degradation of α-synuclein as observed in *HMOX1*-transfected M17 cells [33]. Therefore, HO-1 is highly associated with the therapeutic approach not only by its antioxidant function but also by its influence on proteasomal degradation of tau and α-synuclein.

3. The Role of PGC-1α in Neurodegenerative Diseases

Since its discovery over a decade ago, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) has been implicated in energy homeostasis, adaptive thermogenesis, β-oxidation of fatty acids, and glucose metabolism [34]. The activity of PGC-1α depends on its ability to form heteromeric complexes with a variety of transcription factors including nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2), and the nuclear hormone receptors [35]. In particular, NRF-1 and NRF-2 are transcriptional regulators that act on nuclear genes encoding for constituent subunits of the oxidative phosphorylation system including cytochrome c, complex I-V, and mitochondrial transcription factor A (Tfam) [36]. Tfam, a transcription factor that acts on the promoters within the D-loop region of mitochondrial DNA and regulates the replication and transcription of the mitochondrial genome, contains consensus-binding sites for both NRF-1 and NRF-2 [37].

Mice lacking PGC-1α show a profound spongiform pattern of lesions in the striatum together with hyperactivity,

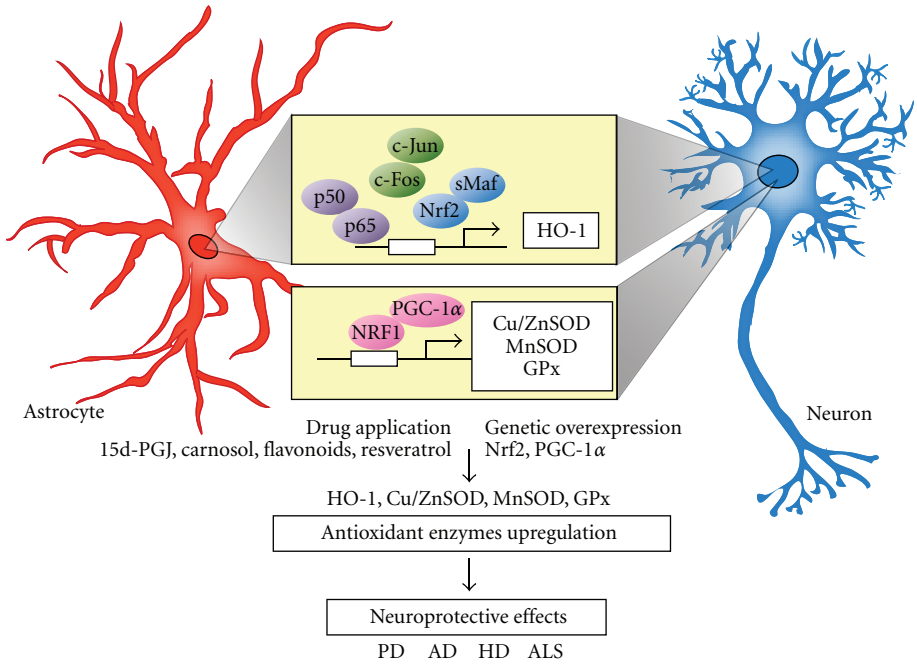

Figure 1: The transcriptional upregulation of antioxidant enzymes in neurodegenerative diseases. Both neurons and astrocytes can increase several antioxidant enzymes including heme oxygenase-1 (HO-1), copper and zinc-containing SOD (Cu/ZnSOD), manganese-containing SOD (MnSOD), and glutathione peroxidase (GPx). By drug application or genetic overexpression of transcription factor, the transcriptional responses via NF-κB (p50/p65), AP-1 (c-Jun/c-Fos), Nrf2/sMaf, and NRF1/PGC-1α in response to oxidative stress and related neurodegenerative disease are activated.

which are the features of human HD [38]. In response to hydrogen peroxide (H_2O_2), there is over a 6-fold increase in PGC-1α expression in mouse embryonic fibroblasts, as well as an increase of the transcription of mRNA encoding ROS defense enzymes such as copper/zinc superoxide dismutase (SOD1), manganese SOD (SOD2), catalase, and glutathione peroxidase (GPx) in association with the transcription factor, cAMP-responsive element binding protein (CREB). PGC-1α expression is reduced by overexpression of mutant Huntingtin through its interference with formation of the CREB/TAF4 complex [39]. The HD striatal cell line, STHdhQ111 also shows reduced expression of PGC1-α target genes encoding mitochondrial cytochrome c and cytochrome oxidase IV. On the other hand, lentiviral overexpression of mitogen- and stress-activated protein kinase-1 increased PGC-1α and protected against striatal lentiviral expression of polyglutamine expansion in huntingtin protein (Exp-Htt) [40]. Moreover, in a postmortem brain tissue study of HD patients, expression levels of 24 out of 26 PGC-1α target genes were reduced, which implies that targeting PGC-1α would be beneficial as a therapeutic approach for HD. PGC-1α might also be beneficial for other neurodegenerative diseases such as PD, as reported in MPTP-induced PD model animals. PGC-1α-deficient mice were more sensitive to neurotoxic insult by MPTP [38], whereas overexpression of PGC-1α protected neurons against MPTP neurotoxicity [41].

PGC-1α activity is regulated by posttranslational modification including direct phosphorylation or deacetylation, which increases PGC-1α activity or expression [42–46]. One such protein is NAD-dependent deacetylase Sir2. Its mammalian or human homologue, SIRT1, has been focused on as a prospective candidate for neuroprotective strategies against AD, PD, HD, and ALS [47–51]. One of the important roles of SIRT1 lies in its deacetylase activity, and its deacetylase substrates such as PGC-1α and forkhead box O3A (Foxo3a) are involved in antioxidant responses and gene transcription [52]. Most especially, overexpression of SIRT1 deacetylase and suppression of GCN5 acetylase increase the transcriptional activity of PGC-1α and prevent mitochondrial loss in neurons induced by expanded Huntingtin protein [53]. A recent study by Martin et al. has revealed that mitogen- and stress-activated kinase (MSK-1), a nuclear protein kinase involved in chromatin remodeling through histone H3 phosphorylation, is linked to the nucleosomal response at the PGC-1α promoter, and transcription via CREB phosphorylation [40].

Among the genes regulated by NRF-1 and/or PGC-1α, SOD1 and 2 are dominant and the first lines of defense against ROS, especially the superoxide anion radical ($O_2^{\bullet -}$), are catalyzed to molecular oxygen and hydrogen peroxide [54]. In humans, three different forms of SOD are reported: SOD1, SOD2 in mitochondria, and extracellular Cu/ZnSOD (SOD3) [55–57]. SOD1 and SOD2 are abundant in the CNS, whereas SOD3 is less abundant than SOD1 and SOD2 [58]. The expression levels of SOD1 and SOD2 are associated with human amyloid precursor protein (hAPP)-/Aβ-induced impairments in aged mouse brain. SOD1 overexpression

protects against the *in vitro* neurotoxicity induced by Aβ [59]. *In vivo*, coexpression of hSOD1 with an APP transgene protects against the lethal effects of APP [60]. SOD2 is enriched around amyloid plaques [61, 62] and brain microvessels [63] in hAPP transgenic mice but decreased in AD brains overall [64]. Esposito et al. has shown that partial reduction in the main mitochondrial superoxide scavenger SOD2 using $SOD2^{+/-}$ mice accelerates the onset of hAPP/Aβ-dependent behavioral abnormalities and worsens a range of AD-related molecular and pathological alterations [65]. On the other hand, overexpression of SOD2 reduces hippocampal superoxide and prevents memory deficits in the Tg2576 mouse model of AD that overexpresses the hAPP carrying the Swedish mutation (K670N:M671L) [66].

4. Reducing ROS by Medical Gases

The generation of ROS and related oxidative damage are believed to be involved in the pathogenesis of neurodegenerative diseases. The main ROS involved in the pathogenesis of neurodegeneration are $O_2^{\bullet -}$, H_2O_2, and the highly reactive hydroxyl radical ($HO^{\bullet}$). Recently, there have been increasing reports showing that medical gases, such as carbon monoxide (CO), nitric oxide (NO), and hydrogen sulfide (H_2S) as well as molecular hydrogen (H_2), might overcome the harmful damage produced by oxidative stress [67, 68]. These gases directly eliminate ROS, or induce resistant proteins and antioxidant enzymes to antagonize oxidative stress Table 1.

4.1. Carbon Monoxide. CO is a diatomic molecule and is soluble in aqueous media and organic solvents [69]. Not only exogenous environmental exposure but also endogenous production during heme metabolism are major sources of CO from primitive prokaryocytes to human [69, 70]. Endogenous production of CO is highly associated with HO-1 activity which induces enzymatic degradation of heme. HO breaks the alpha-methylene carbon bond of the porphyrin ring using NADPH and molecular O_2 in a reaction that releases equimolar amount of biliverdin, iron, and CO [71, 72].

Recent studies have revealed that CO serves as an intrinsic signaling molecule and shows anti-inflammatory and antiapoptotic effects. These effects of CO are mediated by p38 mitogen-activated protein kinase (MAPK) signaling, which is activated in response to physical and chemical stress inducers including oxidative stress, UV light, ischemia, and proinflammatory cytokines [73]. Activation of p38 also mediates the induction of heat shock protein (Hsp72) via its transcriptional factor, heat shock factor-1, leading to the cytoprotective effects [74].

Exogenous CO also activates Nrf2 pathway and decreased infarct size in an ischemia/reperfusion model [75]. Nrf2 activation can coordinately upregulate expression of several antioxidative enzymes recognized to play important roles in combating oxidative stress, including HO-1. Endogenous CO, which is produced by heme degradation, induces ROS-dependent signal transduction in the mitochondrial SOD2 and in HO-1 itself [76].

4.2. Hydrogen Sulfide. H_2S is a flammable, water-soluble gas with a smell of rotten eggs and is known as a toxic gas and as an environmental hazard. The production of H_2S from L-cysteine is catalysed primarily by two enzymes, cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS). Although exposure to higher levels ($\sim$mM) of H_2S is cytotoxic (free radical generation, glutathione depletion, intracellular iron increase, and mitochondrial cell death signal), lower concentration ($\sim\mu$M) of H_2S shows cytoprotective (antinecrotic and antiapoptotic) effects.

Biochemical analysis has revealed that sulphide shows a direct antioxidant reaction with one- or two-electron molecules (one-electron molecules: $\bullet NO_2$, $\bullet OH$, $CO_3^{\bullet -}$, two-electron molecules: peroxynitrite, hydrogen peroxide, hypochlorite, taurine, and chloramine) as well as other low-molecular-weight thiol molecules such as cysteine and glutathione. Although sulphide is not a preferential target for radicals or oxidants due to its low concentration *in vivo*, it can serve as a direct antioxidant [77, 78]. H_2S can also induce upregulation of transcription for anti-inflammatory and cytoprotective genes including HO-1 [79, 80]. By upregulating HO-1 expression, H_2S can trigger the production of CO, which shows anti-inflammatory and antiapoptotic effects.

4.3. Hydrogen. Since the first striking evidence indicating that molecular hydrogen acts as an antioxidant and inhalation of hydrogen-containing gas reduces ischemic injury in brain [81], there have been increasing numbers of reports which support the therapeutic properties of hydrogen against oxidative stress-related diseases and damages in brain [82, 83], liver [84], intestinal graft [85], myocardial injury [86, 87], and atherosclerosis [88]. Hydrogen can be taken up by inhalation of hydrogen-containing air (hydrogen gas) or drinking hydrogen-containing water (hydrogen water). One hour after the start of inhalation of hydrogen gas, hydrogen can be detected in blood, at levels of 10 μM in arterial blood [81]. The content of hydrogen can be measured even after intake of hydrogen water by a catheter, which yields 5 μM in arterial blood calculated after 3 min of hydrogen water incorporation [82]. Taking into account its continuous intake, it is easier and safer to drink hydrogen water than inhaling hydrogen gas.

We have previously showed that H_2 in drinking water reduced the loss of dopaminergic neurons in MPTP-induced Parkinson's disease (PD) mice [89]. The therapeutic effects of H_2 water were observed in another PD model, 6-OHDA-treated rats [90]. In these animal models, administration of neurotoxins decreased the number of dopaminergic neurons in the substantia nigra pars compacta (SNpc), as well as dopaminergic nerve terminal fibers in the striatum. However, taking H_2 water significantly reduced the loss of both neuronal cell bodies and fibers compared with the controls drinking normal water. Mice chronically treated with MPTP using an osmotic pump also showed behavioral impairments observed by open-field test [91], and rats administered with 6-OHDA showed behavioral impairments assessed by the rotarod test. Hydrogen improved these

Table 1: Reducing ROS by medical gases, hydrogen (H_2), carbon monoxide (CO), and hydrogen sulphide (H_2S). These gases can increase endogenous antioxidant enzymes and stress response protein such as HO-1, SOD, and Hsp72. Hydrogen and hydrogen sulphide can directly react with ROS and show radical scavenging effects.

Medical gases	Direct reaction to ROS	Reference	Endogenous cytoprotective protein induction	Reference
Hydrogen (H_2)	Radical scavenging $HO^{\bullet} + H_2 \longrightarrow H_2O + H^{\bullet}$	[67]	Increase superoxide dismutase (SOD)	[81]
			Increase bilirubin $\fallingdotseq$ induction of HO-1	[81]
Carbon monoxide (CO)			Activation of p38 MAPK signaling p38 $\longrightarrow$ HSF1 $\longrightarrow$ Hsp72	[59]
			Activation of Nrf2 pathway Nrf-2 $\longrightarrow$ HO-1	[61]
			Induce superoxide dismutase (SOD2) and HO-1	[62]
Hydrogen sulphide (H_2S)	Radical scavenging	[64]	Upregulation of cytoprotective genes including HO-1	[65]
	$2O_2^{\bullet -} + H_2S \longrightarrow HS\text{–}SH + O_2 + 2OH^-$	[63]		[66]

behavioral impairments in both of these animal models of PD.

In the first report [81], H_2 selectively reduced cytotoxic •OH radicals, whereas the production of other radicals such as superoxide, hydrogen peroxide, and nitric oxide was not altered. This selectivity was verified in a cell-free system, and in particular, the preference for scavenging •OH rather than superoxide was confirmed in PC12 cell culture system. According to Setsukinai et al. [92], both •OH and peroxynitrite ($ONOO^-$) are much more reactive than other ROS. This would explain why H_2 shows a selective reaction with only the strongest radicals, both in the cell-free system and in PC12 cells.

Especially, •OH overproduction in oxidative and neurotoxic reaction by MPTP leads to lipid peroxidation in nigral dopaminergic neurons prior to cell death, observed by 4-hydroxynonenal (4-HNE) immunostaining, the markers of membrane lipid peroxidation. Immunoreactivity of 4-HNE in MPTP-treated mice is increased by three times compared to that in saline-treated mice [89], which is similar to the previous report of 4-HNE protein levels in substantia nigra observed at the same time after MPTP administration using HPLC [93]. H_2 water significantly reduces the formation of 4-HNE in dopaminergic neurons in substantia nigra to the level of control [89]. MPTP-induced loss of dopaminergic neuron is associated with the accumulation of 8-oxo-7, 8-dihydroguanine (8-oxoG) in the dopaminergic neurons. H_2 water significantly decreased this accumulation in the striatum [89]. 8-oxoG is the major oxidized form of guanine in DNA, RNA and nucleotide pool by •OH and is accumulated in both mitochondrial and nuclear DNA; their nomenclature are mt8oxo-G and nu8-oxoG, respectively. Mt8oxoG accumulates in the striatum which is rich in mitochondria in nerve terminals of dopaminergic neurons projecting from the substantia nigra. Although nu8oxoG was not detected in the nigral cell nucleus, H_2 water might prevent the mt8oxoG-induced cellular apoptotic signals, not just reduce •OH in the dopaminergic nerve terminal. On the other hand, the increase in $O_2^{\bullet -}$, which was detected by the $O_2^{\bullet -}$ indicator, dihydroethidium (DHE), was not significantly decreased by H_2 water [89]. Although H_2 prevented superoxide formation in brain slices in hypoxia/reperfusion injury [94], H_2 water might show a preferential reduction of •OH during the protection of dopaminergic neurons.

Initial evidence suggests that H_2 protects cells and tissues against strong oxidative stress by scavenging •OH [81]. Also, H_2 was effective when it was inhaled during reperfusion; when H_2 was inhaled just during the initial ischemia (not in the reperfusion stage), infarct volume was not significantly decreased. It was shown that hydrogen in the brain decreased immediately after stopping inhalation and completely disappeared within 10 min [89], indicating that the effect of hydrogen can be observed only during the period when the oxidative insults occur. According to a previous report [82], H_2 could be detected in the blood 3 minutes after administration of H_2 water into the stomach. However, unpublished data showed that the half-life of H_2 in the muscle in rats was approximately 20 minutes after the administration of H_2 gas. Taking these reports into consideration, H_2 in the brain and other tissues does not stay long enough to exert its ability as an antioxidant to ROS directly. Therefore, it is unlikely that direct reaction of H_2 itself with ROS plays a major role in the neuroprotection, especially with H_2 in drinking water, even though H_2 itself has the ability to reduce •OH preferentially. In accordance with this hypothesis, previous reports from Nakao et al. has demonstrated that drinking hydrogen water increased the amount of antioxidant enzyme, superoxide dismutase (SOD) [95], an endogenous defensive system against ROS-induced cellular damage. It was also reported that H_2-water increases total bilirubin for four to eight weeks compared to baseline. Bilirubin is produced by the catalytic reaction of HO-1, and degradation of heme generates bilirubin as well as CO and free iron. Therefore, taking these observations into consideration, there seem to be other mechanisms for protective effect of H_2 in drinking water, different from that exerted by H_2 inhalation. It is possible that drinking H_2 water has not only the ability to reduce cytotoxic radicals,

but also brings into play novel mechanisms which are related to antioxidative defense system.

5. Conclusion

Recent advance in understanding of the regulation of antioxidant enzyme expression by transcriptional factors has given us the possibility that we can overcome several diseases mediated or induced by oxidative stress. As discussed above, transcriptional upregulation of several antioxidant enzymes like HO-1 and SOD might be beneficial for several neurodegenerative diseases such as AD, PD, HD, and ALS. Several phytochemicals (resveratrol, curcumin, flavonoids, carnosol, etc.) and endogenous mediators (15-deoxy-$\Delta^{12,14}$-PGJ2) can upregulate the antioxidants via transcription factors such as Nrf2, NF-κB, and AP-1 [24]. Surprisingly, HO-1 and SOD are increased by CO, H_2 and H_2S, although we cannot say whether these gases accelerate the stress response signaling or some transcriptional regulation system mediated by Nrf2 and PGC-1α. Together with the fact that H_2, and H_2S themselves have the ability to react with ROS directly, we strongly suggest that these gases can buffer the ROS and in addition might prevent and/or protect the neurons from oxidative stress damages in neurodegenerative diseases.

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Review Article

Recent Advances in the Treatment of Neurodegenerative Diseases Based on GSH Delivery Systems

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Neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease(AD), are a group of pathologies characterized by a progressive and specific loss of certain brain cell populations. Oxidative stress, mitochondrial dysfunction, and apoptosis play interrelated roles in these disorders. It is well documented that free radical oxidative damage, particularly on neuronal lipids, proteins, DNA, and RNA, is extensive in PD and AD brains. Moreover, alterations of glutathione (GSH) metabolism in brain have been implicated in oxidative stress and neurodegenerative diseases. As a consequence, the reduced GSH levels observed in these pathologies have stimulated a number of researchers to find new potential approaches for maintaining or restoring GSH levels. Unfortunately, GSH delivery to the central nervous system (CNS) is limited due to a poor stability and low bioavailability. Medicinal-chemistry- and technology-based approaches are commonly used to improve physicochemical, biopharmaceutical, and drug delivery properties of therapeutic agents. This paper will focus primarily on these approaches used in order to replenish intracellular GSH levels, which are reduced in neurodegenerative diseases. Here, we discuss the beneficial properties of these approaches and their potential implications for the future treatment of patients suffering from neurodegenerative diseases, and more specifically from PD and AD.

1. Introduction

Neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases and amyotrophic lateral sclerosis make up a group of pathologies characterized by a separated etiology with distinct morphological and pathophysiological features. These disorders are defined by a multifactorial nature and have common neuropathological hallmarks such as (a) abnormal protein dynamics with defective protein degradation and aggregation; (b) oxidative stress and free radical formation; (c) impaired bioenergetics and mitochondrial dysfunctions; (d) neuroinflammatory processes [1, 2]. It is difficult to establish the correct sequence of these events, but it has been shown that the oxidative damage to the brains of affected individuals is one of the earliest pathological markers. Oxidative and nitrosative stresses arise from the imbalance between the increased production of both the reactive oxygen species (ROS) and the reactive nitrogen species (RNS) and the cellular antioxidant defense systems [3]. At low levels, ROS function as signaling intermediates for the modulation of cellular activities but, at higher concentrations, they contribute to neuronal membrane damage. The ROS mainly involved in neurodegeneration are the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^\bullet$). RNS, such as nitric oxide (NO), can react with O_2^- to produce peroxynitrite ($ONOO^-$), a powerful oxidant that may decompose itself to form $HO^\bullet$ [4]. Cells normally employ a number of defense mechanisms against free radical such as enzymes (Cu/Zn- and Mn-superoxide dismutase, GSH peroxidase, GSH reductase, catalase, and methionine sulfoxide reductase) and low-molecular-weight antioxidants (vitamin E, ascorbate, and GSH) [5]. Macromolecules such as lipids, proteins, and DNA undergo damage and subsequently cell death mainly

by apoptosis when the antioxidant defense network is not sufficient [6].

2. GSH Depletion in PD and AD

The brain is especially vulnerable to free radical damage because of its high oxygen consumption rate, high content of lipids, and relative paucity of antioxidant enzymes compared with other organs [7]. Significant biological changes, related to a condition of oxidative stress, have been found in brain tissue of individuals affected by PD, AD, and other diseases [5, 8–11]. In particular, data from *postmortem* studies of brains from patients with PD suggest that oxidative stress plays a role in neural degeneration of the pigmented dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) [12]. The normal metabolism of dopamine can generate free radicals and other ROS. Furthermore, in the human SNpc the autooxidation of dopamine leads to neuromelanin and can generate quinone and semiquinone species and ROS [13]. Finally, enzymatic oxidation of dopamine catalyzed by monoamine oxidase leads to formation of H_2O_2, which can react with Fe^{2+} and form the highly reactive radical $HO^{\bullet}$ via the Fenton reaction [14]. All these unfavorable events contribute to alter the antioxidant defenses suggesting that the oxidative stress plays an important role in PD. The strongest alteration in the antioxidant defense is a decrease in GSH concentration [15–17]. According to *postmortem* studies, GSH levels in the SNpc of PD patients are remarkably lower than those of healthy subjects (60% compared to control subjects) while oxidized glutathione (GSSG) levels are slightly increased [18, 19]. Although GSH is not the only antioxidant molecule reported to be altered in PD, it is hypothesized that the magnitude of its depletion is the earliest indicator of nigrostriatal degeneration [20]. Moreover, striatal DA content and GSH levels are not altered in areas of the brain other than SNpc, or in other diseases affecting dopaminergic neurons [21–24]. GSH loss in PD is also accompanied by a reduction in mitochondrial complex I activity, which is regionally selective for the SNpc in PD and does not occur in related basal ganglia degenerative disorders [25]. These findings suggest that decreased nigrostriatal GSH levels can initiate or facilitate a cascade of further oxidative stress with consequent degeneration of dopaminergic neurons in idiopathic PD [26, 27].

AD is characterized by the loss of pyramidal neurons in the hippocampus and cortex, as well as cholinergic neurons in the basal forebrain. The etiology of AD is not completely known yet, although there are different hallmarks that seem to play significant roles in the disease, such as β-amyloid (Aβ) deposits, τ-protein aggregation, oxidative damage in cellular structures, and low levels of acetylcholine (ACh) [28, 29]. In AD patients there is a strong evidence that Aβ-associated free radicals and the resultant oxidative stress are a part of the mechanism that is involved in the pathogenic cascade that leads to neurodegeneration in AD brain [30]. Furthermore, alterations to GSH metabolism have been found in these pathological conditions [16]. In this context, Gu et al. [31] reported that GSH levels are depressed in AD cingulated cortex and AD *substantia innominata*, while Liu et al. [32] found these reduced levels only in red blood cells of male AD patients. Nevertheless, increased GSH levels have been observed by Adams et al. [33] in the midbrain and in the caudate nucleus, while normal GSH contents have been determined by Perry et al. [34]. Presumably, dissenting results are due to differences in techniques or difficulty in sample collection after death of AD patients. In any case, it has been observed that GSH protects cultured neurons against oxidative damage resulting from β-peptide and 4-hydroxynonenal (HNE), a lipid peroxidation product that is increased in AD [35]. A significant decrease in Cu and significant increases in Zn and Fe were found in AD hippocampus and amygdale, while Cu, Fe, and Zn are elevated in senile plaques of AD. These metal ions can catalyze free radical reactions and contribute to oxidative damage observed in AD brain [36]. GSH protects these areas through formation of metal complexes via nonenzymatic reactions and may also be beneficial for normalizing the adverse effects of iron accumulation in the aging brain [37].

3. Antioxidant Neuroprotection in PD and AD

Neuroprotective antioxidants are considered a promising approach to slow down the progression and limit the extent of neuronal cell loss in neurodegenerative disorders [38–41]. These agents were classified by Behl and Moosmann according to their mode of action in (a) compounds that prevent the formation of free radicals; (b) compounds that chemically interfere with formed free radicals; (c) compounds which limit the damage extent to the cell by alleviating the secondary metabolic burden of increased levels of free radicals [42]. N-acetylcysteine, lipoic acid, GSH, and its thiol derivatives belong to the last class of neuroprotective antioxidants. In this context, the GSH system is especially important for cellular defense against ROS in brain cells, acting directly in detoxification of radicals in non-enzymatic reactions and working as a substrate for various peroxidases [43]. Astrocytes appear to play a key role in the GSH metabolism in the brain since astroglial GSH export is essential for providing GSH precursor to neurons. Normally astrocytes release GSH and protect it against oxidation by releasing a protecting factor into the medium. Astroglial release of GSH is the first step in the supply of the GSH precursor cysteine to neurons. The extracellular GSH is processed by γ-glutamyl transpeptidase (γ-GT) and aminopeptidase N (ApN) to generate the cysteine, which limits the synthesis of GSH in neurons. Alterations of the release rate of GSH from astrocytes and reduced activities of the ectoenzymes may contribute to a lowered antioxidant defense in neurons and to an increased susceptibility to oxidative stress, both involved in the progression of neurodegenerative diseases [44]. Alterations of GSH metabolism in brain have been found in neurodegenerative disorders as PD and AD [5, 45–47]. The causes of GSH depletion are not well understood yet, but their consequences are quite serious. GSH depletion can inhibit complex I, E1 ubiquitin ligase, and proteasome activity; it can also exacerbate oxidative stress and activate

the JNK pathway, leading to an inflammatory response. All these effects cause dopaminergic neuronal death and accumulation of proteins into Lewy bodies in patients affected by PD [48]. Furthermore, an emerging evidence indicates that the total antioxidant capacity (including GSH, ascorbic acid, uric acid, and bilirubin) has shown to be reduced by 24% in plasma samples from AD patients [49]. An increased number of mutations in mitochondrial DNA have been found in AD, such as increased concentrations of 8-hydroxy-2-deoxyguanosine, a marker of oxidative damage to DNA. These deletions or point mutations, which may result from oxidative stress, can cause mitochondrial dysfunction and trigger apoptotic cell death. In addition to DNA damage, several mitochondrial key enzymes involved in ROS detoxification are also affected. *In vivo* studies on animal models of AD have also shown the implication of mitochondria in the disease pathogenesis [50]. In this regard, several groups have focused their efforts on developing neuroprotective strategies targeting mitochondria. Some of the major mitochondrial targets used as therapeutics against ROS-mediated damage are members of the quinone family. An ubiquinone derivative, mitoquinone mesylate or MitoQ, has been used to prevent oxidative damage in AD [51]. MitoQ consists of CoQ10 linked to a triphenylphosphonium ion, which has a positive charge; therefore, it accumulates in mitochondria, which have a strongly negative membrane potential (about −120 mV). More precisely, MitoQ is adsorbed in the inner mitochondrial membrane facing the matrix. This ROS-enriched region provides a real potency to MitoQ. In addition, MitoQ prevents AD-like pathology in mouse cortical neurons in cell culture, attenuates β-amyloid-induced neurotoxicity, and prevents increased production of ROS.

Mitochondrial dysfunction, oxidative stress, glutamate excitotoxicity, and formation of high-molecular-weight aggregates also define the most common adult-onset motoneuron disease: amyotrophic lateral sclerosis (ALS) caused by the progressive degeneration of moto-neurons in the spinal cord, brain stem, and motor cortex [52]. Dominant mutation in Cu/Zn-superoxide dismutase (SOD1) causes familial forms of ALS. In order to investigate the role of GSH in this pathology, knockout mice for the glutamate-cysteine ligase modifier (GCLM) subunit were used. Results suggested that the lack of GCLM significantly accelerates disease and mitochondrial pathology in hSOD1 mice [53].

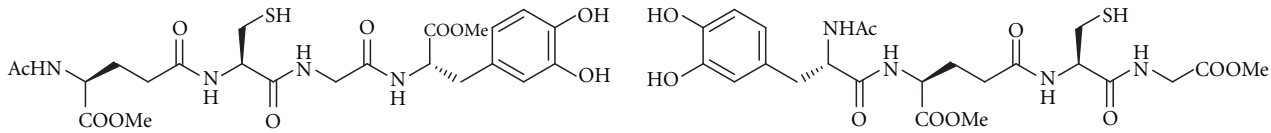
A promising therapeutic intervention in the above reported diseases could be the antioxidant neuroprotection [54]. In this context, the increase of GSH availability in neurons is a logical therapeutical target in neural impairment related to oxidative stress. Due to the difficulty in elevating GSH directly as described by Zeevalk et al. [55], other strategies to raise brain levels of this antioxidant have been investigated [56]. In this paper we will focus on the medicinal chemistry and technological approaches aimed at maintaining or restoring GSH levels in PD and AD patients. Particular attention will be paid to different strategies for increasing GSH levels by supplying GSH codrugs and GSH nanocarrier systems able to cross the cellular membrane more easily than GSH.

4. Medicinal-Chemistry-Based Strategies to Increase GSH Levels

Medicinal-chemistry-based strategies include analogues [57, 58], as well as prodrugs and codrugs approaches [59]. While each of these strategies may be equally promising to increase GSH levels, this paper will mainly focus on codrugs approach since the other medicinal-chemistry-based strategies have been previously discussed [56, 60–62]. The codrug approach consists in linking, via a covalent chemical linkage, two different pharmacophores with similar or different pharmacological activities in order to improve physiochemical, biopharmaceutical, and drug delivery properties of therapeutic agents. The resulting codrug has to be stable at gastrointestinal level and transported to the target site of action where it provides the two parent drugs following hydrolysis [63].

The codrug approach has been used for the treatment of PD and AD joining antioxidant or chelating molecules with a therapeutic compound (antiparkinson or anti-alzheimer's drugs) [64–66]. In particular, codrugs containing antioxidant molecules such as GSH, N-acetyl-cysteine, methionine, and cysteinyl derivatives have been synthesized in order to permit a targeted delivery of antioxidant directly to specific groups of neurons where cellular stress is associated with PD and AD. The dual advantage of these antioxidant molecules lies in the fact that the antioxidant portion, in addition to acting as a scavenger directly or indirectly of free radicals, can be used as a carrier. In fact, GSH and cysteinyl derivatives can be used as BBB shuttles for delivery of antiparkinson or ant-Ialzheimer's drugs since the presence of GSH transporters at the BBB is well documented [67, 68]. In this context, the research of new codrugs for the treatment of PD and AD has gained our attention. L-Dopa-GSH codrugs (LD-GSH, **1-2**), obtained via an amide bond between LD and the C- and N-terminal GSH, respectively, have been synthesized and evaluated as potential anti-Parkinson agents with antioxidant properties (Figure 1) [69]. These codrugs permit a targeted delivery of GSH directly to SNpc neurons of PD patients and contribute in attenuating the damage caused by the prooxidant effects of traditional LD therapies. Codrugs **1-2** showed good stability toward gastrointestinal simulated fluids and released LD in rat and human plasma after enzymatic hydrolysis. Furthermore, they prolonged the plasma LD levels and were able to induce sustained delivery of dopamine (DA) in rat striatum with respect to equimolar dose of LD. Taken together, these results demonstrated the possible therapeutic application of codrugs **1-2** in PD, being able to protect against the oxidative stress deriving from autoxidation and the MAO-mediated metabolism of DA [69].

Later, More and Vince [70] reported two GSH bioconjugates (**3-4**) containing a metabolically stable urea analogue of GSH resistant to the enzyme γ-GT (Figure 2). The antioxidant portion has been covalently joined to the therapeutic drugs, as DA and adamantine, via a heterodisulfide linkage. This suitable junction is stable in plasma and able to release DA or adamantine, and the antioxidant portion due to the abundance of the enzyme disulfide reductases in the



1 2

Figure 1: Chemical structures of GSH codrugs **1-2**.

brain [71]. More importantly, these bioconjugates cross the BBB through recognition by GSH transporters on the luminal side of BBB [68]. Studies successfully confirmed the carrier-mediated transport of conjugates **3-4** in an *in vitro* BBB model and their ability to release the active drug at the target site, thus representing an innovative approach for the targeted delivery of anti-Parkinson drugs into the CNS using the GSH transport system [70]. In particular, the MDCK cell monolayer has been used to study the bioconjugates **3-4** transport. At concentration of 100 μM, the transport of bioconjugates **3-4** from the apical to the basal side was greater than the transport in the reverse direction. Moreover, to ensure that the codrugs **3-4** were not being metabolized as they crossed the MDCK cell monolayer, the integrity of codrugs **3-4** was confirmed by HPLC studies [70]. These experiments successfully demonstrated the GSH-carrier-mediated transport of the bioconjugates **3-4** in an *in vitro* BBB model.

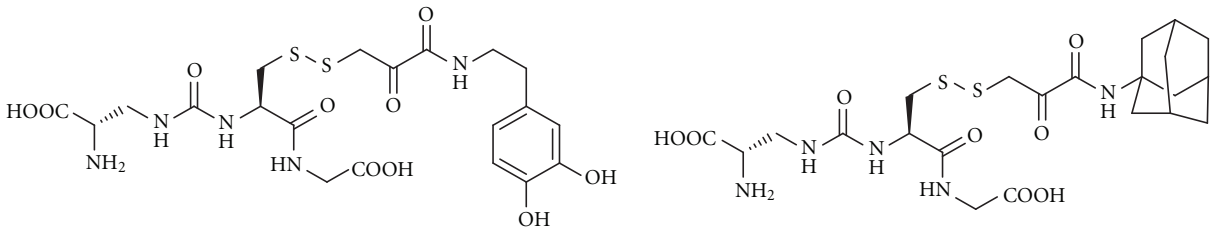
Another bioconjugate that could use the GSH transporters on the luminal side of BBB might be the hybrid **5** (Figure 3). This molecule is characterized by the replacement of cysteine with methionine in order to obtain stable GSH analogue at γ-GT [72]. Furthermore, the GSH analogue has been linked to LD to obtain CNS drug delivery. This compound was demonstrated to cross unaltered the acidic environment of the stomach, to be stable enough to be absorbed from the intestine, to have radical scavenging activity, and to release LD in human plasma after enzymatic hydrolysis. Taken together, these data suggest a therapeutic potential of **5** in pathological events associated with free radical damage and decreasing DA concentration in the brain [72].

Recently, Ehrlich et al. [73] designed and synthesized a library of new GSH codrugs (called UPF peptides) with powerful hydroxyl radical scavenging activities. They have been obtained via an amide bond between GSH and tyrosine derivatives as shown in Figure 4. In particular, the enzyme free hydroxyl radical scavenging assay showed that substitution of γ-glutamyl moiety (UPF1, 4-methoxy-L-tyrosinyl-γ-L-glutamyl-L-cysteinyl-glycine, **6**) with α-glutamyl moiety (UPF17, 4-methoxy-L-tyrosinyl-α-L-glutamyl-L-cysteinyl-glycine, **7**) improved hydroxyl radical scavenging activity of about 500-fold [74]. UPF1 (**6**) is an effective and potential agent that diminishes neuronal injury in global cerebral ischemia [75]; it acts as a free radical scavenger or a modulator of G-protein in frontocortical membrane preparations. Although the exact mechanisms of the protective action of UPF1 still remain unclear, it can possibly act as a scavenger or a signal molecule increasing GSH levels or the GSH redox ratio; UPF1 could be a promising lead for the design of powerful antioxidants for the treatment of conditions associated with reduced GSH levels [76]. Unfortunately, the role of UPF peptides for the treatment of PD has not been studied yet. It could be interesting to investigate the activity of UPF peptides in patients affected by PD since these peptides contain the tyrosine moiety, the metabolic precursor of DA.

Few data are available in the literature about GSH codrugs for the treatment of AD. We recently synthesized Ibuprofen-GSH (IBU-GSH, **8**) obtained via amide bond between GSH and IBU, a nonsteroidal anti-inflammatory drug (NSAID) (Figure 5) [77]. NSAIDs treatment reduces AD risk, delays disease progression, and reduces microglia activation [78]. In particular, Lim et al. [79] reported that six months of treatment of a transgenic animal model of AD with IBU resulted in a significant reduction of amyloid plaque burden and total Aβ peptide levels. Furthermore, IBU treatment led to a reduction of plaque-associated microglia and a corresponding attenuation in proinflammatory cytokine levels in brain [80]. Codrug **8** possessed good stability toward human plasma enzymatic activity and displayed *in vitro* free radical scavenging activity in time- and concentration-dependent manner. More importantly, it antagonizes the deleterious and cognitive effects of β-amyloid(1–40) in a rat model for AD, as also confirmed by behavioral tests of long-term spatial memory. In conclusion, IBU-GSH might permit targeted delivery of IBU and GSH directly to neurons, where oxidative stress and inflammatory processes are associated with AD [77].

Almost all the codrugs (**1-2**, **5**, **9–14**) have been tested for their chemical and enzymatic stabilities in order to check both their stability in aqueous medium and their sensitivity towards enzymatic cleavage in rat and human plasma (Tables 1 and 2) [69, 72, 81]. Stability studies were performed at 37°C in isotonic sodium phosphate buffer (pH 7.4), in simulated gastric fluid (SGF, pH 1.3), and in rat and human plasma diluted to 80% with isotonic sodium phosphate buffer (pH 7.4). All codrugs showed good stability toward gastrointestinal hydrolysis ($t_{1/2}$ > 20 h) (Table 1). On the contrary, in rat and human plasma the codrugs (**1-2**, **5**, **9–14**) underwent rapid bioconversion of the codrugs into their constituents (Table 2).

Sulfur-containing amino acids have gained great attention as source of thiols for GSH synthesis [82]. A series of multifunctional thiol codrugs (**9–14**) were synthesized to overcome the prooxidant effect associated with LD



3 **4**

Figure 2: Chemical structures of GSH codrugs **3-4**.

Table 1: Kinetic data for chemical hydrolysis of codrugs **1-2**, **5**, and **9–14** at 37°C.

	pH 1.3[a]		pH 7.4[a]	
Compd	$t_{1/2}$ (h)	K_{obs} (h^{-1})	$t_{1/2}$ (h)	K_{obs} (h^{-1})
1	20.14 (±0.73)	0.034 ($\pm 1.2 \times 10^{-3}$)	7.22 (±0.31)	0.096 ($\pm 4.1 \times 10^{-3}$)
2	28.12 (±1.21)	0.025 ($\pm 1.1 \times 10^{-3}$)	12.23 (±0.49)	0.057 ($\pm 2.3 \times 10^{-3}$)
5	20.67 (±0.83)	0.094 ($\pm 0.3 \times 10^{-3}$)	10.80 (±0.40)	0.018 ($\pm 0.8 \times 10^{-3}$)
9	301.0 (±10.5)	0.002 ($\pm 0.07 \times 10^{-3}$)	46.2 (±0.90)	0.015 ($\pm 0.3 \times 10^{-3}$)
10	290.6 (±5.8)	0.002 ($\pm 0.04 \times 10^{-3}$)	48.0 (±1.70)	0.015 ($\pm 0.45 \times 10^{-3}$)
11	296.3 (±11.8)	0.002 ($\pm 0.08 \times 10^{-3}$)	30.2 (±1.40)	0.023 ($\pm 1.04 \times 10^{-3}$)
12	292.1 (±4.4)	0.002 ($\pm 0.03 \times 10^{-3}$)	26.9 (±0.70)	0.026 ($\pm 0.65 \times 10^{-3}$)
13	292.8 (±8.8)	0.002 ($\pm 0.06 \times 10^{-3}$)	48.50 (±0.70)	0.005 ($\pm 0.25 \times 10^{-3}$)
14	293.4 (±14.7)	0.002 ($\pm 0.1 \times 10^{-3}$)	21.3 (±0.60)	0.033 ($\pm 0.99 \times 10^{-3}$)

[a] Values are means of three experiments, and standard deviation is given in parentheses.

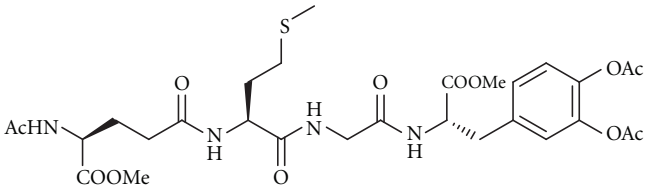


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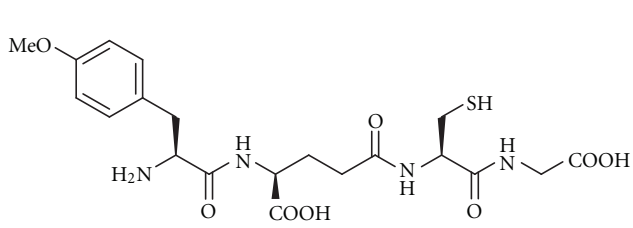
Figure 3: Chemical structure of GSH hybrid **5**.

therapy in parkinsonian models (Figure 6) [81]. In this regard, thiol antioxidants (N-acetyl-L-cysteine, methionine, dithiothreitol) prevent DA autoxidation, production of dopamine-melanin, and inhibition of dopamine-induced apoptosis [83]. Moreover, they increase levels of intracellular cysteine, the limiting amino acid in GSH biosynthesis, thus potentiating the natural cellular defense mechanisms against oxidative damage. The multifunctional codrugs **9**-**14** proved to be good radical scavengers. The LD and DA striatal level profiles indicate that codrugs **11** and **12** were able to induce sustained delivery of both LD and DA in rat striatum with respect to equimolar doses of LD [82].

In addition, Minelli et al. [84] showed that administration of codrug **11** to mice treated with Z-ILeu-Glu(OtBu)-Ala-Leu-CHO (PSI), used as a PD model, resulted in a reduction in dopaminergic neuronal death and a significant raise in GSH levels. In particular, codrug **11** could control the LD-induced oxidative stress in primary mesencephalic cultures and in newborn mice pups since in both cases GSH content results increased. Using newborn mice pups, characterized by incomplete formation of BBB, Minelli et al. [84] found that buthionine-[S,R]-sulfoximine-(BSO-) mediated GSH depletion prevented the increase of GSH levels promoted by codrug **11**, supporting the role of GSH for codrug-**11**-induced protection. To investigate whether heme oxygenase (HO) activity was related to GSH levels, ZnPPIX was used as HO inhibitor. Compared to untreated control, and LD-treated newborn mice, brain GSH levels were increased by ZnPPIX indicating that HO activity was not essential to GSH synthesis. An injection of codrug **11** induced a significant increase in GSH levels that was markedly reduced by BSO indicating the essential role of γ-glutamylcysteine synthetase in increasing GSH brain levels. Codrug **11** exhibited *in vivo* protective effect against LD-induced stress through a mechanism via Nrf2 activation leading to a decrease in ROS generation and an increase in GSH. Therefore, this codrug might offer benefits in the treatment of PD and provide a potential alternative to LD therapy by avoiding nigrostriatal oxidative degeneration [84].

Maher et al. [85, 86] demonstrated that the conjugation of catechins with cysteine generates antioxidant compounds (**15–20**) with enhanced neuroprotective activity (Figure 7). The thiol conjugates **15–20** were active in protecting HT-22 nerve cells (EC_{50} between 36 and 65 μM) from oxidative



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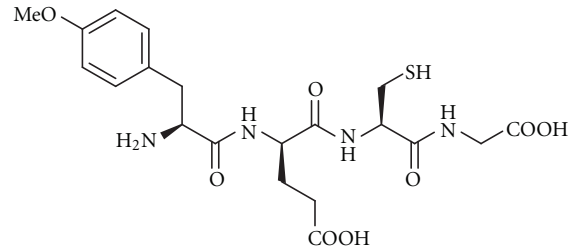

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Figure 4: Chemical structures of GSH codrugs **6-7**.

Table 2: Rate constants for the hydrolysis of codrugs **1-2**, **5**, and **9–14** in 80% rat plasma and 80% human plasma at 37°C.

	Rat plasma[a]		Human plasma[a]	
Compd	$t_{1/2}$ (min)	K_{obs} (min^{-1})	$t_{1/2}$ (min)	K_{obs} (min^{-1})
1	immediate hydrolysis	—	3.2 (±0.1)	0.217 ($\pm 6 \times 10^{-3}$)
2	2.7 (±0.1)	0.257 ($\pm 8 \times 10^{-3}$)	15.1 (±0.4)	0.046 ($\pm 1 \times 10^{-3}$)
5	4.7 (±0.1)	0.150 (±0.01)	7.3 (±0.3)	0.100 (±0.01)
9	46.8 (±1.4)	0.010 ($\pm 0.20 \times 10^{-3}$)	69.6 (±1.4)	0.015 ($\pm 0.44 \times 10^{-3}$)
10	36.6 (±1.6)	0.019 ($\pm 0.85 \times 10^{-3}$)	65.4 (±1.6)	0.011 ($\pm 0.26 \times 10^{-3}$)
11	115.2 (±11.0)	0.002 ($\pm 0.07 \times 10^{-3}$)	315.0 (±4.6)	0.006 ($\pm 0.24 \times 10^{-3}$)
12	93.0 (±10.2)	0.003 ($\pm 0.17 \times 10^{-3}$)	263.4 (±1.9)	0.007 ($\pm 0.14 \times 10^{-3}$)
13	55.8 (±10.5)	0.003 ($\pm 0.11 \times 10^{-3}$)	203.4 (±0.6)	0.030 ($\pm 0.75x10 - 3$)
14	69.6 (±3.5)	0.010 ($\pm 0.50 \times 10^{-3}$)	90.0 (±2.7)	0.008 ($\pm 0.24 \times 10^{-3}$)

[a]Values are means of three experiments, standard deviation is given in parentheses.

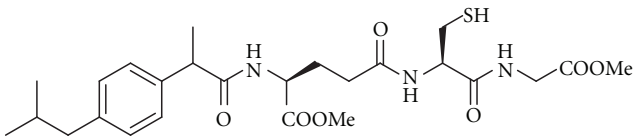

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Figure 5: Chemical structure of GSH codrug **8**.

stress-induced death. Although all the conjugates were able to scavenge mitochondrial generated ROS inside the cells, the majority of their neuroprotective activity seems to be dependent on their ability to maintain GSH levels. These compounds were able to maintain cellular GSH levels by enhancing the uptake of cystine/cysteine into cells by a mechanism that uncouples the uptake from system x_c^-, a Na^+-independent cystine/glutamate antiporter [87]. System x_c^- transports cysteine into cells in a 1 : 1 exchange with glutamate. The importance of this system for the maintenance of the GSH levels in cells is demonstrated by the loss of GSH and subsequent cell death in nerve and other types of cells following exposure to millimolar concentrations of extracellular glutamate. Treatments able to maintain GSH levels, in presence of an induced stress by GSH loss, have a significant potential for the treatment of neurodegenerative diseases.

5. Technology-Based Strategies to Increase GSH Levels

The effectiveness of exogenous antioxidants to protect tissues from oxidative stress *in vivo* depends on the antioxidant used, its physicochemical and biopharmaceutical properties, and its bioavailability at the site of action [88, 89]. With the aim of improving the physicochemical, biopharmaceutical and drug delivery properties of neuroprotective antioxidants, the technology-based strategy could be useful for the treatment of several diseases in which oxidative stress plays an important role [59, 90]. Particularly, this approach could be adopted in order to selectively deliver antioxidants to tissues in sufficient concentrations to reduce the oxidative damage. In order to afford neuroprotection and to facilitate the delivery of GSH across the BBB, several GSH delivery systems, such as liposomes, nanoparticles, and dendrimers, were developed.

Liposomes are considered as carrier systems for therapeutically active compounds due to their unique characteristics such as capability of incorporating hydrophilic and hydrophobic drugs, good compatibility, low toxicity, lack of immune system activation, and targeted delivery of bioactive compounds to the site of action [91]. Liposome technology has been recently used in the treatment of neurodegenerative diseases. In this context, GSH has been encapsulated in liposomes in order to replenish intracellular GSH and provide neuroprotection in an *in vitro* model of PD [92]. The formulation of GSH has been encapsulated in lipid vesicles

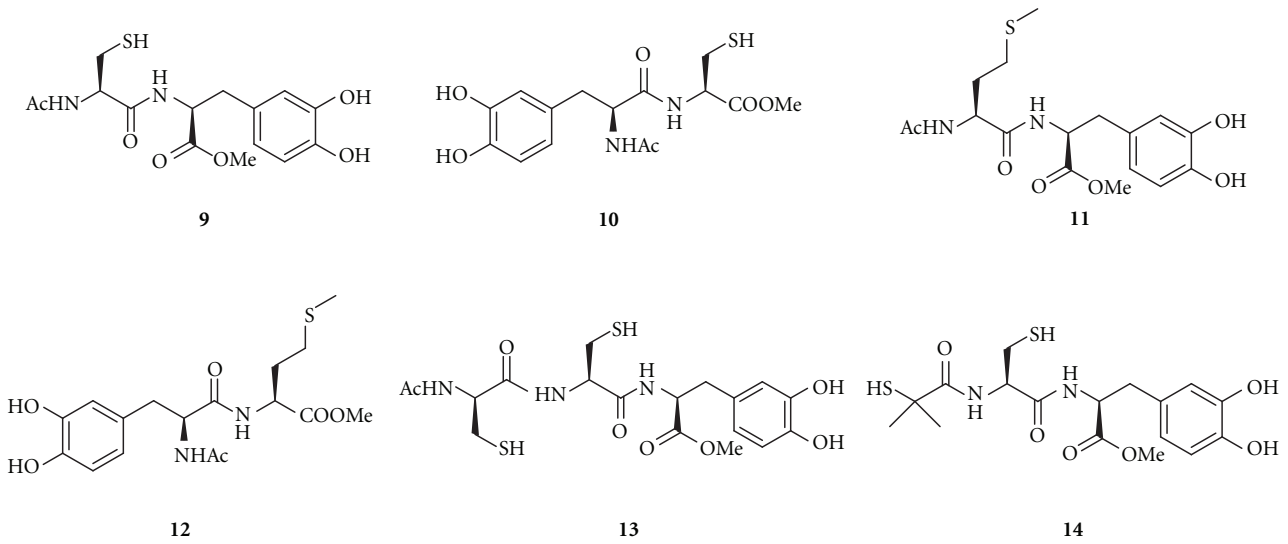


Figure 6: Chemical structures of cysteinyl codrugs **9–14**.

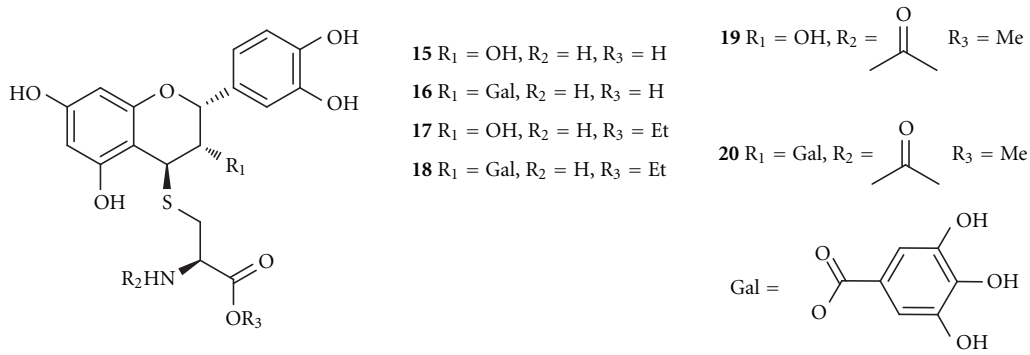


Figure 7: Chemical structures of thiol conjugates **15–20**.

made of lecithin and glycerol and then tested on mixed mesencephalic cultures treated with paraquat plus maneb. Zeevalk et al. [92] observed that liposomal GSH was taken up into neurons and astrocytes via an endosomal process, and subsequently the endosomes containing liposomal-GSH were fused with lysosomes (Figure 8).

In these conditions, GSH was hydrolyzed and its constituent amino acids (glutamate, cysteine, and glycine) released from lysosomes could be used for the GSH biosynthesis. The results obtained by Zeevalk et al. [92] suggested that this formulation was 100-fold more potent than nonliposomal-GSH in providing substrates for the maintenance of intracellular GSH in neuronal cells. Moreover, liposomal-GSH dose-dependently provided complete neuroprotection of dopaminergic neurons treated with paraquat plus maneb with an EC_{50} of 10.5 μM ± 1.08. These findings suggest that liposomal-GSH represents a promising therapeutic strategy for neuronal maintenance in pathologies characterized by GSH depletion.

Nanoparticles (NPs) are solid colloidal particles made of polymeric materials ranging in size from 1–1000 nm. NPs are used as carrier systems in which the drug is dissolved, entrapped, encapsulated, adsorbed, or chemically linked to the surface [93]. The advantages of NPs are high drug-loading capacity and resistance against chemical and enzymatic degradation. Coating NPs with hydrophilic polymer is a promising strategy in order to prolong their presence in plasma and the therapeutic effect. The surface modification of NPs can be achieved using polyethylene glycol (PEG) or polysaccharides such as chitosan, dextran, pectin, and hyaluronic acid [94, 95]. NPs have been employed for delivering GSH to CNS. In this regard, a series of NPs containing GSH (GS-PEG-GS, **21**) were prepared with PEGs of various molecular weights (Figure 9) [96]. PEG was used because of its well-established biocompatibility, low immunogenicity, low antigenicity, and low toxicity [97].

Unfortunately, GS-PEG-GS nanoparticles were not able to exert their antioxidant activity because the thiol groups were consumed during Michael addition. Thus, a disulfide bridge was proposed for antioxidant delivery in order to release GSH when the pH was low enough to enable thiol formation. Disulfide-linked GSH NPs (GS-SPEGS-SG, **22**) were synthesized and tested on SH-SY5Y cells challenged with 100 μM H_2O_2, a compound that induces oxidative

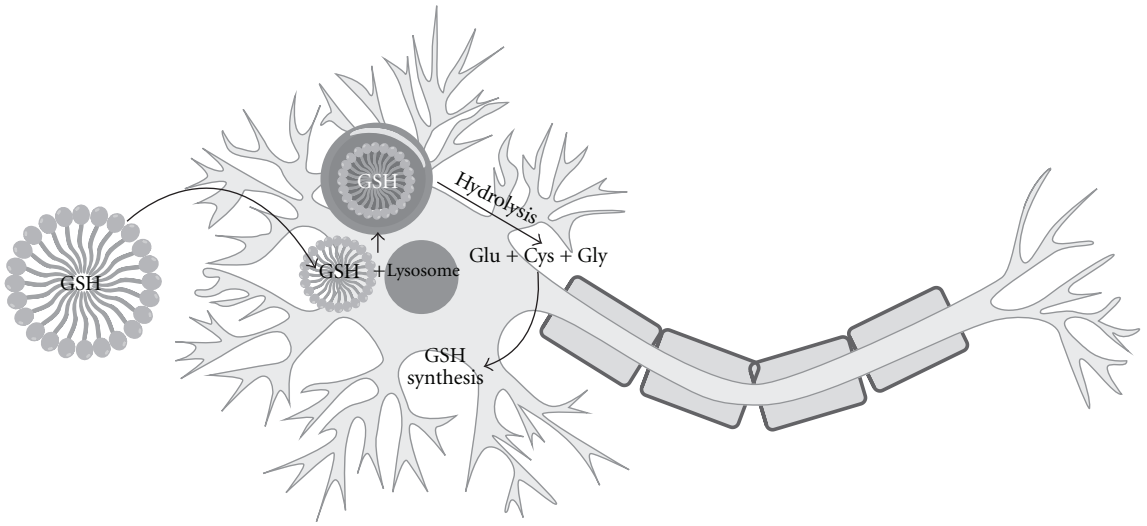
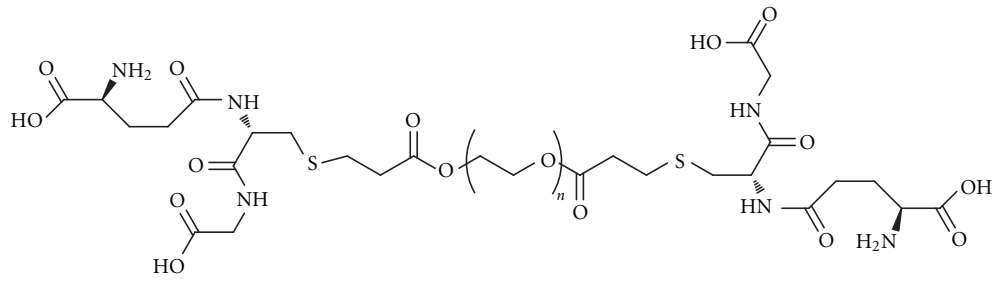


Figure 8: Liposomal GSH delivery to neurons and relative hydrolysis following fusion with lysosome.



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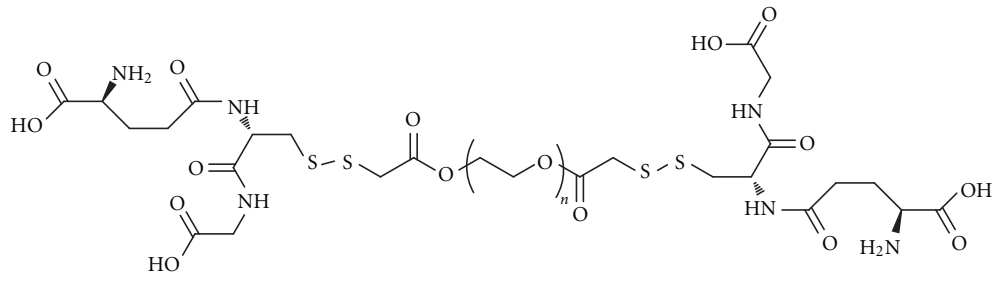

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Figure 9: Chemical structures of GS-PEG-SG (**21**) and GS-SPEGS-SG (**22**).

stress. The GS-SPEGS-SG NPs were 100% at protecting SH-SY5Y cells at 250 μM from oxidative stress, while the GS-PEG-GS did not offer protection [96]. According to these data, this approach could be employed in treating diseases typically associated with increased ROS levels.

Chitosan-GSH nanoparticles (CS-GSH NPs) have been developed by Koo et al. [98] as delivery system for enhancing stability and bioavailability of GSH. Chitosan (CS) nanodelivery system offers many advantages: (a) it is not toxic; (b) it is biodegradable; (c) it is biocompatible; d) it has good bioadhesibility and water dispersibility [99, 100]. Thus, CS-GSH was synthesized using a radical polymerization method, and CS-GSH NPs were prepared by ionic gelation of CS-GSH with sodium tripolyphosphate (TPP) (Figure 10). The resulting NPs showed a good entrapment and loading efficiency. Furthermore, to investigate the CS-GSH NPs stability under oxidative stress, the effect of the presence of H_2O_2 on their thiol groups was evaluated. The reduction of thiol groups of the CS-GSH NPs under oxidative stress resulted in being 1.5-fold lower than that of free GSH [98]. These results suggest that CS-GSH NPs could be used as effective delivery carriers of GSH under oxidative insults, but

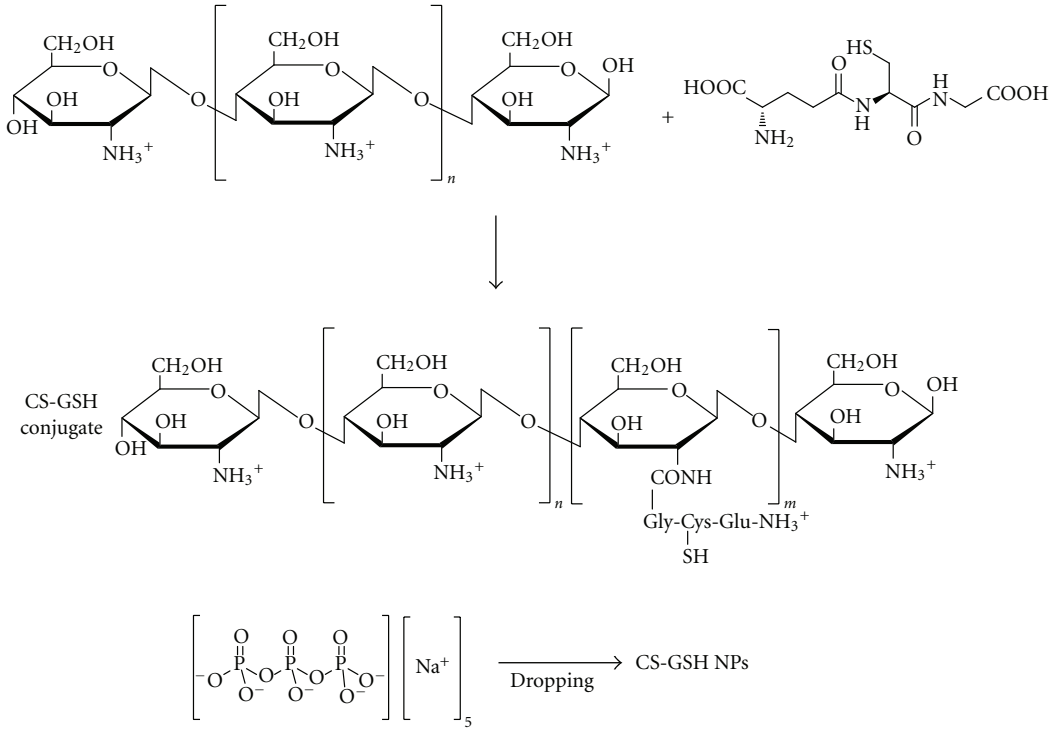

Figure 10: Scheme for GS-GSH NPs preparation as reported by Koo et al. [98].

further studies on animal models of PD and AD are necessary in order to evaluate their true efficacy.

The use of appropriate nanocarrier systems for GSH may be useful because they are noninvasive systems and protect the molecule to be delivered against inactivation mechanism and clearance. However, they are characterized by limiting factors as safety and toxicity. At present, few data are available about the utilization and human application of nanocarrier systems for transport across the BBB and CNS delivery. Thus, the future clinical study of GSH delivery systems for neurodegenerative diseases is strongly recommended.

6. Conclusions

The drug delivery to CNS is a complex and challenging task requiring close collaboration of several scientific areas including pharmaceutical and technological sciences, biological chemistry, and pharmacology. In this context, this paper has investigated multidisciplinary approaches such as the codrug and nanocarriers strategies that could be used to treat neurodegenerative disorders associated with GSH deficiency. GSH and cysteinyl codrugs have been designed on the basis of combining suitable groups of the antioxidant portion with available drugs without altering their inherent pharmacodynamic properties with improved physiochemical properties of drugs. This promising approach has also been used to resolve the issues like permeation, solubility of drug, stability, drug resistance, oral absorption, and brain delivery but still there are very few well-established candidates that have been approved for clinical applications.

Novel experimental neuroprotective strategies include formulations containing GSH, such as nanocarrier systems. This approach could be adopted in order to selectively deliver GSH to tissues in sufficient concentrations to reduce the oxidative damage, but few data are available about clinical studies.

Although the potential use of these strategies needs further exhaustive studies, they may offer a promising therapeutic alternative for reducing the GSH functional loss related to human diseases such as PD and AD.

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Review Article

Preclinical and Clinical Evidence of Antioxidant Effects of Antidepressant Agents: Implications for the Pathophysiology of Major Depressive Disorder

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Major depressive disorder (MDD) is a common mental disorder associated with a significant negative impact on quality of life, morbidity/mortality, and cognitive function. Individuals who suffer with MDD display lower serum/plasmatic total antioxidant potentials and reduced brain GSH levels. Also, F2-isoprostanes circulatory levels are increased in MDD subjects and are correlated with the severity of depressive symptoms. Urinary excretion of 8-OHdG seems to be higher in patients with MDD compared to healthy controls. Despite the fact that antidepressant drugs have been used for more than 50 years, their mechanism of action is still not fully understood. This paper examines preclinical (*in vitro* and animal model) and clinical literature on oxidative/antioxidant effects associated with antidepressant agents and discusses their potential antioxidant-related effects in the treatment of MDD. Substantial data support that MDD seems to be accompanied by elevated levels of oxidative stress and that antidepressant treatments may reduce oxidative stress. These studies suggest that augmentation of antioxidant defences may be one of the mechanisms underlying the neuroprotective effects of antidepressants in the treatment of MDD.

1. Introduction

Despite the fact that antidepressant drugs have been used for more than 50 years, their mechanism of action is still not fully understood. The hypothesis that antidepressants restore noradrenergic and serotoninergic neurotransmitter systems has been dominant [1]. Recently, a new concept of antidepressants action has been suggested, based on growing evidence demonstrating antioxidant effects of antidepressants in the treatment of major depressive disorder (MDD) (Table 1). This paper examines preclinical (*in vitro* and animal models) and clinical literature on oxidative/antioxidant effects of antidepressant agents and discusses the relevance of intracellular oxidative pathways in the pathophysiology of MDD.

2. Oxidative Stress and Antioxidants: Background

Reactive oxygen species (ROS) are continuously generated in physiological conditions and are effectively controlled/eliminated by intracellular and extracellular antioxidant systems [2]. ROS are products of normal cellular metabolism and are well recognized for their dual role as deleterious and essential compounds, given that ROS can be harmful or beneficial [3]. Beneficial effects of ROS occur at low levels and involve cell signalling and signal transduction [4]. ROS also play an essential role in the human immune system helping killing and eliminating infectious organisms. However, elevated or chronic inflammations are major determinants of disease later in the human lifespan, and ROS

Table 1: Antioxidant effects of antidepressant agents: preclinical and clinical studies.

Antidepressant	Oxidative/Antioxidant-related effects			Drug class
	In vitro	Animal models	Human data	
Amitriptyline	+	+		TCA
Bupropion		+		NDRI
Citalopram			+	SSRI
Desipramine	+			TCA
Duloxetine				SNRI
Escitalopram		+	+	SSRI
Fluoxetine	+	+	+	SSRI
Fluvoxamine	+		+	SSRI
Imipramine	+	+		TCA
Maprotiline	+			TCA
Milnacipran			+	SNRI
Mirtazapine	+			NaSSA
Moclobemide			+	MAOI
Nefazodone			+	SNDRI
Nortriptyline	+			TCA
Paroxetine			+	SSRI
Reboxetine	+		+	NRI
Sertraline			+	SSRI
Tianeptine			+	SSRE
Trazodone			+	SARI
Venlafaxine		+	+	SNRI

MAOI: monoamine oxidase inhibitor; NaSSA: noradrenergic and specific serotonergic antidepressant; NDRI: norepinephrine-dopamine reuptake inhibitor; NRI: norepinephrine reuptake inhibitor; SARI: serotonin antagonist and reuptake inhibitor; SNDRI: serotonin-norepinephrine-dopamine reuptake inhibitor; SNRI: serotonin-norepinephrine reuptake inhibitor; SSRE: selective serotonin reuptake enhancer; SSRI: selective serotonin reuptake inhibitor; TCA: tricyclic or tetracyclic antidepressant.

play a critical role in several age-related diseases, particularly cancer, cardiac and neurodegenerative disorders [5]. The major source of ROS in humans is the leakage of superoxide anion ($O_2^{\bullet -}$) from mitochondria during oxidative phosphorylation. Another minor source of ROS is cytoplasmatic, including the $O_2^{\bullet -}$generating enzymes such as xanthine oxidase (XO), NADPH oxidases, and cytochromes P450 (CytP450). The main ROS include $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). $OH^{\bullet}$ is a strong oxidant formed during Fenton ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$) and Haber-Weiss ($H_2O_2 + OH^{\bullet} \rightarrow H_2O + O_2^{\bullet -} + H^{+}$ and $H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + OH^{-} + OH^{\bullet}$) reactions. Additionally, some nitrogen species can be potentially dangerous to the cell, such as peroxynitrite (ONOO–), which is formed in a rapid reaction between $O_2^{\bullet -}$ and nitric oxide (NO) [3].

The main enzymatic antioxidant defences include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SOD enzymes are highly efficient in the catalytic dismutation of $O_2^{\bullet -}$ and generation of H_2O_2 which, in turn, can be removed by two types of enzymes—the catalases (CAT) and peroxidases (e.g., GPx). Importantly, the activity of GPx is closely dependent on glutathione reductase (GR), glutathione tripeptide (GSH), and others cofactors. Moreover, virtually all cells contain nonenzymatic defenses, like GSH, vitamins C (ascorbate) and E (alpha-tocopherol), and metal-binding and related protective proteins [37].

The term "oxidative stress" has been defined as an imbalance between the generation of ROS and antioxidant defenses, favouring the former [3]. In situations of oxidative stress, several biomolecules (e.g., lipid membrane, proteins, and DNA) can be damaged. Because ROS have extremely short half-lives, they are difficult to measure. Therefore, most studies measure products of the damage induced by oxidative stress. For instance, malondialdehyde (MDA) is one of the low-molecular-weight end products formed via the decomposition of primary and secondary lipid peroxidation products [38]. MDA and other thiobarbituric reactive substances (TBARS) condense with two equivalents of thiobarbituric acid that can be assayed spectrophotometrically [39]. Another compound commonly used as a biomarker of oxidative stress is 4-Hydroxynonenal (4-HNE). 4-HNE is generated in the oxidation of lipids containing polyunsaturated omega-6 acyl groups, such as arachidonic or linoleic groups, and the corresponding fatty acids [40]. Perhaps the most accurate markers of lipid peroxidation are the isoprostanes (i.e., F2-isoprostanes). Isoprostanes are prostaglandin-like compounds formed *in vivo* from the free radical-catalyzed peroxidation of essential fatty acids (primarily arachidonic acid) [41]. Proteins are possibly the most immediate targets of cellular oxidative damage. Carbonyl groups (aldehydes and ketones) are produced in protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized, which can be measured by specific techniques [42]. Another method to evaluate levels of oxidation/reduction content in biological samples is the total reduced thiol (–SH) quantification [43]. ROS can also attack and damage the DNA, thereby generating 8-hydroxydeoxyguanosine (8-oxodG) and 8-hydroxyguanosine (8-oxoG) [37].

Additionally, total antioxidant potentials can be measured using various methods such as TAC, total antioxidant capacity; TRAP, total-radical nonenzymatic antioxidant potential; OSI, oxidative stress index; TOS, total oxidant status. Low total antioxidant capacity could be indicative of oxidative stress or increased susceptibility to oxidative damage [44].

3. Oxidative Stress in Major Depressive Disorder

MDD is one of the most common mental disorders among humans and it is associated with a significant negative impact on quality of life, morbidity/mortality, and cognitive function. The pathophysiology of depression is multifactorial and includes changes in brain monoaminergic transmission (e.g., 5-HT, NE, DA), abnormalities in neurotransmitter receptors function (e.g., AC-cAMP pathway), reduced neurotrophic factors (e.g., BDNF), dysregulation of HPA axis (cortisol), increased proinflammatory cytokines (e.g., IL-6, TNF-α, NF-κB), increased NO (e.g., L-arginine-NO-cGMP pathway), and increased oxidative stress (e.g., lipid and DNA damage) [45–47].

Individuals who suffer with MDD display lower serum/plasmatic total antioxidant potentials [28, 32, 48] and reduced brain GSH levels [31] as compared to matched controls. Plasmatic coenzyme Q10 (CoQ10), a strong antioxidant and a key molecule in the mitochondrial electron transport chain, is significantly lower in major depressive patients [34], which indicates lower antioxidant defenses against oxidative stress. Moreover, increased serum XO levels observed in MDD subjects suggest increased systemic ROS production [29]. XO is a widely distributed enzyme involved in later stages of purine catabolism, which catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid, both reactions with potential to generate $O_2^{\bullet -}$ and H_2O_2 [49]. A recent post-mortem study found increased XO activity in the thalamus and putamen patients with recurrent MDD [35].

Dimopoulos et al. (2008) have found that F2-isoprostanes (F2-iso) circulatory levels were increased in major depressive patients and were significantly correlated with the severity of depressive symptoms [50]. The presence of detectable quantities of F2-iso in human biological fluids implies ongoing lipid peroxidation [51]. Furthermore, urinary excretion of 8-OHdG, a marker of oxidative damage to DNA, was found to be higher in patients with MDD than healthy controls [52].

4. Antioxidant Effects of Antidepressants

4.1. Studies In Vitro. The main findings of *in vitro* assays using rat mitochondria and cell culture protocols are depicted in Table 2. Kolla et al. (2005) have demonstrated that pretreatment with amitriptyline and fluoxetine protects against oxidative stress-induced damage in rat pheochromocytoma (PC12) cells. Both drugs attenuated the decrease in cell viability induced by H_2O_2 in PC12 cells. Also, pretreatment with amitriptyline and fluoxetine was associated with increased SOD activity, and no signs of cell death were observed in the treated cells [10]. In another study, pretreatment with imipramine, fluvoxamine, or reboxetine inhibited NO production in a dose-dependent manner in an activated microglia cell culture protocol [11]. The authors suggested that these antidepressant drugs have inhibitory effects on IFN-γ-activated microglia and that these effects are, at least in part, mediated by cAMP-dependent PKA pathway.

Schmidt et al. (2008) examined the effects of desipramine, imipramine, maprotiline and mirtazapine on mRNA levels of various antioxidant enzymes in human monocytic U-937 cells [12]. In this study, short-term treatment with these drugs decreased mRNA levels of SOD and CAT. However, long-term treatment increased mRNA levels of SOD, GST, and GR. These results suggest that the effects of these antidepressants on the expression of antioxidant enzymes are dependent on the duration of the treatment regimen. Zhang et al. (2008) showed for nortriptyline some antioxidant effects using isolated rat liver mitochondria or PCN cell culture. Nortriptyline was able to inhibit loss of mitochondrial membrane potential and the activation of caspase 3 in isolated rat liver mitochondria and decrease cell death induced by oxygen/glucose deprivation on PCN cells [9].

The antioxidant effects of fluoxetine on isolated rat brain and liver mitochondria have been extensively studied. Curti et al. (1999) reported that fluoxetine can indirectly and nonspecifically affect electron transport and F_1F_0-ATPase activity, thereby inhibiting oxidative phosphorylation in rat brain [6]. Two studies that evaluated the effects of fluoxetine in rat liver mitochondria revealed mixed results. Souza et al. (1994) reported that fluoxetine may be potentially hepatotoxic at high doses [7]. However, Nahon et al. (2005) demonstrated that fluoxetine was able to inhibit the opening of the mitochondrial permeability transition (MPT) pore, the release of cytochrome c (cytC) and protected against staurosporine-induced apoptotic cell death [8]. An important difference between these two studies is the fact that Souza et al. used isolated liver mitochondria and tested fluoxetine at different concentrations in order to establish potential toxic doses. On the other hand, Nahon et al. challenged isolated mitochondria against staurosporine-induced damage and showed protective effects of fluoxetine in this model.

In summary, studies *in vitro* not only revealed antioxidant-related effects for antidepressant drugs, but also some potential prooxidant effects specifically in rat liver with fluoxetine at higher dosages. Cell culture and isolated tissues studies are used extensively in research and drug development; however, these techniques have some limitations and studies using live organisms (i.e., rodents) are necessary to better evaluate safety as well as behavioural effects.

4.2. Animal Models. Several animal model protocols have been used to investigate oxidative/antioxidant-related effects of antidepressant drugs. Table 3 summarizes the studies conducted with acute and chronic antidepressant treatments in control and stressed animals.

Réus et al. (2010) reported increased SOD and CAT activity and decreased lipid and protein damage in male rat prefrontal cortex and hippocampus after both acute and chronic treatment with imipramine [17]. Additionally, imipramine treatment increased brain creatine kinase and increased activity of mitochondrial respiratory chain complexes [18, 53]. Katyare and Rajan (1995) showed that long-term administration of imipramine to female rats resulted in significant stimulation of the states 3 and 4 respiration rates. This effect was evident within a week of imipramine administration and was sustained through the second week of treatment [20]. These results suggest that imipramine treatment may induce changes in substrate oxidation pattern, increase rate of ATP synthesis, and can potentially increase mitochondrial ROS production.

Xu et al. (2003) examined dose-dependent effects of amitriptyline and venlafaxine on neuroprotective proteins in male rats. In this study, low dose (5 mg/kg) of amitriptyline and venlafaxine increased the intensity of BDNF immunostaining in hippocampal pyramidal neurons and the intensity of Bcl-2 immunostaining in hippocampal mossy fibers, but did not alter the Cu/Zn-SOD immunoreactivity. High

Table 2: *In vitro* studies with antidepressants.

Method		Antidepressant drugs tested	Main findings	Reference
In vitro	Rat brain mitochondria	Fluoxetine	Indirectly and nonspecifically affects electron transport and F_1F_0-ATPase activity inhibiting oxidative phosphorylation	Curti et al., 1999 [6]
In vitro	Rat liver mitochondria	Fluoxetine	Multiple effects on the energy metabolism of rat liver mitochondria; potentially toxic in high doses	Souza et al., 1994 [7]
In vitro	Rat liver mitochondria	Fluoxetine	Inhibits the opening of the MPT pore, the release of cytC, and protected against staurosporine-induced apoptotic cell death	Nahon et al., 2005 [8]
In vitro	Rat liver mitochondria	Nortriptyline	Inhibits loss of mitochondrial membrane potential and the activation of caspase 3	Zhang et al., 2008 [9]
Cell culture	PCN cells oxygen/glucose deprived	Nortriptyline	Decrease cell death	Zhang et al., 2008 [9]
Cell culture	PC12 cells exposed to H_2O_2	Amitriptyline, fluoxetine	Both agents attenuated cell death induced by H_2O_2, fluoxetine pretreatment increased SOD activity	Kolla et al., 2005 [10]
Cell culture	IFN-γ-activated microglia	Fluvoxamine, imipramine, reboxetine	All drugs inhibited IL-6 and NO production in a dose-dependent manner	Hashioka et al., 2007 [11]
Cell culture	Human monocytic U-937 cells	Desipramine, imipramine, maprotiline, and mirtazapine	Short-term treatment decreased mRNA levels of SOD and CAT after treatment with these drugs; long-term treatment increased mRNA levels of SOD, GST, and GR	Schmidt et al., 2008 [12]

CAT: catalase; cytC: cytochrome C; GR: glutathione reductase; GST: glutathione S-transferase; H_2O_2: hydrogen peroxide; IFN-γ: interferon-gamma; IL-6: interleukin 6; MPT: mitochondrial permeability transition; NO: nitric oxide; SOD: superoxide dismutase.

Table 3: Animal studies with antidepressant drugs.

Animal model		Antidepressant drugs tested	Main findings	Reference
Male albino mice	Acute treatment	Bupropion (10–40 mg/kg), i.p., once, 30 min before brain sample acquisition	Modulated the L-arginine-NO-cyclic cGMP signalling pathway in rat brain	Dhir and Kulkarni, 2007 [13]
Female Swiss mice	Acute treatment	Escitalopram (3 mg/kg), p.o., once, 30 min before behavioural tests	Antidepressant-like effect was mediated by an inhibition of either the NMDA receptor activation or NO-cGMP synthesis	Zomkowski et al., 2010 [14]
Male C57Bl/6J mice	Acute treatment	Imipramine (15 mg/kg), venlafaxine (6 mg/kg), both drugs, i.p., once only	Decreased brain $NO_2 + NO_3$ levels in control mice	Krass et al., 2011 [15]
Male Wistar rats	Acute treatment	Amitriptyline (10 mg/kg), i.p., once only, 3 h before analyses	Drug did not alter $NO_2 + NO_3$ serum levels in control rats	Vismari et al., 2012 [16]
Male Wistar rats	Acute and chronic treatment	Fluoxetine (20 mg/kg once or 10 mg/kg/day), i.p., once only or once a day for 12 days	Showed stimulation of mitochondrial respiration in state 4 in acute or prolonged treatments, indicating uncoupling of oxidative phosphorylation in rat liver mitochondria	Souza et al., 1994 [7]
Male Wistar rats	Acute and chronic treatment	Imipramine (10, 20 and 30 mg/kg), i.p., once only or once a day for 14 days	Decreased MDA and carbonyl content and increased SOD and CAT activity in prefrontal cortex and hippocampus	Réus et al., 2010 [17]
Male Wistar rats	Acute and chronic treatment	Imipramine (10, 20 and 30 mg/kg), i.p., once only or once a day for 14 days	Increased brain creatine kinase and mitochondrial respiratory chain activities	Réus, et al., 2012 [18]
Male Wistar rats	Acute and chronic treatment	Imipramine (10, 20 and 30 mg/kg), i.p., once only or once a day for 14 days	Altered respiratory chain complexes and CK activities; these alterations were different with relation to protocols (acute or chronic), complex, dose, and brain area	Réus, et al., 2012 [53]
Female Swiss mice	Acute and chronic treatment	Fluoxetine (10 mg/kg), p.o., once only or once a day for 28 days	Acute treatment reduced GPx activity in hippocampus; chronic treatment increases GSH in both hippocampus and prefrontal cortex	Lobato et al., 2010 [19]

Table 3: Continued.

Animal model		Antidepressant drugs tested	Main findings	Reference
Female Wistar rats	Chronic treatment	Imipramine (10 mg/kg) twice daily, i.p., 1 or 2 weeks	Promoted stimulation of the states 3 and 4 respiration rates (1 and 2 week treatments) on rat brain mitochondria	Katyare and Rajan, 1995 [20]
Male Sprague-Dawley rats	Chronic treatment	Amitriptyline (5, 10 mg/kg/day), venlafaxine (5, 10 mg/kg/day), both drugs. i.p., for 3 weeks	Both drugs increased SOD immunostaining in the hippocampal neurons	Xu et al., 2003 [21]
Male Wistar Han rats	Chronic treatment	Fluoxetine, 8 and 24 mg/kg/day, p.o., for 4 weeks	Increased levels of carbonyl groups, TBARS, and the uric acid content in the liver, effects more pronounced at high dose	Inkielewicz-Stêpniak, 2011 [22]
Male Swiss albino mice	Acute treatment, with or without previous restraint stress protocol	Fluoxetine, 5 mg/kg/day, i.p., 30 min before restraint stress protocol	Partially reversed the adverse effects of stress (restraint stress significantly increases the generation of ROS in the peripheral defence cells) restoring SOD, CAT, and GSH levels	Novio et al., 2011 [23]
Swiss Albino rats	Chronic treatment, with or without previous restraint stress protocol	Fluoxetine (20 mg/kg/day), imipramine (10 mg/kg/day), venlafaxine (10 mg/kg/day), all drugs, p.o., for 3 weeks	All drugs restored SOD, CAT, GST, and GR activity, increased GSH and decreased MDA and carbonyl in brain samples of stressed animals	Zafir et al., 2009 [24]
Male Wistar rats	Chronic treatment, with or without previous chronic social isolation stress	Fluoxetine, 5 mg/kg/day, i.p., for 3 weeks	Decreased SOD and increased GPx activity in both groups, increased TAC in stressed animals, also induced several hallmarks of apoptosis in the liver of stressed animals	Djordjevic et al., 2011 [25]
Male Swiss-Webster mice	Chronic treatment, stress induced by FST and TST	Venlafaxine (5, 10, and 20 mg/kg/day), i.p. for 3 weeks	Decreased MDA and NO and increased hippocampal GSH and TAC levels and GST activity in the stressed animals, also, reduced both serum and hippocampal 8-OHdG levels	Abdel-Wahab and Salama, 2011 [26]

8-OHdG: 8-hydroxydeguanosine; CAT: catalase; cGMP: cyclic guanosine monophosphate; CK: creatine kinase; FST: forced swimming test; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione; GST: glutathione S-transferase; MDA: malondialdehyde; NO: nitric oxide; $NO_2 + NO_3$, total nitrite + nitrate; ROS: reactive oxygen species; SOD: superoxide dismutase; TAC: total antioxidant capacity; TBARS: thiobarbituric acid reactive species; TST: tail suspension test.

dose (10 mg/kg) of venlafaxine, however, decreased the intensity of BDNF immunostaining in all subareas of the hippocampus and increased the intensity of Cu/Zn-SOD immunostaining in the dentate granular cell layer [21]. More recently, Abdel-Wahab and Salama (2011) showed that long-term venlafaxine treatment at effective antidepressant dosages can protect against stress-induced oxidative cellular and DNA damage in male mice. At all doses tested, venlafaxine decreased MDA and total nitrite levels, increased total antioxidant potential and GSH content, and restored GST activity in hippocampus of stressed animals. Venlafaxine also promoted increased total antioxidant potential and GSH levels in the control, nonstressed group. Finally, this treatment was able to reduce serum and hippocampal levels of 8-OHdG (a marker of DNA damage) in stressed animals [26] showing potential antioxidant effects related to these antidepressant agents.

The effects of chronic (one month) fluoxetine treatment on lipid and protein oxidative damage, uric acid concentration in the liver and the activity of transaminases and transferases in the serum have been investigated in male rats. Chronic fluoxetine treatment increased the levels of TBARS, carbonyl groups, and the uric acid content in the liver. The activities of alanine transaminase (ALT), aspartate transaminase (AST), and GST were increased in the serum. The overall effects are more pronounced in the higher dose (24 versus 8 mg/kg) [22]. More recently, Djordjevic et al. (2011) showed altered antioxidant status and increased apoptotic signalling in male rat liver after 21 days of fluoxetine treatment. Control animals and stressed animals displayed decreased activity of SOD and increased activity of GPx. In addition, in both experimental groups, fluoxetine altered several markers of apoptosis in the liver, including decreased Bcl-2 expression and increased DNA fragmentation [25]. These effects seemed to be associated with liver toxicity induced by high-dose fluoxetine treatment in rats.

Novio et al. (2011) investigated the effects of fluoxetine on intracellular redox status in peripheral blood cells obtained from male mice exposed to restraint stress. They found that restraint stress significantly increased the generation of ROS in the peripheral blood and that acute treatment with fluoxetine partially reversed this effect, possibly through normalization of SOD and CAT activity and GSH content [23]. Using a depression-like rat model, Zafir et al. (2009) examined antioxidant effects of fluoxetine and venlafaxine in the rat brain. The results evidenced a significant recovery in the activities of SOD, CAT, GST, GR, and GSH levels by these antidepressants after restraint stress. Also, fluoxetine and venlafaxine treatment prevented lipid and protein oxidative damage induced by stress [24]. In another study, acute fluoxetine treatment reduced GPx activity in the hippocampus, whereas chronic treatment increased GSH in both hippocampus and prefrontal cortex of female mice [19].

Recent data support that some antidepressants are able to modulate NO synthesis and nitrosative stress-associated signalling cascades. Dhir and Kulkarni (2007) tested different dosages of bupropion in male rats. The antidepressant-like effect of bupropion was prevented by pretreatment with L-arginine (a substrate of nitric oxide synthase, NOS). Pretreatment with 7-nitroindazole (a specific neuronal NO synthase, nNOS inhibitor) potentiated bupropion's effects. In addition, treatment with methylene blue (a direct inhibitor of NOS and soluble guanylate cyclase, sGC) potentiated the effect of the drug in the forced swim test [13]. This study suggests that bupropion possesses antidepressant-like activities in different animal models possibly through dopaminergic and L-arginine-NO-cyclic guanosine monophosphate (cGMP) signaling pathways. This is consistent with a study by Zomkowski et al. (2010) showing similar effects with escitalopram in female mice. The antidepressant-like effect of escitalopram in the forced swim test (FST) was prevented by pretreatment with N-methyl-D-aspartic acid (NMDA), L-arginine, and sildenafil (a phosphodiesterase inhibitor). Also, the administration of 7-nitroindazole, methylene blue or ODQ (i.c.v., a soluble sGC inhibitor) in combination with escitalopram reduced the immobility time in the FST. This study highlights the role of NMDA receptors and L-arginine-NO-cGMP pathway in the mechanism of action of antidepressant agents [14]. Recently, Krass et al. (2011) reported that imipramine decreased brain nitrite + nitrate (NO_2 + NO_3) levels, a marker of nitrosative stress, in male rat brain. This result supports the idea that antidepressants are able to inhibit NO synthesis in the rat brain [16], an effect that could be mechanistically related to the ability of L-arginine to counteract their antidepressant-like effects [15]. In summary, studies in animal models suggest that antidepressant agents modulate antioxidant enzyme activities and decrease oxidative stress markers on liver, brain, and peripheral tissues. In addition, there is a clear association between high dosages of antidepressants and increased hepatic oxidative stress. However, a major limitation of the studies above mentioned is that not all studies measured oxidative stress markers (i.e., MDA, carbonyl); therefore, these prooxidant effects need further investigation.

Consistent with the above-mentioned studies, changes in the blood/brain antioxidant profile have been associated with changes in depressive-like behaviour. More specifically, it has been demonstrated that some classic antioxidants induce antidepressant-like effects in rodents. In one study, treatment with *Ginkgo biloba* extract (10 mg/kg) reduced recorded immobility time in the forced swimming test (FST) to the same extent as imipramine (39% versus 38%). No differences in locomotor activity were observed, suggesting a selective antidepressant-like effect. This antidepressant-like effect of *Ginkgo biloba* extract was associated with a reduction in lipid peroxidation and superoxide radical production (as indicated by a downregulation of SOD activity) [54]. In rats displaying depressive-like behaviour induced by chronic mild stress, administration of liquiritin, an antioxidant derived from *Glycyrrhiza uralensis*, decreased immobility time, increased sucrose consumption, increased SOD activity, and attenuated MDA production in the peripheral blood [55]. These findings are further corroborated by a study showing that Ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one), a substance that mimics the activity of the antioxidant enzyme GPx [56], decreased immobility time

in rodents, an effect that was dependent on its interaction with the noradrenergic and dopaminergic systems [57]. Additionally, alpha-tocopherol (vitamin E) administration produced antidepressant-like effects in animal models of depression. Along with antidepressant-like effects, long-term treatment with alpha-tocopherol enhanced antioxidant defences in the mouse hippocampus and prefrontal cortex, two structures closely implicated in the pathophysiology of depression [19].

4.3. Post-Mortem Studies. A number of *post-mortem* studies reported altered oxidative stress parameters in individuals with MDD (Table 4). Michel et al. (2010) showed increased XO activity in the thalamus and putamen of seven individuals with an ante-mortem diagnosis of recurrent MDD (age range = 61–93 y.o.). Four of these subjects received SSRI and one was medicated with clomipramine in the 6 months before death, while two of them were not antidepressant treatments [35]. These results suggest increased ROS production in brain samples of depressive patients due to increased XO activity. Two recent studies showed reduced oxidized and total GSH in the prefrontal cortex of MDD subjects as compared to controls [31, 36]. In addition, GPx levels were reduced in MDD subjects [31]. Because 10 in 14 patients have taken antidepressants at time of death, we can speculate that antidepressants had limited or no effects on GSH and GPx levels. In a subsequent study with the same cohort, GST levels were also reduced in MDD patients and no effects of antidepressant treatment were observed [36].

In summary, while some changes in antioxidant enzymes have been observed in MDD, these *post-mortem* studies are not conclusive mostly because of small sample sizes, lack of control groups, and lack of relevant information (i.e., treatment duration, specific drugs used).

5. Clinical Data: Human Studies

In the last decade, an increasing number of studies have addressed the potential effects of antidepressant treatments on oxidative stress and antioxidant potential in humans (Table 4). Corroborating with animal data, the majority of these studies revealed that antidepressant agents possess antioxidant properties when used in the treatment of MDD. Increased serum SOD and MDA levels have been found in a cohort of 62 major depressive patients (age 43.8 ± 12.9, mean ± SD; 34/28, female/male ratio) [27]. In another study, plasmatic vitC levels were reduced in patients with MDD compared with age- and sex-matched healthy volunteers ($n = 40$). Oxidative stress markers (SOD, vitC, lipid peroxidation) were reversed after 4 weeks of treatment with fluoxetine (20 mg/day, $n = 32$) and citalopram (20 mg/day, $n = 30$). Notably, these antioxidant effects were persistent after 12 weeks of treatment [27].

Bilici et al. (2001) reported increased oxidative stress in major depressive patients ($n = 32$), indexed by higher antioxidant enzyme activities (erythrocyte SOD, GPx, and plasmatic GR) and MDA levels (erythrocyte and plasmatic). After treatment with four different SSRIs drugs (fluoxetine 20 mg/day, $n = 7$; sertraline 50 mg/day, $n = 13$; fluvoxamine 100 mg/day, $n = 5$; or citalopram 20 mg/day, $n = 5$), for 12 weeks, antioxidant enzyme activities (plasmatic GPx) and MDA levels (plasma and erythrocyte) were restored to control levels. Plasmatic GR and erythrocyte SOD were also significantly decreased in MD patients after 12-week antidepressant treatment [30]. In another study, a group of 50 MDD patients (age 36.7 ± 5.2; 22/28 F/M ratio) who had achieved remission from their first episode of depression after 3 months of treatment with 20 mg of fluoxetine were tested before and after remission [48]. Before treatment, MDD patients displayed increased erythrocyte SOD and CAT activities, increased MDA levels, and decreased plasmatic total antioxidant status (TAS) level. After three months of fluoxetine treatment, MDA levels were normalized [48]. Decreased serum SOD and increased XO were found in 20 individuals with MDD (age range 17–62 years, 19/17 F/M ratio) [29]. Although increased XO levels indicate increased free radical production, no difference was observed in serum total nitrite levels (a marker of nitrosative stress, possible associated to ONOO–) between control and MDD patients before treatment. Also, the authors did not find a significant relationship between the duration of illness and SOD, XO activities, or nitrite levels in this cohort. Treatment with citalopram (20 mg/day, $n = 10$), fluoxetine (20 mg/day, $n = 11$), fluvoxamine (150 mg/day, $n = 7$), or sertraline (50 mg/day, $n = 8$) for 8 weeks increased SOD activity whereas decreased XO levels suggesting that normalization of these enzymes was associated with symptomatic improvement [29].

Cumurcu et al. (2009) investigated whether 3 different total antioxidant parameters (TAC, TOS, and OSI) were associated with MDD and evaluated the impact of antidepressant treatment on these oxidative/antioxidant parameters in a cohort of 57 major depressive patients (age 35.5 ± 12.1, 46/11 F/M ratio). TOS and OSI were higher and TAC level was lower in the MDD group compared with controls ($n = 40$). Furthermore, the authors found a positive correlation between the severity of the disease and serum TOS and OSI ($r = 0.58$, and $r = 0.63$, resp.). Also, a negative correlation was found between the severity of the disease and serum TAC ($r = -0.553$) at the pretreatment stage. After 3 months of treatment with escitalopram, 10–20 mg/day, $n = 10$; paroxetine, 20–40 mg/day, $n = 20$; or sertraline, 50–100 mg/day, $n = 27$, TOS and OSI were decreased and TAC was increased compared with pretreatment values [32]. These further suggest that recovery from a major depressive episode may be associated with normalization of antioxidant potential induced by antidepressants.

More recently, a 24-week follow-up study evaluated the effects of long-term antidepressant treatment on oxidative/antioxidant status in a cohort of 50 MDD subjects (age 33.1 ± 10.0, 39/11 F/M ratio) [33]. Antidepressant treatments included venlafaxine (125 ± 43.3 mg/day, $n = 21$), milnacipran (100 mg/day, $n = 2$), paroxetine 25 ± 7.6 mg/day, $n = 8$, escitalopram 16.3 ± 5.2 mg/day, $n = 8$, sertraline 80 ± 27.4 mg/day, $n = 5$, citalopram 33.3 ± 11.5 mg/day, $n = 3$, fluoxetine 20 mg/day, $n = 1$, tianeptine 37.5 mg/day, and moclobemide 600 mg/day. Plasmatic MDA, serum oxidized LDL (OxLDL) levels, and erythrocyte SOD

Table 4: Antidepressant treatment and oxidative stress markers in major depressive disorder.

Sample (F/M)	Altered oxidative stress markers in MD[a]	Treatment duration	Antidepressant drugs tested	Effect for antidepressants	Reference
34/28	↑MDA ↑SOD ↓VitC	4 weeks and 12 weeks	Citalopram ($n = 30$), fluoxetine ($n = 32$)	↓MDA ↓SOD ↑VitC (effects in both 4 and 12 weeks treatment)	Khanzode et al., 2003 [27]
72/24	↑MDA ↑SOD ↓TAC ↑VitE	6 weeks	Reboxetine, sertraline, venlafaxine	No effects	Sarandol et al., 2007 [28]
19/17	↑XO ↓SOD	8 weeks	Citalopram ($n = 10$), fluoxetine ($n = 11$), fluvoxamine ($n = 7$), sertraline ($n = 8$)	↓XO ↑SOD ↓nitrite	Herken et al., 2007 [29]
21/9	↑MDA ↑SOD ↑GPx ↑GR	12 weeks	Citalopram ($n = 5$), fluoxetine ($n = 7$), fluvoxamine ($n = 5$), sertraline ($n = 13$)	↓MDA ↓SOD ↓GPx ↓GR	Bilici et al., 2001 [30]
28/22	↑MDA ↑SOD ↑CAT ↓TAC	12 weeks	Fluoxetine ($n = 50$)	↓MDA	Galecki et al., 2009 [48]
46/11	↓TAC ↑TOS ↑OSI	12 weeks	Escitalopram ($n = 10$), paroxetine ($n = 20$), sertraline ($n = 27$)	↑TAC ↓TOS ↓OSI	Cumurcu et al., 2009 [32]
39/11	↑MDA ↑OxLDL ↑SOD	24 weeks	Citalopram ($n = 3$), escitalopram ($n = 8$), fluoxetine ($n = 1$), milnacipran ($n = 2$), moclobemide ($n = 1$), paroxetine ($n = 8$), sertraline ($n = 5$), tianeptine ($n = 1$), venlafaxine ($n = 21$)	↓MDA ↓SOD ↓TAC	Kotan et al., 2011 [33]
20/15	↓CoQ10	? weeks	? ($n = 15$)	No effects*	Maes et al., 2009 [34]
5/2	↑XO	Post-mortem study	SSRI ($n = 4$), TCA ($n = 1$)	No effects[#]	Michel et al., 2010 [35]
6/9	↓GPx ↓GSH	Post-mortem study	Trazodone ($n = 1$), nefazodone ($n = 2$, one together SSRI), TCA and/or SSRI ($n = 7$)	No effects[#]	Gawryluk et al., 2011 [31]
	↓GST	Post-mortem study		No effects*	Gawryluk et al., 2011 [36]

Sample (female/male) from MD group; [a]Compared to respective control group. *Compared to unmedicated MD group; [#]speculative. CAT, catalase; CoQ10, coenzyme Q-10; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione S-transferase; MDA, malondialdehyde; OSI, oxidative stress index; OxLDL, oxidized LDL; SOD, superoxide dismutase; SSRI, selective serotonin reuptake inhibitor; TAC, total antioxidant capacity; TCA, tricyclic or tetracyclic antidepressant; TOS, total oxidant status; VitC, vitamin C; VitE, vitamin E; XO, xanthine oxidase.

activity were increased in MDD patients before treatment, and MDA levels were positively correlated with the severity of MDD. After 24-weeks of treatment, MDA and SOD levels decreased. However, TAC was also found decreased after 24-week treatment with antidepressants, indicating that the oxidative stress observed in depressed patients was partly improved during 24 weeks of antidepressant treatment. Patients on venlafaxine were also compared with patients on SSRIs in the aspect of oxidative stress parameters in the follow-up period, but no significant differences were found [33].

Sarandol et al. (2007) found that MDD was accompanied by increased peripheral oxidative stress; however, short-term antidepressant treatment (6 weeks) did not alter oxidative/antioxidant systems in a cohort of 96 MDD patients (age 40 ± 11, 72/24 F/M ratio). In this study, MDD patients had increased plasmatic MDA levels and increased susceptibility of red blood cells (RBCs) to oxidation. Also, SOD activity was increased in patients with MDD, and there was a positive correlation between the severity of depressive symptoms and SOD activity ($r = 0.419$). After 6 weeks of treatment with venlafaxine 75–150 mg/day, sertraline 50 mg/day, or reboxetine 4–8 mg/day, these oxidative parameters were not altered [28].

Maes et al. (2009) investigated plasma concentrations of CoQ10 in 35 depressed patients (age 42.1 ± 10.5, 20/15 F/M ratio) and 22 sex-, age-matched controls. Plasmatic CoQ10 was lower in depressed patients than controls. However, there was no correlation between plasma CoQ10 and the severity of illness or the number of depressive episodes. During the study, part of the depressed patients were on antidepressant treatment at the time of blood sampling ($n = 15$), while the remaining were unmedicated ($n = 20$). There were no differences in plasma CoQ10 between depressed patients who were taking antidepressants and those without [34].

6. Concluding Remarks

This paper examined preclinical (*in vitro* and animal models) and clinical literature on oxidative/antioxidant effects of antidepressant agents. Overall, most animal and human data support that antidepressant drugs exert antioxidant effects during treatment for MDD.

In vitro and animal studies also suggest that some antidepressants may be prooxidant at high doses. The antioxidant effects of antidepressant drugs seem to vary depending on the dose, treatment regimen, and duration. Notably, a number of clinical trials revealed that treatment with antidepressants can reverse the increased oxidative stress observed in individuals with MDD. Short-term treatments (4 to 8 weeks) do not seem to alter antioxidant/oxidative parameters in MDD patients, whereas longer treatments (12 to 24 weeks) seem to induce robust antioxidant effects.

Overall, the literature reviewed does not support differences in antioxidant potential between different antidepressant agents/classes. However, many of these studies were short in duration and likely underpowered to address the question of differences in antioxidant potential amongst particular drugs and larger studies are warranted.

Brain imaging studies have suggested that MDD may be associated with decreased volumes of various brain regions [58–60]. For instance, MDD subjects have smaller normalized frontal lobe volumes when compared with the nondepressed controls after controlling for age, gender and "total cumulative illness rating scale score" [61]. Presence of temporal lobe atrophy and moderate-to-severe white matter lesions can predict occurrence of major depression during a 5-year followup in a population-based sample of elderly [62]. Considering that the presence of oxidative (and nitrosative) stress may cause neurodegeneration and reduced neurogenesis [63, 64], the relationship between oxidative stress and changes in brain structure and function in MDD is a promising area for future studies.

An important issue in biomarker research is the fact that peripheral markers may not necessarily correlate with changes in the central nervous system. For instance, Teyssier et al. (2011) demonstrated that the expression of oxidative stress-response genes was not altered in the prefrontal cortex of individuals with MDD. They concluded that the pathogenic role of oxidative stress in the neurobiology of depression could not be inferred from alterations in the periphery [65]. However, in this *post-mortem* study all of the patients had received antidepressant treatment, which may have normalized oxidative stress parameters. Furthermore, there is also evidence suggesting that BDNF, oxidative stress, and inflammation tend to be abnormal among individuals with multiple mood episodes and correlate with length of illness [51, 66, 67]. Peripheral biomarkers detected during acute mood episodes could in fact constitute markers of disease activity [68]. Studies of peripheral biomarkers in large randomized, placebo-controlled trials will ultimately confirm whether or not normalization of oxidative stress parameters is associated with treatment response.

In conclusion, there is increasing body of evidence supporting that MDD may be associated with changes in oxidative stress markers and that antidepressant agents (especially long-term treatment) may increase antioxidant defences. It is possible that augmentation of antioxidant defences may be one of the mechanisms underlying the neuroprotective effects of antidepressants observed in the treatment of MDD.

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Review Article

The Antioxidant Mechanisms Underlying the Aged Garlic Extract- and S-Allylcysteine-Induced Protection

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Aged garlic extract (AGE) is an odorless garlic preparation containing S-allylcysteine (SAC) as its most abundant compound. A large number of studies have demonstrated the antioxidant activity of AGE and SAC in both *in vivo*—in diverse experimental animal models associated to oxidative stress—and *in vitro* conditions—using several methods to scavenge reactive oxygen species or to induce oxidative damage. Derived from these experiments, the protective effects of AGE and SAC have been associated with the prevention or amelioration of oxidative stress. In this work, we reviewed different antioxidant mechanisms (scavenging of free radicals and prooxidant species, induction of antioxidant enzymes, activation of Nrf2 factor, inhibition of prooxidant enzymes, and chelating effects) involved in the protective actions of AGE and SAC, thereby emphasizing their potential use as therapeutic agents. In addition, we highlight the ability of SAC to activate Nrf2 factor—a master regulator of the cellular redox state. Here, we include original data showing the ability of SAC to activate Nrf2 factor in cerebral cortex. Therefore, we conclude that the therapeutic properties of these molecules comprise cellular and molecular mechanisms at different levels.

1. Introduction

Garlic (*Allium sativum*) has been cultivated and used for culinary and medicinal purposes by many cultures for centuries [1, 2]. Even today, garlic cloves are commonly used in Eastern Europe and Asia, whereas garlic supplements are popular in Western Europe and growing in popularity in the US. Garlic is a source particularly rich in organosulfur compounds, which are responsible for its flavor, aroma, and potential health benefits [3]. Recent studies have demonstrated and validated many of the medicinal properties attributed to garlic [4]. Different types of garlic supplements are commercially available, including garlic powders (tablets), garlic oils (capsules), and aged garlic extracts (tablets, capsules, and liquid); each of these sources provide a different profile of organosulfur compounds [5]. One of the better known garlic preparations is aged garlic extract (AGE), which is formed during garlic aging (up to 20 months). During this time, unstable and highly odorous compounds in fresh garlic are converted into more stable and much less odorous compounds [6].

A considerable number of *in vivo* and *in vitro* studies have been performed so far in order to test the antioxidant properties of AGE and one of its most abundant organosulfur compounds, S-allylcysteine (SAC). In these studies, different antioxidant mechanisms have been reported, such as their ability to (1) scavenge reactive oxygen (ROS) and nitrogen (RNS) species; (2) increase enzymatic and nonenzymatic

antioxidants levels; (3) activate Nrf2 factor; or (4) inhibit some prooxidant enzymes (xanthine oxidase, cyclooxygenase, and NADPH oxidase).

Given that SAC is the most abundant compound in AGE, the present paper brings special attention to the physicochemical characteristics, toxicity, pharmacokinetics, tissue distribution, and metabolism of this compound, as well as on the different antioxidant mechanisms involved in its protective actions in different experimental models of toxicity.

2. Aged Garlic Extract (AGE)

AGE is an odorless product resulting from prolonged extraction of fresh garlic at room temperature; it is highly bioavailable and exerts biological activity in both animals and humans. In AGE, garlic (in 15–20% ethanol) is aged for up to 20 months in stainless steel tanks. The extract is then filtered and concentrated at low temperature [6]. AGE is sold in both dry form and as a liquid containing 10% ethanol [4].

The process of aging gently modifies harsh and irritating compounds from the raw garlic and naturally generates unique and beneficial compounds through both enzymatic and natural chemical reactions (Table 1) [7]. The main changes in AGE during aging process are indicated as follows [4]:

(1) complete hydrolysis of the γ-glutamylcysteines to SAC and S-1-propenylcysteine (the content of SAC remains constant after 3 months, but S-1-propenylcysteine steadily decreases);

(2) increase in cystine due to protein hydrolysis and increase in S-allylmercaptocysteine probably due to the reaction of allicin with protein derived cysteine;

(3) initial loss of alliin to thiosulfinates formation (allicin);

(4) complete loss of thiosulfinates after 3 months due to the fact that they are converted into volatile allyl sulfides (diallyl sulfide, diallyl disulfide, diallyl trisulfide), which evaporate almost completely.

Based on typical total SAC content in cloves (41 μmole/g dry wt.), the total SAC content in commercial aged extracts (7.8 μmole/g dry wt. after correcting for 40% excipients) is only 19%, indicating considerable manufacturing losses or the use of cloves with an unusually low content of γ-glutamylcysteines. The commercial aged products are standardized on SAC content, but a specific or even minimum amount has not been declared yet [4].

Recently, new compounds, such as tetrahydro-beta-carbolines (1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid and 1-methyl-1,2,3,4-tetrahydro-beta-carboline-1,3-dicarboxylic acid) as well as Nα-(1-deoxy-D-fructos-1-yl)-L-arginine, have also been identified in AGE. These compounds increase during the aging process and play an important role as antioxidants [8–10]. Indeed, tetrahydro-beta-carbolines are biologically active alkaloids and are structurally similar to flavonoids. Nα-(1-deoxy-D-fructos-1-yl)-L-arginine is only found in AGE and no other garlic products.

Substantial evidence shows that AGE ameliorates the oxidative damage implicated in aging and a variety of diseases, such as cardiovascular alterations, cancer, stroke, Alzheimer's disease (AD), and other age-related degenerative conditions. In addition, AGE is a commercially available garlic preparation that has been widely studied for its high antioxidant content and its health-protective potential [11–17].

3. S-Allylcysteine (SAC): A Key Compound in Aged Garlic Extract

SAC is formed from γ-glutamyl-S-allylcysteine catabolism (Figure 1) and has been used to standardize commercial AGE [7]. SAC is a white crystalline powder with characteristic odor, it has not hygroscopic ability, and its melting point is 223.3–223.7°C [18]. SAC is a stable compound as it remains unaltered in AGE for up to 2 years [4]. Stored crystal samples show a slight change into a yellowish color, but no transformation or decomposition is observed. Under basic conditions (2 N NaOH, 50°C, 6 days), allylmercaptan and allylsulfide (decomposition products) are observed, suggesting that the C–S bond cleavage occurs. However, under acidic conditions (6 N HCl, 50°C, 5 days), evidence of C–S bond cleavage is not observed. These observations indicate that SAC can be absorbed in gastrointestinal tract after oral administration without any changes [18].

3.1. Toxicity. SAC is 30-fold less toxic than other typical garlic compounds such as allicin and diallyldisulfide. The 50% lethal oral dose (LD_{50}) of SAC in female (9.39 g/kg) or male (8.89 g/kg) mice was higher than allicin (female: 0.363 g/kg and male: 0.309 g/kg) and diallyldisulfide (female: 1.3 g/kg and male: 0.145 g/kg) (Reviewed in [6]). Others studies have confirmed the low toxicity of SAC in male mice, since LD_{50} by oral administration was 8.8 g/kg (Table 2). In male rats, LD_{50} of SAC by *i.p.* administration (3.34 g/kg (<20 mM)) is similar to essential L-amino acids such as methionine (29 mM/kg). Gender differences were observed in acute toxicity tests in mice, since LD_{50} in males was about 1.7 times higher than in females [18].

Negative effects of SAC were observed at high doses ($\geq$500 mg/kg for 1 month, orally) including [18]

(1) decrease of body weight in both genders;

(2) increase of urinary pH in male;

(3) decrease of urinary protein (in female) and urobilinogen (in male) levels. Urobilinogen is a metabolite of bilirubin generated by intestinal bacteria that is reabsorbed. Therefore, these data suggest that SAC may have some effect on the intestinal flora;

(4) increase of serum glucose levels in females, suggesting that SAC induces atrophy of the pancreas and decreases insulin secretion. Atrophy of the pancreatic tissue has been observed only in the females administered with high dosage of SAC;

Table 1: Compositional changes in the AGE.[A]

Compound	Incubation time (months)				
	0	1	3	12	24
	(mg/g dry extract)				
γ-Glutamyl-S-allylcysteine	12.7	5.8	1.1	0	0
S-Allylcysteine	**0.2**	**5.9**	**7.2**	**7.1**	**7.2**
γ-Glutamyl-S-1-propenylcysteine	15.9	3.4	0.5	0	0
S-1-Propenylcysteine	0.5	6.7	8.1	6.5	4.4
S-Allylmercaptocysteine	0.01	0.6	1.2	1.7	1.9

[A] Cloves were chopped into pieces ($2 \times 2 \times 1$ mm) and placed into 20% ethanol (12 mL/g) in a closed container and stored at room temperature. Synthesized from [4].

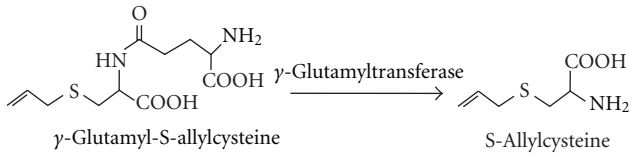

Figure 1: During the process of aging γ-glutamyl-S-allylcysteine is converted to S-allylcysteine (SAC) by a γ-glutamyltransferase.

(5) modifying of renal (decrease blood urea nitrogen and serum creatinine levels) and hepatic (increase serum total cholesterol, protein, lipids, and alkaline phosphatase levels) function in females;

(6) decrease of hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration in both genders.

3.2. Pharmacokinetics. In rats, pharmacokinetics of SAC after oral administration shows a three-phase concentration profile: two very fast phases (absorption and distribution) followed by a slow elimination phase. For *i.v.* administration, SAC pharmacokinetics presents a two-phase concentration profile: a very fast distribution phase and a slow elimination phase [19, 20]. Pharmacokinetic parameters of SAC (100 mg/kg, oral and *i.v.*) are shown in Table 3.

Oral bioavailability of SAC at 100 mg/kg dose is 91% [19], similar to that reported in other studies where bioavailability was 103.0% in mice, 98.2% in rats, and 87.2% in dogs [20].

In addition, a pharmacokinetic study of SAC in humans has been performed by oral administration of garlic preparation containing this compound. The half-life of SAC in humans after oral administration was more than 10 h, and clearance time was more than 30 h [18]. These results are similar to experimental data obtained in dogs, where the half-life of SAC was about 10 h, and clearance time was more than 24 h, albeit these data were different from other experimental results obtained in mice [20]. Total SAC content in the blood of volunteers at T_{max} is about 450 μg (content on T_{max}, 23 ng/mL plasma; body weight: 65 kg; volume of total blood: 1/3 of body weight), suggesting a high bioavailability in humans [18].

Table 2: Toxicity of SAC in mice and rat.[A]

		LD_{50} (g/kg body weight)[B]	
		Oral	*i.p.*
Mice	Male	8.89	6.91
	Female	9.39	3.65
Rat	Male	10.94	3.34
	Female	9.50	3.34

[A] Adapted from [18]. [B] 50% lethal dose of a single dose *i.p.*: intraperitoneal administration.

3.3. Tissue Distribution. After oral intake, SAC is easily absorbed in the gastrointestinal tract and can be detected in several tissues up to 8 h after dosage [19, 20]. In rats, after a single oral dosage of SAC (50 mg/kg), the area under the plasma concentration versus the time curve from zero to the last quantifiable concentration (AUC_{0-t}) is 169.2 mg h/kg, whereas, in tissues, the highest peak concentration is observed in the kidney (C_{max} = 65.7 mg/kg at 10 min). The elimination half-life varies among tissues; for instance, liver shows the longest $T_{1/2}$ value (2.2 h) the shorted value (1.2 h) was observed in the brain (Table 4). For *i.v.* administration, the kidney also exhibits the highest SAC exposure ($AUC_{0-8\,h}$ = 171.9 mg h/kg), whereas the heart shows the longest $T_{1/2}$ value (2.5 h) [19].

3.4. Metabolism. N-Acetyl-SAC has been identified as a metabolite of SAC in urine of rats, dogs, and humans [20, 21]. Also, small amounts of SAC (less than 1%) have been observed in urine of rats. These data suggest that the absorbed SAC seems to be metabolized to N-acetyl-SAC by N-acetyltransferase, which is mainly found in liver and kidney. However, it has been shown that, when SAC is almost completely eliminated from the liver, it is readily retained at a comparatively high concentration in the kidney. Thus, it can be speculated that SAC may be transformed into N-acetyl-SAC by N-acetyltransferase in the liver, and, then, a portion of N-acetyl-SAC may be deacylated to SAC by acylase in kidney, followed by its reabsorption [20].

Table 3: Pharmacokinetic parameters for oral and *i.v.* administration of SAC in the serum of rats.[A]

Parameter	Oral administration	*i.v.* administration
Distribution volume (V_d)	1.2 L	1.3 L
Elimination half-life ($T_{1/2\beta}$)	2.7 h	2.6 h
Total clearance	0.3 L/h	0.3 L/h
Peak concentration (C_{max})	100.1 mg/L at 30 min	
AUC_{0-t} value	293.5 mg·h/L	322.6 mg·h/L

[A] Synthesized from [19]. AUC_{0-t}: area under the plasma concentration versus time curve from time zero to the last quantifiable concentration.

Table 4: Pharmacokinetic parameters after a single oral dose of SAC.[A]

Parameter	C_{max}	AUC_{0-t}	$T_{1/2}$
Tissue	(mg/kg)	mg·h/kg	(h)
Kidney	65.7	169.2	2.1
Liver	58.1	103.5	2.2
Heart	43.3	118.8	2.1
Spleen	43.3	100.6	1.9
Lung	35.1	94.3	1.3
Brain	26.7	70.7	1.2

[A] SAC dose of 50 mg/kg. Adapted from [19].
C_{max}: peak concentration at 10 min; AUC_{0-t}: area under the plasma concentration versus time curve from time zero to the last quantifiable concentration; $T_{1/2}$: elimination half-life time.

4. Antioxidant Mechanisms Associated with the Protective Effect of SAC and/or AGE

Several reports have shown that AGE and SAC inhibit the oxidative damage implicated in aging and a variety of diseases. Derived of these works, different antioxidant mechanisms have been attributed to these compounds and confirmed. Figure 2 shows the antioxidant mechanism associated with the protective effects of SAC.

4.1. AGE and SAC Scavenge Reactive Oxygen Species. SAC contains a thiol group responsible of its antioxidant capacity because this nucleophile can easily donate its proton to an electrophilic species, thereby neutralizing them or making them less reactive.

SAC is the most studied compound of AGE and its antioxidant properties have been reported in several studies. SAC readily prevents lipid [22–26] and protein oxidation [27] and nitration [28], supporting its antioxidant activity. Actually, SAC is known to scavenge superoxide anion ($O_2^{\bullet -}$) [14, 27, 29], hydrogen peroxide (H_2O_2) [22, 27, 29–31], hydroxyl radical ($^{\bullet}OH$) [14, 23, 29, 32], and peroxynitrite anion ($ONOO^-$) [28, 29]. Medina-Campos et al. reported that SAC scavenges hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$). Furthermore, these authors compared the scavenging activity of SAC against reference compounds (molecules that scavenge a specific reactive oxygen species) through the IC_{50} value for each reactive species. SAC scavenged HOCl in a similar manner than lipoic acid, and it was more efficient to scavenge $^1O_2$ than lipoic acid and reduced glutathione [29]. Recently, Maldonado et al. reported that SAC was able to scavenge $^{\bullet}OH$ and peroxyl radical ($ROO^{\bullet}$) in a concentration-dependent manner, and this effect is reduced when SAC is changed by S-propylcysteine, suggesting that allyl group in SAC is necessary for its scavenging activity [33]. To further support this concept, in our laboratory, we have observed that allyl group is necessary to preserve the scavenging activity of SAC on different reactive oxygen species (unpublished data).

On the other hand, AGE is known to scavenge H_2O_2 [22, 30] and $O_2^{\bullet -}$ [34, 35]. Consistently, Wei and Lau observed a protective effect of AGE (1–8 mg/mL) in endothelial cells challenged by H_2O_2 or the $O_2^{\bullet -}$ generator system xantine/xanthine oxidase [36].

Besides SAC, there are other compounds in AGE exhibiting antioxidant properties: (a) S-allylmercaptocysteine scavenges $^{\bullet}OH$ and $^1O_2$ [37]; (b) alliin scavenges $^{\bullet}OH$ [38], $O_2^{\bullet -}$ [32], and H_2O_2 [22] and inhibits lipid peroxidation [22]; (c) Nα-(1-deoxy-D-fructos-1-yl)-L-arginine scavenges H_2O_2 [8, 39] and also inhibits the Cu^{2+}-induced low-density lipoprotein (LDL) oxidation and peroxides release during the coincubation of macrophages with oxidized LDL [39]; (d) tetrahydro-beta-carbolines show a strong H_2O_2 scavenging activity while inhibit 2,2′-azobis(2-amidinopropane) hydrochloride-induced lipid peroxidation and nitrite production induced by lipopolysaccharide in murine macrophages [9, 10].

4.2. AGE and SAC Induce Antioxidant Enzymes and Nrf2 Factor. Hsu et al. evaluated the effect of SAC (1 g/L in drinking water for 4 weeks) on the activity of catalase and glutathione peroxidase in Balb/cA mice. After 4 weeks of treatment, SAC increased glutathione levels in kidney and liver when compared with controls. Moreover, SAC enhanced catalase and glutathione peroxidase activities in kidney and liver [40]. These data suggest that SAC could be acting by recruiting different mechanisms, including radical scavenging and induction of antioxidant enzymes.

Lawal and Ellis reported that AGE prevented death in Cd-treated 1321N1 and HEK293 cells, due to cell AGE reduced Cd-induced lipid peroxidation and LDH leakage, increased GSH levels, expression of the protective enzyme NAD(P)H:quinone oxidoreductase, and the accumulation of the transcription factor Nrf2. The authors suggest that AGE could be beneficial in Cd-induced toxicity, and this protection appears to be mediated via induction of cytoprotective enzymes in a Nrf2-dependent manner. This indicates the AGE potential use as a chemoprevention strategy for Cd toxicity [41].

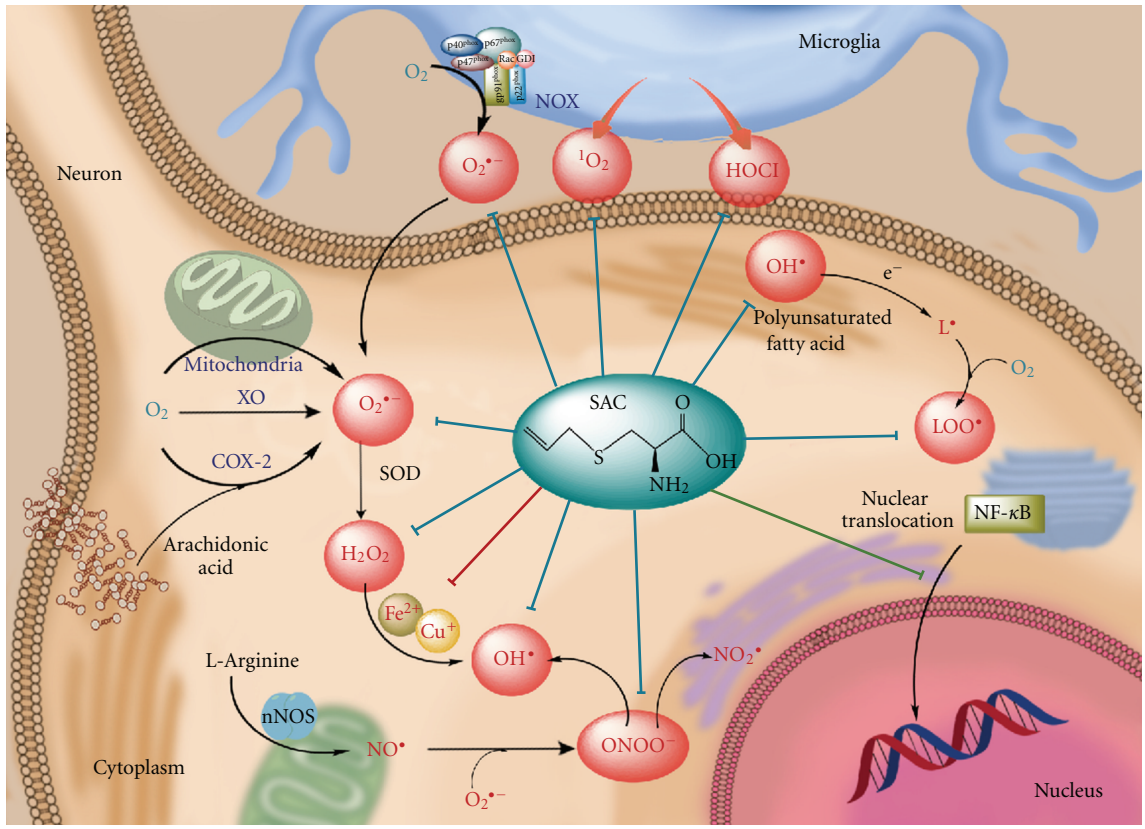

Figure 2: Antioxidant mechanism associated to S-allylcysteine (SAC). SAC can scavenge superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), peroxynitrite radical ($ONOO^-$), and peroxyl radical (LOO•) produced in neuronal cells, as well as hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) produced in microglial cells (blue lines). Moreover, SAC also exhibits chelating properties on Fe^{2+} and Cu^{2+} ions (red line), hence avoiding Fenton reaction. SAC also inhibits NF-kB translocation into the nucleus (green line), thus preventing apoptotic signaling. COX-2: cyclooxygenase-2, NOX: NADPH oxidase, nNOS: neuronal nitric oxide synthase, SOD: superoxide dismutase, XO: xanthine oxidase.

Kalayarasan et al. reported that SAC (100 mg/kg, *i.p.* for 3 days) is also able to activate Nrf2 factor in hepatocytes of Wistar rats exposed to chromium [42]. The transcription factor Nrf2 (nuclear factor-E2-related factor 2) is the guardian of redox homeostasis as it regulates basal and inducible expression of antioxidant and cytoprotective genes, providing the level of protection required for normal cellular activities and against various oxidative stress-related pathologies, including ischemic stroke [43–45]. Lee et al. reported the major functional categories of genes responsible for conferring protection against oxidative stress or inflammation [46]. These genes codify for detoxifying enzymes, antioxidant proteins, NADPH-producing proteins, growth factors, defense/immune/inflammation related proteins, and signaling proteins; thus, the activation of Nrf2/ARE (antioxidant response element) pathway could stimulate a synergistic protective effect [47].

Nrf2 is highly expressed in detoxifying organs—such as liver and kidney—and other organs commonly exposed to external environmental conditions—including the skin, lung, and digestive tract—[48], whereas in the brain its levels are low [49].

To date, there is no available information on Nrf2 induction by AGE or SAC in the brain. Here, we show that SAC administration (100 mg/Kg for 5 consecutive days) is able to activate Nrf2 factor in homogenates of cerebral cortex at 24 h (Figure 3). Although the precise mechanism through which SAC is able to evoke this effect in cerebral cortex still remains unclear, a mechanistic proposal is shown in the Figure 4.

4.3. AGE and SAC Inhibit Prooxidant Enzymes

4.3.1. Nitric Oxide Synthase (NOS). NOS family is formed by three isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed and require the formation of a Ca^{2+}-calmodulin complex for their activation. The third isoform, inducible NOS (iNOS), exerts its activity in a Ca^{2+}-independent manner [50]. In the presence of O_2, these enzymes catalyze the conversion of L-arginine to L-citrulline plus nitric oxide (NO). Despite increased levels of NO have been involved in degenerative events, NO is also a well-known small molecule that acts as a second

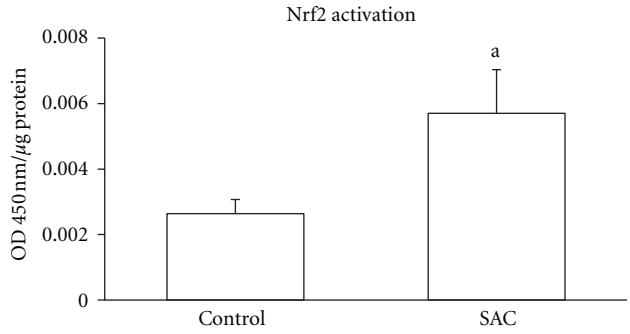

Figure 3: S-Allylcysteine (SAC) induces the activation of the transcription factor nuclear factor-E2-related factor 2 (Nrf2) in cerebral cortex. Animals received SAC 100 mg/kg every day for 5 days. Quantification was made by ELISA at 450 nm in nuclear extracts from frontal cortex of rats at 24 h after the last administration of SAC. Values are expressed as mean ± SEM. $n = 4$-5. $^{a}P < 0.0244$ versus control group. Student's t-test. OD: optical density.

messenger in cellular signaling and in a large variety of diverse physiological processes, including neurotransmission, vascular relaxation, blood pressure regulation, defense mechanisms, and immune regulation [51]. Therefore, a dysregulation of NO concentrations compromises the cell survival mechanisms.

SAC is known to inhibit NO production in LPS/cytokine-stimulated macrophages and hepatocytes by suppression of iNOS gene expression [14]. A SAC-induced inhibition of NO production may be related to its ability to decrease NF-κB activation [13, 52], thus leading to cell protection. On the other hand, reports *in vivo* indicate that AGE increases NO levels through the stimulation of the constitutive NOS isoforms, but not iNOS [15]; however, this mechanism has not been elucidated yet. Altogether, these data suggest that AGE and SAC could be useful in diseases associated with oxidative stress involving dysfunction in NO production.

4.3.2. Xanthine Oxidase (XO). XO and xanthine dehydrogenase (XDH) are interconvertible forms, and both are members of the molybdenum hydroxylase flavoprotein family; they carry out similar reactions in the purine catabolism in mammals, which are often referred as xanthine oxidoreductase activity. The interconversion from XDH to XO can be either reversible by sulphide reagents (oxidation of important protein thiol group) or irreversible by proteolysis (calcium-dependent proteases) [53]. In particular, XO catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid with the concomitant O_2 reduction by a single electron. In turn, XDH catalyzes the same reaction with the reduction of NAD^+ by direct two-electron reduction. O_2 reduction yields $O_2^{\bullet -}$ and H_2O_2 as secondary products [54]. $O_2^{\bullet -}$ generated can then react with NO to generate $ONOO^-$, a strong oxidant capable of damage to proteins, lipids, and DNA.

Demirkaya et al. reported that AGE administration (3 mL/kg during 6 weeks, starting 1 week before doxorubicin treatment) decreased XO activity in a model of doxorubicin-induced cardiotoxicity. However, the protective effect observed in this model was not associated with the decrease in XO activity, but to the ability of AGE to increase the levels of some antioxidant enzymes, while decreasing lipid peroxidation [55].

4.3.3. NADPH Oxidase. NADPH oxidase is an enzymatic complex located mainly in the plasmatic membrane of specific granules of phagocytic leukocytes, such as neutrophils and macrophages. It catalyzes the formation of $O_2^{\bullet -}$, which together with other oxidant species, is produced to kill ingested microorganism (reviewed in [56]). NADPH oxidase overactivation has been related to different pathophysiological events, including renal injury [57], hypertension, atherosclerosis, angiogenesis, and ischemia/reperfusion [56].

Cruz et al. reported a protective effect of SAC (200 mg/kg, *i.p.*) and AGE (1.2 mL/kg *i.p.*) administrated every day for 30 days, in 5/6 nephrectomized rats, a model of renal injury and hypertension. In this work, the protective effect exerted by these compounds was associated with their antioxidant properties and their ability to decrease $gp91^{phox}$ and $gp22^{phox}$ levels [58]. However, the mechanism by which SAC and AGE decrease the abundance of $gp91^{phox}$ and $gp22^{phox}$ is unclear. In animals, $gp91^{phox}$ is the catalytic subunit of NADPH oxidase, whereas $gp22^{phox}$ is a membrane protein that forms a heterodimer with $gp91^{phox}$ to produce $O_2^{\bullet -}$. It is known that NADPH oxidase activation is implicated in the pathogenesis of several renal diseases due to its capacity to produce $O_2^{\bullet -}$, and its inhibition may be useful to ameliorate and delay the progression of renal injury [57].

4.3.4. Cyclooxygenase (COX). COX catalyzes the rate-limiting step in the synthesis of prostaglandins and thromboxanes from arachidonic acid [59]. There are two main COX isoforms: COX-1, a constitutive enzyme expressed in many tissues with functions such as gastric mucose protection and regulation of vascular tone [60]. In contrast, the COX-2 isoform is an inducible enzyme primarily associated with inflammatory processes [59, 60]. Several stimuli induce COX-2, including bacterial lipopolysaccharide, interleukin-1 (IL-1), IL-2, and tumor necrosis factor (TNF-α) [60]. COX-2 activity has also been associated with oxidative stress due to its capacity to produce $O_2^{\bullet -}$ from O_2.

Colín-González et al. reported the effect of AGE on COX-2 protein levels and activity in a model of cerebral ischemia. They found that AGE administration (1.2 mL/Kg *i.p.*, onset of reperfusion) exerted a neuroprotective effect attributable to its ability to decrease the ischemia-induced increase of 8-hydroxy-2-deoxyguanosine (a marker of oxidative damage to DNA) and TNF-α levels (an inflammation marker). Moreover, AGE decreased COX-2 protein expression and activity, although its mechanism of action remains unclear [61].

Noteworthy, only a few studies have paid attention to the effects of AGE and SAC on the main prooxidant enzymes (XO, NADPH oxidase, or COX), so the mechanisms involved in the possible inhibitory actions of these compounds have been not elucidated yet.

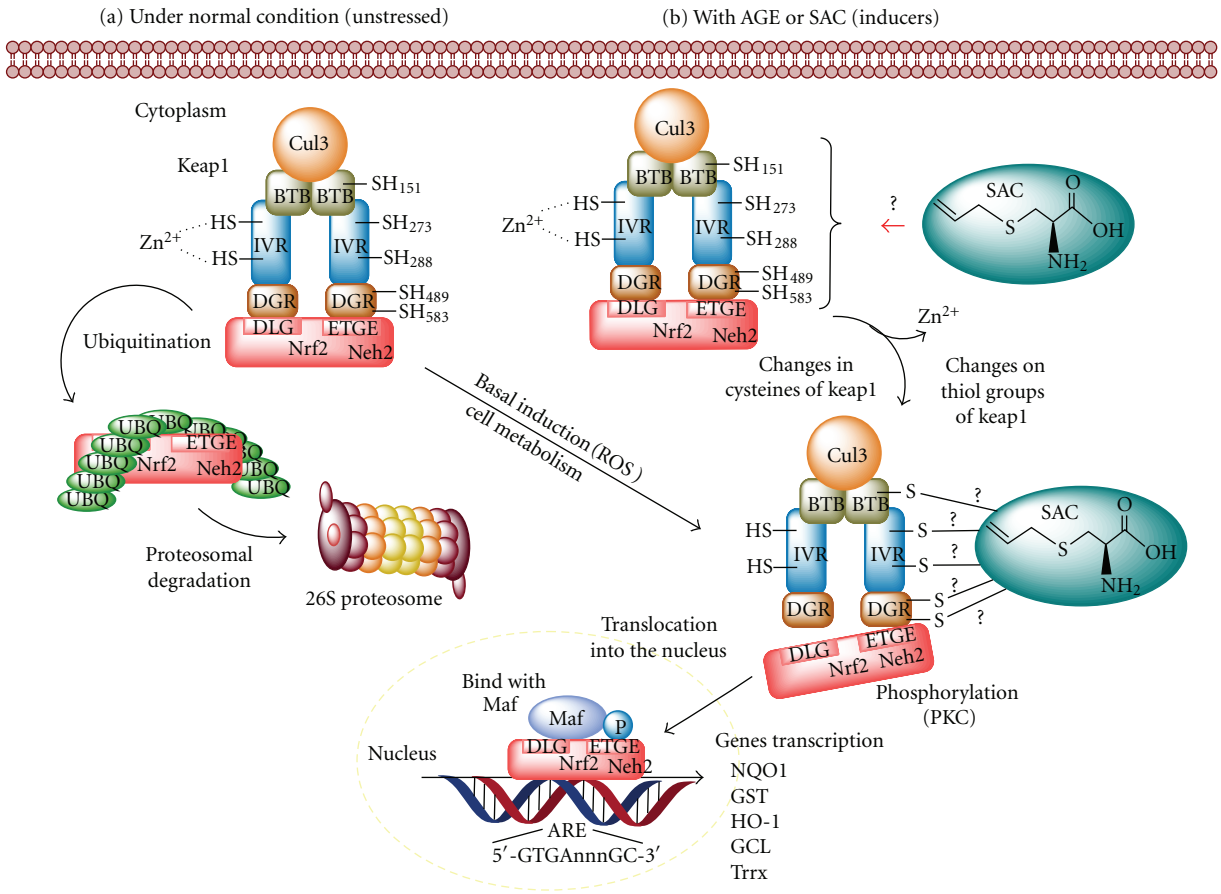

Figure 4: Effect of aged garlic extract (AGE) or S-allylcysteine (SAC) on Nrf2/Keap1 complex. *Left panel*: Upon unstressed conditions, this complex is dissociated and Nrf2 can either suffer proteosomal degradation or respond to stimuli typical of basal cell metabolism. In the later, Nrf2 is phosphorylated and translocated to the nucleus forming heterodimers with Maf and acting on antioxidant response element (ARE). *Right panel*: Under stress oxidative conditions, or in the presence of inducers, several cysteine residues suffer changes inducing its Nrf2 dissociation and further translocation of this factor to nucleus, where it will induce phase 2 genes transcription. SAC could modify cysteine residues on Keap1 domain, hence releasing Nrf2 and allowing its transactivation. Nrf2: transcription factor nuclear factor-E2-related factor 2, Keap1: kelch-related erythroid cell-derived protein with CNC homology (ECH) protein 1, UBQ: ubiquitin, ROS: reactive oxygen species, NQO1: NAD(P)H:quinone oxidoreductase 1, GST: glutathione-S-transferase, HO-1: heme oxygenase-1, GCL: glutamate cysteine ligase.

4.4. Chelating Effect of SAC and AGE. Divalent metals (zinc, iron, copper, cobalt, manganese) are involved in a considerable number of physiological processes. However, some of these metals (mostly iron and copper) are involved in the generation of reactive oxygen radicals. A pathological increase in iron or calcium levels has been related with oxidative stress and mitochondrial damage, further leading to neuronal death. Thus, neurodegeneration can be determined by alterations in ionic homoeostasis and/or changes in prooxidative-antioxidant equilibrium, two conditions that are constantly modified in brain cells (reviewed in [62]). Moreover, the observations that iron induces aggregation of α-synuclein and β-amyloid (Aβ) peptides to form toxic aggregates reinforce its critical role in oxidative stress-induced pathogenesis in neurodegeneration (reviewed in [63]). Furthermore, Fenton and Haber-Weiss reactions (Figure 5) are two mechanisms through which free iron or copper produce reactive oxygen species—particularly $^{\bullet}$OH—to further lead to oxidative degradation of lipids, proteins, and ADN [64–66]. In this regard, it has been reported that the brains of Alzheimer's patients have increased levels of iron, zinc, and copper when compared to control patients [65, 67, 68], whereas the brains of Parkinson's patients show a marked increase in iron and zinc levels [65, 68].

On the other hand, it has been reported that the interaction between Cu^{2+} and Aβ produces Aβ-Cu^{+} and H_2O_2, and this monovalent form of copper catalyzes the oxidation of Aβ and free radicals production through the Fenton reaction [65, 66, 69] as follows:

$$\begin{aligned} Cu^{2+} + A\beta &\longrightarrow A\beta\text{-}Cu^{+} + H_2O_2 \\ &\longrightarrow A\beta\text{-}Cu^{2+} + {}^{\bullet}OH + OH^{-} \end{aligned} \quad (1)$$

Altogether, these data support the concept that a combination of free metal chelation and antioxidant therapies may constitute a valuable approach for neuroprotection.

$$Fe^{3+} + O_2^{\bullet -} \rightleftarrows Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad \text{Fenton reaction}$$
$$O_2^{\bullet -} + H_2O_2 \rightleftarrows O_2 + {}^{\bullet}OH + OH^- \quad \text{Haber-Weiss reaction}$$

Figure 5: The known ways in which iron is directly involved in the generation of reactive oxygen species. The production of these species is potentially harmful for several cell types and tissues. Adapted from [66].

Dairam et al. found that SAC (30 μg/mL) possesses the property of chelating Fe^{2+} and Fe^{3+} in a concentration-dependent manner [70]. Moreover, it has been shown that AGE [34] and SAC [71] are able to inhibit Cu^{2+}-induced LDL oxidation, an effect associated to its ability to chelate Cu^{2+}. In addition, Dillon et al. using a xanthine-XO assay and $CuSO_4$ as inhibitor of XO reported that AGE chelates Cu^{2+} and restores the activity of XO [72].

5. Neuroprotective Effects of AGE

Technology is increasing exponentially impacting on every aspect of life, improving research, diagnosis, equipment, and procedures in medicine. As a result, life expectancy is increasing, and the population is ageing (in 2007, the overall life expectancy at birth in the US was 77.9 years, representing an increase of 0.2 years from that reported in 2006) [73]. As a consequence, the incidence of neurodegenerative diseases has been increased [74, 75] constituting a global problem that will cause profound economical and social challenges. Nowadays, there are no disease-modifying therapies designed for these disorders, so an intense amount of research is justified.

Oxidative stress is widely implicated in the development of neurodegenerative disorders such as AD, Stroke, Parkinson's disease, and Huntington's disease, among others [76]. Therefore, an increasing number of investigations are expected to appear in a near future studying the effects of AGE and SAC in the central nervous system, probably validating these agents as useful therapeutic strategies.

5.1. Neurotrophic Activity of AGE. The neurotrophic activity of AGE was revealed in a primary culture of fetal rat hippocampal neurons. In this study, the addition of AGE significantly promoted neuronal survival and the number of branching points per axon [77]. Later on, the neurotrophic molecular mechanisms of AGE were studied through the screening of genes differentially expressed after the addition of AGE (0.67%) to primary cultured rat fetal hippocampal neurons. Quantitative RT-PCR showed that α2-microglobulin-related protein (α2MRP) mRNA levels were remarkably increased at 24 h after incubation with AGE [78]. α2MRP is a member of the acute phase proteins, molecules whose concentrations increase or decrease in response to inflammation, tissue damage, and maintenance of cell homeostasis [79]. This finding indicates that AGE may directly or indirectly activates the expression of important genes for neuronal survival, an effect that could be relevant for the design of pharmacological interventions in neurodegenerative disorders.

5.2. Attenuation of Ischemic Brain Damage by AGE. In the US, cerebral ischemia is the third leading cause of death and the leading cause of disability. Brain injury following ischemia results in the activation of a series of biochemical events eventually leading to neuronal death. Despite recent medical and surgical advances, we are still unable to prevent acute ischemic brain damage, so the development of effective neuroprotective methods is crucial.

The effects of AGE on brain ischemia have been evaluated using different administration schemes in the middle cerebral artery occlusion paradigm: (1) AGE (0.5 mL/Kg *i.p.*) applied 30 min before the onset of ischemia (1 h ischemia/72 h reperfusion) decreased edema formation [80]; (2) AGE (1.2 mL/Kg *i.p.*) applied at the beginning of reperfusion (2 h ischemia/2 h reperfusion) decreased infarct area, 3-nytrotirosyne levels, and prevented the decrease in antioxidant enzymes (glutathione peroxidase and superoxide dismutase) [81]; (3) AGE (1.2 mL/Kg *i.p.*) applied at the beginning of reperfusion (1 h ischemia/24 h reperfusion) diminished infarct area, neuronal deficit, histological alterations, 8-hydroxy-deoxyguanosine, TNF-α, and COX-2 levels [61]. All the above-mentioned findings show that the complex physiopathological mechanisms leading to neuronal injury in cerebral stroke can be modified by AGE treatment at different doses, administration schedules, and ischemia/reperfusion times, thus emphasizing its broad spectrum of therapeutic applications. However, the protective effect exerted by AGE in this model may be primarily associated to the prevention of oxidative stress and inflammatory responses.

5.3. Antiageing Effect of AGE. Oxidative stress and immune dysfunction in biological systems are among the most important causes of ageing [82, 83]. The senescence-accelerated mouse is a genetic murine model for studying ageing and spontaneous senescence, and these animals show a short life span and exhibit several signs of senescence in early age (impairment of learning and memory performance, neurochemical changes, etc.) [84]. Chronic oral administration (2 month) of AGE-enriched food (2% w/w) to senescence-accelerated mouse (1) prevented skin glossiness, coarseness, and hair loss; (2) increased survival ratio; (3) improved the memory acquisition deficit; (4) avoided the decrease of brain weight and the atrophy of the frontal portion of the brain [85–87].

On the other hand, Zhang et al. raised the question on whether AGE could have any effect as immunomodulator in the central nervous system. To answer this question, they used an oral administration of AGE (2% w/w) in senescence-accelerated mouse and found that this compound increased immune responses, suggesting that AGE may also improve age-associated decline in the immune system. Shortly thereafter, they also observed the effect of AGE in thymectomized mice, a model of immunodeficiency and deterioration of learning performance. AGE treatment prevented the reduction of antibody production responses, improved the deterioration of learning behavior, and restored the contents of noradrenaline, 3,4-dihydroxyphenylacetic acid,

homovanillic acid, and cholineacetyltransferase activity in the hypothalamus. These data suggest that AGE has the capability of reverting thymectomy-induced alterations to normal levels [88].

Clinical studies have demonstrated that brain lesions in Alzheimer's patients may be associated with abnormal immune reactions [89]. Thus, AGE has also been used in Alzheimer animal models. In 2006, Chauhan investigated the anti-inflammatory effects of dietary AGE (2% w/w) in an Alzheimer's transgenic model harboring Swedish double mutation (KM670/671NL; Tg2576). The Tg2576 brains treated with AGE showed reductions in TNF-α levels accompanied by reductions in IL-1β level, and positive plaque-associated microglia, thereby demonstrating a possible anti-inflammatory effect of this compound [90].

AGE contains multiple ingredients with neurotrophic activity that may directly or indirectly prevent the ischemic damage and the age-related morphological changes. Therefore, further studies, including isolation, purification, and identification of its specific compounds and the mechanisms involved, are needed.

6. Neuroprotective Effects of SAC

SAC has been used in several works in order to find effective, therapeutic, and preventive strategies for intervention in neurodegenerative diseases.

6.1. Neurotrophic Effect of SAC. Moriguchi et al. reported the positive actions of garlic compounds (including SAC) with a thioallyl group in rat hippocampal neurons culture. In this work, SAC increased survival and axonal branching from neurons. Based on these findings, this group suggested that thioallyl group is essential for neurotrophic activity [91]. These data suggest that SAC is a compound that not only acts as an antioxidant agent but also as a neurotrophic molecule.

6.2. SAC in Experimental Models of Alzheimer's Disease (AD). AD is a devastating neurodegenerative disorder which causes progressive loss of cognitive abilities, the accumulation of Aβ deposits in the basal forebrain, hippocampus, and cortex, together with oxidative stress, have been consistently implicated in the pathogenesis of this disorder [92]. In this regard, the design of a treatment with antioxidant and/or antiamyloidogenic properties represents an approach of considerable therapeutic value for the prevention of the disease progression.

The effect of SAC on PC12 cells exposed to $A\beta_{(25–35)}$ has also been evaluated. SAC suppressed the generation of ROS; attenuated caspase-3 activation, DNA fragmentation, and PARP cleavage eventually protecting against Aβ-induced cell death [93, 94]. Moreover, SAC attenuated cell death induced by Aβ in organotypic hippocampal culture and cultured hippocampal neurons in a concentration-dependent manner, an effect apparently mediated by the caspase-12-dependent pathway [95–97].

On the other hand, some studies have shown that AD patients exhibit an overstimulation of the N-methyl-D-aspartate receptor in some time points of the disease progress [98]. In order to reproduce this state, Aβ and ibotenic acid (a potent N-methyl-D-aspartate agonist) administrations have been used in organotypic hippocampal cultures resulting in a time-dependent neuronal damage, a feature that SAC administration (10 and 100 mM) significantly attenuated in the CA3 area [95].

AD involves misfolding and aggregation of proteins which increased endoplasmic reticulum stress [99]. Tunicamycin, an inhibitor of N-glycosylation in endoplasmic reticulum, reproduces some severe neurological alterations in animals (resembling neurological disorders) through the induction of endoplasmic reticulum stress. The role of SAC (10^{-8}–10^{-5} M) on tunicamycin-mediated cell death was investigated in PC12 cells and hippocampal neurons, showing a selective neuroprotective effect on the caspase-12-dependent apoptotic pathway [96, 100]. In another work, Aβ plus tunicamycin-induced neurotoxicity was assessed in organotypic hippocampal slice cultures, where SAC (100 μM) improved cell viability in area CA3 and dentate gyrus. Simultaneously, SAC reversed calpain activity as well as the active forms of caspase-12 and caspase-3, without changing the increased levels of endoplasmic reticulum chaperones (GRP94 and −78) or C/EBP homologous protein. The authors suggest that SAC could either directly interact with calpain or alter the environment in the vicinity of the endoplasmic reticulum lumen without affecting unfolded protein responses or the C/EBP homologous protein-mediated signaling pathway induced by Aβ plus tunicamycin [101].

In addition, the protective effect of SAC in Aβ toxicity could not be only related to its antioxidant activity as Gupta and Rao investigated the effect of SAC on Aβ aggregation *in vitro*. SAC (10–50 M) not only inhibited Aβ fibrillation in a dose-dependent manner and disestablished preformed Aβ-peptide fibrils, but also bound to Aβ fibrils. In a docking protocol, the site suitable for accommodating SAC was identified around the $A\beta_{40}$ structure. It was mentioned that SAC could interact with the positively charged Gln15-Lys16 segment. In fact, binding could be induced either by hydrophobic interactions between allyl chain and hydrophobic regions of Aβ (Phe 19 and Val 12), or by the H-bond between the -OH group of the carboxylic group of SAC and donator/acceptor groups of Aβ [102].

Since there is a possibility that AD results from inheritance of an autosomal dominant mutation in the amyloid precursor protein, the introduction of mutant amyloid precursor protein genes into suitable mouse pronuclei is used to build transgenic mouse models of AD. These mutant amyloid precursor protein transgenic models exhibit the progressive Aβ neuritic plaques formation, dystrophic neuritis, and neuroinflammation [103]. Interestingly, the dietary administration of SAC (20 mg/kg for 4 month) in one of these transgenic models decreased Aβ load, IL-1β reactive plaque-associated microglia, Tau2 reactivity, and GSK-3β protein, showing an antiamyloidogenic, anti-inflammatory, and antitangle activity (via GSK-3 β) [90].

Intracerebroventricular injection of streptozotocin to mice impairs brain biochemistry, cerebral glucose, energy metabolism, cholinergic transmission, and increases generation of free radicals, further leading to cognitive deficits; these effects are similar to sporadic dementia in humans. Pretreatment with SAC (30 mg/kg every day for 15 days) in intracerebroventricular streptozotocin-infused mice ameliorated hippocampal neuronal abnormalities, prevented the cognitive and neurobehavioral impairments, restored levels of reduced glutathione and its dependent enzymes (glutathione peroxidase and glutathione reductase), diminished lipid peroxidation, DNA fragmentation, and p53 levels, and increased Bcl2 levels [104].

Finally, SAC (1 g/L for 15 weeks, added to the drinking water) was tested in D-galactose-injected mice. D-Galactose induces AD-like pathological changes in the brain, including increased reactive oxygen species, decreased antioxidant enzyme activity, and enhanced Aβ-peptide expression. SAC decreased the brain levels of A$\beta_{1–40}$ and A$\beta_{1–42}$, lowered amyloid precursor protein level and BACE1 expressions and activities (both of them being factors responsible for Aβ accumulation and AD progression), retained PKC activity and expression of PKC-α and PKC-γ, lowered Aβ accumulation, reduced the levels of advanced glycation end products (carboxymethyllysine, pentosidine), lowered aldose reductase activity and expression (an enzyme that facilitates the production of sorbitol and fructose, which in turn promote advanced glycation end products formation and glycative stress), and displayed antioxidant protection (evidenced by increased reduced glutathione content and glutathione peroxidase, superoxide dismutase, and catalase activities, accompanied by decreased levels of malondialdehyde, reactive oxygen species, and protein carbonyls) [105].

The aforementioned evidence serves to suggest that SAC might prevent the progression of AD by multiple mechanisms: antioxidant, antiamyloidogenic, anti-inflammatory, antitangle, and antiglycative activities (Figure 6). However, further studies are essential to determine if SAC is capable of displaying all these proprieties in humans.

6.3. SAC in Ischemic Brain Damage. The effect of SAC in ischemic brain damage was tested at 100, 300, and 600 mg/kg *i.p*. applied 30 min prior the onset of ischemia in rats. SAC (300 mg/Kg) induced significant reduction of the infarct volume, water content, oxidative stress and improvement in motor performance and memory impairment [23, 80]. Moreover, in a two-vessel occlusion model in gerbils, the oral and *i.p*. administration of SAC 300 mg/kg increased the number of surviving cells/mm^2 of CA1 region [106].

Hypertension is recognized as the most important risk factor for the development of ischemic cerebral infarction in humans [107]. Kim et al. evaluated the effect of SAC in stroke-prone spontaneously hypertensive rats, demonstrating that SAC (5%, 28 day of diet period) reduced mortality and the overall stroke-related behavioral score [108]. In another work, the same authors showed that SAC (300 mg/Kg) reduced the size of infarct area after 2 h occlusion and 22 h reperfusion, prevented neuronal cell death in CA1 region and inhibited the activity of ERK induced by focal ischemia in a middle cerebral artery occlusion model in gerbils, while *in vitro* exerted scavenging activity on peroxynitrite [28].

Additionally, it has been reported that the positive actions of SAC seen in the cerebral ischemia-induced damage to the hippocampus may be due to the possible modulation of mitochondrial dysfunctions. SAC 300 mg/kg *i.p.* administered twice (15 min preocclusion and 2 h postocclusion at the time of reperfusion) in the middle cerebral artery occlusion model in rats, produced a significant decrease in mitochondrial lipid peroxidation, protein carbonyl levels, cytochrome c release, and intracellular H_2O_2 levels. Furthermore, SAC also restored the status of mitochondrial glutathione and glucose 6-phosphate dehydrogenase, ATP content, and the activity of mitochondrial respiratory complexes (I-IV) [109]. In conclusion, the protective effects of SAC on brain ischemic damage may be associated with the decrease of oxidative stress and the modulation of mitochondrial dysfunctions.

6.4. SAC in Experimental Models of Huntington's Disease. Huntington's disease is an autosomal dominant neurodegenerative disorder characterized by the gradual and progressive loss of neurons, predominantly in the striatum. It is caused by a mutation in the huntingtin gen. Its main clinical manifestations are chorea, cognitive impairment, and psychiatric disorders, most of them related to striatal and cortical atrophy. Nowadays, there is no treatment to prevent or reduce the morphological and functional alterations seen in the brains of Huntington's patients. For this reason, it is imperative to look for pharmacological strategies to improve the quality of life of these patients [110].

SAC has been used in two animal models of Huntington's disease: 3-nitropropionic acid and quinolinic acid. SAC (300 mg/kg *i.p.*) administration to rats infused with 3-nitropropionic acid prevented behavioral alterations, increased manganese and copper/zinc superoxide dismutase activities, and decreased lipid peroxidation and mitochondrial dysfunction [111]. Simultaneously, SAC (0.75 mM) also decreased lipid peroxidation and mitochondrial dysfunction induced by 3-nitropropionic acid in synaptosomal fractions [112].

On the other hand, it has been reported that SAC bonds Fe^{2+} and Fe^{3+}, preventing the redox cycling of iron, and consequently, the quinolinic acid induced-lipid peroxidation [70]. Alternatively, the effect of SAC was evaluated in a combined model of excitotoxicity/energy deficit produced by the coadministration of quinolinate and 3-nitropropionate acid in brain synaptosomal membranes. SAC abolished the quinolinic acid plus 3-nitropropionate acid-induced lipid peroxidation [113, 114]. The protective effect of SAC in these models has been attributed to its ability to preserve the cell redox status through its antioxidant properties and probably to its iron-binding properties.

6.5. SAC in Experimental Models of Parkinson's Disease. Parkinson's disease is characterized by progressive degeneration of dopaminergic neurons in the substantia nigra

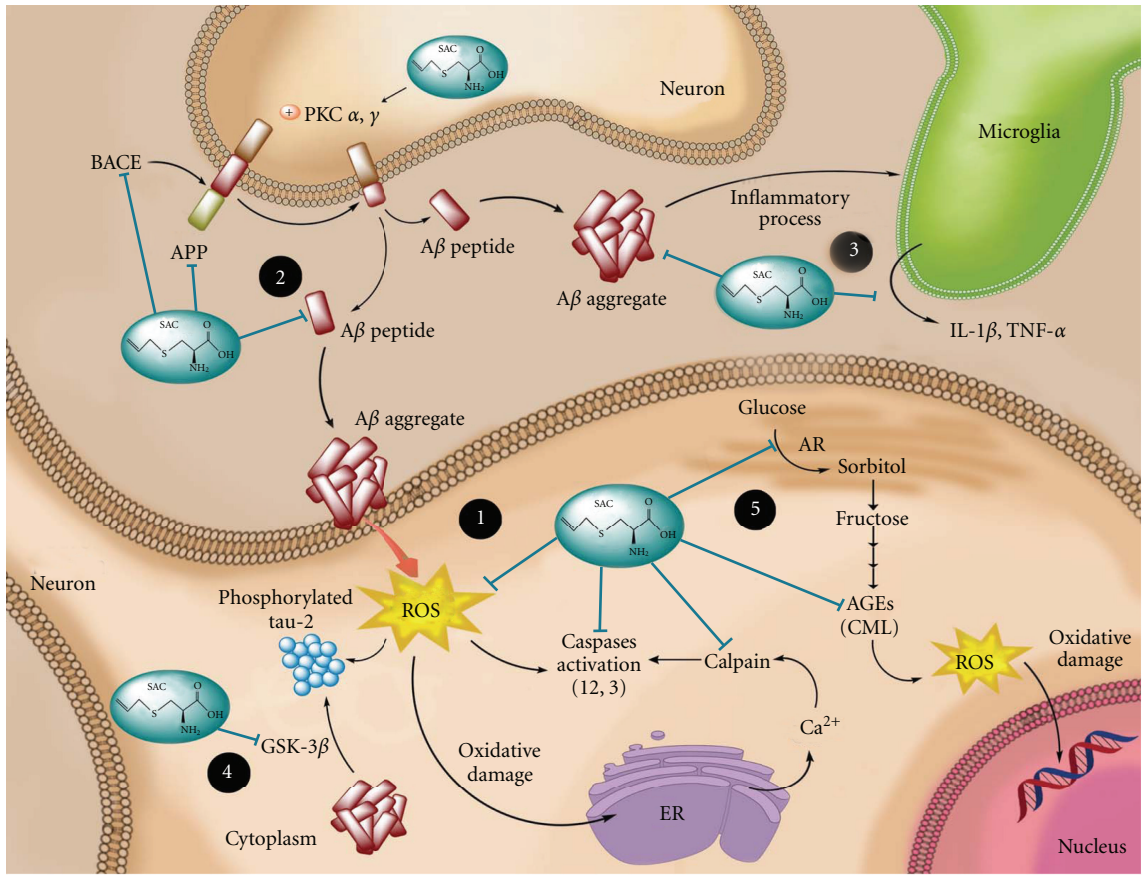

Figure 6: S-allylcysteine (SAC) prevents the progression of Alzheimer's disease (AD) by multiple mechanisms: (1) *antioxidant*, SAC scavenges free radicals and oxidant specie (direct antioxidant) and restores glutathione peroxidase, glutathione reductase, and superoxide dismutase levels (indirect antioxidant). Consequently, SAC diminishes lipid peroxidation, DNA fragmentation, protein oxidation, and endoplasmic reticulum (ER) stress. The decrease in endoplasmic reticulum stress attenuates Ca^{2+} release and the subsequent activation of calpain and the caspase-12-dependent pathway, which altogether decrease the cell death; (2) *antiamyloidogenic*, SAC decreases Aβ formation and/or increases Aβ clearance. SAC lowers amyloid precursor protein (APP) mRNA expression, BACE (β-site APP cleavage enzyme 1) expression and activity and restores PKC activity under AD-like condition, which benefits APP cleavage and decreases the available APP for Aβ. In addition, SAC can bind to Aβ-inhibiting Aβ fibrillation and destabilizing preformed Aβ-peptide fibrils; (3) *anti-inflammatory*, SAC decreases IL-1β and TNF-α levels and IL-1β-positive plaque-associated microglia; (4) *antitangle*, SAC reduces tau2 reactivity and its phosphorylation; this reduction in tau appears to involve GSK-3β protein; (5) *anti-glycative*; SAC declines both activity and mRNA expression of aldose reductase (AR), which subsequently decreases the production of sorbitol and prevents advanced glycation end products (AGEs) formation, such as carboxymethyllysine (CML) and pentosidine, decreasing glycative stress.

pars compacta, with the concomitant formation of intraneuronal fibrillar inclusions (Lewy bodies) and depletion of noradrenaline and serotonin in other brain stem nuclei. In addition, it shows oxidative tissue damage and bioenergetic deficits [115].

1-Methyl-4-phenylpyridinium (MPP^+) is the stable metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyri dine, and it causes nigrostriatal dopaminergic neurotoxicity, which in turn has been the most widely used model of Parkinson's disease. Rojas et al. reported the neuroprotective effects of SAC against the oxidative stress induced by MPP^+ in the mouse striatum. They found that SAC protected dopamine levels, improved hypolocomotion, decreased ROS production and lipid peroxidation, and increased Cu,Zn superoxide dismutase activity in mice injected with MPP^+ [116]. Another Parkinsonian model is produced by 6-hydroxydopamine (6-OHDA). In this model, SAC blocked the oxidative damage, prevented dopamine depletion, and returned behavioral performance to basal levels in rats [117]. The protective actions of SAC observed in these models were mainly attributable to its antioxidant activity.

7. AGE in Humans

Altogether, the data presented in this paper clearly shows that AGE and SAC exert antioxidant activity in a variety of experimental models. Of course, these data could be relevant for humans. Indeed, some groups have reported protective effects after chronic administration of AGE to patients with cardiovascular disease and the mechanisms of protection have been associated with the reduction of multiple cardiovascular risk factors, including blood pressure,

cholesterol, platelet aggregation and adhesion, and vascular calcification (reviewed in [118, 119]). However, only a few works have been performed in humans bringing attention to the antioxidant effects of AGE, and these are reviewed here.

7.1. Effect of AGE in Atherosclerotic Patients. Cardiovascular mortality is frequently associated with atherosclerosis, a chronic multifactorial disease and a leading cause of death worldwide. Lipid peroxidation and LDLs oxidation, produced by free radicals and end-aldehyde products of lipid hydroperoxide breakdown, play an important role in atherosclerosis. Munday et al. reported that LDLs isolated from subjects receiving 2.4 g of AGE daily for 7 days are more resistant to oxidation than those LDL isolated from subjects receiving no supplementation, suggesting that AGE could be useful in preventing the oxidative stress induced in atherosclerotic disease [120]. Later on, Durak et al. demonstrated that oxidative stress is present in atherosclerotic patients and that the consumption of AGE (1 mL/kg daily; 10 g garlic/day) for 6 months decreased plasma and erythrocyte malondialdehyde levels, with no changes in superoxide dismutase and glutathione peroxidase activities [121].

Currently, there is not enough evidence to determine whether antioxidants can reduce atherogenesis or not; however, it is conceivable that combined antioxidant (AGE) and serum cholesterol-lowering therapies may be useful in reducing the progression of atherosclerosis in humans.

7.2. Effect of AGE in Sickle Cell Anemia Patients. Sickle cell disease is one of the most prevalent hereditary disorders with prominent morbidity and mortality. Clinical manifestations are associated to hemolytic processes leading to severe anemia. It has been reported that oxidative stress plays a significant role in the pathophysiology of sickle cell disease, since it contributes to the sickling process with formation of Heinz bodies that are aggregates of insoluble hemochromes [122].

Takasu et al. reported that AGE (5 mL/day for 4 weeks) diminished the number of Heinz bodies in sickle cell anemia patients and suggested that AGE may be evaluated as a potential therapeutic agent to ameliorate complications of sickle cell anemia [123].

7.3. Effect of AGE in Smoking Individuals. F_2-isoprostanes are a family of prostaglandin F_2 isomers that are produced *in vivo* by cyclooxygenase-independent free radical peroxidation of arachidonic acid and are released to plasma in response to cellular activation. Hence, quantification of F_2-isoprotanes in plasma and urine is a sensitive and specific indicator of lipid peroxidation *in vivo*. Smokers are exposed to increased oxidative stress and show increased levels of F_2-isoprotanes in plasma and urine. Dillon et al. found that dietary supplementation with AGE (5 mL in a small volume of fruit juice) for 14 days reduced plasma and urinary levels of F_2-isoprostane 8-iso-prostaglandin $F_{2\alpha}$ in smokers, suggesting that dietary consumption of AGE may be useful in reducing oxidative stress in humans [17].

8. Final Remarks

In summary, the evidence presented in this paper supports the many beneficial health effects attributable to AGE and SAC, as they are able to reduce the risk of stroke and neurodegenerative damage. Moreover, given that SAC is a potent antioxidant agent, a water soluble compound less toxic than other antioxidants, easily absorbed in the gastrointestinal tract, and rapidly detected in several tissues (kidney, liver, lung, brain), it has been suggested that this compound might exhibit prophylactic properties at a clinical level. However, further investigations are still needed to elucidate the precise protective mechanisms exerted by this antioxidant in several toxic paradigms. Finally, further studies in humans are also necessary to support the clinical use of SAC as a drug to prevent the oxidative damage observed in chronic degenerative disorders.

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Research Article

Accumulation of Exogenous Amyloid-*Beta* Peptide in Hippocampal Mitochondria Causes Their Dysfunction: A Protective Role for Melatonin

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Amyloid-*beta* (Aβ) pathology is related to mitochondrial dysfunction accompanied by energy reduction and an elevated production of reactive oxygen species (ROS). Monomers and oligomers of Aβ have been found inside mitochondria where they accumulate in a time-dependent manner as demonstrated in transgenic mice and in Alzheimer's disease (AD) brain. We hypothesize that the internalization of extracellular Aβ aggregates is the major cause of mitochondrial damage and here we report that following the injection of fibrillar Aβ into the hippocampus, there is severe axonal damage which is accompanied by the entrance of Aβ into the cell. Thereafter, Aβ appears in mitochondria where it is linked to alterations in the ionic gradient across the inner mitochondrial membrane. This effect is accompanied by disruption of subcellular structure, oxidative stress, and a significant reduction in both the respiratory control ratio and in the hydrolytic activity of ATPase. Orally administrated melatonin reduced oxidative stress, improved the mitochondrial respiratory control ratio, and ameliorated the energy imbalance.

1. Introduction

The intracellular accumulation of highly amyloidogenic 1–42 residue amyloid-*beta* peptide (Aβ_{1-42}) may result from, (a) decreased Aβ degradation due to disruption of the ubiquitin-proteasome system, (b) increased intracellular generation of Aβ, or (c) increased uptake of Aβ from an external source [1, 2]. The internalization of Aβ_{1-42} peptide by primary neurons is related to a lipid raft-mediated endocytosis [2]. These lipid rafts are dynamic assemblies of proteins and lipids floating freely within the liquid-disordered bilayer of cellular membranes where cholesterol and sphingomyelin play key roles [3]. Reciprocally, cholesterol and sphingomyelin metabolism are strongly related to Aβ [4]. In fact, there are clues indicating that Aβ levels change in response to blood cholesterol content, while the clinical progression of Alzheimer's disease (AD) is commonly associated to hypercholesterolemia and high cholesterol levels in the brain [5, 6].

Another factor gaining more relevance as a mechanism of neuronal damage is oxidative stress, which is a hallmark

feature of Aβ-induced brain damage in AD [7], AD transgenic mice [8], as well as in *in vitro* and other *in vivo* models of this neurodegenerative condition [9, 10]. Mitochondrial dysfunction and associated oxidant stress have been linked to numerous complex diseases and aging [11, 12], such association has been largely established by *in vitro* determination of mitochondrial free radicals overproduction. Membrane-associated oxidative stress in turn has been linked to lipid alterations [13], and the exposure of hippocampal neurons to Aβ, *in vitro,* induces oxidative damage to membranes, accompanied by accumulation of sphingomyelin-derived ceramide species and cholesterol [14]. In fact, by preventing the accumulation of ceramides and cholesterol it is possible to protect neurons from death induced by Aβ; this is achieved with an antioxidant like α-tocopherol or by depleting hippocampal neurons of its sphingomyelin content with an inhibitor of serine palmitoyltransferase, the rate-limiting step in sphingolipid synthesis [15]. Focal demyelination of the cortical grey matter as well as of dystrophic neurites has been observed in AD patients and transgenic mice associated to Aβ plaque core [16]. Thus, it is possible that the breakdown of myelin promotes the buildup of toxic Aβ fibrils, which eventually accumulate in the brain [17].

There is evidence, on the other hand, that the amyloid precursor protein (APP) and Aβ accumulate in mitochondrial membranes, as observed in postmortem AD or in transgenic mice brain sections [18–20]. Aβ extracellularly applied to human neuroblastoma SH-SY5Y cells was found to be internalized and be taken up by mitochondria by using the transporter outer membrane (TOM) [21]. Most of the imported A$\beta_{1–42}$ appeared associated to the inner membrane and only a small fraction was localized to the matrix.

We hypothesized that due to its amphipathic nature [22], its physicochemical composition [23], and being aided by oxidative stress [10, 24], Aβ paves its own pathway from extracellular space to mitochondria where it disrupts membrane fluidity and causes energetic dysfunction. This mechanism of membrane permeabilization induced by Aβ and its own internalization might be the major cause of mitochondrial dysfunction.

Since oxidative stress is considered a key factor in these pathogenic mechanisms, melatonin should reduce the dysfunctional manifestations of Aβ uptake [25]. Melatonin is a proven antioxidant [26, 27], especially in the brain where it reduces molecular damage as demonstrated in animal models of AD [28, 29], as well as in other experimental models of neurodegeneration [30–33]. There are two important clues regarding the role of melatonin in mitochondria rescue, (1) melatonin penetrates mitochondria where it scavenges free radicals [34, 35], (2) melatonin directly inhibits mitochondrial permeability transition pore (MtPTP) [36].

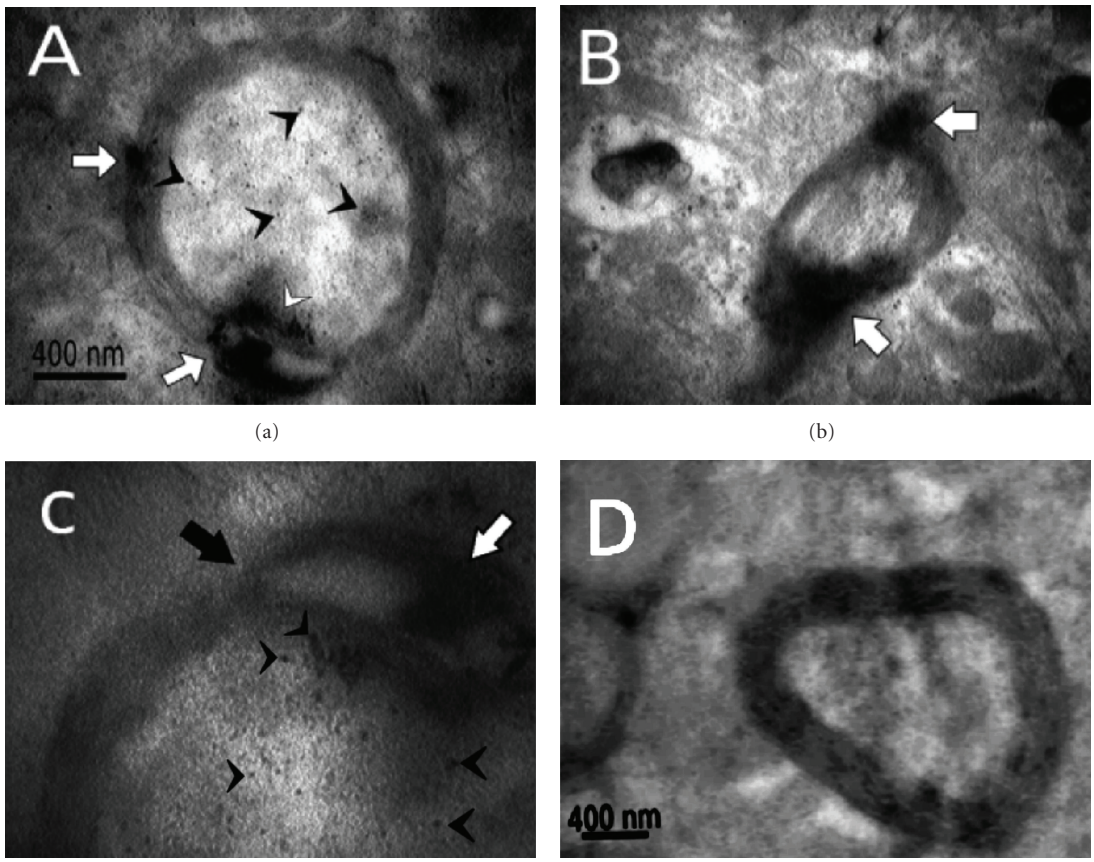
To further document these potential protective effects of melatonin, we worked *in vivo* by injecting fibrillar A$\beta_{1–42}$ directly into hippocampal CA1 pyramidal neurons layer. Aged Wistar rats used in these experiments had no other condition or genetic predisposition to form plaques or other AD features.

2. Results

2.1. Axonal Damage in fAβ Injected Rats. The most striking abnormal change was the intramural accumulation, adhesion, and formation of Aβ aggregates in the myelinated axons. Thus, Aβ invaded the concentric multilamellar myelin sheath of the axon (oligodendrocyte cytoplasm). These Aβ aggregates caused dissection and disruption of myelin layers with mural vacuolization, forming onion bulb-like protuberances as found in chronic neuropathies [37]. In some cases, all layers were disrupted and positive Aβ-immunoreactivity extending from the interstitium to the axonal lumen was observed (Figure 1). Within the onion bulb-like protuberances, Aβ appeared to form aggregates; once inside the axons and mitochondria, the Aβ aggregates were disintegrated, adopting a granular appearance.

2.2. Cholesterol-Enriched Diet in Aβ-Injected Rats Is Related to a Significant Increase in Mitochondrial Membrane Damage. Animals fed with regular Laboratory Rodent Diet showed a normal cholesterol level in blood. When these animals were intracerebrally injected with Aβ showed not only significantly less degree of mitochondrial structural damage as compared to Aβ-injected but also hypercholesterolemic animals due to a cholesterol-enriched diet, as observed by electron microscopy (Figure 2).

2.3. Intracerebrally Injected f A$\beta_{1–42}$ Causes Extracellular Aggregates, Axonal Degeneration, and Accumulates in Mitochondria. Thirty-six hours following the intracerebral injection of f A$\beta_{1–42}$, brain tissue was obtained and subjected to conventional and transmission electron microscopic examination. Using a polyclonal antibody against Aβ for immunohistochemistry, extracellular deposits of this peptide accompanied by an intense microglial response (data not shown) were revealed, as expected (Figure 3(a)). 70–90 nm ultrathin brain sections were incubated with the same anti-Aβ primary antibody, followed by incubation with a gold-labeled secondary antibody. The observation by electron microscopy revealed Aβ immunoreactivity inside mitochondria (Figure 3(b)), accompanied by important swelling, rupture of the outer membrane and cristae dissolution (Figures 3(b) and 3(c)). Aβ was found localized to the cristae of the inner membrane of mitochondria. Both in H_2O_2- and in fAβ-injected brains significant ultrastructural alterations were observed. H_2O_2 was chosen as a positive control of oxidative stress, because of its well-known pathogenic relationship with Aβ (reviewed in [38]). The most prominent change was the peripheral vacuolization of the cristae, as shown in Figure 3. Vacuolization in mitochondria was accompanied by electrodense Aβ-immunoreactivity with inclusions being grouped and bound to the membranes, particularly to the inner cristae membranes. Rupture of the membranes resulted in discontinuity and formation of gaps. Mitochondria looked swollen with disorganized membrane structures and the cristae were lost. In some localized areas, the intermembranous space was absent and the mitochondrion appeared like an irregularly enlarged, single-membrane sac (Figure 3(c)).



(a) (b)

(c) (d)

Figure 1: *Aβ*-induced axonal damage. Following the intracerebral injection of fA*β* into the hippocampus, small blocks of tissue brain containing the lesion area were postfixed in osmium tetroxide 2%, embedded for 48 hours in Embed 812, cut into 70–90 nm ultrathin sections, and then subjected to immunohistochemical procedures using an anti-A*β* antibody. Binding sites of the primary antibody were then revealed by incubating with a gold-labeled secondary antibody. Finally, sections were counterstained with uranyl acetate and lead citrate and observed in an electron microscope. (a) Amyloid tangles within the concentric layers of myelin sheath of axons (white arrows) and a widening of the myelin sheath, corresponding to accumulations of A*β* from a primary pole, are observed. Electrodense spots sparse within the axon (black arrowheads), also seem to enter into the lumen from the primary pole (white arrowhead) (16,700x). (b) A*β* electrodense spots penetrating into the axons from widening onion bulb-like structures (white arrows) (10,000x). (c) Onion bulb detail (35,750x) showing abundant electrodense material corresponding to gold revealed A*β* (white arrow) and the detachment zone (black arrow). Amyloid tangles seem to be granulated once crossing the myelin sheath (black arrowheads). (d) Myelin sheath as observed in PBS-injected rat brain (magnification at 2156x).

2.4. The Incorporation of Intracerebrally Injected Aβ into Mitochondria Is Related to Mitochondrial Free Radical Overproduction. Brain sections were obtained from each group to determine mitochondrial mass density by using Mitotracker Green FM, a selective fluorescent dye whose mitochondria localization is independent of the membrane potential. Mitochondria from PBS-injected brains showed uniform box-like shapes regularly distributed and ranging in size from 318 to 1832 nm (mean 964 ± 365 nm) with an average integrated optical density (IOD) of 41.7 ± 3.5 (Figures 4(a) and 4(b)). In A*β*-injected brains, mitochondria was found to form perinuclear clusters with a size ranging from 529 to 7400 nm (mean 2812 ± 1742 nm, IOD = 54.5 ± 4.6) (Figures 4(c) and 4(d)). Similar clusters were observed in H_2O_2-injected rats ($P < 0.05$), and they reached a size up to 23 μm (Figures 4(e) and 4(f)).

In order to correlate the presence of A*β* within mitochondria and overproduction of free radicals by mitochondria, 36 hours following the application of fAβ_{1-42} and 2 hours before obtaining the tissue sample, CM-H2XRos, a chloromethyl derivative of dihydro-X-rosamine, was intraperitoneally injected; CM-H2XRos is a potential-dependent probe which evaluates the direct production of mitochondrial reactive oxygen species (ROS) in cells. Red CM-H2XRos dye has the ability of diffusing into living cells, labeling only actively respiring mitochondria [39] and it is well retained after fixation. Once located within cells, the reduced CM-H2XRos is oxidized by ROS to a fluorescent mitochondrion-sensitive probe and sequestered in this organelle; thus, its oxidation is useful to detect both ROS (predominantly superoxide anion, $O_2^{\bullet -}$) and possibly reactive nitrogen species (nitric oxide, $^{\bullet}NO$, and peroxynitrite, $ONOO^-$). By comparing the IOD

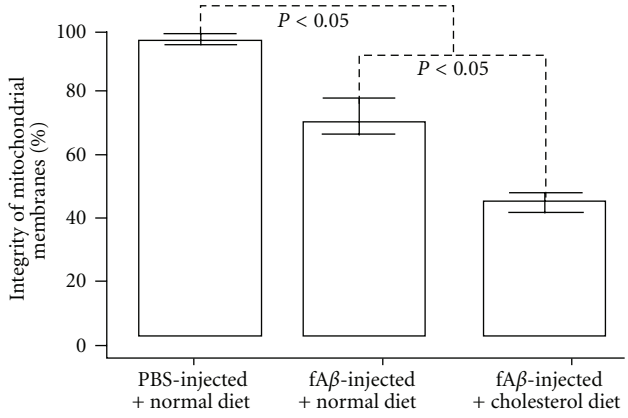


Figure 2: A cholesterol-enriched diet was significantly related to a more important loss of the mitochondrial integrity in animals. The graph shows differences among PBS-injected brains from animals receiving a regular laboratory rodent diet and two other groups of amyloid-injected brains. These latter were from animals receiving a normal diet and from animals receiving a cholesterol-enriched diet. Amyloid-injected brains showed significant loss of mitochondrial membrane integrity as compared to PBS-injected brains. However, those animals additionally fed with a cholesterol-enriched diet were significantly more affected.

of randomly selected regions of equal size within a single optical section, multiplied by the number of CM-H2XRos-stained mitochondria, the overproduction of ROS for each group was estimated. Thus, fAβ-injected brains showed a significant ROS overproduction, as expected (graph in Figure 4).

2.5. Melatonin Significantly Reduces ROS Overproduction. Orally administered (20 mg/kg/day added to the drinking water), melatonin reached 145 ± 8 pg/mL in serum between 01:00 and 02:00 h, which was 84% higher than controls (data not shown). At this dose, melatonin reduced significantly mitochondrial ROS overproduction, as graphically represented in Figure 5. Thus, IOD levels by mitochondrial CM-H2XRos in fA$\beta_{1–42}$-injected brains diminished 35% in rats taking melatonin in the drinking water, whereas in H_2O_2 intracerebrally injected rats, melatonin treatment reduced an average of ROS levels by 69% as compared with H_2O_2 intracerebrally injected rats without melatonin treatment (Figure 5).

Finally, by obtaining the quotient between ROS, estimated by CM-H2XRos, and mitochondrial mass, estimated by Mitotracker green caption, the apparently greater ability of fA$\beta_{1–42}$ to cause oxidative damage with respect to H_2O_2 (graph in Figure 4), was reduced to its basal value (Figure 5). The ability of melatonin to reduce free radicals was significant in both experimental groups.

2.6. Amyloid-Beta Depresses Both the Respiratory Control Ratio and the ATPase Activity. 36 hours following the intracerebral injection of fA$\beta_{1–42}$, we found Aβ positive immunoreactivity in mitochondria, which was closely associated to mitochondrial damage and ROS overproduction, as described above. Mitochondria were then isolated in order to examine a possible correlation between the mentioned findings with functional indicators, such as the mitochondrial respiratory control ratio (RCR) and the hydrolysis activity of F1Fo-ATPase. The former measures mitochondria ability to idle at a low rate yet respond to ADP by making ATP at a high rate [40] in such a manner that is feasible to infer the leaking of electron transfer without concomitant phosphorylation, or how much ATP-synthase is partially uncoupled from respiration; the latter, as a measure of the capacity to maintain an inner-membrane potential by coupling the energy of the electrochemical proton gradient with ATP synthesis [41]. A significant difference was obtained comparing PBS-injected (3.8 ± 0.03) and fAβ groups (2.6 ± 0.05) ($P < 0.05$). This difference was significantly reduced when fAβ-injected animals ingested melatonin in the drinking water (fA$\beta \neq$ fAβ + Mel, $P < 0.05$) (Figure 6).

H_2O_2-injected brains, used as positive controls, showed a more significant reduction in RCR (2.18 ± 0.06), and also responded to melatonin treatment (Figures 8 and 9).

Interestingly, both fAβ and H_2O_2 reduced ATPase hydrolytic activity but, contrary to the RCR response, melatonin did not ameliorate this parameter (fAβ = fAβ + Mel) (Figure 7).

In general, the more extensive oxidative damage was related to the lower the respiratory control ratio and the lower the ATPase hydrolytic activity (Figure 8).

2.7. There Is a Direct Correlation between Oxidative Stress-Induced Low Membrane Fluidity and a Low RCR. After establishing a link between the presence of Aβ in mitochondrion and functional disturbances in this organelle, we searched for changes in membrane fluidity. This parameter correlates with the degree of altered membrane lipid composition and it is a well-known pathogenic factor directly affecting the energetic coupling of Ca^{2+} pumping, with the consequent energetic failure. We found indeed, that following the intracerebral injection of fAβ and its appearance inside mitochondria, a significant reduction in membrane fluidity was evident as compared to control Ie/Im fluorescence values (fAβ 2.6 ± 0.05 versus PBS-injected controls 3.5 ± 0.03, $P < 0.001$). Brains of animals receiving melatonin treatment showed a significant recovery (fAβ 2.6 ± 0.05 versus fAβ + Mel 2.95 ± 0.05, $P < 0.001$) (Figure 9).

3. Discussion

Considerable experimental data favor the hypothesis that amyloid deposition in the brain is one of the etiological factors contributing to AD dementia [43] and the hypothesis of the "intracellular cascade of Aβ" has gained preponderance [18]. It has been demonstrated that A$\beta_{1–42}$ uncouples the mitochondrial respiratory chain and this plays a key role in Alzheimer's pathology [44]. Structurally, Aβ induces swelling of isolated mitochondria [45, 46] and functionally decreases ATP synthesis and the activity of various mitochondrial enzymes, as demonstrated *in vivo* [47] and *in vitro* in cultured neuronal cells or in astrocytes

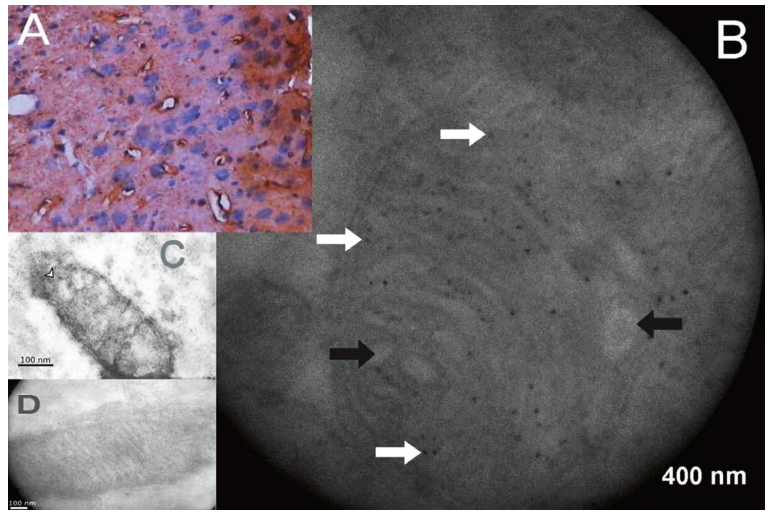


Figure 3: *Aβ* in mitochondria, particularly on cristae of the inner membrane accompanied by severe disruption of the organelle. (a) Deposits of A*β* aggregates into the hippocampal adjacent cortex 36 hours after injection, as shown by DAB-revealed horseradish peroxidase immunohistochemistry (40x). Congophilic amyloid deposits remained visible up to 21 days following the intracerebral injection (data not shown). (b) A*β* immunoreactivity (white arrows) within mitochondria 36 hours following the injection of fA$\beta_{1–42}$ into hippocampus CA1 pyramidal neurons at 35000x magnification. The same anti-A*β* polyclonal antibody used in the regular immunohistochemistry was used in this procedure but revealed with a 6 nm gold-labeled second antibody. Swollen mitochondria with broken cristae and its remnants intermixed with fine electron-dense dusty granule is observed. Additionally, the membrane integrity is lost and some vacuoles became evident (black arrows). (c) Highly swollen mitochondria without cristae, like enlarged sacs, containing fine electrodense granules, rupture of external mitochondrial membranes (white arrow head), and vacuolization. In this case the anti-A*β* antibody was revealed with an irrelevant secondary antibody, used as control. (d) Intact mitochondrial ultrastructure of a PBS-injected control rat (37000x).

exposed to the peptide [45, 47, 48]. Thus, A*β*-induced mitochondrial damage may be an extension of the amyloid cascade hypothesis, which 20 years ago suggested that the altered metabolism of APP was the initiating event in AD pathogenesis, subsequently leading to the aggregation of A*β*, specifically A$\beta_{1–42}$ [43, 49]. Later, different neurotoxic mechanisms for A*β* were proposed, including disruption of mitochondrial function via binding of the A*β*-binding alcohol dehydrogenase (ABAD) protein [19], or formation of ion channels allowing calcium uptake which induces neuritic abnormalities in a dose- and time-dependent fashion [50], or the opening of the mitochondrial permeability transition pore coupled to inhibition of respiratory complexes [51, 52].

A common underlying factor is the overactivation of microglia with the consequent overexpression of proinflammatory cytokines and a significant increase in ROS, which always prevails [53–56]. ROS, in turn, may come from an innate immune response promoted by damaging signals [55, 57] or they may come from the damaged mitochondria [58]. This latter implies a pathological vicious cycle, where mitochondrial dysfunction and ROS leakage from the respiratory chain feed each other. However, there is a pertinent question, is the mitochondrial impairment a consequence of intraneuronal deposits of endogenous oligomeric species of A*β*? Or are the extracellular deposits of fA*β* capable of reaching mitochondria, causing their deterioration before the appearance of plaques?

APP has been localized to the trans-Golgi network, endoplasmic reticulum (ER), and endosomal, lysosomal, and mitochondrial membranes. Thus, the liberation of A*β* and formation of intracellular accumulations could potentially occur wherever APP and the *β*- and *γ*-secretases are localized, which is particularly true when APP is overexpressed (reviewed in [59]); this was not the case for the animals used in the current experimentation. The data presented here, obtained from aged Wistar rat brains, demonstrate that mitochondria undergo major structural and physiological changes following the intracerebral injection of fA$\beta_{1–42}$, which in turn led to the formation of extracellular deposits which eventually appeared in mitochondria.

Mitochondrial genes seem to be activated before plaque formation, in addition to an increase in H_2O_2 content, accompanied by a decrease in cytochrome oxidase activity, as demonstrated in young Tg2576 mice prior to the appearance of A*β* plaques [20]. It is not clear whether a soluble, intramitochondrially produced A*β* could cause impairment of the electron transport chain since, according to the theoretical disposition of APP within the mitochondrial membrane [60], A*β* would have to be imported from outside the mitochondria, from the cytosol. This phenomenon has been revealed and involves the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM), as shown in human cortical brain tissue specimens by Hansson Petersen et al. [21].

However, A*β* has the ability by itself to permeabilize membranes. Without discarding that A*β* from the cytosol may invade mitochondria, it is worth to consider the huge amounts of extracellular A*β*, forming plaques outside the neurons, even visible to conventional light microscopy. A*β* peptides are amphipathic molecules,

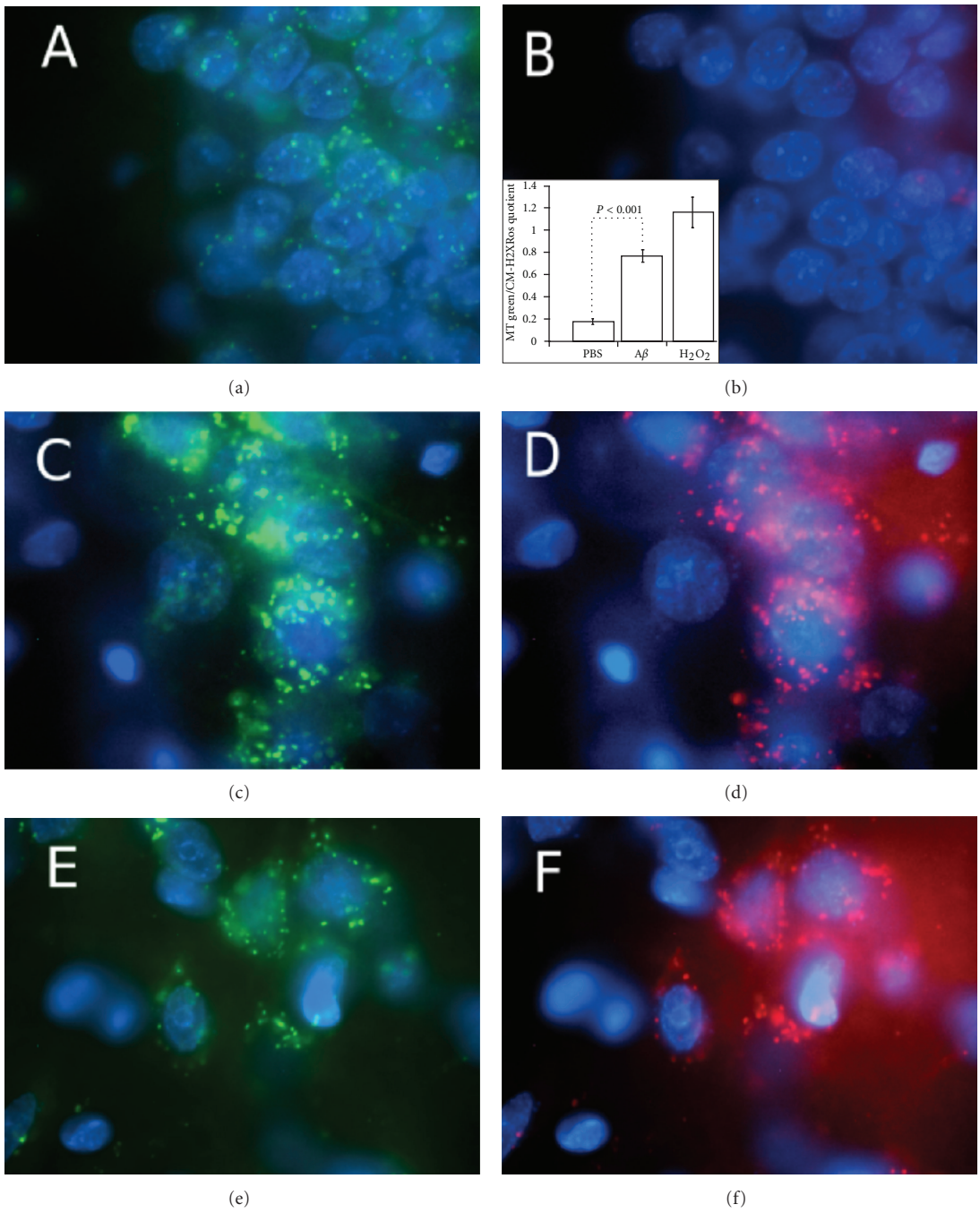


Figure 4: The presence of Aβ within mitochondria is related to ROS overproduction. Control brain exhibited a regular distribution of mitochondria (a) with a size of 964 ± 365 nm, according to Mitotracker Green staining, which is essentially nonfluorescent in aqueous solutions but becomes fluorescent as it accumulates in the mitochondrial membranes. Nuclei appear in blue, stained with DAPI. The amount of mitochondrial ROS in control PBS-injected brain was negligible, as inferred from CM-H2XRos staining, which specifically accumulates inside mitochondria because of the positive charge it acquires upon oxidation by intracellular ROS (b). CM-H2XRos was intraperitoneally injected *in vivo* 2 hours before obtaining the tissue sample. fAβ-injected brains showed a different distribution of mitochondria, predominantly perinuclear, ranging in size from 529 to 7400 nm and forming clusters (c), (d). Integrated optical density (IOD) of CM-H2XRos-stained mitochondria was significantly ($P < 0.001$) elevated in fAβ- and H_2O_2-injected brains (e), as compared with controls (d), (f). In order to estimate how many mitochondria from the mitochondrial mass were overproducing ROS, we divide the IOD of MT-Green by IOD of CM-H2XRos for each group, and we found a significant difference between fAβ-injected brains and controls as shown in the attached graphic ($P < 0.001$).

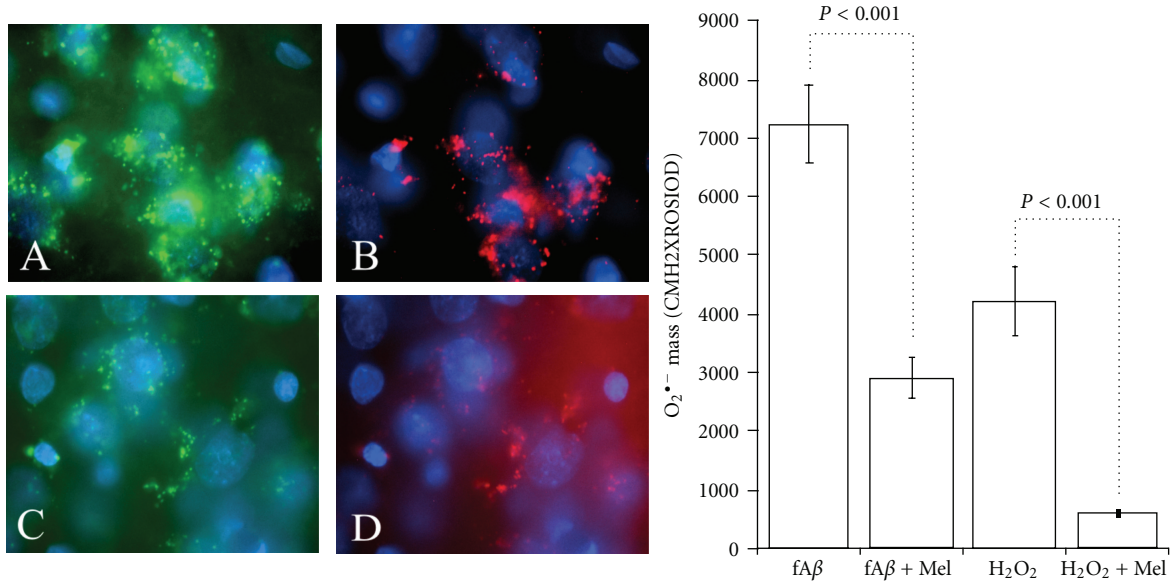


Figure 5: Melatonin reduces significantly mitochondrial ROS overproduction. As expected, melatonin reduced the fluorescence derived from CM-H2XRos oxidation in both the fAβ- and the H_2O_2-injected brains. According to Figure 4 most of the mitochondria in brains injected with Aβ or H_2O_2 were producing free radicals. Now, by comparing the intensity of free radicals produced in brain of animals receiving melatonin treatment against those animals without melatonin, a very significant decrease in the amount of produced free radicals was observed, as shown in the graph.

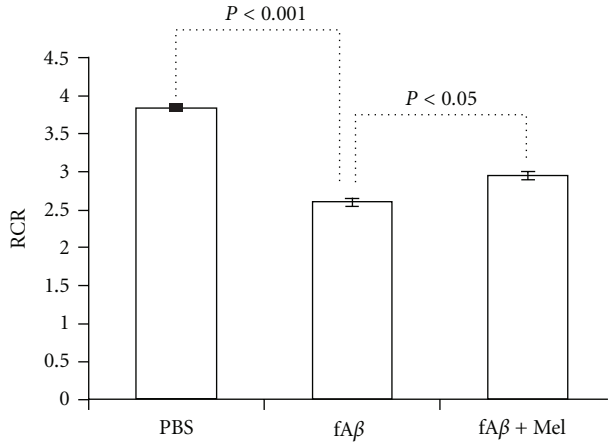


Figure 6: Aβ-induced mitochondrial dysfunction. In order to correlate both the presence of Aβ within mitochondria and the overproduction of ROS with functional alterations in mitochondria, oxygen consumption in isolated mitochondria (1 mg/mL) was measured using a Clark-type oxygen electrode at 34°C in treatment buffer, adding succinate as substrate. The respiratory control ratio [state 3 rate] : [state 4 rate], as an indicator of the appropriate coupling between respiration and phosphorylation, revealed a significant decrease in fAβ- and H_2O_2-injected brains, as compared to controls ($P < 0.001$). However, a significant improvement in the RCR was observed in animals treated with melatonin ($P < 0.05$).

containing a hydrophilic N-terminal stretch (residues 1–28) and a hydrophobic C-terminal domain (residues 29–40/42) partially spanning the APP transmembrane domain. In solution, Aβ peptides display a substantially unfolded conformation with reduced content of secondary structure; however, the latter increases considerably in phospholipid vesicles particularly when enriched in cholesterol and gangliosides [61, 62]. The ability of Aβ to insert into membranes depends more on the cholesterol: phospholipid ratio. By using the same experimental design as in this report, we have found important evidence on Aβ-induced alterations in the lipid content of mitochondrial membranes, in part related possibly to a direct Aβ molecular interaction and in part related to Aβ-induced oxidative stress (unpublished data). Oxidative stress, on the other hand, may induce membrane permeabilization by itself, as revealed by neutron reflectometry in lipid bilayers [63], whereas aging is related to changes in the cholesterol, sphingomyelin, and phospholipid content in membranes [61, 64, 65]. Thus, according to our hypothesis, all these factors are concentrated to facilitate Aβ entry into the cell and eventually to the mitochondria.

In fact, a preferential adsorption, internalization, and resistance to degradation of the major isoform of the Aβ peptide, $A\beta_{1–42}$, has been shown in differentiated PC12 cells. The amount of peptide internalized increases proportionally with the concentration of peptide in the medium and the amount of internalized $A\beta_{1–42}$ is approximately 5-fold higher than the amount of $A\beta_{1–40}$ [66].

Aβ, extracellularly applied to human neuroblastoma SH-SY5Y cells, was demonstrated to be internalized and taken up by mitochondria [21]. In other experiments *in vivo*, fluorescence in isothiocyanate- (FITC-) labeled $A\beta_{1–42}$ introduced via tail vein injection into mice with a blood brain barrier (BBB) rendered permeable by treatment with pertussis toxin, readily crossed the permeabilized BBB and 48 hours later $A\beta_{1–42}$-positive neurons were widespread

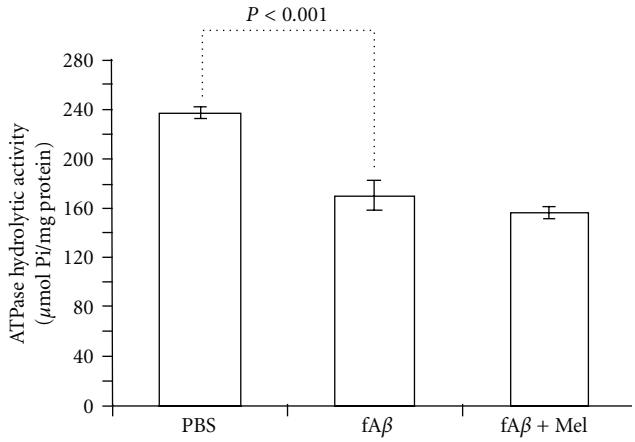


Figure 7: The hydrolytic activity of ATPase did not respond to melatonin. Enzymatic ATP hydrolysis was determined by a colorimetric reaction measuring the release of free orthophosphate (Pi), based on the formation of a phosphomolybdate complex in an acid medium followed by a reduction or complexation with basic dyes that yield colored complexes. When electron transport ceases, the inner-membrane potential is developed at the expense of ATP hydrolysis by the mitochondrial ATP synthase. However, in the presence of Aβ the hydrolytic activity of ATPase decreases significantly as compared to intact brains ($P < 0.001$) and did not show any recovery in melatonin-treated animals (fAβ = fAβ + Mel), which may imply a different mechanism of damage by Aβ. In fact, another report explores the possibility of a direct interaction of Aβ with the alpha subunit of the F1Fo-ATP synthase complex [42].

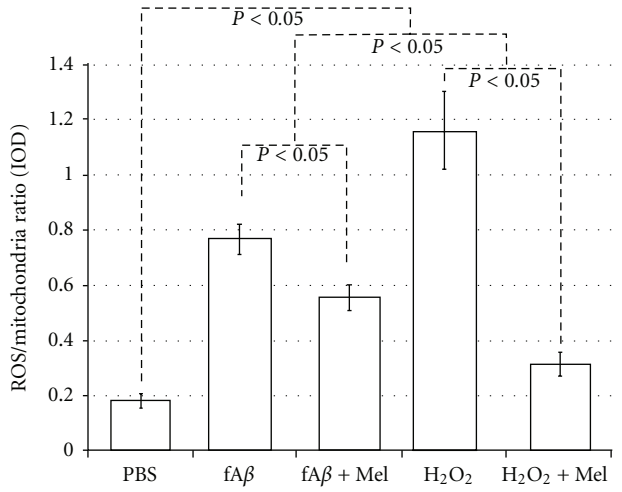


Figure 8: ROS/mitochondria ratio. The amount of mitochondria producing free radicals (ROS/mitochondria ratio) is larger in fAβ and H_2O_2-injected brains without melatonin treatment, but the effect is significantly reduced by using melatonin.

[67]. These experiments demonstrate the possibility that extracellular Aβ, even exogenous A$\beta_{1–42}$, may be the source of the intraneuronal Aβ. Once inside the neurons, Aβ is responsible for mitochondrial damages, as we demonstrated. We have shown in healthy rat brain that following the intracerebral injection of fA$\beta_{1–42}$, this peptide accumulates in plaques outside of the cells. There was not another preexisting pathological condition or predisposition, and experiments have been carried out under *in vivo* conditions. The extracellular accumulation of fA$\beta_{1–42}$ coincided with axonal degeneration and positive Aβ immunoreactivity within mitochondria, accompanied by ultrastructural alterations consistent with mitochondrial dysfunction. All of this also coincide with Saavedra et al. [2] who found that extracellular Aβ contributes to the intracellular pool of Aβ and its internalization is not dependent of ApoE, but it is a lipid raft-mediated mechanism. Additionally, Aβ was shown to be more efficiently internalized by axons than by cell bodies.

We found, as mentioned before, axonal degenerative changes with elevated Aβ immunoreactivity (Figure 1). It is possible that this axonal degeneration could be the origin of demyelination and a pathway to permit the entry of Aβ into the neurons via retrograde transport. Previous reports have revealed increased quantities of Aβ and A$\beta_{1–42}$ in AD white matter accompanied by significant decreases in the amounts of myelin basic protein, myelin proteolipid protein, and 2′,3′-cyclic nucleotide 3′-phosphodiesterase. These observations suggest that extensive white matter axonal demyelination underlies Alzheimer's pathology, resulting in loss of capacitance and serious disturbances in nerve conduction, severely damaging brain function [68]. A new hypothesis suggests that myelin breakdown in the late-myelinating brain regions releases iron, which promotes the development of the toxic amyloid oligomers and plaques, which in turn destroy more myelin [69, 70] (Figure 1). It is worth mentioning that iron is strongly related to oxidative stress and oxidative stress is a key protagonist in neurodegenerative diseases [8, 10, 15, 24, 71, 72]. Additionally, we have also found a significant Aβ-induced rearrangement in membrane cholesterol and fatty acid composition both in cytoplasmic membranes and in mitochondrial membranes (data not shown), which culminate with a significant alteration in membrane fluidity (Figure 9).

It is well known that high levels of circulating cholesterol does not mean high cholesterol concentrations in brain tissue, essentially because cholesterol in the blood does not cross the blood brain barrier (BBB). However, hypercholesterolemia has certain association with accumulation of Aβ peptide in brain. The mechanism is not well understood, but the oxidized cholesterol metabolite 27-hydroxycholesterol likely plays a key role. Our results (Figures 2 and 9) coincide with the results from other reports [73–75].

Once the internalization of Aβ and its ability to infiltrate mitochondria were documented, we proceeded to evaluate the overproduction of mitochondrial free radicals induced by the presence of Aβ, by using CM-H2XRos, a rosamine derivative well retained after fixation, which was intraperitoneally injected *in vivo*. This approach allowed us to examine the free radicals overproduction without changing the natural architecture of brain tissue or the interrelationship between cells. Changes in the mitochondrial mass and Aβ-linked conformational alterations were explored by using Mitotracker green, applied *ex vivo* on tissue brain slices.

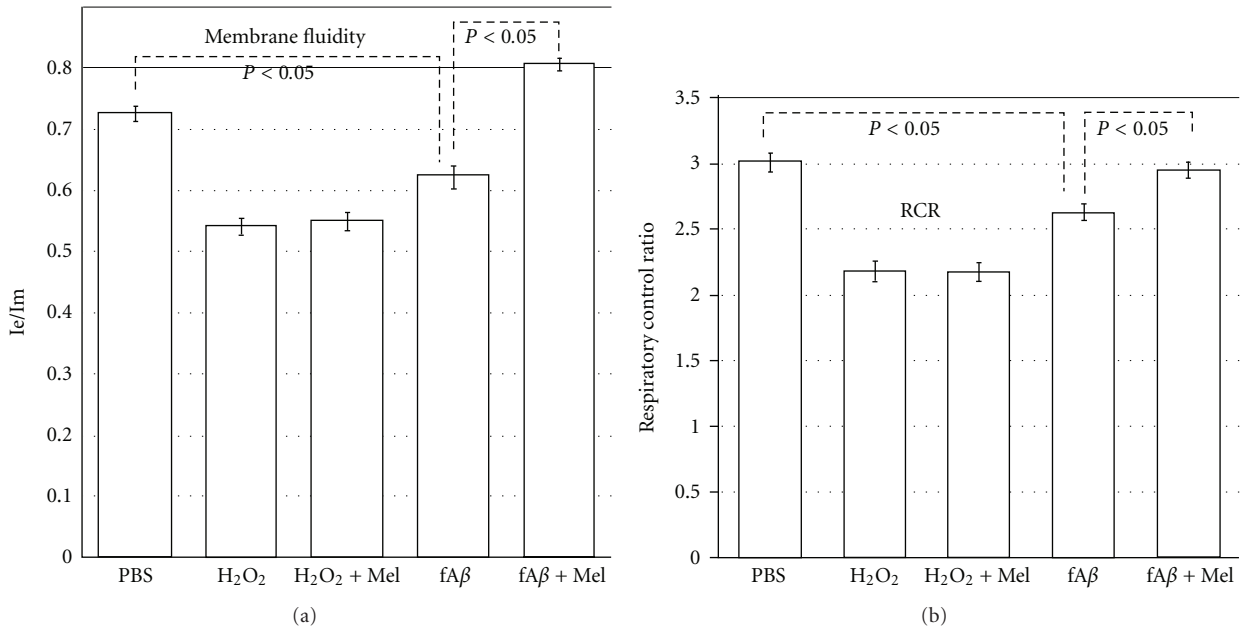


Figure 9: Aβ reduces mitochondrial membrane fluidity closely related to free radical overproduction and to the respiratory control ratio. Measured by estimating the excimer to monomer fluorescence intensity ratio (Ie/Im) of DPP, membrane fluidity in mitochondrial membranes was evaluated in order to show how Aβ, imported from the outside the cell membrane, is able to cause mitochondrial damage once incorporated within this organelle. We found that the groups producing more free radicals (fAβ and H_2O_2 groups), showed more altered membrane fluidity and RCR as compared to PBS-injected brains used as controls ($P < 0.001$). By using melatonin, both membrane fluidity and RCR were significantly ameliorated (fAβ + Mel and H_2O_2 + Mel groups).

Oxidative stress and Aβ toxicity are interdependent phenomena. In fact, the pathological effects of Aβ heavily depend on its capacity to provoke oxidative stress [42]. There are two major means by which Aβ induces oxidative stress, one is through the activation of the NADPH-oxidase probably both in neurons and in glia [76, 77], which links redox control and neuroinflammatory signaling pathways [68]. The other means by which Aβ induces oxidative stress is related to mitochondrial damage, a mechanism closely linked to apoptosis [78]. Reciprocally, oxidative stress induces intracellular accumulation of Aβ through enhancing the amyloidogenic pathway [79]. H_2O_2 is a well-known uncoupler of the mitochondrial respiratory activity, producing a concentration-dependent inhibition of state 3 (ADP-stimulated) respiration and reducing substantially the ADP : O ratio [80]. An evaluation of electron transport chain complexes and Krebs cycle enzymes revealed that *alpha*-ketoglutarate dehydrogenase, succinate dehydrogenase, and aconitase are susceptible to inactivation, which is a reversible process [81]. Melatonin directly detoxifies H_2O_2 with the resulting product being N1-acetyl-N2-formyl-5-methoxykynuramine which is also an efficient free radical scavenger [81, 82]. For this reason H_2O_2 was selected as positive control and the RCR as well as the hydrolytic activity of ATPase in H_2O_2-injected brains responded favorably to melatonin treatment, which implies that oxidative stress may explain those alterations. However, in fAβ-injected brains, melatonin had a favorable influence on the RCR, but it failed to modulate the ATPase hydrolytic activity. This finding explains the energy hypometabolism linked to Aβ, since ATPase hydrolytic activity is responsible for generating a proton gradient at the expense of ATP. Interestingly, Aβ is similar in structure to the ATP synthase-binding sequence of the inhibitor of F1 (IF1), a naturally occurring inhibitor of the ATPase activity of the F1Fo-ATP synthase complex in mitochondria. Thus, as with IF1, Aβ may inhibit ATPase activity presumably by interacting with the alpha subunit of the F1Fo-ATP synthase complex [83]. We speculate that this direct interaction could cause the failure of the antioxidant therapy to restore the ATPase hydrolytic activity in fAβ-injected brains. Even though melatonin has been demonstrated to improve mitochondrial function in both normal and pathological conditions, this effect is related to its antioxidant activity.

Melatonin did not seem to have a direct effect on the ATPase hydrolytic activity. However, this endogenous neurohormone incorporates into mitochondria and may decrease oxygen consumption concomitantly with its concentration, inhibiting any increase in oxygen flux in the presence of an excess of ADP, and reducing the membrane potential, changes that inhibit the production of $O_2^{\bullet -}$ and H_2O_2, as demonstrated in mouse liver cells [84]. Moreover, by using melatonin it is possible to maintain the efficiency of oxidative phosphorylation and ATP synthesis, while increasing the activity of the respiratory complexes (mainly complexes I, III, and IV) [84]. Melatonin also has proven to be effective in preventing mitochondrial nitric oxide synthase induction in parkinsonian mice [85]. However, melatonin also failed to restore the nitrosative stress-induced failure in the ATPase hydrolytic activity in septic mice [86]. These data suggest that

Aβ-induced mitochondrial alterations, as those described at early stages in AD before the appearance of amyloid plaques, are the result of internalization of Aβ. Later, continuous overproduction of amyloid peptide, which induces more oxidative stress [44] and neuroinflammation [55, 87], results in extracellular plaque formation. Thus, the effects of Aβ on mitochondria could be an extension of the amyloid cascade. The results presented here support the hypothesis of the internalization of Aβ as a major cause of mitochondrial dysfunction during AD.

Concluding Remarks. Extracellular deposits of Aβ may access mitochondria because of the Aβ ability to permeabilize cellular membranes and lipid bilayers. Thus, Aβ can pass through the membranes from outside in the extracellular space, where these deposits become so huge they form plaques, visible with conventional microscopy. The role of Aβ-induced membrane damage and the ability of Aβ to pass through different compartments are fundamental to understand the mitochondrial energetic failure. All the above mentioned, in a context where lipids and associated oxidative stress play a key role.

4. Materials and Methods

4.1. In Vivo Aβ-Injected Model. Surgical and animal care procedures were performed with strict adherence to the guide for the care and use of laboratory animals (National Institutes of Health, publication number 86–23, Bethesda, MD, USA). All protocols and procedures were approved by the institution's Animal Care and Use Committee. Male Wistar rats (250–280 grams; 3-month-old) were housed in pairs in a colony room on a 12:12 dark/light cycle with lights off at 20:00 h; food and water were provided *ad libitum*. The rats were divided ($n = 5$) in the following groups: (1) vehicle (PBS) injected rats, (2) fibrillar Aβ_{1-42}-injected rats (fAβ), and (3) H_2O_2 (200 μM) intracerebrally injected rats (H_2O_2). Two additional groups, fAβ + Mel and H_2O_2 + Mel were included. In this case, fAβ or H_2O_2-intracerebrally injected animals received antioxidant treatment with melatonin (Sigma, St. Louis, MO, USA), which was dissolved in the watering vessel at a dose of 20 mg/kg/day [24]. H_2O_2 was used as a positive control because of its powerful oxidizing capacity and its particular ability to alter mitochondria state 3 NADH-linked respiration [88]. The utility of vehicle solutions as controls against Aβ, such as saline solution or PBS has been well established from published data [89, 90]. Aβ exhibits a chemistry that facilitates the formation of reactive free radical peptides. It is acceptable to use PBS instead of Aβ peptides because even non-toxic Aβ derivatives, not excluding the scrambled Aβ, a usual control in this *in vivo* model, possess alkylsulfides which may react with oxygen in a metal-independent manner to produce a sulfoxide [91]. Also, these derivatives may generate PBN adducts, indicating the presence of peptide-derived free radical species [72].

Hippocampal injections of Aβ_{1-42} (2 microliters at a final concentration of 1 mM) were performed as previously described [10, 24, 71]. Lyophilized synthetic Aβ_{1-42} (Sigma, St. Louis, MO, USA) peptide was solubilized (10^{-4} M) in filtered, sterile PBS, then was allowed to incubate with continuous agitation (Teflon stir bar at 800 rpm) at 23°C for 36 h [71, 92] in order to form fibrillar aggregates. Rats, anaesthetized with chloral hydrate (350 mg/kg, i.p.), were placed in a stereotaxic instrument for the intracerebral injections over a 5 min period (coordinate: anterior-posterior = −3.8 mm, medial-lateral = 2.0 mm, dorsal-ventral = 2.6 mm from bregma [93], using 5-microliter Hamilton microsyringe coupled with a 30 gauge needle through flexible tubing. The needle was left in place for 5 min after injection. The same coordinates were used for PBS-injected controls and the H_2O_2 experimental group.

36 hours after the injections, rats were deeply anesthetized and transcardially perfused with 200 mL of PBS. Those animals used for immunohistochemical procedures were additionally perfused with 4% paraformaldehyde. Brain was removed and a piece of tissue (164–180 mg), including the lesioned area, was taken with a punch (diameter 10 mm), at the base of the needle tract. This piece included the hippocampal tissue and adjacent cortical areas.

4.2. Immunohistochemistry. For Aβ immunohistochemistry, 5 mm brain slices were postfixed with paraformaldehyde for 2 hr, washed in PBS, and cut into 25–30 μm thick sections with a Vibratome (Leica). Immunohistochemical staining was carried out using a routine immunoperoxidase technique on free-floating sections. Tissue was first washed in 0.05 M PBS, then rinsed in 1% H_2O_2 in PBS, washed in 0.05 M PBS, and preincubated in PBS containing 0.3% Triton X-100 (PBST); sections were then incubated overnight at room temperature with rabbit anti-Aβ antiserum (anti-βA_{42}, 1 : 1500, from Santa Cruz) in 0.1% PBST. After 15 h, tissue was washed twice in 0.3% PBST followed by incubation with a horseradish peroxidase-bound goat anti-rabbit IgG secondary antibody for 2 hours (Santa Cruz Biotechnology, Inc.). The tissue was washed in 0.3% PBST, then in PBS, and then reacted with 3,3′-diaminobenzidine (DAB) (Sigma Chemical Company).

For the immunoelectron microscopy, hippocampus tissue samples were fixed in 4% paraformaldehyde for 24 hours and immersed in sucrose in 2.3 M for 24 hours. Small blocks were cut and postfixed in osmium tetroxide 2% in PB 0.2 M for 45 minutes, then embedded for 48 hours in Embed 812 (Electron Microscopy Sciences). Ultrathin sections of 70–90 nm were cut with an ultramicrotome (Reichert Om3) and mounted on nickel grids, then incubated for 2 hours in 5% BSA and 0.1% fish gelatin. For immunolabeling experiment, the mounted sections were then incubated for 24 hours at 4°C with the primary polyclonal antibody Anti-Aβ (Santa Cruz Biotechnology) at dilution 1 : 1000 and then washed four times with PBS 0.1 M and 0.1% tween-20, and further incubated for 3 hours at room temperature with a 6 nm gold-conjugated secondary goat anti-rabbit antibody (Jackson Immunoresearch Laboratories) at dilution 1 : 500. After four washes with PBS, sections were counterstained with 2% uranyl acetate for 15 minutes and lead citrate for 5 minutes and examined in a Zeiss EM 906 transmission electron microscope (Oberkochen, Germany).

4.3. Diet. In order to underline the importance of cholesterol in the neuronal Aβ-induced damage, animals intracerebrally injected with fAβ were divided in two groups, one receive regular Laboratory Rodent Diet chows, while the other group was fed with 4% cholesterol-enriched chows plus 1% colic acid (Harlan Teklan TD. 01418). Images were analyzed by using Image Pro-Plus software (v5.1) and structural damages were determined as a percentage of the damaged membranes against complete, healthy mitochondrial membranes per field.

4.4. Analysis of Mitochondrial Free Radical Generation. Mitotracker red CM-H2XRos (Molecular Probes), a rosamine derivative used to detect mitochondrial free radicals, was diluted in DMSO to form a 1 mM stock solution. 100 μL of that solution were diluted in 5 mL of saline physiological solution and stored sterile at 4°C as working solution. Applied at a dose of 0.030 μg/kg, CM-H2XRos did not affect the functional properties of mitochondria after loading, since neither respiratory output nor cell viability was significant changed, as evaluated in a separate study (data not shown). Two hours following the intraperitoneal injection of CM-H2XRos, animals were perfused transcardially with PBS followed by 4% paraformaldehyde. The brains were immediately removed and immersed in the fixative for 8–10 h. Following a brief washing in PBS, brain slices were cut into 25–30 μm thick sections, including the area of interest, with the vibratome and incubated free-floating in MitoTracker Green (Molecular Probes, Ex/Em 490/516 nm), which selectively stains mitochondria both in live cells and in cells that have been fixed [94]. Then sections were mounted on adhesive (Vecta Bond)-coated glass slides, with a DNA dye, 4′,6-diamidino-2-phenylindole (DAPI), containing mounting medium (Vectashield, Vector Laboratories) in order to evaluate mitochondrial mass in cells with nuclear counterstaining in blue (Ex/Em 359/461 nm). The mitochondrial free radicals were analyzed by monitoring the oxidized fluorescence product (Ex/Em 554/576 nm) of CMH2XRos under a fluorescence microscope (Carl Zeiss Axioskop). Integrated optical density (IOD), number of mitochondria, as well as its mitochondrial area was determined by using image analysis software (Image-Pro Plus v5.1).

4.5. Mitochondrial Isolation. Briefly, brain tissue was minced and placed in prechilled Dounce homogenizer with SHE buffer (0.25 M sucrose, 5 mM HEPES and 1 mM EGTA, PH 7.4), followed by centrifugation at 2500 rpm for 10 min, 4°C, recentrifugation of the supernatant (8500 rpm, 10 min) obtain a crude mitochondrial pellet, which in turn, following a 10 min incubation in ice, was resuspended again in SHE plus delipidized bovine serum albumin (Sigma Chemical Company). Albumin was eliminated by centrifugation of the mitochondrial suspension at 9500 rpm, 10 min. The protein content in the mitochondrial fraction was determined using the Lowry method [95].

4.6. Respiratory Control Ratios (RCRs). The measurement of oxygen consumption ratios was performed at 34°C using an incubation chamber with a water jacket and a Clark-type O_2 electrode (Yellow Spring Instrument Co., Yellow Spring, OH, USA) and the respiratory control ratios calculated according to Chance and Williams [96]. 1.9 mL of air-saturated KME buffer (125 mM KCl, 20 mM MOPS, 5 mM MgCl and 0.1 mM EGTA at pH 7.6) was added to the chamber and equilibrated with the oxygen electrode for 3 min with stirring. Freshly prepared mitochondria (1 mg protein/mL) were then added to the buffer in the chamber and incubated for another 3 min with stirring. Respiration was started by adding 5 mM orthophosphate (Pi), 5 mM succinate and 1 mM ADP. For oxidation of succinate, small amounts of ADP stimulated respiratory ratios (State 3) until ADP became exhausted (State 4). The respiratory control ratio (RCR) was calculated from the ratio of state 3/state 4 oxygen consumption ratios.

4.7. ATPase Hydrolytic Activity. ATPase activity was measured at 40°C in a medium (1 mL) containing 125 mm KCl, 40 mM MOPS (pH = 8), 3 mM $MgCl_2$, plus 0.1 mg of mitochondrial protein, and the reaction was initiated with 40 μL of ATP (75 mM) and 10 minutes later stopped with 30% trichloroacetic acid. Free orthophosphate (Pi) delivered from ATP hydrolysis was measured by a colorimetric assay at 660 nm based on the formation of a phosphomolybdate complex in an acid medium (after the addition of 3.3% ammonium molybdate in 3.8 N H_2SO_4 and 10% $FeSO_4$) followed by a reduction or complexation with basic dyes that yield colored complexes [97, 98].

4.8. Fluidity Changes of Mitochondrial Membranes. 1,3-di-pyrenylpropane (DPP) incorporation into membranes forms intramolecular excimers depending mainly on the medium microviscosity and temperature of determination [99]. Membrane fluidity is determined by estimating the excimer to monomer fluorescence intensity ratio (Ie/Im) of this fluorescent probe, a quotient that reflexes the lateral mobility of membrane phospholipids [100]. Briefly, mitochondria were resuspended in Tris-HCl buffer (50 mM, pH 8) and then fragmented by sonication for 15 seconds before being separated by centrifugation at 13000 rpm. The mitochondrial membrane pellet was resuspended and proteins were measured using the Lowry method [95]. 0.1 mg of mitochondrial proteins was mixed in a spectrofluorometric cell containing Tris-HCl (20 mM, pH 7.5). DPP solution in ethanol of spectroscopic grade was diluted (0.02 mg/mL) and mixed with membranes given a molar ratio of fluorescent probe to membrane phospholipids of 1 : 1400 and the mixtures were incubated in the darkness for 4 hours at room temperature. Fluorescence of DPP incorporated into membranes was measured at 24°C on a Perkin Elmer fluorescence spectrometer, LS50B. The fluorophore was excited at 329 nm and the monomer and excimer fluorescence intensities were read at 379 and 480 nm, respectively.

4.9. Statistical Analysis. All data are shown as means ± SE of triplicate experiments. Statistical analysis of the data for multiple comparisons was performed by two-way ANOVA

followed by Student's t-tests. For a single comparison, the significance of any differences between means was determined by unpaired t-tests. The criterion for significance was $P < 0.05$ in all statistical evaluations.

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Research Article

Antiaging Effect of Pine Pollen in Human Diploid Fibroblasts and in a Mouse Model Induced by D-Galactose

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The present paper was designed to investigate the effect of pine pollen against aging in human diploid fibroblast 2BS cells and in an accelerated aging model, which was established by subcutaneous injections with D-galactose daily for 8 weeks in C57BL/6J mice. Pine pollen (1 mg/mL and 2 mg/mL) is proved to delay the replicative senescence of 2BS cells as evidenced by enhanced cell proliferation, decreased SA-β-Gal activity, and reversed expression of senescence-associated molecular markers, such as p53, $p21^{Waf1}$, $p16^{INK4a}$, PTEN, and $p27^{Kip1}$ in late PD cells. Besides, pine pollen reversed D-galactose-induced aging effects in neural activity and inflammatory cytokine levels, as indicated by improved memory latency time and reduced error rate in step-down test and decreased concentrations of IL-6 and TNF-α in model mice. Similar to the role of AGEs (advanced glycation endproducts) formation inhibitor aminoguanidine (AG), pine pollen inhibited D-galactose-induced increment of AGEs levels thus reversed the aging phenotypes in model mice. Furthermore, the declined antioxidant activity was obviously reversed upon pine pollen treatment, which may account for its inhibitory effect on nonenzymatic glycation (NEG) *in vivo*. Our finding presents pine pollen as an attractive agent with potential to retard aging and attenuate age-related diseases in humans.

1. Introduction

Rodent chronically injected with D-galactose has been widely used as an animal aging model for brain aging or antiaging pharmacology research [1–4]. An increased level of advanced glycation endproducts (AGEs) is thought to account at least partially for the underlying mechanism as the AGEs inhibitor aminoguanidine (AG) could block most of the aging phenotypes in the D-galactose-induced mouse model [5]. AGEs are a heterogeneous group of reaction products that form between a protein's primary amino group and a carbohydrate-derived aldehyde group by reducing sugars, such as D-glucose and D-galactose, by nonenzymatic glycation (NEG) *in vitro* and *in vivo* [6]. Accumulating evidence indicates that AGEs exacerbate and accelerate aging process and contribute to the early phases of age-related diseases, including neurodegenerative disease, cataract, renal failure, arthritis, and age-related macular degeneration [7–9]. Moreover, AGEs and their precursors usually contain reactive carbonyl groups, which can be generated by the actions of reactive oxygen species (ROS) [10, 11].

As a kind of Chinese traditional medicine, pine pollen, which is the male spore of pine tree, has been used as a drug and food for thousands of years. Pine pollen has an effect in the treatment of different kinds of diseases such as colds, disease of the prostate, anemia, diabetes, hypertension, asthma, and rhinitis [12–14]. Pine pollen is collected artificially from *Pinus massoniana Lamb.*, *Pinus tabulaeformis Carr.*, and it has the characteristics of a single pollen source, pure quality, and is a stable component. Pine pollen powder, called "natural micronutrient storeroom," is rich in many kinds of body-demanding amino acid, minerals, vitamin, enzyme, and flavonoids [12]. Although it is well proposed that pine pollen may have antiaging effect due to its various benefits on˜human health, the direct supportive experimental evidence linking the drug with aging has rarely been reported so far. So, it is interesting to investigate whether pine pollen possesses any antiaging effect *in vitro* and *in vivo*.

Here, the antiaging effect of pine pollen *in vitro* was firstly investigated by using the human diploid fibroblasts (2BS) cell line, which has been well characterized and widely used as a cellular senescence model [15–17]. Then, the accelerate aging model in mice induced by D-galactose was used to evaluate the effect of pine pollen against aging *in vivo* [1, 5, 18]. We treated a group of 5-month-old C57BL/6J mice daily with D-galactose, D-galactose combined with various dosages of pine pollen (500, 1000, 1500 mg/kg, resp.), D-galactose combined with AGEs formation inhibitor AG, and control buffer for 8 weeks. At the end of the treatment, learning and memory abilities, serum and cerebral AGEs levels, indicators for antioxidant activity, and proinflammatory cytokines levels were determined. Our results demonstrated that pine pollen could retard the aging process in cells and mice thus presents pine pollen as an attractive agent with potential to retard aging and attenuate age-related diseases in humans.

2. Material and Methods

2.1. Reagents. Pine pollen was kindly provided by Zhejiang Yalin Biotechnology Co. Ltd. (Hangzhou, China), and was suspended with 0.5% sodium carboxymethyl cellulose (CMC-Na) before administrating. Bovine serum albumin (BSA), 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal), D-galactose, and aminoguanidine hydrochloride (AG) were purchased from Sigma-Aldrich. Elisa kits for AGEs, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) determination were obtained from R&D Systems, USA. Dulbecco's modification of Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were obtained from Gibco, USA. Kits for malondialdehyde (MDA) assay and total superoxide dismutase (SOD) activity measurement were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). BCA protein assay kit was from Pierce Chemical Co, USA.

2.2. Cell Culture. The 2BS cell line isolated from human fetal lung fibroblasts was originally established by the National Institute of Biological Products (Beijing, China) and has been well characterized and widely used as a cellular senes-cence model [15, 19]. The cells are considered to be young at earlier than population doubling (PD) 30 and fully senescent at PD55 or later. The cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in an incubator at 37°C with 5% CO_2. The cultured cells were split in ratios of 1 : 2 or 1 : 4 when the confluence of the culture was over 85%. The cumulative population doublings (CPDs) were calculated as $\log_2(D/D_0)$, where D and D_0 are defined as the density of cells at the time of harvesting and seeding, respectively.

2.3. Cell Proliferation Monitoring. Cell proliferation was monitored using the XCELLigence system (Roche Applied Science, DP, Switzerland), an impedance-based nondestructive assay of cell proliferation. The presence of the cells on top of the electrodes affects the local ionic environment at the electrode/solution interface, leading to an increase in the electrode impedance. Increasing number of cells increases the impedance detected, displayed as cell index (CI) values. The accuracy of this system has previously been validated for monitoring cell numbers over long periods of incubation [20]. Five thousand cells were seeded in 200 μL of cell culture media in each well of a 16-well plate, in quadruplicate. Pine pollen was supplemented at a final concentration of 1 mg/mL and 2 mg/mL 24 h later, and the cells were cultured for an additional indicated time. Cell culture conditions were identical to that described above, and the cell proliferation was monitored over four days.

2.4. Senescence-Associated Galactosidase (SA-β-Gal) Staining. 2BS cells grown in DMEM with or without pine pollen (1 mg/mL and 2 mg/mL) were stained according to a modified method of Dimri et al. [21]. The percentage of SA-β-gal positive cells out of the total number of cells was counted. Average percentages were obtained from three independent experiments.

2.5. Western Blot Analysis. 2BS cells were washed with iced PBS and then lysed with cell lysis buffer containing protease inhibitors cocktail (Cell Signaling Technology, Inc., USA). Protein concentrations were determined by BCA protein assay kit (Pierce Chemical Co.). Total 50 μg of protein extracts were loaded and electrophoresed on 12% SDS polyacrylamide gel and transferred to the nitro cellulose membrane (Bio-Red, USA). The membranes were subsequently probed with anti-p53, anti-$p16^{INK4}$, anti-$p21^{Waf1}$, anti-PTEN and anti-$p27^{Kip1}$ (Cell Signaling Technology, Inc., USA), and anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology, Inc., USA), respectively. The secondary antibody used for detection was linked with horseradish peroxidase. The enhanced chemiluminescence (ECL) method was used to detect the conjugated horseradish peroxidase.

2.6. Animals and Treatment. Five-month-old C57 BL/6J female mice (Slaccas Laboratory Animal Co. Ltd. Shanghai, China), were randomly divided into six groups of ten each. After one week adaptation period, the animals were given daily one of the following preparations subcutaneously for eight weeks: (I) vehicle control; (II) D-galactose at 100 mg/kg; (III) D-galactose at 100 mg/kg plus pine pollen at 500 mg/kg by intragastric injection; (IV) D-galactose at 100 mg/kg plus pine pollen at 1000 mg/kg by intragastric injection; (V) D-galactose at 100 mg/kg plus pine pollen at 1500 mg/kg by intragastric injection; (VI) D-galactose at 100 mg/kg plus AG at 100 mg/kg by intragastric injection. Mice were sacrificed at the end of treatment, and sera, organs and tissues were immediately collected for experiments or stored at −70°C for later experiments. Sera could be used for detection directly while tissue samples should be performed according to the following treatment.

Tissue Homogenization: tissue samples were weighed and homogenized in normal saline (NS) for studies, and homogenates of 5% were obtained. Tissue homogenates were sonicated two times at 30 sec, intervals. Homogenization and sonication were performed at 4°C. After sonication,

homogenates for biochemical studies were centrifuged at 3 000 rpm for 10 min and at 12 000 rpm for 15 min, respectively. Aliquots of the supernatants were used for the studies. The assayed parameters were expressed per mg protein, and protein content of the aliquots was determined by a BCA protein assay kit (Pierce Chemical Co.). All experimental procedures used in this study had been approved by the ethics committee within our hospital and all animal experiments had been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The authors who performed experiments had given their informed consent prior to the study and had followed "principles of laboratory animal care" published by the National Institutes of Health (NIH publication no. 86-23, revised 1985).

2.7. Step-Through Test for Acquisition of Memory. We used a step-down passive avoidance test to evaluate the learning and memory ability of mice according to the previous description [22]. Briefly, it is consisted of a transparent Plexiglas circular cage (40 cm in height, 30 cm in diameter) with a grid floor and a circular platform (4 cm diameter) in the center. During the training session, animals were placed on the platform and their latency to step down with all four paws was measured. Immediately after stepping down on the grid, animals received an electric shock (0.6 mA, 2 sec). Retention test sessions were carried out 24 h after training. Step-down latency on the test day was recorded as an index of inhibitory avoidance memory. A cut-off time of 300 sec was set, and the number of memory errors during the 5 min was also recorded.

2.8. Measurement of SOD Activity and Lipid Peroxidation in Mouse. The activity for SOD in sera and brains was examined according to xanthine oxidase method provided by the standard assay kit (Nanjing Jiancheng Bioengineering Institute, China) as described [17]. The assay used the xanthine-xanthine oxidase system to produce superoxide ions, which reacted with 2-(4-iodophenyl)-3-(4-nitrophenol-5-phenlyltetrazolium chloride) to form a red formazan dye, and the absorbance at 550 nm was determined. The values were expressed as units per mg protein, and protein concentration was determined by a BCA protein assay kit (Pierce Chemical Co.), where one unit of SOD was defined as the amount of SOD inhibiting the rate of reaction by 50% at 25°C.

Lipid peroxidation was evaluated by measuring MDA concentrations according to the thiobarbituric acid (TBA) method as commercially recommended (Nanjing Jiancheng Bioengineering Institute, China). The method was based on the spectrophotometric measurement of the color produced during the reaction to TBA with MDA. MDA concentrations were calculated by the absorbance of TBA reactive substances (TBARS) at 532 nm.

2.9. Protocols for Other Assays. ELISA assay for AGEs, IL-6, and TNF-α was carried out with commercial kits (R&D Systems, USA) according to the manufacturer's protocol.

Table 1: The life spans of 2BS cells in CPDs, based on the actual number of cells harvested and seeded.

Group	Treatment	Time of transfer to special medium	N	CPDs	Average PDs per week
I	control	—	3	53.7 ± 3.6	1.7 ± 0.1
II	Pine pollen (1 mg/L)	PD30	3	61.8 ± 5.1	2.2 ± 0.2*
III	Pine pollen (2 mg/L)	PD30	3	60.5 ± 4.7	2.1 ± 0.1*

Note. Cells were grown from PD30 in DMEM supplemented with pine pollen at 1 mg/L and 2 mg/L, respectively. The cultured cells were split in ratios of 1 : 2 or 1 : 4 when the confluence of the culture was reached 70–80%. CPDs (cumulative population doublings) were calculated as $\log2(D/D0)$, where D and $D0$ were defined as the density of cells at the time of harvesting and seeding, respectively. The last culture was defined as the subculture that could not be confluent in 3 weeks. Data were obtained from three independent experiments (*$P < 0.05$, versus I).

2.10. Inhibition on AGE Formation In Vitro . AGE-modified BSA was prepared as previously described [23]. Briefly, BSA (100 mg/mL) was incubated under sterile conditions with 0.5 M D-galactose in 0.2 M phosphate-buffered saline (PBS, pH 7.4) at 37°C for eight weeks. For pine pollen or AG inhibition, samples were added with pine pollen (1.2 mg/mL and 2.4 mg/mL) or AG (100 mM) and were incubated under identical conditions. The control BSA sample was incubated under identical conditions but without supplementation of D-galactose. Samples were dialyzed (10 kDa cut-off) against PBS and then the content of BSA-AGEs was determined by a commercial Elisa kit as described previously.

2.11. Data Analysis. All data listed in the figures or the tables were expressed as mean ± SEM. One-way ANOVA analysis (SPSS 15.0) was used for data comparisons within multiple groups. In each case, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of Pine Pollen on Cumulative Population Doublings in 2BS Cells. In this study, we firstly observed the effects of pine pollen on replicative lifespan and biomarkers related to replicative senescence in human fetal lung diploid fibroblasts (2BS). Pine pollen significantly delayed replicative senescence of 2BS cells by at least 7 PDs (see Table 1). The two concentrations of pine pollen (1 mg/mL and 2 mg/mL) showed a similar gain in PDs. The growth rate of pine pollen treatment cells was dramatically increased compared to that of the control cells (Table 1). Meanwhile, pine pollen improved the proliferation of 2BS cells as demonstrated by using the Xcelligence system (Roche Applied Science, Basel, Switzerland), an impedance-based nondestructive assay of cell proliferation. Cells at PD30 incubated with pine pollen at 1 mg/mL and 2 mg/mL for 24–48 h showed a maximum 20% elevation on cell index (CI) compared with that of control (Figure 1).

Table 2: Body weight and AGEs Levels.

Group	Treatment	Body weight		Serum AGEs (ng/mL)	Cerebral AGEs (ng/mg prot)
		Pretreatment	Posttreatment		
I	Young control	22.83 ± 1.13	25.32 ± 1.15	145.34 ± 47.73	24.71 ± 23.42
II	D-gal (100 mg/kg)	22.62 ± 0.98	23.93 ± 0.98	294.65 ± 165.93*	53.46 ± 29.57*
III	D-gal + PP (500 mg/kg)	22.37 ± 0.80	23.28 ± 1.00	163.47 ± 158.04$^{\Delta}$	31.46 ± 14.86
IV	D-gal + PP (1000 mg/kg)	21.84 ± 0.93	22.99 ± 1.42	201.69 ± 118.65	31.67 ± 18.65
V	D-gal + PP (1500 mg/kg)	22.13 ± 1.46	23.52 ± 1.80	202.08 ± 158.40	27.71 ± 19.06$^{\Delta}$
VI	D-gal + AG (100 mg/kg)	22.32 ± 1.32	24.63 ± 2.05	158.29 ± 101.17$^{\Delta}$	23.88 ± 19.17$^{\Delta}$

Note. C57BL/6J female mice (5-month-old, $n = 10$ in each group) were treated daily with PBS (s.c, young control), D-galactose (D-gal, 100 mg/kg, s.c), D-gal (100 mg/kg, s.c) with pine pollen (PP, 500, 1000, 1500 mg/kg, ig) and aminoguanidine (AG, 100 mg/kg, ig). AGEs levels at the end of the treatment were determined by a quantitative AGEs-ELISA kit. Statistical significant difference: $^{*}P < 0.05$, versus I; $^{\Delta}P < 0.05$, versus II.

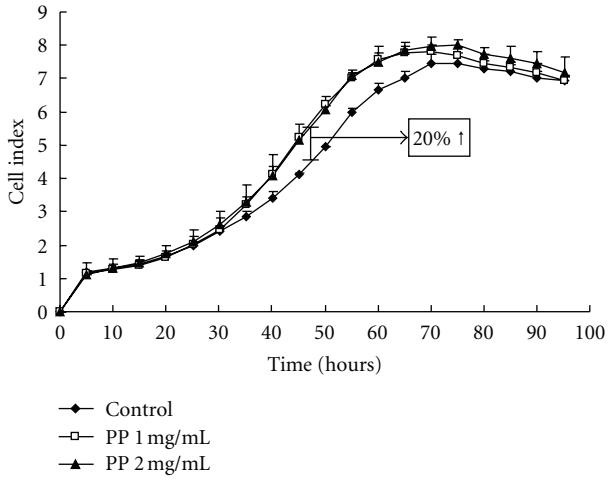

Figure 1: Line graph showing result of impedence-based cell proliferation assay (Xcelligence). The cell index (see Section 2) was plotted against time for the 2BS cells. Pine pollen (PP) was added 24 h after cell-seeding, and an approx. 20% elevation compared with the control was observed between 24–48 h under PP (1 mg/mL and 2 mg/mL) incubation.

3.2. Effects of Pine Pollen on SA-β-Gal Activity in 2BS Cells. As expected, only sporadic SA-β-gal positive cells were observed in young control cells. As shown in Figure 2, the SA-β-gal positive rates of 1 mg/mL and 2 mg/mL pine pollen treatment PD55 cells were 27.2 ± 4.3% and 25.3 ± 4.9%, respectively, which were much lower than that of PD55 control cells (81.7 ± 7.1%). These results indicate indirectly that the pine pollen delayed the population senescence of 2BS cells.

3.3. Effects of Pine Pollen on Expression of Senescence Associated Molecules in 2BS Cells. Activation of p53-p21 and p16-Rb pathways results in replicative cellular senescence in human diploid fibroblasts [17, 24]. Similar to our previous experiment, an elevated protein level of cyclin-dependent kinase (CDK) inhibitors $p21^{Waf1}$ and $p16^{INK4}$ was observed in control late PD 2BS cells. However, the increased expression of senescence-associated molecules p53, p21, and p16 in late PD 2BS cells were significantly reversed to that of young control after pine pollen treatment (Figure 3). Moreover, emerging evidence revealed that the tumor suppressor phosphatase and tensin homolog PTEN and its downstream effector $p27^{Kip1}$ are also critical for replicative senescence [24, 25]. Thus the effect of pine pollen on this signaling pathway was also checked in current experiment. Western blot results indicated that the protein expression of PTEN and $p27^{Kip1}$ was downregulated upon pine pollen treatment in late PD 2BS cells (Figure 3).

3.4. Effects of Pine Pollen on AGEs Formation in Mice. The facts that pine pollen retarding the aging process in 2BS cells promoted us to detect whether pine pollen has any antiaging effect on the D-galactose-induced aging model. Indeed, within the period of pine pollen treatment, no mouse showed a significant abnormality that could be detected by visual examination. All groups of mice gained weight normally throughout the study (Table 2). As expected, mice treated with D-galactose showed a remarkably increased level of serum and cerebral AGEs compared with control ones ($P < 0.05$, Group II versus Group I, see Table 2), and AG, a well-accepted AGEs formation inhibitor, significantly reversed the elevation of serum and cerebral AGEs in D-galactose-treated mice ($P < 0.05$, Group VI versus Group II, see Table 2). It should be noted that among the three different doses of pine pollen, low dosage (500 mg/kg) treatment showed best efficacy, which significantly blocked the increase of serum AGEs in D-galactose-treated mice ($P < 0.05$, Group III versus Group II, see Table 2). Although a dose-dependent inhibitory effect of pine pollen on cerebral AGEs formation was observed in D-galactose-treated mice, the *in vitro* assay suggested that pine pollen had little inhibitory effect on the BSA-AGEs formation (Figure 4).

3.5. Effects of Pine Pollen on Neurological Activities in Mice. Next, we tested whether the decrement of serum and cerebral AGEs induced by pine pollen would reverse any aging effects, such as neurological activities, in D-galactose-induced aging model. Learning and memory abilities were examined by step-down test. D-galactose treatment significantly lowered the learning and memory activity of young mice compared with controls as evidenced by shortened latency of step-down and elevated number of memory errors within five minutes. Consistent with previous studies [5], the lowered

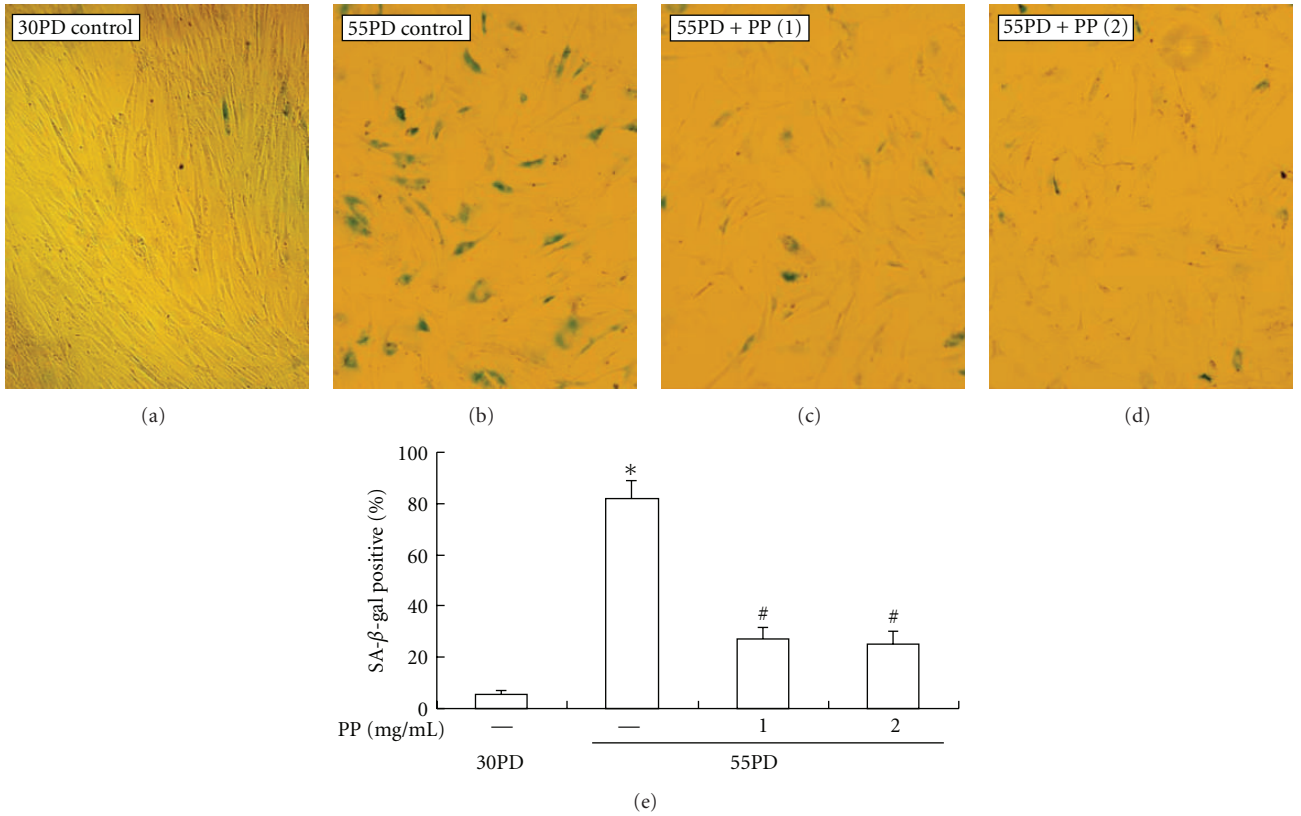

Figure 2: SA-β-gal staining of 2BS cells grown from PD30 in DMEM supplemented with 1 mg/mL or 2 mg/mL pine pollen (PP). Cells of none-confluent state were washed with PBS, fixed with 3% formaldehyde, and stained in staining solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside for 16 h. 2BS cells at PD30 were set as young control. Cells were microphotographed at a magnification of 10×10. $^{*}P < 0.01$ versus 30PD control group; $^{\#}P < 0.01$ versus 55PD control group.

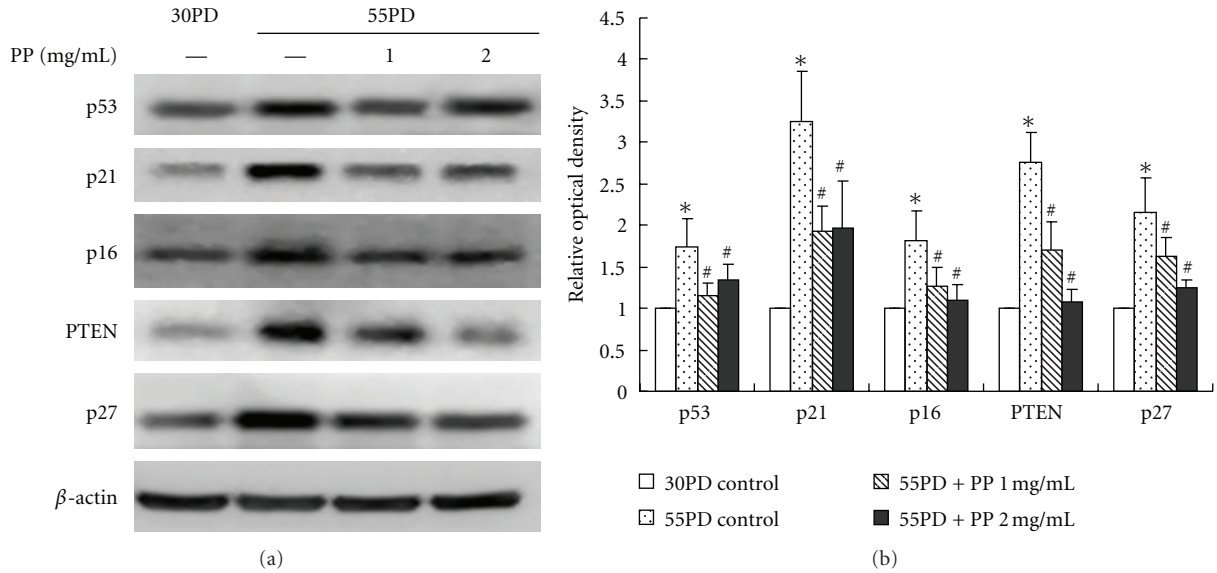

Figure 3: (a) Protein levels of p53, p21, p16, PTEN, and p27 in 2BS cells grown from PD30 in DMEM supplemented with 1 mg/mL or 2 mg/mL pine pollen (PP). Representative images were acquired from three different experiments. (b) Quantitative analysis of the protein levels of p53, p21, p16, PTEN, and p27. Bars represent relative protein levels counted as $D1/D0$ (the value for PD30 control was set as 1.0), where $D0$ and $D1$ stand for the optical density of β-actin ladder and sample ladder, respectively. The optical density for each ladder was calculated by Image J soft ware. Data were obtained from three independent experiments. $^{*}P < 0.05$ versus 30PD control group; $^{\#}P < 0.05$ versus 55PD control group.

Table 3: Effect of pine pollen on SOD activity and MDA levels in mice.

Group	Treatment	Serum MDA (nmol/mL)	Cerebral MDA (nmol/mg prot)	Serum SOD (U/mL)	Cerebral SOD (Ul/mg prot)
I	Young control	7.74 ± 1.04	1.17 ± 0.45	82.91 ± 4.25	128.98 ± 17.78
II	D-gal (100 mg/kg)	17.66 ± 7.93**	2.90 ± 1.28**	69.18 ± 6.00*	91.36 ± 19.83*
III	D-gal + PP (500 mg/kg)	9.27 ± 0.89$^{\Delta}$	1.73 ± 0.59$^{\Delta}$	79.06 ± 2.36	109.27 ± 12.91$^{\Delta}$
IV	D-gal + PP (1000 mg/kg)	10.69 ± 2.44$^{\Delta}$	1.64 ± 0.39$^{\Delta}$	77.73 ± 2.65	110.76 ± 24.02
V	D-gal + PP (1500 mg/kg)	9.14 ± 4.07$^{\Delta}$	1.51 ± 0.22$^{\Delta\Delta}$	78.64 ± 2.44	111.71 ± 16.59$^{\Delta}$
VI	D-gal + AG (100 mg/kg)	5.89 ± 2.65$^{\Delta\Delta}$	1.48 ± 0.24$^{\Delta\Delta}$	79.80 ± 2.75$^{\Delta}$	120.88 ± 25.91$^{\Delta}$

Note. C57BL/6J female mice (5-month-old, $n = 10$ in each group) were treated daily with PBS (s.c, young control), D-galactose (D-gal, 100 mg/kg, s.c), D-gal (100 mg/kg, s.c) with pine pollen (PP, 500, 1000, 1500 mg/kg, ig), and aminoguanidine (AG, 100 mg/kg, ig). SOD and MDA level in sera and brains were determined by commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol. Statistical significant difference: $^{*}P < 0.05$, versus I; $^{\Delta}P < 0.05$, versus II; $^{**}P < 0.01$, versus I; $^{\Delta\Delta}P < 0.01$, versus II.

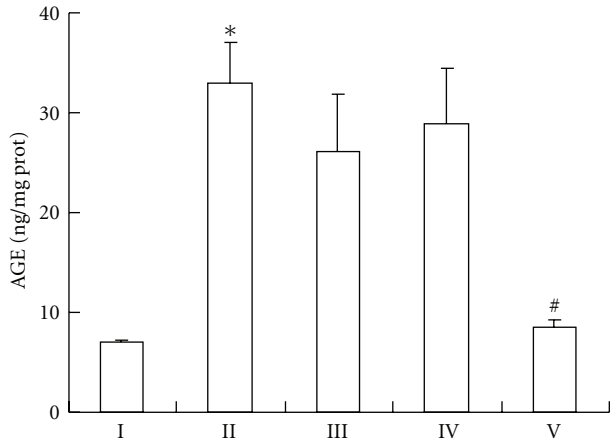

Figure 4: Effect of pine pollen on the BSA-AGEs formation *in vitro*. BSA alone at 100 mg/mL (I), BSA plus D-galactose at 0.5 M (II), BSA plus D-galactose and pine pollen (1.2 mg/mL and 2.4 mg/mL, III and IV), and BSA plus D-galactose and AG at 100 mM (V) were incubated at 37°C for eight weeks under sterile conditions. Samples were dialyzed (10 kDa cut-off) against PBS and then the content of BSA-AGEs was determined by a commercial Elisa kit as described in Section 2. Data were results of three parallel experiments and were expressed as mean± SEM. Statistical significant difference: $^{*}P < 0.01$, versus I; $^{\#}P < 0.01$, versus II.

learning and memory activity of model mice can be reversed by AG treatment (Figure 5). The elevated number of memory errors within five minutes in D-galactose-treated young mice was also prevented by pine pollen treatment. Similarly, pine pollen reversed the shortened latency of step-down in D-galactose-treated mice. Noticeably, the high dosage (1500 mg/kg) of pine pollen exhibited a comparable effect to that of AG treatment (see Figure 5).

3.6. Effect of Pine Pollen on Proinflammatory Cytokines Levels in Mice. It is well documented that aging is associated with increased circulating levels of proinflammatory cytokines. Increased levels of inflammatory serum markers in the elderly are associated with neurodegenerative diseases, such as dementia, Parkinson's disease [26]. As shown in Figure 6, the TNF-α and IL-6 levels in serum and cerebral were significantly elevated in D-galactose-treated mice in comparison to those in control group. Notably, pine pollen treatment significantly prevented the increment of cerebral IL-6 which was comparable to that of AG treatment. A decreased trend of TNF-α level in sera and brains was also observed upon pine pollen treatment in model mice, though the difference was not statistically significant.

3.7. Effects of Pine Pollen on the Antioxidant Enzymes Activity and Lipid Peroxidation in Mice. It has been reported that D-galactose treatment resulted in an elevation of oxidative stress both *in vivo* and *in vitro* [2, 27]. As expected, SOD activity was decreased, while the MDA level, an indicator of lipid peroxidation, increased in serum and brain in D-galactose-treated young mice. However, additional treatment of pine pollen resulted in the increase of cerebral SOD activity and remarkable decrease of serum and cerebral MDA level in D-galactose treated mice (see Table 3). Consistent with previous study [5], a potent reversible effect of AG on SOD and MDA in this aging model was also observed (Table 3).

4. Discussion

Human diploid fibroblast cells can divide for only a limited number of times *in vitro*, a phenomenon known as replicative senescence [28]. The phenotypes of senescent 2BS cells included morphological changes, lower cell proliferation, positive SA-β-gal staining [16], and G1 cycle arrest which was mainly resulted from elevated protein levels of cyclin-dependent kinase (CDK) inhibitors $p21^{Waf1}$ and $p16^{INK4}$ [17]. Thus, we chose these senescence-associated markers to evaluate the antiaging effect of pine pollen in the current study. Our results demonstrated that Pine pollen delayed the replicative senescence of 2BS cells as evidenced by enhanced cell proliferation, decreased SA-β-Gal activity, and reversed expression of p53, p21, and p16 molecules in late PD cells (Figures 1, 2, and 3). Meanwhile, the expression of PTEN-$p27^{Kip1}$ was also attenuated upon pine pollen treatment, which further supported our conclusions. The dosages

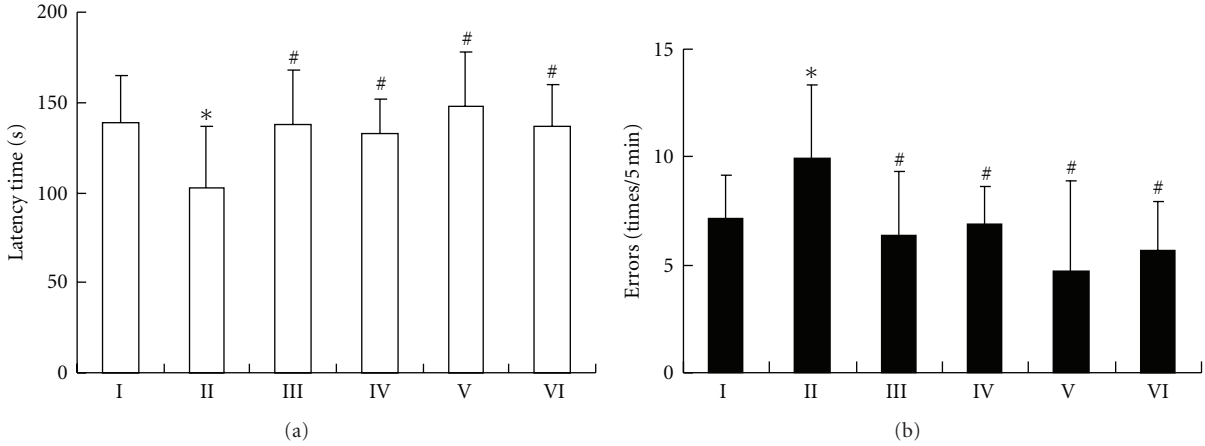

Figure 5: Latency time (a) and memory error rates (b) of young control (I), D-galactose alone(100 mg/kg) treated (II), D-galactose combined with pine pollen (500, 1000, 1500 mg/kg) treated (III, IV, V), and D-galactose combined with AG (100 mg/mg) treated (VI) mice. The step-down method was used to determine the latency time and memory error rates. Each mouse was trained for 5 min first to "remember" the system. Twenty-four hours later, mice were placed in the same cage and latency time (second), and the number of errors within five minutes was recorded. Data were results of ten animals for each group and expressed as mean ± SEM. Statistical significant difference: $^{*}P < 0.05$, versus I; $^{\#}P < 0.05$, versus II.

of pine pollen (1 mg/mL & 2 mg/mL) used in this study were much higher than that in previous studies (10 μg/mL) [14] as they used the ethanol extract of pine pollen (PPE), which was different from ours. We used the pine pollen powder rather than its extract. Since the abundance of active substances in our materials was lower than that of the ethanol extract of pine pollen, higher dosages have to be used in our experiment. However, the cell toxicity of pine pollen among 0~5 mg/mL was undetectable in 2BS cells according to an MTT assay (see Figure 7).

Though pine pollen showed an antiaging effect *in vitro*, whether it possesses any effect against aging *in vivo* remains elusive. Here we selected an accelerated aging model induced by D-galactose for the *in vivo* study as this model resembles their aged control counterparts (16- to 24-month-old) both physiologically and pathologically and has been widely used for antiaging pharmacology research [1, 29, 30]. Our previous study demonstrated that D-galactose injection led to an accelerated aging phenotypes manifested by an increased serum AGEs level [5, 18]. Treatment of AGEs formation inhibitor aminoguanidine (AG) and salidroside in D-galactose-induced aging mice could block most of the aging phenotypes, indicating that AGEs may account at least partially for the mechanism of the accelerated aging [5, 18].

In current experiment, the pine pollen showed different inhibitory effect on serum and cerebral AGEs formation in model mice. When the model mice were treated with a high dosage (1500 mg/kg) of pine pollen, the lowest level of AGEs in brains and a best reversal effect on lowered neurological activity were observed (Figure 5). However, pine pollen at a lower dosage (500 mg/kg) exhibited a best inhibitory efficacy on serum AGEs generation rather than the other two higher doses (1000 mg/kg and 1500 mg/kg). Besides, the *in vitro* assay revealed that pine pollen had little inhibitory effect on the BSA-AGEs formation, which was different from the role of AG, as this compound could inhibit the nonenzymatic glycation (NEG) both *in vivo* and *in vitro* (Table 2 and Figure 4). The possible reasons for the different role between pine pollen and AG were listed as below. Firstly, pine pollen contains various ingredients, including proteins, vitamins, enzymes and coenzymes, fats, flavonoids, nucleic acids, monosaccharides, polysaccharides, phospholipids, and other nutrients [12]. Flavonoids have been reported as potent inhibitors on AGEs formation [31, 32], while the ingredients of protein and monosaccharides are the substrates of nonenzymatic glycation which may accelerate the AGEs formation. Thus the inhibitory effect of pine pollen on AGEs formation would be partly offset by itself. Since higher dosages of pollen contain more nonenzymatic glycation substrates, a better inhibition of pine pollen on serum AGEs formation displayed under low dosage rather than that of higher dosages *in vivo*. Secondly, pine pollen showed an optimal inhibition on cerebral AGEs under high dose (1500 mg/kg); this may be induced by more active substances such as flavonoids in pine pollen going through into brain tissues. Thirdly, pine pollen may indirectly inhibit the AGEs formation *in vivo* by an antioxidant mechanism. Increased AGEs level can induce an elevated level of intracellular oxidative stress levels, which in turn promotes the nonenzymatic glycation [11, 33]. Effective removal of reactive oxygen species (ROS) may block the formation of AGEs. Indeed, the D-galactose-treated mice showed an elevation of oxidative stress in our experiment, and it was significantly reduced by pine pollen, as indicated by the declined MDA concentration, and reversion of decreased SOD activity (Table 3).

On the other hand, aging is associated with increased circulating levels of pro-inflammatory cytokines [26], which at least partly results from the increment of AGEs in elder persons as AGEs increase inflammation through binding

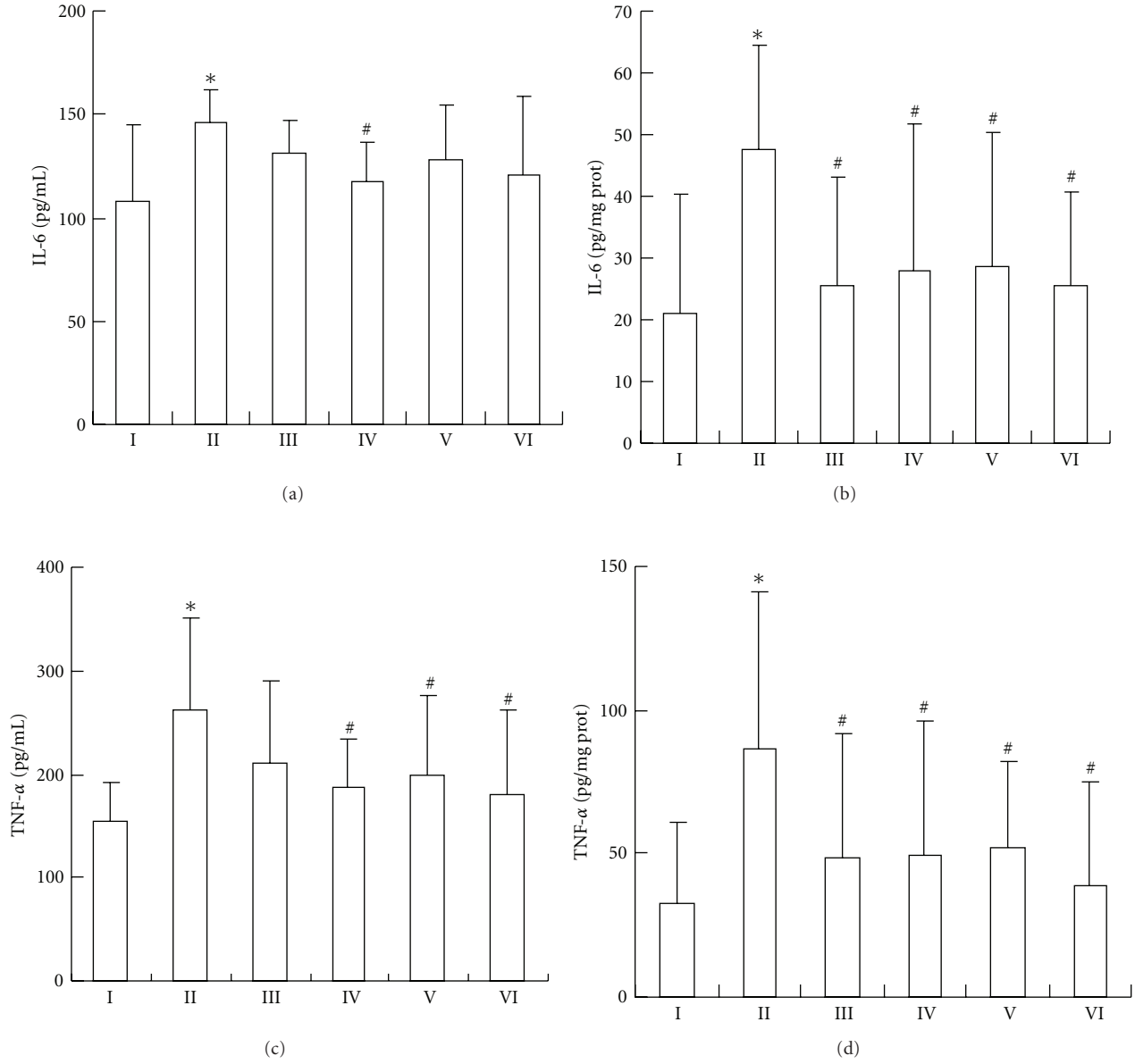

Figure 6: Proinflammatory cytokines levels of young control (I), D-galactose alone (100 mg/kg) treated (II), D-galactose combined with pine pollen (500, 1000, 1500 mg/kg) treated (III, IV, V), and D-galactose combined with AG (100 mg/mg) treated (VI) mice. IL-6 and TNF-α in sera and brains were determined by quantitative ELISA kits. Data were results of ten animals for each group and expressed as mean ± SEM. Statistical significant difference: $^{*}P < 0.05$, versus I; $^{\#}P < 0.05$, versus II.

with the receptor for AGEs (RAGEs). Inflammatory mediators that are upregulated through AGEs pathway include TNF-α, IL-6, and C-reactive protein [34]. Thus a reduction of TNF-α and IL-6 in our model mice treated with pine pollen was partly due to its inhibitory effect on nonenzymatic glycation as a similar result in model mice treated by the well-accepted AGEs inhibitor AG was also observed (Figure 6).

However, our study might be limited by several aspects. As pine pollen is a complex mixture, it is necessary to clarify which kind of composition in pine pollen is responsible for its NEG inhibitory activity, and thus this beneficial effect could be optimized by using corresponding extraction from pine pollen. Further work regarding the relationship between the NEG inhibitory activity and antioxidant property of this drug is also needed.

5. Conclusions

Taken together, pine pollen is proved to delay the replicative senescence of human diploid fibroblasts and block D-galactose-induced increase of serum and cerebral AGEs level in model mouse, which may result in the reversal of D-galactose-induced aging effects in both neural and inflammation system. It is possible that pine pollen exerts its antiaging

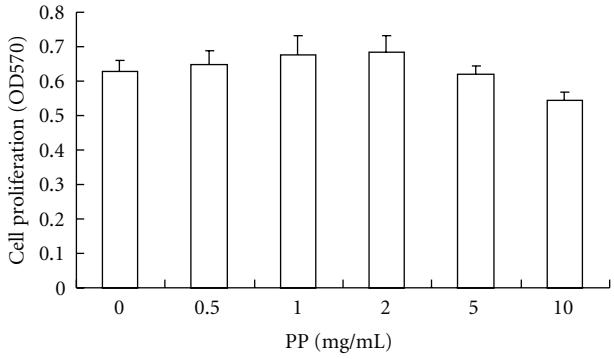

Figure 7: The cell toxicity of pine pollen was evaluated by the cell proliferation detected by the MTT assay. In brief, the 2BS cells were seeded in flat-bottomed 96-well microplates at the density of 3000 cells in 0.2 mL per well. After 20 h, the cells were incubated in the culture medium containing various concentrations of pine pollen (PP) for 48 h.Then 20 μL MTT [3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-diphenlytetrazoliumbromide] of 10 mg/mL was added to each well. After incubation for 4 h, 0.2 mL DMSO was added to stop reactions. The absorbance values of each well were determined spectrophotometrically at 570 nm using the microplate reader (BIOTEK, Rockville, MA, USA). A stimulatory rather than inhibitory effect of pine pollen under 0.5–5 mg/mL on 2BS cell proliferation was observed, which indicated its biosafety under such dosages.

effects at least partially by its NEG-inhibiting effect *in vivo*. Our work first directly proves the antiaging efficacy of the natural agent both *in vivo* and *in vitro* and thus inspires the new application for this drug in gerontological area.

Acknowledgments

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Research Article

Heterogeneous Responses to Antioxidants in Noradrenergic Neurons of the Locus Coeruleus Indicate Differing Susceptibility to Free Radical Content

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The present study investigated the effects of the antioxidants trolox and dithiothreitol (DTT) on mouse Locus coeruleus (LC) neurons. Electrophysiological measurement of action potential discharge and whole cell current responses in the presence of each antioxidant suggested that there are three neuronal subpopulations within the LC. In current clamp experiments, most neurons (55%; 6/11) did not respond to the antioxidants. The remaining neurons exhibited either hyperpolarization and decreased firing rate (27%; 3/11) or depolarization and increased firing rate (18%; 2/11). Calcium and JC-1 imaging demonstrated that these effects did not change intracellular Ca^{2+} concentration but may influence mitochondrial function as both antioxidant treatments modulated mitochondrial membrane potential. These suggest that the antioxidant-sensitive subpopulations of LC neurons may be more susceptible to oxidative stress (e.g., due to ATP depletion and/or overactivation of Ca^{2+}-dependent pathways). Indeed it may be that this subpopulation of LC neurons is preferentially destroyed in neurological pathologies such as Parkinson's disease. If this is the case, there may be a protective role for antioxidant therapies.

1. Introduction

Oxidative stress in neurons arises because of an imbalance between free radical production and antioxidant control. This process leads to cell damage and, when severe, can trigger apoptosis or necrosis [1]. Indeed, such oxidative stress may initiate certain neuropathologies such as Alzheimer's and Parkinson's disease whereby oxidative damage to biomolecules causes cellular dysfunction and neuronal death [1, 2].

The exact mechanisms whereby healthy neurons become sensitive to oxidative stress are unknown, and it is also unclear whether antioxidant treatments limit the spread of oxidative damage. For example, it has been reported that varying levels of oxidants can modulate ion channels and consequently effect important neuronal functions such as pacemaking [3–5]. Thus, antioxidant treatments have been developed and used and have indeed had beneficial effects in preventing or slowing the onset of neurological disease [6].

Antioxidants may modulate transcription factors that ultimately lead to oxidative stress [7]. On the other hand, it has been demonstrated that molecules that were thought to be antioxidants due to their antioxidant capacity *in vitro* (such as vitamin A and retinoids) have different effects in living organisms (*in vivo*) as they can increase oxidative damage to biomolecules and generate oxidative stress [8–10]. In fact free radicals are crucial components of many intracellular signalling pathways (for a review see [11]), and an exaggerated decrease in their levels could lead to undesired cellular events. Thus, many factors contribute to the varied effectiveness of antioxidants in both human and

animal trials including differences in dose and timing of antioxidant administration.

The present paper explores the effect of two different antioxidants (Trolox and DTT) on pacemaking in Locus coeruleus (LC) neurons. These antioxidants can chelate different reactive species. Both compounds are membrane permeable, so they can easily access intracellular compartments. Trolox is a water-soluble vitamin E analogue with a broad antioxidant spectrum [12], whereas DTT is a reducing agent that acts on thiol (–SH) groups [13]. Here, we demonstrate that LC neurons exhibit three different types of electrophysiological responses to these antioxidants. We believe these responses may represent three neuronal populations that have differential sensitivity to free radicals and are involved in the massive loss of LC neurons observed in pathologies such as Parkinson's disease.

2. Experimental Procedures

2.1. Preparation of Brain Slices. All procedures used in this study were approved by the University of Newcastle Animal Care and Ethics Committee. Brain slices containing the LC were prepared from Swiss mice (P7–12, both sexes) rendered unconscious with Ketamine (100 mg/kg i.p.) according to a previous established protocol [14]. Mice were decapitated, and the brain was rapidly removed and immersed in ice-cold "modified sucrose ringer" containing (in mM): 25 $NaHCO_3$, 11 Glucose, 235 Sucrose, 2.5 KCl, 1 NaH_2PO_4, 1 $MgCl_2$, and 2.5 $CaCl_2$, bubbled with 95% O_2/5% CO_2 [15]. The cerebellum and brain stem were isolated and slices (270–300 μm thick) were cut with a vibrating tissue slicer (Leica VT1000S). Slices were kept in a recovery chamber (containing ACSF) at room temperature and high oxygen for 1.5–2 h before experiments commenced. The slice containing the LC was identified and transferred to a recording bath where it was visualized with an upright microscope (Olympus BX50) and superfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 120 NaCl, 25 $NaHCO_3$, 11 Glucose, 2.5 KCl, 1 NaH_2PO_4, 1 $MgCl_2$ and 2.5 $CaCl_2$, constantly bubbled with 95% O_2/5% CO_2. LC neurons were visualized using infrared video microscopy with differential interference contrast optics and identified according to their large size and location near the ventrolateral border of the fourth ventricle [16]. Most experiments were performed on neurons in slices, whereas our Ca^{2+} imaging and JC-1 experiments used isolated LC neurons.

2.2. Preparation of Fresh Dissociated LC Neurons. Neurons were isolated using an adapted protocol according to [17]. Brain slices were cut using the same protocol described above in "*Preparation of brain slices*" and allowed to recover for 1 h in the recovery chamber. Slices were then placed in the ACSF-containing recording chamber, and neurons were dissociated and isolated using a custom-made vibrating device. This instrument dissociated neurons by vibrating a fine glass electrode with a sealed tip just above the tissue surface. This method usually provided about 10 healthy neurons per slice. After isolation, neurons were left for 15 min to settle on the glass bottom of the recording chamber before gentle ACSF perfusion was commenced.

2.3. Electrophysiology. Electrophysiological recordings were made using the whole cell attached voltage clamp or current clamp recording modes (Axopatch-1C amplifier) with data sampled at 100 kHz and low-pass filtered at 5 kHz. Electrodes had resistance ranging from 1.8 to 2.5 MΩ. Neurons were considered to be adequately voltage clamped when no unclamped spikes were observed during voltage ramp application [14, 16].

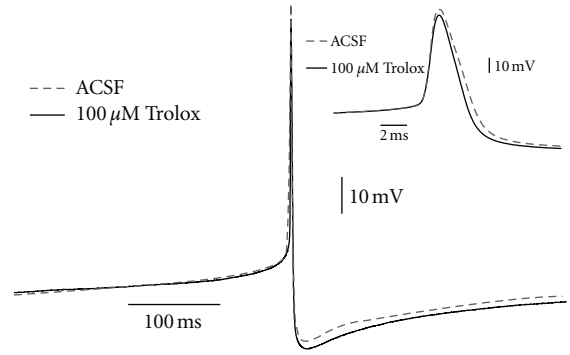
2.4. Solutions and Pharmacology. To avoid perfusion-associated washout during whole cell recording, we used the same experimental protocol as described previously [16]. In brief, 10 s after intracellular access was obtained, the first voltage protocol was applied, with the treatment perfusion started immediately using a rapid exchange (>95% change over time ~2 s) local-perfusion system [18]. The second voltage protocol was then applied 180 s after commencement of treatment. Total incubation time for voltage clamp experiments was 180 s; the incubation time for other experiments is indicated in the respective figures. Tetrodotoxin (TTX; 1 μM) was added to the perfusate to block voltage-dependent Na^+ channels when step pulse protocols were applied. The standard internal pipette solution contained (in mM): 135 K methylsulphate, 8 NaCl, 10 HEPES, 2 Mg_2ATP, 0.3 Na_3GTP, 0.1 EGTA, pH: 7.3. The standard external solution used was ACSF. All experiments were carried out at 33 ± 2°C.

2.5. Acquisition and Analysis. Data were acquired using Axograph 4.8 software (ITC-16 interface and a Mac G4 computer) and analysed using Axograph X 1.1.0 software. Input resistance, cell capacitance, and series resistance were measured by the software according to the response to a −5 mV pulse delivered shortly after obtaining the whole cell recording mode. A correction of −8.5 mV, calculated using JPCalc [19], was applied to account for the junction potential between ACSF and K methyl sulphate in the recording pipette. *I-V* plots were constructed from depolarizing pulse protocol recordings. Individual currents were normalised to cell size based on the whole cell capacitance to obtain current density values (pA/pF). Current density values were multiplied by 100 to normalise values to the measured current. Recordings obtained by ramp and depolarizing pulse protocols were filtered at 1 kHz using Axograph X software prior to analysis.

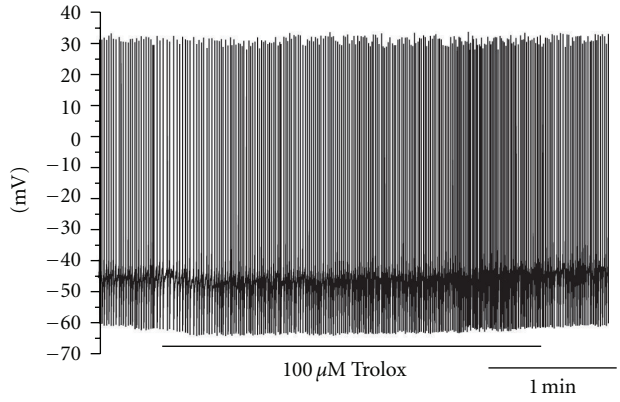
2.6. Measurement of Cytosolic Ca^{2+} and Mitochondrial Membrane Potential (Ψm). Relative intracellular [Ca^{2+}] or Ψm was measured in freshly dissociated LC neurons. After isolation (Methods), LC neurons were incubated in ACSF at room temperature containing the fluophores 10 μM Oregon green/AM for 45 min or 5 μg/mL JC-1 for 1 h [20]. The recording chamber containing the isolated and loaded neurons was then placed on the stage of a Nikon TE200 inverted microscope connected to a BIORAD 1000 confocal scanner system. The isolated neurons were viewed with a 60X

Impact on spontaneous firing		Action potential values		
	100 μM Trolox		ACSF	100 μM Trolox
No effect	6 of 11	AP amplitude (mV)	92.6 ± 0.8	89.9 ± 1.3
Increased (depolarization)	2 of 11	AHP amplitude (mV)	23.1 ± 0.3	24.4 ± 0.4
Stopped (hyperpolarization)	3 of 11			

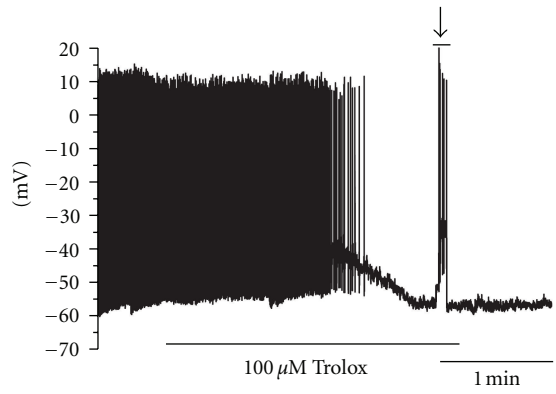
(a)



(b)



(c)



(d)

Figure 1: Impact of Trolox treatment on pacemaker activity in LC neurons. (a) Table demonstrating the effect of 100 μM Trolox on the spontaneous firing of LC neurons and comparison of values obtained for action potentials (APs) before (ACSF) and 180 s after 100 μM Trolox treatment ($n = 11$ for spontaneous firing and $n = 23$ for AP comparison). (b) Comparison of the waveshape of averaged APs before (ACSF) and 180 s after application of 100 μM Trolox in ACSF ($n = 23$). Hyperpolarizing group was excluded from comparison demonstrated in a and b due to lack of APs at 180 s treatment. (c) and (d) Recordings demonstrating opposite effects induced by 100 μM Trolox which in a small number of cases depolarized neurons and increased firing (c) or hyperpolarized neurons which led to abolition of AP firing (d) ($n = 11$). AP firing recommenced in response to depolarization induced by current injection (d, Arrow).

water immersion objective. ACSF was perfused for at least 10 min before experiments commenced. The Oregon green fluophore was excited using a 488 nm 20 mW Argon laser with intensity set to 3% and recordings made using a 522 nm emission filter. JC-1 experiments were made using the Argon laser set to 1% intensity with a filter combination of 488 nm for excitation and 522 nm for emission to record the green fluorescence and 514 nm for excitation and 585 nm for emission to record the red fluorescence. Relative fluorescence plots were analysed offline using ImageJ software.

2.7. Statistics. All data are presented as mean ± SEM. GraphPad Prism 4.02 was used to prepare graphics. Statistical analysis was performed with SPSS version 17.0 using one-way ANOVA.

3. Results

3.1. Effect of Trolox and DTT on Spontaneous Firing and Voltage-Dependent Currents of LC Neurons. We first examined the effects of antioxidants on spontaneous firing in LC neurons. Application of 100 μM Trolox had no significant effect on 6 of 11 neurons (Figure 1(a)). Curiously, under the same conditions, 2 neurons increased their firing rate (Figures 1(a) and 1(c)) and 3 neurons exhibited strong hyperpolarization, which abolished spontaneous firing. In such cases firing could be reinstated by repolarizing the membrane potential via current injection (Figures 1(a) and 1(d)). Comparison of averaged action potentials (APs) from test (100 μM Trolox) and control (ACSF) neurons showed that there were no differences in AP shape after 180 s of treatment (Figures 1(a) and 1(b)). The electrophysiological properties of neurons that did not respond and those that hyperpolarized were compared to examine whether the hyperpolarizing neurons were damaged. This comparison showed there were no differences in resting membrane potential, input resistance, firing frequency, and after hyperpolarization amplitude. This indicates that the hyperpolarizing neurons were not simply damaged or unhealthy neurons (See Supplementary Table in Supplementary Material available online at doi:10.1155/2012/820285).

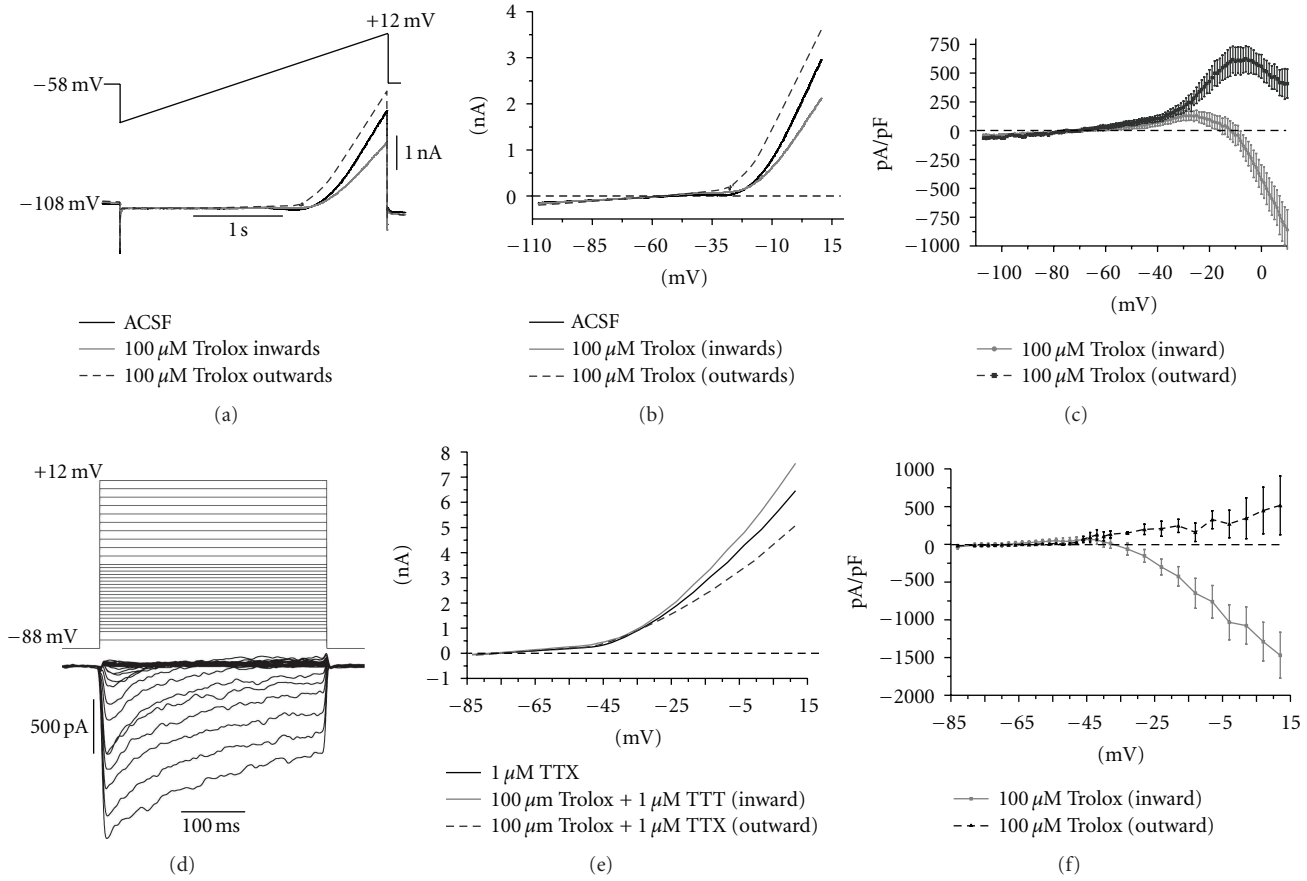


Figure 2: Effect of Trolox treatment in voltage-dependent currents evoked by ramps and pulses. (a) Representative recording of the currents evoked by depolarizing ramps showing the current evoked in ACSF (control solution; solid black line) and the inward (solid light grey) and outward (dashed grey) currents evoked at 180 s after 100 μM Trolox application ($n = 14$). (b) I-V plot for the currents presented in (a). (c) Mean differential (i.e., Trolox-control) with records obtained 180 s after rapid application of Trolox showing the inward (solid light grey, 8 of 14 neurons) and outward (dashed grey, 6 of 14 neurons) currents. (d), (e), and (f) were performed in 1 μM TTX background. (d) Representative depolarizing pulse-evoked differential currents obtained by taking the currents in test solution (i.e., 100 μM Trolox + 1 μM TTX) and subtracting the corresponding currents in control (i.e., 1 μM TTX) 180 s after Trolox application. (e) Averaged I-V plot showing the currents evoked by pulses for control (1 μM TTX—solid black) and the inward (solid light grey) and outward (dashed grey) currents after 180 s of 100 μM Trolox application. (f) Mean differential I-V plots (i.e., Trolox—TTX) inward (solid light grey, 6 of 8 neurons) and outward (dashed grey, 2 of 8 neurons) after 180 s application of 100 μM Trolox. Graph shows mean ± SEM with $n = 8$ for (d), (e) and (f).

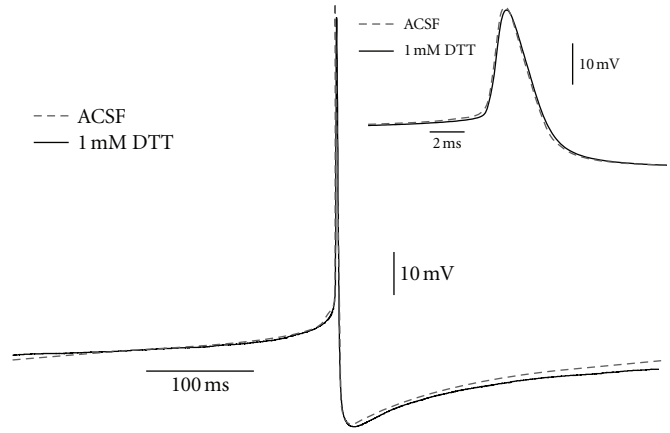
Voltage-dependent currents were first investigated using depolarizing ramps (slope of 40 mV/s) that were designed to mimic the natural depolarization during the interspike interval (see [16]). In these experiments ($n = 14$ LC neurons) Trolox induced a heterogeneous response (Figure 2(a)) with 8 neurons presenting a differential outward current followed by an inward current at depolarized potentials. In contrast, the remaining 6 neurons only exhibited a differential outward current (Figures 2(b) and 2(c)). The second protocol applied voltage steps designed to rapidly activate/deactivate a broad range of ionic channels. When this protocol was applied to neurons held at a hyperpolarized potential of −88 mV, similar results were observed, but in this case, more polarized towards the inward current (6 of 8 neurons, Figures 2(d) to 2(f)). Importantly, the step pulse protocols only generated either an outward or inward current (Figure 2(f)), versus the presence of both currents for ramp protocols (Figure 2(c)). These results indicate that a mix of different voltage-dependent channels are directly modulated by Trolox and/or by reactive species chelated by this antioxidant, and this effect is heterogeneous within the population of LC neurons.

Dithiothreitol (DTT; 1 mM) also produced varied effects on spontaneous firing activity of 19 LC neurons (Figure 3(a)). AP firing was not significantly altered in 10 neurons and was abolished in 4 due to marked hyperpolarization (Figure 3(c)), and 3 neurons ceased AP firing with no apparent hyperpolarization (Figures 3(a) and 3(d)). A small portion of the neurons (2/19) increased their AP firing rate after depolarization (2 of 19; Figure 3(a)). AP comparison demonstrated no differences in AP shape (Figure 3(b)) as was the case for Trolox.

The voltage-dependent current responses that were observed after DTT application were more heterogeneous than

Impact on spontaneous firing		Action potential values		
	1 mM DTT		ACSF	1 mM DTT
No effect	10 of 19	AP amplitude (mV)	95 ± 1.2	95.9 ± 1
Increased (depolarization)	2 of 19	AHP amplitude (mV)	24.9 ± 0.3	26.2 ± 0.2
Stopped (hyperpolarization)	4 of 12			
Stopped (no hyperpolarization)	3 of 12			

(a)



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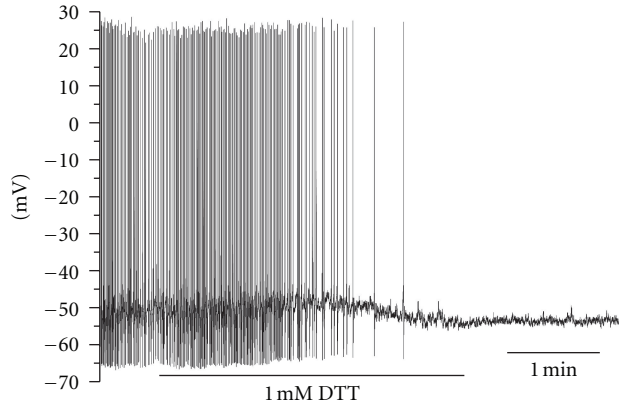

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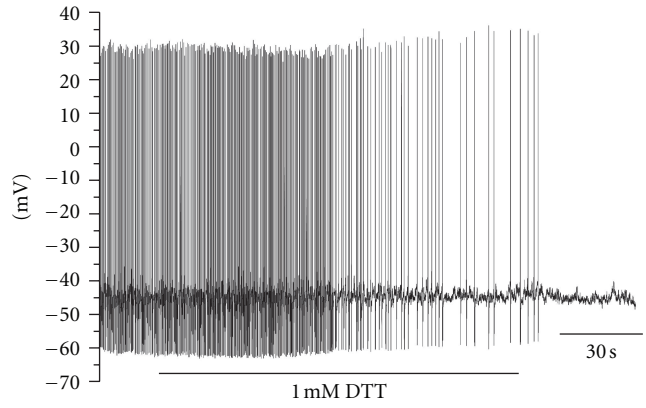

(d)

Figure 3: Impact of DTT treatment in the pacemaker process of LC neurons. (a) Table demonstrating the effect of 1 mM DTT in the spontaneous firing of LC neurons and comparison of values obtained for AP before (ACSF) and 180 s after 1 mM DTT treatment ($n = 19$ for spontaneous firing and $n = 28$ for AP comparison). (b) Averaged APs demonstrating comparison before (ACSF) and after 180 s after 1 mM DDT treatment ($n = 28$). Hyperpolarizing group was excluded from comparison demonstrated in (a) and (b) due to lack of APs at 180 s treatment. (c) and (d), Example for recordings demonstrating the two minor effects induced by 1 mM DTT where neurons hyperpolarized and ceased firing (c) and stopped firing without hyperpolarization (d) ($n = 19$).

those for Trolox application. Application of depolarizing ramps to 9 LC neurons indicated that 7 exhibited a large inward current at depolarized potentials with minimal if any initial outward current, and 2 neurons exhibited only outward currents (Figures 4(a) to 4(c)). Voltage step protocols applied to further 10 LC neurons produced the same characteristic depolarization-induced responses with 5 exhibiting inward and 5 exhibiting outward differential currents (Figures 4(d) to 4(f)). The size of the currents induced by both voltage ramp and step protocols during DTT treatment was larger than the currents induced by Trolox treatment (compare Figures 2(c) and 2(f) with Figures 4(c) and 4(f)).

3.2. Effect of Trolox and DTT Cotreatment with the Mitochondrial Protonophore CCCP during Ramp-Elicited Currents. We next investigated a role of mitochondria in the antioxidant actions as these organelles are the main source of reactive oxygen species in neurons and also play an important role in Ca^{2+} buffering [21]. To do this, we perturbed mitochondrial metabolism using the mitochondrial protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) without and with cotreatment with Trolox or DTT.

Ramp protocols were used as they can reveal voltage-dependent currents, including those that flow during the interspike interval [16]. CCCP (1 μM) induced an initial differential outward (up to ~ −20 mV) followed by an inward current during the ramp depolarization (Figure 5). Cotreatment with Trolox (100 μM) caused a small reduction in the CCCP-induced differential outward current between membrane potentials ~ −40 mV to ~−30 mV (Figure 5(a)). DTT cotreatment in contrast had a more profound impact on the CCCP-induced differential outward current. It partially reversed the outward current and reduced it to less than half at its peak (Figure 5(b)). DTT co-treatment also shifted the reversal potential from ~80 mV to ~−60 mV, suggesting recruitment of different channels and/or modulation of the channel's selectivity to ions. These results

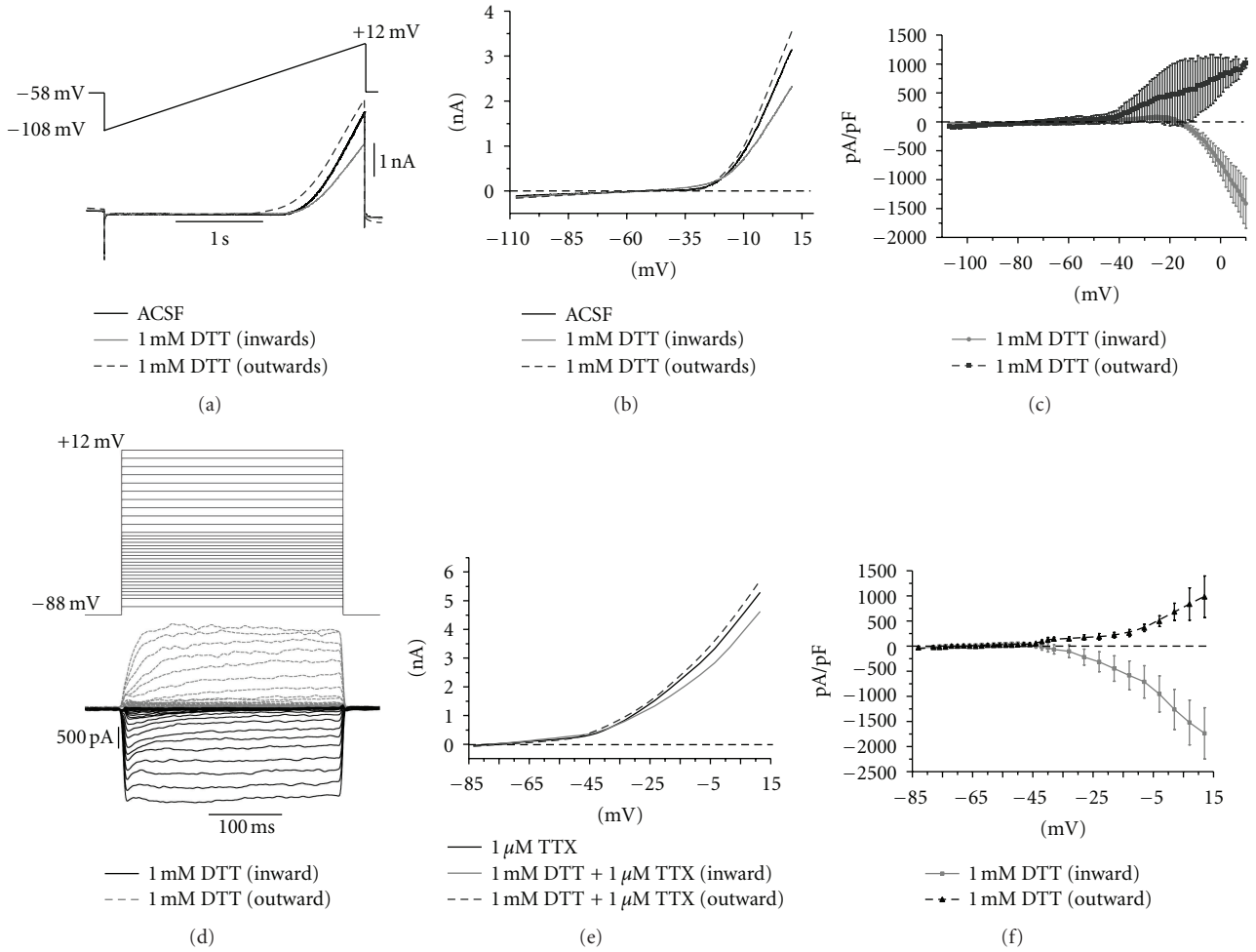

Figure 4: Effect of DTT treatment on voltage-dependent currents evoked by ramps and pulses. (a) Representative recording of the currents evoked by depolarizing ramps showing the current evoked in ACSF (control solution; solid black line) and the inward (solid light grey) and outward (dashed grey) currents evoked at 180 s after 1 mM DTT application ($n = 9$). (b) *I-V* plot for the currents presented in a. (c) Mean differential (i.e., DTT-control) with records obtained 180 s after rapid application of DTT showing the inward (solid light grey, 7 of 9 neurons) and outward (dashed grey, 2 of 9 neurons) currents. (d), (e), and (f) were performed in 1 μM TTX background. (d) Representative depolarizing pulse-evoked differential currents obtained by taking the currents in test solution (i.e., 1 mM DTT + 1 μM TTX) and subtracting the corresponding currents in control (i.e., 1 μM TTX) 180 s after DTT application. (e) Averaged *I-V* plot showing the currents evoked by pulses for control (1 μM TTX—solid black) and the inward (solid light grey) and outward (dashed grey) currents after 180 s of 1 mM DTT application.(f) Mean differential *I-V* plots (i.e., DTT—TTX) inward (solid light grey, 5 of 10 neurons) and outward (dashed grey, 5 of 10 neurons) after 180 s application of 1 mM DTT. Graph shows mean ± SEM with $n = 10$ for (d), (e), and (f).

suggest that the antioxidants Trolox and DTT can affect mitochondrial function either directly or by altering existing neuronal free radicals.

3.3. Impact of Trolox and DTT Treatment on Cytosolic Ca^{2+} and Mitochondrial Membrane Potential under Control Conditions and When Mitochondrial Function Is Impaired. Due to the heterogeneous nature of the responses produced by the two antioxidant treatments, we next investigated if the activation of different currents was related to the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in both control conditions and those where mitochondrial function was impaired. Figure 6(a) demonstrates that under control conditions, neither Trolox or DTT treatments were able to increase $[Ca^{2+}]_c$, suggesting that Ca^{2+}-activated pathways are not involved in the induction/modulation of ionic currents by these antioxidants. When CCCP was added to impair mitochondrial function, Trolox (100 μM) caused a further enhancement of the CCCP-induced increase in $[Ca^{2+}]_c$ (Figure 6(b)). In contrast, cotreatment with DTT (1 mM) had no impact on the CCCP-induced increase in $[Ca^{2+}]_c$ (Figure 6(b)). The consequences of the Trolox-induced increase in $[Ca^{2+}]_c$ are unclear given that Trolox was less effective than DTT in modulating CCCP-related voltage dependent currents (Figure 5(a)).

Given that mitochondrial function is generally dependent on mitochondrial membrane potential (Ψm) [22], we

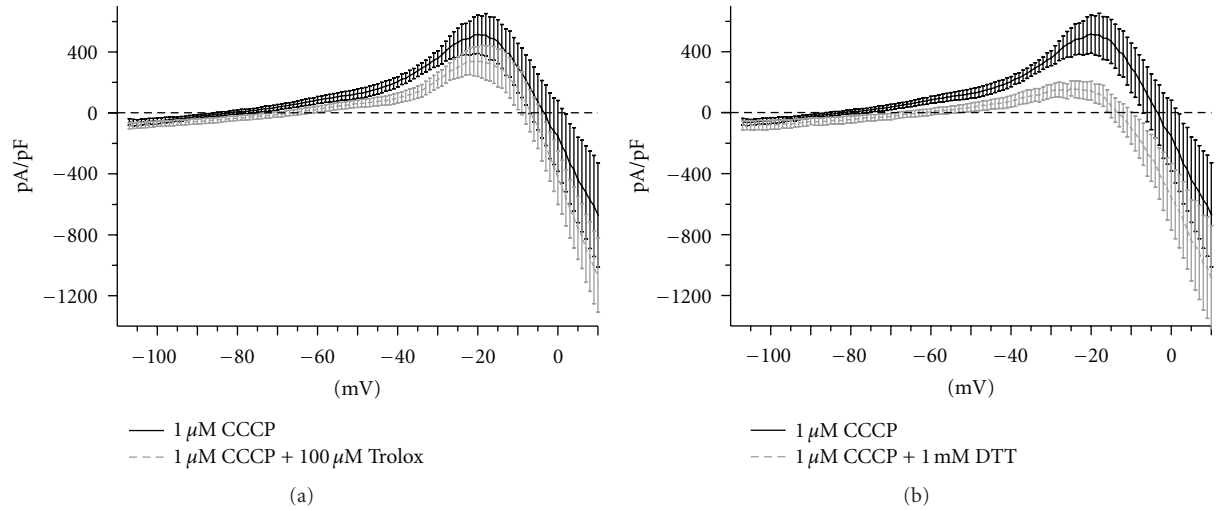

Figure 5: Effect of Trolox and DTT treatment on ramp-evoked currents when mitochondrial membrane potential was impaired with CCCP. (a) and (b), Mean differential I-V plots (i.e., Antioxidants-control) demonstrating the impact of 100 μM Trolox (a) and 1 mM DTT (b) cotreatments with 1 μM CCCP. Graph shows mean ± SEM with $n = 13$ for (a) and $n = 6$ for (b).

investigated whether antioxidant treatments were able to modulate Ψm. Application of CCCP (1 μM) reduced Ψm (Figure 6(c)). Trolox (100 μM) or DTT (1 mM) also reduced Ψm to an extent that was indistinguishable from that produced by impairing mitochondrial function with CCCP alone (Figure 6(c)). Application of the antioxidants together with CCCP appeared to be synergistic as it caused a profound decrease Ψm, with the effect of being most pronounced for DTT where a ~70% reduction in Ψm occurred (Figure 6(c)). Importantly, fluorescence levels partially recovered to the same levels after 10 min of washout for both antioxidants in control and/or impaired conditions (Figure 6(c)). Taken together, these results suggest that mitochondria could be involved in the currents induced by Trolox and DTT treatments, but such actions are unlikely to be dependent on $[Ca^{2+}]_c$.

4. Discussion

Many neuronal populations in the CNS are spontaneously active, including nuclei such as the LC and Substantia nigra (SN) [23, 24]. Such sustained AP firing is tightly controlled and, given that nuclei such as the LC project widely over the brain [25], has major implications for brain function.

Free radicals and oxidative stress are known to be involved in the initiation and/or progression of a variety of neurological conditions, such as Alzheimer's and Parkinson's diseases [26–28], and disturbances in mitochondrial metabolism seem to be a key event in the occurrence of such pathologies [29]. Here, we demonstrate that antioxidant treatment of LC neurons, a neuronal population that is suggested to be involved in progression of many neurological diseases [30, 31], produced a heterogeneous response on the pacemaking activity in these neurons. Our results indicate that within the LC there is a subpopulation of neurons, which are more sensitive to free radical content and consequent modulation of the pacemaker activity. This opens the possibility that this neuronal subpopulation is particularly vulnerable and likely to undergo apoptosis or necrosis during the onset of specific neurological diseases.

The LC is the largest concentration of noradrenergic neurons in the brain, and it projects to and releases noradrenaline across a multitude of brain regions [32, 33]. LC neuron involvement in neurological pathologies, especially Parkinson's disease (PD), has been proposed because of the high levels of LC neuron degeneration observed in PD patients and because the LC plays a fundamental role in the early stages of the disease [31, 34]. Further support for this hypothesis is provided by the fact that lesions to LC neurons increase the sensitivity of SN neurons to a range of insults [35, 36] and that simultaneous partial destruction of SN and LC neurons slows recovery in animal models of Parkinson's disease [30]. Furthermore, pharmacological stimulation of LC neurons imparts some resistance to SN neurons in animal models; Parkinson's disease [37] and genetically modified animals with increased noradrenergic innervation appear to be relatively immune to insults on SN neurons [38]. Therefore neuronal death in the LC may be a primary factor in the onset of PD with the consequent loss of noradrenergic signalling contributing to the loss of dopaminergic neurons in the SN. The fact that oxidative stress is one of the main causes of neuronal death in PD [39] makes the finding that there are subpopulations of LC neurons that are highly sensitive to free radicals of considerable interest.

Our results indicate that both Trolox and DTT induce heterogeneous responses in LC neuronal pacemaking (Figures 1–4), suggesting a differential sensitivity to antioxidants (or free radical content) in neuronal subpopulations within the LC. The majority of the neurons exhibited no detectable alteration in their firing rates (Figures 1(a) and 3(a)). However, Trolox and DTT revealed two other responses and by

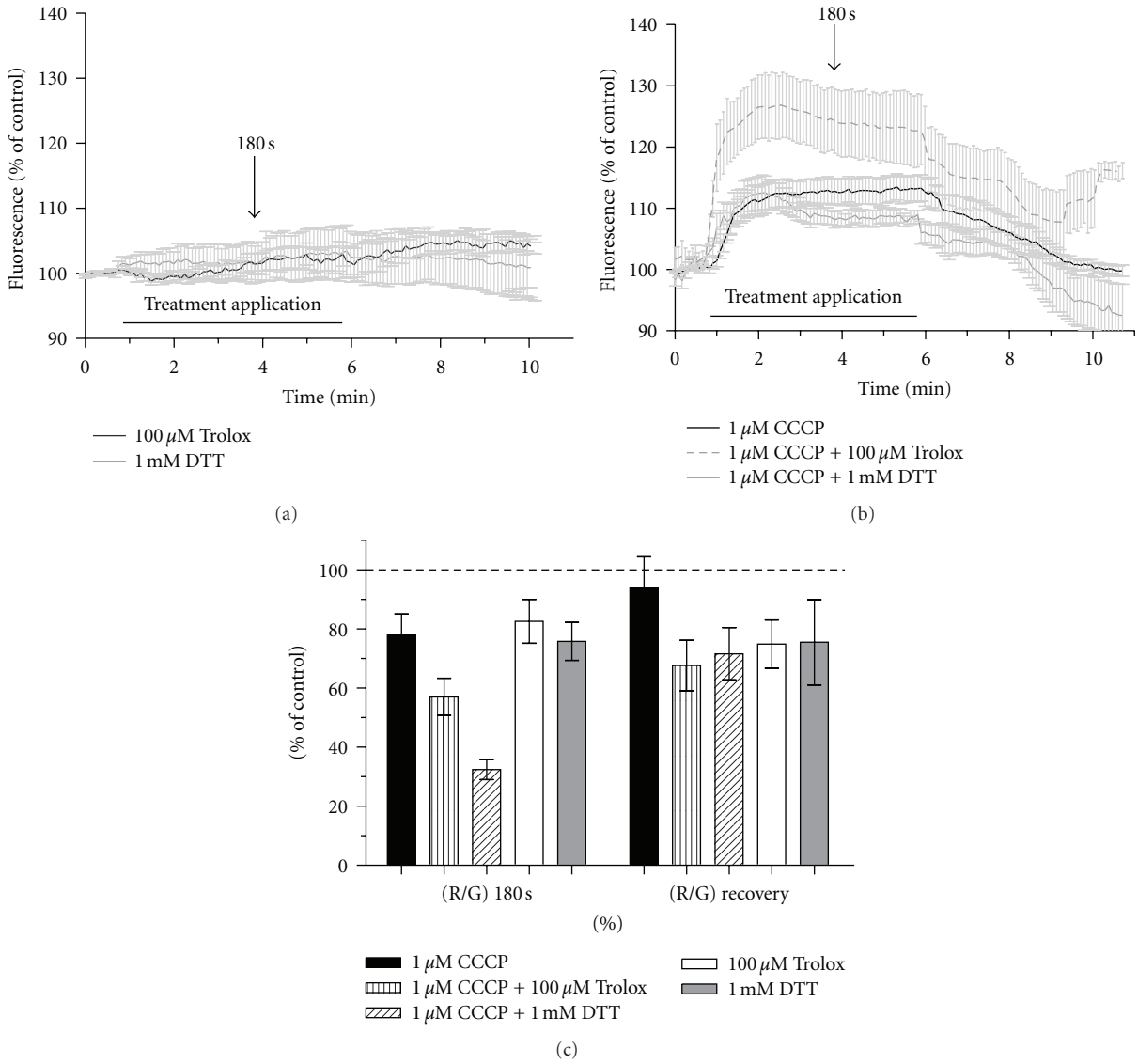

Figure 6: Effect of Trolox and DTT treatment in $[Ca^{2+}]_c$ and Ψm in control and impaired conditions. (a) Graph demonstrating that both antioxidants did not impact on $[Ca^{2+}]_c$ in control conditions ($n = 5$ for both). (b) Graph demonstrating that 100 μM Trolox co-treatment with 1 μM CCCP increased $[Ca^{2+}]_c$ and 1 mM DDT did not effect the normal CCCP-induced increase in $[Ca^{2+}]_c$ ($n = 6$ for Trolox $n = 5$ for DTT cotreatments, and $n = 7$ for CCCP by itself). (c) JC-1 fluorescence demonstrating the impact of Trolox and DTT in Ψm in control and impaired (1 μM CCCP cotreatment) conditions ($n = 8$ for Trolox and $n = 9$ for DTT by themselves; $n = 12$ for Trolox and $n = 10$ for DTT cotreatments; $n = 9$ for CCCP by itself). All graphs show mean ± SEM.

inference neuronal subpopulations: the first featured hyperpolarization and slowing down or cessation of pacemaking activity, whereas the second caused depolarization and an increase in firing rate (Figures 1 and 3). These observations are surprising given that LC neurons have generally been considered to be fairly homogeneous with respect to their electrophysiological properties, and previous results from our group detected no electrophysiological differences in the neuronal population on which our experiments were made [14, 16, 40]. Voltage clamp experiments confirmed the heterogeneity we observed in our experiments on LC neurons, as application of the antioxidants also revealed two types of responses when ramps or pulses were applied (Figures 2 and 4). Another important finding came from our Ca^{2+} imaging experiments where, in contrast, application of antioxidants produced no change in $[Ca^{2+}]_c$ (Figure 6(a)). This suggests that the effects we observed were caused by changes in the neuronal free radical content and not by changes in $[Ca^{2+}]_c$. Trolox treatment had a small impact on voltage-dependent currents (Figure 5(a)) however DTT treatment had a profound impact. DDT partially reversed the currents induced by CCCP coapplication (Figure 5(b)) and this effect was unlikely to be mediated by $[Ca^{2+}]_c$ (Figure 6(b)). A role for mitochondria was indicated as the antioxidants altered Ψm when applied alone or combined with CCCP (Figure 6(c)).

The finding of two neuronal subpopulations within LC that are responsive to antioxidant treatment may be of considerable importance for our understanding of the environment and physiological conditions that lead to development of neurological diseases. It is difficult to delineate which subpopulation will be most adversely affected by changes in free radical content. The subpopulation that responded to antioxidants with hyperpolarization will be protected by antioxidants, as pacemaking and hence firing rate would slow down or stop, thus saving energy and accelerating recovery time in cases of hypoxia [41]. Abolition of firing would also prevent activation of voltage-dependent Ca^{2+} channels and the subsequent increase in $[Ca^{2+}]_c$. This would slow activity in all Ca^{2+}-dependent pathways.

Control of $[Ca^{2+}]_c$ is of fundamental importance given apoptotic and/or necrotic pathways can be initiated by uncontrolled increases in $[Ca^{2+}]_c$ [42, 43]. In contrast, the neuronal subpopulation where antioxidants produced depolarization and elevated firing rates would be stressed by such treatment. We do not know the mechanism whereby some cells respond with inward or outward currents following antioxidant application. However, if these actions were due to altering free radical content, then "mopping up" free radicals may not necessarily protect cells. Given that specific neurological pathologies have been associated with an increase in free radicals, then it highlights the fact that antioxidant treatments may need to be carefully evaluated.

In summary, our findings indicate that there is a small neuronal subpopulation within LC that responds to antioxidants, and, on the basis that this is caused by altering free radicals, members of this subpopulation may be more subject to apoptosis or necrosis. We believe that the results presented here represent an important step for our understanding of how free radicals contribute to neuropathologies (such as LC loss in Parkinson's diseases) and suggest that antioxidant therapies must be carefully evaluated before administration.

Abbreviations

ACSF: Artificial cerebrospinal fluid
AP: Action potential
$[Ca^{2+}]_c$: Cytosolic Ca^{2+} concentration
CCCP: Carbonyl cyanide m-chlorophenyl hydrazone
CNS: Central nervous system
DTT: Dithiothreitol
LC: Locus coeruleus
PD: Parkinson's disease
TTX: Tetrodotoxin
Vm: Membrane potential
Ψm: Mitochondrial membrane potential.

Acknowledgments

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