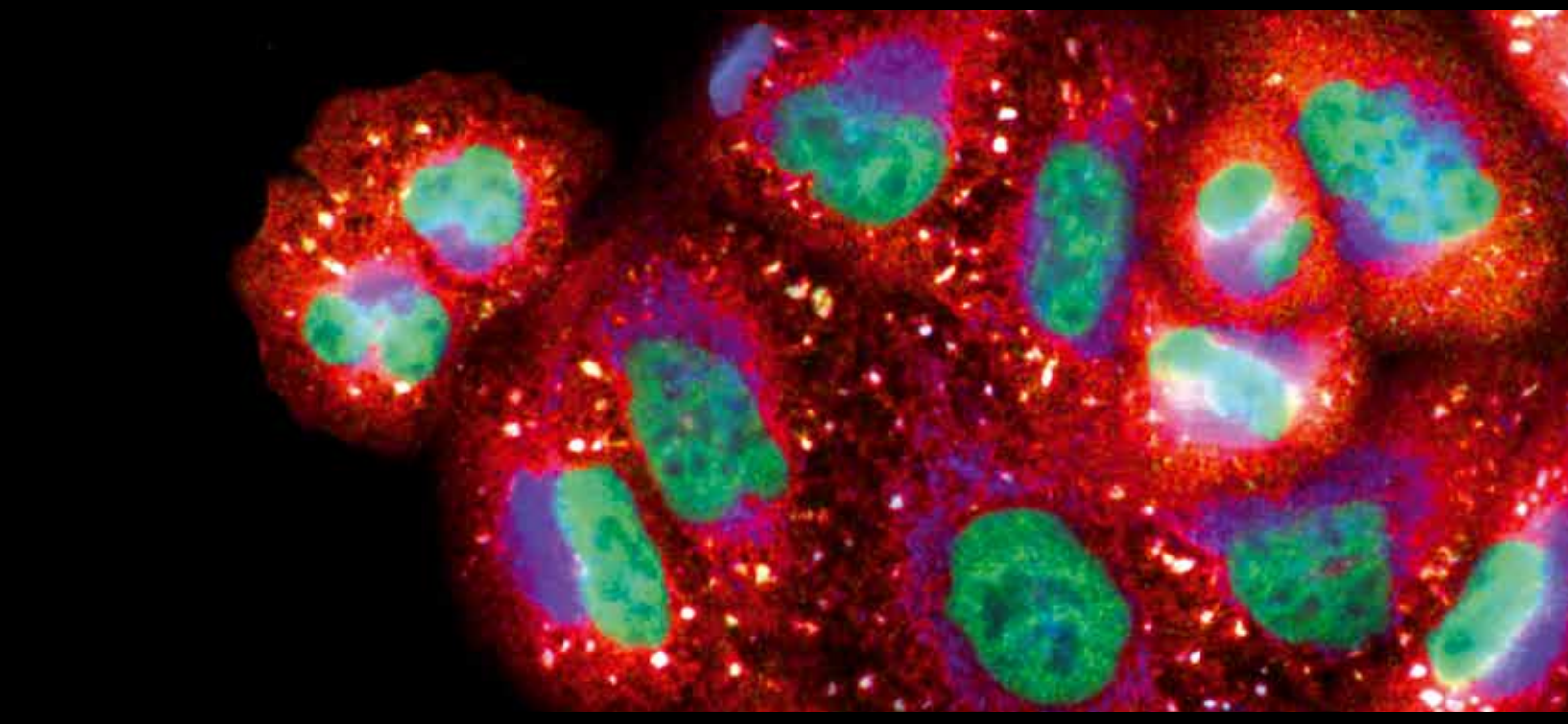
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Neurodegenerative Diseases and Ageing

Guest Editors: Marcos D. Pereira, Krzysztof Ksiazek, and Regina Menezes



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Editorial
Oxidative Stress in Neurodegenerative Diseases and Ageing

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Since Denham Harman has proposed the free radical theory of ageing, the knowledge and the understanding of the mechanisms of ageing process have increased remarkably. Over decades, this unavoidable phenomenon has also been linked, both causatively and consequently, with the development of several abnormalities, currently known as the "age-related diseases." Oxidative Medicine and Cellular Longevity dedicates this special issue to illustrate the foremost findings concerning the role of oxidative stress in the vital aspects of a progressive dysfunction of human body during ageing. The purpose of this thematic issue is to provide an up-to-date text covering the most recent discoveries in the area, focusing on a deeper understanding of the signaling mechanisms that trigger neurodegenerative diseases, a multidisciplinary approach combining several scientific areas and finally an issue for a broad group of professionals, including physicians, pathophysiologists, biologists, biochemists, molecular biologists, and geneticists. This special issue provides a selection of review as well as original articles that emphasize the source and the engagement of free radicals in ageing and neurodegenerative diseases, other systemic diseases (e.g., Niemann-Pick disease type C and cancer), the biomarkers of oxidative radicals, the characterization of mechanisms underlying an abnormal iron metabolism, oxidative stress signaling related to age-related diseases, the emerging issue of the consequences of a mobile phone radiation, and the genetic link between the efficacy of DNA repair and the development of renal failure. The new therapeutical approaches for preventing age-related diseases and the use of different and relevant experimental models to decipher the relevance of oxidative stress to age-related neurodegenerative diseases will be also presented. Collectively, we hope that the scientific problems covered by this special issue highlight the advances of seminal Harman's theory stimulating the creation of a platform for a fruitful cooperation and discussion within the scientific community.

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Review Article

Oxidative Stress and Epilepsy: Literature Review

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Backgrounds. The production of free radicals has a role in the regulation of biological function, cellular damage, and the pathogenesis of central nervous system conditions. Epilepsy is a highly prevalent serious brain disorder, and oxidative stress is regarded as a possible mechanism involved in epileptogenesis. Experimental studies suggest that oxidative stress is a contributing factor to the onset and evolution of epilepsy. *Objective*. A review was conducted to investigate the link between oxidative stress and seizures, and oxidative stress and age as risk factors for epilepsy. The role of oxidative stress in seizure induction and propagation is also discussed. *Results/Conclusions*. Oxidative stress and mitochondrial dysfunction are involved in neuronal death and seizures. There is evidence that suggests that antioxidant therapy may reduce lesions induced by oxidative free radicals in some animal seizure models. Studies have demonstrated that mitochondrial dysfunction is associated with chronic oxidative stress and may have an essential role in the epileptogenesis process; however, few studies have shown an established link between oxidative stress, seizures, and age.

1. Introduction

Oxidative stress (OS) is the condition that occurs when the steady-state balance of prooxidants to antioxidants is shifted in the direction of the former, creating the potential for organic damage. Prooxidants are by definition free radicals, atoms, or clusters of atoms with a single unpaired electron [1].

Initially, oxidative stress was described as an imbalance between generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive species were originally considered to be exclusively detrimental to cells, but now it is considered that redox regulation involving ROS is essential for the modulation of critical cellular functions (mainly in astrocytes and microglia), such as mitogene-activated protein (MAP) kinase cascade activation, ion transport, calcium mobilization, and apoptosis program activation [2].

Oxidative stress has been shown to be associated with alterations in ROS, RNS, and nitric oxide (NO) signaling pathways, whereby bioavailable NO is decreased and ROS and RNS production are increased [3]. Oxidative and nitrosative stress pathways are induced by inflammatory

responses, and subsequent mitochondrial metabolic processes generate highly reactive free radical molecules. Indeed, ROS and RNS consist of active moieties that can react with other substrates. Examples of ROS and RNS are superoxide anion, hydroxyl radical, and peroxynitrite. Under physiological conditions defense pathways counterbalance ROS and RNS production, thus in these conditions reactive species have physiological roles that include signaling. In conditions of excessive production or if body defenses are compromised, ROS and RNS may react with fatty acids, proteins, and DNA, thereby causing damage to these substrates [4].

Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, are defined by progressive loss of specific neuronal cell populations and are associated with protein aggregates. A common feature of these diseases is extensive evidence of oxidative and nitrosative stress (O&NS), which might be responsible for the dysfunction or death of neuronal cells which contributes to disease pathogenesis [4, 5].

These neurodegenerative diseases affect distinct population groups: children, young adults, and the elderly. These diseases are much more prevalent in the elderly as a result of aging, environmental factors and to a lesser extent genetic factors [6].

Age, in turn, is an independent risk factor both for neurodegenerative diseases and for epilepsy [7, 8]. Epilepsy occurs in about 1% of patients aged over 65 years (about one quarter of newly diagnosed epilepsies) [8–13]. In this population, poststroke epilepsy is predominant, but tumor-associated, traumatic and neurodegenerative pathologies are also commonly associated with epilepsy. [8–14]. In some conditions such as stroke, trauma, or a tumor, the association with the onset of epilepsy may be immediately apparent. However, with insidious neurodegeneration with no clear markers of disease, the link with epilepsy may be less obvious.

Thus, given the fact that (i) old age is an important risk factor for epilepsy and neurodegenerative disorders, (ii) neurodegenerative disorders are risk factors for epilepsy, and (iii) O&NS are related to both pathological conditions (epilepsy and neurodegenerative disorders), we decided to conduct a literature review of studies regarding O&NS and age as risk factors for epilepsy and also discuss the role of O&NS pathways in seizure induction and propagation.

2. Oxidative and Nitrosative Stress

Oxidative stress is defined as an imbalance between oxidants (free radicals); nitrosative stress (NS) refers to processes in which the fluxes of NO become high enough to result in nitrosation of amines and thiols and antioxidants which results in a relative or actual excess of oxidative species and this leads to disruption in signaling, redox control, and/or molecular damage [15]. Free radicals consist of chemical structures which contain one or more unpaired electrons in their outer layer. This property is associated with a highly reactive state and a propensity for chemical reactions. In 1956, Harman proposed the "free radical theory" of the ageing process. He suggested that free radicals produced during aerobic respiration had damaging effects on cell components and connective tissues, causing cumulative damage which results in the process of ageing and eventually death. He initially speculated that free radicals were probably produced by reactions involving molecular oxygen and catalyzed in cells by oxidative enzymes [16]. In 1972, Harman included the involvement of mitochondria in physiological ageing processes. Approximately 90% of all oxygen in a cell is consumed in the mitochondrion, especially in the inner membrane where oxidative phosphorylation occurs [17]. Oxygen is involved in the oxidation of organic compounds and the production of energy for cell metabolism. However, only a very small amount of consumed oxygen (between 2 and 5%) is reduced, which leaves a variety of highly reactive chemicals known as oxygen-free radicals or ROS, as well as RNS. The production of free radicals is associated with damage caused to cell structures and the pathogenesis of central nervous system (CNS) conditions, such as Parkinson's disease, stroke, dementia, and epilepsy [18, 19]. The CNS is highly sensitive to O&NS due to its high oxygen consumption and the low activity of antioxidant defenses [20, 21].

The CNS has an extraordinary metabolic rate consuming approximately 20% of all inhaled oxygen at rest; however, it only accounts for 2% of body weight [22]. This enormous metabolic demand is due to the fact that neurons are highly differentiated cells and need large amounts of ATP in order to maintain ionic gradients across cell membranes and for neurotransmission. Since most neuronal ATP is generated by oxidative metabolism, neurons depend critically on mitochondrial function and on oxygen supply [23].

The mitochondria have critical functions which influence neuronal excitability, including the production of adenosine triphosphate (ATP), fatty acid oxidation, excitotoxicity, apoptosis and necrosis control, amino acid cycle regulation, biosynthesis of neurotransmitters, and regulating the homeostasis of cytosolic calcium. Mitochondria are the main site of ROS production and are therefore extremely vulnerable to oxidative damage [24].

3. Reactive Oxygen Species and Reactive Nitrogen Species

Hydroxyl ($HO^{\bullet}$) is the most damaging free radical to cells. It is unstable, with an average life of milliseconds, and therefore it is rarely captured *in vivo*. These radicals often attack molecules by hydrogen abstraction and addition to unsaturation. Intensive and frequent attacks promoted by this radical cause damage to DNA, RNA, proteins, lipids and cell membranes of the nucleus and mitochondria [25, 26].

Superoxide ($O_2^{-\bullet}$) production occurs mainly inside the mitochondrion during the electron transport chain (ETC) when a small number of electrons escape forming $O_2^{-\bullet}$ anion. Measurements of submitochondrial particles demonstrate that between 1–3% of all ETC electrons escape to generate $O_2^{-\bullet}$ instead of contributing to reduce oxygen to water. ETC complexes I and III are responsible for producing $O_2^{-\bullet}$ [27]. Superoxides are relatively unstable, with a half-life

of only milliseconds. Because they are charged, they do not easily cross cell membranes although it may reduce ionic iron and its protein complexes and cause damage to amino acids or loss of protein function [28]. On the other hand, hydrogen peroxide (H_2O_2) molecules do not contain an unpaired electron and thus they are not a free radicals species. In physiological conditions, the production of H_2O_2 is estimated to account for about ~2% of the total oxygen uptake by the organism [25]. Although H_2O_2 is not a free radical, it is extremely harmful because it works as an intermediate in $HO^{\bullet}$ producing reactions, such as Fenton's reaction [29]. Hydrogen peroxide has a long half-life and is able to cross several lipid layers and react with transition metals and some hemoproteins. It can also induce chromosomal alterations, break the deoxyribonucleic acid (DNA) column and, in the absence of catalysts, oxidize sulfhydryl compounds (–SH) [30].

Nitric oxide is a relatively abundant free radical that operates as an important biological signal in several physiological processes, including neurotransmission, blood pressure regulation, body defense mechanisms, smooth muscle relaxation, and immune regulation [25]. The NO has low reactivity with most biomolecules but reacts easily with other free radicals. Nitric oxide is not sufficiently reactive to attack DNA directly, but it may react with $O_2^{-\bullet}$ produced by phagocytes generating peroxynitrite.

Peroxynitrite, on the other hand, is the product of the diffusion-controlled reaction of NO with $O_2^{-\bullet}$ radical. Peroxynitrite is a short-lived oxidant species that is a potent inducer of cell death [31]. As for NO, it may undergo secondary reactions forming agents that may nitrate aromatic amino acids, for instance, tyrosine generating nitrotyrosine and DNA bases, especially guanine [32].

The harmful effects of free radicals to the organism induce several defense mechanisms against O&NS. Such mechanisms include removal of free radicals by catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and nonenzymatic antioxidants [33]. Under normal conditions there is a balance between O&NS and antioxidant action, both with respect to action at the intracellular level. This is essential for organism survival and health [27, 34].

4. Enzymatic Antioxidants

The role of SOD is to protect aerobic cells against $O_2^{-\bullet}$ action. It catalyzes $O_2^{-\bullet}$ dismutation reaction into H_2O_2 and $O_2^{-\bullet}$. There are three known types of SOD: copper-zinc SOD (CuZnSOD), manganese SOD (MnSOD), and extracellular SOD (ECSOD) [33].

Copper-zinc SOD is present mainly in cytoplasm and in some organelles called peroxisomes. This enzyme specifically catalyzes the dismutation of $O_2^{-\bullet}$ anion into H_2O_2 and $O_2^{-\bullet}$ in a pH-independent medium (5–9.5) [35]. Manganese SOD is the mitochondrial form of this dismutase. Its active site contains manganese and reduces the $O_2^{-\bullet}$ generated during the ETC. The amount of MnSOD inside the cell varies according to the number of mitochondria found in each cell type. This enzyme has antitumor activity [25, 33]. Extracellular SOD also contains copper and zinc in its structure and is the main extracellular SOD. It is synthesized inside the cells and secreted into the extracellular matrix [36].

Catalase is an enzyme that reacts very effectively with H_2O_2 to form water and molecular oxygen and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity. Catalase protects cells against H_2O_2 generated inside them. Although CAT is not essential to some cell types under normal conditions, it has an important role in the acquisition of tolerance to O&NS in cellular adaptive response [36, 37].

Glutathione peroxidase is an enzyme that contains a single selenocysteine (Sec) residue in each of four identical subunits, which are essential to the enzyme's activity. Humans have four different GPX types: (1) a classic cytosolic form; (2) a membrane-associated GPx phospholipid H_2O_2; (3) another cytoplasmic enzyme, gastrointestinal GPx; and (4) an extracellular type. All GPx enzymes are known to add two electrons to reduce peroxides by selenols forming (Se-OH) [28]. GPX antioxidant properties allow them to eliminate peroxides as potential substrates for Fenton's reaction. Glutathione peroxidase works together with glutathione tripeptide (GSH), which is present in cells in high (micromolar) concentrations. The substrate for the GPx catalytic reaction is H_2O_2 or organic peroxide ROOH. Glutathione peroxidase catalyzes hydroperoxide reduction using GSH, thus protecting mammalian cells against oxidative damage. Glutathione metabolism is one of the most important antioxidant defense mechanisms [25, 34, 36]. Together with classic H_2O_2-removing enzymes (CAT and GSH-Px), the enzyme thioredoxin reductase (TrxR) is a selenoflavoprotein which forms the thioredoxin system together with the protein thioredoxin (Trx) and NADPH. This is an effective system to reduce proteins in disulfide form and it also participates actively in the removal of H_2O_2 and other peroxides [38]. Thioredoxin reductase catalyzes the reduction of Trx especially, but in humans it can also reduce other substrates, such as vitamin C. This reductase also catalyzes the reduction of disulfide proteins and it is involved in countless vital processes, such as DNA synthesis and the regulation of apoptosis [39]. Additionally, this system also donates electrons during DNA synthesis [40], and Bjornstedt et al. have discovered that NADPH and human TrxR by themselves or with Trx are efficient electron donors to this human plasma peroxidase [41], which allows this enzyme to reduce hyperoxides even when there are low levels of GSH available [42].

There are three identified TrxR isozymes: cytosolic (TrxR-1), mitochondrial (TrxR-2), and a third isoenzyme which has been isolated from the mitochondrion of rat testes (TrxR-3) [43]. TrxR-1 has a wide substrate specificity, since it is responsible for reducing not only Trx but also hydroperozides [44], lipoic acid, ubiquinone, and dehydroascorbate [43]. Thus, the Trx system is regarded as having a crucial role maintaining a cell's redox state. It may also have a role in the system which regulates the expression of redox-sensitive genes through the activation of transcription factors [43].

Coenzyme Q10, in turn, is a liposoluble ubiquinone which has a long isoprenoid side-chain. Ubiquinone is an endogenously-synthesized lipid which has a redox function [45]. Although it is unique and specific, coenzyme Q10 is biosynthesized by all cells and is the main component of the internal mitochondrial membrane, Golgi complex membrane and lysosome membrane. However, its concentration in the membrane of low-density lipoprotein (LDL) particles is low [46]. This variation in distribution suggests different functions for different biological membranes. Ubiquinone taken as a food supplement is distributed mainly between the liver and blood plasma; it is not absorbed by membranes which have high concentrations of this compound. Its reduced form ubiquinol-10 (CoQH2) is a hydroquinone which is found predominantly in the heart, kidneys, and liver. Its oxidized form ubiquinone (CoQ10) is abundant in the brain and intestines [47]. Ubiquinone's main function occurs in the internal mitochondrial membrane where it is involved in the electron transport chain and H+ proton translocation in the mitochondrion, together with cytochromes and mitochondrial dehydrogenases. Dehydrogenases oxidize NADH, NADPH, and FADH2 and transfer protons and electrons to ubiquinone, converting it into ubiquinol. The latter then transfers protons to the mitochondrial matrix and electrons to cytochromes. Thus, cytochromes reduce $O_2^{-\bullet}$ to H_2O with electrons and protons from the matrix. This entire process is essential to produce ATP [46].

However, ubiquinone's redox cycle can also transfer unpaired electrons to acceptors that do not take part in the respiratory chain. Ubiquinol's oxidation occurs through the donation of hydrogen to a free radical, thus generating the respective semiquinone. When oxidation continues, it leads to the formation of ubiquinone with final deactivation of two free radicals. Therefore, this compound is high in antioxidant powers through free radical scavenging; it is also efficient interrupting free radical chain reactions. Such activity is limited to the liposoluble medium due to its long side chain [46].

5. Nonenzymatic Antioxidants

Vitamin C is a hydrosoluble antioxidant, which facilitates its diffusion into intra- and extracellular matrices. Its antioxidant potential is related to direct removal of $O_2^{-\bullet}$ and $HO^{\bullet}$. Furthermore, it contributes to regenerating oxidized vitamin E; however, vitamin C also has prooxidant activity. It may be the one compound, in addition to $HO^{\bullet}$, that can convert Fe^{3+} into Fe^{2+}, which then reacts with H_2O_2 to form OH [25, 33].

Vitamin E (α-tocopherol) scavenges the chain-carrying peroxyl radicals rapidly and interrupts the chain propagation [48, 49]. During this reaction, vitamin E becomes a free radical called tocopheryl, which is less reactive than the lipid radical and migrates to the surface of the membrane to be transformed again into tocopherol through the action of ascorbic acid. However, in elevated concentrations the tocopheryl radical may act as prooxidant [25, 33].

On the other hand, β-carotene is a hydrophilic precursor of vitamin A and large concentrations accumulate in the membranes of certain tissues. Its antioxidant activity is related to the removal of $O_2^{-\bullet}$ and free radicals formed during lipid peroxidation [33, 49]. This activity is due to its conjugated double-bonded structure that can dislocate unpaired electrons, which enables β-carotene to physically quench singlet oxygen without degradation [25].

GSH is present in cytosol, in the mitochondrion, as a cofactor in glutathione reduction cycle through hydrogen atom donation during peroxide reduction by GSHPx, transforming into oxidized glutathione (GSSG). GSH in the nucleus maintains the redox status of sulfhydryl proteins which are necessary for DNA expression and repair [50].

Flavonoids have the ideal structure for radical scavenging. They are more efficient antioxidants than vitamins C and E. Flavonoid antioxidant activity depends on its structure and may be determined by five factors: reactivity as a donor agent of H^+ and electrons, stability of formed flavanol radical, reactivity compared with other antioxidants, capacity to chelate transition metals, and solubility and interaction with membranes [48]. Sequestering activity is directly linked to the flavonoid oxidation potential and to the species to be scavenged. The smaller the flavonoid oxidation potential, the greater its activity as a free radical scavenger [48].

6. Epilepsy

Epilepsy is one of the most common and serious brain disorders in the world. It affects at least 50 million people worldwide. Approximately 100 million people will have at least one epileptic seizure during their lifetime. It causes serious physical, psychological, social, and economic consequences [51]. The median prevalence of lifetime epilepsy for developed countries is 5.8 per 1,000 and 10.3 per 1,000 for developing countries [52].

6.1. Epilepsy: Classification and Etiology. Epilepsy can be classified as idiopathic, provoked or symptomatic. Symptomatic epilepsies may have several causes (trauma, tumor, infection, malformation or a systemic genetic disease); provoked seizures are predominantly caused by specific environmental or systemic factors and there are no significant neuroanatomical or neuropathological anomalies. Idiopathic epilepsy is defined as having a predominantly or presumably genetic cause and there are no significant neuroanatomical or neuropathological anomalies [52, 53]. From neuroimaging techniques (computed tomography and magnetic resonance imaging) it is possible to identify the possible structure or anatomy associated with epilepsy, such as tumors, hydrocephalus, congenital lesions, vascular accidents, hippocampal sclerosis. Progress in the field of genetics, with techniques such as the development of sequencing methods, karyotype analysis and DNA amplification methods, has produced the identification of several genes and genetic conditions which include epilepsy in their phenotype. With progress in neuropharmacological studies it is possible to identify

the involvement of neurotransmitters (GABA and glutamate), as well as other alterations in membrane functions, receptors, ionic changes and alteration of neural networks that are involved in epileptogenesis [54].

6.2. Epilepsy and Oxidative and Nitrosative Stress. Production of free radicals has a role in the regulation of biological function, damage to cell structures, as well as in the pathogenesis of central nervous system neurodegenerative diseases, such as Parkinson's disease, stroke, and dementias [18, 19]. Studies suggest that neurodegenerative diseases may develop characteristics of epilepsy with time [55, 56]. Oxidative and nitrosative stress are regarded as possible mechanisms in the pathogenesis of epilepsy [57]. Studies have already verified that status epilepticus changes redox potential and decreases the level of ATP, which can lead to a collapse in brain energy production and supply [58]. Liang and Patel have demonstrated oxidative damage to susceptible targets (protein, lipids, and DNA) caused by persistent seizures (status epilepticus) [59]. Several studies (animal models and genetic studies) have demonstrated an increase in mitochondrial O&NS and subsequent cell damage after persistent seizures [24, 59–64].

Myoclonic epilepsy with ragged red fibers (MERRF) is a rare syndrome characterized by myoclonus, muscle weakness, cerebellar ataxia, heart block, and dementia. MERFF is the first type of epilepsy in which a molecular defect has been identified and linked to the epileptic syndrome [65]. An A to G transition mutation (A8344G mutation) of nucleotide pair 8344 in human mitochondrial DNA (mtDNA) has been identified as the cause of MERRF. This mutation affects the biosynthesis of mitochondrial oxidative phosphorylation proteins [66]. Furthermore, it has been documented that MERRF causes inefficient ATP generation, increased ROS production, and unbalanced genetic expression of antioxidant enzymes [67]. There are data on generalized seizures associated with mitochondrial mutations in several forms of epilepsy including mitochondrial DNA polymerase γ (POLG1) [68] and tRNAPhe (MT-TF) [69]. Several mitochondrial DNA mutations which compromise the mitochondrial respiratory chain or mitochondrial ATP synthesis have been associated with epileptic phenotypes [70].

The use of animal models has made important contributions to our understanding of seizures. For example, the injection of a single dose of the glutamatergic agonist kainic acid (KA) in rats has been shown to provoke status epilepticus (SE). It has been demonstrated that 16 hours after KA injection the enzyme aconitase, which takes part in the Krebs cycle, becomes inactive decreasing the availability of reducing agents, NADH, and FADH2, for the mitochondrial electron transport chain and compromising ATP synthesis [64]. Systemic or intracerebral KA injections may result in consistent epileptic activity. During an experiment in which KA was injected directly into the CA3 area of the hippocampus, an increase in NO synthesis was demonstrated, contributing to cell death by apoptosis in the CA3 area of the hippocampus after the induction of an SE in the experimental temporal lobe [71]. Therefore in the KA induction model there is an increase in ROS production, mitochondrial dysfunction, and apoptosis of neurons in several areas of the brain, especially those in the hippocampus [72]. Another study which used KA in the CA3 region produced seizures and decreased activity of nicotinamide adenine dinucleotide cytochrome c reductase (NCCR), a marker for ETC's complexes I and III. This was observed in the entire hippocampus 180 minutes after induction [73].

Pilocarpine (a muscarininc agonist) is another chemical induction model. Through excitotoxic stimulation it results in excessive ROS production, formation of lipid peroxidation and nitrite in the hippocampus, striatum and frontal cortex. Pilocarpine is regarded as an appropriate model to study temporal lobe epilepsy (ELT). Animals are systematically treated with a dose of pilocarpine which induces an acute crisis of the limbic system. Status epilepticus usually resolves with the administration of diazepam. This acute intoxication is followed by a period of "latency" (i.e., seizure-free), which usually lasts between 1-2 weeks. It is soon followed by a condition of chronic spontaneous seizures, similar to human ELT. From the pathological perspective, animals treated with pilocarpine show alterations that are very similar to hippocampal sclerosis, a condition that is similar to most ELT patients. There is evidence to support an increase in ROS production in SE induced by pilocarpine or KA, producing considerable amounts of $O_2^{-\bullet}$ and overloading endogenous protection mechanisms (GPx, SOD, and CAT). This results in oxidative damage to proteins, phospholipids, and mitochondrial DNA [74]. Furthermore, there are recent data demonstrating the involvement of mitochondrial OS in oxidative damage to DNA, which can occur in different stages of epileptogenesis triggered by pilocarpine or KA [24].

A model with knockout animal shows the connection between OS and epilepsy. It shows the importance of $O_2^{-\bullet}$ endogenous mitochondrial detoxification when an animal (MnSOD-null) has the MnSOD enzyme removed and shows severe pathologies, while animals with MnSOD superexpression (SOD2) have shown better neuronal survival to KA-induced SE [75].

Recently Waldbaum et al. investigated whether acute lesions induced by ROS formation contribute mechanically to the formation of chronic epilepsy. They have questioned whether mitochondrial and cellular alterations might occur during the "latency period" between the initial brain lesion and the appearance of recurring spontaneous seizures, inducing progression to chronic epilepsy. An adaptive increase of mtDNA repair occurs immediately after ROS increase induced by acute SE. However, chronic increase in ROS production is accompanied by failure in the induction of mtDNA repair [76]. Although mitochondrial production of H_2O_2 returns to control levels during the "latency period," measurements of more sensitive OS indexes suggest the occurrence of ongoing OS, especially in the mitochondrial compartment during the "latency period" [24]. Oxidative stress (GSH) markers and specific markers of redox status in the mitochondrion (coenzyme A) have recently been demonstrated to decrease in the hippocampus after lithium-pilocarpine induced SE and to become permanently damaged during epileptogenesis and chronic epilepsy, even when

H_2O_2 production measurements and mtDNA damage return to control levels [73]. This may contribute to significant mitochondrial dysfunction, harming neuronal excitability through ETC dysfunction and decreased ATP production. Damage to mtDNA and abnormal mitochondrial H_2O_2 production has been observed in the hippocampus of rats three months after SE. Such data suggest there is evidence to support the involvement of mitochondrial OS in epilepsy and also suggest that mitochondrial lesions might contribute to epileptogenesis [76]. Such evidence raises an intriguing possibility that mitochondrial dysfunction caused by the production of free radicals may increase susceptibility to seizures [77].

Mitochondrial dysfunction and O&NS mechanisms during epileptogenesis remain obscure. Since mitochondrial oxidative phosphorylation is the main source of ATP for neurons and the mitochondrion has a role in the homeostasis of intracellular calcium, its dysfunction may strongly affect neuronal excitability and synaptic transmission [77]. Thus, decreased intracellular ATP levels and changes to the homeostasis of neuronal calcium may be factors that contribute to increased susceptibility to epileptic seizures associated with mitochondrial dysfunction. Those changes strongly affect neuronal excitability and synaptic transmission, whose purpose is to be highly relevant to the generation of seizures [78]. Walbaum and Patel propose a model linking acute alterations to chronic epilepsy, while Costello and Delanty believe that epilepsy is a dynamic process characterized by a "latency" period of epileptogenesis after brain damage in, for example, a head injury that occurs prior to the first unprovoked seizure. Subsequently, the risk of new seizures is increasingly higher and, therefore, "seizures could generate seizures" [79].

6.3. Antiepileptic Drugs. The use of antiepileptic drugs (AEDs) with possible neuroprotective effects has been investigated in human or animal models of excitotoxic/non-excitotoxic insults [80]. Classically, the primary objective of epilepsy control has focused on suppressing seizure activity after epilepsy has developed, but the challenge remains to control acquired epilepsy by preventing epileptogenesis, the process by which the brain becomes epileptic [81].

In a review article about the effects of antiepileptic drugs in experimental models of epileptogenesis, Augustín Legido used the kindling model, which involves repeated subconvulsive electrical stimulation to the brain, leading to spontaneous seizures. Classic drugs such phenobarbital, diazepam and valproic acid were more effective attenuating epileptogenesis than phenytoin and carbamazepine (which was practically ineffective). Ethosuximide only had a positive effect on a single model (PTZ). The new antiepileptic drugs, vigabatrin, levetiracetam, tiagabine, and zonisamide, attenuate seizures. In corneal kindling of rats, levetiracetam even protects against epileptogenesis. Felbamate has a slight effect; lamotrigine and topiramate are ineffective [82]. Animal studies about the effect of phenytoin on brain lipid peroxidation initiated by a free radical generating mechanism have shown that phenytoin treatment prevents the occurrence of convulsive and EEG seizures; however, lipid peroxidation was unaffected [83].

Temkin conducted a meta-analysis on the effects of AEDs on seizure prevention and contrasting their effectiveness on provoked versus unprovoked seizures. Data on seven drug trials or combinations for preventing seizures associated with fever, alcohol, malaria, perinatal asphyxia, contrast media, tumors, craniotomy, and traumatic brain injury were evaluated. In conclusion, AEDs were effective or had promising results predominantly for provoked (acute, symptomatic) seizures. For unprovoked (epileptic) seizures, no drug has been shown to be effective, and some have had a clinically important effect ruled out [84].

On the other hand, Hamed and Abdellah reviewed the relation between essentials elements of brain homeostasis (trace elements, electrolytes, membrane lipid peroxidation and antioxidants), neuronal excitotoxicity, and AEDs. The authors identified different effects among AED treatments in which carbamazepine (CBZ) was found to be a better antiepileptic for the control of free radical-related seizures and the level of trace elements were better regulated with CBZ than with valproate (VPA) and phenytoin (PHT) therapies [85].

In a review article of neuroprotection, antioxidants, free radicals, oxidative stress and AEDs, Azam et al. concluded that the use of free radical scavengers in the treatment of epilepsy has provided important perspectives that will be the driving force for future drug design of novel antiepileptics. Although there have been new drug developments for epilepsy, the failure rate of neuroprotective therapies in clinical trials is high [80].

According to Schmidt and Löscher, there have been a number of clinical trials that have failed to prove any significant antiepileptogenesis effects of a several AEDs in posttraumatic epilepsy. Such results may indicate the need to improve understanding of the basic mechanisms of epilepsy. Mechanisms involved in ictogenesis (i.e., initiation, amplification, and propagation of seizures) differ from those involved in epileptogenesis. As for prevention of epilepsy, it is important to identify diagnostic and surrogate markers that help identify who needs prophylaxis, that is, which patients will develop epilepsy after an insult [86].

In [81] Temkin et al. concluded that until some drugs demonstrate a clear antiepileptogenic effect in clinical trials, the best course to reduce the incidence of epilepsy is primary prevention (wearing helmets, wearing seat belts, or decreasing the risk of stroke by reducing smoking) [81].

6.4. Epilepsy and Antioxidants. Induced seizures may be partially prevented with treatment using antioxidant substances, such as SOD mimetics, melatonin e vitamin C [18]. Kong et al. have investigated the role of RNA oxidation in epileptogenesis. Using pilocarpine to induce SE, they observed a significant increase in RNA oxidation in [18] vulnerable neurons in rat brains immediately after SE followed by neuronal death. However, a daily supplement of antioxidants (coenzyme Q10) significantly reduced RNA oxidation and protected rats from SE and neuronal loss. These results

suggest that RNA oxidation may be an important factor that contributes to the degeneration process in seizures induced by neuron and epileptogenesis [18].

Catalytic antioxidants have been shown to reduce oxidative damage in animals with epilepsy, although they have been unable to reduce the seizure's duration or latency. Pretreatment with EUK-134 (a synthetic superoxide dismutase/catalase mimetic) prevents neuronal damage and decreases levels of markers of oxidative damage, including protein nitration, resulting from KA-induced seizures. However, EUK-134 does not affect seizure latency or duration [87].

Sudha et al. studied parameters of oxidative stress (lipid peroxidation, superoxide dismutase (SOD), glutathione peroxidase (GP), glutathione reductase (GR) and catalase), and levels of antioxidant substances (vitamin C, vitamin E, vitamin A, and ceruloplasmin activities) were determined in epileptic patients and normal controls. Patients who were treated with phenobarbital and who did not suffer convulsions for one year were considered for followup. Lipid peroxidation in patients with epilepsy was significantly higher when compared to controls. Moreover, plasma ceruloplasmin concentrations were also markedly increased in these cases. Plasma vitamin C and A concentrations were significantly lower in epileptics when compared to controls. In the follow-up patients, GR levels were significantly higher than in their pretreated condition. Furthermore, plasma vitamin A, E, and C concentrations remained within normal ranges. The results indicate that antioxidant status in the blood of epileptic patients, which was low compared to controls, improved after treatment with AED, suggesting that free radicals may be implicated in epilepsy [88].

Wojtal et al. reviewed the role of NO in the anticonvulsant action of AEDs. The influence of various NO synthase inhibitors (NOSIs) on AED anticonvulsant activity was tested in experimental animal epilepsy models. The results showed that some NOSIs were able to modify (through potentiation, inhibition or lack of effect) the anticonvulsive properties of AEDs, but the effects of NOSI were not reversed by L-arginine, an NO precursor [89].

6.5. Oxidative and Nitrosative Stress Pathways, Inflammation, and Neurogenesis in Epilepsy and Ageing. As reported in the introduction section O&NS pathways are induced by inflammatory responses, and subsequent mitochondrial metabolic processes generate highly reactive free radical molecules.

Inflammation, in turn, appears to play a central role among the various processes that have been connected to brain aging. Age-related increases in the activation of glial cells [90–92] as well as age-related increase in cytokines and their receptors [93] documented by histology and gene expression analysis [94–96] indicate widespread inflammatory responses in the aged brain. The increase in inflammatory response observed in the aged brain is associated to structural changes (reduction in neuronal size and a loss of white matter) and impaired functions in areas such as the prefrontal cortex [97] and temporal lobe [98], which is related to a progressive decline of the cognitive and memory functions as well as epilepsy [98].

Prospective studies suggest that inflammatory markers (e.g., high-sensitivity C-reactive protein, interleukin-6, fibrinogen) are important predictors of adverse cognitive outcomes and recent reports link inflammatory biomarkers to age-accelerated cerebral atrophy as well [99]. Indeed, chronic epilepsy appears to be associated with an increased risk of exposure to inflammatory risk factors linked with abnormal cognitive aging and dementia. Evidence that persons with epilepsy may be particularly vulnerable to inflammation comes from both human and animal studies. For example, experimentally-induced seizures trigger a prominent inflammatory response in neural areas involved in the onset and propagation of seizures [100, 101]. Increased inflammatory markers have been detected in serum, CSF, and brain of people with epilepsy. There are relevant findings of increased IL-6 following recent tonic-clonic seizures [102, 103]. This cytokine has also been reported to be elevated secondary to carbamazepine but not valproic acid treatment [104] and elevated levels of fibrinogen have been reported in chronic epilepsy [105].

According to Ekdahl et al., 2003, the suppression of hippocampal neurogenesis by microglia activation contributes to cognitive dysfunction in aging, dementia, epilepsy, and other conditions leading to brain inflammation. These authors' report suggests anti-inflammatory treatment as a possible novel strategy to improve the efficacy of neuronal replacement from endogenous precursors in stroke and other neurodegenerative disorders [106].

Overall, there is an intrinsic relationship between oxidative stress and inflammation in aged people, as a source of hippocampal neurogenesis decline, leading to neurodegenerative diseases, such as epilepsy. Thus, these mechanisms must be further explored in the clinical management of these conditions.

6.6. Epilepsy and the Elderly. Several studies have demonstrated increased incidence and prevalence of epilepsy in older age groups [107–109]. Epilepsy is the third most common type of brain disease in old age, after stroke and dementias [110, 111].

Epilepsy in the elderly is usually the expression of an underlying brain condition. Symptomatic epilepsy in young adults is usually the result of trauma during birth, congenital malformations or brain development anomalies, encephalitis, head trauma or a brain tumor. In elderly individuals epilepsy is caused by stroke or a neurodegenerative disease. However, etiology remains unclear in at least a third of all elderly cases. Elderly individuals (60 or older) who have no other risk factor (prior stroke, trauma, or dementia) have a risk of 1.1%. This might seem small, but it is double the risk of corresponding young adults. Stroke is the main etiology of epilepsy in elderly individuals. Epidemiological studies have demonstrated that a stroke increases the likelihood of a seizure by 23 times and the risk of epilepsy in the first year after stroke increases by 17 times when compared with the risk in the comparable average population [112]. When the

seizure occurs within the first hours to two weeks after the stroke, this is due to acute biochemical abnormalities, for instance, the action of excitatory neurotransmitter glutamate [113]. Late seizures are usually due to chronic processes, such as the removal of inhibitory influences, scars and the formation of new synaptic connections [112].

Stroke and other vascular disasters are the most common risk factors for epilepsy in the elderly [114]. Epidemiological studies show that incidence of neurodegenerative diseases (e.g., Alzheimer's dementia) is increasing. In Europe, the prevalence of dementia is estimated to be approximately 6–8% after 65 years of age and may rise to 20–30% in subjects older than 85 years. [115–117]. A diagnosis of Alzheimer's dementia or other dementia types was associated with at least a six-fold increased risk of unprovoked seizure [118].

Investigating the links between stroke, epilepsy, and dementia, Cordonnier et al. confirmed the hypothesis that patients with stroke who have epileptic seizures without dementia have an increased risk of new-onset dementia [119]. In another study they showed that stroke patients with preexisting dementia have an increased risk of late seizures [120].

A stroke can be caused by rupture of atherosclerotic plaques in the arterial wall. The development of atherosclerotic lesions is the result of a cascade of cellular and molecular events that can be well characterized as a chronic immune-mediated inflammation [121].

Lipid metabolism is of particular interest due to its high concentration in the CNS. In a review article about the effects of altered lipid metabolism on the mechanism of brain injury and disorders, Adibhatla and Hatcher describe the importance of atherosclerosis that results from accumulation of LDL-derived lipids in the arterial wall. Lipids have been associated with the physiopathology of many neurological disorders and neurodegenerative diseases [122].

The sequence of events of cerebral ischemia start with loss of energy, which results in excessive release of neurotransmitters; elevated stimulation of glutamate receptors results in elevated intracellular Ca++ and activation of phospholipase A2 (PLA2) [123]. The activation of PLA2 results in hydrolysis of membrane phospholipids and release of free fatty acids including arachidonic acid, a precursor of important cell-signaling eicosanoids [124]. ROS is produced by the metabolism of arachidonic acid reacting with cellular lipids to generate lipid peroxides. ROS can also be formed nonenzymatically (autoxidation of catecholamines) [122]. ROS produces oxidization of the polyunsaturated fatty acids, resulting in the production of conjugated aldehydes. The most studied aldehyde is 4-hydroxy-2-nonenal (HNE). [125]. HNE is considered a potential inducer of apoptotic cell death and induces cellular dysfunction by many mechanisms (extracellular calcium uptake, GSH depletion, alteration of mitochondrial function leading to the release of cytochrome c and subsequent activation of the caspase cascade and loss of proteasome function) [126]. In a study demonstrating that cellular apoptosis may activate an inflammatory response, resulting in more oxidative damage, Rong et al. investigated the effect of a synthetic superoxide dismutase/catalase mimetic (EUK-134) on indices of oxidative stress as well as on pathological manifestations produced by kainic acid-induced seizure (KA). EUK-134 prevented oxidative stress and attenuated rat brain damage induced by KA and showed that kainate-induced excitotoxicity is caused, at least in part, by the action of reactive oxygen species. Also, oxidative stress occurs before significant neuronal death in the hippocampus [87]. In summary, increased ROS production started a pathological cycle with loss of antioxidant defenses leading to progressive cell damage, which further increase the production of free radicals provoking damage to all components of the cell (proteins, carbohydrates, nucleic acids, and lipids). This leads to cellular death, producing an increase in oxidative stress. This cycle can lead to progressive decline in physiological function and ultimately cell death [122].

Epidemiological studies showed that increased circulating levels of lipoprotein-associated phospholipase A2 predict an increased risk of stroke [127]. Previous studies showed the importance of lipid peroxidation in the pathogenesis of AD. There is evidence of increased levels of lipid peroxidation and neurotoxic byproducts of lipid peroxidation (HNE) in vulnerable regions of the Alzheimer's disease (AD) brain and increased levels of HNE in the brain tissue from patients affected by mild cognitive disorder and early AD [128].

Oxidative and nitrosative stress has an important effect on onset and maintenance of seizures, as previously discussed. However, this effect seems to have different impacts on groups of different ages (children, young adults, and the elderly). We know that seizures are more prevalent in old age than in children [108, 109]. This may be due to an increased excitability of primary hippocampal neurons seen with age [129, 130]. The CNS is highly sensitive to oxidative stress, especially in elderly patients. This implies that the elderly have a higher risk of neural diseases such as epilepsy. However, future experimental studies need to confirm the relationship between oxidative stress, the elderly, and epilepsy.

7. Conclusions and Future Directions

Oxidative stress and mitochondrial dysfunction are involved in neuronal death and seizures. There is evidence that suggests that antioxidant therapy may reduce lesions induced by oxidative free radicals in some animal seizure models. Recent studies have shown that an association between mitochondrial dysfunction and chronic oxidative stress may play an important role in epileptogenesis. However, further preclinical and clinical studies are required to further investigate the relationship between oxidative stress, seizures, and age.

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Review Article

Oxidative Stress in Genetic Mouse Models of Parkinson's Disease

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There is extensive evidence in Parkinson's disease of a link between oxidative stress and some of the monogenically inherited Parkinson's disease-associated genes. This paper focuses on the importance of this link and potential impact on neuronal function. Basic mechanisms of oxidative stress, the cellular antioxidant machinery, and the main sources of cellular oxidative stress are reviewed. Moreover, attention is given to the complex interaction between oxidative stress and other prominent pathogenic pathways in Parkinson's disease, such as mitochondrial dysfunction and neuroinflammation. Furthermore, an overview of the existing genetic mouse models of Parkinson's disease is given and the evidence of oxidative stress in these models highlighted. Taken into consideration the importance of ageing and environmental factors as a risk for developing Parkinson's disease, gene-environment interactions in genetically engineered mouse models of Parkinson's disease are also discussed, highlighting the role of oxidative damage in the interplay between genetic makeup, environmental stress, and ageing in Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is a highly prevalent neurodegenerative disorder, second to Alzheimer's disease in terms of incidence [1]. Its prevalence increases with age, being approximately 1% in people over the age of 60 and increasing to about 4% over the age of 85 [2]. It affected about 4.5 million people over the age of 50 in 2005, and by the year 2030, this number will have doubled [3]. The etiology is still unknown [4], but it is hypothesized that it may result from a complex interaction between environmental factors, genetic susceptibility and ageing [5, 6].

Diagnosed patients are characterized by motor and non-motor clinical manifestations. The motor symptoms include resting tremor, bradykinesia, akinesia, muscular rigidity, and a loss of balance [7] and are predominantly attributed to a lack of dopamine (DA) in the striatum, and the resulting dysfunction of the basal ganglia, a cluster of nuclei involved in the initiation and execution of movement [1, 7]. The non-motor symptoms include impaired olfaction, sleep disorders, constipation, urinary incontinence, orthostatic hypotension, and various neuropsychiatric manifestations (e.g., depression, hallucinations, dementia), and can appear both before and during the motor symptomatology of PD [7].

The pathological hallmark of PD comprises loss of nigrostriatal dopaminergic neurons in the substantia nigra (SN) pars compacta (SNc) and the presence of insoluble protein inclusions termed Lewy bodies (LBs) and Lewy neurites (LNs), located in either the neuronal cell bodies or neuronal processes, respectively [2, 6, 8]. The major constituent of LBs and LNs is a misfolded version of the protein alpha-synuclein (α-syn) [8]. There are also non-dopaminergic neurons affected in PD, leading to more wide-spread neuronal changes that cause a complex and heterogeneous clinical picture [7, 9]. Recently, Braak and colleagues hypothesized a six-stage pathological process in which PD pathology emerges in the olfactory bulb and the dorsal motor nucleus of the vagal nerve and only in later stages extends to the midbrain and other brainstem regions [10, 11]. Current therapies of PD are symptomatic, targeting the lack of DA in the striatum with DA replacement strategies. Although these therapies provide symptomatic

relief at the beginning, they become gradually inefficient as the disease progresses. Therefore, there is an urgent need of therapeutic strategies that can tackle the disease progression [12].

Despite the fact that PD has long been considered as a *non-genetic* disorder of sporadic origin, research performed during the past decade has led to the identification of genes linked to rare monogenic forms of PD. This resulted in the identification of 16 "PARK" loci, with the autosomal dominant genes SNCA (PARK1/4), and LRRK2 (PARK8), and the autosomal recessive genes parkin (PARK2), DJ-1 (PARK7), and PINK1 (PARK6) being the most common ones [2, 3, 5]. Although monogenic forms account for <10% of PD cases, these genes also play a role in the much more common sporadic form of the disease [5]. Unraveling the molecular mechanisms underlying the familial forms of PD will contribute to our understanding of sporadic PD, since both share clinical and neuropathological features [5]. Moreover, several cellular abnormalities which may underlie the neurodegeneration displayed in sporadic PD, such as mitochondrial dysfunction, oxidative stress, excitotoxicity, proteasomal stress, neuroinflammation, and protein aggregation, are also associated with mutations in the familial PD genes [13, 14].

Multiple animal models have been developed in order to study the pathogenesis and progression of PD and to test potential therapeutic strategies [15]. Currently, focus is being put on integrating genetic, environmental, and age-related influences in more relevant preclinical animal models, which can reproduce PD pathology with high fidelity.

Since there is extensive evidence of oxidative stress in the pathogenesis of PD [14, 16–20], and a link between oxidative stress and some of the monogenically inherited PD associated genes has been described, this paper focuses on its importance and potential impact on neuronal function. It is interesting that despite all the evidence for a role of oxidative stress in PD, there have been relatively few studies that have extensively characterized oxidative stress in animal models of PD [21]. In this paper, an overview is given of oxidative stress findings in monogenic mouse models of PD. In addition, a focus will be put on gene-environment interactions in these mouse models.

2. Genetic Forms of Parkinson's Disease

The PD genes can show both recessive and dominant modes of inheritance. The autosomal dominant PD genes include SNCA, leucine-rich repeat kinase 2 (LRRK2), microtubule-associated protein tau (MAPT), Htr serine peptidase 2 (HtrA2), and glucocerebrosidase (GBA), while the autosomal recessive PD genes include parkin, PTEN-induced kinase 1 (PINK1), and DJ-1 [5, 22]. In the context of this paper, only SNCA, parkin, PINK1, and DJ-1 are discussed in further detail.

Studies of PD-linked genes have brought to light several pathways involved in neuronal death in the SNc (protein aggregation, defects in the ubiquitin-proteasomal pathway, impaired defense against oxidative stress, abnormal protein phosphorylation, mitochondrial and lysosomal dysfunction, apoptosis), thus improving our understanding of the more common sporadic form of the disease. "Sporadic" cases may, in fact, be monogenic as well, because mutations may have occurred *de novo* [5].

2.1. SNCA. The SNCA gene encodes α-syn, a small 140 amino acid protein that can be both lipid associated and free in the cytoplasm [23]. It has been shown that α-syn promotes PD pathogenesis either by dominantly inherited mutations in the SNCA gene (A30P, A53T, and E46K) or by factors that enhance α-syn expression, such as gene multiplication (e.g., duplication, triplication) of the SNCA gene or by SNCA promoter polymorphisms that increase α-syn expression [2, 7]. Although the three missense mutations in SNCA are very rare, a normal expression of a mutant form of α-syn, or overexpression of the wild-type (WT) α-syn, can contribute to the pathogenesis of PD [2]. Furthermore, a clear evidence for an SNCA dosage effect was also revealed in one familial case of PD [2].

The phenotype of patients with SNCA mutations resembles sporadic PD, but with earlier onset and atypical features, including cognitive decline, psychiatric problems, and autonomic dysfunction. The same mutations have been found in sporadic PD patients, suggesting significant frequency of *de novo* mutations [5].

2.2. Parkin. Parkin is a 465 amino acid E3 ubiquitin ligase, which transfers ubiquitin to target proteins for degradative (i.e., via the proteasome system) or non-degradative (i.e., signaling) purposes. Mutations in parkin account for the majority of early-onset familial PD cases [24]. Loss-of-function of parkin can contribute to the etiology of PD either by disrupting the normal function of the ubiquitin proteasome system (UPS) in the clearance of aggregated proteins or by disabling a mitochondrial protective mechanism mediated by parkin's signaling function, contributing to mitochondrial dysfunction [24].

2.3. PINK1. PINK1 encodes a 581 amino acid serine/threonine kinase that is localized to the mitochondria. Mutations in PINK1 are the second most common cause of autosomal recessive early-onset familial PD after parkin, with most mutations occurring in and disrupting the activity of the kinase domain [18, 24, 25]. As with parkin, loss-of-function of PINK1 leads to decreased mitochondrial protection against oxidative stress, causing enhanced mitochondrial dysfunction [26]. Although the biological function of the PINK1 protein is not fully understood, studies show an important role in the maintenance of mitochondrial function and protection against oxidative stress [27–30].

2.4. DJ-1. DJ-1 encodes a 189 amino acid protein of still-uncertain function. Mutations in DJ-1 represent a rather rare cause of early-onset familial PD [24]. Still, insights into putative roles of DJ-1 brought this protein under investigation, and it seems that DJ-1 is involved alongside parkin and PINK1 in protecting the mitochondria against

oxidative stress [7]. Interestingly, reduced DJ-1 expression is also associated with proteasome inhibition [23], highlighting an additional role of DJ-1 in the normal function of the UPS. The DJ-1 protein was found to be oxidatively damaged and significantly increased in brains of sporadic PD patients [5].

Patients carrying a mutation in parkin, PINK1, or DJ-1 show marked clinical overlap, generally suffering from early-onset parkinsonism with a slower disease progression, good levodopa response, and early development of motor fluctuations [2].

3. Oxidative Stress in Parkinson's Disease

Oxidative stress can be defined as a condition in which the cellular antioxidant defense mechanisms are insufficient to keep the level of reactive oxygen species (ROS) below a toxic threshold [7]. This may be either due to an overproduction of reactive free radicals or to a failure of cell buffering mechanisms [14]. ROS can damage all types of biomolecules, and oxidative damage to nucleic acids, lipids, and proteins can be deleterious [31]. Several of the genes linked to familial forms of PD appear to be involved in the protection against or in the propagation of oxidative stress [14, 32]. Furthermore, oxidative stress and mitochondrial dysfunction have been linked to the pathogenesis of PD, ever since exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a complex I inhibitor, was found to induce parkinsonism in humans [30, 33]. Data from postmortem studies of brains from patients with PD (Table 1) suggest that oxidative stress plays an important role in neuronal degeneration of the dopaminergic nigral neurons [34, 35]. Early and profound loss of glutathione (GSH) levels, a reduction in mitochondrial complex I activity, increased oxidative damage to lipids, proteins, and DNA, increased superoxide dismutase (SOD) activity, and elevated free iron levels in the SN of PD patients have been demonstrated [9, 15, 35–39]. Moreover, *in vivo* observations (Table 1) demonstrated that several markers of oxidative stress are altered in the cerebrospinal fluid (CSF) and blood samples of PD patients [40, 41].

Nevertheless, it should be noted that many clinical trials are done in chronically treated PD patients, and drug intake may influence the outcome of the study. For instance, a well-described effect of chronic levodopa intake is the elevation of the homocysteine plasma levels, which is a risk factor for various pathological conditions possibly due to homocysteine-mediated increase in oxidative stress [47–50]. Furthermore, Buhmann et al. [43] have shown that levodopa monotherapy results in an increase in auto-oxidation and in a decrease of plasma antioxidants with significance for ubiquinol-10, while DA agonist monotherapy was associated with higher alpha-tocopherol levels [43]. Müller and Muhlack [48] have also shown that acute levodopa/carbidopa application reduces free cysteine/glycine levels in plasma and that this decline may be linked to prior appearance of oxidative stress with concomitant consumption of antioxidants like GSH and subsequent conversion of this molecule to oxidized GSH (GSSG) [48]. Therefore, the study design of the clinical trial should be carefully considered when investigating oxidative stress in PD patients.

It is interesting to note that the brain in particular is more vulnerable to oxidative stress and oxidative damage compared to other organs. For instance, the brain consumes more oxygen on a per weight basis under physiological conditions than any other organ, thereby increasing its susceptibility to oxidative stress. Also, the brain contains a relatively low level of antioxidants and free radical scavenging enzymes compared to other tissues [51–53], as well as a high amount of substances, such as phospholipids and unsaturated fatty acids, which are vulnerable to oxidative modifications [33, 54]. Indeed, markers of lipid peroxidation, such as elevated 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), have been observed in postmortem brain tissue and CSF of PD patients [42, 51]. Moreover, the vulnerability of neurons to oxidative damage, which accumulates in ageing neurons, might also be due to their postmitotic nature [55].

ROS are produced by a number of different pathways, including direct interactions between redox-active metals and oxygen species via reactions such as the Fenton and Haber-Weiss reactions, or by indirect pathways involving activation of enzymes, such as nitric oxide synthase (NOS). All the initial free radical reactions require activation of molecular oxygen [51]. It is important to realize that ROS are being continuously generated *in vivo* as a result of oxygen metabolism, with about 1–5% of the oxygen consumed being converted to ROS [30]. The univalent reduction of molecular oxygen leads to the formation of superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals. The $O_2^{\bullet -}$ can lead to the generation of hydroxyl radicals via Fe^{2+} catalyzed Haber-Weiss and Fenton reactions. The generation of reactive nitrogen species (RNS) is due to NOS-mediated conversion of arginine to citrulline and the subsequent generation of nitric oxide (NO), which reacts with $O_2^{\bullet -}$ to produce peroxynitrite ($ONOO^-$) [51].

The high concentration of DA in the nigrostriatal pathway is presumed to be an essential determinant for the high vulnerability of dopaminergic cells to oxidative stress. DA itself does not exert direct toxic effects, but toxic intermediates derived from its catabolism [54] may contribute to the oxidative stress pathogenic pathway in PD. The catabolism of DA includes auto-oxidation into toxic DA-quinone species, $O_2^{\bullet -}$ and H_2O_2, and the enzymatic conversion via monoamine oxidase B (MAO-B) into the inert 3,4-dihydroxyphenylacetic acid (DOPAC) and H_2O_2 [56]. Furthermore, the H_2O_2 and $O_2^{\bullet -}$ produced by DA catabolism can be further converted into highly toxic hydroxyl radicals as described above [17, 36]. It is important to note that these hydroxyl radical generating pathways are heavily dependent on the presence of iron, which have been found to be elevated in the SN of PD patients [36]. The particular reason for this is not yet understood, but it seems to be related with the age-dependent accumulation of neuromelanin in the nigral neurons [51]. Neuromelanin is a dark-brown pigment that concentrates metal ions, in particular iron, and that makes the nigrostriatal dopaminergic neurons seem dark colored. The age-dependent accumulation of neuromelanin will lead to a higher local concentration of iron in dopaminergic

Table 1: Oxidative stress parameters in Parkinson's disease patients.

Observation	Marker for oxidative stress	Sampled region	Outcome (versus control)	References
In vivo	MDA	Blood, CSF	↑	[40]
	GSH reductase	Blood	↑	
	Cu/Zn-SOD	Blood	↑	
	Superoxide	Blood	↑	
In vivo	HNE	Plasma, CSF	↑	[42]
In vivo	Lipoprotein oxidation	Plasma, CSF	↑	[43]
	Protein sulfhydryl groups	Plasma, CSF	↓	
	α-Tocopherol	CSF	↓	
In vivo	Cu/Zn/Mn-SOD activity	Skin fibroblasts	↔	[44]
	GPx activity	Skin fibroblasts	↔	
	Catalase activity	Skin fibroblasts	↔	
In vivo	Lipid oxidation products	Plasma	↑	[19]
	8-OHdG	Urine	↑	
In vivo	Markers of oxidative stress	iPSCs	↑	[45]
	Sensitivity to oxidative stress	iPSCs	↑	
In vivo	MDA	Blood	↑	[41]
	SOD activity	Blood	↑	
	GPx	Blood	↔	
Postmortem	Lipid peroxidation	SN	↑	[38]
	Reduced GSH	SN	↓	
	Total iron	SN	↑	
	Ferritin	SN	↓	
	GPx activity	SN	↓	
	Catalase activity	SN	↓	
Postmortem	Iron	SN	↑	[39]
	MAO-B	SN	↑	
	Cu/Zn-SOD	SN	↑	
	Heme oxygenase	SN	↑	
	GSH	SN	↓	
	Vitamin C	SN	↓	
Postmortem	GPx 4	SN	↑	[46]
Postmortem	Protein oxidation	Caudate nucleus	↑	[35]
	Lipid peroxidation	Frontal cortex	↑	
	Protein nitration	Caudate nucleus, putamen, frontal cortex	↔	
	Total GSH	Caudate nucleus, putamen, frontal cortex	↑	
	Catalase	Caudate nucleus, putamen, frontal cortex	↔	
	SOD	Caudate nucleus, putamen, frontal cortex	↔	
	GSH reductase	Caudate nucleus, putamen, frontal cortex	↔	
	GPx	Caudate nucleus, putamen	↑	

Abbreviations: 4-HNE: 4-hydroxynonenal, 8-OhdG: 8-hydroxydeoxyguanosine, CSF: cerebrospinal fluid, GPx: GSH peroxidase, GSH: glutathion, iPSCs: induced pluripotent stem cells, MDA: malondialdehyde, SN: substantia nigra, SOD: sodium dismutase, ↑: increased, ↓: decreased, ↔: not different.

neurons, and this will expose the neurons to increasing concentrations of highly toxic hydroxyl radicals. Moreover, several studies have indicated that one of the consequences of normal aging is the increase in the concentrations of copper and iron in brain tissue [51]. Due to this, the current view is that the hydroxyl-radical-mediated oxidative stress induced by dysregulation in the neuromelanin and iron homeostasis is one of the most important contributors to the oxidative damage in PD [17, 36, 51]. The generation of ROS also initiates excitotoxicity, which is modulated by the overactivation of N-methyl-D-aspartate (NMDA) receptors [51]. In response to activation of the NMDA receptors, NO is produced, due to the interaction of NOS with these receptors [54].

Numerous studies have reported the involvement of mitochondria, neuroinflammation via activated microglia, and other ROS-mediated pathways in the pathogenesis of PD.

3.1. Impaired Mitochondrial Function. Several lines of evidence, some obtained from PD patients, indicate that the mitochondrial dysfunction plays a major role in the pathogenesis of PD, with defects in complex I of the mitochondrial electron chain transport gaining the most attention [9, 57, 58]. Complex I is located in the inner mitochondrial membrane and constitutes a part of the oxidative phosphorylation system responsible for the generation of cellular adenosine-5′-triphosphate (ATP) [59, 60]. Mitochondria exert both vital and lethal functions in physiological and pathological conditions. On the one hand, they are indispensable for energy production and hence for the survival of eukaryotic cells; on the other hand they are crucial regulators of the intrinsic pathway of apoptosis [61]. Furthermore, mitochondria are main sources of ROS in the central nervous system (CNS) [62]. Mitochondria contain redox carriers that can transfer single electrons to oxygen, thus generating $O_2^{\bullet -}$. Enzymes in the tricarboxylic acid cycle and the electron transport chain (complexes I, II and III) and MAOs are among the mitochondrial carriers generating $O_2^{\bullet -}$. The generated $O_2^{\bullet -}$ can be converted by the cellular defense mechanism into H_2O_2, which further can react with other molecules, as described above [30, 62].

An impairment of normal mitochondrial function leads to an excessive production of ROS and a general decrease in ATP levels. Furthermore, there is a concomitant loss of mitochondrial membrane potential [63]. Under physiological conditions, mitochondria harbour a robust mitochondrial transmembrane potential and a low-conductance state of the permeability transition pore complex (PTPC) might contribute to exchange of small metabolites between the cytosol and the mitochondrial matrix, a process that is mainly controlled by mitochondrial solute carrier. In response to proapoptotic stimuli, such as ROS and calcium (Ca^{2+}) overload, the PTPC assumes a high conductance state that allows the deregulated entry of small solutes into the mitochondrial matrix along their electrochemical gradient. This phenomenon, which is known as mitochondrial permeability transition, results in the immediate dissipation of the mitochondrial membrane potential and osmotic swelling of the mitochondrial matrix. As a consequence, this may lead to mitochondrial outer membrane permeabilization and to the release into the cytosol of cytotoxic proteins normally confined within the mitochondrial intermembrane space. The cytotoxic proteins include caspase activators, such as cytochrome c and DIABLO, as well as caspase-independent cell death effectors like apoptosis-inducing factor and endonuclease G [61, 63].

Next to being the main source of free radicals and having a role in the electron transport chain and oxidative phosphorylation, the mitochondria are also involved in Ca^{2+} homeostasis [9]. Mitochondria are known to play an important role as Ca^{2+} buffer, thereby preventing sustained high cytosolic Ca^{2+} levels, via the uptake of these ions through a membrane potential-dependent uniporter [64]. Saturation of this buffering system can be prevented, by releasing mitochondrial Ca^{2+} again into the cytosol via the H^+/Ca^{2+} exchanger and a yet unidentified Na^+/Ca^{2+} exchanger [65].

3.2. Neuroinflammation. The neurotoxicity associated with inflammatory processes in the CNS seems to be mainly mediated by overactive microglia [66]. Microglia are components of the CNS innate immune system, normally display a resting phenotype, and only become activated upon brain injury or immune challenge [66]. In relation to oxidative stress, microglia have been identified as an important source of ROS [62]. Microglia, which are phagocytic cells, have a dual role in the CNS. They can be either neuroprotective through the elimination of exogenous and endogenous substances or promote neurodegeneration by producing potentially toxic agents [66–68]. Overactive microglia were shown to release toxic compounds to dopaminergic cells, such as $O_2^{\bullet -}$, H_2O_2, and NO, as well as proinflammatory cytokines, such as the tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) [69, 70], and it is currently well known that microglial activation results in dopaminergic cell death in PD patients [71]. $O_2^{\bullet -}$, released by overactive microglia, seems to be the critical mediator of cell death [71]. Its production is mediated by the NADPH oxidases, which comprise several multicomponent enzyme complexes, that transfer electrons across biological membranes [62, 71–73]. Although the major source of NADPH oxidase induced oxidative stress is of activated microglia, NADPH oxidases from neuronal origin might also contribute to cell death [74]. The activation of NADPH oxidase is also mediated via interactions between the dopaminergic system and the central renin-angiotensin system [75]. We recently showed that stimulation of central angiotensin 1 (AT1) receptors leads to the activation of NADPH oxidase, while stimulation of central AT2 receptors leads to inhibition of NADPH oxidase activation [75, 76]. Factors driving microglial overactivation in PD might be linked to environmental toxins, endogenous disease proteins, and even the neurodegenerative process itself. Indeed, it was shown that PD toxins such as MPTP, rotenone, paraquat, and particulate matter from polluted air are potent inducers of microglial activation, suggesting environmental triggers in PD [66].

Lastly, dopaminergic cell death releases proteins in the extracellular space, such as histones, oxidized lipids, DNA, and ATP, that are interpreted by microglial cells as damage-associated molecular pattern molecules, leading to their activation [77]. This finding is particularly interesting, as it implies that neuronal cell death, which may be increased by microglial neurotoxicity, further reinforces the overactive phenotype of the microglia. This would create a self-perpetuating neurotoxic cycle, whereby microglial cells are capable of enhancing neurodegeneration, while this in turn would further enhance microglial activation [66]. Such a cycle would be particularly damaging to dopaminergic neurons in the SN, as it contains 4.5 times more microglial cells compared to other areas of the brain [71].

Understanding the role of oxidative stress as a primary event in the pathogenesis of PD is complicated, as different pathways themselves can play a primary role and, in turn can initiate the formation of ROS/RNS. The above findings demonstrate how complex the interaction between oxidative stress, mitochondrial impairment, and neuroinflammation is, making it problematic to determine which occurs first. Furthermore, impairment in the UPS system, which is the primary mechanism responsible for eliminating mutant, damaged, and misfolded intracellular proteins, and for regulating the levels of short-lived proteins, can result in the accumulation of abnormal proteins, leading to the disruption of the cellular homeostasis and integrity, and creating a state referred to as proteolytic stress [78]. Again, oxidative stress may contribute to this impairment, and in turn, UPS impairment can induce oxidative stress [17].

3.3. Cellular Antioxidant Mechanisms. To maintain a proper redox balance, all aerobic organisms utilize series of sophisticated antioxidant defense mechanisms in an attempt to protect themselves against oxidative damage by eliminating ROS, thereby balancing the ratio between generation and detoxification of ROS. Numerous antioxidant enzymes, such as catalases, SOD, peroxiredoxins, and GSH peroxidase (GPx), and low-molecular weight compounds such α-tocopherol, ascorbic acid, and GSH have been characterized [31, 37, 79, 80]. The removal of H_2O_2 is accomplished by two enzyme families: GPx and catalases, both reducing it to oxygen and water. GPx utilizes the power of reduced GSH that is converted, via reduction of H_2O_2, to oxidized GSH [79]. The continuous action GPx is permitted by GSH reductase that recycles reduced GSH from its oxidized form. Another enzyme, quinone reductase, catalyzes the direct divalent reduction of quinones to quinols and avoids the formation of reactive semiquinone radical intermediates [79]. Furthermore, two nonenzymatic proteins, ferritin and ceruloplasmin, have an important role in transition metal storage [79].

In addition, a rise in ROS may also constitute a stress signal that activates redox-sensitive signaling pathways, which once activated may have either damaging or potentially protective functions [80]. The major signaling pathways activated in response to oxidative stress are the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinase [PI(3)K]/protein kinase B (AKT), nuclear factor kappa B (NF$\kappa\beta$), p53 and p38 pathways. NF$\kappa\beta$ and p53 are transcription factors, while PI(3)K/AKT and mitogen-activated protein kinase (MAPK) pathways regulate transcription factors through phosphorylation. The degree to which a given pathway is activated is highly dependent on the nature and on the duration of the stress [80]. Furthermore, the activation of peroxisome proliferator-activated receptors (PPAR), whose natural ligands are polyunsaturated fatty acids and their oxidation products, may be involved in the pathogenesis of PD. PPAR is also considered as a regulator of mitochondrial biogenesis genes that upregulates genes known to protect against oxidative stress [81]. Numerous protective genes, such as heme-oxygenase, have been shown to be inducible in mammalian cells following exposure to oxidative stress [79]. The exact ROS sensing mechanisms are not well understood, but a number of transcription factors that regulate the expression of antioxidant genes are well characterized [37]. Major signaling pathways activated in response to oxidative stress include (i) transcription factors, such as NFκb, activator protein 1 (AP1), and antioxidant response elements (AREs) binding proteins that can interact directly with specific DNA motifs on promotors of target genes (ii) or via MAPK cascades, which in turn activate transcription factors that trigger target gene transcription [82]. The transcription of the cytoprotective proteins is under control of the nuclear transcription factor NF-E2-related factor 2 (Nrf2). Upon normal homeostatic conditions, Nrf2 is repressed by its negative regulator Keap1. Upon exposure to ROS, Nrf2 dissociates from cytosolic Keap1 and translocates to the nucleus, where it binds to the AREs in the promoter region of the genes encoding antioxidant enzymes [37]. These findings suggest that an increase in ROS results in two critical effects: (i) damage to cell components via an excessive production of ROS and also (ii) activation of specific signaling pathways regulated via ROS, influencing various cellular processes leading either to cell death or to proper cell function [82, 83].

Mitochondria are highly dynamic organelles that can undergo repeated cycles of fusion and fission to regulate their shape and length, and to control the total number of mitochondria per cell. The fusion of two mitochondria serves as a protective mechanism by forming tubular networks, leading to the exchange of lipid membranes, mitochondrial DNA (mtDNA), and soluble metabolites to compensate for individual deficiencies, as well as to the dilution of toxic intermediates, such as ROS. On the other hand, mitochondrial fission allows division and sequestration of damaged parts of this organelle by autophagy [84]. Several levels of defense mechanisms have been shown in mitochondria to ensure their integrity. A first line of defense comprises molecular chaperones, which assist folding and assembly of mitochondrial proteins and prevent oxidative-stress-mediated mitochondrial injury. A second protective mechanism involves the degradation of damaged proteins by mitochondrial proteases [85]. However, once the mitochondrial quality control is overwhelmed, a third pathway is activated and induces selective autophagic clearance of damaged mitochondria (mitophagy) [85–87].

4. Evidence of Oxidative Stress in Genetic Animal Models of Parkinson's Disease

A better understanding of the pathophysiology of PD is only possible with the development of reliable experimental models, which can mimic disease processes with good fidelity. Initially, PD was modeled by administration of neurotoxins, such as 6-hydroxydopamine (6-OHDA) and MPTP. However, the identification of PD-associated genes has led to the development of new genetic animal models of PD [88]. While many models have been created, to date no single model, either based on toxins or genetics, has been able to recreate all the key features of the disease [89]. Nevertheless, knockout (KO), knockdown (KD), knock-in, overexpression, and/or mutations in single genes provide a powerful tool to study the etiology of PD [89]. Thus far, several genetically engineered animal models of PD have been generated [55, 90]. Transgenic systems are widely used to study the cellular and molecular basis of human neurodegenerative diseases. A wide variety of model organisms have been utilized, including bacteria (*Escherichia coli*), nematodes (*Caenorhabditis elegans*), arthropods (*Drosophila melanogaster*), fish (zebrafish, *Danio rerio*), and rodents (mouse, *Mus musculus* and rat, *Rattus norvegicus*), as well as nonhuman primates (rhesus monkey, *Macaca mulatta*). These transgenic systems have enormous value for understanding the pathophysiological basis of disorders and have, in some cases, been instrumental in the development of therapeutic approaches to treat these conditions [91]. Once a causative mutation has been identified, transgenic systems can be generated to model the human disease or aspects of the altered gene function. All the models can provide further confirmation of the genetic basis of the disease and contribute to the identification of cellular and molecular mechanisms responsible for the disease phenotype. The available transgenic models vary in terms of easiness of manipulation and phylogenetic relatedness to humans, but have all been useful for the study of neurodegenerative diseases [91].

Among the invertebrate systems, the arthropod fruit fly *Drosophila melanogaster* and the nematode worm *Caenorhabditis elegans* have been the most widely used. The advantage of using invertebrates is their rapid production and analysis of transgenic lines expressing variants of disease-associated proteins. A disadvantage is that they lack critical factors, such as immune function and myelination [91, 92]. Despite the notable advances that have occurred in the above-mentioned species, the mouse remains the most widely used system for modeling human neurodegenerative diseases, since it is closely related to humans and offers advantages such as the relatively low cost, short generation time, high-environmental control, and the possibility for genome manipulation that allow virtually any disease-associated genetic alteration in humans to be introduced into the mouse [91].

Since, as described above, there is a prominent role for oxidative stress in the pathogenesis of PD, while deficiencies in products of several PD-associated genes, including SNCA, parkin, PINK1, and DJ-1, may be linked to increased oxidative stress and/or higher vulnerability to oxidative damage, an overview is given of several oxidative stress hallmarks that have been observed within these genetically modified animal models of PD, with an emphasis on the mouse models (Table 2).

4.1. DJ-1 Models. No significant differences were detected in protein nitration, nucleic acid oxidation, and lipid peroxidation in the SN of aged DJ-1 KO mice [95]. Similarly, protein oxidation was not altered in the whole brain of DJ-1 KO mice at different ages [93, 94]. These findings demonstrate that DJ-1 KO does not lead to increased oxidative damage in basal conditions [93–95]. Nevertheless, several groups observed increased mitochondrial ROS levels in the SN as well as in the whole brain, of DJ-1 KO mice at different ages [94, 96]. Furthermore, decreased mitochondrial aconitase activity was described in young, but not in aged KO mice [94]. It is interesting to note that although Andres-Mateos et al. [94] observed a 2-fold increase in the mitochondrial H_2O_2 production in DJ-1 KO brains, the mice did not show any dopaminergic degeneration or mitochondrial damage. They did note, however, that compensatory mechanisms (upregulation of MnSOD and GPx levels and increase in GPx activity) have been activated to compensate for the elevated H_2O_2 in mitochondrial, but not in cytosolic fractions from aged mice. These compensatory increases in GPx levels and activity appear to compensate for the lack of DJ-1 because the cytosolic levels of reduced GSH and GSSG and the ratio GSSG/GSH in DJ-1 KO brains do not differ significantly compared with their respective WT littermates [94]. These results taken together suggest that the absence of DJ-1 leads to an increase in mitochondrial H_2O_2 and a compensatory increase in mitochondrial GPx activity in aged DJ-1 KO mice, suggesting that DJ-1 plays an important role in mitochondrial elimination of H_2O_2 [94]. Furthermore, the decreased mitochondrial aconitase activity (an enzyme which is highly sensitive to ROS), as a consequence of an increase of mitochondrial H_2O_2 production, was only detected in young DJ-1 KO mice, and not in aged mice that showed an increased expression in GPx [94].

Recently, Guzman et al. [96] have shown, using transgenic mice that expressed a redox-sensitive variant of green fluorescent protein targeted to the mitochondrial matrix, that the engagement of plasma membrane L-type Ca^{2+} channels during normal autonomous pacemaking created an oxidative stress that was specific to the nigral neurons, and that the oxidative stress engaged defenses that induced transient, mild mitochondrial depolarization or uncoupling. This demonstrates that in mature SNc dopaminergic neurons a basal mitochondrial oxidant stress is present, and that the oxidative stress is not a consequence of old age, pathology, or the experimental preparation, but rather one of a neuronal design that engages L-type Ca^{2+} channels during autonomous pacemaking. Furthermore, SNc dopaminergic neurons lacking DJ-1 were shown to have an elevation in mitochondrial oxidative stress, and additional experiments confirmed that this was not due to lowered expression of antioxidant enzymes, but rather to diminished levels of

Table 2: Oxidative stress parameters in transgenic mouse models of Parkinson's disease.

Model	Age (mo.)	Marker for oxidative stress	Sampled region	Technique	Outcome (versus control)	References
DJ-1 KO	5, 11	Protein oxidation	Entire brain	Oxyblot	↔	[93]
DJ-1 KO	2-3,18–24	Protein oxidation	Entire brain	Oxyblot	↔	[94]
		Mitochondrial ROS	Entire brain	Amplex red assay	↑	
		Mitochondrial aconitase activity	Entire brain	Mitochondrial aconitase activity assay	↓ at 2-3 mo., ↔ at 18–24 mo.	
DJ-1 KO	24–27	DNA/RNA oxidation	SN	IHC	↔	[95]
		Nitrotyrosine	SN	IHC	↔	
		4-HNE	SN	IHC	↔	
DJ-1 KO	1	Mitochondrial ROS	SN	2PLSM	↑	[96]
		Mn-SOD	SN	RT-qPCR	↔	
		GPx	SN	RT-qPCR	↔	
		Catalase	SN	RT-qPCR	↔	
DJ-1 KO	4–6	Mitochondrial ROS	Entire brain	PHPA assay	↑	[97]
Parkin KO	22	Protein oxidation	Striatum, cortex	Oxyblot	↔	[98]
Parkin KO	11	Total GSH	Striatum	Method of Tietze	↑	[99]
		GSSH	Striatum	Method of Griffith	↔	
Parkin KO	8	Peroxiredoxin 1	Ventral midbrain	2DGE + MS	↓	[100]
		Peroxiredoxin 2	Ventral midbrain	2DGE + MS	↓	
		Peroxiredoxin 6	Ventral midbrain	2DGE + MS	↓	
		Lactoylglutathione lyase	Ventral midbrain	2DGE + MS	↓	
	3, 6, 18	4-HNE	Brain	IHC	↔ at 3 or 6 mo., ↑ at 18 mo.	
	5–12	Total antioxidant capacity	Serum	Serum antioxidant capacity assay	↓	
	3, 18–20	Protein oxidation	Entire brain	Oxyblot	↔ at 3 mo., ↑ at 18–20 mo.	
Parkin KO	2, 12	Mitochondrial aconitase	Striatum, cortex	2DGE + MS	↑ in cortex at 2 mo.	[101]
		GSH S-transferase P 2	Striatum, cortex	2DGE + MS	↓ in striatum and cortex at 2 mo.	
		Carbonyl reductase [NADPH] 1	Striatum, cortex	2DGE + MS	↑ in striatum at 2 mo.	
		Lactoylglutathione lyase	Striatum, cortex	2DGE + MS	↑ in striatum at 2 mo.	
		Thioredoxin reductase	Striatum, cortex	2DGE + MS	↑ in striatum at 2 mo.	
		Protein oxidation	Striatum, cortex	Oxyblot	↔	
Parkin KO	2, 24	Total GSH	Striatum, midbrain, limbic system	Method of Tietze	↑ at 2 mo., ↓ at 24 mo.	[102]
		GSH reductase activity	Striatum, midbrain, limbic system	GSH reductase activity assay	↔ (within KO group, ↑ at 24 mo. versus 2 mo.)	
		GPx activity	Striatum, midbrain, limbic system	Method of Flohe and Gunzler	↔ (within KO group, ↑ at 24 mo. versus 2 mo.)	
		Catalase activity	Striatum, midbrain, limbic system	Reaction with methanol in presence of H_2O_2	↔	

Table 2: Continued.

Model	Age (mo.)	Marker for oxidative stress	Sampled region	Technique	Outcome (versus control)	References
Parkin KO	19–21	Nitrotyrosine	SN	ELISA	↑	[103]
		Nitrated α-synuclein	SN	IHC	Only KO stain positive	
PINK1 KO	3-4	Lipid peroxidation	Striatum, cortex	TBARS levels	↔	[29]
	24	Protein oxidation	Striatum	Oxyblot	↔	
		4-HNE	SN	IHC	↔	
		Mitochondrial ROS	Striatum, cortex	Amplex red assay	↔	
	22–24	Mn-SOD	Striatum	WB	↔	
		CuZn-SOD	Striatum	WB	↔	
		Catalase	Striatum	WB	↔	
		G6-PDH	Striatum	WB	↔	
PINK1 KO	2	Mitochondrial ROS		DCF assay	↔	[104]
PINK1 KO	4	GSH synthetase	Striatum, midbrain, cortex	2DGE + MS	↑ in cortex and striatum	[28]
		Peroxiredoxin 1	Striatum, midbrain, cortex	2DGE + MS	↑ in cortex	
		Protein oxidation	Striatum, midbrain, cortex	Oxyblot	↔	
PINK1 KO	2-3	4-HAE	Left ventricle	MDA/4-HAE kit	↑	[105]
		8-OHdG	Left ventricle	8-OHdG kit	↑	
		GSH/GSSG	Left ventricle	GSH/GSSG kit	↓	
		SOD activity	Left ventricle	SOD activity kit	↓	
		Aconitase activity	Left ventricle	Aconitase activity kit	↓	
SNCA KO	N.R.	Hydroxyl radicals	Striatum	4-HBA trapping	↔	[106]
SNCA OExp A30P	N.R.	Oxidized/nitrated α-syn	Brain	IHC	Only OExp stain positive	[107]
SNCA OExp A30P	6–14	Protein oxidation	Entire brain	2DGE + WB	↑ (oxidized mitochondrial proteins Car2, Eno1, Ldh2)	[108]
SNCA OExp A53T	12	Oxidized α-syn	Spinal cord, locus coeruleus	IHC	OExp stain positive	[109]
		Nitrated α-syn	Pons	IHC	OExp stain positive	
SNCA OExp A53T	N.R.	Mitochondrial DNA damage	Brainstem, neocortex, spinal cord	TUNEL technique	↑	[110]
		Nitrated α-syn	Spinal cord	IHC	OExp stain positive	
SNCA OExp A53T A30P	3, 6	GPx 3	Striatum	Microarray	↓	[111]
		SOD 2	Striatum	Microarray	↓	
SNCA OExp WT	12	Oxidized α-syn	Entire brain	WB	↑	[112]

Abbreviations: 2DGE: 2D gelelectrophoresis, 2-HBA: salicylate, 4-HNE: 4-hydroxynonenal, 4-HAE: 4-hydroxyalkenal, 8-OhdG: 8-hydroxydeoxyguanosine, α-syn: α-synuclein, GPx: *GSH peroxidase*, GSH: glutathion, GSSH: oxidized glutathion, H_2O_2: hydrogen peroxide, IHC: immunohistochemistry, IF: immunofluorescence, KO: knockout, mo: months, MS: mass spectrometry, OExp: overexpression, PHPA: p-hydroxyphenylacetate, ROS: reactive oxygen species, SN: substantia nigra, SOD: sodium dismutase, WB: Western blot, WT: wildtype, ↑: increased, ↓: decreased, ↔: not different.

UCP4 and UCP5, comprising mitochondrial uncoupling in response to Ca^{2+}-induced stress [96].

Furthermore, DJ-1 interacts with DAXX, a protein that binds to a fas death receptor and activates the JNK apoptotic pathway. By binding to DAXX, DJ-1 sequesters the protein into the nucleus and prevents it from gaining access to the cytoplasm and from inducing apoptosis at the fas death receptor. DAXX upregulation has been found to occur in cells that have been treated with H_2O_2, linking oxidative stress to this pathway. These data show that DJ-1 is involved in inactivating the apoptotic JNK pathway that is activated by oxidative stress, indicating its role in guarding against oxidative-stress-induced cell death [113].

4.2. Parkin Models. In line with the findings on DJ-1 deficient mice, no significant differences in protein oxidation have been found in different brain structures of parkin KO mice at the age of 2, 3, 12, and 22 months [100, 101, 114]. However, when the entire brain was analyzed for protein oxidation, an increase was described in parkin KO mice at 18 to 20 months [100]. The same pattern was observed for lipid peroxidation in aged parkin KO mice [100].

Total striatal GSH levels were elevated in parkin KO mice at the age of 11 months, while no differences were observed in GSSG levels [99]. Rodríguez-Navarro et al. [102] also noticed increased total GSH levels in the striatum, midbrain, and limbic system of young parkin KO mice; however, total GSH levels were decreased at 24 months old of age in all the brain regions. No changes were observed in the activity of GSH reductase, catalase and GPx in the same brain regions when comparing age-matched parkin KO, but an age-dependent increase was found for GPx and reductase within the parkin KO group [102].

It is interesting to note that the endogenous levels of DA were increased in the limbic regions of the above-mentioned mutant mice, while the DOPAC/DA and DOPAC/3-methoxytyramine (3-MT) ratios were also increased. The increased DA levels and increased DA metabolism to DOPAC via MAO, an enzyme considered to be mostly intraneuronal, and to 3-MT via COMT, an enzyme considered mostly extraneuronal, suggest that parkin dysfunction impairs the release of DA and increases intraneuronal DA metabolism via MAO. To determine in which cellular compartment DA is shifted to intraneuronal oxidation, the turnover of catecholamines after inhibition of their synthesis by α-methyltyrosine (α-MT) was investigated. The rate of depletion of catecholamines in α-MT-treated animals was similar in both mutant and WT animals, suggesting that the turnover is similar in both groups. Since the short-term effects of α-MT on catecholamines are mostly related to the turnover of the newly synthesized intracytoplasmic pool of catecholamines, rather than the vesicular pool of catecholamines, these data suggest that the increased production of DOPAC in the absence of parkin is not mediated by changes in DA turnover, but due to intracytoplasmatic DA being unprotected against metabolism by MAO. Hence, monoamine metabolism in parkin mutant mice was shifted towards the H_2O_2 producing MAO pathway, with a compensatory increase in GSH levels. Although there was no evidence for a reduction of nigrostriatal dopaminergic neurons in the parkin mutant mice, the level of DA transporter (DAT) protein was reduced in these animals, suggesting a decreased density of dopaminergic terminals or adaptative changes in the nigrostriatal system. Such an adaptive change was revealed by Itier et al. (2003) who noticed reduced amphetamine-induced DA release in neuronal cultures from parkin mutant mice compared to WT [99]. A similar decrease in evoked DA release in striatal slices was demonstrated by Kitada et al. [115]; however, the levels of total DA and striatal DAT were comparable between the experimental groups [115]. On the contrary, another study showed higher striatal levels of DA, DOPAC, and homovanillic acid (HVA) in parkin KO mice [116]. Similarly, increased DA levels in the midbrain, alongside a decrease in endogenous DA release after methamphetamine (METH) challenge shown by *ex vivo* autoradiography, and an upregulation of DA D1 and D2 receptor binding in the striatum were shown in parkin KO mice [117].

A proteomic analysis, performed by two-dimensional gel electrophoresis coupled with mass spectrometry, revealed several markers of oxidative stress to be upregulated or downregulated in either the ventral midbrain, or the striatum and cortex of parkin KO mice. In the ventral midbrain of 8-month-old parkin KO mice, a decrease in peroxiredoxin 1, 2, 6 and lactoylglutathione lyase was observed [100]. Furthermore, a decreased antioxidant capacity was observed in the serum of parkin KO mice [100]. Several proteins involved in detoxification, stress-related chaperones, and components of the UPS were also altered in parkin KO mice [101]. These differences might reflect adaptive mechanisms aimed at compensating for the presence of ROS and the accumulation of damaged proteins in parkin KO mice. This study provides clues into possible compensatory mechanisms that protect dopaminergic neurons from death in parkin KO mice and may help to understand the preclinical deficits observed in parkin-related parkinsonism [101].

No gross mitochondrial abnormalities in striatal mitochondria from parkin KO mice were demonstrated, but reductions in the respiratory capacity were found [100]. Additional findings, which might be related to increased ROS, are an increased astrogliosis in the striatum and increased levels of microglial markers in the midbrain of aged parkin KO mice [102]. Furthermore, in aged parkin KO mice, higher levels of nitrotyrosine and nitrated α-syn were found in the SN [103]. However, in another parkin KO model, immunohistochemical analysis of olfactory bulb, cortex, basal ganglia, midbrain, and brainstem of α-syn, glial fibrillary acidic protein, and ubiquitin, as well as staining with thioflavin S, hematoxylin/eosin, and silver, revealed no differences between parkin null mice compared with WT mice [114, 118]. Only one group demonstrated age-dependent dopaminergic degeneration in the SN of parkin KO mutant determined by a significant loss of dopaminergic neuron terminals in the striatum and a reduction of striatal DA levels. Furthermore, age-dependent accumulation of proteinase-K-resistant endogenous α-syn in the SN was found, which was colocalized with 3-nitrotyrosine (3-NT) [103]. However, earlier it was demonstrated that the ratio

of prosurvival/proapoptotic proteins, as measured by the fraction Bcl2/Bax, is reduced in the striatum and the midbrain of young mature and of aged parkin KO mice with respect to their respective age-matched controls. This was associated with a greater number of apoptotic cells with fragmented DNA in the striatum and SN of 24-month-old parkin KO mice than in any other group [102].

4.3. PINK1 Models. Similarly, as with the other mouse models, no significant differences in protein oxidation and lipid peroxidation have been found in different brain structures of PINK1-deficient mice [28, 29]. However, in the left ventricle of PINK1 KO mice, increased lipid peroxidation and DNA oxidation were observed [105].

The role of PINK1 in the maintenance of basic functions of mitochondria was investigated by several groups [30, 119]. In this perspective, PINK1 KO mice did not reveal any changes in the ultrastructure or the total number of mitochondria [29, 120]. Nevertheless, some of the functional aspects of the mitochondria were found altered in PINK1 KO mice. Reduced mitochondrial respiration was shown in a brain-region-specific manner in young PINK1 KO mice, with the impairment present in the striatum, which is rich in dopaminergic nerve terminals, but absent in the cerebral cortex, suggesting that high concentrations of DA in the striatum may render the striatum more sensitive to the loss of PINK1 than in the cerebral cortex [29]. Consistent with the presence of oxidative stress in the striatum, and independent of the genotype, an increased lipid peroxidation was present in the striatal mitochondria relative to cortical mitochondria [29]. Furthermore, in the striatum of PINK1 KO mice, no differences in different antioxidant proteins could be observed [27, 29].

However, with a large proteomic screening, increased levels of GSH synthetase in the striatum and cortex and increased levels of peroxiredoxin 1 in the cortex were observed in PINK1 KO mice [28]. Within the left ventricle of PINK1 KO mice, decreased activities of SOD, and of mitochondrial and cytoplasmic aconitase were observed, associated with a decreased ratio GSH/GSSG [105].

Mitochondria isolated from PINK1 KO mice had significantly reduced membrane potential [55]. As has been noted previously, under conditions of oxidative stress, the cysteines of the mitochondrial permeability transition pore undergo oxidation, thus increasing the probability for the pore to open and collapse the transmembrane potential. In conditions of PINK1 deficiency, the changes in mitochondrial transmembrane potential are independent of the mitochondrial permeability transition pore regulation, suggesting that other pathways are involved [29]. PINK1 KO mice do not show any loss of dopaminergic neurons and have normal levels of striatal DA and DA receptors [88, 121]. Although silencing of PINK1 does not cause degeneration, it induces some functional deficits, such as a decrease in evoked extracellular striatal DA levels, which may lead to a decreased activation of postsynaptic DA 1 and DA 2 receptors and, as a consequence, to defects in striatal plasticity and in spontaneous locomotor activity [121, 122]. It has also been observed that silencing of PINK1 expression in mice using RNA interference did not change the striatal DA content and the nigral dopaminergic neuron number [123]. These observations strengthen the hypothesis that PINK1 has a critical role in DA release and that loss of PINK1 may play a role in mechanisms preceeding nigrostriatal degeneration.

It has been demonstrated that PINK1 can protect mitochondria via the activation of mitochondrial chaperones TRAP-1 and HtrA2. *In vitro* studies lacking these chaperones, such as the study by Gautier et al. [29], only support a direct protective role of PINK1 [29]. The neuroprotective mechanisms of PINK1 might involve the phosphorylation of these key signaling proteins in pathways governing cell death and survival. Upon phosphorylation, these proteins may mediate neuroprotective signals of PINK1 by different mechanisms. Upon phosphorylation, TRAP-1 is activated and blocks generation of mitochondrial ROS. HtrA2 phosphorylation protects neuronal cells from undergoing apoptosis induced by neurotoxins and oxidative stress [124].

Several studies in PINK1-deficient neuronal cells and fibroblasts from patients carrying PINK1 mutations also revealed increased levels of mitochondrial ROS and lipid peroxidation [125]. The oxidative stress observed in these patients or in PINK1-deficient cells can also result from reduced antioxidant defense mechanisms. Indeed, Gegg et al. [126] demonstrated that silencing PINK1 resulted in decreased cellular levels of reduced GSH, as reduced GSH levels were decreased in human PINK1 KD cells, as well as in human fibroblasts derived from PINK1 patients [126]. Mitochondrial Ca^{2+} overload and a concomitant impaired buffering capacity were also observed due to PINK1 deficiency and appeared to be caused by a defective Na^{+}/Ca^{2+} antiporter function [27, 65]. An indirect role for PINK1 in Ca^{2+} signaling in zebrafish has been proposed [64]. Using a yeast two-hybrid screen, the interaction of PINK1 and a Ca^{2+} sensing molecule, neuronal Ca^{2+} sensor 1, has been demonstrated [64]. This sensor may be involved in the regulation of DA 2 receptor expression [127] and the release of neurotransmitters [128]. An increased Ca^{2+}-induced mitochondrial permeability transition was shown in purified brain mitochondria of PINK1 KO mice [29]. Accumulation of phospho-c-jun in neurons of the SN of PINK1 KO mice was shown, suggesting that JNK activity in PINK1 KO mice is increased. Therefore, the increased mitochondrial Ca^{2+} sensitivity and JNK activity are early defects in PINK1 KO mice that precede reduced DA levels and abnormal DA homeostasis, and that may contribute to neuronal dysfunction. Differential gene expression in the nigrostriatal system of PINK1 KO mice supports early dopaminergic dysfunction and shows that PINK1 deletion causes aberrant expression of genes that regulate innate immune responses [104]. Striatal gene expression profiles between 2-month-old WT and PINK1 KO mice have been performed. Several of the upregulated genes encoded stress-inducible transcription factors of the activating transcription factor (ATF,) and AP1 families, including ATF-3, c-fos, FosB, JunB and Egr-2. ATF3 is induced by multiple signals, including inflammatory cytokines, DNA-damaging agents, and physiological stressors. Interestingly, increased

striatal expression of Fos-related antigens and JunB has been observed following neuronal injury and degeneration in the DA system. Moreover, striatal c-fos expression is regulated by DA and, in the DA-depleted striatum, may be induced via compensatory supersensitivity of DA receptors [104].

Apart from complex I inhibition, recent genetic data point to PD-associated genes that are linked with normal mitochondrial function and resistance against oxidative stress, such as parkin, PINK1, and DJ-1. All these genes are believed to play an important protective role within the mitochondria by preventing mitochondrial dysfunction mediated by oxidative stress [125]. This protective role might be mediated by parkin's ubiquitin ligase function. Indeed, production of ROS is a physiological consequence of the mitochondrial respiratory chain, and parkin might play a role in removing oxidatively damaged proteins that can impair normal mitochondrial function. However, it is equally possible that parkin's protective action may be mediated via its signalling function [24]. DJ-1 can be considered as a redox-active molecule that can sense oxidative stress via oxidation of an internal cysteine residue, followed by translocation to the mitochondrial matrix and intermembrane space [9]. Once localized to the mitochondria, DJ-1 can act as a molecular chaperone to protect against oxidative stress [129]. Furthermore, DJ-1 localized in the intermembrane space can act as a free radical scavenger, preventing the accumulation of free radicals derived from the mitochondrial respiratory chain [63].

Interestingly, recent insights indicate the collaboration of all mitochondrial PD genes, DJ-1, PINK1, and parkin, in protecting the mitochondria against oxidative stress [7]. Furthermore, parkin and PINK1 seem to act in concert to mediate the autophagic degradation of terminally damaged mitochondria, functioning as a global mitochondrial quality control mechanism [119, 130, 131]. Such a quality control mechanism is important to the cell, as damaged mitochondria are sensitive to rupture and release of proapoptotic mediators via a selective form of macroautophagy known as mitophagy [130], and loss of this protective function may be an important cause of neurodegeneration in PD, as neuronal death in PD appears to occur by apoptosis rather than necrosis [17]. Consistently parkin can partially compensate for PINK1 loss when being overexpressed [132]. Nevertheless, a recent study demonstrated that even the inactivation of the genes PINK1, DJ-1, and parkin is insufficient to cause significant nigral degeneration within the lifespan of the mice, suggesting that these genes may be protective to external stressors rather than being essential for the survival of dopaminergic neurons during the normal aging process [133].

4.4. SNCA Models. Under physiological conditions, there was no difference in the basal hydroxyl radical production in the striata of WT and homozygous SNCA KO mice, suggesting that α-syn deficiency does not affect basal hydroxyl radical production [106]. As has been noted previously, α-syn is a specific target of nitration in the SNc of PD patients, suggesting the role of oxidative damage in the formation of α-syn inclusions [134]. Similarly, an increased oxidative damage, as shown by the presence of oxidized and nitrated α-syn, was shown in mice that overexpress mutant or WT α-syn [107, 109, 110, 112]. In addition, in the A30P mutant mouse model, increased levels of oxidized mitochondrial proteins were found [108], whereas in mice overexpressing the mutant A53T form of α-syn, increased mtDNA damage was demonstrated [110]. The presence of mtDNA damage is possibly related to oxidative stress, since the presence of nitrated α-syn was shown in the same mouse model [110].

Moreover, in double mutant α-syn transgenic mice, decreased expression levels of GPx 3 and SOD-2 were described, yet this still needs to be confirmed at the protein level [111]. Furthermore, an A53T α-syn transgenic mouse model was found to have higher DJ-1 levels at 3 months of age, suggesting an attempt to preserve mitochondrial function and reduce oxidative burden [55]. Furthermore, it was shown that α-syn is colocalized with the JNK interacting protein (JIP). This proposes a protective role for α-syn against oxidative stress, due to its ability to inactivate the JNK pathway via upregulation of JIP. The protective activity of α-syn may be decreased when the protein is mutated, misfolded, or aggregated and therefore unable to interact with JIP [113]. Growing evidence indicates a connection between α-syn, oxidative stress, and mitochondrial dysfunction in PD [54, 81]. In this connection, α-syn can bind to mitochondrial complex I and inhibit its activity, illustrating one way in which excess α-syn can aberrantly induce mitochondrial dysfunction [55]. The oxidative events seem to be fairly target specific, concerning the modified amino-acid residue. Indeed, for instance, the nitration of tyrosine residues within the α-syn protein is found to accumulate in the LBs [36].

The main involvement of SNCA in PD is via α-syn aggregation, as α-syn oligomers are considered as an important source of cell toxicity for dopaminergic neurons [129]. Selective damage to both soluble and insoluble α-syn, which increases the tendency to aggregate, has been demonstrated in the brains of PD patients [81]. Free radicals may lead to the stabilization of a toxic protofibril of α-syn, and this stabilization appears to depend on the oxidative ligation of α-syn to DA [81]. Furthermore, α-syn is involved in synaptic vesicle homeostasis and the storage of DA in synaptic vesicles, and the loss of normal α-syn function following aggregation can lead to increased levels of oxidative stress [56]. Furthermore, the described mutations reduce the affinity of α-syn for lipids, therefore increasing the cytoplasmic pools of the protein, and this in turn can be affected by several mechanisms [23]. Furthermore, it is known that lipid-bound WT α-syn is a potent inhibitor of phospholipase D2, an enzyme that hydrolyzes phospholipids, and that is involved in synaptic vesicle recycling [56]. As a consequence of the mutations, a decreased phospholipase D2 inhibition will in turn lead to enhanced and dysregulated synaptic vesicle recycling and reduced synaptic vesicle formation. The inability of the neurons to from synaptic vesicles from the early endosome may result in a decreased number of vesicles available for DA storage, leading to accumulation of DA in the cytoplasm [56]. It was shown that toxic oligomeric aggregates of α-syn can also bind to and form pores in

the vesicle membrane, thereby releasing the remaining DA vesicular stores in the cytoplasm [63]. As a consequence, mutations in SNCA can lead to both loss of normal α-syn function in synaptic vesicle dynamics, and gain-of function of toxic α-syn aggregation [56], both pathways leading to a higher content of cytoplasmic DA, which in turn considerably increases cellular oxidative stress [23]. Mutations in SCNA have also been shown to have a modulatory action on tyrosine hydroxylase (TH) activation, inhibit of vesicular monoamine transporter 2 activity, interact with septin4 [135], and alter the expression of dihydropteridine reductase [51]. Furthermore, a complex formation with the human DAT has been shown, thereby inhibiting the uptake of DA by its transporter [51].

Recently, induced pluripotent stem-cell-derived dopaminergic neurons obtained from a PD patient with SNCA triplication showed increased levels of oxidative stress and were more sensitive to H_2O_2-induced oxidative stress [45]. Additionally, α-syn levels increase within the dopaminergic neurons with aging, an important etiological factor of PD [24]. These findings and the intrinsic potential for the oxidative metabolism of DA to generate ROS have suggested that oxidative stress may be involved in the degeneration of the dopaminergic neurons in the SNc of PD patients [134].

Altogether, these findings indicate that genetic alterations in the PD genes parkin, PINK1, DJ-1, and SNCA can contribute to enhanced levels of oxidative stress. Even though the precise mechanistic subtleties are yet to be revealed, the working hypothesis based on the previously presented data is that they could enhance cellular oxidative stress by contributing to oxidative stress generating pathways in dopaminergic neurons, such as improper DA handling, mitochondrial dysfunction, and neuroinflammation (Figure 1).

5. Gene-Environment Interactions in Mouse Models of Parkinson's Disease

Besides the genetic background, environmental factors, such as environmental toxins or lifestyle factors, may have a role in PD pathogenesis [24].

5.1. Environmental Toxins. Hints that environmental toxins might play a role in the molecular pathology of PD first appeared after the accidental administration of MPTP in a group of young intravenous drug users, who eventually developed a clinical phenotype reminiscent of late-stage PD, albeit in the absence of LB pathology [69]. It was the first proof that the exposure to an environmental toxin could produce parkinsonism in humans. MPTP was subsequently identified as a potent neurotoxin that can easily cross the blood-brain barrier, being metabolized to 4-phenyl-2,3-dihydropyridinium ion (MPP^+) in astrocytes, a potent mitochondrial complex I inhibitor, that is then selectively transported into dopaminergic neurons via DAT, ultimately leading to cell death via mitochondrial impairment [24]. In addition to MPTP, other environmental toxins, such as the herbicide paraquat and rotenone have been identified as contributors to dopaminergic neuronal cell death and parkinsonism, supporting further the link between environmental exposure to pesticides and a risk of developing PD. Interestingly, a recent meta-analysis of 19 studies on the involvement of environmental pesticides in the pathogenesis of PD found an estimated doubling of the risk to develop PD [7]. Four individual pesticides were found to increase the risk of PD: dieldrin, maneb, paraquat, and rotenone [24], the latter two behaving as mitochondrial toxins, in a similar manner to MPTP [7].

5.2. Lifestyle Factors. Lifestyle factors and dietary habits have also been shown to influence the risk of developing PD. Epidemiological data showing that consumption of coffee, tobacco, and nonsteroidal anti-inflammatory drugs reduces the risk of PD are intriguing [1, 55]. A high lipid consumption and caloric intake have been investigated as potential risk factors in PD. This was confirmed by a study showing that eating foods high in animal fat is associated with increased risk of PD [136].

5.3. Gene-Environment Interactions. As previously noted, the current view is that the ethiopathogenesis of PD is fueled by an interplay of genetic, environmental, and age-related factors, with gene-environment interactions playing a critical role [24]. In certain cases, genetic susceptibility is insufficient to lead on its own to PD [24]. For instance, the penetrance of some monogenic forms of PD is incomplete and variable, such as the penetrance of the Gly2019Ser mutation in LRRK2 [24]. Therefore, other factors, such as age and environmental exposure to certain toxins, might contribute to the pathology. At the same time, the converse is also true, as the risk of developing PD after toxin exposure also seems to be determined in part by genetic factors. Indeed, it seems that the presence of mutations that predisposes to spontaneous neurodegeneration are able to increase the endogenous toxicity of environmental toxins [24]. Recent studies also suggest that environmental factors may contribute to neurodegeneration through the induction of epigenetic modifications, such as DNA methylation and chromatin remodeling, which may induce alterations in gene expression [137]. In PD, a direct relation between epigenetics and neurodegeneration has not been widely exploited yet [137]. It has been shown that methylation of the α-syn intron 1 was reduced in DNA from SN, putamen, and cortex in patients with sporadic PD. Methylation of this intron region regulates the α-syn expression in experiments using a luciferase reporter, suggesting that hypomethylation of α-syn intron 1 contributes to increased expression of α-syn in PD. In turn the toxicity induced by α-syn can be rescued by administration of histone deacetylase (HDAC) inhibitors in both cell culture and a transgenic fruit fly Drosophila model. Furthermore, HDAC inhibitors may be neuroprotective in dopaminergic neurons and in rodent models of PD [138]. Epigenetics may constitute the missing link in the interplay between genes and environment, despite this, it still remains largely unexplored [137].

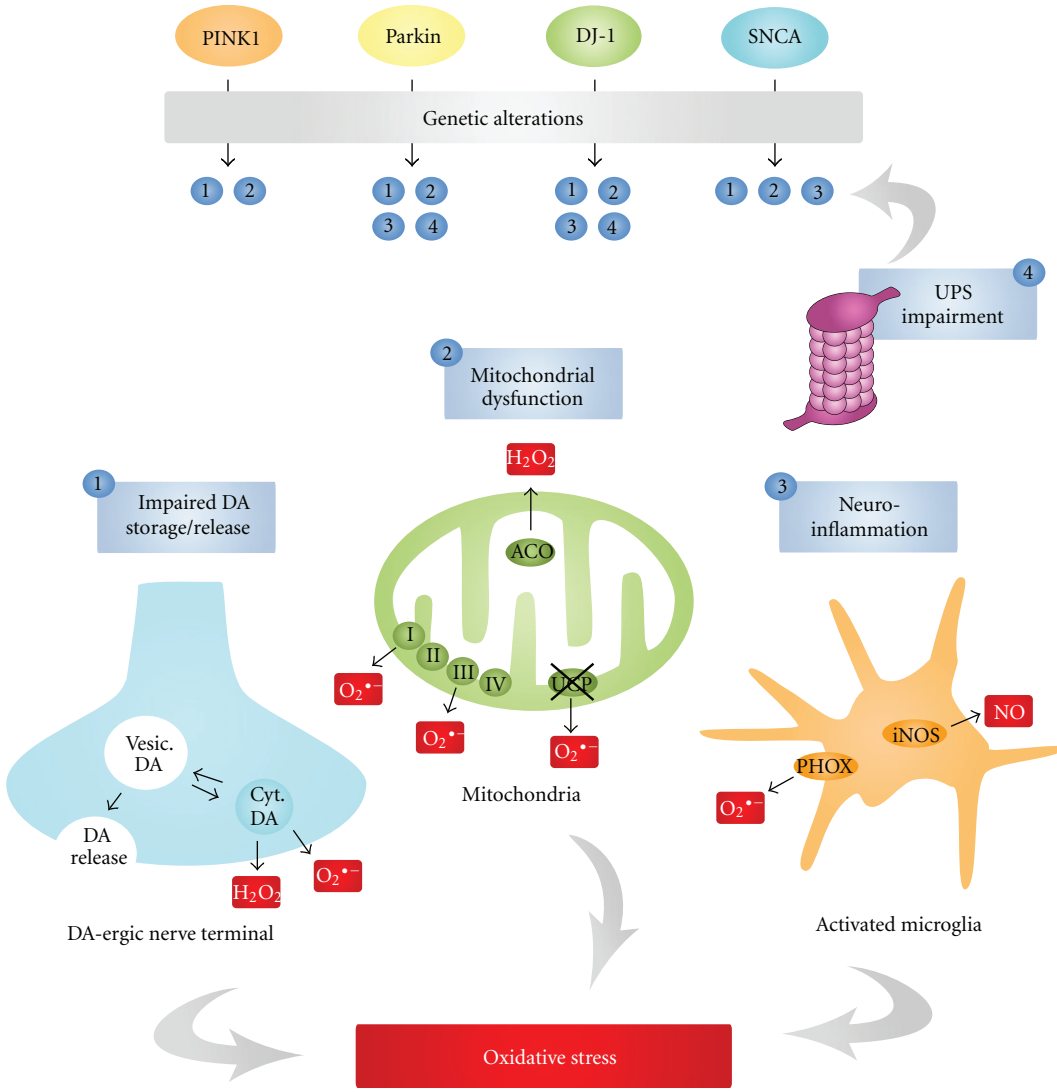

Figure 1: Pathways leading to oxidative stress in PD and the modulation by PD-related genes. Different pathways contribute to high levels of oxidative stress in dopaminergic neurons, including impaired DA handling (1), mitochondrial dysfunction (2), and neuroinflammation (3). As further discussed in the text, alterations in PD genes can potentiate all these impairments and, therefore, lead to enhanced levels of oxidative stress (see Table 2). UPS dysfunction (4), another important pathogenic pathway in PD, can contribute to the damaging effects of α-syn, due to improper degradation of α-syn mutants or oligomers. Abbreviations: ACO: aconitase, CYT: cytosolic, DA: dopamine, iNOS: inducible nitric oxide synthase, PHOX: NADPH oxidase, UPS: ubiquitin proteasome system, UCP: uncoupling proteins, VESIC: vesicular.

5.4. Toxin-Induced Models. The classical animal models of PD are based on the use of neurotoxins that reproduce some of the pathological and behavioural changes of the disease by inducing selective degeneration of the nigrostriatal system. The prototypical toxin-induced PD model is the 6-OHDA model, which is still the most commonly used procedure for obtaining PD-like loss of dopaminergic neurons in the SNc [139]. Until now, the neurotoxicity of 6-OHDA has been linked to (auto-) oxidation and the formation of quinones, H_2O_2 and oxygen radicals, intra- and extracellularly, and to inhibition of complex I and complex IV of the respiratory chain. Next to this, it has been shown to induce a massive release of DA in the extracellular space of the striatum, which in turn may contribute to oxidative stress. An increased level of DA may be correlated with an increased DA metabolism, which in turn may lead to an enhanced basal production of H_2O_2 and a depletion of GSH. It has also been demonstrated that nigrostriatal infusion of 6-OHDA is associated with an increased inflammatory response, due to activated microglia, which produce and release a broad spectrum of free radicals and inflammatory cytokines [139].

5.5. Genetic Models and Environmental Challenge. To complete our understanding of the genetic contribution to PD, a full characterization of the genetic architecture of the disease is needed, together with an exploration to the complex interaction between genetic and environmental factors. As the

current genetic models of PD do not reproduce the complete disease pattern, the influence of genes, environment, and the interaction between both on different aspects of PD should be explored through the use of appropriate animal models. Nevertheless, genetic models can still provide interesting insights into the role of different genes in the pathogenic pathways of PD.

Integrating the use of neurotoxins, thereby simulating the environmental etiology, and the genetically engineered mice models, enables to simulate the gene-environment interactions that might occur during the development of PD. Consequently, a literature search was performed for studies where the susceptibility of genetic PD models to environmental stressors has been investigated (Table 3). Of course, not all environmental stressors used in the experimental settings are widely available to human exposure but should be considered as surrogate environmental stressors, as there is considerable overlap in pathogenic pathways. For example, 6-OHDA can be used as a surrogate stressor, as it has a similar mechanism of action as the more common environmental toxins, such as rotenone and paraquat. Furthermore, to increase the predictive validity of the animal model, the main etiological risk factor of PD, namely, ageing, can be integrated within the model, thereby representing more realistic conditions.

6. Transgenic Models in Conjunction with a Neurotoxin

6.1. DJ-1 Mouse Models. DJ-1 KO mice of 2 to 4 months old were found to be significantly more susceptible to nigrostriatal deficits following systemic MPTP injection [140, 141]. Furthermore, in WT mice, adenoviral-mediated overexpression of DJ-1 blocks MPTP-induced neuronal loss and protects the animals against neurodegeneration in the SNc [140, 142]. Consequently, these findings point to a physiological role for DJ-1 in the protection of neurons against oxidative stress and environmental neurotoxins [140].

However, exposure of MPTP to differently generated DJ-1 KO mice resulted in striatal DA depletion with no differences in dopaminergic nigral cell loss [141]. Furthermore, enhanced DAT levels in mice deficient in DJ-1 protein were revealed when the transporter was evaluated in cell preparations enriched for synaptosomal membranes [141]. The discrepancy with the findings of Kim et al. [140] is likely due to differences among the various lines of DJ-1-deficient mice that differ in their sensitivity to MPTP [140, 141]. One explanation could be that the C57BL/6 background strain of the DJ-1 KO mice confers higher sensitivity to MPTP than the B6/129 background of transgenic animals of Manning-Bog et al. [140, 141]. Indeed, since a couple of years, several reasons to avoid the use of MPTP have been proposed, including its strain-, age-, and gender-dependency in mice [150, 160–164].

6.2. Parkin Mouse Models. Parkin KO mice of 3 months of age challenged with the neurotoxins METH or 6-OHDA are not more sensitive compared to WT mice, as demonstrated by analysis of striatal DA levels [98]. METH is a toxin that releases vesicular DA into the cytoplasm and extracellular space and can inhibit mitochondrial function [98].

These findings suggest that the absence of a robust parkinsonian phenotype in parkin deficient mice is not due to the lack of exposure to environmental triggers with mechanisms of action similar to METH or 6-OHDA. Nevertheless, parkin-deficient mice could be more sensitive to other neurotoxins, such as rotenone or MPTP, which have different mechanisms of action [98]. Indeed, overexpression of parkin in combination with MPTP exposure appears to provide some protective benefits [142], whereas parkin delivery rescues α-synucleinopathy in a rat model [89, 142].

6.3. PINK1 Mouse Models. The exposure of mitochondria isolated from the striatum or the cerebral cortex of aged PINK1 KO mice to mitochondrial toxins, such as paraquat, 6-OHDA, DA, and rotenone, does not lead to a different level of H_2O_2 production as in basal conditions [29]. PINK1 KO mice challenged with a systemic, acute lipopolysaccharide (LPS) injection and killed 8 hours after LPS administration demonstrated higher striatal expression levels of IL-1β, IL-12, and IL-10. Striatal levels of 12 cytokines were not different versus WT without LPS challenge. Furthermore, a tendency for increased striatal expression of IL-2, IL-4, and TNF-α was observed after the LPS challenge. No differences were found in striatal CD3 expression before and after the LPS challenge. Isolated and cultured neonatal microglia from forebrain of PINK1 KO mice were also challenged with LPS. Higher expression of IL-10 and similar expression of IL-6, TNF-α, and granulocyte-colony stimulating factor (G-CSF) were found in cultured microglia after an LPS challenge [104]. In a recently published study, a conditional PINK1 RNA interference (RNAi) transgenic mouse model was exposed to paraquat. Mice were exposed to paraquat at the age of 14 days and further at the age of 12 weeks for 3 consecutive weeks. One week after the last paraquat injection, RNAi-mediated PINK1 KD was activated by injection with 4-hydroxytamoxifen to induce Cre activity. At the age of 20 months, significant loss of dopaminergic neurons and loss of striatal DA was found in these mice [143]. Consistently, locoregional overexpression of PINK1 protects dopaminergic neurons from degeneration caused by MPTP [144]. Taken together, these data reveal an important *in vivo* role of PINK1 in protecting dopaminergic neurons against toxin-induced cell death.

6.4. SNCA Mouse Models. As shown by different groups, α-syn-deleted mice are more resistant to MPTP neurotoxicity than their WT littermates, as demonstrated via striatal DA content determination and/or staining of the nerve terminals and/or dopaminergic nigral neurons [106, 145–149]. However, the intrastriatal administration of 3-nitropropionic acid resulted in a significant increase in the striatal hydroxyl radical production in the WT mice, which was significantly attenuated in heterozygous and homozygous SCNA KO mice in a dose-dependent manner, suggesting that α-syn deficiency results in neuroprotection

Table 3: Gene-environment interactions in transgenic mouse models of Parkinson's disease.

Model	Age (mo.)	Marker for susceptibility to oxidative stress	Sampled region	Technique	Outcome (versus control)	References
DJ-1 KO	2-3	Lesion after MPTP	SN, striatum	TH/DAT IHC, DA HPLC	↑	[140]
DJ-1 KO	3-4	Lesion after MPTP	Striatum	TH WB, DA HPLC	↑	[141]
DJ-1 OExp	N.R.	Lesion after MPTP	SN	TH IHC	↓	[142]
Parkin KO	3	Lesion after 6-OHDA	Striatum	DA HPLC	↔	[98]
		Lesion after METH	Striatum	DA HPLC	↔	
Parkin OExp	N.R.	Lesion after MPTP	SN	TH IHC	↓	[142]
PINK1 KO	24	Mitochondrial ROS after paraquat, 6-OHDA, DA, or rotenone	Striatum, cortex	Amplex red assay	↔	[29]
PINK1 KO	6	IL-1β, IL-12, and IL-10 after LPS	Striatum	ELISA	↑	[104]
PINK1 KD	20	Lesion after paraquat	SN, striatum	TH IHC, DA HPLC	↑	[143]
PINK1 OExp	2-3	Lesion after MPTP	SN	TH IHC	↓	[144]
SNCA KO	2	Lesion after MPTP	SN, striatum	TH IHC	↓	[145]
SNCA KO	2-3	Lesion after MPTP	Striatum	DA HPLC	↓	[146]
SNCA KO	2-3	Lesion after MPTP	Striatum	DA HPLC	↓	[147]
SNCA KO	9	Lesion after MPTP	SN	TH IHC	↓	[148]
SNCA KO	2-3	Lesion after MPTP	SN, striatum	TH IHC, DA HPLC	↓	[149]
SNCA KO	N.R.	Lesion after MPTP	SN, striatum	DAT IHC, DA HPLC	↓	[106]
		Hydroxyl radicals after 3-NP	Striatum	4-HBA trapping	↓	
SNCA KO	2-3	Lesion after 6-OHDA	SN	TH IHC	↓	[150]
		Lesion after 6-OHDA	Striatum	DA HPLC	↔	
SNCA OExp A30P	6	Lesion after MPTP	SN, striatum	TH IHC, DA HPLC	↔	[151]
SNCA OExp A30P	3-4, 6–8	Lesion after MPTP	SN	TH IHC	↑	[152]
		Lesion after MPTP	Striatum	DA HPLC	↔ at 3-4 mo., ↑ at 6–8 mo.	
		Lesion after rotenone	SN, striatum	TH IHC, DA HPLC	↔	
SNCA OExp A53T	2	Lesion after MPTP	SN, striatum	TH IHC, DA HPLC	↔	[153]

Table 3: Continued.

Model	Age (mo.)	Marker for susceptibility to oxidative stress	Sampled region	Technique	Outcome (versus control)	References
SNCA OExp A53T	9-10	Lesion after MPTP	Midbrain	DA HPLC	↔	[154]
		3-NT after MPTP	Olfactory bulb	WB	↑	
SNCA OExp A53T	2, 12, 23	Lesion after iron and/or paraquat	SN	TH IHC	↔ at 2 mo. and 12 mo., ↑ at 23 mo.	[155]
	2, 23	3-NT after iron and/or paraquat	SN	TH 3NT IF	↔ at 2 mo., ↑ at 23 mo.	
	12	Nitrated α-syn after iron and/or paraquat	SN	TH nSYN IF	↑	
SNCA OExp A53T	7	Lesion after LPS	SN, striatum	TH IHC	↑	[156]
		Nitrated α-syn after LPS	Midbrain	WB	OExp are positive for nitrated α-syn only after LPS	
SNCA OExp WT or A53T	3-4	Lesion after paraquat	SN	TH IHC	↓	[141]
SNCA OExp WT	3-4	Lesion after paraquat	SN	TH IHC	↔	[157]
SNCA OExp A53T A30P	2-3	Lesion after MPTP	Striatum	DAT IHC	↑	[158]
SNCA OExp A53T A30P	6-7	Lesion after paraquat+maneb	SN, striatum	TH IHC, DA HPLC	↑	[159]

Abbreviations: 2-HBA: salicylate, 3NT: 3-nitrotyrosine, 6-OHDA: 6-hydroxydopamine, α-syn: α-synuclein, DA: dopamine, DAT: dopamine transporter, HPLC: High Pressure Liquid Chromatography, IHC: immunohistochemistry, IF: immunofluorescence, KO: knockout, KD: knockdown, LPS: lipopolysaccharide, METH: methamphetamine, mo: months, MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, OExp: overexpression, ROS: reactive oxygen species, SN: substantia nigra, TH: tyrosine hydroxylase, WB: Western blot, WT: wildtype, ↑: increased, ↓: decreased, ↔: not different.

by reducing oxidative stress [106]. Consequently, Klivenyi et al. [106] concluded that the protective effects of α-syn deletion were due to reduced hydroxyl radical formation [106]. One drawback of using 3-nitropropionic acid is its nonselectivity for dopaminergic neurons. Alvarez-Fischer et al. [150] checked the susceptibility of SNCA KO mice to 6-OHDA and found that deletion of α-syn rendered the mice more resistant to 6-OHDA and suggested that the toxicity of 6-OHDA was at least partially mediated by α-syn [150]. These findings demonstrate that deletion of α-syn results in neuroprotection, which might be mediated via inhibition of ROS formation [106, 150, 165, 166]. Another hypothesis suggests that aggregated, misfolded, and oxidized α-syn, released or secreted from dying dopaminergic neurons, induces ROS formation, providing another mechanism by which ROS production could be attenuated in α-syn-deleted mice [150]. Mice overexpressing the mutant α-syn A30P under the control of a neuron-specific Thy-1 or a TH promoter are not more susceptible to MPTP compared to their WT littermates at the age of 6 months old [151]. These findings are in contrast with the increased susceptibility to MPTP of α-syn A30P Tg5093 transgenic mice at 6–8 months of age. Furthermore, in the same mice, the lesion degree was not different from their nontransgenic littermate controls at the age of 3-4 months and a chronic rotenone treatment did not result in a different lesion degree [152].

Mice overexpressing unilaterally the mutant human α-syn A53T in the SN are not more vulnerable to MPTP when compared to control [153]. The authors argue from their results against a direct function of (mutant) α-syn in oxidative stress or apoptotic pathways [153]. On the contrary, transgenic mice expressing human A53T α-syn are more sensitive to the neurotoxicity of MPTP when compared to non-transgenic littermates. Furthermore, MPTP treatment of the A53T transgenic mice was associated with a marked elevation in the 3-NT levels, which colocalized with α-syn [154].

In another study by Peng et al. [155], the combined effects of neonatal iron feeding and environmental paraquat exposure on age-related nigrostriatal degeneration in transgenic mice expressing the A53T familial mutant form of human α-syn was assessed in three age groups, namely, 2, 12, and 23 months old. Paraquat exposure reduced dopaminergic neurons in all three age groups to the same extent in WT and A53T α-syn expressing transgenic mice, suggesting that paraquat toxicity is age-independent [155]. However, by 23 months of age, paraquat induced loss in dopaminergic cell numbers was more pronounced in those animals that had previously received elevated oral iron during the neonatal period, demonstrating that older iron-fed animals are more susceptible to exposure to the paraquat than younger iron-fed animals and that paraquat administration accelerates nigral dopaminergic neuronal loss in these animals as a consequence of early iron exposure [155]. Furthermore, the neurodegeneration could be attenuated by a systemic treatment with the bioavailable antioxidant compound EUK-189. In addition, EUK-189 administration appeared to reduce the amount of colocalization of nitrated α-syn within the nigral dopaminergic neurons, suggesting a preeminent role for oxidative stress in neurodegeneration and impairment of dopaminergic function in an experimental model of PD, which combines environmental exposures with genetic factors known to separately be involved in the disease [155]. On the contrary, paraquat neurotoxicity assessed in mice overexpressing α-syn, either the human WT or the A53T mutant form of the protein, displayed paraquat-induced protein aggregates, but were completely protected against neurodegeneration. Furthermore, increased levels of heat shock protein 70 were found, which could, therefore, contribute to neuroprotection in the transgenic mice [141].

In young and old double mutant (A53T and A30P) transgenic mice, the vulnerability against MPTP [158] and paraquat plus the fungicide maneb [159] was assessed. The enhanced toxicity of MPTP in the young double mutant mice could be due to a higher density of the DAT [158]. Double mutant mice expressing human A53T and A30P mutant forms of α-syn were more sensitive to paraquat and maneb-induced toxicity, when compared to mice expressing human WT alone [159].

In another study, 7-month-old transgenic mice expressing the A53T familial mutant form of human α-syn were injected with LPS. When compared to WT mice, only the transgenic mice displayed a persistent neuroinflammation, chronic progressive nigrostriatal degeneration, accumulation of aggregated, nitrated α-syn, and formation of LB like inclusions in nigral neurons. In addition, a treatment with two inhibitors of inducible NOS and NADPH oxidase blocked the neurodegeneration in LPS-injected transgenic mice [156].

So how can we interpret the accumulating data on gene-environment interactions in mouse models of PD? Although literature findings are not always consistent, most point towards a consensus, where parkin, PINK1, and DJ-1 are important in protecting the neurons in the face of oxidative damage, whereas α-syn seems to play an intriguingly dual role, enhancing the sensitivity to oxidative stress in conditions of overexpression, and decreasing the sensitivity to oxidative stress in conditions of deletion (Figure 2). Altogether, there is significant evidence confirming that gene-environment interactions are relevant to PD pathogenesis, and that the above-mentioned PD genes are involved in pathways linking oxidative stress to cell survival.

7. Concluding Remarks

Recent advances in understanding the genetics of PD in humans, as well as the use of animal models of PD, have enabled us to make important steps not only in identifying factors involved in the pathogenesis, but also in determining how these factors interact [9]. However, the lack of a clear picture regarding the pathogenesis of PD means that rational neuroprotective therapies are still missing. Therefore, there is currently an urgent need for the elucidation of the exact molecular architecture behind the pathogenesis of PD, in order to provide sound bases for a rational pharmacological intervention that can prevent, slow down, or stop the progressive neuronal degeneration.

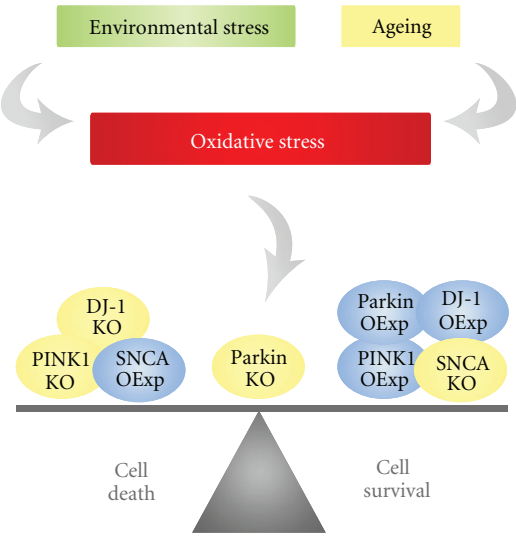

Figure 2: Gene-environment interactions involving oxidative stress in genetic mouse models of PD. Environmental factors and ageing converge to induce cellular oxidative stress, and this can either lead to cell death, or cell survival, based on genetic background. As shown in various gene-environment interaction studies in mouse models of PD (see Table 3), the PD genes can tilt the balance either to cell death, or to cell survival, thereby modulating the survival of the neurons following oxidative damage. Abbreviations: KO: knockout, OExp: overexpression.

Not only the genetic findings, but also compelling data from epidemiological studies, indicate that PD is a strong candidate for studying gene-environment interactions [167]. The susceptibility of an individual to develop PD probably involves a complex interplay of genetic factors, environmental factors and ageing, with the consequence that within a population of individuals with PD, considerable heterogeneity exists with regard to function of specific cellular pathways [9]. Despite the importance of this interplay, not much data are available on gene-environment interaction studies in animal models of PD. Although different genetic animal models of PD have failed to fully reproduce the PD pathology, they still provide an interesting platform to investigate the role of the different PD-associated genes on the integrity of the nigrostriatal dopaminergic and other neuronal systems. The exposure of animal models to external stressors may accelerate the disease process and distinguish them more from animal models that are kept in well-controlled laboratory conditions away from stressors. We suggest that challenging genetically engineered animal models of PD might be of important value to predict the influences of the genetic alterations on the readouts. Furthermore, most of the studies that characterize animal models of PD only take into account the phenotype of the disease, without considering presymptomatic manifestations of PD. Investigating pre-symptomatic aspects, such as oxidative stress, might allow a more in-depth characterization of animal models.

Since biomarkers are important tools to monitor the disease status of an individual, efforts should be made to develop, standardize, and harmonize the different techniques to allow experimental studies to be performed across different centers ensuring power, allowing replication, and increasing their successfulness. Several promising approaches for PD biomarkers, such as proteomics, are heading towards promising directions (reviewed in [168–171]. Furthermore, it should be emphasized that the detection of more than one marker for oxidative stress is key, because a single marker might give misleading results [31]. More efforts should be made to extrapolate findings from PD patients to animal models and vice versa. Not only findings, but also the developed methodologies should be more applicable and/or easy adjustable for application in both animal models and PD patient. As mentioned above, it is wellknown that PD is a heterogeneous neurodegenerative disorder and results in the occurrence of a variety of symptoms, which forms the basis for classifying PD patients into different subtypes [1, 172, 173]. Since, the severity and type of the symptoms among these different subtypes may vary from patient to patient and may likely reflect distinct underlying etiologies, their identification and biochemical characterization may facilitate our understanding of their pathogenesis, which consequently may lead to more tailored therapies [1, 173]. To our knowledge and after extensive literature research, no data have been found that investigated the different subtypes of PD in terms of impact of oxidative stress and pathogenesis.

Within this review, we focused on the complex interaction and complementary interrelationship between oxidative stress and neurodegeneration and the possible connection between oxidative stress and PD-associated genes. Although not always consistent, literature findings point to an important involvement of the PD genes parkin, PINK1, DJ-1, and SNCA in the oxidative stress pathogenic pathway and in protection against oxidative stress, confirming again that oxidative stress is an important pathogenic pathway in PD.

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Review Article

Alterations in Nitric Oxide Synthase in the Aged CNS

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Aging is associated with neuronal loss, gross weight reduction of the brain, and glial proliferation in the cortex, all of which lead to functional changes in the brain. It is known that oxidative stress is a critical factor in the pathogenesis of aging; additionally, growing evidence suggests that excessive nitric oxide (NO) production contributes to the aging process. However, it is still unclear how NO plays a role in the aging process. This paper describes age-related changes in the activity of NADPH-diaphorase (NADPH-d), a marker for neurons containing nitric oxide synthase (NOS), in many CNS regions. Understanding these changes may provide a novel perspective in identifying the aging mechanism.

1. Introduction

During normal aging, the brain changes morphologically and functionally in terms of brain weight, protein quantity, number of neurons, and neurotransmitter synthetic enzyme concentrations, leading to impairments and changes in sleep patterns, emotions, appetite, neuroendocrine function, exercise, and memory [1, 2]. Reactive oxygen species (ROS) have particularly deleterious effects on the nervous system because the brain is relatively deficient in antioxidant systems [3]. It is widely believed that the long-term effects of oxidative stress drive aged-related deficits in brain function [4]. This aging process, which is related to the effects of increased oxidative damage, is thought to involve the production of free radicals [5], including superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($ONOO^-$) [1]. In particular, the diffusible gas, NO, is involved in essential functions of the central nervous system (CNS), including neurotransmitter release, synaptic plasticity, and the regulation of neuronal electrical activity [6, 7]; it is also associated with learning and memory in both the cerebrum and cerebellum [1, 8]. NO plays a significant role in both normal aging and neurodegenerative processes [9, 10]. Increased NO production during aging suggests that NO may contribute to the aging process [10–12]. Although these free radicals do not have deleterious effects on the human body in a general environment, the enhanced production of NO aggravates aging process in the CNS [1]. Mitochondria and nitric oxide synthase (NOS) are two major sources of free radicals [13]. To identify the effects of NO in aging, various studies have been made use of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry, which is a simple and selective method for the visualization of neurons containing NOS [14]. In this paper, we highlight the age-related changes in NOS in many CNS regions via several NADPH-d immunohistochemistry studies.

2. NADPH-d-Positive Neurons

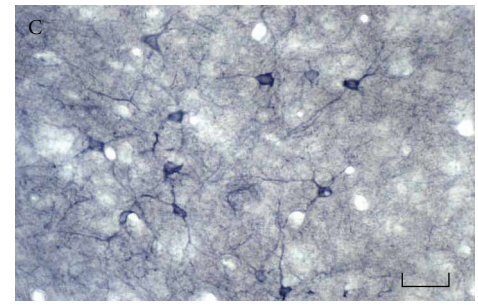
NO is produced by the oxidation of L-arginine through NOS using NADPH-d as the electron donor [15]. NADPH-d histochemical staining has been used to identify neuronal NOS (*n*NOS) because many studies show that NADPH-d may correspond to *n*NOS [16, 17]. Thus, NADPH-d is a specific histochemical marker for NOS in the brain [18, 19], and NADPH-d-positive neurons have been used to evaluate NO-positive neurons [20]. NOS-immunoreactive (IR)/NADPH-d-positive neurons have been localized in the brain structures of various mammalian species. Many studies have shown the presence of NO and NOS expression in several aging brain areas, such as the cerebral cortex [21], cerebellum [22], amygdala [23], hippocampus [16], striatum [21], tegmental nuclei [24], and periaqueductal gray [25].

To varying degrees, *n*NOS has been colocalized with choline acetyltransferase in the basal forebrain and brainstem [26]. Excessive NO production has been shown to be associated with some neurotoxic and neurodegenerative characteristics during aging [27]. Some studies have suggested that NADPH-d-positive neurons are relatively resistant to various neurodegenerative diseases such as Alzheimer's disease [28, 29] and Huntington's disease [30], and to toxic insults such as *N*-methyl-d-aspartate (NMDA) agonist [31, 32] and quinolinic acid [32, 33]. Unger and Lange [29] reported that there was no significant reduction in the number of NADPH-d-positive neurons in the amygdala and temporal cortex of aged humans although Benzing and Mufson [28] demonstrated an increased number of NADPH-d-positive neurons within the substantia innominata of Alzheimer's disease patients. In many studies, age-related changes in the NADPH-d-positive neurons had different significances in different regions of the brain. Thus, the significance of increased or decreased numbers of NADPH-d-positive neurons in aged rats remains unclear because, in studies by our group, increased NOS-IR/NADPH-d-positive neurons increased resistance to the aging process more than neurodegenerative events in brain [15, 34].

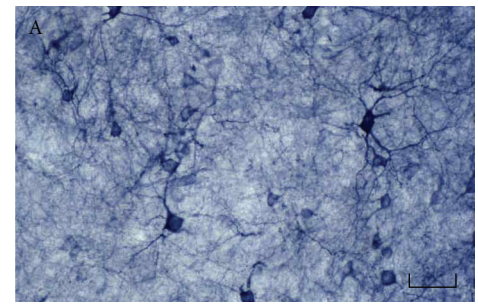
3. Regional Changes in NADPH-d in the Aging Brain

Thomas and Pearse determined that neurons with high NADPH-d activity existed and were dispersed throughout the cerebral cortex and basal ganglia [35]. In this section, we will briefly review the age-related alterations that occur in various CNS regions.

3.1. NOS and the Auditory System. In the central auditory nervous system, auditory information is delivered from the cochlear nucleus (CN) to the superior olivary complex, lateral lemniscus, inferior colliculus (IC), medial geniculate body (MGB), and auditory cortex (AC), in that order. Of these structures, the auditory cortex is considered the most important for hearing. To identify the mechanism of aging in the auditory system, various studies suggested the involvement of oxidative stress in auditory processing. In the central auditory system, age-related changes in NOS-IR/NADPH-d-positive neurons were found in the cochlea [36], the superior olivary complex [37], the inferior colliculus (IC) [38], and the auditory cortex (AC) [39]. In previous studies, a significant increase in NADPH-d-positive neurons was reported in the superior olivary nucleus in aged hamsters [37] and rats [40]. Sánchez-Zuriaga et al. showed evidence of a decreased area of NADPH-d-positive neurons in the dorsal cortex (DC) of the IC and an age-related loss of NADPH-d-positive neurons in the IC and primary cortical auditory area (Te1) in rats [41]. These changes were related to hearing impairments associated with increasing age. Our group showed that the number of NADPH-d-positive neurons in the inferior colliculus was significantly increased in aged rats (Figure 1), whereas the area of NADPH-d-positive neurons in all regions did not differ significantly between aged and younger rats [42]. Thus, age-related alterations in the NADPH-d-positive neurons of the auditory system may be region-specific.



(a)



(b)

Figure 1: NADPH-d-positive neurons in the rat inferior colliculus. The number of NADPH-d-positive neurons in the inferior colliculus was significantly higher in aged than in younger rats. NADPH-d-positive neurons are dark purple. Frozen sections of 40 μm thickness were made in the coronal plane. The histochemical detection for NADPH-d activity was performed as follows: after free-floating sections were incubated for 10 min at room temperature in 0.05 M Tris buffer (pH 8.0), sections were incubated for 60 min at 37°C in the 0.05 M Tris buffer containing 0.3% (v/v) Triton X-100, 0.1 mg/mL nitroblue tetrazolium, and 1.0 mg/mL β-NADPH. C: control; A: aged rat. Scale bar = 50 μm. Modified from Huh et al. [42].

Interestingly, NO has been reported to inhibit the activity of *N*-methyl-D-aspartate (NMDA) receptors, reducing the effects of glutamate and inducing changes in neural transmission [43]. This reduction in NMDA receptors (NMDARs) expression may be involved in the change of synaptic plasticity driven by the age-related decease in sensory input, resulting in age-related impairment in the function of the NMDAR/NO signaling pathway in the CNS [44]. However, in our study, the expression of NMDARs was increased in the CN, MGB, and AC during aging, although it was found that the expression of NMDARs was decreased in the superior olivary nucleus and IC [45]. Thus, these findings also indicated that age-related changes in the NMDARs in the central auditory system were region-specific.

Another interesting observation was the activation of voltage-gated K^+ currents through excessive NO production in rat auditory cortical neurons [46]. In a previous study, NO-stimulated potassium channels induced long-term potentiation in slices prepared from the rat auditory cortex layer IV [47]. Using patch-clamp electrophysiology,

our group evaluated the effects of NO on modulating K^+ currents by assaying for NADPH-d, a marker of NOS, and by examining the effects of S-nitro-*N*-acetylpenicillamine (SNAP), an NO donor. The modulatory effects of NO on the K^+ currents of acutely isolated rat auditory cortical neurons showed gradual increases in the K^+ currents [46]. This NO-induced activation of K^+ currents hyperpolarizes the membrane potential of a neuron, inhibiting neuronal excitability. The increased NO activation of K^+ currents suppress neuronal activity in the auditory cortex. Therefore, excessive NO production may be involved in the hearing impairment caused by aging.

3.2. NOS and the Visual System. In the visual system, retinal ganglion cells project to the lateral geniculate nucleus (LGN) of the thalamus. Visual input is delivered from the retina and the occipital cortex to the ventral lateral geniculate nucleus (vLGN), a thalamic visual nucleus, of the rat [48]. During normal aging, visual function decreases partly because of neural changes in the retina and central visual pathways. Ahmad and Spear [49] suggested that aging produced a statistically significant reduction in neuron density in both the magnocellular and parvicellular layers, which are parts of the visual system, although there was no significant loss of neurons. Uttenthal et al. [50] observed a several fold increase in NOS-positive bands in aged rats using Western blotting of brain extracts. In the rat vLGN, most NADPH-d-positive neurons are geniculotectal projection neurons, although a smaller proportion acts as local circuit inhibitory neurons [51]. Yu et al. [40] showed a significant enhancement in NADPH-d activity in the supraoptic nucleus of aged rats. Villena et al. showed a decrease in the number of NADPH-d-positive neurons in aged old rat vLGN compared with controls [17]. Our group showed that the number of NADPH-d- and *n*NOS-positive neurons did not change significantly in the dorsal LGN (dLGN) and vLGN of aged rats. Additionally, no age-related changes were observed in the superior colliculus. The staining intensities of NOS-IR/NADPH-d-positive neurons increased significantly in the dLGN and vLGN aging processes [52]. However, although each group published different results characterizing NAPDH-d-positive neurons in the central visual system all, these results showed that increased NO production may be associated with alteration in visual function during aging. Further studies are required to clarify this relationship between NADPH-d-positive neurons and *n*NOS in age-related changes in central visual system.

3.3. NOS and Spinal Cord. During advanced aging, the pelvic visceral organs physically and functionally changes. The lower lumbar and sacral spinal cord are essential for controlling the function of the bowel, bladder, and sexual organs [53]. Ranson et al. reported that the dorsal commissural nucleus (DCN) and the intermediolateral nuclei (ILN) of the lumbosacral spinal cords in aged rats exhibit significant decreases in neurotransmitter levels [54]. Yoon et al. reported that a reduction in the number of *n*NOS-IR neurons occurred in the central autonomic nucleus and the superficial dorsal horn of the spinal cord in aged rats [55]. The number of NADPH-d-positive neurons in the motor nucleus at the L4–L6 levels of the spinal cord decreased in aged rats [56]. Tan et al. found that NADPH-d-positive neurons are present in the lumbosacral spinal cords of aged rats. However, no colocalization of NADPH-d-positive and *n*NOS-IR neurons was detected in the lumbosacral spinal cords of aged rats [57]. Thus, NADPH-d activity does not always coincide with the NO-containing neural structures, and NADPH-d function in spinal cord is also unclear.

3.4. NOS and Hormones. Magnocellular neurosecretory neurons in the rat supraoptic nucleus (SON) have been found to synthesize oxytocin (OXY) in the dorsal part and the arginine vasopressin (AVP) in the ventral region [58]. OXY is a mammalian hormone that functions as a neuromodulator in the brain. OXY is best known as a reproduction-related hormone, facilitating childbirth and breastfeeding after childbirth. Arginine vasopressin (AVP), also known as antidiuretic hormone (ADH), is a neurohypophysial hormone produced in most mammalian brains. AVP is responsible for increasing the effective circulating volume by increasing water absorption, water permeability, reabsorption, and peripheral vascular resistance. Yu et al. observed no significant age-related changes in the number of the OXY-IR/NADPH-d-positive neurons in the dorsal part of the SON, but they did observe an age-related increase in NADPH-d-positive neurons in the SON [40]. Because the concentration of OXY in the plasma decreased in aged rats [59], it is thought that the age-related increase in the NADPH-d-positive neurons may inhibit the secretion of the OXY and reduce the concentration of OXY in the plasma. The AVP-expressing neurons exhibited an increased area in the SON of aged rats [60]. Yu et al. confirmed this increase and the existence of AVP-IR/NADPH-d-positive neurons in aged rats [40]. However, because the circulating levels of AVP did not change in aged rats compared with young rats [61], this result corresponded to inhibition of the secretion of AVP by NO in NOS-expressing neurons [62].

Neuropeptide Y (NPY) is a widely distributed neurohormone associated with food intake [63] and the release of gonadotrophins [64]. NOS colocalizes with both somatostatin and neuropeptide Y (NPY) in the corpus striatum and the cerebral cortex [65, 66]. During aging process, the decrease in NPY levels in neural tissues is drastic [67, 68]. Our group demonstrated that, in the aged group, the number of NPY-IR/NADPH-d-positive neurons did not significantly decrease in the cerebral cortex and striatum compared to the control group. However, the number of NPY-IR/NADPH-d-negative neurons significantly decreased in all cerebral cortical areas, except the nucleus accumbens, and the caudatoputamen in the aged group [21]. In a study of aged Fischer 344 rats, which are more resistant to aging than other rats strain [69, 70], the aged group showed that the number of NPY-IR/NADPH-d-positive neurons did also not significantly change in all regions of the cerebral cortex compared to the control group [71]. However,

the number of NPY-IR/NADPH-d-negative neurons significantly decreased in the frontal association, primary motor, secondary somatosensory, insular, ectorhinal, perirhinal and auditory cortexes in the aged group [71]. These studies demonstrated that the NADPH-d containing NPY-IR neurons in the cerebral cortex and striatum of rats were less influenced by aging than those of the control. Therefore, the relative stability of the selective population of NPY-IR/NADPH-d-positive neurons within the cerebral cortex and striatum helped protect against atrophy during aging.

Vasoactive intestinal polypeptide (VIP) is a peptide hormone produced in the suprachiasmatic nuclei (SCN) of the hypothalamus in the brain [72, 73]. The SCN is the specific location of the "master circadian pacemaker," an area that daily modulates timekeeping in the body. VIP plays an important role in the communication between individual brain cells in this area [74]. Andreose et al. [75] suggested that there is a marked decrease in VIP-IR in the cerebral cortex and other brain regions in rats during the aging process. Chee et al. [76] reported that the size of VIP-IR neurons in the suprachiasmatic nucleus of the aged rats increased in comparison with that of controls. Our group showed that VIP and NADPH-d did not coexist in any single neuron in the cerebral cortex of either group [77]. However, a significant decrease was found in the number of VIP-IR/NADPH-d-negative neurons in the cerebral cortex of aged rats. This selective depletion and atrophy of VIP-IR neurons from the cerebral cortex of aged rats shows an increased vulnerability of VIP-IR neurons to the aging process compared with NADPH-d-positive.

3.5. NOS and the Salivary System. Salivary secretion is controlled by the combined action of the parasympathetic and sympathetic nervous system. Several areas of the SON and the limbic system, which influence the endocrine and autonomic nervous systems, participate in the regulation of water and sodium balance [78, 79]. Inhibition of salivary secretion during aging has been demonstrated in rats [80], and many reports have demonstrated that NO influences the regulation of salivary secretion [81, 82]. In the SON and the medial septal area of rats, NOS-expressing neurons were identified, and NO-related inhibition of salivary secretion was demonstrated [83, 84]. Tanaka et al. showed that cell number, cell size and reactive density of the NADPH-d-positive neurons significantly increased in the SON of aged rats [85]. These results suggest an inhibitory role of NO in salivary secretion during aging. However, there are not many studies which have contributed to evaluating the inhibitory role of NO in salivary secretion in age-related changes.

3.6. NOS and Stress. The periaqueductal gray (PAG) surrounding the cerebral aqueduct is important for the organization of responses to stress and pain. NADPH-d is well represented in the neurons of the dorsolateral neuronal column of the periaqueductal gray (dlPAG) [86]. Lolova et al. demonstrated that, with aging, the total dendritic length of NADPH-d-positive neurons was increased, and the total cell number in the dlPAG was significantly decreased [87]. Several stress models using immobilization, cold and hot stimuli in wild-type rats increased NOS expression [88] and the number of NADPH-d neurons in the PAG [89]. Smalls and Okere reported that acute restraint increases varicosity density and decreases intervaricosity length in NADPH-d-positive neurons in the rat dlPAG [90]. The capacity to defend against stress is decreasing with aging. Although there are many reports of relationship between stress and NOS, or NOS and aging, the direct relationship between NO and aging in dlPAG has not been identified. Thus, through further studies, it is expected that the decreased stress response in aging may be related to the action of NO in dlPAG.

4. Concluding Remarks

NO and aging are closely related, but more intensive research is necessary to understand this relationship and the functional, anatomical, and molecular mechanisms of age-related alteration by NO in the CNS. This information will help us understand the mechanisms of both the aging process and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Alterations in the NO system in the aged CNS influence many regions in the rats brains, such as the neuroendocrine system, the visual system, the auditory system, the spinal cord, cognition and learning, the stress response, the salivary system, and the autonomous nervous system. The NO system in each region may influence chemical and structural changes in the CNS during aging. Consecutive neurophysical changes may cause symptoms associated with the CNS aging process, such as change in orientation, attention, and memory. In different parts of the CNS, NADPH-d-positive neurons have different distributions and different effects on the aging process. Additionally, NADPH-d-positive neurons have both protective and toxic effects on the CNS during aging. Therefore, clarifying age-related and NO-related alterations in the CNS may be helpful in the identification of new therapeutic targets for aging and neurodegenerative diseases.

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Research Article

Reactive Oxygen Species Formation and Apoptosis in Human Peripheral Blood Mononuclear Cell Induced by 900 MHz Mobile Phone Radiation

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We demonstrate that reactive oxygen species (ROS) plays an important role in the process of apoptosis in human peripheral blood mononuclear cell (PBMC) which is induced by the radiation of 900 MHz radiofrequency electromagnetic field (RFEMF) at a specific absorption rate (SAR) of ~0.4 W/kg when the exposure lasts longer than two hours. The apoptosis is induced through the mitochondrial pathway and mediated by activating ROS and caspase-3, and decreasing the mitochondrial potential. The activation of ROS is triggered by the conformation disturbance of lipids, protein, and DNA induced by the exposure of GSM RFEMF. Although human PBMC was found to have a self-protection mechanism of releasing carotenoid in response to oxidative stress to lessen the further increase of ROS, the imbalance between the antioxidant defenses and ROS formation still results in an increase of cell death with the exposure time and can cause about 37% human PBMC death in eight hours.

1. Introduction

Mobile phones have been widely used in popular telecommunication and medical telemetry systems. The tremendous use of mobile phone has drastically increased the amount of radiofrequency electromagnetic field (GSM RFEMF) exposure in our daily lives. To ensure telecommunication in anywhere, various kinds of mobile phone relay stations or devices need to be placed inside or near living/working and residential areas. It makes people have the possibility to be exposed to the RFEMF radiation almost every moment. Thus there is a major concern about the effects of RFEMF radiation exposure on human health. Despite previous studies, our knowledge on these effects is still inadequate and strong debates continue [1–5].

Among the various health effects of GSM RFEMF exposure, the formation of reactive oxygen species (ROS) and increased oxidative stress are those proposed mechanisms that can explain the link between RFEMF radiation and possible harmful effects on human health. It was found that RFEMF could induce ROS formation in animal brain, cortical neurons, spleen, blood serum, and human semen [6–10]. The purpose of this study was to investigate the extent of ROS formation and oxidative DNA damage as well as cell apoptosis caused by RFEMF on human peripheral blood mononuclear cell (PBMC). PBMC cells are a critical component in the immune system to fight infection and adapt to intruders. They also play significant roles in neurodegenerative diseases and aging [11–14]. Therefore, investigation of whether and how oxidative stress activates in PBMC under the exposure of RFEMF radiation can help to further clarify its effects on human health.

In this study, isolated fresh human peripheral blood mononuclear cells were exposed to the radiation of 900 MHz GSM RFEMF at a specific absorption rate (SAR) of 0.4 W/kg for 1 h, 2 h, 4 h, 6 h, and 8 h. The specific absorption rate was chosen to mimic the situation that people usually may absorb in an environment within a distance of 20 meters from mobile phone relay stations, or occupationally in an equipment room of microwave communication, or around a

surveillance radar [15–17]. It is also the occupational exposure restriction suggested by the International Commission on Non-ionizing Radiation Protection and some national radiological protection boards [18, 19].

To detect the intracellular ROS activation in the exposed cells, fluorescent dye DCFH was used as the probe in flow cytometry. The caspase-3 activity of the cells was assessed by colorimetric assay, while the cell apoptosis was analyzed by flow cytometry with FITC-Annexin V/Propidium Iodide (PI) double staining. To assess DNA damage of human PBMC and reveal the mechanism of the effect of RFEMF radiation, confocal Raman microspectroscopy was also employed.

2. Material and Methods

2.1. Sample and Reagents. Study on blood of volunteers (providing informed written consent) was proved by Jinan University Animal Care and Use Committee conforming to the Chinese Public Health Service Police on Human Care and Use of Laboratory Animals.

Normal peripheral blood was obtained from healthy nonsmoking adult volunteers aging 25.3 ± 0.8 by venipuncture and poured into heparinized tubes. The blood samples were anticoagulated with heparin lithium. After centrifugation, the peripheral blood monocytes in the middle cloud layer were taken out, washed twice repeatedly, and then resuspended. The cell survival rate was >98% estimated by Trypan blue staining.

Annexin V/PI double-staining kit was purchased from Bender Company, USA. The fluorescent dye DAPI was from Roche, USA. The mitochondrial membrane potential detection kit (JC-1), ROS detection kit, Bradford protein concentration assay kit, and caspase-3 colorimetric assay kit were all purchased from Beyotime Institute of Biotechnology, China.

2.2. Exposure of Human PBMC Samples to RFEMF. 200 μL of PBMC samples with cell density of 1.5×10^6/L was placed in each well of a culture plate. Then they were exposed to the radiation emitted by a VS401A RF RFEMF emitter (Shenzhen Weikete Technology Company, Ltd. China) at a specific absorption rate of 0.43 W/kg at 37°C for 1 h, 2 h, 4 h, 6 h, and 8 h. The radiation distributed uniformly on the sample and the SAR was determined using the conductivity of the PBMC sample σ, the RFEMF electric field strength E at the determined point, and the mass density of the sample ρ_m in the follwoing form: $\text{SAR} = \sigma E^2/\rho_m$. In the experiment, σ was found to be 0.229 ± 0.001 (S/m), E was 43.42 (V/m), and ρ_m was 1.011 ± 0.006 (g/mL). Therefore, SAR was estimated to be 0.43 W/kg.

2.3. Cell Apoptosis Detection. 5 μL FITC-Annexin V and 10 μL PI were added to 100 μL cell suspension with cell concentration of 1×10^6/mL. The mixture was incubated for 15 minutes in dark at room temperature. Then they were washed with binding buffer twice and adjusted again to the cell concentration of 1×10^6/mL. The cell apoptosis was analyzed using an FACS Aria flow cytometry (BD company, USA) within 1 hour.

2.4. ROS Detection. The exposed cells were collected and the supernatant was removed by centrifugation. Thereafter the cells were resuspended and 5×10^5 cells were collected. They were centrifuged again to remove the supernatant and then added into 500 μL diluted DCFH-DA. The mixture was incubated for 20 minutes at 37°C and then washed twice. The samples were later analyzed with flow cytometer within 1 hour. An Ar^+ laser with 488 nm wavelength was used as the excitation light and 525 nm was the receiving wavelength to obtain the proportion of the fluorescent cells.

2.5. Caspace-3 Activity Detection. The caspace-3 activity of the exposed cells was evaluated using the caspase-3 colorimetric assay kit and the assessment was performed according to the manufacturer's recommendations. The ratio of the OD value of the sample and that of the control group were taken to evaluate the caspase-3 activity.

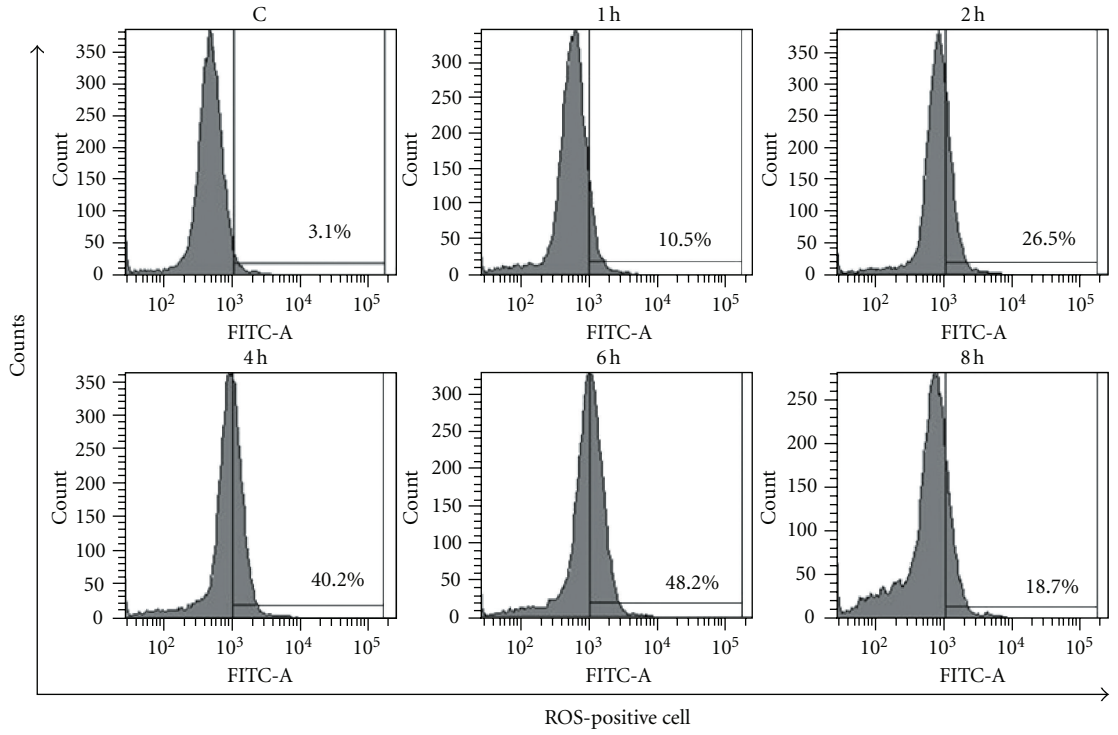
2.6. Mitochondrial Membrane Potential Determination. (10–60) $\times 10^5$ exposed cells were resuspended and mixed with the JC-1 staining working solution. The mixture was incubated at 37°C for 20 minutes and then centrifuged for 3-4 minutes to remove the supernatant. The mixture was washed twice with buffer solution and then the cells were resuspended with the buffer solution. The fluorescence of the cells was imaged using a Nikon TE300 inverted fluorescence microscope.

2.7. DNA Damage Detection by Raman Spectroscopy. The Raman spectra of PBMC were recorded by a JY RAM INV system using 514.2 nm excitation line from an Ar^+ ion laser through an inverted Olympus optical microscope with a ×60 objective. The acquisition band was 600~1800 cm^{-1} with a spectrum resolution of 1 cm^{-1}. At least 35 cells were measured for each group of the exposed PBMC sample.

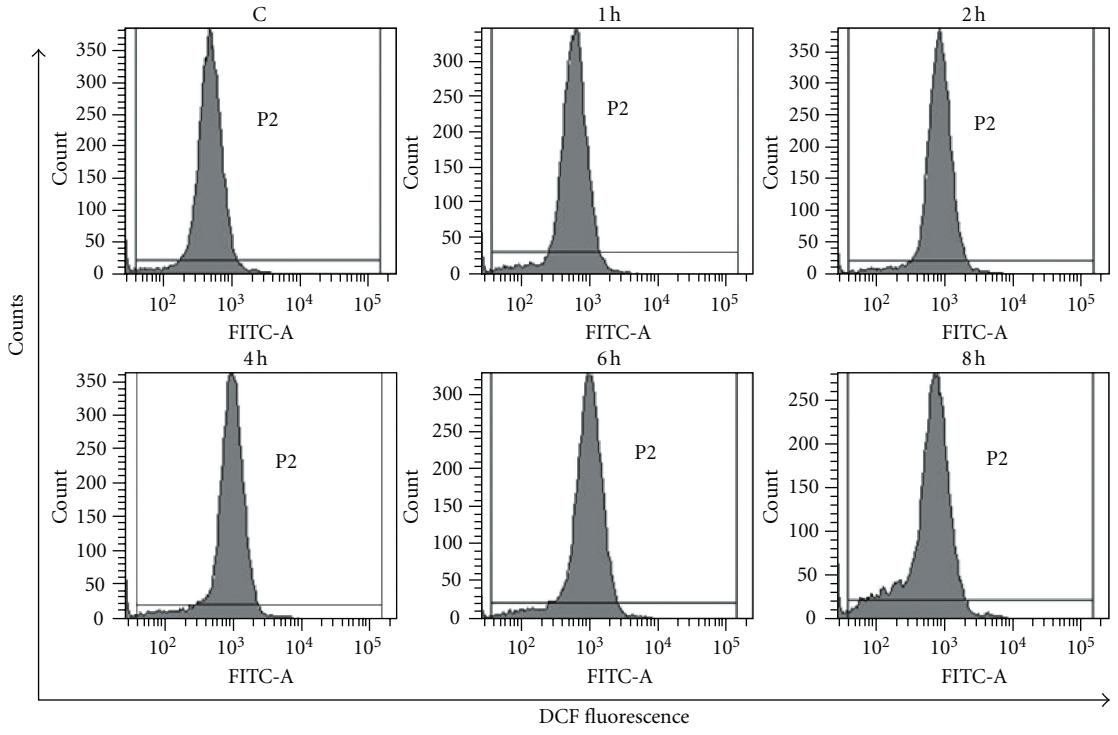
2.8. Data Processing. The PBMC cells were from the blood samples of 6 volunteers (3 males and 3 females). Each sample contained 10000 cells. All data were averaged from the results of five parallel samples; each sample was detected three times. The final result is denoted by $\bar{x} \pm s$. SPSS 13.0 was used for statistical analysis of the data, in which $P < 0.05$ was regarded as significantly different.

3. Results

3.1. ROS Activation. The flow cytometric results of human PBMCs' ROS activation are shown in Figure 1. Figure 1(a) indicates the histograms of ROS-positive cells, and Figure 1(b) shows the histograms of mean DCF fluorescence intensity (indication of ROS level). Figure 2 shows how the ROS-positive cells and the ROS level vary with radiation time. We can see that just 1 h radiation can activate ROS in PBMC ($P < 0.05$, versus control). The ROS level continuously rose in the period from the 2nd h to the 6th h. After 6 h exposure, both the number of ROS-positive cells and ROS level reached their maximum and then declined.

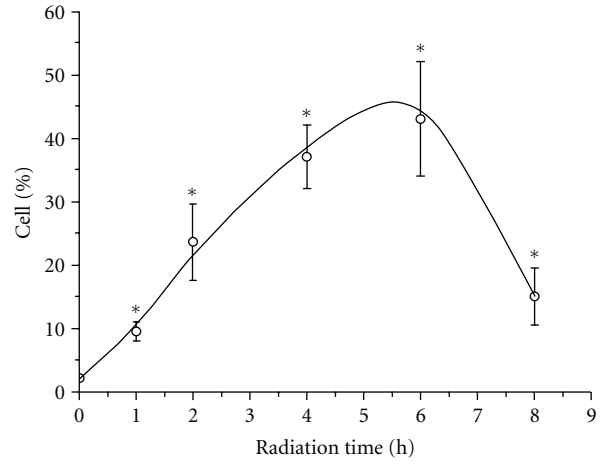


(a)

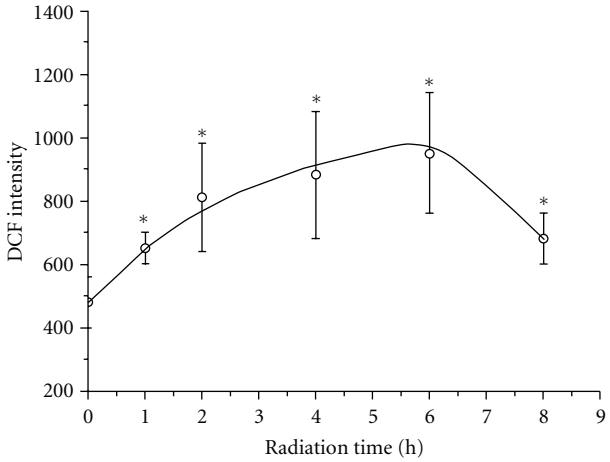


(b)

Figure 1: The flow cytometric results of human PBMCs' ROS activation. (a) Histograms of ROS-positive cell percentage. (b) Histograms of mean DCF fluorescence intensity.



(a)



(b)

Figure 2: The number of ROS-positive cells (a) and DCF intensity (b) versus radiation time.

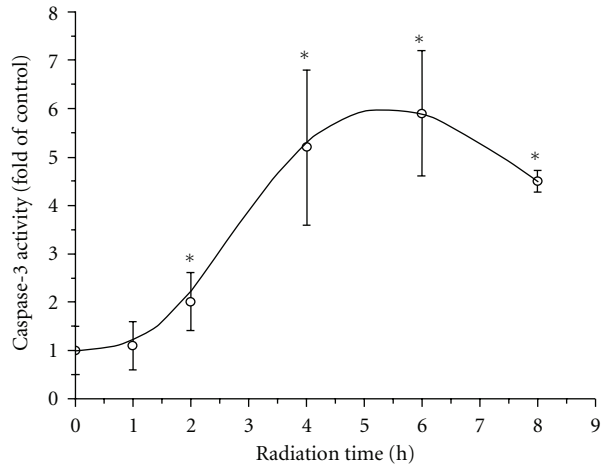


Figure 3: The variation of caspacse-3 activity in human PBMCs with radiation time.

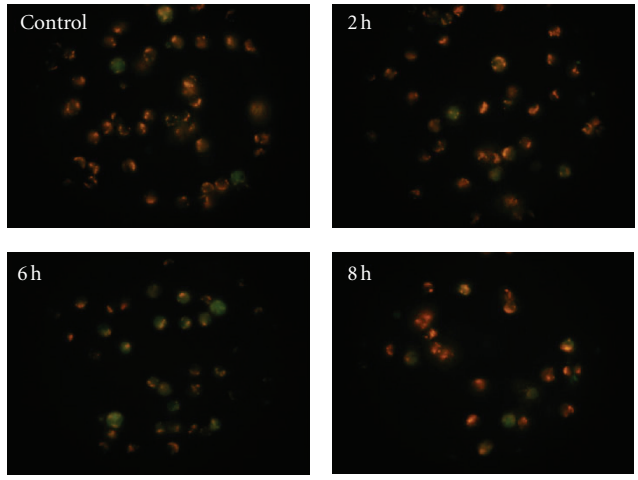


Figure 4: The mitochondrial staining images of human PBMCs.

3.2. Caspace-3 Activity. The variation of caspacse-3 activity in human PBMCs with radiation time is shown in Figure 3. Within the first 2 h radiation, the change of caspase-3 content was not evident ($P > 0.05$ versus control). However, when the cells were radiated longer than 2 h, the caspase-3 activity became significantly increased ($P < 0.05$ versus control). The activity of caspase-3 at the 6th h was 6 times as that of control group. But at the 8th h, the caspase-3 activity declined significantly compared with that at the 6th h ($P < 0.05$).

3.3. Mitochondrial Potential. Figure 4 illustrates the mitochondrial staining of human PBMCs. The PBMCs in the control group (not exposed to electromagnetic radiation) emitted bright orange-red fluorescence with few emitting green fluorescence. The red fluorescence intensity of the cells weakened while the proportion of the green fluorescence cells increased in the images taken from the 2nd h to the 6th h, indicating a decline of mitochondrial potential in the cells during the period. However, it slightly went up at the 8th h.

3.4. Human PBMC Apoptosis. Figure 5 shows the flow cytometric analysis of apoptosis in human PBMC using FITC-annexin V and PI double staining, and Figure 6 illustrates the apoptotic rates of the exposed cells. It can be seen that neither early apoptosis (Annexin V+/PI−) nor late apoptosis (Annexin V+/PI+) was evident ($P > 0.05$ versus control) in the 1st h. When the exposure lasted longer than 2 h, the apoptotic rates increased evidently. The early apoptotic rate increased to $12.2\% \pm 3.3\%$ and $21.5\% \pm 5.2\%$ ($P < 0.05$ versus control), respectively, at the 2nd h and 4th h. At the same time, the late apoptotic rate increased to $2.2\% \pm 0.8\%$ and $5.0\% \pm 1.6\%$ ($P < 0.05$ versus control). Compared with the early apoptotic rate at the 4th h, there was no significant increase ($P > 0.05$ versus the 4th h) at the 6th h, whereas the late apoptotic rate began to decrease at the time. At the 8th h, the early apoptotic rate decreased from $21.2\% \pm 4.9\%$ (at the 6th h) to $10.4\% \pm 5.0\%$ ($P < 0.05$ versus the 6th h). Figures 6(a) and 6(b), respectively, show the detail

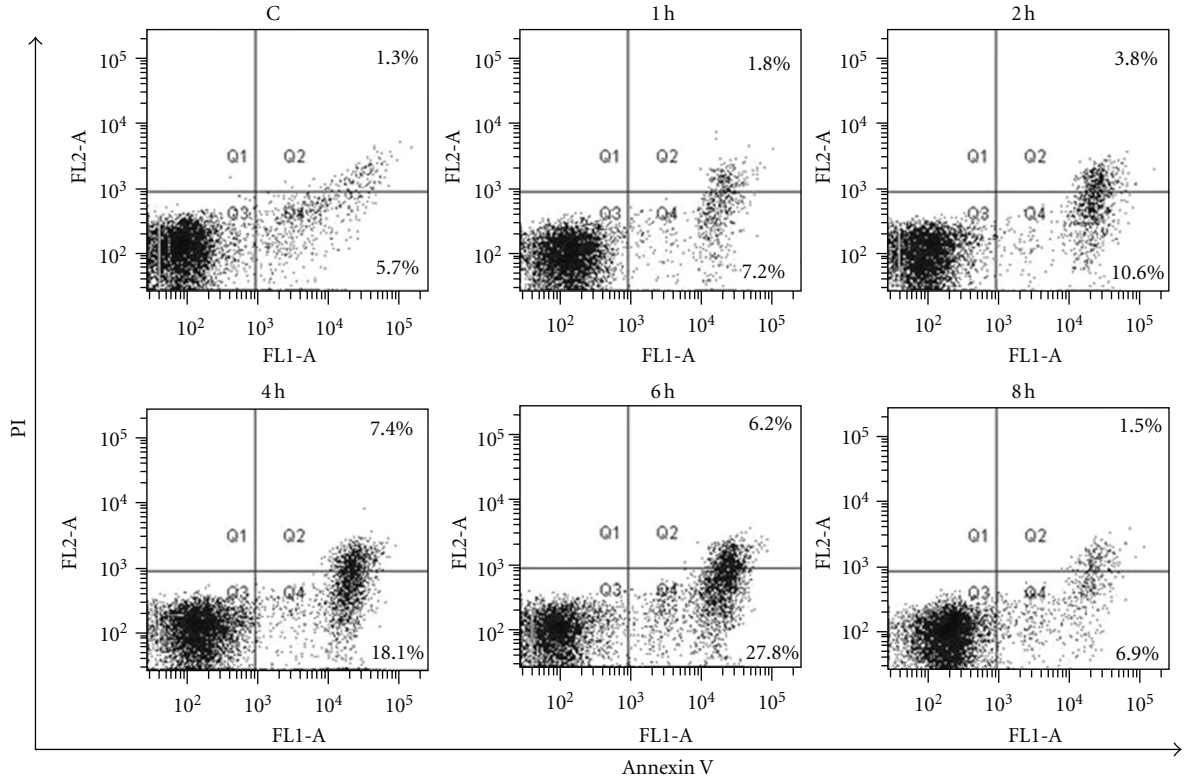


Figure 5: The flow cytometric analysis of apoptosis in human PBMCs using FITC-annexin V and PI double staining. Quadrant analysis of the gated cells in FL-1 versus FL-2 channels was from 10,000 events. Annexin V+/PI− (lower right quadrant) areas stand for early apoptotic cells, and Annexin V+/PI+ (upper right quadrant) areas stand for late apoptotic or necrotic cells.

information about the variations of early and late apoptotic rates of human PBMC with radiation time.

3.5. Raman Spectra. Two kinds of Raman spectra were obtained from the exposed cells. One contains weak signal of carotenoid but the other one contains strong signal of carotenoid. Both of them are shown in Figure 7, with peaks at 1157 and 1525 cm^{-1} being the bands of carotenoid. The spectra with strong signal of carotenoid were observed only in the samples being exposed longer than one hour but not found in the control group. The proportion of the spectra with strong signal of carotenoid thereafter increased with radiation time and become 60% of all the observed ones in the 4th h. This indicates that the carotenoid releasing in the exposed cells was a reaction to the exposure.

Besides the bands assigned to carotenoid, the bands assigned to DNA (787, 1258, and 1579 cm^{-1} in Figure 7(a); 787, 952, 1491, and 1581 cm^{-1} in Figure 7(b)) and the bands assigned to protein (1004, 1450, 1616, 1661, and 1678 cm^{-1}) also change evidently with radiation time.

3.6. Cell Counting. Figure 8 illustrates the result of cell counting on the exposed human PBMC as a function of radiation time. We can see that the number of cells constantly decreases within six hours and then significantly reduces from the 6th h to the 8th h. At the 8th h, about 37% of the exposed cells had died.

4. Discussion

From the results we can see obviously that cell apoptosis can be induced in human peripheral blood mononuclear cell (PBMC) by the radiation of 900 MHz GSM RFEMF at a specific absorption rate of ~0.4 W/kg when the exposure lasts longer than two hours. Using the data about ROS activation, caspacse-3 activity, mitochondrial potential and the Raman spectra of DNA and proteins, we can figure out the mechanism of the cell apoptosis as follows. The exposure to the radiation of 900 MHz GSM RFEMF can induce a series changes in the protein, lipid, and DNA structure. These changes include (1) broken carbon-hydrogen bond of lipid and protein (indicated by the intensity decrease at 1130 cm^{-1} in the Raman spectra), (2) damage of the protein side chain (Phe, Tyr, indicated by the intensity decrease at 1616 cm^{-1} in the Raman spectra), (3) destruction of the protein secondary structure such as reducing α-helix and β-sheet and increasing random coil (indicated by the intensity decreases at 1264 cm^{-1} and 1678/1680 cm^{-1} in the Raman spectra), and (4) DNA damage (indicated by the intensity decreases at 952 cm^{-1} and 1491 cm^{-1} and intensity increase at 1579 cm^{-1} in the Raman spectra). All these changes influence the stability of the protein conformation, so that

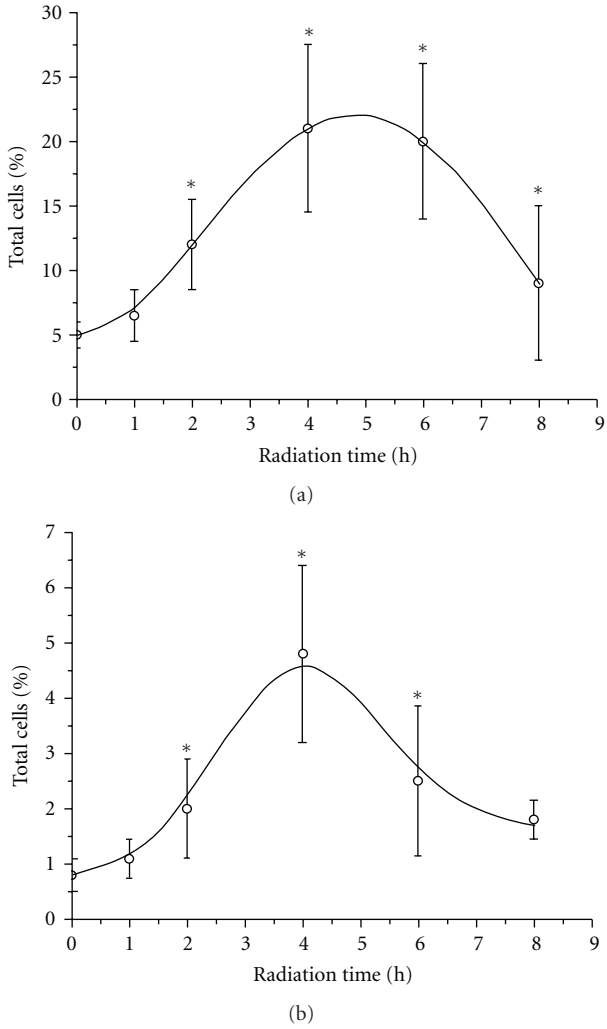


Figure 6: The apoptotic rates of exposed human PBMCs:(a) Early apoptotic cells and (b) late apoptotic cells.

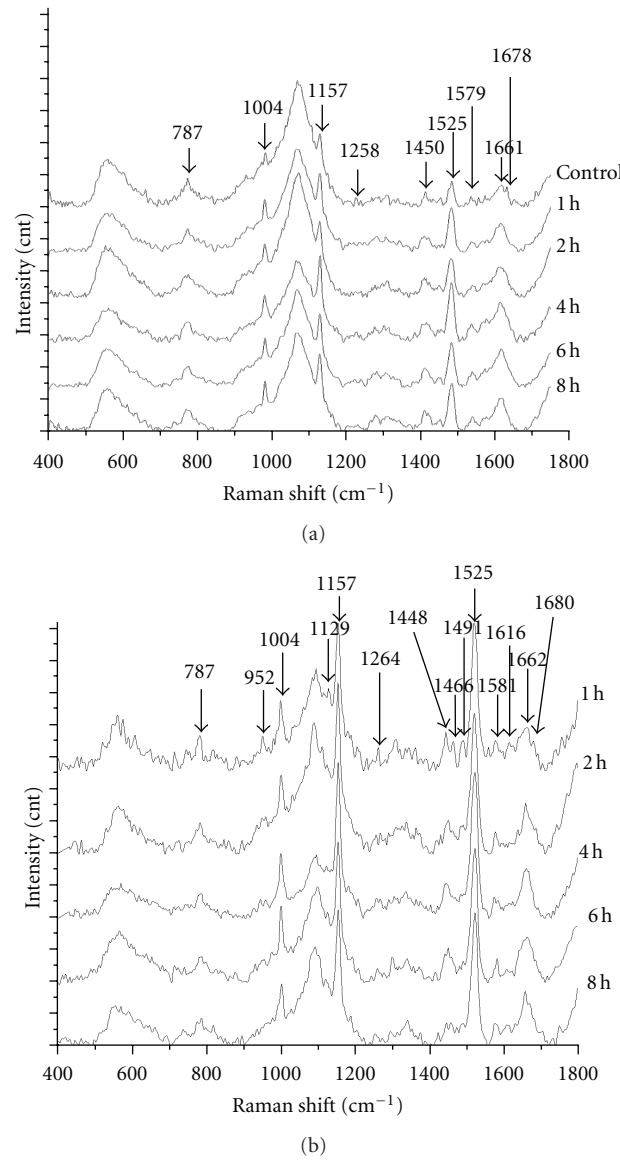


Figure 7: The Raman spectra of PBMC which were exposed for different time. (a) Raman spectra with weak signal of carotenoid. (b) Raman spectra with strong signal of carotenoid.

the proteins cannot perform their normal function to get rid of the excess ROS. The imbalance between ROS formation and antioxidant defenses results in oxidative stress in human PBMC, thus inducing mitochondrial permeability transition pore (mPTP) opening [20, 21]. The opening of mPTP declines the mitochondrial potential, thereby triggering the caspacse-3 activity and finally inducing cell apoptosis [22–26]. The apoptosis was mainly early apoptosis, with less than 6% of the cells being late apoptosis.

This is the so-called mitochondrial pathway of apoptosis and has been demonstrated step by step by our experimental results. As described previously, the ROS activation was induced by DNA damage and the disturbance on protein and lipid conformation, suggesting that DNA, protein, and lipid probably are the targets of the GSM RFEMF radiation on human PBMC. On the other hand, human PBMC seems to have a self-protection mechanism of releasing carotenoid in response to oxidative stress to inhibit the further increase of ROS. However, it cannot stop the process of cell death if the exposure continues. The number of cells even decreased faster in the period from the 6th h to the 8th h as shown in Figure 8. A possibility is that the amount of releasing carotenoid was not enough to against the excessive ROS generation. Another possibility is that, besides cell apoptosis, human PBMC has another cell death process induced by the GSM RFEMF exposure. It is oncosis [27–29] and was proved by our experiment of cell morphological observation on the exposed human PBMC (data not shown). Therefore, the cell number continuously decreased from the 6th h to the 8th h even though the number of apoptotic cells had already decreased in the period. We will not discuss the mechanism of the cell oncosis in human PBMC here but will leave it for a future paper. Finally, we strongly ask for more concern on the possible hazardous health effects of exposure to the radiation of GSM RFEMF emitted from the mobile phone

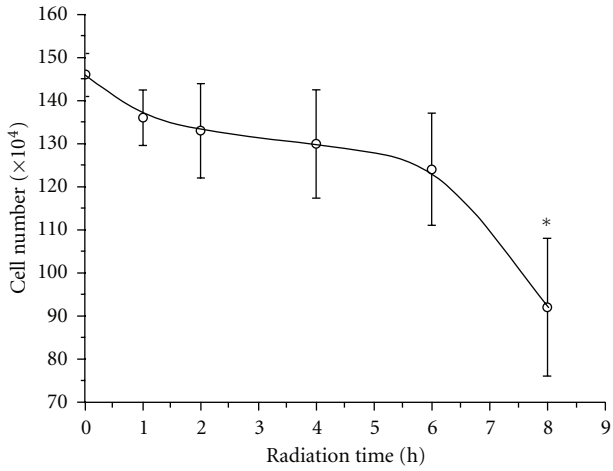


Figure 8: The cell counting of the exposed human PBMC versus radiation time.

relay stations or devices as it can cause 37% human PBMC death in eight hours.

5. Conclusions

We have demonstrated that cell apoptosis can be induced in human PBMC by the radiation of 900 MHz GSM RFEMF at a specific absorption rate of ~0.4 W/kg when the exposure lasts longer than two hours. The apoptosis is induced through the mitochondrial pathway and mediated by activating ROS and caspase-3, and decreasing the mitochondrial potential. The activation of ROS is triggered by the conformation disturbance of lipids, protein, and DNA induced by the exposure to GSM RFEMF. Although human PBMC has a self-protection mechanism of releasing carotenoid to inhibit further increase of ROS, if the exposure continues, the imbalance between the antioxidant defenses and ROS formation still results in an increase of cell death with the exposure time. These findings not only clarify the effect of GSM RFEMF on human health but also reveal its mechanism. We hope that it will help people to realize the possible hazardous health effects of exposure to GSM RFEMF radiation emitted from the mobile phone relay stations or devices in their living/occupational environment.

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Review Article

Molecular Hydrogen as an Emerging Therapeutic Medical Gas for Neurodegenerative and Other Diseases

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Effects of molecular hydrogen on various diseases have been documented for 63 disease models and human diseases in the past four and a half years. Most studies have been performed on rodents including two models of Parkinson's disease and three models of Alzheimer's disease. Prominent effects are observed especially in oxidative stress-mediated diseases including neonatal cerebral hypoxia; Parkinson's disease; ischemia/reperfusion of spinal cord, heart, lung, liver, kidney, and intestine; transplantation of lung, heart, kidney, and intestine. Six human diseases have been studied to date: diabetes mellitus type 2, metabolic syndrome, hemodialysis, inflammatory and mitochondrial myopathies, brain stem infarction, and radiation-induced adverse effects. Two enigmas, however, remain to be solved. First, no dose-response effect is observed. Rodents and humans are able to take a small amount of hydrogen by drinking hydrogen-rich water, but marked effects are observed. Second, intestinal bacteria in humans and rodents produce a large amount of hydrogen, but an addition of a small amount of hydrogen exhibits marked effects. Further studies are required to elucidate molecular bases of prominent hydrogen effects and to determine the optimal frequency, amount, and method of hydrogen administration for each human disease.

1. Introduction

Molecular hydrogen (H_2) is the smallest gas molecule made of two protons and two electrons. Hydrogen is combustible when the concentration is 4–75%. Hydrogen, however, is a stable gas that can react only with oxide radical ion ($\bullet O^-$) and hydroxyl radical ($\bullet OH$) in water with low reaction rate constants [1]:

$$\begin{aligned} \bullet O^- + H_2 &\longrightarrow H\bullet + OH^- \qquad k = 8.0 \times 10^7\ M^{-1} \cdot s^{-1} \\ \bullet OH + H_2 &\longrightarrow H\bullet + H_2O \qquad k = 4.2 \times 10^7\ M^{-1} \cdot s^{-1} \\ H\bullet + \bullet OH &\longrightarrow H_2O \qquad k = 7.0 \times 10^9\ M^{-1} \cdot s^{-1}. \end{aligned} \tag{1}$$

The reaction rate constants of $\bullet O^-$ and $\bullet OH$ with other molecules are mostly in the orders of 10^9 to $10^{10}\ M^{-1}\cdot s^{-1}$, whereas those with H_2 are in the order of $10^7\ M^{-1}\cdot s^{-1}$. Hydrogen, however, is a small molecule that can easily dissipate throughout the body and cells, and the collision rates of hydrogen with other molecules are expected to be very high, which is likely to be able to overcome the low reaction rate constants [2]. Hydrogen is not easily dissolved in water, and 100%-saturated hydrogen water contains 1.6 ppm or 0.8 mM hydrogen at room temperature.

In 1995, hydrogen was first applied to human to overcome high-pressure nervous syndrome in deep sea diving [3]. Hydrogen was used to reduce nitrogen (N_2) toxicity and to reduce breathing resistance in the deep sea. In 2001, being prompted by the radical-scavenging activity of hydrogen, Gharib and colleagues examined an effect of molecular hydrogen on a mouse model of schistosomiasis-associated chronic liver inflammation [4]. Mice were placed in a chamber with 70% hydrogen gas for two weeks. The mice exhibited decreased fibrosis, improvement of hemodynamics, increased nitric oxide synthase (NOS) II activity,

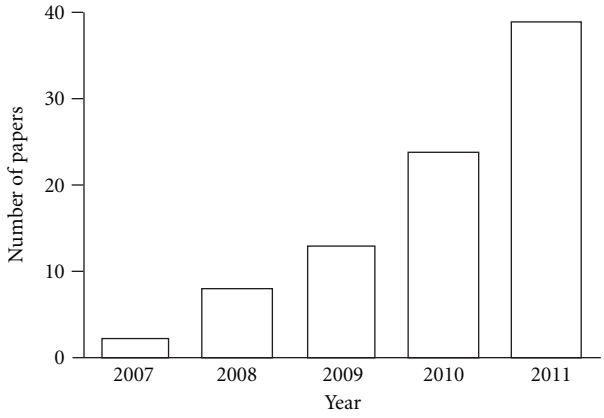

Figure 1: Number of papers that report effects of molecular hydrogen since 2007 shown in Table 1.

increased antioxidant enzyme activity, decreased lipid peroxide levels, and decreased circulating tumor-necrosis-factor-(TNF-) α levels. Although helium gas also exerted some protective effects in their model, the effect of helium gas was not recapitulated in a mouse model of ischemia/reperfusion injury of the liver [5].

2. Effects of Hydrogen Have Been Reported in 63 Disease Models and Human Diseases

A major breakthrough in hydrogen research occurred after Ohsawa and colleagues reported a prominent effect of molecular hydrogen on a rat model of cerebral infarction in June 2007 [6]. Rats were subjected to left middle cerebral artery occlusion. Rats placed in 2–4% hydrogen gas chamber showed significantly smaller infarction volumes compared to controls. They attributed the hydrogen effect to the specific scavenging activity of hydroxyl radical (•OH). They also demonstrated that hydrogen scavenges peroxynitrite ($ONOO^-$) but to a lesser extent.

As have been previously reviewed [7, 8], effects of molecular hydrogen on various diseases have been reported since then. The total number of disease models and human diseases for which molecular hydrogen has been proven to be effective has reached 63 (Table 1). The number of papers is increasing each year (Figure 1). Among the 87 papers cited in Table 1, 21 papers showed an effect with inhalation of hydrogen gas, 23 with drinking hydrogen-rich water, 27 with intraperitoneal administration or drip infusion of hydrogen-rich saline, 10 with hydrogen-rich medium for cell or tissue culture, and 6 with the other administration methods including instillation and dialysis solution. In addition, among the 87 papers, 67 papers showed an effect in rodents, 7 in humans, 1 in rabbits, 1 in pigs, and 11 in cultured cells or cultured tissues.

Two papers, however, showed that hydrogen was ineffective for two disease models (Table 2). One such disease was moderate to severe neonatal brain hypoxia [9], although marked effects of hydrogen gas [10, 11] and intraperitoneal administration of hydrogen-rich saline [12] on neonatal brain hypoxia have been reported in rats [10, 12] and pigs [11]. We frequently observe that therapeutic intervention that is effective for mild cases has little or no effect on severe cases, and hydrogen is unlikely to be an exception. Another disease is muscle disuse atrophy [13]. Although oxidative stress is involved in the development of muscle disuse atrophy, oxidative stress may not be a major driving factor causing atrophy and thus attenuation of oxidative stress by hydrogen may not be able to exhibit a beneficial effect.

Effects of molecular hydrogen have been observed essentially in all the tissues and disease states including the brain, spinal cord, eye, ear, lung, heart, liver, kidney, pancreas, intestine, blood vessel, muscle, cartilage, metabolism, perinatal disorders, and inflammation/allergy. Among them, marked effects are observed in ischemia/reperfusion disorders as well as in inflammatory disorders. It is interesting to note, however, that only three papers addressed effects on cancers. First, molecular hydrogen caused growth inhibition of human tongue carcinoma cells HSC-4 and human fibrosarcoma cells HT-1080 but did not compromise growth of normal human tongue epithelial-like cells DOK [14]. Second, hydrogen suppressed the expression of vascular endothelial growth factor (VEGF), a key mediator of tumor angiogenesis, in human lung adenocarcinoma cells A549, which was mediated by downregulation of extracellular signal-regulated kinase (ERK) [15]. Third, hydrogen protected BALB/c mice from developing radiation-induced thymic lymphoma [16]. Elimination of radical oxygen species by hydrogen should reduce a probability of introducing somatic mutations. Unlike other disease models, cancer studies were performed only with cells in two of the three papers. Hydrogen is likely to have a beneficial effect on cancer development by suppressing somatic mutations, but an effect on cancer growth and invasion needs to be analyzed further in detail.

3. Effects of Molecular Hydrogen on Rodent Models of Neurodegenerative Diseases

Parkinson's disease is caused by death of dopaminergic neurons at the substantia nigra pars compact of the midbrain and is the second most common neurodegenerative disease after Alzheimer's disease. Parkinson's disease is caused by two mechanisms: excessive oxidative stress and abnormal ubiquitin-proteasome system [17]. The neurotransmitter, dopamine, is a prooxidant by itself and dopaminergic cells are destined to be exposed to high concentrations of radical oxygen species. An abnormal ubiquitin-proteasome system also causes aggregation of insoluble α-synuclein in the neuronal cell body that leads to neuronal cell death. We made a rat model of hemi-Parkinson's disease by stereotactically injecting catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) in the right striatum [18]. *Ad libitum* administration of hydrogen-rich water starting one week before surgery completely abolished the development of hemi-Parkinson's symptoms. The number of dopaminergic neurons on the toxin-injected side was reduced to 40.2% of that on the

Table 1: Sixty-three disease models and human diseases for which beneficial effects of hydrogen have been documented.

Diseases	Species	Administration
Brain		
Cerebral infarction [6, 30, 55, 56]	Rodent, human	Gas, saline
Cerebral superoxide production [75]	Rodent	Water
Restraint-induced dementia [22]	Rodent	Water
Alzheimer's disease [23, 24]	Rodent	Saline
Senile dementia in senescence-accelerated mice [25]	Rodent	Water
Parkinson's disease [18, 19]	Rodent	Water
Hemorrhagic infarction [34]	Rodent	Gas
Brain trauma [76]	Rodent	Gas
Carbon monoxide intoxication [52]	Rodent	Saline
Transient global cerebral ischemia [66]	Rodent	Gas
Deep hypothermic circulatory arrest-induced brain damage [57]	Rodent	Saline
Surgically induced brain injury [77]	Rodent	Gas
Spinal Cord		
Spinal cord injury [78]	Rodent	Saline
Spinal cord ischemia/reperfusion [51]	Rabbit	Gas
Eye		
Glaucoma [79]	Rodent	Instillation
Corneal alkali-burn [61]	Rodent	Instillation
Ear		
Hearing loss [80–82]	Tissue, rodent	Medium, water
Lung		
Oxygen-induced lung injury [53, 60, 83, 84]	Rodent	Saline
Lung transplantation [85]	Rodent	Gas
Paraquat-induced lung injury [86]	Rodent	Saline
Radiation-induced lung injury [87–89]	Rodent	Water
Burn-induced lung injury [90]	Rodent	Saline
Intestinal ischemia/reperfusion-induced lung injury [44]	Rodent	Saline
Heart		
Acute myocardial infarction [36, 65, 91]	Rodent	Gas, saline
Cardiac transplantation [46]	Rodent	Gas
Sleep apnea-induced cardiac hypoxia [48]	Rodent	Gas
Liver		
Schistosomiasis-associated chronic liver inflammation [4]	Rodent	Gas
Liver ischemia/reperfusion [5]	Rodent	Gas
Hepatitis [43]	Rodent	Intestinal gas
Obstructive jaundice [47]	Rodent	Saline
Carbon tetrachloride-induced hepatopathy [62]	Rodent	Saline
Radiation-induced adverse effects for liver tumors [31]	Human	Water
Kidney		
Cisplatin-induced nephropathy [92–94]	Rodent	Gas, water
Hemodialysis [20, 28]	Human	Dialysis solution
Kidney transplantation [95]	Rodent	Water
Renal ischemia/reperfusion [54]	Rodent	Saline
Melamine-induced urinary stone [96]	Rodent	Water
Chronic kidney disease [37]	Rodent	Water

Table 1: Continued.

Diseases	Species	Administration
Pancreas		
Acute pancreatitis [97]	Rodent	Saline
Intestine		
Intestinal transplantation [41, 45, 59]	Rodent	Gas, medium, saline
Ulcerative colitis [42]	Rodent	Gas
Intestinal ischemia/reperfusion [63]	Rodent	Saline
Blood vessel		
Atherosclerosis [98]	Rodent	Water
Muscle		
Inflammatory and mitochondrial myopathies [29]	Human	Water
Cartilage		
NO-induced cartilage toxicity [38]	Cells	Medium
Metabolism		
Diabetes mellitus type I [32]	Rodent	Water
Diabetes mellitus type II [26]	Human	Water
Metabolic syndrome [27, 99]	Human, rodent	Water
Diabetes/obesity [33]	Rodent	Water
Perinatal disorders		
Neonatal cerebral hypoxia [10–12]	Rodent, pig	Gas, saline
Preeclampsia [58]	Rodent	Saline
Inflammation/allergy		
Type I allergy [64]	Rodent	Water
Sepsis [100]	Rodent	Gas
Zymosan-induced inflammation [101]	Rodent	Gas
LPS/IFNγ-induced NO production [67]	Cells	Gas
Cancer		
Growth of tongue carcinoma cells [14]	Cells	Medium
Lung cancer cells [15]	Cells	Medium
Radiation-induced thymic lymphoma [16]	Rodent	Saline
Others		
UVB-induced skin injury [49]	Rodent	Bathing
Decompression sickness [102]	Rodent	Saline
Viability of pluripotent stromal cells [103]	Cells	Gas
Radiation-induced cell damage [104, 105]	Cells	Medium
Oxidized low density lipoprotein-induced cell toxicity [50]	Cells	Medium
High glucose-induced oxidative stress [35]	Cells	Medium

control side, whereas hydrogen treatment improved the reduction to 83.0%. We also started giving hydrogen-rich water three days after surgery, and hemi-Parkinson's symptoms were again suppressed, but not as much as those observed in pretreated rats. The number of dopaminergic neurons on the toxin-injected side was 76.3% of that on the control side. Pretreated rats were also sacrificed 48 hrs after toxin injection, and the tyrosine hydroxylase activity at the striatum, where dopaminergic neurons terminate, was decreased in both hydrogen and control groups. This indicated that hydrogen did not directly detoxicate 6-OHDA but exerted a delayed protective effect for dopaminergic cells. Fujita and colleagues also demonstrated a similar prominent effect of hydrogen-rich water on an MPTP-(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-) induced mouse model of Parkinson's disease [19]. MPTP is a neurotoxin that blocks complex I of the mitochondrial electron transport system and causes Parkinson's disease in mice and humans.

Table 2: Two disease models for which hydrogen has no effect.

Diseases	Species	Administration
Brain		
Moderate to severe neonatal brain hypoxia [9]	Rodent	Gas
Muscle		
Muscle disuse atrophy [13]	Rodent	Water

It is interesting to note that the concentration of hydrogen that they used for the MPTP mice was only 0.08 ppm (5% saturation), which is the second lowest among all the trials published to date for rodents and humans. The lowest hydrogen concentration ever tested is 0.048 ppm in the dialysis solution for patients receiving hemodialysis [20].

Alzheimer's disease is the most common neurodegenerative disease and is characterized by abnormal aggregation of β-amyloid (Aβ) and tau, the large aggregates of which are recognizable as senile plaques and neurofibrillary tangles, respectively [21]. Effects of molecular hydrogen on Alzheimer's disease have been studied in three rodent models. First, Nagata and colleagues made a mouse model of dementia by restricting movement of mice for 10 hrs a day [22]. They analyzed cognitive functions through passive avoidance learning, object recognition tasks, and the Morris water maze and demonstrated that *ad libitum* administration of hydrogen-rich water efficiently ameliorated cognitive impairment. They also showed that neural proliferation in the dentate gyrus was restored by hydrogen. Second, Li and colleagues made a rat model of Alzheimer's disease by intracerebroventricular injection of Aβ1-42 [23]. They analyzed cognitive functions by the Morris water maze open field tasks, and electrophysiological measurement of long-term potentiation (LTP) and found that intraperitoneal injection of hydrogen-rich saline for 14 days efficiently ameliorated cognitive decline and preserved LTP. The same team later reported that the protective effects were mediated by suppression of abnormal activation of IL1β, JNK, and NFκB [24]. Third, Gu and colleagues used a senescence-accelerated mouse strain (SAMP8) that exhibits early aging syndromes including impairment in learning ability and memory [25]. *Ad libitum* administration of hydrogen-rich water for 30 days prevented cognitive decline, which was examined by the Morris water maze. Additionally, *ad libitum* drinking of hydrogen water for 18 weeks showed efficient amelioration of hippocampal neurodegeneration.

Cerebrovascular diseases are the most frequently reported neurological diseases for which hydrogen has prominent effects. As stated in Section 2, current hydrogen research has broken out after Ohsawa reported a prominent effect of 2–4% hydrogen for a rat model of left cerebral artery occlusion in 2007 [6].

In addition to neurodegenerative disorders of Parkinson's disease and Alzheimer's disease, effects of molecular hydrogen have been reported in eight other brain diseases listed under the categories of "brain" and "perinatal disorders" in Table 1. The brain consumes a large amount of oxygen and is predisposed to be exposed to a large amount of radical oxygen species especially under pathological conditions. Molecular hydrogen is thus likely to exert a prominent beneficial effect on brain diseases.

4. Molecular Hydrogen Is Effective for Six Human Diseases

As in other therapeutic modalities, effects of molecular hydrogen have been tested mostly on rodents but have also been studied in six human diseases. The reported human diseases include diabetes mellitus type II [26], metabolic syndrome [27], hemodialysis [20, 28], inflammatory and mitochondrial myopathies [29], brain stem infarction [30], and radiation-induced adverse effects for liver tumor [31]. These studies are reviewed in detail here. In addition, a therapeutic trial for Parkinson's disease is currently in progress and exhibits favorable responses as far as we know, but the details are not yet disclosed.

First, Kajiyama and colleagues performed a randomized, double-blind, placebo-controlled, crossover study in 30 patients with diabetes mellitus type II and 6 patients with impaired glucose tolerance [26]. The patients consumed either 900 mL of hydrogen-rich water or placebo water for 8 weeks, with a 12-week washout period. They measured 13 biomarkers to estimate lipid and glucose metabolisms at baseline and at 8 weeks after hydrogen treatment. All the biomarkers were favorably changed with hydrogen, but statistical significance was observed only in improvement of electronegative charge-modified low-density lipoprotein-(LDL-) cholesterol, small dense LDL, and urinary 8-isoprostanes. In four of six patients with impaired glucose tolerance, hydrogen normalized the oral glucose tolerance test. Lack of statistical significance in their studies was likely due to the small number of patients and the short observation period. Lack of statistical significance, however, may also suggest a less prominent effect in human diabetes mellitus compared to rodent models [32, 33].

Second, Nakao and colleagues performed an open-label trial in 20 subjects with potential metabolic syndrome [27]. Hydrogen-rich water was produced by placing a metallic magnesium stick in water, which yielded 0.55–0.65 mM hydrogen water (70–80% saturation). The participants consumed 1.5–2.0 liters of hydrogen water per day for 8 weeks and showed a 39% increase in urinary superoxide dismutase (SOD), an enzyme that catalyzes superoxide anion (O_2^-); a 43% decrease in urinary thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation; an 8% increase in high-density-lipoprotein-(HDL-) cholesterol; a 13% decrease in total cholesterol/HDL-cholesterol. The aspartate aminotransferase (AST) and alanine transaminase (ALT) levels remained unchanged, whereas the gamma glutamyl transferase (GGT) level was increased by 24% but was still within a normal range. Although the study was not double blinded and placebo controlled, improvements in biomarkers were much more than those in other hydrogen studies in humans. As this study used a large amount of hydrogen water, the amount of hydrogen might have been a

critical determinant. Alternatively, excessive hydration might have prevented the participants from excessive food intake.

Third, Nakayama and colleagues performed an open-label placebo-controlled crossover trial of 12 sessions of hemodialysis in eight patients [28] and an open-label trial of 78 sessions of hemodialysis in 21 patients [20]. In both studies, continuous sessions of hemodialysis with hydrogen-rich dialysis solution decreased systolic blood pressure before and after dialysis. In the short-term study, plasma methylguanidine was significantly decreased. In the long-term study, plasma monocyte chemoattractant protein 1 and myeloperoxidase were significantly decreased.

Fourth, we performed an open-label trial of 1.0 liter of hydrogen water per day for 12 weeks in 14 patients with muscular diseases including muscular dystrophies, polymyositis/dermatomyositis, and mitochondrial myopathies, as well as a randomized, double-blind, placebo-controlled, crossover trial of 0.5 liter of hydrogen water or dehydrogenized water per day for 8 weeks in 22 patients with dermatomyositis and mitochondrial myopathies [29]. In the open-label trial, significant improvements were observed in lactate-to-pyruvate ratio, fasting blood glucose, serum matrix metalloproteinase-3 (MMP3), and triglycerides. Especially, the lactate-to-pyruvate ratio, which is a sensitive biomarker for the compromised mitochondrial electron transport system, was decreased by 28% in mitochondrial myopathies. In addition, MMP3, which represents the activity of inflammation, was decreased by 27% in dermatomyositis. In the double-blind trial, a statistically significant improvement was observed only in serum lactate in mitochondrial myopathies, but lactate-to-pyruvate ratio in mitochondrial myopathies and MMP3 in dermatomyositis were also decreased. Lack of statistical significance with the double-blind study was likely due to the shorter observation period and the lower amount of hydrogen compared to those of the open-label trial.

Fifth, Kang and colleagues performed a randomized placebo-controlled study of 1.5–2.0 liters of 0.55–0.65 mM hydrogen water per day for 6 weeks in 49 patients receiving radiation therapy for malignant liver tumors. Hydrogen suppressed the elevation of total hydroperoxide levels, maintained serum antioxidant capacity, and improved the quality of life (QOL) scores. In particular, hydrogen efficiently prevented loss of appetite. Although the patients were randomly assigned to the hydrogen and placebo groups, the study could not be completely blinded because hydrogen was produced with a metallic magnesium stick, which generated hydrogen bubbles.

Sixth, Ono and colleagues intravenously administered hydrogen along with Edaravone, a clinically approved radical scavenger, in 8 patients with acute brain stem infarction and compared MRI indices of 26 patients who received Edaravone alone [30]. The relative diffusion-weighted images (rDWIs), regional apparent diffusion coefficients (rADCs), and pseudonormalization time of rDWI and rADC were all improved with the combined infusion of Edaravone and hydrogen.

No adverse effect of hydrogen has been documented in the six human diseases described above. Among the six diseases, the most prominent effect was observed in subjects with metabolic syndrome, who consumed 1.5–2.0 liters of hydrogen water per day [27]. The amount of hydrogen water may be a critical parameter that determines clinical outcome. It is also interesting to note that lipid and glucose metabolisms were analyzed in three studies and all showed favorable responses to hydrogen [26, 27, 29].

5. Molecular Bases of Hydrogen Effects

Effects of hydrogen on various diseases have been attributed to four major molecular mechanisms: a specific scavenging activity of hydroxyl radical, a scavenging activity of peroxynitrite, alterations of gene expressions, and signal-modulating activities. The four mechanisms are not mutually exclusive and some of them may be causally associated with other mechanisms.

The first molecular mechanism identified for hydrogen was its specific scavenging activity of hydroxyl radical [6]. Indeed, oxidative stress markers like 8-OHdG, 4-hydroxyl-2-nonenal (4-HNE), malondialdehyde (MDA), and thiobarbituric acid reactive substances (TBARSs) are decreased in all the examined patients and rodents. As hydrogen can easily dissipate in exhalation, hydrogen in drinking water is able to stay in human and rodent bodies in less than 10 min (unpublished data). Hydrogen, however, can bind to glycogen, and the dwell time of hydrogen is prolonged in rat liver after food intake [33]. A question still remains if mice and humans can take a sufficient amount of hydrogen that efficiently scavenges hydroxyl radicals that are continuously generated in normal and disease states.

Another molecular mechanism of hydrogen effect is its peroxynitrite-($ONOO^-$-) scavenging activity [6]. Although hydrogen cannot eliminate peroxynitrite as efficiently as hydroxyl radical *in vitro* [6], hydrogen can efficiently reduce nitric-oxide-(NO-) induced production of nitrotyrosine in rodents [34–38]. NO is a gaseous molecule that also exerts therapeutic effects including relaxation of blood vessels and inhibition of platelet aggregation [39]. NO, however, is also toxic at higher concentrations because NO leads to $ONOO^-$-mediated production of nitrotyrosine, which compromises protein functions. A part of hydrogen effects may thus be attributed to the reduced production of nitrotyrosine.

Expression profiling of rat liver demonstrated that hydrogen has a minimal effect on expression levels of individual genes in normal rats [40]. Gene ontology analysis, however, revealed that oxidoreduction-related genes were upregulated. In disease models of rodents, expression of individual genes and proteins is analyzed. In many disease models, hydrogen downregulated proinflammatory cytokines including tumor necrosis-factor-(TNF-) α, interleukin-(IL-) 1β, IL-6, IL-12, interferon-(IFN-) γ, and high mobility group box 1 (HMGB1) [4, 23, 24, 36, 41–59]. Hydrogen also downregulated nuclear factors including nuclear factor kappa B (NFκB), JNK, and proliferation cell nuclear antigen (PCNA) [24, 44, 50, 55, 60–63]. Caspases were also downregulated [10, 55–57, 62, 64, 65]. Other interesting molecules studied to date include vascular endothelial growth factor (VEGF)

[15]; MMP2 and MMP9 [34]; brain natriuretic peptide [48]; intercellular-adhesion-molecule-1 (ICAM-1) and myeloperoxidase [36]; B-cell lymphoma 2 (Bcl2) and Bcl2-associated X protein (Bax) [60]; MMP3 and MMP13 [38]; cyclooxygenase 2 (COX-2), neuronal nitric oxide synthase (nNOS), and connexins 30 and 43 [66]; ionized calcium binding adaptor molecule 1 (Iba1) [52]; fibroblast growth factor 21 (FGF21) [33]. Most molecules, however, are probably passengers that are secondarily changed by hydrogen administration, and some are potentially direct targets of hydrogen effects, which need to be identified in the future.

Using rat RBL-2H3 mast cells, we demonstrated that hydrogen attenuates phosphorylation of FcεRI-associated Lyn and its downstream signaling molecules [64]. As phosphorylation of Lyn is again regulated by the downstream signaling molecules and makes a loop of signal transduction pathways, we could not identify the exact target of hydrogen. Our study also demonstrated that hydrogen ameliorates an immediate-type allergic reaction not by radical-scavenging activity but by direct modulation of signaling pathway(s). In addition, using murine RAW264 macrophage cells, we demonstrated that hydrogen reduces LPS/IFNγ-induced NO production [67]. We found that hydrogen inhibits phosphorylation of ASK1 and its downstream signaling molecules, p38 MAP kinase, JNK, and IκBα without affecting ROS production by NADPH oxidase. Both studies point to a notion that hydrogen is a gaseous signal modulator. More animal and cells models are expected to be explored to confirm that hydrogen exerts its beneficial effect as a signal modulator.

6. Enigmas of Hydrogen Effects

Two enigmas remain to be solved for hydrogen effects. First, no dose-response effect of hydrogen has been observed. Hydrogen has been administered to animals and humans in the forms of hydrogen gas, hydrogen-rich water, hydrogen-rich saline, instillation, and dialysis solution (Table 1). Supposing that a 60-kg person drinks 1000 mL of saturated hydrogen-rich water (1.6 ppm or 0.8 mM) per day, 0.8 mmoles of hydrogen is consumed by the body each day, which is predicted to give rise to a hydrogen concentration of 0.8 mmoles/(60 kg $\times$ 60%) = 0.022 mM (2.8% saturation = 0.022 mM/0.8 mM). As hydrogen mostly disappears in 10 min by dissipation in exhalation (unpublished data), an individual is exposed to 2.8% hydrogen only for 10 min. On the other hand, when a person is placed in a 2% hydrogen environment for 24 hrs, body water is predicted to become 2% saturation (0.016 mM). Even if we suppose that the hydrogen concentration after drinking hydrogen water remains the same for 10 min, areas under the curves of hydrogen water and 2% hydrogen gas are 0.022 mM $\times$ 1/6 hrs and 0.016 mM $\times$ 24 hrs, respectively. Thus, the amount of hydrogen given by 2% hydrogen gas should be 104 or more times higher than that given by drinking hydrogen water. In addition, animals and patients are usually not able to drink 100%-saturated hydrogen water. If the hydrogen concentration is 72% of the saturation level, the peak concentrations achieved by drinking hydrogen water and 2% hydrogen gas should be identical (0.022 mM $\times$ 72% = 0.016 mM). Nevertheless, hydrogen water is as effective as, or sometimes more effective than, hydrogen gas. In addition, orally taken hydrogen can be readily distributed in the stomach, intestine, liver, heart, and lung but is mostly lost in exhalation. Thus, hydrogen concentrations in the arteries are predicted to be very low. Nevertheless, marked hydrogen effects are observed in the brain, spinal cord, kidney, pancreas muscle, and cartilage, where hydrogen is carried via arteries.

The second enigma is intestinal production of hydrogen gas in rodents and humans. Although no mammalian cells can produce hydrogen endogenously, hydrogen is produced by intestinal bacteria carrying hydrogenase in both rodents and humans. We humans are able to make a maximum of 12 liters of hydrogen in our intestines [68, 69]. Specific-pathogen-free (SPF) animals are different from aseptic animals and carry intestinal bacteria that produce hydrogen. The amount of hydrogen taken by water or gas is much less than that produced by intestinal bacteria, but the exogenously administered hydrogen demonstrates a prominent effect. In a mouse model of Concanavalin A-induced hepatitis, Kajiya and colleagues killed intestinal bacteria by prescribing a cocktail of antibiotics [43]. Elimination of intestinal hydrogen worsened hepatitis. Restitution of a hydrogenase-negative strain of *E. coli* had no effects, whereas that of a hydrogenase-positive strain of *E. coli* ameliorated hepatitis. This is the only report that addressed a beneficial effect of intestinal bacteria, and no human study has been reported to date. Kajiya and colleagues also demonstrated that drinking hydrogen-rich water was more effective than the restitution of hydrogenase-positive bacteria. If intestinal hydrogen is as effective as the other hydrogen administration methods, we can easily increase hydrogen concentrations in our bodies by an α-glucosidase inhibitor, acarbose [70], an ingredient of curry, turmeric [71], or a nonabsorbable synthetic disaccharide, lactulose [68, 72, 73]. The enigma of intestinal bacteria thus needs to be solved in the future.

7. Summary and Conclusions

Effects of hydrogen have been reported in 63 disease models and human diseases (Table 1). Only two diseases of cerebral infarction and metabolic syndrome have been analyzed in both rodents and humans. Lack of any adverse effects of hydrogen enabled clinical studies even in the absence of animal studies. Some other human studies including Parkinson's disease are currently in progress, and promising effects of hydrogen are expected to emerge for many other human diseases. We also have to elucidate molecular bases of hydrogen effects in detail.

8. Added Note in Proof

We recently reported a line of evidence that molecular hydrogen has no dose-response effect in a rat model of Parkinson's disease [74].

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Research Article

Association of Base Excision Repair Gene Polymorphisms with ESRD Risk in a Chinese Population

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The base excision repair (BER) pathway, containing *OGG1, MTH1* and *MUTYH*, is a major protector from oxidative DNA damage in humans, while 8-oxoguanine (8-OHdG), an index of DNA oxidation, is increased in maintenance hemodialysis (HD) patients. Four polymorphisms of BER genes, OGG1 c.977C > G (rs1052133), *MTH1* c.247G > A (rs4866), *MUTYH* c.972G > C (rs3219489), and *AluYb8MUTYH* (rs10527342), were examined in 337 HD patients and 404 healthy controls. And the 8-OHdG levels in leukocyte DNA were examined in 116 HD patients. The distribution of *MUTYH* c.972 GG or *AluYb8MUTYH* differed between the two groups and was associated with a moderately increased risk for end-stage renal disease (ESRD) ($P = 0.013$ and 0.034, resp.). The average 8-OHdG/10^6 dG value was significantly higher in patients with the *OGG1* c.977G, *MUTYH* c.972G or *AluYb8MUTYH* alleles ($P < 0.001$ via ANOVA). Further analysis showed that combination of *MUTYH* c.972GG with *OGG1* c.977GG or *AluYb8MUTYH* increased both the risk for ESRD and leukocyte DNA 8-OHdG levels in HD patients. Our study showed that *MUTYH* c.972GG, *AluYb8MUTYH*, and combination of *OGG1* c.977GG increased the risk for ESRD development in China and suggested that DNA oxidative damage might be involved in such process.

1. Introduction

Oxidative stress is characterized by an excess of reactive oxygen species (ROS) and leads to cellular injury via reactions with proteins, nucleic acids, and lipids [1, 2]. The DNA bases, especially guanine (G), are particularly susceptible to oxidation, for which ROS frequently lead to a plethora of oxidized guanine products [3]. 8-hydroxy-2′deoxyguanosine (also known as 8-oxoguanine; 8-OHdG) is one of the most common mutagenic products and pairs with adenine in double-stranded DNA during DNA replication [3, 4]. If the mispairing is not repaired, it will lead to a G : C to T : A transversion mutation in cells [5].

Several repair pathways are involved with the DNA insults that result from either endogenous sources or exogenous sources, including the direct reversal pathway, the mismatch repair (MMR) pathway, the nucleotide excision repair (NER) pathway, and the base excision repair (BER) pathway [6]. Base excision repair (BER) is the primary DNA repair pathway that corrects base lesions that arise due to oxidative, alkylation, deamination, and depurinatiation/depyrimidination damage, such as 8-OHdG [7]. Actually, the BER pathway specifically prevents those G : C-to-T : A mutations by the repair of 8-OHdG. It includes the *MTH1*, *OGG1,* and *MUTYH* genes that prevent, recognize and remove the misincorporated oxidized nucleotide, 8-OHdG, and the adenine paired with 8-OHdG, respectively, when initiated by the BER pathway.

Increasing evidence has shown that genetic polymorphisms in DNA repair genes may modulate DNA repair capacity, result in DNA damage accumulation, and then contribute to some complex diseases [8, 9]. Kasahara et al. have reported that *MUTYH* Gln324His (c.972G > C) is associated with increased risk of colorectal cancers [10]. Marchand et al. have described the effect of *OGG1* Ser326Cys (c.977C > G) on the risk of lung cancer [11]. We have also shown that

Table 1: Sequences of PCR Primers used for genotyping.

Polymorphisms	Primer sequence (5′-3′)	Annealing temperature (°C)	Product length (bp)
rs1052133:	F: 5′-actgtcactagtctcaccag-3′	55	200
OGG1 c.977C > G (Ser326Cys)	R: 5′-ggaaggtgcttggggaat-3′		
rs4866:	F: 5′-gagcggtctgacagtgga-3′	58	168
MTH1 c.247G > A (Val83Met)	R: 5′-tggcactcagagatggtttg-3′		
rs3219489:	F: 5′-cccattccagttcttcctct-3′	58	208
MUTYH c.972G > C (Gln324His)	R: 5′-cctttctggggaagttgacc-3′		
rs10527342:	F: 5′-tcttgacctggagaccttcc-3′	60	500 or 826
AluYb8MUTYH	R: 5′-agctgcttcctccaaacagc-3′		

the *AluYb8* insertion in *MUTYH* (*AluYb8MUTYH*) might be a risk factor for age-related diseases and type 2 diabetes mellitus [12, 13].

The kidney is highly vulnerable to any of the results caused by ROS, and leukocyte 8-OHdG content is a surrogate biomarker for oxidation-induced DNA damage in patients with end-stage renal disease (ESRD), especially those on maintenance hemodialysis (HD). Oxidative injury is thought to alter the structure and function of glomeruli and is suggested to be related to renal diseases risk and eventual ESRD as well as atherosclerosis, dialysis-related amyloidosis and anemia in incident dialysis patients [14, 15]. The primary role of DNA repair in ESRD may be complex. Fukushima et al. [16] demonstrated that the polymorphism of the *hOGG1* (Ser326Cys) was associated with progression of IgA nephropathy. Most recently, Trabulus et al. [17] showed that *XRCC1 Arg399Gln* polymorphism may confer increased risk for the development of ESRD in Turkey, which is the first report showing an association between DNA repair gene polymorphisms and ESRD development. However, the genetic variations involved in antioxidant defense still need to be clarified in this disease, especially in China.

Based on the association of BER polymorphisms, oxidative DNA damages, and ESRD, we hypothesized that genetic variation in the BER genes might lead to repair impairment or disability, oxidative DNA damage accumulation, and pathogenesis of ESRD. Given the potential roles of *OGG1* c.977C > G, *MTH1* c.247G > A, *MUTYH* c.972G > C, and *AluYb8MUTYH* in the oxidative DNA repair pathway; we examined the association between these four polymorphisms in the BER pathway and ESRD in a Chinese cohort. We also assessed the leukocyte DNA 8-OHdG levels in HD patients to reveal the correlation between oxidative damage and end-stage renal disease arises.

2. Materials and Methods

2.1. Subjects. The allelic frequency of *OGG1* (NG_012106.1) c.977C > G, *MTH1* (NC_000007.13) c.247G > A, *MUTYH* (NG_008189.1) c.972G > C, and *AluYb8MUTYH* (AluYb8 insertion at intron 15 of *MUTYH* [12]) was investigated in 337 HD patients, regardless of cause, in Nanjing, Jiangsu province, China, between October 2009 and February 2010. All patients had been maintained on hemodialysis protocols for >3 months and were reviewed for age, sex, and presentation of clinical and laboratory data. Hypertension was defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg and/or use of antihypertensive medication [18]; anemia was defined as an Hgb <11 g/dL or use of recombinant human erythropoietin [19].

Healthy individuals with normal renal function were recruited from volunteers receiving health checkups in the same region. Detailed interview and various laboratory analyses were made upon every individual, including albumin excretion rate (AER) and serum creatinine. The subjects were excluded if their albumin excretion rate (AER) ≥30 mg/24 h, serum creatinine ≥1.2 mg/dL and ultrasound of the kidney and ureter was abnormal in size and appearance. They were ruled out if they suffering from certain diseases, such as acute inflammation, and diabetes, hypertension, autoimmune diseases or cancer according to past history and the clinical or laboratory characteristics. A total of 404 sex and age matched subjects were selected for inclusion in the control cohort. The Institutional Ethics Committee of Nanjing University School of Medicine approved this study, and written informed consents were obtained from all participants.

2.2. High-Resolution Melting Analysis. In this study, *OGG1* c.977C > G, *MTH1* c.247G > A, and *MUTYH* c.972G > C were genotyped using the dsDNA dye LCGreen in combination with HRM analysis. DNA was extracted from peripheral blood samples, and PCR was performed to amplify the target sequences. The PCR primers were designed by LightScanner primer design software (Idaho Technology) (Table 1). Each PCR reaction was initially performed in a final reaction volume of 10 μL, using 25 ng of genomic DNA, 0.2 pmol of each primer, 0.8 μL 2.5 mM dNTPs, 1 μL 25 mM $MgCl_2$, 1 μL 10 × Taq buffer with $(NH_4)_2SO4$, 0.4 U Taq DNA Polymerase (Fermentas), and 0.4 μL dimethyl sulfoxide (DMSO). The reaction mixture was incubated at 95°C for 5 min and then subjected to 40 cycles of 95°C for 30 sec, 55–58°C (Table 1) for 30 sec, and 72°C for 30 sec, followed by 72°C for 7 min using a PTC-200 thermal cycler (Bio-Rad).

The 9 μL reaction was supplemented with 1 μL 1 × LCGreen PLUS (Idaho Technology), and the 96-well plate (Bio-Rad) was transferred to the Light Scanner (Idaho

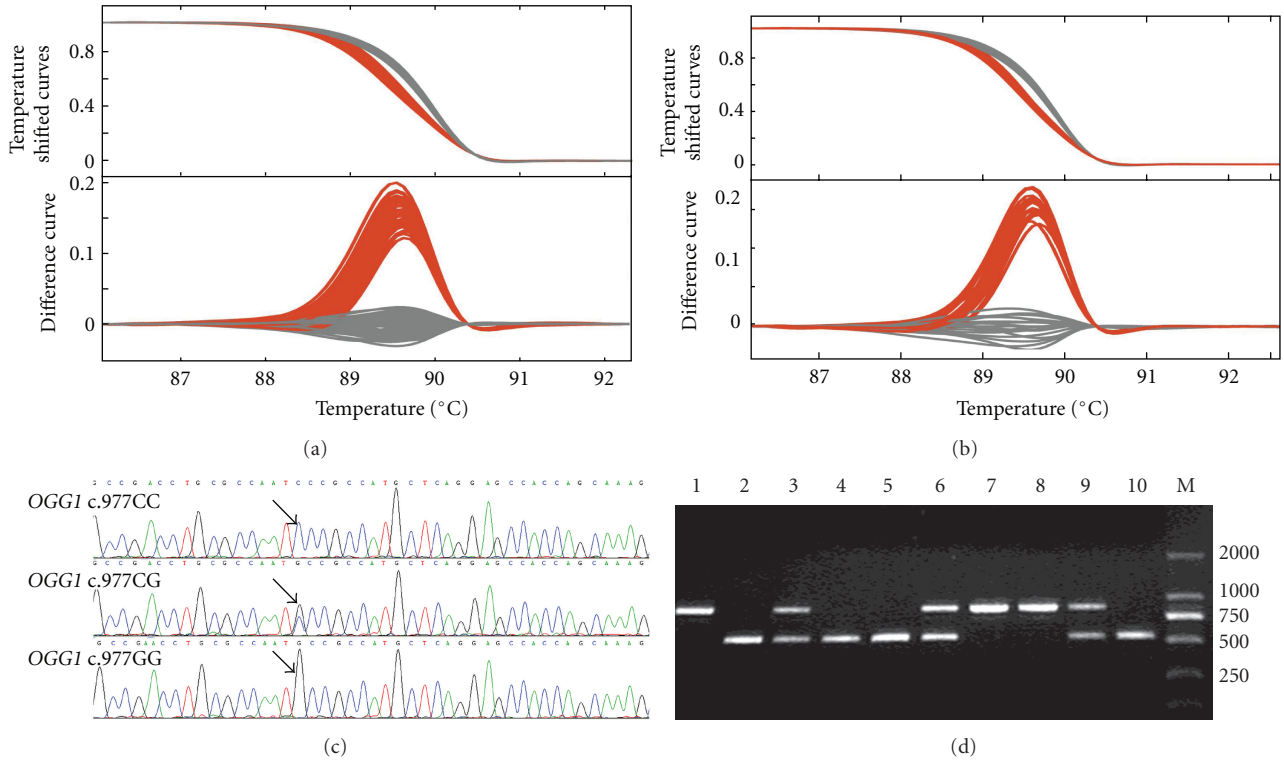

Figure 1: Demonstration of genotyping and sequence. (a) HRM directly discriminated the heterozygotes (*OGG1* c.977 CG) and homozygotes (*OGG1* c.977 CC or GG). (b) homozygous PCR products (CC or GG) were measured by LightScanner after being mixed with an equal amount of a known product (CC), which distinguished the wild homozygous samples (CC) from the variant ones (GG), as the mutational homozygotes (GG) were converted into heterozygotes (CG). (c) random samples from *OGG1* c.977C > G testing were sequenced for confirmation. (d) The PCR products were separated using 1% agarose gels to assess the pattern of *AluYb8* insertion into the *MUTYH* gene. Lanes 2, 4, 5, and 10: absence/absence (*A*/*A*); 3, 6, and 9: absence/presence (*A*/*P*); 1, 7, and 8: presence/presence (*P*/*P*); M: DNA Marker 2000.

Technology). Fluorescence data was collected over a temperature range of 70°C–97°C, as the samples were melted. Melting curve analysis was performed according to the manufacturer's software. HRM could directly discriminate the heterozygote (CG) and homozygote (CC or GG) genotypes of *OGG1* c.977C > G through melt scanning (Figure 1(a)). After mixing homozygous DNA with an equal amount of known PCR products (e.g., CC), it further distinguished between the CC and GG genotypes (Figure 1(b)). For further confirmation, 10% of samples from each group detected by HRM were randomly selected and subjected to DNA sequencing (Figure 1(c)). Similarly, the *MTH1* c.247G > A and *MUTYH* c.972G > C polymorphisms were genotyped by HRM.

2.3. Agarose Gel Assay for AluYb8MUTYH Polymorphism. The PCR primers were listed in Table 1, and the PCR condition was carried out with an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 50 sec, and then a final extension at 72°C for 10 min. The PCR products were run out on 1% agarose gels (Invitrogen, Carlsbad, CA, USA). The *AluYb8MUTYH* genotypes were classified as homozygous absence of this variation (only 500 bp products, absence/absence, *A*/*A*), homozygous presence of this variation (only 826 bp products, presence/presence, *P*/*P*), and heterozygote (500 bp and 826 bp products, absence/presence, *A*/*P*), according to the variant fragment absence or presence (Figure 1(d)).

2.4. Measurement of 8-OHdG Levels in Genomic DNA of Blood Cells. For measuring the level of 8-OHdG, 116 patients were randomly rerecruited from the HD cohort and investigated by the method reported previously [12]. Briefly, DNA extraction from fasting venous whole blood (10 mL, with EDTA added to prevent coagulation) was performed within 1 h of collection, using the salting out method [20]. The purity of the DNA sample was checked by OD260 nm/OD280 nm and OD260 nm/OD230 nm using an Eppendorf BioPhotometer Plus (Eppendorf, North America). Acceptable DNA stored frozen at −80°C until all samples could be assayed at the same time.

The DNA (200 μg) of each sample was dissolved in 135 μL of water. Sodium acetate (15 μL, 200 mM) and Nuclease P1 (15 μL, 6 units, Sigma, USA) were added to the DNA solution and incubated at 37°C for 30 min. Tris-HCl buffer (15 μL, 1 M, pH 7.4) and alkaline phosphatase (7 μL, 2 units, TAKARA, Shiga, Japan) were added and incubated at 37°C

for another 30 min. The hydrolysate was filtered through Millipore Microcon columns at 14000 rpm for 10 min, and 50 μL of digested DNA was applied to one well of an ELISA kit (Highly Sensitive 8-OHdG Check, JaICA, Fukuroi, Shizuoka, Japan). Results were measured in nanograms per milliliter, and then 1 ng/mL was converted to 4.8 8-OHdG/10^6 dG based on Halliwell [21].

2.5. Statistical Analysis. All statistical analyses were carried out using the statistical program SPSS, version 15.0. Descriptive statistical values included mean ± SD values for continuous data and percentages for categorical data. Chi-squared tests were used to compare the genotype and allelic frequencies for patients and healthy controls. Odds ratios (OR) are shown with 95% confidence intervals (CIs). Separate comparisons of variables among subjects with different genotypes were conducted with ANOVA and followed by post hoc analysis. Since 8-OHdG levels in leukocyte DNA were positively skewed, a natural logarithmic transformation was used to normalize the distributions for analyses. In all cases, a P value of less than 0.05 was considered statistically significant.

3. Results

Of the 337 HD patients, 212 (62.9%) were men, and 125 (37.1%) were women. The average age was 53.1 ± 15.8 yrs (ranging from 22 to 85 yrs), and duration of hemodialysis was 4.0 ± 3.5 yr. Primary glomerulonephritis (GN) was the most prevalent kidney disease in the HD group: 207 (61.4%) developed ESRD as a result of GN, 51 (15.1%) as a result of hypertensive nephropathy (HN), 36 (10.7%) as a result of diabetic nephropathy (DN), 16 (4.7%) as a result of congenital or inherited causes, 6 (1.8%) as a result of systemic lupus erythematosus (SLE) and 21 (6.2%) as a result of other causes. Additionally, 267 (79.2%) of the patients had anemia, and 250 (74.2%) had hypertension. The 404 healthy individuals were age and sex matched, with a mean age of 53.1 ± 16.2 yrs, and 254 (62.9%) were males.

3.1. Genotyping of BER Polymorphisms in HD Patients. The frequencies of the *OGG1* c.977C > G, *MTH1* c.247G > A, *MUTYH* c.972G > C, and *AluYb8MUTYH* genotypes associated with HD were shown in Table 2. The distribution in the healthy controls of these polymorphisms was consistent with Hardy-Weinberg equilibrium ($P > 0.05$ for all).

Compared to healthy controls, the distribution of the genotypes in *OGG1* c.977 C > G (namely, CC, CG, and GG) and the allele frequencies were not significantly different in the patients ($P > 0.05$, χ^2 test). For the c.247 G > A in *MTH1*, the frequency of heterozygous *MTH1* c.247 G > A was only 6.2% and 7.7% in the patients and controls ($P = 0.444$), while the homozygote was not detected. Thus, the *MTH1* polymorphism (c.247 G > A) was not included in further analysis.

Interestingly, both of the polymorphisms in the *MUTYH* gene showed an individual risk effect for ESRD (Table 2). For *MUTYH* c.972 G > C, the distribution of the three genotypes, namely CC, CG, and GG, and the allele frequencies were significantly different in HD patients ($P = 0.046$ and 0.026) compared with healthy controls. Furthermore, the frequency of the *MUTYH* c.972GG genotype was statistically higher in the HD cases (40.9%) than in the controls (32.2%), and the OR of GG adjusted by age and gender was 1.46 (95% CI: 1.08–1.98; $P = 0.013$). For *AluYb8MUTYH*, the distribution of the three genotypes and alleles in the HD patients was almost identical to that in the controls. Compared to the *A*/*A* genotype, the *AluYb8MUTYH* insertion carriers (*A*/*P* or *P*/*P*) were significantly higher in HD patients, and the OR was 1.40 (95% CI, 1.03–1.90; $P = 0.034$).

Regarding the effect of the *MUTYH* c.972GG genotype and the *AluYb8MUTYH P* allele on ESRD, a combined risk analysis was performed and shown in Table 3. Individuals carrying the *MUTYH* c.972GG genotype might have a higher risk for ESRD, and the OR of *MUTYH* c.972GG adjusted by age and gender was 2.23 (95% CI: 1.37–3.64; $P = 0.001$) among those with the *OGG1* c.977GG genotype. Meanwhile, the presence of *MUTYH* c.972GG also added to the risk of *AluYb8MUTYH A/P* or *P/P* genotypes for ESRD development (OR, 1.46; 95% CI, 1.07–1.99; $P = 0.017$).

3.2. BER Polymorphisms in the Patients with Different Clinical Characteristics. The HD patients were stratified into six subgroups on the basis of the primary diagnoses (i.e., GN, HN, DN, congenital or inherited causes, SLE, or other causes). Similar to ESRD, the effects of BER polymorphisms on HD risk were confirmed in the 207 patients with primary diagnosis of glomerulonephritis when compared to the whole cohort (Table 4). The frequency of the *MUTYH* c.972 G > C GG genotype was significantly higher in cases than in controls, and the OR was 1.75 (95% CI: 1.24–2.47; $P = 0.001$). The frequency of the *MUTYH AluYb8MUTYH A/P* or *P/P* genotype was significantly higher in cases than in controls, and the OR was 1.73 (95% CI: 1.20–2.52; $P = 0.003$).

In addition, the association of BER polymorphisms with risk of HD complication status was further analyzed (Table 4). Among 267 patients with anemia, the frequency of *MUTYH* c.972G > C GG was markedly higher in patients than controls (42.7% versus 32.2%; OR (95% CI) = 1.57 (1.14–2.16); $P = 0.006$), whereas the *AluYb8MUTYH* insertion (*A*/*P* or *P*/*P*) significantly increased the risk for patients with anemia (73.4% versus 62.6%; OR (95% CI) = 1.78 (1.22–2.60); $P = 0.003$). A similar relationship was detected among 250 patients with hypertension. The frequency of *MUTYH* c.972GG carriers was higher in cases than controls (41.2% versus 32.2%; OR (95% CI) = 1.48 (1.07–2.05); $P = 0.019$). The frequency of *AluYb8MUTYH* insertion carriers (*A*/*P* or *P*/*P*) was higher in cases than controls (70.4% versus 62.6%; OR (95% CI) = 1.42 (1.01–1.99); $P = 0.042$).

3.3. Predictor Effect of BER Polymorphisms to 8-OHdG. The 8-OHdG levels in leukocyte DNA were evaluated in 116 HD patients divided into different subgroups according to the polymorphism genotypes and compared (Figure 2(a)). The genotypic frequencies of the three polymorphisms were

Table 2: Genotypes of *OGG1*, *MTH1,* and *MUTYH* and the risk for HD.

	Patients ($n = 337$)	Controls ($n = 404$)	P value[a]	OR (95%CI)
OGG1 c.977 C > G			0. 394	
CC	56 (16. 6%)	77 (19.1%)		
CG	160 (47.5%)	200 (49.5%)		
GG	121 (35.9%)	127 (31.4%)	0.199	1.22 (0.90–1.66)
CC or CG[b]	216 (64.1%)	277 (68.6%)		1.00
C allele	0.404	0.438		1.00
G allele	0.596	0.562	0.180	1.15 (0.94–1.42)
MTH1 c.247 G > A			0.444	
GG	316 (93.8%)	373 (92.3%)		1.00
GA	21 (6.2%)	31 (7.7%)		
AA	0	0		
GA or AA[b]	21 (6.2%)	31 (7.7%)	0.444	0.80 (0.45–1.42)
G allele	0.969	0.962		1.00
A allele	0.031	0.038	0.453	0.81 (0.46–1.42)
MUTYH c.972 G > C			0.046	
CC	44 (13.1%)	63 (15.6%)		
CG	155 (46.0%)	211 (52.2%)		
GG	138 (40.9%)	130 (32.2%)	0.013	1.46 (1.08–1.98)
CC or CG[b]	199 (59.1%)	274 (67.8%)		1.00
C allele	0.361	0.417		1.00
G allele	0.639	0.583	0.026	1.27 (1.03–1.57)
AluYb8MUTYH			0.099	
A/A	101 (30.0%)	151 (37.4%)		1.00
A/P	164 (48.7%)	172 (42.6%)		
P/P	72 (21.3%)	81 (20.0%)		
A/P or *P/P* [b]	236 (70.0%)	253 (62.6%)	0.034	1.40 (1.03–1.90)
A allele	0.543	0.587		1.00
P allele	0.457	0.413	0.092	1.19 (0.97–1.47)

Note: CI: confidence interval; OR: odds ratio. [a]P value for comparison using χ^2 test to assess correlation between HD risk and predicted high-risk *OGG1*, *MTH1,* and *MUTYH* genotypes and alleles; [b]genotypes were combined properly to assess their association with HD and the genotype 1.00 as the reference category.

similar between the 116 patients tested for leukocyte DNA 8-OHdG levels and all 337 patients investigated in the present study. In a parallel investigation of healthy controls in our laboratory, the HD patients exhibited increased 8-OHdG levels compared to the healthy individuals [12].

For the *OGG1* c.977 C > G polymorphism, the genotypic frequencies (CC/CG/GG ratios of 15.5%/43.1%/41.4%) for the 116 patients whose leukocyte DNA 8-OHdG levels had been analyzed did not vary significantly from the whole study population of 337 patients (16.6%/47.5%/35.9%). The leukocyte 8-OHdG levels for patients carrying GG ($26.7 \pm 4.7/10^6$ dG) or CG ($26.6 \pm 5.5/10^6$ dG) were significantly higher than the patients carrying CC ($18.4 \pm 8.9/10^6$ dG) ($P < 0.001$ via ANOVA). For the *MUTYH* c.972G > C polymorphism, the genotypic frequencies (CC/CG/GG ratios of 13.8%/49.1%/37.1%) for the 116 patients did not vary significantly from the whole study population. The leukocyte 8-OHdG levels for patients carrying GG ($27.6 \pm 5.5/10^6$ dG) or CG ($25.3 \pm 6.1/10^6$ dG) were significantly higher than the patients carrying CC ($19.5 \pm 7.1/10^6$ dG) ($P < 0.001$ via ANOVA). For the *AluYb8MUTYH* polymorphism, out of the 116 patients, 37, 52, and 27 showed the *A*/*A*, *A*/*P* and *P*/*P* genotypes, which did not differ from the whole population. The patients carrying *P*/*P* ($29.2 \pm 3.9/10^6$ dG) or *A*/*P* ($25.4 \pm 5.6/10^6$ dG) had significantly higher 8-OHdG levels than the patients carrying *A*/*A* ($22.6 \pm 7.9/10^6$ dG) ($P < 0.001$ via ANOVA).

The combined impacts of these polymorphisms on 8-OHdG levels were further investigated (Figures 2(b) and 2(c)). Based on the risk for HD, 43 patients carrying the *MUTYH* c.972GG genotype were analyzed; 6, 21, and 16 showed the CC, CG, and GG genotypes of *OGG1* c.977C > G, respectively (Figure 2(b)). The *OGG1* c.977C > G GG or CG genotypes significantly increased the 8-OHdG level when compared with patients with the *OGG1* c.977C > G CC genotype among patients with the *MUTYH* c.972GG genotype ($29.2 \pm 3.2/10^6$ dG, $28.1 \pm 4.8/10^6$ dG versus $21.8 \pm 9.0/10^6$ dG; $P = 0.01$ via ANOVA). This indicates that

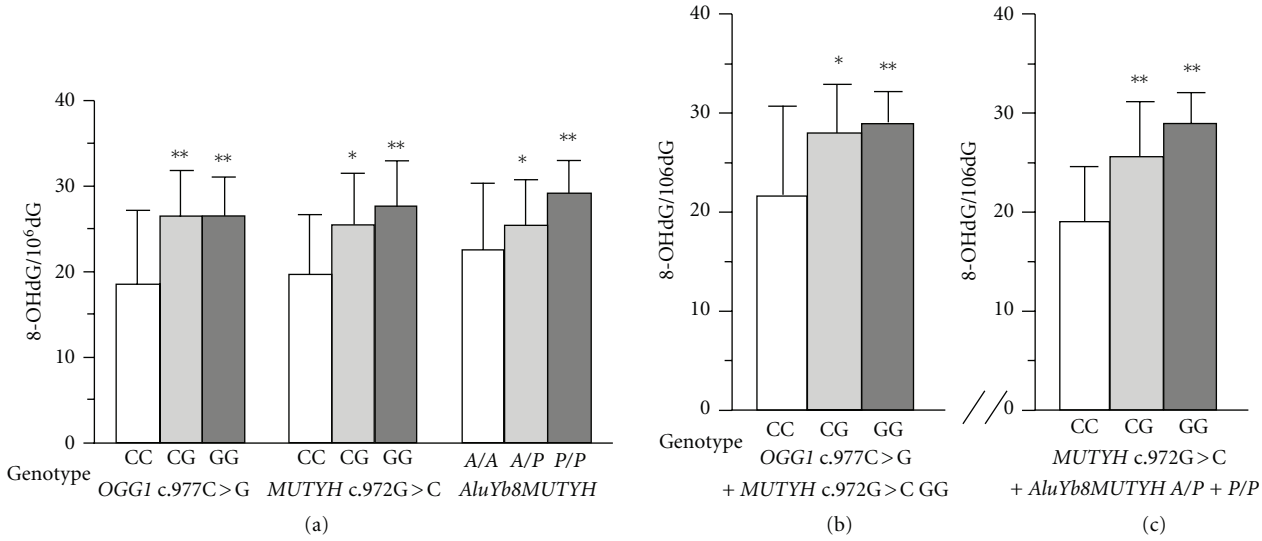

Figure 2: Individual (a) and combined [(b) accompanied with *MUTYHc.972GG* genotype; (c) accompanied with *AluYb8MUTYH A/P* or *P/P* genotype] analysis of the effect of BER polymorphism on mean levels of leukocyte DNA 8-OHdG in 116 HD patients. Every group includes three different bars stratified according to the polymorphism genotypes. Statistical significance was calculated using one-way ANOVA testing followed by post hoc analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus subjects with the genotype in the blank bar.

Table 3: Combined analysis of genetic polymorphisms in *OGG1* and *MUTYH* with HD risk.

Genotypes	Patients ($n = 337$)	Controls ($n = 404$)	P value[c]	OR (95% CI)
MUTYH c.972GG[a]				
OGG1				
c.977CC	23 (6.8%)	27 (6.7%)	0.939	1.02 (0.58–1.82)
c.977CG	67 (19.9%)	75 (18.6%)	0.650	1.09 (0.75–1.57)
c.977GG	48 (14.2%)	28 (6.9%)	0.001	2.23 (1.37–3.64)
AluYb8MUTYH				
A/A	18 (5.3%)	19 (4.7%)	0.691	1.14 (0.59–2.22)
A/P	58 (17.2%)	54 (13.4%)	0.146	1.35 (0.90–2.02)
P/P	62 (18.4%)	57 (14.1%)	0.113	1.37 (0.93–2.03)
AluYb8MUTYHA/P or P/P[b]				
OGG1				
c.977CC	38 (11.3%)	42 (10.4%)	0.701	1.10 (0.69–1.74)
c.977CG	120 (35.6%)	136 (33.7%)	0.579	1.09 (0.80–1.48)
c.977GG	78 (23.1%)	75 (18.6%)	0.125	1.32 (0.93–1.89)
MUTYH				
c.972CC	11 (3.3%)	12 (3.0%)	0.818	1.10 (0.48–2.53)
c.972CG	105 (31.2%)	130 (32.2%)	0.766	0.95 (0.70–1.30)
c.972GG	120 (35.6%)	111 (27.5%)	0.017	1.46 (1.07–1.99)

Note: CI: confidence interval; OR: odds ratio. [a]Trend test assessing correlation between HD risk and predicted high-risk *OGG1* and *MUTYH* genotypes combined with the *MUTYH c.972GG* genotype. [b]Trend test assessing correlation between HD risk and predicted high-risk *OGG1* and *MUTYH* genotypes combined with the *AluYb8MUTYH A/P* or *P/P* genotype. c: P value for comparison using χ^2-test between patients and controls.

MUTYH and *OGG1* may have synergistic roles in the prevention of DNA oxidative damage. Similarly, out of 79 patients carrying the *AluYb8MUTYH* insertion (*A*/*P* or *P*/*P*), 6, 41, and 32 showed the *MUTYH* c.972G > C CC, CG, and GG genotypes, respectively (Figure 2(c)). The 8-OHdG levels of individuals carrying the GG or CG genotypes were higher than in individuals carrying the CC genotype among patients with the *AluYb8MUTYH* insertion ($29.5 \pm 3.4/10^6$ dG, $25.6 \pm 5.3/10^6$ dG versus $19.4 \pm 5.3/10^6$ dG; $P < 0.001$ via ANOVA).

4. Discussion

End-stage renal disease (ESRD) is a troublesome health problem worldwide, and the mortality rate for ESRD patients

Table 4: Genotypes of genetic polymorphisms in *OGG1* and *MUTYH* and their effects on HD risk of patients with primary diagnoses of glomerulonephritis, hypertension, and anemia.

Genotype	Controls ($n = 404$)	Primary glomerulonephritis ($n = 207$)			Anemia ($n = 267$)			Hypertension ($n = 250$)		
			P value[a]	OR (95% CI)		*P* value[b]	OR (95% CI)		*P* value[c]	OR (95% CI)
OGG1 c.977 C > G										
CC or CG	277 (68.6%)	132 (63.8%)		1.00	178 (66.7%)		1.00	158 (63.2%)		1.00
GG	127 (31.4%)	75 (36.2%)	0.233	1.24 (0.87–1.76)	89 (33.3%)	0.607	1.09 (0.78–1.52)	92 (36.8%)	0.158	1.27 (0.91–1.78)
MUTYH c.972 G > C										
CC or CG	274 (67.8%)	113 (54.6%)		1.00	253 (57.3%)		1.00	147 (58.8%)		1.00
GG	130 (32.2%)	94 (45.4%)	0.001	1.75 (1.24–2.47)	114 (42.7%)	0.006	1.57 (1.14–2.16)	103 (41.2%)	0.019	1.48 (1.07–2.05)
AluYb8MUTYH										
A/A	151 (37.4%)	53 (25.6%)		1.00	71 (26.6%)		1.00	74 (29.6%)		1.00
A/P or *P/P*	253 (62.6%)	154 (74.4%)	0.003	1.73 (1.20–2.52)	196 (73.4%)	0.004	1.65 (1.18–2.31)	176 (70.4%)	0.042	1.42 (1.01–1.99)

Note: CI: confidence interval; OR: odds ratio. *P* value for comparison using χ^2-test and trend test assessing correlation with HD risk among subgroup of primary glomerulonephritis[a], anemia[b], and hypertension[c], compared with the healthy controls.

is 10 to 20 times higher than similarly aged individuals from the general population [22]. Maintenance hemodialysis (HD) is an efficient way to treat ESRD, and its use is increasing due to the epidemic of ESRD. New epidemiological studies show that China is also anticipating an increasing burden from ESRD and HD in the near future, although it used to be severely underestimated. It was reported that the number of patients with chronic kidney disease was 119.5 million in China [23], and the annual incidence of HD was estimated to be as high as 36.1 per million population (pmp) [24].

In this study, we investigated polymorphisms of base excision repair (BER) genes in a case-control Chinese population and demonstrated that individual and combined BER variations, mainly *MUTYH* polymorphisms, might increase the risk for ESRD. The underlying mechanical linkage might be an increase in 8-OHdG levels in leukocyte DNA, which was confirmed to be genetically determined. Oxidative DNA damage is unavoidable and is continuously generated by oxidative byproducts of normal cellular metabolism. The BER pathway is a critical process for genomic maintenance, as highlighted by the severe phenotypes seen in cells and animals deficient in BER function. *MUTYH* and *OGG1* double knockout cells are more sensitive to oxidants, and the double knockout resulted in a reduction of S phase and an increase in G2/M phase than wildtype cells, suggesting multiple roles of *MUTYH* and *OGG1* in the maintenance of genome stability [25].

The effectiveness of DNA repair is subject to modulation by gene polymorphism. In the hemodialysis population, *OGG1* c.977C > G, *MUTYH* c.972G > C, and *AluYb8MUTYH* showed significant effects on 8-OHdG levels in peripheral leukocytes, both individually and in combination. Our previous study illustrated that leukocyte 8-OHdG levels are variable among the Chinese population, regardless of *AluYb8MUTYH* variations [12, 13]. In this study, we confirmed a relationship among ESRD patients. Patients carrying the *OGG1* c.977 GG or CG genotypes had significantly higher 8-OHdG levels than those with the *OGG1* c.977CC genotype. Similarly, Kohno et al. previously reported that 326Ser-containing (c.977CC) OGG1 has a seven-fold higher activity for repairing 8-oxoguanine than 326Cys-containing (c.977GG) OGG1 [26]. In a background of the *MUTYH* c.972GG genotype, *OGG1* c.977C > G still showed a significant increase in 8-OHdG levels in peripheral leukocytes. The same results were detected for the *MUTYH* c. 972C > G combined with the *AluYb8MUTYH P* allele. However, the patients carrying *MUTYH* c.972GG had significantly higher 8-OHdG levels than the patients carrying *MUTYH* c.972CC. In contrast, Ali et al. reported that the glycosylase and DNA-binding activity was partially impaired in the *MUTYH* c.972CC genotype [27], whereas Shinmura and Yokota showed the same activity levels despite variation in the *MUTYH* c.972 G > C polymorphism [28]. Therefore, the genetic variations in the BER pathway may be enough to maintain 8-OHdG levels in nuclear DNA, although the underlying mechanisms are not extensively studied or understood.

Intriguingly, the genotype frequencies of *MUTYH* c.972GG or *AluYb8MUTYH* carriers (*A*/*P* or *P*/*P*) in the HD patients were markedly higher than those in the controls. The combined analysis showed that the risk of HD was further increased among the individuals carrying both the *MUTYH* c.972GG and *OGG1* c.977GG genotypes as well as those with both the *AluYb8MUTYH* (*A*/*P* or *P*/*P*) and *MUTYH* c.972GG genotypes. The findings from Trabulus et al. [17] confirmed the association between DNA repair gene polymorphisms and ESRD development in Turkish population. And the combined effect of DNA repair variants added to such association, which was similarly illustrated in the present study.

It has been noted that the disease profile of ESRD is different in China from Western countries. Zuo and Wang showed that the glomerulonephritis remained the leading cause and accounted for nearly 50% of cases [24]. In this study, 61.4% of HD cases were the result of primary glomerulonephritis (GN). Additionally, the *MUTYH GG* genotype also significantly increased the risk for ESRD from GN (OR = 1.75). This association remained in persons with the *AluYb8MUTYH P* allele, which increased the risk for ESRD from GN by a factor of 1.73. Thus, *MUTYH* c.972GG or *AluYb8MUTYH* could be the novel genetic risk factor for ESRD, and screening for these genetic variants or combined analysis may have predictive value in assessing potential risk in China.

Based on the correlation between BER polymorphisms and 8-OHdG levels, patients with different BER genetic polymorphisms were found to be at increased risk of cumulative oxidative DNA damage. Thus, we proposed that the relationship between genetic factors and ESRD and the effect of increased 8-OHdG levels underly this process. Increasing evidence has shown that the accumulation of 8-OHdG in DNA could increase the risk of DNA mutations and cancer development [29, 30]. We have also demonstrated that increased DNA oxidation might contribute to age-related diseases [12]. 8-OHdG levels in leukocyte DNA of HD patients are significantly higher than healthy controls, which has been confirmed by other groups [31, 32]. However, to date, there has been no direct evidence demonstrating a cause-and-effect relationship between oxidative DNA damage and the development of GN and ESRD. Our study using genetic analysis supports such a relationship, but further studies are needed to elucidate the pathologic significance of oxidative DNA damage among people with respect to ESRD development.

Anemia and hypertension are the most frequent complications of ESRD and related to the increased mortality rates [33, 34]. Increased DNA damage is responsible for depressed production of erythropoietin (EPO), hypertension formation, and cardiovascular disease (CVD) [35, 36]. Observational studies have revealed a strong association between the severity of anemia and the risk of morbidity and mortality from cardiovascular disease and other causes in HD patients [19, 37]; hypertension is also likely to be a major contributing factor to these events [38]. In this study, we illustrated that mutations in BER genes were tightly linked with the complications of anemia or hypertension among HD patients. As cardiovascular events are the primary cause of death in HD patients, these data suggest that oxidative

DNA damage might be involved in the risk for complications and long-term outcomes.

However, not all relationships regarding BER polymorphisms in the present study can be explained thoroughly. It was demonstrated that the BER polymorphisms were related to the disability to repair oxidative DNA and then accumulation of the levels of 8-OHdG in leukocyte DNA. The high levels of 8-OHdG, therefore, contribute to the development of ESRD. The connection between genetic variations, oxidative DNA damage, and disease condition were not consistent. Take *OGG1* c.977C > G for instance, the *GG* and/or *CG* variations significantly increased the 8-OHdG levels, which predicts a high risk for oxidative DNA damage. But the *OGG1* c.977C > G polymorphism did not appear to be related to ESRD among the investigated Chinese population. Tarng et al. also showed similar results among patients undergoing HD, but did not provide a detailed interpretation [31].

In summary, our study showed that the polymorphisms in BER system, including *MUTYH* c.972GG and *AluYb8MUTYH,* increased the risk for ESRD development in China, especially their combined effect with *OGG1* c.977GG. It suggests that oxidative DNA damage might be one common risk factor for related renal diseases, and the genes in BER pathway may be involved in the progress of renal function deterioration and complications. Those homozygous or heterozygous for BER polymorphisms might be candidate genetic factors for ESRD development. Screening those polymorphisms would be helpful for preventing progression of chronic kidney disease and improving the patients' long-term outcomes of hemodialysis.

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Review Article

Oxidative Stress: A Pathogenic Mechanism for Niemann-Pick Type C Disease

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Niemann-Pick type C (NPC) disease is a neurovisceral atypical lipid storage disorder involving the accumulation of cholesterol and other lipids in the late endocytic pathway. The pathogenic mechanism that links the accumulation of intracellular cholesterol with cell death in NPC disease in both the CNS and the liver is currently unknown. Oxidative stress has been observed in the livers and brains of NPC mice and in different NPC cellular models. Moreover, there is evidence of an elevation of oxidative stress markers in the serumof NPC patients. Recent evidence strongly suggests that mitochondrial dysfunction plays an important role in NPC pathogenesis and that mitochondria could be a significant source of oxidative stress in this disease. In this context, the accumulation of vitamin E in the late endosomal/lysosomal compartments in NPC could lead to a potential decrease of its bioavailability and could be another possible cause of oxidative damage. Another possible source of reactive species in NPC is the diminished activity of different antioxidant enzymes. Moreover, because NPC is mainly caused by the accumulation of free cholesterol, oxidized cholesterol derivatives produced by oxidative stress may contribute to the pathogenesis of the disease.

1. Introduction

Niemann-Pick type C disease (NPC) is a neurovisceral atypical lipid storage disorder that is mainly characterized by unesterified cholesterol accumulation in late endosomal/lysosomal (LE/Lys) compartments [1]. Several other kinds of lipids, such as lactosylceramide, glucosylceramide and GM2 and GM3 gangliosides, also accumulate in these compartments [2–5]. NPC is a fatal autosomal recessive disease that is caused by mutations in the *Npc1* or *Npc2* genes [6]. The *Npc1* gene encodes a 1278-amino-acid lysosomal transmembrane protein with 13 transmembrane domains that have homology to the sterol-sensing domains (SSDs) of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, SREBP cleavage-activating protein (SCAP), and Patched [7, 8]. NPC1 protein localizes to the Rab7-positive and mannose-6-P-receptor-negative late endosomal compartment [9–11]. The *Npc2* gene encodes a soluble lysosomal protein that binds cholesterol [12] with a 1 : 1 stoichiometry and submicromolar affinity [13, 14]. Both proteins are involved in cholesterol trafficking from lysosomes. The current model for NPC1- and NPC2-mediated cholesterol efflux is as follows: after lysosomal hydrolysis of LDL-cholesteryl esters, cholesterol binds NPC2, which transfers it to NPC1 [15]; subsequently, NPC1 mediates the exit of cholesterol from lysosomes [16]. Mutations in the *Npc1* gene account for approximately 95% of NPC cases, whereas mutations in the *Npc2* gene explain the remaining 5% [17].

The disease is often diagnosed in early childhood, with patients typically displaying cerebellar ataxia, speaking and swallowing difficulty, and progressive dementia [1, 17]. The main neurological symptom of this disease is vertical

supranuclear ophthalmoplegia, and its other prominent neurologic features include cataplexy, dysarthria, dysphagia, dystonia, and seizures [18, 19]. These symptoms are associated with damage to the central nervous system (CNS), especially in the cerebellum, where extensive and progressive neuronal death is observed [20].

Although neuronal damage is a major feature of NPC, most patients present considerable damage in the liver [2]. Indeed, NPC disease is recognized as a relatively common cause of liver disease in early life [21]. Approximately half of NPC patients suffer from liver disease, and NPC may be the most common metabolic disorder that is responsible for neonatal cholestasis [17].

The pathogenic mechanism linking the accumulation of intracellular cholesterol with cell death in NPC disease is currently unknown. However, increasing evidence indicating the presence of oxidative damage in NPC neurons and data connecting oxidative damage with fibrosis and apoptosis in the liver support the possibility that oxidative damage may induce these pathways in NPC disease. The question that remains unsolved is how oxidative damage is induced.

2. Cell Death and Oxidative Damage in NPC

The CNS is especially sensitive to oxidative stress damage [22]. This sensitivity can be explained by several features of the CNS: the high concentration of polyunsaturated fatty acids that are susceptible to lipid peroxidation, the relatively large amounts of oxygen consumed for energy production, and the fewer antioxidant defenses available to the CNS compared with other organs. In this sense, neurons are particularly vulnerable to oxidative stress because they have low levels of reduced glutathione (GSH) [23]. Oxidative stress has been observed in different NPC cellular models [24] as well as in the brains of NPC mice [25]; however, the functional relevance of oxidative stress to the disease process has not yet been established. In this context, we have reported increased levels of nitrotyrosinilated (N-Tyr) proteins in the cerebella of NPC mice by western blot analysis and an accumulation of N-Tyr-positive cells by immunofluorescence [26]. Furthermore, Smith et al. detected positive staining for N-Tyr in the thalami of NPC mice [25]. We have also found that, in a neuronal model of the disease, treatment with an antioxidant compound diminished c-Abl kinase activation and prevented cellular death and apoptosis [26]. This observation is particularly relevant because oxidative stress is a potent activator of the c-Abl/p73 proapoptotic pathway [27]. In fact, c-Abl/p73 activation kinetics correlate with the kinetics of the appearance of oxidative-stress markers in rat hippocampal neurons exposed to U18666A (U18), an inducer of cholesterol accumulation in lysosomes and a drug that has been widely used to induce the NPC phenotype in different cell types [28–30]. Highlighting the role of oxidative stress in NPC, we have shown that treatment with the antioxidant N-Acetyl Cysteine (NAC) prevented c-Abl/p73 activation and apoptosis in NPC-like neurons, suggesting that oxidative stress is the main upstream stimulus activating the c-Abl/p73 pathway in NPC neurons [26].

Livers of NPC mouse models present apoptosis, inflammation, and fibrosis. There is also functional damage, as evidenced by the large increases in the levels of liver disease markers such as plasmatic alanine and aspartate aminotransferases in NPC mice [31–33]. In support of the participation of oxidative stress in NPC mouse livers, we have recently found an increase in oxidative stress markers, such as protein carbonyls and oxidative-stress related genes, and a decrease in protective species, such as reduced glutathione [34].

Moreover, NPC patients present decreased antioxidant capacity (expressed as Trolox equivalents) and reduced Coenzyme Q10 in serum, which indicates a decrease in antioxidant defenses [35]. In addition, an increase in cholesterol oxidation products was detected in the plasma of NPC patients [36]. Additionally, microarray studies have shown that human NPC fibroblasts exhibit an activation of the apoptotic cascade and induction of genes related to oxidative stress [37, 38]. Oxidative damage is present in animal and cellular NPC models and in NPC patients; therefore, it may have a relevant role in the pathogenesis and progression of the disease.

3. Increase in Mitochondrial Cholesterol and Dysfunction in NPC Disease

One possible element associated with oxidative damage in NPC tissues could be mitochondrial dysfunction. In fact, mitochondrial dysfunction appears to be a key element in many neurodegenerative diseases and pathologies associated with liver and cardiac damage as a significant source of oxidative stress [39–44].

However, the role of mitochondrial dysfunction in the NPC disease phenotype remains undefined. Recent studies suggest that mitochondrial dysfunction and subsequent ATP deficiency may be responsible for the neuronal impairment in NPC disease [45]. These studies have demonstrated that NPC mouse brains contained smaller and more rounded mitochondria, with translucent matrices and irregular cisternae. Moreover, the levels of ATP in the brain, muscle, and livers of NPC mice are significantly decreased compared with those in WT mice [45]. Interestingly, several groups have shown that the mitochondria of NPC neurons and hepatocytes have a higher cholesterol content [45–48], and increased cholesterol has also been shown in the mitochondria of HeLa cells treated with U18 [49]. These data should be interpreted with caution, as it is difficult to obtain pure mitochondria uncontaminated by lysosomes because of the interaction between these organelles as a result of the increased autophagy in NPC cells [50, 51]. However, treatment with the cholesterol chelator cyclodextrin restores ATP synthesis and mitochondrial function [45], supporting the idea that the increase in mitochondrial cholesterol causes dysfunction *per se*. Therefore, a potential hypothesis is that the increase in mitochondrial cholesterol content and dysfunction may contribute to this damage (Figure 1).

Mitochondrial glutathione (mGSH) is the main line of defense against the reactive oxygen species (ROS) generated physiologically through the activity of the electron transport

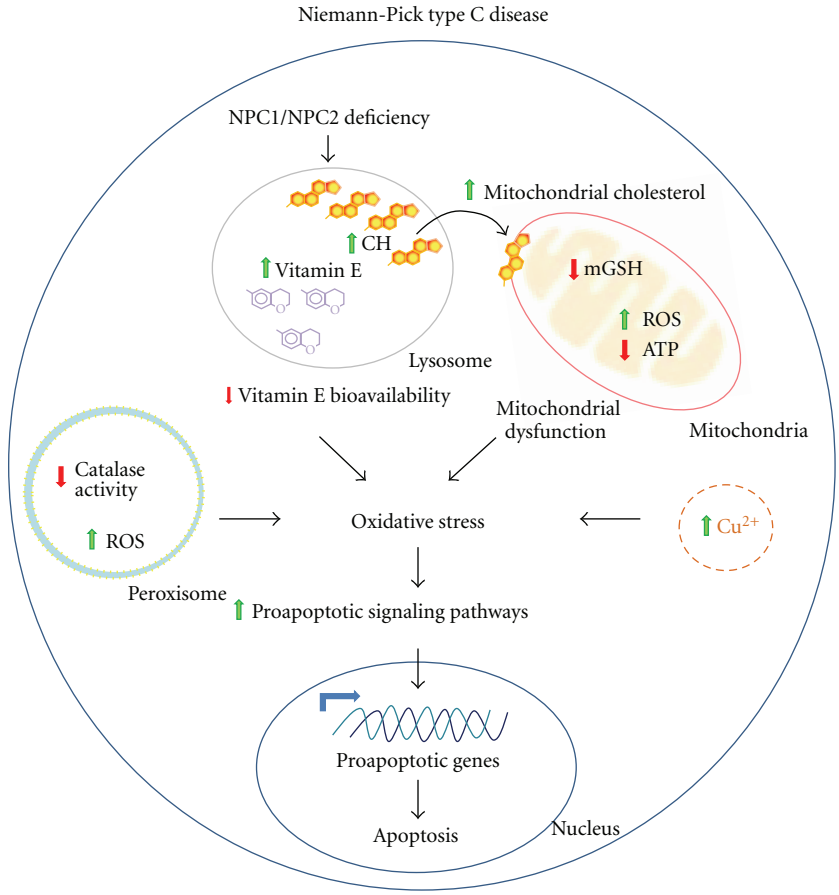

Figure 1: Oxidative stress as a pathogenic mechanism in Niemann-Pick C disease. NPC disease arises from deficiencies in one of two lysosomal proteins, NPC1 and NPC2, which are involved in the proper export of free cholesterol (CH) from lysosomes to different cellular compartments. Therefore, in NPC disease, free cholesterol accumulates inside the lysosomes and intracellular cholesterol transport is impaired. Remarkably, vitamin E also accumulates inside the lysosomes, possibly diminishing its bioavailability and decreasing the antioxidant capacity of the cell. Additionally, mitochondrial cholesterol is also increased, leading to a decrease in mGSH levels and diminishing mitochondria antioxidant capacity. In this scenario, there is an increase in mitochondrial ROS production and a decrease in ATP production. These two phenomena are well-known features of mitochondrial dysfunction. Furthermore, decreases of catalase activity and increases in ROS production inside the peroxisomes have been reported. In summary, in NPC disease, alterations in several different organelles, including lysosomes, mitocondria, and peroxisomes, along with disturbances in copper (Cu) transport, trigger oxidative stress damage, activating proapoptotic pathways and proapoptotic gene expression and inducing apoptotic cell death. Symbols: green up-arrows indicate increase or activation; red down-arrows indicate decrease or inhibition; bold black arrows represent multistep pathways; angled blue arrows indicate gene transcription activation.

chain in the mitochondria and is therefore a key player in the maintenance of the appropriate mitochondrial redox environment, preventing mitochondrial dysfunction, and cell death. We have detected reduced GSH content in the livers of NPC mice [34], and Marí et al. reported a specific decrease of mGSH in NPC hepatocytes without changes in cytosolic GSH [46]. Interestingly, the increase in mitochondrial cholesterol seems to have a direct effect on the transport of GSH to the mitochondria, because the activity of its transporter depends on membrane fluidity [52, 53]. A decrease of membrane fluidity in mitochondria or an increased cholesterol : phospholipid molar ratio, as is observed in rat liver mitochondria after chronic alcohol intake, results in an impairment of mGSH transport through the 2-oxoglutarate carrier and, therefore, in a decrease in mGSH levels [54]. Supporting a connection between mitochondrial cholesterol content and reduced mGSH levels, evidence suggests that the hepatocytes of ob/ob mice or rats fed high-cholesterol diets have reduced mGSH levels and increased mitochondrial cholesterol content. Furthermore, hepatocytes from these mice and those obtained from NPC mice have decreased GSH content and increased sensitivity to TNF-α cell death signaling [46].

In summary, regarding the role of mitochondrial dysfunction in NPC disease, it has been observed in NPC models that important mitochondrial characteristics such as ATP synthesis, mitochondrial morphology, and the input of GSH to the mitochondrial matrix are altered. These alterations are correlated with increased mitochondrial cholesterol content and together result in a reduction in energy production,

increased ROS production, and increased energy demand in NPC cells.

4. Peroxisomes Alterations in NPC Disease

Another relevant source of ROS are the peroxisomes [55]. In fact, peroxisomal catalase is one of the most important antioxidant enzymes that regulate intracellular H_2O_2 levels. Interestingly, different studies have shown early alterations of several peroxisomal enzymatic activities, such as catalase, and β-oxidation enzymes in the livers and cerebella of NPC mice (Figure 1) [56]. There is also one clinical case report of defective peroxisomal β-oxidation in an 18-month-old NPC patient [57]. Moreover, treatment of NPC mice with the peroxisomal inducers perfluorooctanoic acid or clofibrate rescued peroxisomal and lysosomal enzyme activities and decreased cholesterol content [58]. Furthermore, several groups have demonstrated that the activity and protein levels of catalase are regulated in a biphasic fashion by c-Abl kinase. At low H_2O_2 levels, catalase interacts with c-Abl, which stabilizes catalase by phosphorylating it at Tyr residues. Meanwhile, at high H_2O_2 levels or after sustained proapoptotic stimulus, c-Abl translocates to the nucleus to perform its proapoptotic function without phosphorylating catalase, which in turn is degraded [59–61]. Although it is still unknown where the interaction between intraperoxisomal catalase and c-Abl (which is located in cytosol, endoplasmic reticulum and the nucleus) occurs, this regulation has been demonstrated in several cell types, including fibroblasts and 293 cells [61]. As was mentioned above, we have demonstrated that in NPC neurons, the c-Abl kinase is activated and induces apoptosis [26, 62]. These antecedents suggest that reduced catalase activity in NPC cells could be related to alterations in c-Abl regulation mechanisms, contributing to the oxidative damage observed in the disease.

5. Intracellular Accumulation of Vitamin E in NPC Disease

Intracellular accumulation of vitamin E and decreased bioavailability of this antioxidant has emerged as one of the possible causes of oxidative damage in NPC. Vitamin E, and in particular α-tocopherol, its most important biological derivative, is one of the most relevant antioxidant defense molecules in addition to GSH. Both react directly with free radicals to form inactive, nonradical products [63–67].

Data from the literature and results from our lab show increased levels of α-tocopherol in the cerebella and a tendency to increase in the livers of NPC mice, and α-tocopherol accumulation was observed in several NPC *in vitro* models in a filipin-positive compartment (Figure 1) [68, 69]. Interestingly, one of the most damaged regions in NPC disease, the cerebellum, contains high levels of vitamin E [70]. Furthermore, mutations in the gene coding for the α-tocopherol transporter protein (α-Ttp) result in a neurologic syndrome of spinocerebellar ataxia called *Ataxia with Vitamin E Deficiency* or AVED. This condition is characterized by progressive ataxia, dysarthria, sensory loss, and severe damage of Purkinje cells [70, 71], sharing some symptoms with NPC patients. In addition, vitamin E supplementation has been demonstrated to protect against age-related deficits in Purkinje cell-β adrenergic receptor function and ethanol-induced Purkinje cell loss in rats [72, 73]. Moreover, interestingly, NPC1-like 1 (NPC1L1), which is the closest NPC1 homologue, mediates α-tocopherol transport in rat intestines [74].

Collectively, these data suggest that vitamin E transport through lysosomes could be mediated by NPC proteins and that it might therefore be altered in NPC cells. Because vitamin E is essential for health and increasing evidence suggest that antioxidants have a protective role in oxidative damage-associated diseases, studies on the accumulation and bioavailability of vitamin E in NPC cells are relevant for understanding the pathophysiology of this disease and the possible benefits of vitamin E therapy.

Interestingly, in addition to its antioxidant properties, α-tocopherol also induces signaling in cells [75]. It has been shown that α-tocopherol activates phosphatase 2A (PP2A), causing dephosphorylation and diminishing PKC alpha activity in various types of cells [75]. In addition, α-tocopherol inhibits TNF-α-induced ERK1/2 and p38 MAPKs activation [76]. These results suggest an anti-inflammatory action of α-tocopherol in addition to its antioxidant properties.

Our hypothesis is that vitamin E is trapped in LE/Lys and that it has a decreased bioavailability in NPC cells (Figure 1). However, we cannot disregard a different scenario in which the excess vitamin E compounds acting as prooxidants in NPC cells. In fact, α-TOH can play diverse roles in lipoprotein oxidation, displaying neutral, anti-, or, indeed, prooxidant activities under various conditions [77]. For example, in the presence of determinant Cu^{2+}/LDL ratios, α-TOH acts as a mediator of LDL lipid peroxidation [78]. Therefore, an alternative hypothesis is that the lysosomal buildup of vitamin E found in NPC cells could actively contribute to the pathology, acting as a pro-oxidant molecule and increasing the levels of toxic cholesterol oxidation products.

It is therefore important to mention that a previous *in vivo* study [79] showed that treatment with vitamin E exerts a small but significant beneficial effect on locomotor performance in NPC mice. It may be assumed that the effects of this treatment were small because part of the vitamin E was trapped in the lysosomes and was not available as cell antioxidants, making it less likely that the small protective effect is due to its behavior as a pro-oxidant molecule. Furthermore, another study with vitamin C failed to obtain a significant benefit in NPC mice [25]; this failure might be related to the lack of neuroprotective properties of vitamin C in neurodegenerative diseases in which oxidative stress plays a key role and vitamin E serves as a neuroprotectant [80]. On the other hand, there is evidence for other potential NPC treatments that can be neuroprotective. Early treatment with the neurosteroid allopregnanolone also improved neurological symptoms and survival in NPC mice by correcting the neurosteroidogenic abnormalities [81]. Interestingly, allopregnanolone was demonstrated to

work as a potent antioxidant in *in vitro* NPC models [82]. Treating NPC mice *in vivo* with curcumin, a potent activator of the antioxidant Nrf2 pathway [83], also improves the neurological symptoms and survival of NPC mice [5]. All these studies indicate a relevant role for oxidative stress in NPC-related neurodegeneration.

6. Glycosphingolipid Accumulation in NPC Disease

It is important to note that in NPC disease, there is not only cholesterol accumulation in the LE/Lys compartment but also an increased accumulation of other kinds of lipids. The improvement observed in patients treated with miglustat (Zavesca; Actelion Pharmaceuticals Ltd., Allschwil, Switzerland) can be attributed to its capacity to decrease the levels of glycosphingolipids. The accumulation of glycosphingolipids leads to defective intracellular calcium signaling, as has been demonstrated in several gangliosidoses [84]. Defective intracellular calcium could be detrimental for the cell because calcium plays vital roles in regulating a variety of cellular events, with impaired calcium homeostasis leading to endoplasmic reticulum (ER) stress, oxidative stress, and cell death. Moreover, Platt's group has demonstrated that sphingosine storage, an early event in NPC LE/Lys, causes a unique defect, inhibiting the filling of the LE/Lys calcium store [5]. Indeed, treatment of NPC1 CHO cells with thapsigargin elevated cytosolic calcium and corrected the NPC cellular phenotype, reducing glycosphingolipid and cholesterol accumulation. Additionally, the chelation of LE/Lys calcium content in healthy cells induces a set of cellular phenotypes identical to NPC, including defective endocytic transport and the subsequent storage of cholesterol, glycosphingolipids, and sphingomyelin [5, 85].

The depletion of glycosphingolipids by miglustat treatment reduces pathological lipid storage, improves endosomal uptake, and normalizes lipid trafficking in peripheral blood B lymphocytes [86]; however, the incomplete success of miglustat treatment in patients may be because it addresses only one aspect of the pathological cascade of NPC disease. Fu and colleagues [87] showed that miglustat therapy does not significantly improve oxidative stress in NPC patients. This result suggests that oxidative stress is an independent pathological process and that combination therapies that include an antioxidant may have an additional benefit in NPC patients.

7. Other Sources of Reactive Species in Oxidative Damage in NPC

Another possible source of reactive species in NPC is the diminished activity of different antioxidant enzymes. For example, catalase is one of the most important antioxidant enzymes participating in the regulation of intracellular H_2O_2, and, as mentioned previously, its levels are decreased in the livers and brains of NPC mice [56]. In addition, another antioxidant enzyme located in the cytosol and mitochondria, glutathione peroxidase 1, or GPx1, is regulated in the same way by the proapoptotic kinase c-Abl [61, 88], which is activated in NPC neurons. The activity of GPx1 is thought to be relevant to antioxidant defenses at the neuronal level, and $GPx1^{-/-}$ mice showed greater apoptosis-related sensitivity and damage in the brain when faced with extreme oxidative damage, such as that produced by ischemia-reperfusion [89].

Oxygen species are not the only inducers of oxidative damage to biomolecules. There is evidence for nitric-oxide- (NO-) mediated damage in NPC. Peroxynitrite binds a nitro group to tyrosines, inducing protein nitrotyrosination, which negates the physiological function of the proteins [90]. Interestingly, NO levels are elevated in neural stem cells (NSCs) from NPC mice and NSC self-renewal is decreased [91], suggesting that oxidative stress damage could be relevant in NPC neurodegeneration, even at the early stages of development [92]. Moreover, microarray analyses of human NPC fibroblasts have revealed an increase in NO synthase mRNA expression [37] and an increase in N-Tyr staining has been reported in fibroblasts [93].

Considering other additional sources for oxidative stress, we previously reported copper accumulation (Figure 1) in the liver [34] and plasma, along with a decrease of copper excretion into the bile of $Npc1^{-/-}$ (NPC) mice (unpublished results). These data are in agreement with previous reports of copper increase and ceruloplasmin alterations in a cellular NPC model [94, 95]. Copper is an important micronutrient that plays an essential role in human physiology [96]. Increases in copper, iron, or zinc levels have been described as risk factors for oxidative stress damage in neurodegenerative pathologies, such as amyotrophic lateral sclerosis, Alzheimer's Disease, and Parkinson's Disease [97]. Furthermore, mutations in copper-binding proteins have been linked to those devastating disorders [98] and to Wilson disease and Menkes disease [99, 100]. Therefore, appropriate levels of copper are essential to avoid cellular damage by oxidative stress due to the rapid oxidation of copper, which causes damage to biomolecules and generates ROS, leading to cell death [101]. The liver plays a major role in this delicate maintenance of copper homeostasis, because most of the newly absorbed copper enters the liver after absorption from dietary sources in the small intestine. Additionally, the liver regulates the distribution of copper through its release into the plasma when bound to ceruloplasmin or by its excretion via bile [102]. Moreover, the expression of genes involved in metal homeostasis and transport, including iron, copper, and zinc, was reported to be altered in NPC fibroblast microarray studies [37, 38], suggesting alterations in metal levels in NPC disease.

As NPC is mainly caused by the accumulation of free cholesterol, it can be expected that oxidized cholesterol derivatives are produced in the presence of oxidative stress. In fact, an increase in cellular cholesterol oxidation products, such as 7-ketocholesterol (7-KC), 7β-hydroxycholesterol (7β-HC), and cholestane-3β,5α,6β-triol (3β,5α,6β-triol), has been described in NPC mouse tissues, plasma, and macrophages [36, 103, 104]. They are produced by the nonenzymatic oxidation of cholesterol with ROS and several of them have been reported to be cytotoxic *in vitro* [105]. Although the

mechanism of oxysterol production in NPC is unclear, and most of the cholesterol should accumulate in lysosomes, the elevation of oxysterol levels in the disease has been consistently reproduced in mice and humans, and they have been proposed as biomarkers for NPC [36]. Moreover, 7-KC signaling has been shown to be modulated by vitamin E. It has been demonstrated that α-tocopherol impedes the cellular signaling of 7-KC, inhibiting its incorporation in lipid rafts [106]. As such, if there is less bioavailability of vitamin E in NPC cells, the proapoptotic effect of 7-KC, through its inhibition of the phosphatidylinositol 3-kinase/Akt survival pathway [107], could be further increased.

Remarkably, a study by Porter et al. [36] showed that cholesterol oxidation product levels correlated with the age of disease onset and disease severity in NPC mice and humans. Thus, cholesterol oxidation products have been proposed as blood-based biochemical markers for NPC disease that may prove informative for the diagnosis and treatment of this disorder and as outcome measures to monitor the response to therapy. These results suggest that oxidative damage is an important element in the pathology of NPC disease.

8. Putative Treatments for NPC Disease

Unfortunately, there is no fully effective treatment for this devastating and fatal disorder to date, only supportive measures for the relief of specific manifestations of the disease. Interventions to slow disease progression are the most promising therapies. Several experimental disease-specific therapies based on the molecular pathology of NPC have been tested in cell culture and animal models, including neurosteroids, cholesterol-binding agents (e.g., cyclodextrin), and molecules with antioxidant properties, such as curcumin and miglustat [108].

Among the cholesterol-binding agents, cyclodextrin seems to be promising [109], although data suggesting limited or no blood-brain barrier penetration of cyclodextrin following systemic administration have to be considered [110]. Treatment of NPC mice with cyclodextrin reduces the neurodegeneration and markedly extends the life span of NPC mice, suggesting a potential therapeutic approach for the treatment of individuals with NPC disease [111]. The mechanisms by which cyclodextrin mediates these beneficial effects are still unknown. The evidence shows that a single injection of cyclodextrin at p7 in NPC mice caused a marked increase in cholesteryl esters and the suppression of cholesterol synthesis in many organs, as well as changes in the expression of genes responsive to cholesterol levels [112], suggesting that cyclodextrin acutely reverses the lysosomal transport defect observed in NPC disease. However, although cyclodextrin has promising effects in the brain and liver, it has little or no effect at all on lung dysfunction, another important issue in NPC pathology [113]. Nonetheless, the positive data on cyclodextrin reported in model animals have encouraged its application as a potential treatment in NPC patients. In fact, last year, the European Medicines Agency (EMA) granted Hydroxypropyl-beta-cyclodextrin (HPbCD) orphan drug status and designated the compound as a potential treatment for NPC disease. In addition, the National Institutes of Health (NIH) in collaboration with the Therapeutics for Rare and Neglected Diseases Program (TRND) announced that they are developing a clinical trial utilizing cyclodextrin for NPC patients. This clinical trial is in the planning phase and is not yet officially approved by the FDA.

Regarding the use of antioxidant molecules, it has recently been reported that NPC patients showed significant decreases in the fractions of reduced coenzyme Q10 (CoQ10) [87]. This decrease in CoQ10 levels can cause changes similar to those reported in patients with deficiencies in CoQ10. CoQ10 deficiency is a rare human genetic condition that has been associated with a variety of clinical phenotypes: decreased activities of complex II + III, complex III and complex IV, reduced expression of the mitochondrial proteins involved in oxidative phosphorylation, decreased mitochondrial membrane potential, increased ROS activation of mitochondrial permeability transition (MPT), and reduced growth rates [114]. In fibroblasts from patients with CoQ deficiency, these abnormalities were partially restored by CoQ supplementation [114]. However, supplementation with CoQ10 does not correct the abnormal fraction of reduced CoQ10 found in NPC patients [87]. Although the treatment did not restore the levels of reduced CoQ10 in NPC patients, the authors did not analyze the effect of the treatment on mitochondrial function and ROS production.

Currently, there is an active clinical protocol to test the safety and efficacy of the antioxidant NAC in NPC patients at the National Institute of Health Clinical Center in the U.S.A. (http://clinicaltrials.gov/ct2/show/NCT00975689). NAC is a powerful antioxidant that acts by increasing cellular glutathione levels [115–117]. The advantage of using NAC lies in the fact that it has been approved by the FDA to treat acetaminophen poisoning or to reduce mucous stickiness in patients with cystic fibrosis. Plasma oxysterol analysis will be used as a biomarker in the treatment, because Ory's group has previously demonstrated an increase of oxysterols in the plasma of NPC mice [103]. Interestingly, NAC treatment shows beneficial effects not only in neurodegenerative diseases such as Alzheimer's but also in myocardial dysfunction [116]. In addition, we have reported a decrease of foamy cells on the livers of 7-week-old NPC mice after 2 weeks of treatment with NAC [34], and we also have shown a decrease in apoptosis in U18-treated neurons incubated with NAC [26]. Although the acute use of NAC has demonstrated clear beneficial effects [86], its chronic use for the treatment of diseases associated with oxidative damage is controversial [118], mainly because, to date, there have not been positive results reported in this clinical trial, so it is not clear if this treatment could be effective. A possible explanation for this lack of effectiveness is that the antioxidant effect of NAC results from its ability to restore the cytosolic levels of GSH, which is transported to mitochondria, where it exerts its detoxifying function. The mitochondrial transport of GSH is highly sensitive to membrane dynamics, and this transport is delayed in hepatocytes from NPC mice and is correlated with mitochondrial GSH (mGSH) depletion [46]. Therefore, NAC therapy would not be effective in NPC disease, because

although the levels of cytosolic GSH are increased, GSH transport into mitochondria remains defective.

Following this line of evidence, it has recently been shown that a curcumin derivative, J147, has the ability to prevent memory deficits in AD transgenic mice, and this effect is correlated with the reduction of markers for oxidative stress damage and inflammation [119]. Furthermore, it has previously been reported that curcumin treatment can normalize cytosolic calcium levels, prolonging the survival of NPC mice [5]. However, this explanation might not be the only one for the curcumin protective effect, as it is also an antioxidant molecule.

Another putative drug that may be useful in ameliorating NPC symptomatology is imatinib, a specific c-Abl kinase inhibitor. Imatinib treatment increased the quality of life of NPC mice by delaying body weight loss and neurological symptoms, decreasing cerebellar apoptosis and inflammation, and increasing Purkinje cell survival. However, the inhibition of c-Abl with imatinib did not affect oxidative stress levels, suggesting that c-Abl/p73 activation in NPC is downstream of oxidative stress. Nonetheless, inhibition of the c-Abl/p73 module is still an interesting therapeutic target in NPC and perhaps in other neurological disorders as well [62]. Interestingly, imatinib (Gleevec, STI571) is a U.S. Food and Drug Administration-approved drug used in patients with chronic myelogenous leukemia, in which the therapeutic target is the aberrant oncogenic fusion protein Bcr-Abl [120, 121].

Finally, it seems that the path to an effective therapy for NPC disease could be a multidrug approach that combines antioxidant and anti-inflammatory compounds with other drugs that improve lipid metabolism. In this sense, it is important to note that the first and only approved therapy for patients with NPC, miglustat [122–124], is a reversible inhibitor of glucosylceramide synthase, which catalyzes the first committed step of glycosphingolipid synthesis. Miglustat is currently approved in the European Union (EU), USA, Canada, Brazil, Australia, Turkey, Israel, Switzerland, South Korea, and New Zealand for the treatment of patients with Gaucher disease. In January 2009, the EU Commission extended miglustat's indication to include the treatment of progressive neurological manifestations in adult pediatric patients with NPC. This extension was followed by authorization in Brazil and South Korea [108, 125].

9. Concluding Remarks

NPC disease is a fatal disorder without an efficient treatment available. Several approaches have attempted to at least delay the disease progress. A better understanding of the mechanisms involved in the pathogenesis of NPC is necessary to design appropriate and effective therapeutic approaches. Increasing evidence indicates that oxidative stress damage has an important role in the pathophysiology of NPC disease. Therefore, drugs that can decrease or ameliorate oxidative stress damage and apoptosis must be used in combination with other pharmacological strategies that restore the proper metabolism and transport of lipids, especially cholesterol, in NPC patients.

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Review Article

Oxidative Stress in Alzheimer's and Parkinson's Diseases: Insights from the Yeast *Saccharomyces cerevisiae*

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Alzheimer's (AD) and Parkinson's (PD) diseases are the two most common causes of dementia in aged population. Both are protein-misfolding diseases characterized by the presence of protein deposits in the brain. Despite growing evidence suggesting that oxidative stress is critical to neuronal death, its precise role in disease etiology and progression has not yet been fully understood. Budding yeast *Saccharomyces cerevisiae* shares conserved biological processes with all eukaryotic cells, including neurons. This fact together with the possibility of simple and quick genetic manipulation highlights this organism as a valuable tool to unravel complex and fundamental mechanisms underlying neurodegeneration. In this paper, we summarize the latest knowledge on the role of oxidative stress in neurodegenerative disorders, with emphasis on AD and PD. Additionally, we provide an overview of the work undertaken to study AD and PD in yeast, focusing the use of this model to understand the effect of oxidative stress in both diseases.

1. Introduction

Misfolded proteins are typically insoluble and tend to form long linear or fibrillar aggregates known as amyloid deposits. Amyloid-like protein fibrils are a well-known pathological hallmark of age-related neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD). Alzheimer's and Parkinson's diseases are the most common forms of dementia, currently affecting 30 and 4 million people worldwide, respectively. In AD, the beta-amyloid (Aβ) peptide accumulates mainly extracellularly, whereas in PD, the α-Synuclein (α-Syn) protein accumulates, within neurons, inside the Lewy bodies (LB) and Lewy neurites (LN). Although there is a plethora of factors interfering in those pathological depositions, it is clear that oxidative stress may play a crucial role in neuronal death in neurodegenerative disorders [1–3].

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen and are produced in all aerobic cells. Oxidative stress occurs when the generation of ROS in a system exceeds that system's ability to neutralize and to eliminate them. All organisms have developed adaptive responses to oxidative stress that involve defensive enzyme and molecular chaperones—the expression of both being orchestrated by stress-responsive transcription factors—as well as antioxidant molecules [4]. Excessive production of ROS, and the consequent disruption of cellular redox balance, drives the oxidation of biological macromolecules, such as DNA, proteins, carbohydrates, and lipids, potentially leading to failure of biological functions [4].

Many ROS possess unpaired electrons and are therefore free radicals. The generation of free radicals is closely linked with the involvement of trace metals, particularly copper and irons [5, 6]. To cope with this potential hazard, the concentration of cytosolic free metals is accurately controlled through regulation of their uptake, storage, and mobilization, in order to maintain redox-active metals in normal physiological limits [7, 8]. Inside cells, "free pools" of copper and iron are avoided through their effective sequester by metal-binding proteins [5, 9]. The chelatable redox-active

iron constitutes the so-called labile iron pool (LIP), which serves as a transient source of iron [5, 9, 10]. Nevertheless, whenever cells are subjected to stress conditions, an excess of superoxide anion radical acts as an oxidant of Fe-S clusters of several enzymes, releasing "free iron." The released iron can in turn participate in Fenton type reactions, producing the highly reactive hydroxyl radical [11].

During the oxidative burst triggered during inflammatory processes, cells of the immune system produce both superoxide anion and nitric oxide (NO) free radicals. Nitric oxide is produced by the NO synthase family of enzymes. NO may directly react with its biological targets, as it is known to regulate the catalytic activity of various enzymes primarily by interacting with Fe-S clusters, oxidized copper centres, heme, and tyrosyl radicals [12]. NO also reacts with superoxide ion (O_2^-) or oxygen to form the nitrogen radical peroxynitrite ($ONOO^-$). Reactive nitrogen species (RNS) are highly reactive towards biological macromolecules and are thought to be responsible for NO-mediated cell death.

The aim of this paper is to provide an overview on the role of oxidative stress in neurodegenerative disorders, with emphasis on AD and PD. Despite the absence of a nervous system in yeast, several studies have shown that this eukaryotic unicellular organism is a suitable model system to understand the molecular mechanisms underlying neurodegenerative diseases. The knowledge from those studies is summarized herein. Finally, we discuss how yeast models have been or may be used to extend our understanding on the role of oxidative stress in AD and PD.

2. Oxidative Stress and Neurodegenerative Diseases

The human brain is responsible for approximately 20% of our body oxygen consumption and thus subjected to a high metabolically derived level of ROS [13, 14]. An increasing body of evidence suggests that oxidative stress is involved in the etiology and pathogenesis of neurological disorders. The lipid bilayer of the brain is rich in polyunsaturated fatty acids and oxygen and is therefore highly susceptible to lipid peroxidation, a complex process involving the interaction of polyunsaturated fatty acids with free radicals that results in production of reactive electrophilic aldehydes. Lipid peroxidation occurs in several neurodegenerative diseases [15]. Evidence of oxidative stress in these diseases is further supported by increased DNA (and often RNA) base oxidation products and oxidative protein damages in specific regions of the brain [4]. The destruction of cellular components can induce a diversity of cellular responses through generation of secondary reactive species and ultimately lead to cell death via apoptosis and necrosis [16–18]. Mitochondrion is the center of ROS production. About 90% of mammalian oxygen consumption is mitochondrial, making mitochondria particularly important in neurons due to their high demands for energy. This fact, together with the observation that mitochondrial perturbation occurs in multiple neurodegenerative disorders [19], suggests that neurodegenerative diseases are mitochondrial diseases.

3. Alzheimer's Disease and Oxidative Stress

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disease caused by severe neurodegeneration in the hippocampus and neocortical regions of the brain of affected individuals [20].

AD pathological hallmarks include extracellular amyloid plaques and intracellular aggregates (neurofibrillary tangles). The major component of the amyloid plaques is the amyloid peptide Aβ, which results from the proteolysis of the amyloid precursor protein (APP). APP is a ubiquitously expressed transmembrane protein exerting a critical role in neuron growth and survival [21, 22]. APP proteolysis to form the Aβ peptide involves the sequential action of aspartic protease BACE1 (β-secretase) and of γ-secretase, a multiprotein complex [23]. The length of Aβ peptide may range from 39 to 43 aminoacid residues, due to different γ-secretase cleavage sites. Aβ appears to be unfolded, under physiological conditions [24]. In amyloid plaques, the most frequent species are Aβ40 and Aβ42, the latter being the most prone to aggregation [23]. Neurofibrillary tangles are composed of hyperphosphorylated tau protein, a microtubule-binding protein thought to be involved in microtubules stabilization and in regulation of axonal transport in the brain [25].

The causes of Alzheimer's disease are not well understood, except for a small percentage of cases that are linked to familial genetic mutations [26]. Several hypotheses have been put forward with the aim to explain the cause of the sporadic form of the disease. One widely discussed of those hypotheses assumes that amyloid deposits of Aβ peptides are the causative agents of AD [27]. The "amyloid" theory is further supported by the link between mutations in the APP gene and some inherited forms of the disease [26].

Aβ toxicity is dependent on Aβ's conformational state, peptide length, and concentration [28, 29]. Moreover, it has been described that Aβ toxicity is also related to Aβ's ability to form hydrogen peroxide and free radicals [30, 31]. These findings are supported by the significant lipid peroxidation, protein oxidation, and DNA oxidation observed in AD brains [29, 32, 33]. In addition, two factors reinforce the role of oxidative stress in AD pathogenesis: pro-oxidants increase Aβ production, whereas several antioxidants, namely vitamin E, melatonin, and several free radical scavengers, can protect neurons from Aβ-induced toxicity [34].

Interestingly, the Aβ peptide is not toxic in the absence of redox metal ions, and many recent studies implicate biometals in the development or progression of Alzheimer's disease [6, 35–37]. Accordingly, sophisticated techniques have shown an overaccumulation of copper, iron, and zinc within the amyloid plaques compared with the surrounding tissues [38]. Aβ has high affinity for redox-active metals being able to reduce them and consequently lead to the formation of hydrogen peroxide and oxidized amyloid [6]. Butterfield and Bush proposed that a single methionine residue (Met35) of Aβ42 is critical for the oxidative and

neurotoxic properties of this peptide [39, 40]. Substitution of Met35 renders the Aβ peptide nonoxidative and nonneurotoxic [40]. The sulphur atom of Met35 is highly susceptible to oxidation, forming the sulfide radical $MetS^{\bullet+}$ and reducing copper(II) to its high-active low-valency form [5, 40]. The $MetS^{\bullet+}$ radical is able to undergo very fast reactions with superoxide ion, leading to the formation of methionine sulfoxide (MetO). In AD senile plaques, a significant fraction of Aβ has Met35 in the form of MetO [41].

Another well-studied source of oxidative stress in AD is mitochondria damage and its consequent functional abnormality that favors the production of ROS. Indeed, it was shown that neurons in AD exhibit a significantly higher percentage of damaged mitochondria compared to an aged-matched group [42]. Furthermore, mitochondrial dysfunction has been widely implicated in the etiology of AD, since early impairments of mitochondrial function and oxidative stress may precede Aβ overproduction and deposition [43]. Also inflammation can induce oxidative damage in AD, especially via microglia, leading to increased ROS and RNS formation and the resulting damage to lipid, proteins, and nucleic acids [44–46].

4. Parkinson's Disease and Oxidative Stress

Parkinson's disease is an age-related neurodegenerative disorder affecting the central nervous system. It is characterized by the progressive degeneration of nigrostriatal dopaminergic neurons within the *substantia nigra pars compacta*, which is the pathological process responsible for the motor symptoms attributed to PD [47]. The pathological hallmark of the disease is the presence of proteinaceous cytoplasmic inclusions designated as Lewy bodies and Lewy neurites. These are predominantly composed of the presynaptic protein α-Synuclein (α-Syn) [48] together with proteasomal and lysosomal subunits as well as molecular chaperones [49].

The ubiquitous α-Syn brain protein is implicated in both hereditary and sporadic PD. Its encoding gene, *SNCA,* was the first genetic determinant associated with the disease and, for this reason, much of the work on PD converges on α-Syn [50]. α-Syn was shown to interact with lipids and membranes, accelerating amyloid fibril formation [51], and it has been proposed to regulate the dynamics of synaptic vesicles at the synapse [52]. Indeed, α-Syn exhibits a remarkable conformational plasticity being its structure largely dependent on the surrounding environment. The monomeric α-Syn is a typical natively unfolded protein under physiological conditions [53, 54]. However, under specific conditions, such as the increase of its intracellular levels, α-Syn can adopt different conformations, including several α-helical and β-sheet species folded to different degrees in both monomeric and oligomeric states [55].

Although PD is a recognized multifactorial disease, a large body of evidence has implicated oxidative stress in the pathogenesis of PD. The conclusive connection between PD and oxidative stress is supported by the increased oxidative damage of sugars, lipids, nucleic acids, and proteins observed in *postmortem* dopaminergic neurons within the *substantia nigra pars compacta* of PD brains [6, 56, 57].

Auluck et al. proposed that the impairment of α-Syn function leads to its local accumulation, favoring the formation of toxic oligomeric species that interfere with ER-to-Golgi trafficking, mitochondria turnover—through the abrogation of mitophagy—and generate oxidative stress. Moreover, the abnormal interaction of α-Syn with membranes has been implicated in the cytoplasmic retention of catecholaminergic neurotransmitters yielding cytotoxicity through the generation of dopamine adducts and ROS [52]. This effect is potentiated in the presence of iron-rich environments, as it is the case of Lewis bodies in the neurons decorating the *substantia nigra* of PD patients [58–61]. Indeed, it is known that dopamine is able to coordinate iron and regenerate Fe^{2+}, possibly providing an equally important source of hydroxyl radical production [62].

Mitochondria have been claimed as dominant sites for oxidative stress-driven initiation and propagation in PD. The direct implication of this organelle in PD was first suggested by the use of the mitochondrial complex I (CI) inhibitor MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [63, 64]. This chemical mimics human PD in animal models and is associated with development of Parkinsonism in humans subjected to accidental exposure [65]. Further corroborating the relevance of mitochondria in PD, it was shown that the well-known CI inhibitor rotenone induces death of dopaminergic cells [66–69]. In addition, depletion of the antioxidant peptide glutathione (GSH) in PD cells, which may be caused by a decrease in its synthesis and recycling [70], has been associated with a decrease of mitochondrial CI activity, resulting in mitochondrial dysfunction [71, 72]. Moreover, defective mitochondrial CI function is observed in the *substantia nigra* of PD brains [73, 74]. Recent studies have also demonstrated that α-Syn monomers and oligomers associate with the inner mitochondrial membrane where they can physically associate with CI, thereby interfering with the mitochondrial function and increasing free radicals production [72, 75]. Further highlighting the relevance of mitochondrial dysfunction and oxidative stress in the pathological process of the disease, several genes associated with familial cases of PD were shown to encode either mitochondrial proteins or mitochondria-associated proteins [6, 76]. Among the latter is DJ-1, a protein that shares structural homology with the *Escherichia coli* chaperone Hsp31 and is thought to have a protective role against oxidative stress [77]. Under conditions of oxidative stress, DJ-1 is relocated to the mitochondria, affecting the sensitivity of specific neuron populations to compounds targeting mitochondrial CI, namely, rotenone, paraquat, and MPTP [6, 78–80].

Several evidences support as well an unbalanced generation of RNS as a feature of PD pathology. First, nitrated α-Syn accumulates in LB of PD cells. Secondly, the treatment of GSH-depleted dopaminergic cells with RNS inhibitors prevents mitochondrial CI inhibition [6], indicating that RNS itself has a role in mitochondrial dysfunction and ROS generation in PD. Lastly, glial cells within the *substantia nigra* exhibit increased NO levels [81], possibly due to the accumulation of interferon-γ (IFN-γ) [82], a cytokine which was shown to promote induction of RNS in brain.

5. *S. cerevisiae* as Model Organism to Study Neurodegenerative Diseases

Budding yeast *Saccharomyces cerevisiae* has been introduced as an experimental organism in the midthirties of the 20 century. Since then, its potential as a model organism has been exploited in many areas of biology [83]. Despite lacking the physiological complexity of the mammalian nervous system, yeast was recently used in the study of neurodegenerative disorders, such as Alzheimer's and Parkinson's Diseases. This became possible due to the development of powerful yeast genetic tools as well as the high conservation of fundamental biological processes and pathways associated with neurodegeneration including protein folding, cellular trafficking, and secretion [84]. It is noteworthy that about one-fifth of yeast genes are members of orthologous gene families associated with human diseases [85]. This is an important aspect to consider when studying human diseases in yeast. If a homologue of the gene implicated in the disease is present in yeast genome, a unique opportunity to directly study its function is offered, either through its deletion or overexpression. Otherwise, if the disease-associated gene does not have a yeast counterpart, functional analysis can still be performed via heterologous expression [86, 87]. Equally important "humanized" yeasts are being used as platforms for high-throughput screenings of compounds with therapeutic potential.

6. Yeast as a Model for Studying Alzheimer's Disease

Yeast models have been extensively used to study several molecular aspects of AD, even though yeast lacks for some AD-associated genes. Studies in yeast have been mainly focused on the *in vivo* APP processing, Aβ oligomerization, and toxicity.

The usage of heterologous expression of human secretases in yeast has greatly contributed to the understanding of human APP processing. It has allowed the discovery of BACE1 inhibitors and prompted the study of the individual function of each component of the γ-secretase complex [88].

Growing evidence suggests that the oligomeric forms of the Aβ peptide, rather than amyloid fibrils, are the most toxic forms [89–92]. These findings have shifted the focus of investigation towards the earliest stages of Aβ oligomerization. As a result, the following described yeast systems were developed and are now useful tools not only in the study of Aβ-oligomerization, but also in the understanding of the molecular events triggered by aggregation as well as in the screening of potential therapeutic compounds that affect the aggregation process.

The first yeast study on Aβ oligomerization used a two-hybrid approach to analyze Aβ dimerization. Protein-protein interactions were measured by fusing the Aβ peptide to a LexA DNA-binding domain and also to a B42 transactivation domain, and then monitoring the expression of a *lacZ* reporter driven by a LexA-dependent promoter [93]. The authors showed that Aβ peptide was able to form dimers, *in vivo*, in the yeast cell nucleus.

Bagriantsev and Liebman and von der Haar et al. implemented a different yeast model system that may constitute a valuable tool to seek for agents that interfere with the initial steps of Aβ42 oligomerization [94, 95]. In this study, the ability of Aβ42 peptide to aggregate was monitored by fusing it to the middle and C-terminal domain of Sup35. The Sup35 yeast translational termination factor can undergo spontaneous conversion into a prion state, losing its function [96]. Sup35 loses the ability to aggregate when its prion-forming (N-terminal) domain is deleted. However, the insertion of Aβ peptide sequences in place of the original prion domain of Sup35 protein restores its ability to aggregate [94, 95]. Using this reporter system, it was possible to confirm *in vivo* the impact of point mutations, previously shown to inhibit Aβ42 aggregation *in vitro* [94, 97]. Furthermore, it was shown that the Hsp104 yeast chaperone, a chaperone known to rescue proteins from the aggregated state in other yeast models of neurodegenerative diseases [98, 99], appears to have a contrary function in AD, protecting Aβ-fusion protein from disaggregation and degradation [94, 95].

Oligomerization of Aβ was also the subject of a third yeast study, by the use of a reporter consisting of Aβ fragment fused to GFP [100]. The assay was based on the premise that aggregates of the fusion protein suppressed green fluorescence. The Aβ-GFP fusion was shown to cause slight but significant yeast growth reduction and to induce a heat shock response (HSR), as indicated by the cotransformation of yeast with Aβ-GFP and HSE2 element fused to a downstream *lacZ* gene. The authors put forward the hypothesis that HSR could arise from Aβ inducing ROS and/or the presence of misfolded proteins and suggested that HSR might be a target for further studies seeking for inhibitors of Aβ effects [100].

Recently, Treusch et al. engineered a yeast model for studying Aβ toxicity [101]. The overexpression of a construct harboring the Aβ42 fragment fused at the N-terminus to an endoplasmatic reticulum targeting sequence was driven by a galactose-inducible promoter. Aβ oligomers localized to secretory compartments and, like in neurons, contributed to toxicity in yeast. A screen for genetic modifiers allowed the identification of 40 genes that were able to modulate Aβ toxicity. Among those, 12 had homologues in humans, 3 being related to clathrin-mediated endocytosis, and 7 functionally associated with the cytoskeleton. Interestingly, all the former genes behaved as Aβ toxicity suppressors and had been previously shown to be or interact with validated risk factors for AD. The authors further showed that Aβ affects clathrin-mediated endocytosis and proposed that Aβ oligomers may interact with transmembranar receptors and prevent their correct destination [101].

7. Yeast as a Model for Studying Parkinson's Disease

As a common feature of sporadic and familial cases of PD, the understanding of the pathological processes associated with α-Syn has attracted special attention. In order to gain insight into α-Syn pathobiology, Outeiro and Lindquist

exploited a myriad of advantages of using *S. cerevisiae* as a model organism, by developing a powerful "humanized" yeast system. As a means to study the α-Syn dynamics *in vivo*, the authors overexpressed in yeast cells a construct harboring the wild type or the mutant versions of human *SNCA* gene fused to GFP [102]. This pioneering system faithfully reproduces several features of PD in yeast, allowing to thoroughly investigate the pathological processes involved in the disease. Three strains, designated as NonTox, InTox, and HiTox, were created to express α-Syn at different levels [52]. As it happens in complex eukaryotic models, the appearance of cytoplasmic foci, cytotoxicity, and α-Syn-decorated vesicle accumulation was shown to be dose dependent [102, 103]. Moreover, high doses of α-Syn lead to increased toxicity, accumulation of cytoplasmic lipid droplets, vesicle trafficking defects, ER stress, activation of the heat-shock response, impairment of the ubiquitin-proteasome pathway, and mitochondrial dysfunction in the HiTox strain, therefore recapitulating the pathological features displayed by PD patients whose genome encodes duplications or triplications of *SNCA* locus [52].

The α-Syn yeast model developed by Outeiro and Lindquist has been the basis of several genome wide and high-throughput analyses aimed at unveiling the intricacies of PD. The systematic screening of a galactose-inducible overexpression library in the InTox strain revealed the Rab GTPase Ypt1 (Rab1) as suppressor of α-Syn toxicity [104], reinforcing the role of α-Syn in vesicle formation and delivery. In addition, an unbiased genome-wide screen for modifiers of α-Syn toxicity was performed in the InTox strain, allowing the identification of the polyamine transporter Tpo4 [105] and highlighting the significance of polyamine pathway in PD pathogenesis. Using the ResponseNet algorithm to integrate α-Syn mRNA profiling and genome-wide genetic data, it was found that trehalose might be involved in the protection pathway against α-Syn toxicity possibly promoting misfolded protein clearance. In addition, mitochondrial dysfunction and oxidative/nitrosative stress also appeared as consequences of α-Syn overexpression in yeast [106]. Comparison of the transcriptome of HiTox and NonTox strains provides further evidence supporting the assumption that mitochondrial dysfunction and oxidative stress are associated to conditions in which α-Syn is expressed at high levels. It has also been verified that mitochondria morphology is affected and ROS is accumulated in the HiTox strain further suggesting that high levels of α-Syn elicit global mitochondrial dysfunction [107]. This may suggest that α-Syn accumulation is the origin of oxidative damage of specific neuronal cells in PD.

More recently, overexpression of α-Syn in yeast revealed that the knockout of genes encoding lipid elongases, namely, *ELO1, ELO2, and ELO3*, impairs cell growth, dramatically decreases the survival of aged cells, and leads also to ROS accumulation and aberrant protein trafficking [108]. A similar strategy, using a different plasmid to drive galactose-inducible α-Syn expression in distinct *S. cerevisiae* backgrounds, disclosed the significance of fatty acid synthase activity and intracellular redox status in the mechanisms of α-Syn toxicity [109].

α-Syn-humanized yeasts have also been exploited to search for compounds with therapeutic potential. In this context, the HiTox strain was used in a high-throughput chemical screen to identify agents capable of rescuing the robust toxicity of this strain. A class of small molecules of 1,2,3,4-tetrahydroquinolinones were identified and shown to revert the formation of α-Syn foci, to reestablish ER-to-Golgi trafficking, to ameliorate mitochondria damage, to limit ROS production, and consequently to reduce α-Syn toxicity not only in yeast but also in other more complex PD models [107].

8. Concluding Remarks

Although AD and PD have been extensively studied, the exact mechanism of disease progression or pathogenesis remains largely unknown. As outlined in this paper, several *in vivo* and *in vitro* studies point towards a role of oxidative stress in AD and PD pathogenesis. Nevertheless, whether it is a primary cause or simply a consequence of the neurodegenerative process is still an unanswered question. In addition, specifically concerning AD, there are quite a few contradictory reports regarding the role of oxidative stress in the disease. Indeed, it has been described that oxidative stress may as well lead to an increase in Aβ [14, 110], and *in vivo* studies showed a negative correlation between oxidative stress and Aβ, indicating an antioxidant role for Aβ [111].

Yeast can be a powerful tool as a means to clarify several of these issues. Within this context, yeast models of AD may in the future be used to monitor Aβ oligomerization and toxicity under an oxidative environment or in the absence of ROS (hypoxia). Interestingly, a yeast model consisting of Aβ peptide fused to GFP has been successfully used to test whether folate, an antioxidant, was able to prevent Aβ aggregation [112]. To better understand the relationship between oxidative stress and α-Syn aggregation, in the pathological processes triggering PD, it would be interesting to assess both the behavior of α-Syn in the "humanized" NonTox strain under oxidative environments and in the InTox and HiTox strains under hypoxia conditions.

Future studies combining yeast and animal models of AD and PD will certainly provide valuable insights into the role of oxidative stress in these neurodegenerative diseases.

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Review Article

Iron and Neurodegeneration: From Cellular Homeostasis to Disease

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Accumulation of iron (Fe) is often detected in the brains of people suffering from neurodegenerative diseases. High Fe concentrations have been consistently observed in Parkinson's, Alzheimer's, and Huntington's diseases; however, it is not clear whether this Fe contributes to the progression of these diseases. Other conditions, such as Friedreich's ataxia or neuroferritinopathy are associated with genetic factors that cause Fe misregulation. Consequently, excessive intracellular Fe increases oxidative stress, which leads to neuronal dysfunction and death. The characterization of the mechanisms involved in the misregulation of Fe in the brain is crucial to understand the pathology of the neurodegenerative disorders and develop new therapeutic strategies. *Saccharomyces cerevisiae*, as the best understood eukaryotic organism, has already begun to play a role in the neurological disorders; thus it could perhaps become a valuable tool also to study the metalloneurobiology.

1. Iron Neurotoxicity

Iron (Fe) is the most important element for almost all types of cells, including brain cells. It is an essential cofactor for many proteins involved in the normal function of neuronal tissues, such as the non-heme Fe enzyme tyrosine hydroxylase required for the synthesis of myelin and the neurotransmitters dopamine, norepinephrine, and serotonin [1]. In a normal brain, Fe appears widely distributed by region and cell-type and it accumulates progressively during aging and neurodegenerative processes [2]. Fe is an originator of reactive oxygen species (ROS). Ferric iron (Fe^{3+}) can be reduced to ferrous iron (Fe^{2+}) by the superoxide radical ($O_2^{\bullet -}$) ($Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$). Fe^{2+} can also react with H_2O_2 generating the highly reactive hydroxyl free radical ($^{\bullet}OH$) ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$, Fenton reaction) [3]. The combination of these reactions results in the so-called Haber-Weiss reaction ($O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$), which together with dopamine oxidation can trigger neurotoxicity [4]. Therefore, the control of Fe homeostasis is essential to keep a healthy brain.

1.1. Iron Homeostasis. In mammals, the regulatory mechanism for Fe homeostasis is mediated by the iron-regulatory proteins IRP1 and IRP2, which posttranscriptionally modulate the expression of specific mRNAs in response to intracellular Fe [5, 6], mainly transferrin (Tf) and ferritin. Tf is an Fe-binding blood plasma glycoprotein that controls the level of free Fe, and ferritin is an Fe storage protein composed of H and L subunits that assemble to form a hollow sphere in which ferric iron (Fe^{3+}) precipitates are sequestered [7]. The ferritin subunits have different functions. The H chains are involved in the rapid oxidation of Fe^{2+} to Fe^{3+}, and the L chains function in the nucleation of Fe^{3+} within the protein shell. While the L-rich ferritins are associated with iron storage, the H-chain ferritins are associated with responses to stress [8].

In Fe-depleted cells, IRPs bind to the IREs cis-elements in the 5′UTR (untranslated region) of ferritin and in the 3′UTR of TfR1. By binding to the IRE in the 5′UTR, of ferritin, IRPs prevent translation, whereas by binding to IRE in the 3′UTR of TfR1, the IRPs protect the transcript from degradation [9]. In Fe-replete cells, IRPs do not bind to IREs, and

ferritin and other transcripts are freely translated, whereas TfR1 undergoes cleavage and subsequently degradation [6, 10, 11].

Although Fe metabolism in mammals is mainly regulated at the level of absorption, changes in gene expression in response to Fe overload have been observed in a variety of eukaryotes from yeast to mammals [12, 13].

Ferroportin (Fpn), the basolateral membrane Fe exporter, is the only Fe exporter to date identified in mammals [14–16]. Fpn mediates the release of the Fe in conjunction with ceruloplasmin (Cp), which must oxidize the Fe^{2+} transported by Fpn to Fe^{3+} before release into the extracellular medium [17]. Fpn expression has also been detected on the blood-brain barrier (BBB) endothelial cells, neurons, oligodendrocytes, astrocytes, the choroid plexus and ependymal cells. Since Cp is essential for stabilization of Fpn, under conditions of Cp deficiency or malfunction Fpn is not expressed, which results in a decreased Fe efflux potentiating cellular Fe overload [18]. These observations indicate that Cp plays a major role in maintaining Fe homeostasis in the brain and in protecting it from Fe-mediated free radical injury.

The Fe uptake pathway starts in the intestines, where Fe^{3+} is reduced to Fe^{2+} that then is transported to the blood by Fpn. In the blood, Cp oxidizes Fe^{2+} to Fe^{3+} and promotes its binding to the serum iron carrier, Tf [19]. In order to enter the brain, Fe needs to cross two distinct barriers, the BBB and BCSF (blood-cerebrospinal fluid) [20]. The most common pathway for Fe transference across the BBB is through the TfRs expressed in the endothelial cells. The circulating Fe bound to Tf is captured by TfR, entering the brain by endocytosis and then is translocated across the endosomal membrane, probably through the divalent metal transporter 1 (DMT1) [21]. In addition to the Tf-TfR pathway, it has been suggested that the lactoferrin receptor-lactoferrin (LfR-Lf) pathway might also play a role in Fe transport across the BBB. Fe^{2+} in the cytoplasm can also be transported inside the mitochondria by mitoferrin or participate in electron exchange reactions [22, 23]. Figure 1 summarizes the brain Fe uptake pathways.

Fe-related neurodegenerative disorders can result from both iron accumulation in specific brain regions or defects in its metabolism and/or homeostasis.

As the brain ages, Fe accumulates in regions that exhibit pathologic characteristics of Alzheimer's disease (AD) [24], Parkinson's disease (PD) [25], or Huntington's disease (HD) [26, 27]. In younger individuals, the largest amounts of Fe are in the oligodendrocytes whereas in older individuals over 60 years old most of the Fe is found in the microglia and astrocytes of the cortex, cerebellum, hippocampus, basal ganglia, and amygdala [27]. In these regions Fe is either bound to neuromelanin, a dark brown pigment that accumulates essentially iron, or to ferritin [28]. Interestingly, neurons express mostly H-ferritin, microglia express mostly L-ferritin, and oligodendrocytes express similar amounts of both subunits [29, 30]. Additionally neurons excrete the nonrequired Fe through the carrier Fpn (Figure 1).

It has been widely accepted that abnormal high concentrations of Fe contribute to neurodegenerative processes; however, a major question has not yet been answered. Is the excessive Fe accumulation in the brain an initial event that causes neurodegeneration or a consequence of the disease process?

Fe accumulation has been shown to lead to neuronal death [31]. Available Fe interacts with molecular oxygen and generates reactive oxygen species (ROS) through Fenton and Haber-Weiss reactions [32, 33], which leads to oxidative stress. Mitochondrial dysfunction has also been raised as a common cause for a number of neurodegenerative diseases. Since mitochondria has an important role in the Fe-S clusters formation [34], malfunction can result in a low Fe-S cluster synthesis and consequent activation of DMT1 and decrease of Fpn1, Fe accumulation, and oxidative stress [4]. Oxidative injury induces lipid peroxidation, nucleic acid modification, protein misfolding and aggregation, and cell dysfunction and death [35].

1.2. Alzheimer's Disease (AD). AD is the most common cause of age-related neurodegeneration and is characterized by the progressive loss of memory, task performance, speech, and recognition of people and objects. AD is characterized by the accumulation of aggregates of insoluble amyloid-β protein (Aβ), and neurofibrillary tangles (NFTs) consisting of precipitates/aggregates of hyperphosphorylated tau protein [36].

In AD, Fe accumulation has been observed in and around the amyloid senile plaques (SP) and neurofibrillary tangles (NFTs) [37]. The excessive Fe can lead to alterations in the interaction between IRPs and their IREs [38] and disruption in the sequestration and storage of Fe by ferritin [39]. Further studies have suggested that high Fe toxicity may be due to the propensity of Fe^{2+} to generate ROS [40], and *postmortem* analysis of AD patients' brains has revealed activation of two enzymatic indicators of cellular oxidative stress: heme oxygenase-1 (HO-1) [41] and NADPH oxidase [42]. In addition, other evidence suggests that in AD the Fe metabolism is disrupted. Tf is not found in the oligodendrocytes but rather trapped within senile plaques and ferritin is expressed within reactive microglial cells that are present both in and around the senile plaques [43]. A decade ago Rogers et al. [44] provided another link between iron metabolism and AD pathogenesis by describing the presence of an IRE in the 5′UTR of the amyloid precursor protein (APP) transcript. APP 5′UTR is responsive to intracellular iron levels, which regulate translation of APP holo-protein mRNA by a mechanism similar to the translation of ferritin-L and -H mRNAs via IREs in their 5′UTRs. Recently, Duce et al. [45] have described that APP is a ferroxidase that couples with Fpn to export Fe. In AD APP ferroxidase activity appears inhibited, thereby causing neuronal Fe accumulation.

1.3. Parkinson's Disease (PD). PD is a progressive disorder that manifests as tremor at rest, bradykinesia, gait abnormalities, rigidity, postural dysfunction, and loss of balance [46]. It is the most prevalent neurodegenerative disorder after AD affecting about 2% of people over 65 years old. PD is characterized by the loss of the *substantia nigra* dopaminergic neurons [47] and the deposition of intracellular inclusion bodies known as Lewy bodies. The principal protein

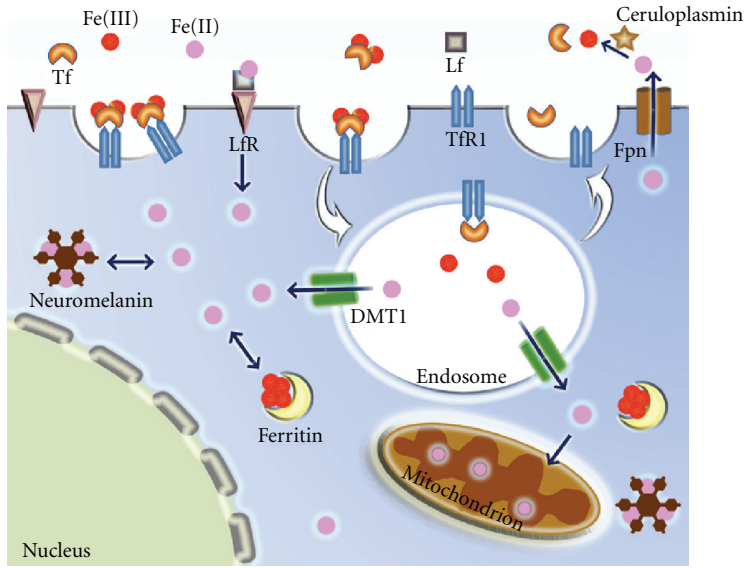

Figure 1: The brain iron homeostasis. Iron (Fe) binds to transferrin (Tf), enters the brain through the transferrin receptors (TfR) by endocytosis, and translocates across the endosomal membrane through the divalent metal transporter 1 (DMT1). Lactoferrin receptors (LfR) provide another pathway to transport Fe from Fe containing lactoferrin across the cell membranes. Inside the cell Fe binds to H-ferritin and accumulates around the neuromelanin. Ferroportin (Fpn) transports Fe^{2+} outside the neuron that is oxidized to Fe^{3+} by ceruloplasmin promoting its binding to Tf.

component of these bodies is α-synuclein (α-syn) that is ubiquitously expressed in the brain [48].

Several studies have confirmed an increase of Fe in the *substantia nigra* of most severe cases of PD [49–51]; however, there are still some conflicting reports about the stage during disease progression at which nigral Fe changes occur.

Nevertheless, there is a general agreement that total nigral Fe levels increase in PD, possibly leading to nigrostriatal dopamine neuron degeneration as a result of its ability to produce ROS and cause lipid peroxidation [52, 53].

The elevated Fe content, besides contributing to the increase of oxidative stress, also enhances α-syn aggregates [54]. It has been shown that α-syn harbors an IRE in its 5′-UTR. Thus high intracellular Fe might also regulate α-syn aggregation through the IRE/IRP system, therefore, causing the death of dopaminergic neurons [55]. As Fe deposits are commonly found in the Lewy bodies, Fe might play a role on the pathogenicity of α-syn in PD.

1.4. Huntington's Disease (HD). HD is a neurodegenerative disorder characterized by progressive motor, cognitive, and psychiatric deterioration. Typically, onset of symptoms is in middle-age (30 and 50 years old), but the disorder can manifest at any time between infancy and elderliness. HD is caused by a dominant glutamine expansion (CAG repeat coding) within the N-terminal of the huntingtin protein that initiates events leading to neuronal loss primarily within the striatum and cerebral cortex. Full-length huntingtin is large (~350 kD), but it is the smaller N-terminal fragments that are the main mediators of disease progression [56]. These fragments have aberrant interactions with themselves and other biomolecules that lead to the molecular hallmarks of HD including aggregates, transcriptional repression [57], oxidative damage, and metabolic dysfunction [58].

For individuals with HD, increased Fe levels have primarily been observed in the basal ganglia, namely, in the *striata* and the *globus pallidus* [59]. In addition, ferritin-Fe levels are increased in *striata* of early clinical HD patients as measured by magnetic resonance imaging (MRI) [60]. Fe levels increase early stage in HD and continue to increase with age, which suggests that Fe may play a role in the progression of the disease. However, the mechanisms involved in this process are not yet understood. Although both AD and PD are characterized by Fe accumulation, the Fe regulation patterns seem to be different from HD [59]. For instance, Parkinson's disease is characterized by Fe accumulation in the *substantia nigra*, which has not been observed in HD. It is possible that in HD Fe accumulation occurs because after neuronal loss, cells with higher Fe content replace the dead cells. Thus Fe accumulation in HD is most probably a secondary effect of the disease [60].

1.5. Other Neurological Disorders. The accumulation of Fe has also been implicated in a series of other neurological diseases, such as Neuroferritinopathy, Hallervorden-Spatz syndrome, and Aceruloplasminemia that are characterized by mutations in genes that encode for ferritin light polypeptide (*FTL*), pantothenate kinase (*PANK2*), and ceruloplasmin, respectively.

Neuroferritinopathy is dominantly inherited and is a late-onset disease of the basal ganglia that presents extrapyramidal features similar to HD and PD. It is caused by a single adenine insertion at position 460-461 that is predicted to change the C-terminal residues of the gene encoding L-chain ferritin [61]. Brain histochemistry of patients with neuroferritinopathy showed abnormal aggregates of ferritin and Fe and low serum ferritin concentrations. The C-terminus of the aberrant L chain might interfere with the formation of

the hollow sphere allowing inappropriate release of Fe from the loaded ferritin [62].

Another evidence for the involvement of Fe in neurodegeneration is provided by the study of Hallervorden-Spatz syndrome (HSS), also referred to as neurodegeneration with brain-iron accumulation 1 (NBIA) or pantothenate-kinase-2-associated neurodegeneration (*PANK2*) [63]. HSS is an autosomal recessive disorder characterized by dystonia, pigmentary retinopathy in children and neuropsychiatric defects in adults. The HSS patient's MRI has a characteristic pattern in the *globus pallidus*, known as "the eye of the tiger" because of its appearance [64]. Zhou et al. [63] identified the genetic basis for this neurodegenerative disease in which Fe accumulation is most dramatic by detecting the underlying mutations in the gene that encodes for pantothenate-kinase. This enzyme is essential for the coenzyme A biosynthesis [65], which in turn catalyzes the phosphorylation of pantothenate (vitamin B_5), *N*-pantothenoyl-cysteine, and pantetheine [63]. The product of this reaction, 4′-phosphopantothenate, is then converted to 4′-phosphopanthetheine in a reaction that consumes cysteine. HSS results from 4′-phosphopantothenate deficit, which is caused by genetic defects in *PANK2*. Given that cysteine is consumed in the conversion of 4′-phosphopantothenate, an absence of functional *PANK2* might explain the observed accumulation of cysteine in the degenerating brain areas of HSS patients. Consequently the cysteine Fe-chelating properties might account for the observed regional Fe accumulation, and cysteine-bound Fe may promote Fe-dependent oxidative damage in these regions [66]. Even though *PANK2* is not directly involved in Fe metabolism, its absence may contribute for Fe accumulation in the brain, leading to neuronal death via oxidative stress.

Finally, aceruloplasminemia, an autosomal recessive disorder caused by mutations in the ceruloplasmin gene, also results in Fe overload in the brain characterized mainly by retinal neurodegeneration [17]. Cp is a multicopper ferroxidase responsible for the Fe homeostasis by promoting Fe incorporation into Tf, therefore, playing a key role in releasing Fe from the cells [67]. Consequently, mutations in the ceruloplasmin gene may cause Fe metabolism misregulation in the brain. Due to the low release of cellular Fe and the high nontransferrin-bound Fe uptake, the intracellular Fe concentration becomes abnormally high. This induces oxidative stress and formation of ROS triggering a cascade of pathological events that lead to neuronal death.

1.6. Fe-Chelation Therapies. Oxidative stress, protein aggregation, and active redox Fe have been considered promising pharmacological targets for the treatment of AD and PD. BBB permeable Fe chelators can be used as potential therapeutic agents in the treatment of neurodegenerative diseases. A promising Fe chelator is desferrioxamine (Desferal), which has been shown to prevent up to 60% of dopaminergic neurons from death in a rat model of PD [68]. The main disadvantage of desferrioxamine is that it cannot cross the BBB, due to its size and hydrophobicity [65]. Clioquinol, a small lipophilic Fe chelator that can cross the BBB, has also proved to have beneficial effects in patients with AD [69]. However, clioquinol is not iron selective and has very toxic effects. Aroylhydrazones are the new nontoxic lipophilic Fe chelators that can form a neutral complex with Fe and diffuse out of the membrane [70]. Other important class of compounds proposed for therapy is the polyphenols that have antioxidant properties and can bind Fe [71]. A major limitation is their capacity to be absorbed at the gastrointestinal tract and subsequently be transported through the BBB.

The development of an effective non-toxic therapeutic agent for such complex brain disorders still represents a challenging task.

2. The Yeast Model

In the last decade, the budding yeast *Saccharomyces cerevisiae* has been used as a model system to gain insights about the mechanisms of neurodegenerative disorders such as Parkinson's, Huntington's, and Alzheimer's [72]. Yeast cells are generally used to study key proteins involved in the etiology and/or pathology of these diseases. When a yeast homologue exists, the corresponding gene can be easily disrupted or overexpressed to determine the loss or gain of function phenotypes, respectively. When a yeast homologue is not present, the human gene can be expressed in yeast and any relevant phenotype that results from this expression can be analyzed. The latter has been called humanized yeast models [73]. Despite their simplicity, yeast cells possess most of the same basic cellular machinery as neurons in the brain, including pathways required for protein homeostasis and energy metabolism. Also their easy genetic manipulation makes these cells an ideal tool for molecular biology.

Many of the genes and biological systems that function in yeast Fe homeostasis are conserved throughout eukaryotes to humans [74]. *S. cerevisiae* expresses three genetically distinct transport systems for Fe, two reductive systems and one nonreductive system. The reductive Fe uptake system consists in a low-affinity pathway defined by Fet4, that can also transport other metals and in a high-affinity pathway that is mediated by a protein complex composed of a multicopper ferroxidase Fet3, the mammalian Cp homologue, and a permease Ftr1. The Fet3-Ftr1 complex is specific for Fe and is regulated both transcriptionally and posttranscriptionally by this metal [75–77]. The nonreductive Fe uptake system is mediated by the ARN family (Arn1-4) of membrane permeases that transport siderophore-Fe^{3+} complexes [78, 79]. Additionally Harris et al. [80] showed for the first time that Fet3 can functionally replace ceruloplasmin in restoring Fe homeostasis.

Moreover, cells are able to spare Fe through the regulation of Tis11 homologues and Cth1/2-mediated degradation of mRNAs coding for Fe-binding proteins, thereby facilitating the utilization of limited cellular Fe levels [81, 82].

Since *S. cerevisiae* lacks the Fe storage protein, ferritin, during Fe overload this is sequestered into the vacuole by the Ccc1 transporter, which is under the control of the Yap5 transcription factor [13]. On the other hand, Fet5/Fth1 complex mobilizes Fe out of the vacuole for use during Fe limitation [83].

Given the similarities between yeast and mammals and the availability of humanized *S. cerevisiae* strains, yeast could potentially become an effective model to dissect the molecular pathway associated with the misregulation of Fe homeostasis in the neurodegenerative diseases.

One good example of the use of yeast to study Fe accumulation in a neurodegenerative disease was first reported for Friedreich's ataxia (FRDA). FRDA is an autosomal recessive mitochondrial disorder that causes progressive damage to the nervous system, resulting in gait disturbance, speech problems, heart disease, and diabetes. It is caused by GAA triplet expansion in the first intron of the frataxin gene (*FA*) [84].

A gene with high sequence similarity to *FA* was initially identified in yeast, the yeast frataxin homologue, *YFH1* [85] and later it was shown that the two proteins were both located in the mitochondria. Moreover, human *FA* could complement for the absence of the yeast *yfh1* [86]. However, *FA* function was only discovered when Lodi and coworkers [87] showed that the *YFH1* knockout strain led to an excessive Fe accumulation in the mitochondria resulting in the generation of ROS and consequently oxidative damage. The yeast frataxin homologue provided the evidence that FRDA is indeed a mitochondrial disorder. The yeast model allowed a better understanding of the FDRA pathophysiology and provided a tool for assaying therapeutic targets.

3. Concluding Remarks

In this paper, we have summarized the role of Fe, a redox-active transition metal, in neurodegenerative disorders. Despite a considerable investigation already performed, it is still not clear whether excessive Fe accumulation in the brain is an initial event that causes neuronal death or is a consequence of the disease process. The growing evidence suggests that the abnormal high Fe levels in the brain may have genetic causes, as found in patients with aceruloplasminemia, or sporadic causes that can disrupt the normal mechanisms of Fe transport into the brain. In addition, elevated Fe levels generate ROS and increase the levels of oxidative stress, which is considered one of the pathways leading to neuronal death. A new study from Lei et al. [88] shows that loss of Tau impairs the Fpn Fe export by preventing the proper trafficking of APP ferroxidase to the neuronal surface, leading to Fe accumulation, which results in degeneration of dopaminergic neurons in PD. These findings suggest the involvement of a new mechanism associated with Tau's role in PD. However, the precise role of Fe transport proteins in the brain is not completely understood, which impairs the success of therapeutic strategies to prevent the damaging effects of the Fe in the brain.

Finally, we believe that the use of the yeast neurodegenerative disease models might provide valuable insights into key aspects of the Fe pathology in the brain and pave the way towards the discovery of promising therapeutic targets.

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Review Article

Relationship between Human Aging Muscle and Oxidative System Pathway

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Ageing is a complex process that in muscle is usually associated with a decrease in mass, strength, and velocity of contraction. One of the most striking effects of ageing on muscle is known as sarcopenia. This inevitable biological process is characterized by a general decline in the physiological and biochemical functions of the major systems. At the cellular level, aging is caused by a progressive decline in mitochondrial function that results in the accumulation of reactive oxygen species (ROS) generated by the addition of a single electron to the oxygen molecule. The aging process is characterized by an imbalance between an increase in the production of reactive oxygen species in the organism and the antioxidant defences as a whole. The goal of this review is to examine the results of existing studies on oxidative stress in aging human skeletal muscles, taking into account different physiological factors (sex, fibre composition, muscle type, and function).

1. Human Aging Muscle: An Overview

Aging represents an inevitable and complex biological process that is characterized by a general time-dependent decline in the physiological and biochemical functions of the major systems [1]. Several changes can be observed during aging, which include a reduced capacity to use oxygen along with impaired cardiocirculatory capacity and respiratory adaptation, deterioration of nervous system (decrease in the form, width, and rate of conduction of evoked potential), and degeneration in muscle mass characterized by a reduction in muscle fiber diameters and by a qualitative and quantitative alteration in muscle fibres [2].

Also at the cellular level, morphological and biochemical changes are involved in this process. Skeletal muscle can be considered the largest organ in the body [3] and the age-associated loss of skeletal muscle mass and strength (i.e., sarcopenia) seems an unavoidable part of the aging process. After about the age of 50 years, there is a progressive decrease of muscle mass at the rate of 1-2% per year. Similarly but with different decline rate and timing, muscle strength also decreases by about 3% yearly after 60 years of age [4] while the cross-sectional area of skeletal muscle is reduced by 25–30% after age 70 [4–6]. Sarcopenia is, therefore, a multidimensional phenomenon of aging (someone indicates it as a syndrome) and represents a powerful risk factor for the development of negative health-related events in the elderly. In fact, the relationships of sarcopenia with impaired physical performance, frailty, loss of functional independence, and increased risk of falls are all well established in literature [7]. Moreover, decreased muscle strength is also highly predictive of incident disability, and all-cause mortality in older persons [8]. An important aspect regards the different functional decline associated with sarcopenia, which is more evident in men than in women [9]. Moreover, the extent of sarcopenia, and thus age-related atrophy, are higher in glycolytic muscles compared to oxidative muscles [10, 11]; Type I fibers are slow contracting, mainly oxidative, while type II fibres are fast contracting, mainly glycolytic with a lower number of mitochondria. In humans, the structural changes of responsible for age-related atrophy and decline in muscle strength are correlated to the progressive impairment of the cross-sectional fibre area [12] and to fibre denervation and fibre number loss, with type II fibres being the most

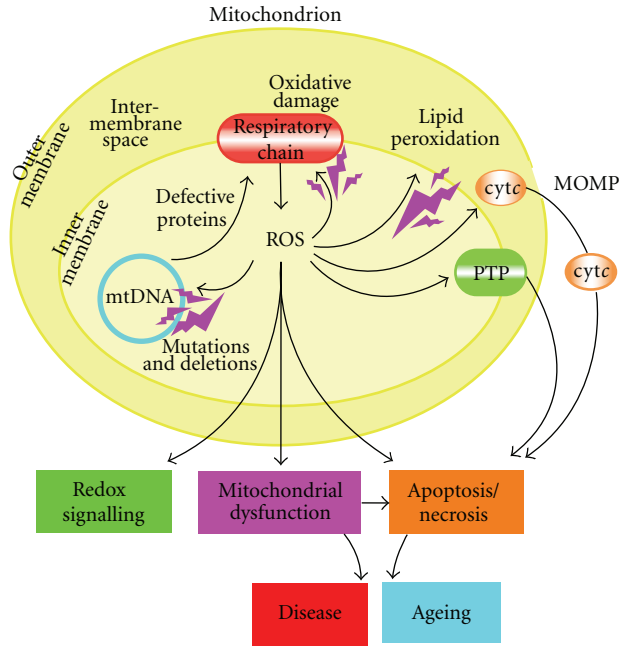

Figure 1: mitochondria ROS production by [28].

affected by aging [13, 14]. The remaining type II fibres seems to maintain their efficiency probably by adjusting their capability to produce energy, as suggested by the absence of age-related changes in the enzymatic activities of the anaerobic machinery for energy production [15–17].

One of the most important endogenous causes of sarcopenia is likely correlated to the loss of a motor neuron input to the muscle [18]. This decline of muscle innervation may be one of the key events in the sarcopenic process since innervation is crucial to the maintenance of muscle mass, as well as strength. In the elderly, there is a decrease in the number of functional motor units associated with a concomitant enlargement of the cross-sectional area of the remaining units [19]. Together with neurological factors, a decline in anabolic hormones may also play a key role in the sarcopenic process. This reduction of anabolic hormones, namely, growth hormone (GH) and sexual steroid hormones, could be implicated in the aetiopathogenesys of the sarcopenic process. Many studies have demonstrated that GH levels begin to decline in the fourth decade and progressively continue to decline over ensuing years. Interestingly, it seems that sex hormones are an important factor in maintaining muscle mass and strength in men but not in women [20–23]. Regarding the multifactorial aetiology in recent years many assumptions were made about the causes of Sarcopenia that can be very schematically summarized as follows [24].

(a) Mitochondrial deletion: a failure of replication of mitochondrial DNA (mtDNA) may be the cause of a significant deletion in the mitochondrial genome; the shorter genome is replicated more quickly by inducing the formation of malfunctioning or completely inactive mitochondria.

(b) Alteration of protein synthesis.

(c) Loss of the ability of reparative satellites cells (SC): the proliferation and fusion of the SC is regulated by specific growth factors protein (mainly IGF-1, mIGF-1, HGF) but is also influenced by hormones such as GH, testosterone, and estrogen. Moreover, satellite cells are also activated by mechanical stretch, and muscle or sarcolemmal damage. One of the factors that could play a key role in triggering sarcopenia is the accumulation of reactive oxygen species (ROS) that have produced throughout one's lifetime [2]. ROS, which are generated by the addition of a single electron to the oxygen molecule, are formed in all tissues including muscle fibers and, especially, in the mitochondrial respiratory chain.

2. Mitochondrial ROS Production, mtDNA Mainteinance, and Skeletal Muscle Aging

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a normal process in the life of aerobic organism; ROS and RNS, in fact, are continually produced as a consequence of aerobic metabolism: up to 5% of oxygen reacting with the respiratory chain is incompletely reduced to ROS [15, 25, 26]. This production contributes to mitochondrial damage in a range of pathologies and is also important in redox signalling from the organelles to the rest of the cell. A schematic overview of the mitochondrial ROS production is shown in the Figure 1: ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes, and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions, including the tricarboxylic acid cycle, fatty acid oxidation, the urea cycle, amino acid

metabolism, haem synthesis, and FeS centre assem bly that are central to the normal operation of most cells. Mitochondrial oxidative damage can also increase the tendency of mitochondria to release intermembrane space proteins such as cytochrome *c* (*cyt c*) to the cytosol by mitochondrial outer membrane permeabilization (MOMP) and consequently activate the cell's apoptotic machinery. It is well established that apoptosis is elevated during aging of cells and tissues and significantly contributes to cell loss and the pathogenesis of several age-related diseases [27]. In fact, mitochondrial ROS production also leads to induction of the mitochondrial permeability transition pore (PTP), which renders the inner membrane permeable to small molecules in situations such as ischaemia/reperfusion injury. Consequently, it is unsurprising that mitochondrial oxidative damage contributes to a wide range of pathologies. Moreover, mitochondrial ROS may act as a moduletable redox signal, reversibly affecting the activity of a range of functions in the mitochondria, cytosol, and nucleus [28].

ROS include oxygen free radical, the superoxide radical anion ($O_2^{\bullet -}$, primary product of one-electron dioxygen reduction, precursor of most other reactive oxygen species and involved in the propagation of oxidative chain reactions, even though it is not a strong oxidant), the extremely aggressive hydroxyl radical (deriving from subsequent chemical reaction), and the strong nonradical oxidants, singlet oxygen and hydrogen peroxide [15]:

$$O_2 + 1e^- \longrightarrow O_2 + 1e^- \longrightarrow H_2O_2 + 1e^- \longrightarrow HO + 1e^- \longrightarrow H_2O \quad (1)$$

ROS-mediated damage is the result of an increase in electron flux and the corresponding leakage from the mitochondrial respiratory chain [15].

In muscle fibres, ROS, in particular the superoxide anion, can be produced in several cellular sites, including plasma membrane, mitochondria, sarcoplasmic reticulum (SR), T tubules, sarcolemma and cytosol, and they are generally released in the cytosol of the cell muscle [29].

The skeletal muscle is the largest consumer of oxygen in the body, and in the literature, there are several debates about the relationship between the aging in this tissue and the changes of the cellular antioxidant defence mechanism with a progressive ROS increase [30]. Oxidative damage has been proposed as one of the major contributors of the skeletal muscle decline occurring with aging [31, 32]. In general, this lack of results homogeneity in literature could be related to the variety of mechanisms that can lead to free radical production and that can be modulated by aging, sex, fibre composition, muscle type, and the physiological state of the animals [15]. Summarizing the findings of the last years, if oxidative stress plays an important role in the aging of skeletal muscle, in particular of the satellite cells, that are usually recruited to replace damaged fibers and promote their regeneration, the susceptibility against oxidative stress seems to be less prominent in this kind of cells with respect to other cell systems, since oxidative stress is counteracted by an increase in the antioxidant defence system [20]. Cellular antioxidant defence, in fact, has been shown to play an interesting role in advanced age: in the liver, brain, heart, kidney, and rat skeletal muscle aging seems to be associated with reduced capacity of enzymatic and nonenzymatic antioxidant system to convert ROS into more inert species [33, 34]. The redox imbalance is this disparity between ROS generation and the counter-acting antioxidant forces, and it constitutes the major trigger of the imbalance between protein synthesis and degradation that in turn leads to muscle atrophy [35, 36]. How such an oxidative insult plays a role in the age-related decrease of muscle performance and mass has yet to be defined. In aged muscle, there is an increase in the presence of the products of oxidative damage to lipids, proteins, and DNA [37] together with a marked increase in the number and variety of mitochondrial DNA rearrangements [38]. On the other hand, a causal relationship between oxidative macromolecules modifications, mitochondrial DNA mutations, mitochondrial dysfunction and aging is now clearly demonstrable [39]. The increased ROS production in mitochondria, hence, can subsequently lead to a vicious cycle of exponentially increasing levels of mtDNA damage.

The lack of protective histones and close proximity to the ETC, whose complexes I and III are believed to be the predominant sites of ROS production inside the cell, make mtDNA extremely vulnerable to oxidative stress [38]. Although various genetic problems in mitochondria cause phenotypes that resemble premature aging [40], additional support for this theory was provided by studies showing a direct link between mtDNA mutations and mammalian aging. In particular, mice with a proofreading-deficient version of PolgA, the catalytic subunit of mitochondrial DNA polymerase γ (POLG), accumulate mtDNA mutations that are associated with impaired respiratory-chain function and increased levels of apoptosis. These mtDNA-mutator mice, with accelerated levels of mutations, had a shorter life span and displayed age-related phenotypes such as hair loss, kyphosis (curvature of the spine), osteoporosis, and sarcopenia at an early age [40]. Interestingly, these changes were not accompanied by increased levels of oxidative stress, a finding that has also been confirmed in humans [41]. This has resulted in much controversy regarding the idea that mtDNA mutations contribute to aging through increased ROS production and enhanced levels of oxidative stress in mitochondria [42]. However, it is possible that the accumulation of mtDNA mutations that occur with age leads to alterations in cell-signaling pathways that can induce cell dysfunction and initiate apoptosis, irrespective of increased ROS production and oxidative stress in mitochondria. Previous studies have provided strong, though correlative, experimental support for an association between mtDNA mutations and tissue dysfunction, particularly in long-lived postmitotic cells such as cardiomyocytes, skeletal muscle fibers, and neurons. Moreover, mtDNA mutations have been shown to accumulate with aging in several tissues, including skeletal muscle of various species. Therefore, it has been established that many of these mtDNA mutations start to occur after the mid-thirties and they accumulate with age in postmitotic tissues of the human body [43–45]. Beside this, it was observed that the extent of mtDNA

mutation strongly correlates with the progressive decrease of *cytochrome c oxidase* activity in aging human muscle [46]. Actually, whether mtDNA mutations play a causal role in the aging process is still an ongoing debate; however, the fact that a functional decline in mitochondria occurs with age and that properly functioning mitochondria are crucial for longevity and minimizing age-related diseases cannot be refuted [42]. Another important aspect to be considered is the motility of mitochondria which continuously undergo fusion and fission events that actively alter their morphology [43]. Therefore, genetic defects in the proteins involved in mitochondrial fusion and fission lead to severely altered mitochondrial shape, loss of mtDNA integrity, increased oxidative stress, and apoptotic cell death; it has been shown that these alterations can subsequently cause developmental abnormality, neuromuscular degeneration, and metabolic disorders in humans [44]. On the other hand there are only few in-depth study confirming this relationship between the mitochondrial fusion and fission mechanism and aging, mostly because the molecular events that underlie the aging process have not yet been completely elucidated. But an important example that supports this concept is provided by recent studies of mitochondria in muscle [42]. The subcellular localization of mitochondria and the overall mitochondrial network in multinucleated muscle fibers are tightly controlled by mitochondrion-shaping proteins, and an imbalance in mitochondrial fusion and fission dynamics probably impairs their function and contributes to the age-related loss of muscle. Indeed, it has recently been shown that the expression of two genes codifying for proteins involved in mitochondrial fusion (*Mfn1* and *Mfn2*) is reduced in the skeletal muscle of aging individuals [45]. Furthermore, the role of mitochondrion-shaping proteins has recently been investigated in mice with a muscle-specific deficiency in genes codifying for two key protein (*Fis1* and *Drp1)* of the regulation of mitochondrial fission in mammalian cells: diminished mitochondrial fission was associated with reduced muscle atrophy and attenuated activation of atrophy-related genes during fasting [47]. Interestingly, the level of PGC-1α, a transcriptional coactivator that enhances the activity of specific transcription factors and that consequently stimulates the expression of *Mfn2*, is decreased in various models of muscle atrophy [48–50], whereas overexpression of PGC-1α protects skeletal muscle from atrophy, mainly by inhibiting the induction of genes that are crucial for the atrophy process [51, 52]. Many other studies indicate that alterations in mitochondrial dynamics are a key component of the mitochondrial adaptations that occur in response to mitochondrial biogenesis. These mitochondrial adaptations seem to be driven by regulatory pathways that can be activated by several physiological stimuli, including exercise and calorie restriction. However, these studies are not sufficient to reveal the functional relationship between mitochondrial dynamics and biogenesis, or to explain how fusion and fission events contribute to mitochondrial turnover [42]. Nevertheless, as the results of several studies on mammalian cells showed, there is a relationship between mitochondrial dynamics and genomic stability [50, 51]. Furthermore, following confirmation of the role of some genes like *Mfn1* and *Mfn2* in mtDNA maintenance, loss of mitochondrial fusion in skeletal muscle resulted in increased mtDNA point mutations and deletions as well as severe mtDNA depletion, which preceded the phenotypic changes observed in mice mutated for the genes above mentioned (*Mfn1* and *Mfn2*) [53–55]. These studies support the hypothesis that mitochondrial fusion through intramitochondrial exchange increases the tolerance of a cell to mutant mtDNA and protects the integrity of the mitochondrial genome [42].

In general, several lines of evidence indirectly implicate mtDNA in longevity. The Framingham Longevity Study of Coronary Heart Disease has indicated that longevity is more strongly associated with age of maternal death than that of paternal death, suggesting that mtDNA inheritance might be involved [56]. On the other hand, longevity was shown to be associated with certain mtDNA polymorphisms, which also may play a role in aging process [57]. In the opinion of Alexeyef, the mitochondrial theory of aging can be considered as an extension and refinement of the free radical theory. Its major premise is that mtDNA mutations accumulate progressively during life and are directly responsible for a measurable deficiency in cellular oxidative phosphorylation activity, leading to an enhanced ROS production. In turn, increased ROS production results in an increased rate of mtDNA damage and mutagenesis, thus causing a "vicious cycle" of exponentially increasing oxidative damage and dysfunction, which ultimately culminates in death [58].

Regarding the genetic modifications induced by ROS and various other oxidants, it was also found that radicals induce MnSOD mRNA levels to a moderate extent in several cell types. This increase in SOD level is expected to result in increased generation of hydrogen peroxide which can in turn affect the expression of many redox-sensitive genes (such as MMP-1 and MMP-2 codifying for extracellular matrix-degrading metalloproteinases [59]) and consequently a displaced equilibrium in the steady-state levels of ROS. This process is associated with massive macrophage apoptosis and contributes thereby to the formation of the atherosclerotic lesions. The process may be further enhanced by cytokines and other factors such as TNF, interleukin-1β, angiotensin II, and interferon-γ [60–63].

3. Biochemical Alterations and Oxidative Damage Induced by ROS

Therefore, currently, it is generally accepted that free radicals or ROS play a primary role in the aging process, especially in those tissues in which the generation of free radicals is more pronounced, such as skeletal muscle. This is a consequence of the high level of oxygen consumption, seen in skeletal muscle compared to other tissues, which results in higher concentrations of ROS [24], which are not only involved in muscle damage but are also able to modulate the contractile function of skeletal muscle [39]. Studies of reduced preparations have identified a number of regulatory sites that exhibit altered function when exposed to exogenous ROS donors *ex vivo*. There are insights have

correlated oxidative damage to the mechanisms underlying EC (excitation-contraction) coupling. Since Ca^{2+} release channels are one of the major targets of oxidative damage [64, 65], the unbalance of Ca^{2+} transport that is present in the sarcopenic muscle could be due to the modified oxidative status of those structures involved in Ca^{2+} release and uptake [66]. Several studies have shown the presence of an enzyme, NAD(P)H-oxidase, that is associated with skeletal muscle SR (sarcoplasmic reticulum) [67]. In fact, the superoxide anion generated by this enzyme can stimulate the proper release of calcium from SR through oxidation of the Ryanodine receptor [67]. Ryanodine receptor 1 (RyR1) is the calcium release channel of the skeletal muscle SR required for muscle contraction. Related experiments showed as RyR1 from aged (24-month-old) rodents resulted oxidized and cysteine-nitrosylated compared to RyR1 from younger (3–6-month-old) adults. RyR1 channel activity is dramatically altered by redox modifications of critical thiols group contained in the protein (oxidation, S-nitrosylation, or alkylation); an oxidation of approximately 20% of these free thiols is enough to produce a significant effect on the channel activity that in some case can lead to an irreversible inactivation of the channel. These data indicate that leaky RyR1 contributes to age-related loss of muscle function [68, 69].

The involvement of other enzymes, such as Phospholipase A2 (PLA2) and xanthine oxidase, has been suggested. The activation of PLA2 may stimulate NAD(P)H oxidase, while it has been shown that increases in PLA2 activity stimulate ROS production in mitochondria and in cytosol, and subsequent release in extracellular space [70]. PLA2 enzyme is present in cells as two isoforms: calcium-dependent PLA2 (cPLA2) and non-calcium-dependent PLA2 (iPLA2). It has been suggested that iPLA2 is mostly responsible for ROS production at rest, while isoform cPLA2 seems to be activated during all processes that increase intracellular Ca-concentration, such as muscle contraction [70]. Xanthine dehydrogenase (XDH) is a cytoplasmic enzyme that is converted to Xanthine oxidase, which in turn is able to produce superoxide anion and, accordingly, hydrogen peroxide. Skeletal muscle generates superoxide and nitric oxide at rest, and this generation is increased by contractile activity. Proteins, such as superoxide dismutases (SODs), catalase (CAT), and heat shock proteins (HSPs), prevent oxidative damage to tissues.

The mechanisms underlying these adaptive responses to contraction include activation of redox-sensitive transcription factors, such as nuclear factor-kappaB (NF-κ B), activator protein-1 (AP-1), and heat shock factor 1 (HSF1) [1]. Moreover the literature reports that postcontraction attenuation of the pathways involved in mitochondrial biogenesis in fast muscle fibers (type II) is far more severe in old rats than in those of young rats. ROS are known to play an important role in the activation of signalling cascades [71]. These latter suggest that ROS affect mitochondrial biogenesis via upregulation of transcriptional regulators, such as peroxisome proliferator-activated receptor-gamma coactivator-1 protein-alpha (PGC-1alpha) and that excessively high ROS generation subsequent to contractions may be responsible for diminished mitochondrial biogenesis in the muscles of old rats. The inability of skeletal muscle from old rats to activate redox-sensitive transcription factors such as NF-κ B or AP-1 in response to stress is characterized by chronic activation of transcription factors at rest and by an inability to further activate these factors subsequent to muscle contraction. This lack of activation of redox-sensitive transcription factors with contractile activity is associated with an inability to increase the expression of various cytoprotective proteins and appears to influence the susceptibility of skeletal muscle from old animals to oxidative damage [72, 73].

3.1. Lipid Damage. One of the principal indicators of free radical damage during aging is lipid peroxide (LPO). Polyunsaturated fatty acids (PUFAs) of the biomembranes are attacked by free radicals in presence of the oxygen; malondialdeyde (MDA) and 4-hydroxy-2,3-trans-nonenal (4-HNE) are the main products of PUFA peroxidation because they bear numerous double bonds such as linoleic acid and arachidonic acid. The main effect on lipid peroxidation of biological membranes is the overall decrease in their fluidity, which makes it extremely easy for the two monolayers to exchange phospholipids, increases the permeability of the membrane to substances that are not normally able to cross other than by specific channels, and ultimately results in severe damage to membrane proteins. These latter tend to aggregate and to form high-molecular-weight complex [74].

In human skeletal muscle, LPO levels are significantly higher in aged than in young subjects [75]. There are works that have demonstrated that there is an age-dependent increase in MDA content in skeletal muscle [76, 77], and also data obtained from our research group in these years agree with those reported by previous authors. Furthermore, LPO levels in young women are significantly lower (by about 50%) than in young men [36, 75]. Possibly, the difference in lipid peroxidation between young and aged women could be caused by the reduction of estrogens' protective role after menopause. In any case, the level reached in elderly women is lower compared to men [31, 75]; in particular, MDA values in the VL (*vastus lateralis*) muscle in aged females (over 70) were comparable to those obtained from young males (under 40) [77].

In human skeletal muscle, the level of peroxidation also depends on muscle fiber composition and on muscle function. Fiber composition indicates that muscle with more than 40% type II fiber content shows statistically lower LPO levels than does muscle with less than 40% type II fiber content [75]. This suggests that increased lipid peroxidation prevails in type I fibers (which are mainly oxidative), where most ROS production probably takes place by mitochondrial alteration [1]. An increase in the LPO level during aging is evident in the VL [75, 77, 78] and in the external intercostal [75], but not in the RA (*rectus abdominis*) muscle [77]. Fiber I distribution is very similar in RA and VL, which suggests that functional differences in these muscles could be the cause of major lipid peroxidation. There are two possible explanations for the observation that oxidative lipid levels in RA differ from those in VL: the greater contractile capacity

of VL muscle compared with RA muscle and/or the higher levels of peroxidable substrates present in VL compared with RA. On the base of these results, it seems that lipid peroxidation level are influenced by a variety of different factors: sex, fiber composition, and muscle specificity [1].

4. Protein Carbonylation

A great number of studies have shown an increase in oxidized proteins at the intracellular level during senescence. For the most part, oxidative-modified proteins are not repaired and must be removed by proteolytic degradation carried out by proteosome, which selectively degrades damaged proteins [15]. The molecular mechanism that causes the accumulation of oxidative-modified proteins is not completely clarified. Nevertheless, the oxidative damage to proteins has several physiological implications related to the loss of function in the affected proteins with deducible consequences for the organism. Protein oxidative damage involves both the loss of thiol groups, and modifications to amino acids that constitute the polypeptide chain, in particular, histidine. Protein carbonylation (PC) is used as a marker of oxidative damage, which acts, in particular, on the side chains of the amminoacid residues lysine, arginine, proline, and threonin; this may occur either directly or indirectly as a consequence of lipid peroxidation [79].

Since oxidized protein is harmful to the maintenance of cellular homeostasis, it requires rapid removal by proteolytic digestion. PC content increases drastically in the last third of the life span [80] and reaches a level such that on average one out of every three protein molecules carries modification. Both in men and in women, PC content tends to increase during aging, but in women these changes are not statistically significant [31]. Increasing amounts of PC content were observed and compared during aging in male skeletal muscles, but only in subjects younger than age 40 and older than age 70 [31]. Muscles with different functions in humans have been compared [77], and some authors reported an increase in PC level during aging in both the VL [81] and the external intercostal [78]; in other cases, the increase was not statistically significant in the VL [77, 78] and in the RA [77]. The difficulty in showing significant changes in PC in the chronic physiological process could be due to the dynamic features of the process: during aging, myofibrillar carbonyl content has been shown to increase significantly three hours after acute oxidative stress, only to return to its basic level within a few hours, in contrast with the normal condition in skeletal muscle as characterized by a relatively slow protein turnover [82]. Finally, protein carbonyl content tend to increase during aging and although data recently obtained from our research do not reach statistical significance, they are in line with those obtained in other studies on the brain, heart, and kidney [83] and skeletal muscle [84].

5. Mitochondrial DNA Damage

Mitochondrial DNA (mtDNA) is a double-stranded ring (about 16.5 kb) encoding 13 subunits of the ETC (electron transport chain) as well as 2rRNA and 22tRNA protein necessary for their translocation. Mitochondrial DNA lacks introns and is devoid of histones and other DNA-associated proteins; it is located in mitochondrial matrix, close to the major source of ROS [15]. For all these reasons, the probability of the oxidative modification of a coding region of mtDNA is very high. Moreover, the repair devices of mtDNA, even present, are largely insufficient to overcome extensive DNA damage which, furthermore, persist longer than that nuclear DNA, as demonstrated by *in vitro* experiments with cell cultures [85, 86].

The high turnover of mitochondria and the not sufficient mtDNA repair systems cannot counteract the oxidative damage that accumulates. This involves a progressive accumulation of mitochondrial functional impairment and, therefore, a lower availability of energy and consequent impairment of cell functions. Therefore, ROS can damage DNA either by an indirect mechanism, or by direct interaction with several molecules. Indirect mechanism involves the peroxides, which can oxidize-SH groups of Ca^{2+} channels in the endoplasmic reticulum, which contains ion cell, and thus determines its release [87]. Increases in intracellular calcium can stimulate Ca^{2+}-dependent endonucleases and it can results in excessive and incorrect DNA fragmentations. The hydroxyl radical bears the greatest responsibility for the damage caused by ROS with a direct mechanism. $HO^{\bullet}$ reacts indiscriminately with both sugars, ribose and deoxyribose, and with both nucleobases, purine and pyrimidine, by addition and H-abstraction. In this case, modified bases induce DNA strand breakage, during replication, and pairing errors with consequent mutations [88]. $HO^{\bullet}$ reacts predominantly with Guanine in C8 and C4 of the ring, thus generating Guanine radical. In the presence of oxygen or other oxidizing agents, the Guanine radical is converted to stable product 8-hydroxy-2′-deoxyguanosine (8-OHdG) [89], which is considered a good biomarker of oxidative DNA damage [90].

Data obtained in male skeletal muscle indicate an increase in DNA damage during aging [32, 81] for pooled male and female data [37]. In fact, investigations of human skeletal muscle have established a correlation between age and the accumulation of mtDNA deletions an mutations [91]. It has also been suggested that mtDNA mutations start to occur after the fourth decade of life, and that they accumulate with age in postmitotic tissues [92]. There is strong evidence for continuous age-related increase in the levels of oxidative stress and oxidative damage on mitochondrial biomolecules, damage that becomes progressively more apparent with advancing age [93]. However, progressive loss of mitochondrial redundancy may not limit human skeletal muscle's capacity to supply the cellular energetic demands at basal metabolic conditions. This is important, because such limits might constrain the functioning of myofibers during situations with higher energy requirements. Moreover, an increase in MDA content is significantly correlated with an increase in 8-OHdG, a finding which suggests a direct correlation between lipid peroxidation and DNA damage in human skeletal muscles [37]. It has been demonstrated that deletion patterns of mtDNA over a lifetime are tissue specific and more pronounced in the skeletal muscle and heart [94].

It is also important to note that in human skeletal muscle oxidative damage is probably lower in aged women compared with aged men [32]. In particular, it seems that women are more protected against molecular oxidative stress during premenopausal life period suggesting a significative antioxidant role of estrogen, present in high levels in women. These hormones could contribute, therefore, to enhancing antioxidant defences in female muscles [95]. During the fertile age, these hormones probably induce a less accumulation of the oxidative damage with respect to males, determining a more favourable condition in the aging process [15].

5.1. The Antioxidant Defence in Aged Muscle. Antioxidants are substances able to inhibit the rate of oxidation [96, 97]. Biological antioxidant elements are present under diverse and numerous forms and they can be categorized as enzymatic (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR)) and nonenzymatic (ascorbate, tocopherol, beta-carotene, reduced coenzyme Q, or ubiquinol, reduced glutathione, uric acid, and minerals like selenium and zinc). These antioxidant systems act as primary intracellular defences against reactive oxygen species before their interaction with macromolecules protecting cells from free radical damage. Nevertheless, since free radicals are necessary for correct functioning of human organism, efficient mechanisms of enzymatic antioxidant defence had to be developed, especially in cells highly exposed to oxidation processes like those of skeletal muscle with the aim of protecting cellular constituents. The equilibrium between ROS production and the antioxidant defences at the level of the cellular structures determines whether a cell exposed to ROS increase will survive or will be seriously damaged or will die inducing apoptosis [98]. The enzymatic antioxidants serve mainly intracellularly, being absent or present in small amounts extracellularly, and they prevent free radical chain propagation by interrupting initiation. This is particularly important in the intravascular and connective-tissue spaces [99]. Nonenzymatic antioxidants can be divided into lipid- and water-soluble molecules. Among the nonenzymatic antioxidant molecules, the most important and studied are the vitamins C and E.

5.2. Non-Enzymatic Antioxidants. Endogenous non-enzymatic elements with antioxidant properties contribute to the maintenance of homeostasis by primarily acting as cofactors for the antioxidant enzymes. A major source of antioxidants is diet [100]. Among dietary antioxidants, the most important (and also largely available as supplements) are vitamin C, vitamin E, and carotenoids. Tocopherol (vitamin E) and beta-carotene, a precursor of vitamin A, are the main lipid-soluble nonenzymatic antioxidants. They are mainly localized to cell membranes and LDL. Alpha-tocopherol, the most active form of vitamin E, breaks free radical chain reactions. Given its location, tocopherol inhibits already initiated lipid peroxidation, in addition to having several other antioxidant effects. Beta-carotene interacts primarily with singlet oxygen by "de-exciting" it. Moreover it has been reported a significantly high correlation of α-tocopherol (the most diffuse form of circulating vitamin E) with physical performance, and of γ-tocopherol with skeletal muscle strength [101].

Ascorbate (AA) is the main water-soluble nonenzymatic antioxidant. AA interacts with a wide variety of free radicals intracellularly and forms a front-line extracellular defence against free radicals in plasma [102]. AA is moreover able to interact with superoxide and hydroxyl-free radicals, in addition to singlet oxygen. Ascorbate free radical, with its relatively low redox potential, does not propagate free radical chain reaction [103, 104]. One additional important antioxidant role ascorbate plays is in the regeneration of alpha-tocopherol (the most active form of vitamin E) from the tocopherol radical [105, 106]. Thus, AA does not only directly protect membranes and LDL from ROS generated in the aqueous phase but also indirectly protects them by reduction of vitamin E radical. Moreover, ascorbic acid concentrations are inversely associated with isoprostanes, a marker of lipid peroxidation [107] and it has a number of well-defined biological functions, including collagen, catecholamine, and carnitine biosynthesis [101–103].

Recent investigations are emerging that show that low serum/plasma carotenoids are independently associated with poor skeletal muscle strength and impaired physical performance. Among 669 women aged 70–79 years in the Women's Health and Aging Studies (WHAS) I and II, low serum carotenoid levels were associated with poor muscle strength [108]. Also, other studies showed carotenoids provide a balance to reactive oxygen species [109].

The diet is, moreover, a very important source of carotenoid. The six major dietary carotenoids, up-taken mostly from fruits and vegetables (α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene) comprise an important component of the antioxidant defense system in humans protecting against oxidative stress by quenching singlet oxygen, scavenging free radicals, inhibiting lipid peroxidation, and modulating redox-sensitive transcription factors that are involved in the upregulation of proinflammatory cytokines [103, 107]. Carotenoids are hydrophobic molecules, and thus, interact with lipophilic elements of the cell, such as the lipid membrane bilayer. They are commonly located within cell membranes, and the location of specific carotenoids within the membrane structure depends upon the chemical structure of the carotenoid [109].

Other important and powerful nonenzymatic antioxidant is glutathione (GSH/GSSG). It is representing also the base material for several other important antioxidant enzymes including glutathione-peroxidase, glutathione-reductase, and glutathione-transferase. Glutathione is a compound classified as a peptide made of three amino acids: cysteine, glutamic acid, and glycine. Glutathione is also found in every part of the body, especially the lungs, intestinal tract, and liver. The body produces and stores the largest amounts of GSH in the liver, where it is used to detoxify harmful compounds so that they can be removed from the body through the bile. The liver also supplies GSH directly to red and white blood cells in the bloodstream; it helps keep red blood and white blood cells healthy to maximize the disease-fighting power of the immune system. Glutathione

also appears to have an anti-aging affect on the body. Strong evidence that glutathione depletion causes cell death comes from cell culture studies by Li and colleague [110]. Also other works showed that direct depletion of mitochondrial and cytoplasmic GSH resulted in increased generation of ROS, disruption of the mitochondrial transmembrane potential, and rapid loss of mitochondrial function [111, 112].

The CoQ10 (Q10 coenzyme) is also an important endogenous compound in preserving mitochondrial function and it cooperates in maintaining a proper energy level, which serves to prevent the aging skin from switching to anaerobic energy production mechanisms. Furthermore, the antioxidant capacity of CoQ10 contributes to a positive effect against UV-mediated oxidative stress [103].

5.3. Enzymatic Antioxidants. Probably the main physiological mechanism by which cells regulate ROS activities (and thus protect against oxidative damage) consists, firstly, in modification of the expression and activities of the regulatory enzymes such as SOD, CAT, GPx, haem oxygenase-1 (HO-1) and, secondly, in increases in other cytoprotective proteins, such as HSPs [29]. These enzymes work to maintain a state of balance preventing the transformation of ROS and to convert them into more stable molecules (like water and molecular oxygen). There is a great controversy in the literature as to whether or not aging is associated with an increase or decrease of enzymatic antioxidant defence in the cell. Results reported in the literature need to be carefully interpreted taking into account sex, muscle fiber composition, and specificity [15].

5.4. Superoxide Dismutase (SOD). SOD is one of the primary enzymatic antioxidant defences, and it readily converts superoxide radicals (O^{2-}) in hydrogen peroxyde (H_2O_2). An increase in total SOD activity corresponds with enhanced resistance to oxidative stress [113]. SOD exists in two forms, cytosolic (Cu-ZnSOD) and mitochondrial (MnSOD). MnSOD activity in skeletal muscle, both in rats [114] and in men [2, 114, 115], is reported to increase significantly with aging and to respond to age-related oxidative stress on the mitochondria by upregulation [15]. In human, moreover, as in the rats, the age-dependent enhancement in MnSOD activity is particularly evident in the RA [77] and in external intercostal muscle [78] rather than in VL [75, 77, 82], suggesting a marked increase in ROS production mainly in the oxidative muscle respect to glycolytic muscle. Furthermore, MnSOD activity value is comparable between young men and women, doubling during aging in both sexes [31]. The enzymatic level seems to play an active role in both women and men during aging [15]. This regulatory mechanism, therefore, partially counteracts the increase of free radicals, in particular O^{2-}, in the mitochondria in senescent tissues. This reinforces the hypothesis that mitochondrial stress, due to an increased leakage in ETC during aging, is associated with production of superoxide. Accordingly, cytoplasmic SOD did not show any change or decrease in activity during aging [2, 32, 75].

In human skeletal muscle, the relationship between SOD activity, aging, and sex was investigated [2], and the obtained results showed that enzymatic activity in this kind of muscle tissue in young women is about four-to-five times higher than in men of the same age, suggesting that total SOD activity could be a limiting factor in muscle defence against oxidative damage in humans [15, 116]. Moreover, total SOD activity appears to be significantly higher in enriched type II fiber muscle [31]. Strength training, which results in muscle hypertrophy and improved muscular strength and power [117, 118], could be an important factor in delaying the progressive loss of type II fibers in elderly subjects as well [118, 119].

5.5. Catalase (CAT) and Glutathione Peroxidase (GPx). The decomposition of hydrogen peroxide to form water and oxygen is carried out in the cell by GPx and CAT. H_2O_2 has the ability to freely cross the membrane and has relatively long half life [120]. Like SOD, GPx and CAT are located in both mitochondria and cytosol, where they provide important cellular protection against free radical damage to membrane lipids, protein, and nucleic acids [15]. Most of the data on rats report an increase in CAT activity both in senescent oxidative muscles and glycolytic muscles [121]. This finding is consistent with the possibility that nonmitochondrial ROS production increases. In contrast, few and contradictory data are available on human skeletal muscle. Some authors report no change in CAT activity in men during aging [116]. No differences are observed in CAT activity with aging in all condition tested: between men and women, between muscles with different fiber compositions, and between oxidative and glycolytic muscles [75], while other studies describe a significant increase [76] or decrease in CAT activity in the VL [32]. Nevertheless, these contradictory data could be due to the dynamics of CAT activity during aging: in men, as in rats, a two-phase trend was described: an initial decrease in adult animal followed by a significant enhancement in aged animals [114, 122].

In human skeletal muscles, GPx does not change during aging, whether in glycolytic or oxidative muscle [123]. In young rats, skeletal muscle fibers with greater oxidative capacity (like the soleus muscle) have higher levels of GSH (reduced glutathione), the substrate for GPx to detoxify hydrogen peroxide, and total glutathione content than those with lower oxidative potential. In addition, one study reports that GPx activity was higher in oxidative muscles and thus caused less vulnerability and more resistance to an exercise-induced oxidative stress, while the modest levels of GSH and GPx in the deep or superficial VL muscle were associated with greater lipid peroxidation under exercise stress [15]. Probably, GSH and GSSG (oxidized glutathione) muscle levels, as well as the GSH/GSSG ratio, are closely correlated with differing muscle functions. Moreover, since some data showed that in type I fibers of both glycolytic and oxidative muscle in aging the GSH and GSSG levels remained unchanged [75], there is suggested that during aging there are no alterations of membrane glutathione transport and

that there is fiber-specific adaptation of the GSH system in the skeletal muscle [123].

5.6. Role of Nutrition against Muscle Oxidative Damage. The beneficial effects of nutrition on the prevention of oxidative stress have been attributed to the presence of antioxidants contained mainly in vegetables: carotenoids, plant polyphenols, tocopherols, ascorbate, phytic acid, and selenium [1]. Recent epidemiological studies suggest that carotenoids or carotenoid-rich foods are protective against a decline in muscle strength and walking disability among older community-dwelling adults. As report above, the carotenoids protect against oxidative stress by quenching singlet oxygen, scavenging free radicals, and inhibiting lipid peroxidation [109]. The literature provides little evidence of the possible effects of antioxidant supplementation on skeletal muscle aging [124–126]. On an animal aged model, it has been showed that vitamin E and C supplementation as well as a blend of polyphenols and carotenoids for 10 months resulted in significantly increased activity of the GSH system in muscle cell [125]; another study indicated that mixed supplementation with rutin, vitamin E, vitamin A, Zinc, and selenium restores the ability to stimulate protein synthesis subsequent to leucine administration [124]; some authors showed that vitamin C and E supplementation attenuated the increase in markers of oxidative stress (H_2O_2, MDA, and 8-OHdG) in response to chronic repetitive muscle loading, with an improvement in the work output of aged muscles [125]. Altogether, the above studies suggest that a diet supplemented with a combination of antioxidants may possibly increase antioxidant defences, lower muscle oxidative damage, and improve muscle protein balance during senescence [1]. But if, overall, results consistently report significant improvements of antioxidant biomarkers after a period of specific supplementation, differently, the effect of antioxidant supplementation on muscle performance is still and largely controversial. In fact it is necessary to underline that there are currently no trials verifying the effects of antioxidant supplementation on sarcopenia (as identified by one of several the consensus definitions provided by international groups of experts). Interestingly, a recent statement from the society on sarcopenia, cachexia, and wasting disease does not even mention antioxidant supplementation as a possible tool to manage sarcopenia in older persons [127]. On the other hand, in order to explain these controversial findings, we must consider that the modification of a biomarker concentrations not automatically changes clinical parameters. It is more likely that subclinical effects are more sensible to changes than clinically evident manifestations.

6. Conclusions

During organism aging, production of reactive oxygen species is increased as a result of the functional deterioration of mitochondria. There is strong evidence that increased free radical generation may be the underlying reason for several age-related pathogeneses. However, few studies have actually measured aging-induced free radicals in human skeletal muscles. It has been suggested that age-related oxi dative stress may be a function of a reduction in enzymatic antioxidant capacity, but this has not been demonstrated in human skeletal muscle.

The increased production of free radicals with aging plays a major role in targeting adaptive responses in human skeletal muscle. The adaptive mechanisms involve the antioxidant scavenger system and appear to be sex-, fiber-, and muscle-specific. Furthermore, the amount of introduced dietary antioxidants could play a specific and important role even if further trials in humans are required to clearly establish the hypothesized relationship between antioxidant and sarcopenia [15].

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Review Article

Mechanism of Oxidative Stress in Neurodegeneration

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Biological tissues require oxygen to meet their energetic demands. However, the consumption of oxygen also results in the generation of free radicals that may have damaging effects on cells. The brain is particularly vulnerable to the effects of reactive oxygen species due to its high demand for oxygen, and its abundance of highly peroxidisable substrates. Oxidative stress is caused by an imbalance in the redox state of the cell, either by overproduction of reactive oxygen species, or by dysfunction of the antioxidant systems. Oxidative stress has been detected in a range of neurodegenerative disease, and emerging evidence from in vitro and in vivo disease models suggests that oxidative stress may play a role in disease pathogenesis. However, the promise of antioxidants as novel therapies for neurodegenerative diseases has not been borne out in clinical studies. In this review, we critically assess the hypothesis that oxidative stress is a crucial player in common neurodegenerative disease and discuss the source of free radicals in such diseases. Furthermore, we examine the issues surrounding the failure to translate this hypothesis into an effective clinical treatment.

1. Introduction

1.1. Oxidative Stress and Neurodegeneration. It has been long recognised that oxidative stress may be important in the aetiology of a variety of late onset neurodegenerative diseases. Aging has been established as the most important risk factor for the common neurodegenerative diseases, Alzheimer's disease (AD), and Parkinson's disease (PD). Most theories of aging centre are on the idea that cumulative oxidative stress leads to mitochondrial mutations, mitochondrial dysfunction, and oxidative damage [1]. However, as the role of ROS becomes increasingly recognised in aging and age-related diseases, a number of controversies begin to emerge in this field. Is oxidative stress an epiphenomenon of dysfunctional and dying neurons, or does oxidative stress itself cause the dysfunctionality/death of neurons? How does a global event such as oxidative stress result in the selective neuronal vulnerability seen in most neurodegenerative diseases? And finally, if oxidative stress is truly fundamental to pathogenesis then why has the use of antioxidant therapy been thus far largely unsuccessful in such diseases?

In order to address these questions, we first outline the definition of oxidative stress and show how ROS is generated in the human brain (Box 1), as well as the antioxidant defence mechanisms that exist to counteract it (Box 2). We present the evidence that oxidative stress can be found in neurodegenerative disease. Next we address the issue of whether oxidative stress is truly pathogenic in disease models. In order to prove a crucial of ROS, it is necessary to observe oxidative stress as an early event in the disease process, and to further demonstrate that inhibition of ROS production is able to prevent the pathogenic process. We describe the evidence from animal and cellular models of the role of ROS in the major neurodegenerative diseases. We present hypotheses for the interplay between oxidative stress and selective cell death. Finally we study the rationale for the use of antioxidant therapy and the outcome of its use in human disease. Although oxidative stress has been implicated in a range of chronic neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis, the two commonest of these diseases, AD and PD, will be discussed in detail

in this review, and other neurodegenerative diseases will be referenced where relevant.

2. What Is Oxidative Stress?

Oxygen is essential for the normal function of eukaryotic organisms. Its role in survival is linked to its high redox potential, which makes it an excellent oxidizing agent capable of accepting electrons easily from reduced substrates. Different tissues have different oxygen demands depending on their metabolic needs. Neurons and astrocytes, the two major types of brain cells, are largely responsible for the brain's massive consumption of O_2 and glucose; indeed, the brain represents only ~2% of the total body weight and yet accounts for more than 20% of the total consumption of oxygen [2]. Despite the essentiality of oxygen for living organisms, the state of hyperoxia produces toxicity, including neurotoxicity [3, 4]. The toxicity and chemical activity of oxygen depends on its electronic structure. The identical spin states of its two outer orbital electrons render oxygen kinetically stable, except in the presence of appropriate catalysts that scramble electron spin states to produce partially reduced forms of oxygen. Partially reduced forms of oxygen are highly active because the free radical is very unstable and must either accept or be a donor of electrons. There are many different varieties of partially reduced reactive oxygen species (ROS) including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$). The modern use of the term "ROS" includes both oxygen radicals and nonradicals that are easily converted into free radicals (O_3, H_2O_2, $^1O_2$) [2]. ROS have different reactive abilities, and one of the most reactive ROS is the hydroxyl radical $OH^{\bullet}$. Due to the high reactive activity of ROS, they chemically interact with biological molecules leading to changes in cell function and cell death. As a result, oxygen has the potential to be poisonous, and aerobic organisms survive its presence only because they contain antioxidant defences [5]. Brain cells and especially neurons require effective antioxidant protection for several reasons.

(1) They exhibit higher (about 10-fold) oxygen consumption compared to other tissues.

(2) Nondividing cells such as neurons have a long life duration.

(3) Nitric oxide has a prominent role in the brain and can form reactive nitrogen species such as peroxynitrite, in combination with some forms of oxygen such as superoxide. Nitric oxide may take part in nitrosylation of proteins; however, peroxynitrite is a highly reactive nitrogen species that can nitrate tyrosine residues of proteins and alter their function.

Oxidative stress is a condition in which the balance between production of ROS and level of antioxidants is significantly disturbed and results in damage to cells by the excessive ROS. ROS contribute to the development of neurodegeneration by modulating the function of biomolecules. ROS may target several different substrates in the cell, causing protein, DNA, RNA oxidation, or lipid peroxidation (Figure 1). The oxidation products of polyunsaturated fatty acids, especially arachidonic acid and docosahexanoic acid which are abundant in brain, are malondialdehyde and 4-hydroxynonenal. ROS attacks protein, oxidising both the backbone and the side chain, which in turn reacts with amino acid side chains to form carbonyl functions. ROS attacks nucleic acids in a number of ways, causing DNA-protein crosslinks, breaks in the strand, and modifies purine and pyridine bases resulting in DNA mutations.

2.1. Oxidative Stress Occurs in Neurodegenerative Disease. Alzheimer's disease is the most common neurodegenerative disease, affecting approximately 16 million people worldwide. It is characterised by progressive neuronal loss associated with aggregation of protein as extracellular amyloid (βA) plaques, and intracellular tau tangles. AD brains also show evidence of ROS mediated-injury; there is an increase in levels of malondyaldehyde and 4-hydroxynonenal in brain and cerebrospinal fluid of AD patients compared to controls [6]. Protein carbonyl moieties are increased in the frontal and parietal cortices, and hippocampus in AD brain, with sparing of the cerebellum where no AD pathology occurs [7, 8]. There is also an increase in hydroxylated guanine in AD samples compared to age-matched controls [9, 10]. This data from human brain is also supported by data from transgenic animal models of AD in which markers of protein and lipid peroxidation are increased in the cortex and hippocampus prior to the appearance of plaques or tangle pathology [11].

Parkinson's disease is the second most common neurodegenerative disease and is characterised by progressive loss of dopaminergic neurons in the substantia nigra, and aggregation of the protein α-synuclein. In PD brain, the concentration of polyunsaturated free fatty acids in the substantia nigra is reduced, while the levels of lipid peroxidation markers (malondialdehyde and 4-hydroxynonenal) are increased [12]. Protein oxidative damage in the form of protein carbonyls [13] is also evident in PD brain compared to controls, and there is some evidence to suggest a role for nitration and nitrosylation of certain proteins due to reactive nitrogen species in PD brain [14]. In addition to increased levels of 8-hydroxydeoxyguanosine in PD brain [15], it has been reported that there is an increase in the common deletions in mitochondrial DNA in the surviving dopaminergic neurons in PD substantia nigra. Such deletions are believed to be the result of oxidative stress [16].

2.2. Mechanisms of Oxidative Stress: ROS Production by Mitochondrial Dysfunction. Mitochondrial pathology is evident in many neurodegenerative diseases including AD, PD, Huntington's disease, ALS, PSP, Friedreich's ataxia, Neurodegeneration with brain iron accumulation, and optic atrophy. The spectrum of mitochondrial dysfunction is vast and includes respiratory chain dysfunction and oxidative stress, reduced ATP production, calcium dysregulation, mitochondrial permeability transition pore opening, peturbation in mitochondrial dynamics, and deregulated mitochondrial clearance. As many of the functions of the mitochondria are interdependent, many of these pathologies exist together in varying degrees in the different disorders.

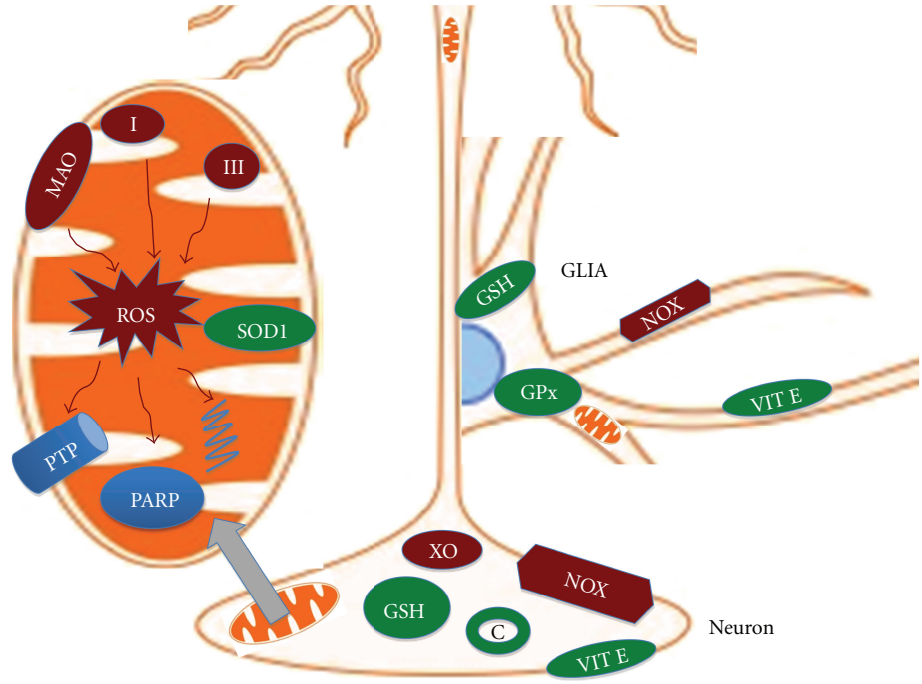

Figure 1: Schematic diagram of main producers of ROS and antioxidant system in neurons and glia. Main ROS producers are shown in red: monoamine oxidase (MAO), complex I and III are major sources within mitochondria. ROS generated in mitochondria target the permeability transition pore (PTP), PARP, and mitochondrial DNA. In the cytosol, NADPH oxidase (NOX) and xanthine oxidase (XO) are the main producers of ROS. The major antioxidant systems are shown in green and include superoxide dismutase (SOD) in the mitochondria, glutathione (GSH), catalase (C), and glutathione peroxidase (GPx).

The greatest interest in mitochondrial dysfunction and mitochondrial ROS production has been in PD, as demonstrated in human brain, as well as both the toxin and genetic disease models. A reduction in complex I activity has been demonstrated in the substantia nigra, lymphocytes, and platelets of PD patients (reviewed in [17]). The neurotoxin 1-methyl-4-phenyl-l,2,3,6-tetrahydropyridine (MPTP) has been shown to produce parkinsonian symptoms in primates and rodents, and it has therefore been extensively used as an animal model of Parkinson's disease. Studies have indicated that l-methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP, can block electron transport by binding to the same site as the classic Complex I inhibitor, rotenone, leading to a loss of ATP production. Rotenone or MPP^+ also produces superoxide anions in submitochondrial particles, adding further support to the basic premise that MPP^+ acts primarily as a mitochondrial toxin [18, 19]. The neurotoxic effects of MPP^+ and rotenone are likely to be due to oxidative stress rather than metabolic changes because they can be effectively prevented by antioxidants [20]. Mild uncoupling of mitochondria with UCP2 overexpression reduces ROS production in toxic (MPP^+, rotenone) mouse models of Parkinson's disease. UCP2 deficiency also increases the sensitivity of dopamine neurons to MPTP, whereas UCP2 overexpression decreases MPTP-induced nigral dopamine cell loss [21].

The identification of a number of PD-related genes that are strongly associated with mitochondrial function (PINK1, DJ-1, Parkin) further adds weight that mitochondrial dysfunction with resultant oxidative stress is a primary event in PD pathogenesis. Loss of function of DJ-1 results in oxidative stress, and DJ-1 exerts neuroprotection via its antioxidant mechanism in mitochondria [22, 23]. Mutations in PINK1 cause a recessive form of PD. PINK1 is a mitochondrial kinase, and we and other authors have previously demonstrated that PINK1 deficiency results in impaired respiration with inhibition of complex 1, reduced substrate availability, and rotenone-like increased production of ROS in mitochondria [24–26]. PINK1 deficiency also results in an inability to handle cytosolic calcium challenges due to an impairment of mitochondrial calcium efflux, that leads to mitochondrial calcium overload. A combination of ROS production and mitochondrial Ca^{2+} initiates opening of mitochondrial permeability transition pore (PTP), which allows translocation of proapoptotic molecules from the mitochondria to the cytosol, in order to trigger apoptotic cell death [27, 28]. Early opening of the PTP has been demonstrated in PD models, for example, the toxic (MPP^+) model and the genetic PINK1 deficiency model [24, 29]. PINK1 deficiency also results in abnormal mitochondrial morphology reflecting an altered balance of mitochondrial fission/fusion, and in Drosophila models PINK1 appears to genetically interact with the mitochondrial fission/fusion machinery proteins. More recently an emerging function of PINK1 has been its concerted action with parkin in the regulation of clearance of damaged mitochondria via autophagy.

Loss of the mitochondrial membrane potential stabilises PINK1 expression, leading to PINK1-dependent recruitment of parkin to the mitochondria. Ubiquitination of a number of substrates by parkin then activates the autophagy of damaged mitochondria. Mutations in either parkin or PINK1 thus result in a failure of mitophagy, and an accumulation of dysfunctional mitochondria with increased ROS production, further increasing the oxidative stress on the neuron [30–32].

Abnormal aggregation of protein is a characteristic feature of neurodegenerative disease, and it appears that mitochondrial dysfunction and ROS production may influence the aggregation of the protein, α-synuclein, which accumulates in all PD brain. Mutations in α-synuclein gene cause a familial form of autosomal dominant PD. Expression of mutant α-synuclein in mouse models or neurons results in mitochondrial dysfunction and increased ROS production [33, 34]. However, protein oxidation induced by mitochondrial ROS production is also required for α-synuclein oligomerization [35] and toxicity. α-synuclein has recently been shown to bind to mitochondria and induce mitochondrial fragmentation through the inhibition of membrane fusion, a phenotype that interestingly can be rescued by coexpression of the mitochondrial PD proteins PINK1, DJ-1, and parkin [36].

Mitochondrial dysfunction and ROS production may play a role in the pathogenesis of AD. A reduction in complex IV activity has been demonstrated in mitochondria from the hippocampus and platelets of AD patients, as well as in AD animal models and AD cybrid cells in reviewed [37]. Accumulation of βA leads to oxidative stress, mitochondrial dysfunction, and energy failure prior to the development of plaque pathology [38]. Deregulation of calcium homeostasis has been demonstrated in AD, with βA causing increased cytoplasmic calcium levels and mitochondrial calcium overload, resulting in increase in ROS production and opening of the PTP [39, 40]. βA is able to induce opening of PTP in isolated mitochondria [41, 42] and primary astrocytes [43–45]. Furthermore βA may directly interact with cyclophilin D (a PTP component) forming a complex in the mitochondria that has reduced threshold for opening in the presence of mPTP inducers. Prevention of PTP opening by inducing cyclophilin D deficiency (molecular inhibition of PTP opening) is also able to improve mitochondrial function and learning/memory in an aging Alzheimer's disease mouse model [46]. A peturbation in mitochondrial dynamics has also been described in AD human brain and cell models. Fragmented mitochondria are seen in AD hippocampus in association with a downregulation of mitochondrial fusion proteins (MFN-1, MFN-2, OPA-1), with an increase in expression of the mitochondrial fission protein Fis-1 [47].

2.3. Mechanisms of Oxidative Stress: ROS Production via NADPH Oxidase. Although there are several different proposed mechanisms of neurodegeneration in Alzheimer's Disease (AD), there is good evidence for the presence of oxidative stress and the involvement of NADPH oxidase in this disease. Activation of NOX2 has been demonstrated in brains of AD patients [48], with upregulation of NOX1 and NOX3 in early stage postmortem AD brain [49].

The crucial role for NADPH oxidase in AD has also been confirmed in animal models. Thus, NOX2 deficiency improved the outcome in a mouse model of AD. Mice that overexpress the Swedish mutation of APP (Tg2576, which leads to Aβ fragment accumulation) were crossed with NOX2-deficient mice. In this model, the absence of functional NOX2 was protective and prevented the negative effects of βA deposition. Neuronal oxidative stress was abrogated, and behavioural deficits improved in both young and aged Tg2576/NOX2-deficient mice [50, 51].

The role of NADPH oxidase in AD has also been suggested at a cellular level. Amyloid-beta (βA) induced direct activation of NADPH oxidase in rat primary culture of microglial cells and human phagocytes [52]. βA activates microglial NOX through B-class scavenger receptor CD36 [53]. Active NADPH oxidase transfers protons across the membrane and, for normal functioning, requires opening of an ion/anion channel for charge compensation [54]. In βA-activated microglia, inhibition of CLIC1 channel inhibited superoxide production and protected cells by blocking the charge compensatory mechanism of NADPH oxidase [55]. βA-induced stimulation of NADPH oxidase may damage surrounding cells because it occurs in combination with massive NO production and the generation of peroxynitrate [56, 57]. Neuroprotective effect of some endogenous compounds, such a hormone melatonin is induced by its antioxidant properties [58, 59].

βA also activates NADPH oxidase by inducing calcium entry into astrocytes but not neurons [39, 40]. The activation of NADPH oxidase results in the generation of oxidative stress, which depolarises the mitochondrial membrane and, in combination with calcium, induces opening of the mitochondrial permeability transition pore (mPTP; Figure 1) [43, 45] as well as changing membrane structure through activation of phospholipase C [60]. This oxidative stress signal is passed to neighbouring neurons, which are more vulnerable than astrocytes. The mechanism by which this occurs is not certain although neuronal production of glutathione, a key antioxidant, requires glutathione precursors derived from extracellular cleavage of glutathione released from astrocytes. It has therefore been suggested that depletion of GSH in astrocytes due to increased oxidant production by NADPH oxidase could diminish GSH release from astrocytes and consequently deplete GSH in neurons [43, 44]. There is much less information available regarding the direct activation of NADPH oxidase in neurons in AD models although there is some evidence that βA and the presenilins exhibit the ability to activate NADPH oxidase in primary neurons [61–63].

In Parkinson's disease (PD), oxidative stress has been demonstrated in both the rotenone and MPTP-induced toxin models. In these models, activation of NADPH oxidase (NOX2) in microglia occurs. [64, 65]. Furthermore, pharmacological inhibition of NADPH oxidase is able to protect mesencephalic dopaminergic neuronal (N27) cells against MPP^+-mediated dopaminergic degeneration [66]. Genetic models of PD also exhibit increased oxidative stress. In one such model, loss of PINK1 function is associated with increased ROS production by NADPH oxidase in midbrain neurons. Interestingly, the NADPH oxidase is activated by

high cytosolic calcium concentration, leading to overproduction of superoxide. ROS production from NADPH oxidase inhibits the plasmalemmal glucose transporter resulting in deregulation of mitochondrial metabolism [24, 26].

Much less information is available about the role of Xanthine Oxidase (XO) in Alzheimer's or Parkinson's diseases. Oxypurinol was able to reduce the production of ROS and protect neurons in the genetic presenilin 2 mouse model of AD [67]. In addition, βA is able to activate production of H_2O_2 in cytosol of neocortical neurons [68]. Inhibitor of XO allopurinol significantly suppressed *OH generation in rat striatum of toxic models of Parkinson's disease induced by paranonylphenol and MPP($^+$) [69], suggesting a potential role for XO in the oxidative stress associated with PD.

2.4. Oxidative Stress Results in Selective Neuronal Degeneration. One of the major features of neurodegenerative disease is the selective vulnerability of different neuronal populations that are affected in a progressive and often stereotyped manner. However, the susceptible neuronal population varies between diseases, despite oxidative stress being implicated as the major pathogenic process in all of them. Thus, the most vulnerable regions of the brain affected in AD such as the entorhinal cortex and the hippocampus CA1 region differ from the most vulnerable regions affected in PD which includes the dopaminergic neurons in the substantia nigra. Even within a region, the subset of neurons affected will be adjacent to a subset of neurons that are spared; for example, in PD, the SNpc dopaminergic neurons are affected while the VTA neurons are spared. Therefore, in addition to a global oxidative stress that affects all neurons, there must be additional factors that determine the selective cell death in each disease.

Certain neuronal groups have high intrinsic levels of oxidative stress and are therefore more vulnerable to additional disease-related oxidative stress. Neurons that have long axons and multiple synapses have high bioenergetic requirements for axonal transport or long-term plasticity. A high ATP demand combined with relative mitochondrial dysfunction will render these groups of neurons far more sensitive to degeneration than other neuronal groups. Different neuronal groups exhibit different degrees of oxidative stress. For example, in the hippocampus CA1 neurons generate higher levels of superoxide anion than CA3 neurons and exhibit higher levels of expression of both antioxidant and ROS-producing genes [70]. Neurons that are exposed to higher levels of cytosolic dopamine; that is, dopaminergic neurons are also exposed to additional oxidative stress produced by the metabolism of dopamine by MAO (which generates hydrogen peroxide) as well as the autooxidation of dopamine (which generates superoxide). Thus endogenous dopamine, as well as exogenous treatment with levodopa (used in PD) may be a further source of oxidative stress that may worsen pathogenesis [71, 72]. Higher levels of intrinsic oxidative stress may result in mitochondrial dysfunction, which further results in the production of more free radicals and an exacerbation of the cycle of oxidative stress. However, it should be noted that the MAO-induced metabolism of dopamine and production of hydrogen peroxide have an important role in physiological calcium signaling in astrocytes and is not solely a pathological process [73]. Therefore, it is possible that antioxidant treatment may also have an effect on normal signal transmission in the brain.

One interesting hypothesis for the vulnerability of specific neuronal groups in Parkinson's disease has emerged from the discovery that adult substantia nigra pars compacta dopaminergic neurons have an autonomous pacemaker mechanism that utilizes L-type calcium channels resulting in intracellular calcium oscillations. This creates a metabolic stress for such neurons as the repeated and persistent entry of calcium into cells needs to be counterbalanced by ATP demanding pumps to restore the calcium concentration. In fact it has been demonstrated that the opening of these L-type ion channels results in higher levels of oxidative stress in the mitochondria of such neurons [74]. Moreover, other nondopaminergic groups of neurons that are also selectively affected in PD such as the dorsal motor nucleus of the vagus, the serotonergic neurons of the raphe nuclei, and neurons of the locus coeruleus all engage the same L-type calcium channel pacing mechanism. Therefore, this represents an example of a cell-specific factor that renders certain neuronal groups highly vulnerable to the disease process.

2.5. Use of Antioxidant Therapy in Neurodegenerative Disease. Based on the hypothesis that oxidative stress is pathogenic in neurodegenerative disease, the rationale for the use of antioxidants as therapies is clear. And indeed the initial demonstration of the benefits of antioxidants in animal and cell models of disease was promising. Perhaps the most widely studied of these antioxidant therapies have been vitamin E (the major scavenger of lipid peroxidation in brain), vitamin C (intracellular reducing molecule), and coenzyme Q10 (transfers electrons from complexes I and II to complex III in respiratory chain).

Vitamin E supplementation in an AD mouse model resulted in improved cognition and reduced βA deposition [75]. The reduction of amyloid deposition was particularly noted in young AD mice [76]. Daily injections of vitamin C in the APP/presenilin 1 mouse model significantly reduced memory deficits.

Conversely, vitamin E has not been shown to have a protective effect in the commonly used toxin-based model of MPTP-induced PD. However, the molecule coenzyme Q10 has been shown to have multiple protective effects within the mitochondria and therefore has been widely tested as a potential therapy. Administration of CoQ10 protects MPTP-treated mice from dopaminergic neuronal loss and also attenuated α-synuclein aggregation. Neuroprotection by CoQ10 in an MPTP-primate model has also been reported [77].

Despite the promise of these animal studies, there has been no proven benefit for the use of vitamin E and/or vitamin C in either AD or PD from large randomised controlled trials [78]. These trials have been reviewed in detail in [79, 80]. Furthermore, a large meta-analysis of vitamin E clinical trials, CoQ10 trials, and a glutathione trial in PD concluded that there were only minor treatment benefits in the CoQ10 trials that may have been due to improvement in

the respiratory chain deficit rather than a direct antioxidant action [81]. None of the trials have shown significant benefit to warrant recommendation for use in the clinical setting.

The disappointing translation of the oxidative stress hypothesis into useful therapy in human disease raises several issues regarding extrapolation of results from animal studies to the clinical setting. All animal models are limited in recreating the human disease as they do not recapitulate the long-time frame and gradual accumulation of age-related changes that characterise late onset sporadic neurodegenerative diseases in humans. From much of the animal model data, it appears that antioxidants must be administered at an early stage in the disease where the process influences pathogenesis most, and therefore the use of antioxidants in established late disease in humans may be ineffective. There are several pharmacodynamic and pharmacokinetic considerations such as the bioavailability of reducing molecules in the human brain in the doses used in animal models and furthermore the effective targeting of such molecules to the mitochondria in human brain. Many therapies have used antioxidants that act as scavengers, rather than blocking the source of the ROS production, and this may be less effective. Finally it is possible that there are several different producers of oxidative stress in each disease, and that these may need to be targeted separately but simultaneously by multiple therapies in order to effectively reduce oxidative stress and slow disease progression.

There are many processes that have been implicated in the pathogenesis of neurodegeneration including protein misfolding and aggregation, abnormal kinase-signalling pathways, neuronal calcium dysregulation, and impaired synaptic transmission. Many of these interact with, and are exacerbated by, oxidative stress. With the mounting robust evidence of the role of oxidative stress in pathogenesis, it remains likely that the hypothesis that oxidative damage is critical in disease is indeed true, and moreover that careful targeting of this process should serve to ameliorate neurodegenerative disease.

3. BOX 1: ROS Producers in Mammalian Brain

3.1. NADPH Oxidase. NADPH oxidase is a multisubunit enzyme complex that was first described in phagocytes [82]. NADPH oxidase is a member of the NOX gene family, also called NOX2 and phagocytic oxidase (PHOX). Seven NOX genes have been identified: NOX1 to 5 and DUOX1 and 2. Very little is known about the role of NOX5 and DUOX1 and 2 in the CNS. The majority of the NOX enzyme expressed in the brain is NOX2 although some evidence exists for the CNS localization of NOX1, NOX4, and possibly also NOX3. The NOX2 enzyme complex consists of the membrane-bound cytochrome b558 ($p22^{PHOX}$ and the enzymatic subunit, $gp91^{PHOX}$), several cytosolic proteins ($p47^{PHOX}$, $p67^{PHOX}$, and $p40^{PHOX}$), and the Rac G-protein. NADPH oxidase is activated when the cytosolic subunits are phosphorylated and Rac is activated in the cytosol, resulting in their translocation to the membrane and formation of the active NADPH oxidase complex with cytochrome b558 [83]. The enzyme transfers the proton across the membrane, and the end product of the enzyme is superoxide. The NOX family of proteins is expressed on diverse cell types, and NADPH oxidase is present in microglia [84], neurons, and astrocytes [85, 86]. The physiological role of NOX enzymes in brain cells is still unclear and described mostly as an effect of superoxide produced by NADPH oxidase [87].

3.2. Xanthine Oxidase. Xanthine oxidase or xanthine dehydrogenase are two convertible forms of Xanthine oxidoreductase. It is a complex molybdoflavoenzyme that is readily available from milk and widely distributed in mammalian tissues and is generally accepted to be a key enzyme of purine catabolism [88]. XO catalyses the oxidation of a wide range of substrates and can pass electrons to molecular oxygen to produce uric acid, superoxide, and hydrogen peroxide. Under normal conditions the enzyme exists in the form of xanthine dihydrogenase. Ca^{2+}-stimulated proteases cause the irreversible partial cleavage of xanthine dehydrogenase to xanthine oxidase, which in turn catalyzes the oxidation of hypoxanthine to xanthine.

The role of XO in ischaemic cell injury was demonstrated in the 1980's. Deprivation of oxygen leads to the metabolism of ATP and accumulation of hypoxanthine. Hypoxia induces a rise in the levels of intracellular calcium, which activates a protease that converts XDH, predominantly in vivo, into XO. Concomitantly, purines are catabolised, and hypoxanthine accumulates. On reperfusion, oxygen is again available and, in the presence of XO and hypoxanthine, is reduced to hydrogen peroxide and superoxide [88, 89].

3.3. Mitochondria. It has become dogma that mitochondria are a major source of ROS. In pathological conditions, this organelle actually produces less-free radicals than cytosolic enzymes such as NADPH oxidase. However, mitochondria (electron transport chain-ETC), in contrast to other cellular producers of ROS, generate free radicals all the time. Mitochondria, which harbor the bulk of oxidative pathways, are packed with various redox carriers and centers that can potentially leak single electrons to oxygen and convert it into superoxide anion, a progenitor ROS. The initial observations of ROS production in mitochondrial fragments came as early as 1966 but passed almost unnoticed until 1971 when Loschen, Flohe, and Chance demonstrated for the first time that succinate supported H_2O_2 production by intact pigeon heart mitochondria [90–92]. Depending on the metabolic conditions, isolated mitochondria produced superoxide in respiratory complex I (in the direction of the matrix), complex III (in direction of matrix and to outside of matrix) [90]. ROS in mitochondria can also be generated in several enzymes including aconitase and α-ketoglutarate dehydrogenase complex [90, 93]. The production of superoxide by the ETC in mitochondria is dependent on the value of mitochondrial membrane potential.

Because of the constant production of free radicals, mitochondria possess a very efficient antioxidant system in the matrix. Overproduction of ROS in mitochondria or in other sources and changes in the antioxidant system lead to

imbalance and induce oxidative stress and neurodegeneration.

Inhibition of neuronal respiration by oxygen deprivation or chemical ischemia leads to a significant increase in the generation of ROS in mitochondria [94]. This effect can be reduced by the application of mitochondrial uncouplers, and a number of reports have demonstrated significant neuroprotection by mild uncoupling with UCP2 in cerebral stroke [95]. In addition, mutations in mitochondrial complexes I–IV induce inhibition of respiration and activation of ROS production [96, 97] and selective neuronal cell death [98].

3.4. Monoamine Oxidase. The mitochondrially located (outer membrane) flavoenzymes monoamine oxidase A (MAO A) and monoamine oxidase B (MAOB) represent one of the most extensively studied enzyme group. This long-term interest stems from their role in the oxidative catabolism of important amine neurotransmitters, including serotonin, dopamine, and epinephrine [99]. MAO-A and MAO-B are encoded by separate genes that correspond to different amino acid sequences that are ~70% identical. In the CNS they are expressed in neurons (MAO-A) and glial cells (MAO A and B). MAO breaks down monoamines using FAD and results in the production of aldehydes. The FAD-$FADH_2$ cycle generates hydrogen peroxide.

4. BOX 2: The Antioxidant System

4.1. Antioxidant System. Cellular levels of ROS are controlled by antioxidant enzymes and small-molecule antioxidants.

4.1.1. Superoxide Dismutase. As major antioxidant enzymes, superoxide dismutases (SODs), play a crucial role in scavenging $O_2^{\bullet -}$. The superoxide dismutase family is specialized in eliminating superoxide anion radicals derived from extracellular stimulants, including ionizing radiation and oxidative insults, together with those primarily produced within the mitochondrial matrix as byproducts of oxygen metabolism through the electron transport chain [100]. Three distinct isoforms of SOD have been identified and characterized in mammals: copper-zinc superoxide dismutase (Cu/ZnSOD; encoded by the *sod1* gene), manganese superoxide dismutase (MnSOD; encoded by the *sod2* gene), and extracellular superoxide dismutase (ECSOD; encoded by the *sod3* gene). These forms of SOD exhibit similar functions, but characteristics of their protein structure, chromosome localization, metal cofactor requirements, gene distribution, and cellular compartmentalization are distinctly different from one another [100].

4.1.2. Glutathione Peroxidases. Glutathione peroxidase is the general name for a family of multiple isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor ($H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O$). In mammalian tissues, there are four major selenium-dependent glutathione peroxidases (GPX) and phospholipid hydroperoxide glutathione peroxidase, which incorporates cysteine instead of selenocysteine in the conserved catalytic motif [101]. GPX1 is known to localize primarily in glial cells, in which GP activity is tenfold higher than in neurons [101].

4.1.3. Catalase. Catalase is a ferriheme-containing enzyme that is responsible for the conversion of hydrogen peroxide (but not other peroxides) to water [5]. It is localised in peroxisomes and may also be found in cytoplasm and mitochondria. It has a minor role at low levels of hydrogen peroxide generation but becomes more important at higher levels of hydrogen peroxide production.

4.2. Nonenzymatic Antioxidants.

4.2.1. GSH. The main antioxidant in CNS, glutathione (GSH), is the most abundant small molecule, nonprotein thiol in cells (present in millimolar concentration in the brain) [102]. It consists of a tripeptide of glutamate, cysteine and glycine characterized by a reactive thiol group and γ-glutamyl bond. Reduced GSH can nonenzymatically act directly with free radicals, notably superoxide radicals, hydroxyl radicals, nitric oxide, and carbon radicals for their removal. GSH peroxidase and GSH reductase can act enzymatically to remove H_2O_2 and maintain GSH in a reduced state [102].

4.2.2. Vitamin E. The role of vitamin E in the central nervous system is not fully understood although it is a lipid soluble molecule with antioxidant function. It appears to neutralize the effect of peroxide and prevent lipid peroxidation in membranes.

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Review Article

Schisandrin B as a Hormetic Agent for Preventing Age-Related Neurodegenerative Diseases

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Oxidative stress and mitochondrial dysfunction have been implicated in the pathogenesis of neurodegenerative diseases, with the latter preceding the appearance of clinical symptoms. The energy failure resulting from mitochondrial dysfunction further impedes brain function, which demands large amounts of energy. Schisandrin B (Sch B), an active ingredient isolated from Fructus Schisandrae, has been shown to afford generalized tissue protection against oxidative damage in various organs, including the brain, of experimental animals. Recent experimental findings have further demonstrated that Sch B can protect neuronal cells against oxidative challenge, presumably by functioning as a hormetic agent to sustain cellular redox homeostasis and mitoenergetic capacity in neuronal cells. The combined actions of Sch B offer a promising prospect for preventing or possibly delaying the onset of neurodegenerative diseases, as well as enhancing brain health.

1. The Role of Mitochondrial Dysfunction and Mitoenergetic Failure in the Development of Age-Related Neurodegenerative Diseases

Although the etiologies of age-related neurodegenerative diseases are different and multifactorial, mitochondrial dysfunction has been recognized as a common factor in the pathogenesis of these diseases [1, 2]. The common mechanistic features of most age-related neurodegenerative diseases involve the mitochondrial-derived free radical generation and the existence of a hypometabolic state (i.e., a cellular energy deficit) which results from mitochondrial functional impairment [3–6].

The brain is critically dependent on energy supply in order to sustain various neuronal processes such as induction of action potentials and neurotransmission, [7]. In this regard, mitochondria generate approximately 90% of the required energy through oxidative phosphorylation, in which the electron transport process unavoidably results in reactive oxygen species (ROS) generation [8]. Thus, while being the sites of ATP generation, mitochondria are also a significant source of ROS such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot -}$) [9]. Functional impairment of mitochondria, resulting in excessive ROS production and mitoenergetic failure, can result in subtle pathological alterations to neuronal cells. In this regard, aberrations at the level of organelles involved in cellular energetics have been implicated in more than 40 different pathological conditions [10].

Emerging evidence has shown that mitochondrial ROS-induced oxidative stress is involved in the pathogenesis of neurodegenerative diseases [2, 11, 12]. Mitochondrial dysfunction involving electron transport chain (ETC) failure and ROS-mediated cellular damage is common features of Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [13–16]. ROS can harm cells by causing random oxidative damage to essential cellular components including DNA, proteins, and lipids. The high susceptibility of the brain to oxidative stress is mainly due to the relative deficiency of antioxidant enzymes, such as superoxide dismutase (SOD), Se-glutathione peroxidase (GPX), glutathione reductase (GR), and catalase (CAT) in this tissue [17, 18]. Furthermore, brain mitochondria are particularly sensitive to oxidative damage and show

a slow turnover rate; the accumulation of dysfunctional mitochondria, therefore, can further exacerbate the oxidative stress in brain tissue [19].

In addition to serving as a cellular source of energy to brain tissue, mitochondria also play a critical role in other important cellular processes, including intermediary metabolism, calcium homeostasis, intracellular signaling, and apoptosis through the generation of intracellular oxidants such as H_2O_2 [9]. Mitochondria-derived ROS can affect overall cellular and mitochondrial function by altering glutathione redox status and/or the posttranslational modification of proteins structure and function via oxidative processes [20, 21]. Redox-sensitive signaling pathways, such as glycogen synthase kinase (GSK) insulin signaling, the C-Jun-NH_2-terminal kinase (JNK) proapoptotic, and protein kinase B (Akt) prosurvival pathways, are found to be dysregulated during neurodegeneration associated with enhanced mitochondrial ROS production [22–24]. The release of oxidants ($O_2^{\cdot -}$, H_2O_2, NO) from mitochondria into the cytosol further results in chemical (posttranslational) modification of intracellular proteins subsequent to the changes in cellular redox status. Under conditions of oxidative/nitrosative stress, exposure of proteins to ROS or reactive nitrogen species (RNS) can result in oxidation/nitrosylation of protein thiols, nitration of tyrosine residues, and S-glutathionylation involving the formation of mixed disulfides between protein sulfhydryls and glutathione, all of which can lead to protein structural and functional alterations. For instance, the posttranslational modification of key enzymes involved in energy metabolism, such as pyruvate dehydrogenase (PDH), aconitase, and succinyl-CoA transferase (SCOT), often causes a loss of protein function and results in glucose hypometabolism and mitoenergetic failure [3, 23, 25]. In this regard, several clinical studies have shown that before the occurrence of any pathological changes in the brain, impaired glucose metabolism in cerebral tissues is the earliest and consistent abnormality observed in AD and mild cognitive impairment (MCI) [26]. At the molecular level, the oxidative stress-induced impairment in mitochondrial energy-transducing capacity can lead to the opening of mitochondrial permeability transition (MPT) pores [27]. This process, which is accompanied by a collapse of mitochondrial membrane potential and energy production, can result in the reverse operation of ATP synthase, thereby further accelerating ATP depletion resulting in the loss of ion homeostasis, and ultimately, necrotic cell death [28]. MPT pore opening also causes the leakage of cytochrome *c* from mitochondria into the cytoplasm and triggers a cascade of events such as caspase-9 activation that eventually leads to mitochondrion-driven apoptosis [29]. In summary, enhanced oxidative stress and mitoenergetic failure resulting from mitochondrial dysfunction can all collectively contribute to the pathogenesis of neurodegenerative diseases.

In this paper, we will review the neuroprotective effects of schisandrin B (Sch B) and its potential application as a hormetic agent for favorably influencing the course of neurodegenerative diseases. In addition, we will discuss the biochemical basis of the Sch B-afforded neuroprotection and its possible role in preventing mitoenergetic failure, thereby improving brain health.

2. Dietary Schisandrin B Mitigates Age-Related Impairment in Mitochondrial Antioxidant Status and Functional Capacity in Brain Tissues

Sch B is the most abundant dibenzocyclooctadiene derivative found in Fructus Schisandrae (FS), a traditional Chinese herb commonly used for the treatment of viral and chemical hepatitis. According to Traditional Chinese Medicine (TCM) theory, FS is classified as a "Qi-invigoration" herb under the "Yang" family. Holistically, while "Yang" is viewed as a manifestation of body function supported by various organs, "Qi" is regarded as a vital substance, which is fundamental to life and provides energy for the human body [30]. FS is believed to nourish the "Qi" of the heart, kidney, liver, lung, and spleen, with improved energy utilization and increased longevity. In order to validate this time-honored TCM theory, various scientific investigations have been undertaken in several laboratories, including our own, to investigate the effectiveness of Sch B in ameliorating impairments in mitochondrial antioxidant status and functional capacity. Early findings have confirmed the beneficial effect of Sch B on liver function, particularly in enhancing the detoxification of xenobiotics and the regeneration of damaged liver [31]. Emerging evidence demonstrates the protective effect of Sch B against free-radical-induced tissue injury in various tissues, including the brain [32–36].

Data from aging studies provide additional information on the ability of Sch B to mitigate age-related impairments in brain mitochondrial antioxidant status and functional capacity. Progressive impairment in mitochondrial antioxidant status and a decline in mitochondrial function have been documented in brain tissue of aging rodents [37, 38]. Decreases in the levels of mitochondrial reduced glutathione (GSH) and α-tocopherol (α-TOC), as well as the activities of GPX and manganese SOD (MnSOD) in brain tissue are invariably associated with the increased mitochondria-driven production of ROS as a function of aging in experimental animals [37, 38]. In agreement with TCM theory, experimental data has shown that long-term dietary supplementation with Sch B significantly enhances mitochondrial antioxidant status, stimulates mitochondrial respiratory function, and maintains mitochondrial structural integrity, as well as decreasing ROS production in the brain of experimental animals [37, 38]. Correlation analysis has further indicated that the protection afforded by Sch B against age-dependent decline in mitochondrial antioxidant components, particularly MnSOD, may be relevant to survival enhancement [37]. MnSOD has been recognized as a major antioxidant enzyme protecting the brain against both oxidative and nitrative stress by eliminating $O_2^{\cdot -}$ and preventing the generation of highly reactive peroxynitrite ($ONOO^-$) arising from the reaction between NO with $O_2^{\cdot -}$. High levels of oxidative and nitrative

stress have been implicated in pathogenesis of neurodegenerative conditions such as AD, PD, and Huntington's disease (HD) [39]; animals with MnSOD deficiency have been shown to be more vulnerable to the mitochondrial neurotoxin malonate, 3-nitropropionic acid, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), both of which can induce oxidative stress [40]. Furthermore, the alteration of redox status as a result of MnSOD-mediated ROS elimination has also been proposed to regulate specific stress-responsive genes related to antiapoptotic substances, such as cyclin B1, cyclin A, GADD153, and 14-3-3 zeta [41].

The enhancement of mitochondrial function (i.e., respiratory activity) by Sch B has been shown to stimulate mitochondrial ATP generation in aging mouse brains [37]. Improvement in respiratory function has been proposed to stimulate the activity of sirtuin [42], which can activate antiapoptotic, anti-inflammatory, and antistress responses, as well as modulate the aggregation of proteins associated with neurodegenerative conditions, thereby preventing or delaying the onset of neurological damage [43]. Enhanced regeneration of NAD as a result of Sch B-induced respiratory functional improvement can also be beneficial to neuronal health. Studies have demonstrated that exposure of neuronal cells to NAD can prevent axonal degeneration, a pathophysiological process that often precedes the death of neuronal cell bodies in PD and AD, through the activation of the NAD-dependent deacetylase sirtuin-1 (SIRT1) pathway [44, 45].

3. Neuroprotection Afforded by Schisandrin B

In addition to the aforementioned aging research, studies utilizing various models of brain oxidative challenge have further advanced our understanding of the neuroprotective effect of Sch B against oxidative insult. In the mouse model of cerebral damage induced by the prooxidant tertbutylhydroperoxide (t-BHP), Sch B treatment prevented the increase in cerebral lipid peroxidation and impairment in GSH antioxidant status produced by intracerebroventricular injection of t-BHP [34]. The restoration of GSH levels by Sch B is important for neuronal viability because an imbalanced glutathione redox status can trigger the sulfhydryl groups of cysteine sulfinic acids to react with other sulfhydryl groups, including that of glutathione, with the resultant formation of disulfides or mixed disulfides. The modification of protein sulfhydryl groups can lead to alterations in protein function, with disruption in normal cellular physiology, as has been shown in the inactivation of the SH-group containing enzyme glucose 6-phosphate dehydrogenase (G6DPH) by ROS [46]. Transgenic overexpression of G6PDH in mouse dopaminergic nigrostriatal neurons has been shown to reduce their sensitivity to MPTP, presumably through increasing the activity of this rate-limiting enzyme in the hexose monophosphate shunt, which provides NADPH required for the regeneration of GSH from its oxidized form and sulfhydryl groups of oxidatively modified antioxidant enzymes [47].

In a rat model of cerebral ischemia/reperfusion (I/R) injury induced by clamping of both carotid arteries in anesthetized animals, the neuroprotective property of Sch B has been shown to be associated with the maintenance of antioxidant status as well as the structural integrity of mitochondria [32]. The neuronal injury caused by I/R challenge involves pathophysiological mechanisms such as ROS production and intracellular calcium overload; the resultant perturbation of cellular redox homeostasis and mitochondrial permeability transition pore opening eventually leads to necrotic and/or apoptotic cell death [48]. Long-term treatment with Sch B (10 mg/kg/day, p.o., for 15 days) has been shown to afford protection in the abovementioned model of I/R-induced cerebral injury; as evidenced by a significant increase the percentage of viable tissue after I/R challenge, which was determined by quantifying the area of 2,3,8-triphenyl tetrazolium chloride-stained tissue in brains of control and Sch B-pretreated rats [32]. The protection was associated with enhancement of mitochondrial antioxidant components and preservation of mitochondrial integrity, as evidenced by increased GSH, α-TOC levels, and MnSOD activity as well as a reduced sensitivity of the Ca^{2+}-induced permeability transition and a reduction in cytochrome *c* release [32]. The preservation of mitochondrial integrity by long-term Sch B treatment is of crucial importance for the mitoenergetic capacity of cerebral mitochondria. This notion is supported by the observation that the ATP-generating capacity of cerebral mitochondria was increased by long-term Sch B treatment in brain tissue of both control and I/R-challenged rats [32].

Recently, Giridharan et al. have demonstrated that Sch B prevents memory deficits in mice induced by scopolamine, a muscarinic antagonist that induces central cholinergic blockade [36]. Since cholinergic neurotransmission in the basal forebrain is believed to play an important role in learning and memory, scopolamine toxicity may provide a useful model of cognitive impairment in rodents and humans [36]. The scopolamine-induced cognitive deterioration resembles the memory disturbances observed in AD, which are associated with an increase in acetylcholinesterase (AChE) activity and decreased acetylcholine levels [49]. In the aforementioned study, Sch B treatment significantly inhibited AChE activity, resulting in increased acetylcholine levels in the brain, accompanied by an amelioration of the learning and memory impairment caused by scopolamine. Although the detailed inhibitory mechanism of Sch B on AChE remains to be investigated, it was postulated that metabolites of Sch B (see below) may be responsible for the AChE inhibition [36]. The neuroprotective effects of Sch B were also found to be closely related to its antioxidant activity, in which the antioxidant depletion associated with scopolamine toxicity was ameliorated by Sch B treatment, as indicated by enhancements in GSH levels, GPX, and SOD activities as well as a reduction in malondialdehyde and nitrite levels in brain tissue [36].

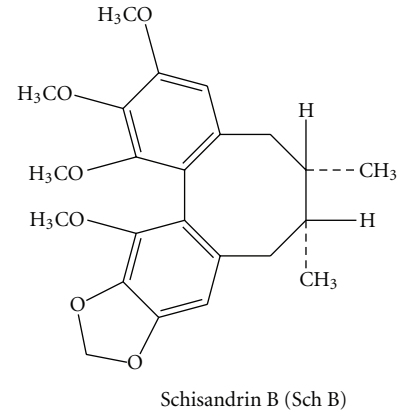
Taken together, the beneficial effects of Sch B on mitochondrial antioxidant status and mitochondrial function may suggest a new therapeutic approach for the prevention

and/or treatment neurodegeneration, thereby promoting brain health in aging individuals.

4. Biochemical Mechanism Underlying Schisandrin B Cytoprotection Against Oxidant Injury in Neuronal Cells

Sch B is a dibenzocyclooctadiene derivative with one methylenedioxy group which can be dealkylated by a cytochrome P-450 (CYP-) catalyzed reaction (Figure 1). In vivo, Sch B was metabolized to yield three main phase I metabolites, in which several oxidation routes appeared to be involved: (1) hydroxylation of an alkyl substitute and (2) demethylation of the OCH3 groups on the aromatic rings [50]. The metabolites were detectable in bile and urine of rats. Sch B could also be demethylated by demethylase presented in red blood cells and then further metabolized to produce phenolic hydroxyl group [51]. The metabolism of Sch B ultimately leads to the formation of a quinone and a subsequent low level ROS production via redox cycling [52, 53]. The modest amount of ROS generated during Sch B metabolism has been shown to elicit a glutathione antioxidant response, which results in an enhancement of glutathione antioxidant status that is closely associated with a decreased susceptibility of various tissues, including the brain, to oxidative injury [32, 54, 55].

The causal relationship between Sch B-induced GSH enhancement and its cytoprotection against oxidative injury in neuronal cells is further supported by a recent study in differentiated PC12 dopaminergic neuronal cells [56]. Paraquat (PQ), a Parkinsonism-inducing agent, was used to induce oxidative injury in differentiated PC12 cells, and the extent of neuronal damage, as indicated by GSH depletion and cell death, was significantly reduced by pretreatment with (−)Sch B, a potent Sch B stereoisomer [56]. The cytoprotective effect of (−)Sch B in PQ-challenged cells was abrogated by inhibitors of γ-glutamylcysteine ligase (GCL) and GR, suggesting the crucial involvement of GSH regeneration and GSH synthesis in the protection against oxidative stress [56]. Further investigations in PC12 cells subjected to an acute t-BHP challenge have revealed that while the initial GSH depletion induced by the peroxide was reduced through the GR-catalyzed regeneration of GSH in (−)Sch B-pretreated cells, the subsequent enhancement of GSH recovery was mainly mediated by the GCL-catalyzed synthesis of GSH [56]. The results suggested that (−)Sch B treatment may increase the resistance of dopaminergic cells to oxidative stress both by reducing the extent of oxidant-induced GSH depletion and enhancing GSH recovery. A comprehensive study using cultured cell lines derived from various tissues has further demonstrated that the enhancement of GR-mediated GSH regeneration is a universal protective mechanism afforded by Sch B in response to an acute oxidant challenge. The study has also shown that acute t-BHP-induced GSH depletion is significantly reduced by Sch B treatment, in which the activity of GR was found to be enhanced [57]. This finding is consistent with the postulation that GR-catalyzed GSH regeneration is crucial for the maintenance of cellular glutathione redox status, and hence cell survival, even in the absence of oxidative stress [58, 59].



Schisandrin B (Sch B)

Figure 1: Chemical structures of schisandrin B.

Although the molecular mechanism underlying Sch B-induced neuroprotection remains to be elucidated, the low levels of ROS produced during CYP-mediated Sch B metabolism have been shown to activate several different redox-sensitive signal transduction pathways, with the eliciting of protective cellular responses [60, 61]. Our laboratory has shown recently that (−)Sch B caused a dose-dependent and sustained increase in ROS production as well as a time-dependent activation of mitogen-activated protein kinase (MAPK), particularly extracellular signal-regulated kinases (ERK)1/2 [60, 61]. The MAPK activation was followed by an enhanced translocation of NF-E2-related factors 2 (Nrf2) to the nucleus and the eliciting of a glutathione-dependent antioxidant response in cultured hepatocytes and cardiomyocytes [60, 61]. Nrf2 functions as a redox-sensitive transcription factor that is translocated from the cytosol to the nucleus upon phosphorylation by upstream kinase and then binds to the antioxidant response element consensus sequence to induce a glutathione antioxidant response through the expression of antioxidant proteins such as GR, GPX, and glutathione transferase (GST). Since the eliciting of adaptive responses to oxidative stress often requires one or more members of the MAPK cascade, the possible involvement of oxidative stress-sensitive c-Jun N-terminal kinases (JNK) and p38 MAPK (p38) kinases in the neuronal model cannot be excluded. In addition to MAPK-mediated phosphorylation, Nrf2 has also been shown to be phosphorylated by protein kinase C (PKC) or phospoinositol-3 kinase (PI3K) under conditions of oxidative stress, and the activation of two or more of these pathways may be required to achieve cytoprotection in a cell type- and stimulant-dependent manner [62]. Therefore, the information obtained from hepatocytes and cardiomyocytes, when viewed in the light of established biochemical mechanism of the Sch B-induced glutathione-dependent protection observed in neurons, suggests a new approach for future investigation of the involvement of JNK, p38, PKC, and/or

PI3K in the Nrf2-mediated signaling pathways in Sch B-induced neuroprotection.

A recent study of Sch B protection against cisplatin (cis-diamminedichloroplatinum II, cDDP)-induced neurotoxicity has revealed the involvement of nuclear factor Kappa B (NF-κB) [35]. cDDP is a chemotherapeutic agent used for the treatment of various solid tumors. However, its clinical use is limited by its adverse effects, particularly ROS-mediated neurotoxicity which can produce severe cognitive dysfunction [63]. As assessed by the passive avoidance performance task test, cDDP-treated mice were found to develop both short-term and long-term memory deficits that could be significantly improved by Sch B treatment. In this study, the ability of Sch B to ameliorate memory deficits was associated with its ability to suppress the activation of NF-κB. The inhibition of NF-κB by Sch B was further demonstrated in this study by the reduction of the downstream activation of p53 and caspase 3. NF-κB has been well established as a common mediator of cDDP-induced cytotoxicity and has been implicated in many other neurodegenerative diseases such as AD, PD, and HD [35]. Therefore, the ability of Sch B to modulate NF-κB, p53, and caspase-3 signaling pathways further strengthens the prospect of its potential use for preventing or ameliorating neurodegenerative disorders.

5. Potential Role of Schisandrin B as a Hormetic Agent in Preventing Mitoenergetic Failure and Improving Brain Health

To date, the use of antiapoptotic agents to prevent neurodegenerative disorders has not been successful, mainly due to the toxicity and the associated risk of carcinogenesis of the compounds in question. On the other hand, the effectiveness of free radical scavenging antioxidants in achieving neuroprotection requires high concentrations that are not readily achievable in brain tissue. Therefore, in recent years, there has been a growing interest, as supported by a large volume of experimental evidence, in the possible use of hormetic agents as a novel therapeutic alternative in preventing various pathological conditions, including those associated with neurodegeneration [64].

By definition, hormesis refers to an adaptive response of cells and organism to a moderate stress and a hormetic response is a biphasic dose-response phenomenon in which a chemical/stimulus (e.g., ionizing radiation, heat stress, or ROS) has a beneficial effect on maintaining cellular homeostasis at low doses/levels but causes a toxic effect at high doses/levels [65, 66]. A compelling body of evidence suggests that the low-level mitochondrial ROS-induced oxidative stress produced by caloric restriction, hypothermia, or hyperbaric oxygen conditions elicits adaptive cellular signaling/responses that can result in an increased stress resistance, which maintains homeostasis and promotes longevity, thus providing the basis of mitochondrial hormesis or "mitohormesis" [67–70]. However, all the aforementioned conditions are generally impractical to achieve in humans. Therefore, the approach to achieving mitohormesis using hormetic agents to enhance endogenous mitochondrial antioxidant status and functional capacity may offer a promising prospect for preventing or possibly delaying the onset of age-related neurodegenerative diseases.

In this regard, Sch B acts as a hormetic agent in cultured cells, with cytoprotective effects predominating at low concentrations and cytotoxicity occurring at high concentrations [71, 72]. Pharmacokinetic study on Sch B showed that a mean value of 96.1 ± 14.1 ng/mL ($\sim 0.25\,\mu$M) of maximum plasma drug concentration and a half-life of about 2 hours can be achieved by oral administration of 15 mg Sch B to healthy male subjects [73]. Toxicology study also demonstrated that the LD_{50} values of orally or intraperitoneally administered petroleum ether extract of FS, which contains 40% (w/w) lignans, were 10.5 and 4.4 g/kg, respectively [74]. No death was observed by administration of a single oral dose of Sch B at 2 g/kg in rats [75]. Furthermore, an intragastric dose of 200 mg of Sch B for 30 days did not significantly affect body weight, blood parameters, and histological parameters of major organs in mice [75]. When given at 10 mg/kg daily for 4 weeks, Sch B also did not affect appetite, liver, or kidney functions, as well as liver histological parameters in dogs [75]. Taken together, Sch B treatment is generally considered to be safe; a single oral dose (0.8 g/kg), multiple doses (200 mg/kg $\times$ 30) as well as dietary supplementation (0.012%, w/w, starting from 9 months of age until death) did not cause any undetectable adverse effects in rodents [34, 37].

As far as bioavailability is concerned, the delivery of therapeutic agents across the blood-brain barrier (BBB) represents a major challenge to therapeutic agents aimed at treating brain disorders. This leads to the failure of therapeutic agents that might otherwise be effective if they can penetrate the BBB. Although whether or not Sch B or its metabolites can penetrate the BBB requires further investigation; the aforementioned observations from Sch B in preventing different types of oxidative stress-induced neuronal injury have provided substantial evidence to support the postulation that Sch B can cross the BBB and exert its actions in brain tissue.

In addition to safety and bioavailability concerns, the ability of a hormetic agent to enhance mitoenergetic capacity is another indispensable property for achieving mitohormesis in brain and promoting brain health. Mitoenergetic failure has emerged as a focus of research on the pathology of various age-related neurodegenerative diseases, with numerous investigations attempting to identify potential therapeutic agents that might enhance mitochondrial functional capacity. While health supplements, such as lipoic acid, curcumin, and resveratrol, have all been shown to increase antioxidant capacity by functioning as free radical scavengers, their effectiveness in enhancing mitochondrial function and thereby helping to maintain neuronal viability has not been fully investigated. On the other hand, the well-established mitochondrial antioxidant and mitoenergetic sparing properties of Sch B make it a particularly promising candidate for promoting brain health. Results from our recent investigation have further demonstrated that

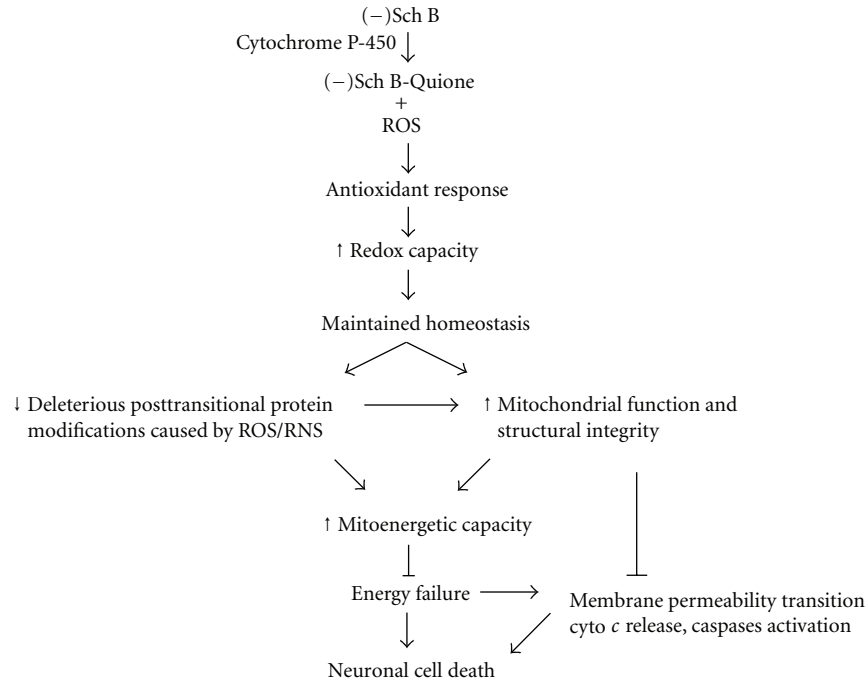


Figure 2: The role of Schisandrin B as a hormetic agent in preventing mitoenergetic failure and neurodegeneration (please refer to the text for details).

Sch B treatment can attenuate the oxidative stress induced by 3-nitroproponic acid, a potent irreversible inhibitor of mitochondrial succinate dehydrogenase that has been used in experimental models of HD, in differentiated PC12 cells and prevent the oxidative stress-induced energy crisis by suppressing the activation of the JNK signaling pathway and the consequent inhibition of PDH [76], a key bioenergetic enzyme which bridges anaerobic and aerobic brain energy metabolism. This observation supports the role of Sch B in enhancing glucose utilization in the brain. Abnormalities in brain glucose metabolism have been implicated in the early stages of various neurodegenerative disease development [77].

As of today, investigations on the beneficial effects of Sch B on neurodegenerative disorders remain predominantly preclinical. However, substantial evidence of Sch B on reducing oxidative stress-induced neuronal injury has shed light on its potential use as a therapeutic agent for neurodegenerative diseases. Recently, clinical studies have adopted the concept of disease modification, in which the improvement on long-term clinical outcomes, presumably by slowing down disease progression, is evaluated in patients. In this connection, the reduction of free radical-induced oxidative stress represents a novel therapeutic approach for retarding the progression of neurodegenerative diseases, such as PD, in that MAO-B inhibitors were used in the therapy in order to prevent oxidative stress generated by the transformation of dopamine to its metabolites 3,4-dihydroxyphenylacetaldehyde and 3,4-dihydroxyphenylacetic acid [78]. This approach has favorably influenced the progression of PD, as assessed by both motor and nonmotor symptoms [79], suggesting the causal relationship between reduction of oxidative stress and neuroprotection. As such, the ability of Sch B to retard disease progression would be a primary endpoint in clinical trial. Clinical parameters that are more relevant to pathogenesis of neurodegenerative diseases should be assessed in the trials.

Taken together, we posit that Sch B can function as a hormetic agent by eliciting a cellular antioxidant response, with resultant enhancement of cellular redox capacity and mitochondrial functional integrity, thereby maintaining cellular homeostasis against oxidative challenge. The occurrence of deleterious posttransitional modification of key bioenergetic enzymes can also be reduced by the improved cellular redox status. The abilities of Sch B to enhance mitoenergetic capacity and fortify antioxidant defense offer a promising prospect in preventing or delaying the onset of neurodegenerative disorders, possibly by inhibiting cell apoptosis (see Figure 2).

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Review Article

The Human Tripeptide GHK-Cu in Prevention of Oxidative Stress and Degenerative Conditions of Aging: Implications for Cognitive Health

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Oxidative stress, disrupted copper homeostasis, and neuroinflammation due to overproduction of proinflammatory cytokines are considered leading causative factors in development of age-associated neurodegenerative conditions. Recently, a new mechanism of aging—detrimental epigenetic modifications—has emerged. Thus, compounds that possess antioxidant, anti-inflammatory activity as well as compounds capable of restoring copper balance and proper gene functioning may be able to prevent age-associated cognitive decline and ward off many common neurodegenerative conditions. The aim of this paper is to bring attention to a compound with a long history of safe use in wound healing and antiaging skin care. The human tripeptide GHK was discovered in 1973 as an activity in human albumin that caused old human liver tissue to synthesize proteins like younger tissue. It has high affinity for copper ions and easily forms a copper complex or GHK-Cu. In addition, GHK possesses a plethora of other regenerative and protective actions including antioxidant, anti-inflammatory, and wound healing properties. Recent studies revealed its ability to up- and downregulate a large number of human genes including those that are critical for neuronal development and maintenance. We propose GHK tripeptide as a possible therapeutic agent against age-associated neurodegeneration and cognitive decline.

1. Introduction

Today the most widespread neurological problems are considered neurodegenerative diseases of aging such as Alzheimer's and Parkinson's that rob people from their golden years causing early debilitation and dependency on special care. Despite all advances in neuroscience in recent decades, age-associated cognitive decline resulting from neurodegenerative processes in the brain remains a challenge for researchers and clinicians due to the highly complex nature of its pathogenesis.

Currently, the key processes leading to neurodegeneration are thought to be oxidative stress, disruption of transitional metal homeostasis, and neuroinflammation [1, 2].

There is also growing evidence that neurodegenerative diseases such as Alzheimer's and Parkinson's may be caused by detrimental environmental and dietary factors that alter gene expression by means of DNA methylation and histone modification [3].

Since the importance of oxidative stress in the development of age-related neurodegeneration is well established, a number of antioxidant compounds are considered promising in prevention of neurodegenerative disorders, including vitamin E, melatonin, green tea polyphenols, resveratrol, and others [4]. However, so far the clinical studies produce mixed results with many promising approaches such as vitamin E therapy failing to slow down the progression of age-related neurodegenerative conditions [5]. There are also studies investigating an effect of metal chelators and dietary approaches aimed at reducing intake of iron and copper [6]. Finally, researchers are investigating diverse compounds capable of favorably altering gene expression (epigenetic modifiers), reversing effects of environmental perturbagens [7].

The human copper-binding tripeptide glycyl-L-histidyl-L-lysine (GHK) is a compound with a long history of safe use in wound healing and antiaging skin care. Since its discovery in 1973, almost four decades of extensive research have established its diverse beneficial actions in many organs and tissues including nervous tissue, skin, intestine, bone, and blood vessels. The molecule has a high affinity for Cu (II) and forms the chelate GHK-Cu. The GHK copper complex (or GHK-Cu) has been proven to exhibit antioxidant, anti-inflammatory, regenerative, and wound healing actions [8]. Recent studies demonstrated that the GHK tripeptide up- and downregulates a large number of human genes, which may contribute to the pleiotropic health promoting effects of its copper complex [9]. We propose that the GHK-Cu complex may act therapeutically against age-associated neurodegeneration and cognitive decline.

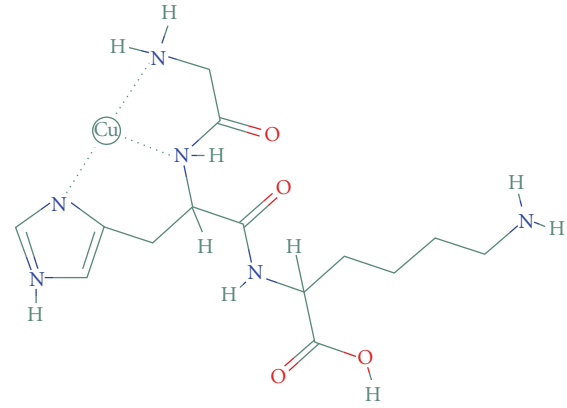

Figure 1: Molecular structure of the tripeptide GHK-Cu. In solution lysine carboxyl groups of neighboring complexes may participate in a complex formation.

2. Copper Binding Properties of the Human Tripeptide GHK

Human peptide GHK was isolated in 1973 as an activity in human plasma that caused old human liver tissue to synthesize proteins like younger tissue [10]. In human plasma GHK is present at about 200 micrograms/liter in men of age 20–25 but declines to 80 micrograms/liter by age 60–80. Subsequent studies established this activity as a tripeptide with an amino acid sequence glycyl-L-histidyl-L-lysine with a strong affinity for copper that readily formed the complex GHK-Cu. Since GHK-Cu promotes cell growth, it was proposed that the GHK acts by delivering copper required for the cellular functions into the cell in a form that is nontoxic and can be utilized by the cell [11].

The molecular structure of the GHK copper complex (GHK-Cu) has been extensively studied using X-ray crystallography, EPR spectroscopy, X-ray absorption spectroscopy, and NMR spectroscopy as well as other methods such as titration. In the GHK-Cu complex, the Cu (II) ion is coordinated by the nitrogen from the imidazole side chain of the histidine, another nitrogen from the alpha-amino group of glycine, and the deprotonated amide nitrogen of the glycine-histidine peptide bond (Figure 1). Since such a structure could not explain a high stability constant of the GHK-Cu complex ($\log_{10}$ = 16.44 versus 8.68 of the GH copper complex, which is similar to the GHK-Cu structure), it was proposed that another amino group participates in the complex formation. According to the recent study by Hureau et al., the Cu (II) is also coordinated by the oxygen from the carboxyl group of the lysine from the neighboring complex. Another carboxyl group of lysine from a neighboring complex provides the apical oxygen, resulting in the square-planar pyramid configuration. Many researchers proposed that, at the physiological pH, GHK-Cu complexes can form binary and ternary structures which may involve amino acid histidine and/or the copper binding region of the albumin molecule. Lau and Sarkar found also that GHK can easily obtain copper 2+ bound to other molecules such as the high affinity copper transport site on plasma albumin (albumin binding constant $\log_{10}$ = 16.2 versus GHK binding constant 16 $\log_{10}$ = 16.44). It has been established that copper (II) redox activity is silenced when copper ions are complexed with the GHK tripeptide, which allows the delivery of nontoxic copper into the cell [12–14].

3. The Copper Paradox

The brain is exceptionally rich in copper, which plays an important role in its physiology and pathology. Copper deficiency caused by bariatric surgery or gastrointestinal bleeding led to myelopathy (human swayback), paralysis, blindness, and behavioral and cognitive changes [15–17]. Mice born and maintained on a copper-deficient diet had 80% reduction in brain copper level at 6–8 weeks and had neuronal and glial changes typical for neurodegenerative disorders [18].

Despite unquestionable essentiality of copper, unregulated copper ions may increase oxidative damage. It is known that amyloid precursor protein (APP) implicated in development of Alzheimer's disease (AD) can convert Cu (II) into Cu (I) potentially increasing oxidative damage [19]. However, it remains unclear whether copper accumulation in senile plaques of AD patients is a cause or a consequence of pathological processes observed in AD. According to Exley followed by Bolognin et al., only aluminum, but not copper or iron, is capable of triggering amyloid precipitation and APP and tau181 protein overproduction [20, 21]. The study by Kawahara et al. showed that copper and carnosine attenuate neurotoxicity of another compound involved in neurodegeneration—prion protein [22]. Bishop and Robinson observed that amyloid beta protein may be neuroprotective when combined with copper. According to these authors, amyloid beta injected simultaneously with copper was not toxic, while iron and zinc complexed with amyloid beta were more toxic than amyloid beta alone [23].

Since copper accumulates in senile plaques of AD patients, some authors proposed dietary restriction of copper or intake of copper chelators as a preventive therapy for the elderly. However, several studies demonstrated that AD

patients have reduced, not elevated, brain and cerebrospinal fluid copper level [24, 25]. Currently, many authors suggest mild copper deficiency as a causative factor in AD and possibly other neurodegenerative disorders [26, 27]. In a placebo-controlled, double-blinded, randomized clinical trial, oral copper supplementation (8 mg/day) in 68 AD patients had a beneficial effect on relevant AD biochemical markers. The authors concluded that long-term intake of copper can be excluded as a causative factor in AD and may in fact be protective [28]. In addition, several studies revealed molecular mechanisms underlying beneficial effects of copper in AD, such as an inhibition of beta-amyloid peptide production [29].

Due to high importance of copper in the brain metabolism and a possible role of copper deficiency in the development of Alzheimer's and other neurodegenerative disorders, limiting of the dietary copper intake in elderly may actually increase their chance of developing neurodegenerative disorders. Another option is to use compounds that can form nontoxic complexes with Cu (II), preventing its accumulation in senile plaques and increasing its bioavailability. According to Narahara et al., β-citryl-L-glutamate—a compound that is abundant in the developing brain—has SOD-like activity when complexed with copper and may be neuroprotective [30]. Rózga and Bal propose that human serum albumin (HSA) may be neuroprotective due to its ability to bind both copper and amyloid beta protein [31]. Perrone at al. reported copper transfer from amyloid beta to a copper binding domain of HSA that is similar to the tripeptide GHK's structure with the same copper-binding histidine residue (DAHK) [32]. Thus GHK peptide, a natural copper-binding and copper-regulating molecule with well-established regenerative and protective actions in different organs and tissues, should be considered a promising therapeutic agent in preventing and correcting copper imbalance in neurodegenerative disorders.

4. Antioxidant and Anti-Inflammatory Properties of GHK-Cu

The brain's high metabolic activity results in elevated oxygen consumption and constant production of reactive oxygen species (ROS) in mitochondria. At the same time, the brain tissue is rich in unsaturated fatty acids and transition metal ions yet has relatively fewer antioxidants comparing to other organs creating favorable conditions for oxidative damage. Since the blood-brain barrier prevents many dietary antioxidants from entering the brain, it largely relies on endogenous antioxidants such as Cu- and Zn-dependent superoxide dismutase (Cu, Zn SOD1). This enzyme requires metal ions copper and zinc in order to be active. Hence, copper deficiency can lead to reduced SOD activity and increased oxidative brain damage [33]. When pregnant rats were kept on a copper-deficient diet, the embryos displayed low SOD activity, increased super oxide anion radical level, and higher incidence of DNA damage and malformations [34]. Amyloid precursor protein (APP) that is implicated in Alzheimer's disease (AD) development has copper binding activity and can trap copper, rendering brain tissue copper deficient. Transgenic mice overexpressing APP had reduced SOD1 activity in the brain. SOD1 activity was restored by copper supplementation [35]. It has been shown that GHK-Cu increases the level of antioxidant enzymes and SOD activity, supposedly by supplying copper necessary for its function [36].

GHK-Cu also reduces oxidative damage by modulating iron levels. The presence of iron complexes in damaged tissues is detrimental to wound healing, due to the increased lipid peroxidation in the presence of iron ions, as well as microbial infection mediated by iron. Pickart demonstrated that GHK-Cu inhibited lipid peroxidation if the iron source was ferritin. It was proposed that GHK-Cu binds to the channels of ferritin involved in iron release and physically prevents the release of Fe (II). Thus, GHK-Cu exhibits antioxidant function in wounds by inhibiting ferritin iron release in damaged tissues, preventing inflammation and microbial infections [37]. GHK-Cu produced a 75% reduction of gastric mucosa homogenates of lipid peroxidation in the range 10–100 mM suggesting that copper-peptide complexes are able to effectively neutralize damaging oxygen-derived free radicals [38].

GHK (in this experiment the peptide alone, not its copper complex, was used) has been proven to quench alpha,beta-4-hydroxy-trans-2-nonenal—a toxic product of fatty acids' lipid peroxidation that play important role in the pathogenesis of several age-related conditions including Alzheimer's disease, neuropathy, and retinopathy [39]. GHK peptide was also able to quench acrolein—another toxic product of lipid peroxidation involved in the development of many age-related degenerative disorders. The authors proposed that GHK may be used for prevention of some age-related pathologies, including Alzheimer's disease [40].

Inflammation and oxidative damage due to the overproduction of proinflammatory cytokines play an important role in the development of AD and other neurodegenerative conditions [41]. In 2001 McCormack et al. established that GHK-Cu decreased proinflammatory cytokine TGF-beta in human fibroblast culture [42]. In 2003 Canapp et al. demonstrated that GHK-Cu improved healing of ischemic wounds and suppresses inflammation by lowering the level of acute-phase inflammatory cytokines such as TGF-beta and TNF-alpha [43].

5. GHK Stimulates Blood Vessel Growth

Vascular factors play an important role in the development of many neurodegenerative diseases of aging. Since the brain is a highly metabolically active organ and requires constant supply of oxygen and nutrients, a well-developed, adequate vascular network is essential for its health [44].

From wound healing studies, it is known that GHK-Cu helps reestablish blood flow into damaged tissues through a mixture of three actions: angiogenesis (new blood-vessel formation), anticoagulation, and vasodilation. GHK-Cu increases the expression of basic fibroblast growth factor and vascular endothelial growth factor, both of which aid blood vessel formation [45]. In addition GHK-Cu's ability

to stimulate synthesis of collagen and elastin is useful in restoring integrity of blood vessel walls.

Sage et al. observed that endothelial cells at the site of an injury produce a protein called SPARC that contains GHK sequence. SPARC protein turned out to be abundant in all tissues that undergo rapid remodeling such as skin or embryonic tissues. When the tissue is damaged, tissue proteases break down SPARC, releasing an array of GHK and GHK-containing copper-binding peptides, which stimulate cell proliferation and new vessels growth. When blood flow is sufficiently restored, SPARC inhibits cell proliferation and growth, controlling new vessels progression [46].

6. GHK Increases Neurotrophins

Among compounds that have a protective effect and can reduce oxidative damage are some neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [47]. There is evidence that GHK increases production of neurotrophic factors. Both Sensenbrenner et al. and Lindner et al. found that GHK stimulates the outgrowth of cultured nerves [48, 49]. Ahmed et al. established that nerve stubs placed in a collagen tube impregnated with GHK (used without copper) had an increased production of nerve growth factor and the neurotrophins NT-3 and NT-4 increased migration of cells into collagen tube and sped up the regeneration of nerve fibers. In addition, GHK also increased axon count and proliferation of the Schwann cells compared to the control group [50].

7. GHK as a Gene Regulator

Epigenetic modification of gene expression is currently considered a link between the environment, aging, and neurodegeneration. It has been shown that some dietary and environmental factors, such as certain toxins, may result in abnormal DNA methylation and histone modification, altering gene activity. Recent studies revealed that some well-known antioxidant and anti-inflammatory substances such as plant flavonoids may counteract these deleterious changes by modulating activity of certain genes, reducing glial inflammation and inhibiting production of neurotoxins [51, 52].

First evidence that GHK-Cu may regulate an activity of certain genes came from wound healing and skin remodeling studies. By 1983, Pickart had established that GHK-Cu accelerates wound healing and contraction, improves the take of transplanted skin, and also possesses anti-inflammatory actions [53, 54].

Subsequent studies directed by Maquart et al. (France) demonstrated that GHK-Cu at a very low, nontoxic concentration (1–10 nanomolar) stimulated both the synthesis and breakdown of collagen and glycosaminoglycans [55, 56]. It modulated an expression of both metalloproteinases and their inhibitors (TIMP-1 and TIMP-2), improving wound healing and facilitating skin remodeling processes [57]. In 2000 the same group demonstrated that GHK-Cu increased mRNA for collagen, dermatan sulfate, chondroitin sulfate, and a small proteoglycan decorin [58].

In 2009, a group of researchers from the Seoul National University (Republic of Korea) demonstrated that GHK-Cu in concentrations of 0.1–10 microM increases expression of integrins and p63. Since these molecules are considered proliferative markers of epidermal stem cells, the authors concluded that GHK-Cu helps to maintain an active proliferative state of epidermal stem cells [59].

Iorio et al. used a repository of transcriptional responses to compounds, the Connectivity Map (cMap) [60], and MANTRA software (http://mantra.tigem.it/) to explore networks of compounds producing similar transcriptional responses. GHK, as one of the compounds studied, increased mRNA production in 268 genes while suppressing 167 [61].

Also, Hong et al. used genome-wide profiling to identify genetic biomarkers (genetic signature) for metastasis-prone colorectal cancer as well as their perturbagens—substances that modulated their expression. GHK suppressed RNA production in 70% of 54 human genes overexpressed in patients with an aggressive metastatic form of colon cancer and was active at a low nontoxic 1 micromolar concentration [62].

8. The Connectivity Map and the GHK-Affected Genes

Our own studies using the Broad Institute's Connectivity Map (cMap) showed that GHK activates numerous genes involved in nervous system physiology, development, and maintenance. The Connectivity Map holds three GHK gene expression profiles created using the GeneChip HT Human Genome U133A Array. Two of the profiles emerged from the treatment of the PC3 cell line; the other profile came from the cell line MCF7. All cell lines were treated with GHK at 1 micromolar. By selecting all three gly-his-lys instances we determined which genes are affected. Most interesting are the genes that are remarkably up- and downregulated. Using the cMap one can enter amplitude thresholds for both up- and downregulated genes in order to find the genes whose expression are significantly altered. With an up gene amplitude threshold of 0.40 (equivalent to a 1.5-fold induction) and a down gene amplitude threshold of −0.40 (equivalent to a 1.5-fold repression), we discover that 76 genes lay above the up threshold and 6 lay below the down threshold.

In the cMap genes are represented by probe set IDs in the tag lists, which identify the up- and downregulated genes. Running the probe set IDs in the tag lists through the "Batch query" in the NetAffx Analysis Center (http://www.affymetrix.com/), annotations for each probe set are retrieved. Reviewing the annotations we observe that 5 genes associated with nerves are stimulated (mRNA expression is increased) by GHK while none are suppressed (mRNA expression is decreased). These 5 genes are listed in Table 1 along with their corresponding average fold change.

9. GHK May Reverse Gene Silencing

Epigenetic silencing of certain genes is currently considered the main reason for age-associated increase in

Table 1

Probe set ID	Gene symbol	Gene products and functions (from GENE database; http://www.ncbi.nlm.nih.gov/gene)	Fold change*
214484_s_at	SIGMAR1	Sigma nonopioid intracellular receptor 1, plays an important role in the cellular functions of various tissues associated with the endocrine, immune, and nervous systems. Mutations are implicated in early-onset dementia and neurodegeneration.	2.46
205231_s_at	EPM2A	Laforin, a dual-specificity phosphatase associates with polyribosomes. This gene is defective in a neurodegenerative disorder associated with epileptic seizures (Lafora disease). Possibly a repair enzyme.	2.32
204860_s_at	NAIP	Apoptosis inhibitory protein; functions include suppression of neuronal apoptosis	2.13
208229_at	FGFR2	Fibroblast growth factor receptor 2, influences mitogenesis and differentiation, important in embryonic brain development.	1.9
209897_s_at	SLIT2	Slit homolog 2, neuronal repellent factor, nervous system development; downregulation of this gene by neuronal differentiation factor promotes tumor growth in neuroblastomas [63].	1.67

* This represents the average fold change of gene expression of the three GHK instances profiled in the cMap. Furthermore, it should be noted that all instances were performed using doses of GHK at 1 micromolar. Peak cellular responses to GHK have been recorded at 1 nanomolar; higher doses can reduce the cellular response [56].

tumorogenesis, oxidative stress, and inflammation. It is commonly accepted that human health is at its best until approximately the age of 20–25. It then begins to decline in later years. Recent genetic studies demonstrated accumulation of altered gene products in various tissues starting from the age of 20–25 [64]. In later decades of life, the genes produce less regenerative proteins but more inflammatory and oncogenic genes. The key enzymes implicated in gene silencing are the family of histone deacetylase proteins (HDACs). Inhibitors of selected HDACs possess neuroprotective and neuroregenerative properties in animal models of brain diseases and have been suggested as promising therapeutic drugs [65]. Results from the Broad Institute's Connectivity Map and ChemBank found that GHK is a strong inhibitor of several HDACs. Although the data was collected for GHK without copper, we cannot exclude the possibility that the actual gene regulator was the GHK copper complex formed in the culture media, since, as it was mentioned above, GHK can easily obtain copper from other biological molecules such as albumin. It is also possible that GHK and GHK-Cu have complimentary effects on gene activity. At present, it is not always possible to track gene effects to its protein product; however, we may conclude from all experimental data on GHK-Cu effects that the reversing of gene silencing by GHK has protective and health-promoting benefits.

10. Therapeutic Administration of GHK-Cu

It is possible that administration of GHK-Cu could be used as a preventive and regenerative therapy for senescent or damaged brain tissue. Using GHK-Cu has an advantage over using just GHK, since it alleviates copper deficiency without the risk of oxidative damage. Even though it is yet not clear whether or not the GHK-Cu peptide can pass the blood-brain barrier, there is a high possibility that it will do so, since GHK-Cu has a very high uptake into human skin, easily passing through the lipids of the epidermal barrier [66, 67]. The peptide could be administered intravenously or orally when encapsulated into liposomes. Strong systemic wound healing was induced in pigs at about 1.1 mg GHK-Cu per kilogram body weight which would correspond to about 75 mgs in humans. This is about 300-fold below GHK-Cu's toxic action (lowering of blood pressure). Much lower dosages may also be effective since GHK-Cu's actions on cells generally occur at a 1 nanomolar concentration [68].

11. Conclusion

The multifaceted nature of age-associated cognitive decline calls for complex approaches that address all key factors involved in the development of neurodegenerative disorders such as oxidative stress, neuroinflammation, disrupted bioavailability of copper, impaired circulation, and altered gene expression.

The human tripeptide GHK has a long history of safe use in wound healing and skin care; it is naturally occurring, nontoxic, and is active at a very low nanomolar concentration. It readily forms complexes with copper, regulating its metabolism and improving its bioavailability. It possesses antioxidant, anti-inflammatory, and regenerative properties, improves circulation, supports stem cell functions, and promotes nerve outgrown and synthesis of neurotrophic factors. Recent studies demonstrated its ability to regulate a large number of human genes. At 1 micromolar it was able to suppress 70% of genes overexpressed in metastatic colon cancer. It upregulates p63 and integrins in epidermal stem cells, increases collagen, glycosaminoglycans, and decorin expression. Our studies with the Broad Institute's Connectivity Map revealed its ability to regulate a large number of human genes including those that are involved in nervous system physiology, development, and maintenance.

Even though it is not always possible to distinguish between activity of GHK peptide and its copper complex

GHK-Cu, we strongly believe that for the future therapeutical applications GHK-Cu should be used. Numerous studies demonstrating pleiotropic health promoting and antiage activity of the GHK-Cu peptide together with recent studies revealing gene regulating activity of GHK suggest that this compound may belong to a class of epigenetic modifiers capable of exhibiting broad protective and restorative actions, reducing harmful epigenetic changes caused by environmental perturbagens. The GHK-Cu peptide should be considered a promising neuroprotective agent capable of preventing the development of common age-associated neurodegenerative disorders.

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Review Article

The Bad, the Good, and the Ugly about Oxidative Stress

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Alzheimer's disease (AD), Parkinson's disease (PD), and cancer (e.g., leukemia) are the most devastating disorders affecting millions of people worldwide. Except for some kind of cancers, no effective and/or definitive therapeutic treatment aimed to reduce or to retard the clinic and pathologic symptoms induced by AD and PD is presently available. Therefore, it is urgently needed to understand the molecular basis of these disorders. Since oxidative stress (OS) is an important etiologic factor of the pathologic process of AD, PD, and cancer, understanding how intracellular signaling pathways respond to OS will have a significant implication in the therapy of these diseases. Here, we propose a model of minimal completeness of cell death signaling induced by OS as a mechanistic explanation of neuronal and cancer cell demise. This mechanism might provide the basis for therapeutic design strategies. Finally, we will attempt to associate PD, cancer, and OS. This paper critically analyzes the evidence that support the "oxidative stress model" in neurodegeneration and cancer.

1. The Verdict: Oxygen Is Guilty, Not Guilty

Oxidative stress (OS) has become a major topic in all areas of medical knowledge. Entry of the term "oxidative stress" in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) shows that the number of publications has dramatically increased from none in the early 1970's to cover ~90,000 peer-reviewed articles in 2011 (Figure 1(a)). A similar trend is recorded for Alzheimer's disease (AD), Parkinson's disease (PD), and cancer when searched jointly with OS (Figure 1(b)). Since the discovery of the superoxide dismutase (SOD) in 1969 by McCord and Fridovich ([1], for a historical perspective see [2–4]), our understanding of the molecular defense mechanisms, which include catalase [5], glutathione peroxidase (GPx), and peroxiredoxin [6] and thioredoxin reductase [7], against diverse stress stimuli and pathogens [8] has dramatically changed (reviewed in [9, 10]). Moreover, given the phylogenetic distribution and subcellular localization of the SOD isozymes, the discovery has provided strong support for the hypothesis that the chloroplasts and mitochondria of eukaryotic cells arose from prokaryotic endosymbionts [11]. SOD is an enzyme that catalyzes the dismutation of the superoxide radical ($O_2^{\cdot -}$) very efficiently ($k_2 \sim 2 \times 10^9\,M^{-1}\,s^{-1}$) through a redox reaction of its copper centre enzyme into oxygen (O_2) and hydrogen peroxide (H_2O_2). Today, it is clear that decrease of enzymatic activity of the defense system or an overwhelming production of $O_2^{\cdot -}$ and/or H_2O_2 is linked to neurodegenerative disorders (e.g., familial amyotrophic lateral sclerosis [12], AD [13], PD [14], and cancer [15]). The idea that oxygen might not only be involved in the beginning of life and evolution [16–18] but also it might be a toxic molecule [19] was further popularized by Halliwell and Gutteridge in their book entitled "Free Radicals in Biology and Medicine" [20] and some important follow-up papers [21–23]. The chemistry of oxygen is well known. Basically, O_2 is classified as a free radical. By definition, a free radical is an atom or group of atoms with at least one unpaired electron. Indeed, the electronic configuration of the oxygen diatom is $[2He^4]2s^42p^8$ with the first ten electrons placed into σ, σ^*, π, orbitals, and two unpaired electrons each located in a different π^* antibonding orbital. Removal of an electron

from O_2 results in a superoxide cation radical ($O_2^{\cdot+}$). In contrast, if a single electron is added, the product is the superoxide anion radical ($O_2^{\cdot-}$). Addition of one more electron will yield the peroxide ion, O_2^{2-}, which is not a radical. Since this reaction may take place in solution, it is quite likely that this ion became protonate ($2H^+$) and converted into H_2O_2. This last compound represents a potential danger. In the presence of metal ions such as iron (Fe^{2+}) and copper (Cu^+), H_2O_2 decomposes into more reactive free radical specie, the hydroxyl radical ($^{\cdot}OH$). In sharp contrast with $O_2^{\cdot-}$, there is not an antioxidant system to protect cells against $^{\cdot}OH$. Indeed, this last radical can provoke a whole series of radical chain reactions involving damage of lipids, proteins, and nucleic acids. Therefore, an excessive generation or accumulation of $O_2^{\cdot-}/H_2O_2$ may lead to a biochemical phenomenon known as OS. Simply, this term refers to an atypical state in which exaggerate production of reactive species overwhelms the antioxidant defense systems of the cell [24]. Interestingly, $O_2^{\cdot-}$ and H_2O_2 are recognized to play signaling functions (reviewed in [25, 26]). However, H_2O_2 best fulfills the requirements of being a second messenger, that is, its enzymatic production, along with the requirements for the oxidation of thiols by this molecule, provides the specificity for time and place that are required in signaling, whilst $O_2^{\cdot-}$ is more likely as a precursor of H_2O_2. Although efforts have been made to explain the complexities of OS in cancer [27, 28] and neurodegeneration [29–31], several questions still remain unanswered, mainly because of two key issues. First, except for a few causative genetic mutations, the underlying pathogenic mechanism(s) of Parkinson's and Alzheimer's cases is not yet well understood. Consequently, this makes it difficult to identify potential therapeutic targets to stop their progression. Therefore, it is imperative to elucidate the precise molecular mechanism and/or identify the molecular "switches" that trigger neuronal death [32]. Clearly, identifying the precise steps/"switches" in the pathological cascade has been proven difficult since multiple death signaling pathways are often activated in response to a single stimulus. Thus, the questions what kills neurons and how do they get deteriorate in neurodegenerative diseases [33, 34] are still unresolved. Second, it is not surprising that some neuroprotective clinical trials had been completely unsatisfactory [35–38]. This last outcome is even aggravated by either technical incongruities [39], the challenging task of recruitment and retention of subjects in clinical trials (e.g., AD, [40]), limited knowledge on antioxidant bioavailability [41, 42], or that they have failed because they have not been aimed at the right target [43–45].

2. The Bad Touch of Oxidative Stress: Involvement in Alzheimer's and Parkinson's Disease

AD and PD are the two most common progressive neurodegenerative disorders worldwide [46, 47] affecting all ethnicities but especially some genetically isolated groups, such as the "paisa community" living in the Antioquia region of Colombia [48–52]. AD and PD are neuropathologically characterized by abundant insoluble protein deposits (e.g., $A\beta_{[1-40/42]}$ and hyperphosphorylated tau in AD [53], α-Synuclein in PD [54], metal deposition (e.g., iron [55–57]), specific neuronal and synaptic loss of the hippocampal pyramidal neurons (AD), and dopaminergic neurons of the *substantia nigra* (PD), probably via OS [58]. Despite the fact that both of these types of cells are vulnerable to OS, it is still unknown the complete cascade of molecular events at a single cell level responsible for neural deterioration. Consequently, no effective and/or definitive therapeutic treatment aimed at reducing or delaying clinical and pathological symptoms is currently available. Therefore, it is urgently needed to elucidate the molecular cell death signaling pathway involved in these processes to identify potential pharmacological target(s).

To get insight into these issues, we initially selected peripheral blood lymphocyte (PBL) culture as model system in AD and PD. Indeed, these cells display striking biochemical similarities to neurons (e.g., [59–63]). Lymphocytes therefore represent a remarkable nonneural cell model for understanding the molecular machinery and metabolic regulation of apoptosis associated with cell survival signaling against stressful stimuli. Apoptosis is a controlled and regulated form of programmed cell death defined by specific morphological features such as rounding-up of the cell, reduction of cellular volume, chromatin condensation (i.e., stage I nuclei morphology composed of high molecular weight DNA), nuclear fragmentation (i.e., stage II nuclei morphology composed of low molecular weight DNA, highly chromatin condensation packed in round masses), classically little or no ultrastructural modifications of cytoplasmic organelles, and plasma membrane blebbing [64]. Although morphologically similar, apoptosis can be triggered through different intrinsic or extrinsic signaling biochemical routes [65–67]. Because H_2O_2 is more stable reactive oxygen specie (ROS), it can work either as a second messenger in prosurvival [68] or in prodeath intracellular signaling pathways. During the last decade, we have focused on investigating the H_2O_2-induced cell death signaling in PBLs. We have consistently shown that $A\beta_{[25-35]}$ [69], dopamine (DA, [70]), and its related neurotoxins (e.g., 6-hydroxidopamine (6OHDA), 5,6 and 5,7-dyhydroxy-tryptamine (5,6- and -5,7-DHT, [71]), paraquat (PQ, [72]), and rotenone (ROT, [73]) induce apoptosis in lymphocytes in a concentration- and time-dependent fashion by OS mechanism involving several steps: $O_2^{\cdot-}$ and H_2O_2 generation (Figure 2, step 1, *numbers in red*), activation of the nuclear factor kappa-B (NF-κB, step 2)/p53 (step 3)/c-Jun N-terminal kinase (JNK, step 4)/c-Jun (step 5) transcription factors, mitochondrial depolarization (step 6), and caspase-3 activation (step 7). As a result we observed the typical nuclei morphological feature of apoptosis including chromatin condensation and fragmentation (step 8). Remarkably, this cell death subroutine can be blocked by the action of antioxidants (e.g., N-acetyl-cysteine (NAC) [69, 71], vitamin C (VC, [71]), testosterone [70], 17β-estradiol [70, 74], cannabinoids (e.g., CP55940 and JWH-015 [72, 75]), mitochondria permeabilization transition pore inhibitor (e.g., cannabinoids

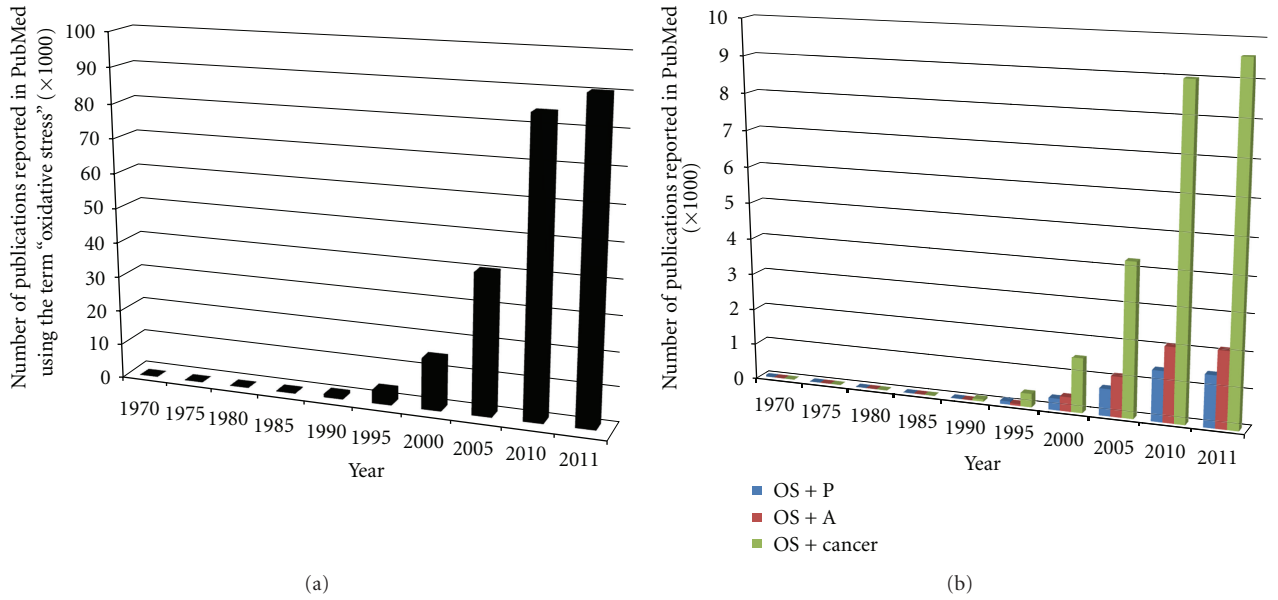

Figure 1: Number of articles reported in PubMed by using the term "oxidative stress" (OS) alone (a) or together (b) with the term "Parkinson" (P), "Alzheimer" (A), and "cancer".

[76]), insulin-like growth factor-1 [72, 73, 77]), high glucose [72, 73], specific pharmacological inhibitors (e.g., PDTC, pifithrin-α, SP600125, Ac-DEVD-cho inhibitor of NF-κB, p53, JNK, and caspase-3, resp.) and inhibitors of protein (e.g., cycloheximide [71]), and RNA (e.g., actinomycin D [69, 71]) synthesis. These findings may be explained by the following assumptions. H_2O_2 might indirectly activate NF-κB through phosphorylation of the IκBα (i.e., the inhibitor of the complex NF-κB or p50/p62) either by the spleen tyrosine kinase protein (Syk, step 9, *number in blue*) at tyrosine 42 [78, 79] or at serine 32 and 36 via SH2 (Src homology 2)-containing inositol phosphatase-1 (SHIP-1, step 10)/IκB-kinase (IKK) complex pathway [80]. Alternatively, H_2O_2 might activate NF-κB through activation of the IKK complex by mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1, step 11, [81]). Once the IκB is phosphorylated, the release of active NF-κB dimmer (p50/p63) translocates into the nucleus and transcribes several antiapoptotic genes (e.g., Bcl-2, cIAP-1-2, and Bcl-xL) (step 12) and pro-apoptotic genes, amongst them the *p53* [82]. At this point, a vicious cycle is established wherein p53 plays a critical role by balancing the cell to a death decision because of its many actions. First, p53 transcribes proapoptotic genes such as *Bax* (step 13), which in turn might contribute to the permeabilization of the outer mitochondrial membrane by antagonizing antiapoptotic proteins (e.g., Bcl-2, cIAP-1-2, and Bcl-xL). Second, p53 not only induces prooxidant genes (e.g., *p53-induced gene-3 (PIG3), proline oxidase (PO),* step 14), which generate more H_2O_2 but also represses the transcription of antioxidant genes (e.g., *NAD(P)H: quinone oxidoreductase-1*) [83]. Elevated stress stimuli (i.e., H_2O_2 production, step 1) and further activation of NF-κB induce upregulation of proapoptotic genes (e.g., *p53*), which in turn amplify the initial H_2O_2-induced cell death signal. Formation of the mitochondrial permeabilization transition pore allows the release of apoptogenic proteins (by a not fully established mechanism, step 15 [84, 85]) such as the apoptosis-inducer factor (AIF, [86]) responsible for causing DNA fragmentation and chromatin condensation (i.e., stage I nuclei morphology) and cytochrome C, which together with Apaf 1, dATP, and procaspase-9 (i.e., the apoptosome) elicits caspase-3 protease activation [87]. This protease is essential for the fragmentation and morphological changes associated with apoptosis [88]. Indeed, caspase-3 activates the endonuclease DNA fragmentation factor 40 (DFF40) or caspase-activated DNAse (CAD) by cutting the nuclease's inhibitor DFF45/ICAD [89]. Finally, DFF40/CAD causes nuclear chromatin fragmentation (i.e., stage II nuclei morphology), typical of apoptosis [90]. Interestingly, the apoptosis signal-regulating kinase (ASK1; step 16, [91]) and MEKK1 (step 11, [92]) phosphorylate MKK4/MAPK kinase (step 17). MEKK1 kinase therefore represents a cross-talk between the JNK and NF-κB pathway. Indeed, MEKK1 kinase phosphorylates IKK and MKK4. This last kinase phosphorylates JNK/stress apoptosis protein kinase (SAPK [93], step 4), which in turn phosphorylates the c-Jun transcription factor [94], also involved in transcription of death signaling [95]. Interestingly, it has also been shown that JNK1/2 cooperates in the activation of p53 apoptotic pathway [96, step 3]. Alternatively, high concentration of metal ions (e.g., Fe^{2+}; Cu^{+}, Mn^{2+}) alone or in combination with H_2O_2 are able to directly induce mitochondria damage and apoptotic morphology by caspase-3-dependent mechanism [70, 97]. In conclusion, NF-κB, p53, c-Jun and caspase-3 activation, and mitochondrial depolarization are crucial events in mediating cell death by apoptosis.

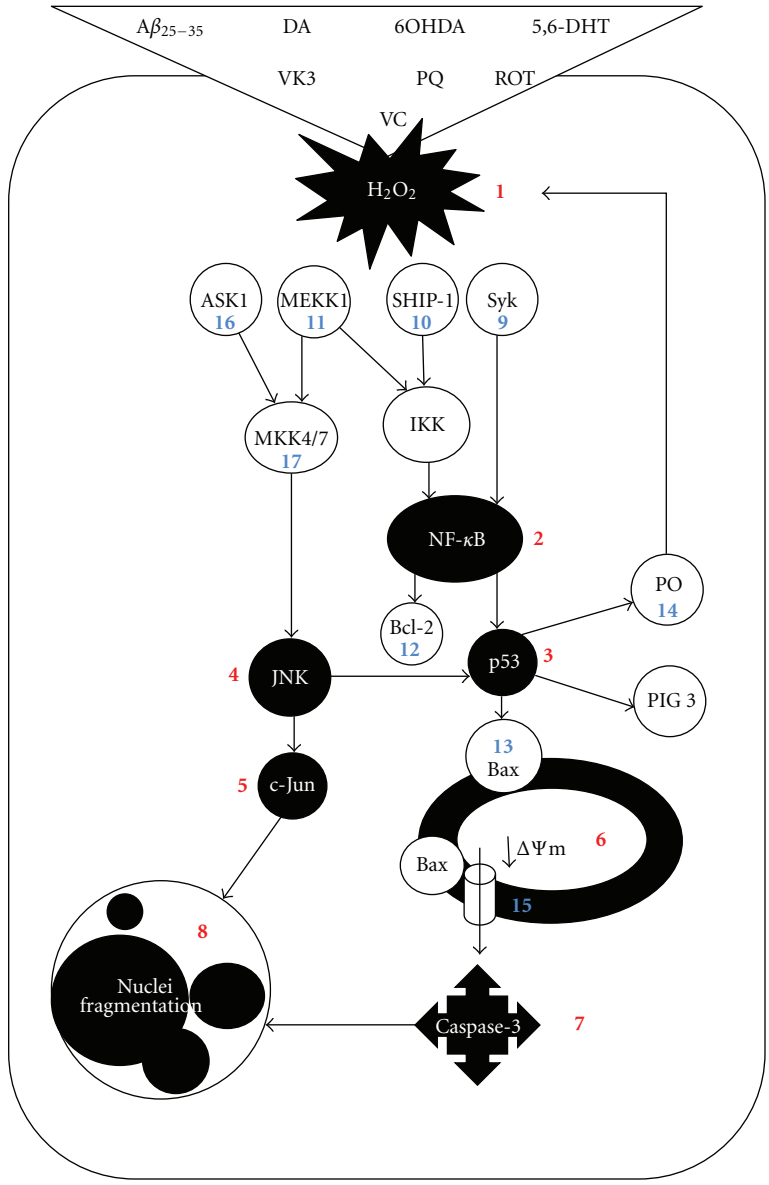

Figure 2: Proposed model of minimal completeness of cell death signaling induced by oxidative stress as a mechanistic explanation of neuronal and cancer cell demise.The neurotoxins A$\beta_{[25-35]}$, dopamine (DA) and its related neurotoxins (6OHDA, 5,6- and 5,7-DHT), paraquat (PQ), and rotenone (ROT) trigger a cell death subroutine in lymphocytes, a well-established model of AD and PD. This mechanism is characterized by $O_2^{\cdot -}/H_2O_2$ generation (step **1**, *numbers in red*), activation of the transcription factors NF-κB (step **2**), p53 (step **3**), and c-Jun (step **5**), activation of the JNK kinase (step **4**), mitochondrial depolarization (step **6**), caspase-3 activation (step **7**), and nuclei chromatin condensation/fragmentation (step **8**). These findings may be explained by the following assumptions. H_2O_2 might indirectly activate NF-κB through phosphorylation of its inhibitor IκBα either by Syk (step **9**, *numbers in blue*) or via SHIP-1 (step **10**)/IKK complex pathway. H_2O_2 might also activate NF-κB through activation of the IKK complex by the MEKK1 protein (step **11**). Once NF-κB is activated, it translocates into the nucleus and transcribes several antiapoptotic genes (step **12**) and proapoptotic genes, amongst them the *p53* (step **3**). At this point, a vicious cycle is established. First, p53 transcribes proapoptotic genes such as Bax (step **13**), contributing to the permeabilization of the outer mitochondrial membrane by antagonizing antiapoptotic proteins. Second, p53 induces prooxidant genes (e.g., *p53-induced gene-3 (PIG3), proline oxidase (PO)*, step **14**), which generate more H_2O_2 (step **1**) and represses the transcription of antioxidant genes. H_2O_2 overproduction and further activation of NF-κB induce upregulation of proapoptotic genes (e.g., *p53*), which in turn amplify the initial H_2O_2-induced cell death signal (step **2–8**). Mitochondrial damage allows the release of apoptogenic proteins (step **15**) responsible for the formation of apoptosome and activation of caspase-3 protease. This protease in turn activates the endonucleases DFF40/CAD, by cutting the nuclease's inhibitor DFF45/ICAD. Finally, DFF40/CAD causes nuclear chromatin fragmentation, typical of apoptosis. Alternatively, ASK1 (step **16**) and MEKK1 (step **11**) phosphorylate MKK4/MAPK kinase (step **17**). MEKK1 kinase also phosphorylates IKK. This last kinase phosphorylates JNK1/2/SAPK (step **4**), which in turn phosphorylates c-Jun, also involved in death signaling. Noticeably, vitamin C (VC) and vitamin K3 (VK3) alone or in combination induce apoptosis in Jurkat and K562 cells by a similar mechanism as described. This mechanism might provide the basis for therapeutic design strategies in AD, PD, and cancer (leukemia).

Over the years, not only *in vitro* (e.g., [98–107] or *in situ* (e.g., [55, 108–115]) but also *in vivo* studies have validated the findings highlighted in Figure 2, step 1–8. Of note, McLellan et al. [116] have shown directly that a subset of amyloid plaques (e.g., dense core plaques) produce ROS, that is, H_2O_2, in animal Alzheimer's models (e.g., Tg2576 APP overexpressing transgenic mice) and in human postmortem Alzheimer tissue. Wang et al. [117] found that $A\beta_{[1–42]}$ injection in Sprague-Dawley male rats increased JNK and NF-κB protein levels in brain. This effect was prevented by hydrogen-rich saline implicating OS. Likewise, Mogi et al. [118, 119] showed significant increase in the levels of p53, NF-κB, and caspase-3 reflecting apoptosis in the Parkinsonian brain. In agreement with these human brain data, Liang et al. [120] have shown that NF-κB activation contributes to 6-OHDA OS-induced degeneration of dopaminergic neurons through a NF-κB-dependent p53-signaling pathway in rat model of PD. Interestingly, Li et al. [121] have shown that bilobalide (an active component of *Gingko biloba*) and the peptide inhibitor of NF-κB, SN50 inhibit 6-OHDA-induced activation of NF-κB and loss of dopaminergic neurons in rat *substantia nigra*. Muñoz et al. [122] have shown that systemic administration of NAC protects dopaminergic neurons against 6-OHDA-induced degeneration in rats. Remarkably, Braithwaite et al. [123] have shown that SP600125 inhibition of JNK provides neuroprotection in a Tg2576/PS^{m146L} transgenic mice model of AD. To establish *in vivo* relevance of our *in vitro* findings, we showed that SP600125 increased the survival and locomotor activity of *Drosophila melanogaster (D. melanogaster* [124]), used as a valid model of PD [125, 126], against acute exposure to PQ [127]. Furthermore, the cannabinoid CP55,940 prolongs survival and improves locomotor activity in *Drosophila* against acute exposure to PQ [124]. We also demonstrated that pure polyphenols such as gallic acid (GA), ferulic acid (FA), caffeic acid (CA), coumaric acid (CouA), propyl gallate (PG), epicatechin (EC), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) protect, rescue, and, most importantly, restore the impaired movement activity (i.e., climbing capability) induced by PQ in the fly [128]. Remarkably, PG and EGCG protected and maintained movement abilities in flies cotreated with PQ and iron [128]. Recently, Ortega-Arellano et al. [129] have demonstrated that chronic polyphenols prolong life span and restore locomotor activity of *D. melanogaster* chronically exposed to PQ compared to flies treated with PQ alone. These observations support the notion that polyphenols might be potential therapeutic compounds in the treatment of PD [130, 131]. Moreover, Bonilla-Ramirez et al., [132] have found that desferrioxamine (DFO), ethylenediaminetetraacetic acid (EGTA), and D-penicillamine chelators were able to protect but not rescue *D. melanogaster* against acute or chronic metal intoxication. Taken together, *in vitro* and *in vivo* data suggest that antioxidants (e.g., NAC [133]), polyphenols, cannabinoids, metal chelators [134], mitochondrial targeted antioxidant compounds [135, 136], pharmacological inhibition of NF-κB [137, 138], p53 [139, 140], JNK [141], and caspase-3 may be of therapeutic value in AD and PD.

3. The Good Touch of Oxidative Stress: A Perspective for Cancer Cell Death

Oxidative stress has two opposite outcomes in cancer cells: on one side, OS has been associated to initiation, promotion, progression, and maintenance of tumor cell phenotypes [26, 27]. Specifically, H_2O_2 stimulates proliferation, migration, and adhesion of these cells [142–144]. However, the causative relationship of ROS increase, and oncogene activation remains unclear. On the other side, OS has been associated with antitumorigenic actions, senescence, and apoptosis [145, 146]. Strikingly, NF-κB has been found to play pro- and antiapoptotic roles, which might depend on the type of cell [147–151], intracellular level of ROS, induced or constitutive expression of NF-κB, quantity of cellular antioxidant defenses, and absence or presence of growth factors or metabolic sources (e.g., glucose). Therefore, NF-κB constitutes a critical molecule in cell survival/death decision. Based on our previous experience with OS mechanism and cell death, we hypothesize that cancer and neurodegeneration processes share common cellular foundations. In contrast to the unsatisfactory results of the antioxidant therapy in AD [152, 153] and PD [154], generation of ROS to kill cancer cells is currently not only an idea but has already been effective as treatment in cancer patients (e.g., procarbazine, doxorubicin, and arsenic). We reasoned that the OS mechanism depicted in the Figure 2 might be operative in both neurodegeneration and cancer processes but with opposite therapeutic approaches: while it might be used to destroy malignant cells, it might also be stopped with antioxidants or signals to retard or delay neural cell death. Concerning the former consideration, we found that low-dose (10 μM) vitamin K3 (VK3, also known as menadione or 2-Methyl-1,4-naphthoquinone) or high-dose (10 mM) vitamin C (VC, also known as ascorbate, $AscH^-$) alone or in combination induced apoptosis in Jurkat (model of acute lymphoblastic T-cell leukemia [155]) and K562 (model of myelogenous leukaemia cells) cells by OS mechanism [156]. This data provided, for the first time, *in vitro* evidence supporting a causative role for OS in VK3- and VC-induced apoptosis in Jurkat and K562 cells in a domino-like mechanism similar to the mechanism identified in lymphocytes and neuronal cells under OS (Figure 2). The VC/VK3 observations can be explained because the synthetic VK3 can be reduced via one- or two-electron transfer by intracellular reductases or by VC. The two electron reductions of VK3 to hydroquinone VK3 ($VK3QH_2$) can slowly autoxidise to reform VK3. The single-electron reduction of the VK3 by VC^- ($AscH^-$) gives semiquinone anion radical ($VK3Q^{\cdot -}$), which in turn reduces O_2 to $O_2^{\cdot -}$ and regenerates the VK3. Consequently, redox cycling of VK3 can ensue and produce large amounts of $O_2^{\cdot -}$, which can dismutate via SOD to form H_2O_2 and O_2. As mentioned, H_2O_2 can take part in metal-catalyzed reactions to form more toxic species of active oxygen such as $^{\cdot}OH$. Therefore, if the single-electron reduction pathway predominates and the rate of redox cycling of VK3 exceeds the capacity of the detoxifying enzymes (e.g., catalase, GPx, and SOD), OS occurs, ultimately triggering a specific subroutine of cell death signaling (Figure 2 and

[156]). Altogether these data suggest that VK3 and VC or any molecule capable of producing excessive amount of $O_2^{\cdot -}/H_2O_2$ can be useful in the treatment of leukemia (e.g., arsenic [157], taxol [158]).

4. *Dangerous Liaisons*: Oxidative Stress as Central Aspect for Neurodegeneration and Cancer

Up-to-date, >200 pathogenic mutations distributed in 3 (*Aβ amyloid precursor protein* (*APP*), *presenilin-1* (*PSEN1*), *presenilin-2* (*PSEN2*)), and 6 genes (*α-Synuclein (SNCA*), *Leucine-rich repeat kinase 2* (*LRRK2*), *PARKIN*, *PTEN-induced putative kinase 1* (*PINK1*), *DJ-1*, and *P-type ATPase 13A2 (ATP13A2)*) have been conclusively shown to cause familial Alzheimer and Parkinsonism, respectively (http://www.molgen.ua.ac.be, reviewed in [159, 160]). Interestingly, mutations in those genes are directly related to OS and mitochondrial alterations [161, 162]. Specifically, Vinish et al. [163] have found increase in malondialdehyde content and SOD activity in peripheral blood parameters in PD patients with *PARKIN* mutations in comparison to controls. Ramsey and Giasson [164] found that the p.E163K DJ-1 mutant loses the ability to protect against OS while demonstrating a reduced redistribution towards mitochondria. Moreover, Ren et al. [165] have shown that DJ-1 protects cells against UVB-induced cell death dependent on its oxidation and its association with mitochondrial Bcl-X(L). Heo et al. [166] have shown that the p.G2019S mutation in LRRK2 generates H_2O_2 and induces neurotoxicity via its kinase activity. Last, the Butterfield's group has shown that mutation in *APP* and *PSEN1* (e.g., $APP^{NLH}/PS\text{-}1^{P264L}$ mice) induces brain OS [167, 168]. Taken together, these data support the notion that environmental and genetic pathways converge in the pathogenesis of AD [169] and PD [170–172]. It is interesting to note that iron accumulation is linked with the brain pathology in AD [55] and familial PD [56, 57]. These observations suggest that iron might play a toxic role in the pathophysiology of both neurologic disorders [173, 174], most probably linked to a common molecular mechanism of cell death via generation of intermediate ROS and mitochondrial damage [97, 175, 176]. Therefore, it is not unusual that PD patients develop dementia [164, 177, 178] concomitantly with AD pathology [179]. Moreover, recent data suggest that exposition to ethacrynic acid, a compound that induces cellular glutathione (GSH) depletion therefore causing OS, increases presenilin-1 protein levels in human neuroblastoma SH-SY5Y cells [180]. Furthermore, the *γ*-secretase protein complex mediates OS-induced expression of *β*-site APP cleaving enzyme I (BACE1) resulting in excessive A*β* production in AD [181]. Remarkably, extensive analysis of the effects and interactions of the AD [182, 183] and PD [184, 185] pathogenic genes in *D. melanogaster* has shown that mutations in *parkin* [186, 187], *pink-1* [188], *α-synuclein* [189], *Lrrk* [190] genes, or overexpression of normal *α*-synuclein [189] cause death of dopaminergic neurons in *Drosophila* probably via OS [166, 191–195]. Accordingly, it has been shown that DJ-1 and parkin are essential for mitochondrial function and rescue *pink-1* loss of function [196, 197]. Since these genes are conserved in invertebrates (insects) and vertebrates (mammals) [198], we believe that *D. melanogaster* could provide new insights into the relationship between gene mutations, OS, and mitochondria [184]. Taken together, these data suggest that OS is at the pathobiological basis of PD and AD and that its generation and detrimental effects can be exacerbated by environmental factors and mutation in causative genes.

Surprisingly, epidemiological studies have consistently shown the cooccurrence of PD and melanoma [199, 200] and this association is strongly increased by mutations in *PARKIN, LRRK2*, and *α-Synuclein* (for a review, see [201]). Moreover, Veeriah et al. [202] have shown that point mutations and exon rearrangements of *PARKIN* are linked to glioblastoma multiform, colon cancer, and lung cancer. Although, the exact mechanism(s) underlying the observed cancer-PD association is not clear, it has been suggested that genes (e.g., *PARKIN*) that cause neuronal dysfunction when mutated in the germline may instead contribute to oncogenesis when altered in nonneuronal somatic cells [202]. Whether OS is involved in these malignancies needs further investigation. However, based on the assumption that cancer and neurodegeneration share some of the same genes and molecular mechanisms of OS-induced cell death, one may anticipate a positive correlation between OS, cancer and PD. Recently, Zhang et al. [203] have found that Parkin is a p53 target and Parkin contributes to the role of p53 in regulating antioxidant defense. Indeed, ectopic Parkin expression significantly reduced ROS levels in H460p53siRNA treated with or without H_2O_2. Simultaneous knockdown of p53 and Parkin results in higher intracellular ROS levels than individual knockdown of p53 and Parkin. Moreover, ectopic Parkin expression significantly increased GSH (reduced) levels, thus altering the GSH : GSSG (oxidized) ratio in human lung cancer line, H460p53siRNA. Interestingly, Parkin knockdown in H460 (control) cells and Parkin knockout in mouse embryonic fibroblast (MEF) cells significantly decreased GSH levels and the GSH : GSSG ratio. Given that Parkin has also been reported to repress p53 [204], together these data suggest that the regulation of Parkin by p53, or vice versa, could be cell type or tissue specific. Further investigation is warranted in this topic.

5. Oxidative Stress: *Quo Vadis?*

In conclusion, there is enough support evidence for the role of OS in AD, PD, and cancer. Clearly, the relationships between some causative genes of Parkinson's such as *PARKIN* and *LRRK2* and cancer will challenge the medical research for designing new therapeutic approaches and the necessity to bring new proposals of unified models of disease and molecular mechanisms. In this respect, the model of minimal completeness of cell death induced by H_2O_2 (see Figure 2, steps 2–8) might provide a platform to evaluate new natural or synthetic antioxidants, pharmacological agents which target the mitochondria, transcription factor(s), and/or caspase-3, or it simply might be used as a model to test other novel hypothesis (e.g., [205, 206]). In this regard,

plant polyphenols has been suggested as promising compounds for the prevention of neurodegenerative diseases and treatment of cancer (For reviews see [130, 207–209]). Yet, whether polyphenols might function as effective antioxidant compounds *in vivo* is still a controversial issue [210–213]. One of the most urgent issues is to clarify the many studies reported to show failed clinical benefit or persuasive evidence of neuroprotection [214]. Most importantly, we will need to definitely establish the molecular mechanism(s) of cell death in neurodegenerative disorders before novel treatments can be available. Undoubtedly, there are still many unresolved issues. Perhaps, studying the biology of cancer cells might provide understanding of the underlying pathogenic mechanisms of cell death in neurodegeneration and help developing new treatment strategies.

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