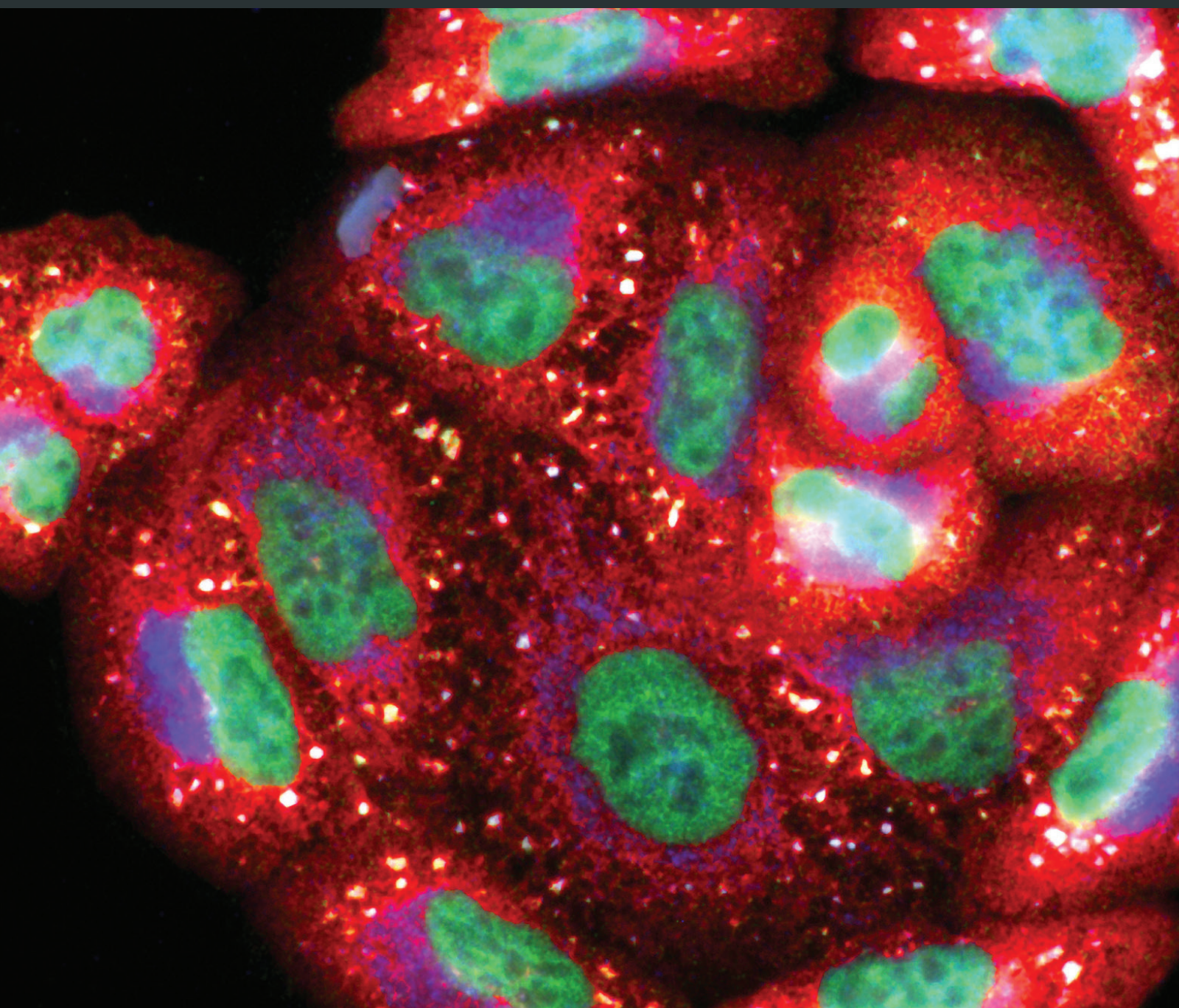


# Stresses, Aging, and Age-Related Disorders

Guest Editors: Xiaotao Li, Robb E. Moses, Jianping Jin, Weiguo Cao,  
and Carlos Caulin





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Oxidative Medicine and Cellular Longevity

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## Editorial

# Stresses, Aging, and Age-Related Disorders

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This special issue focuses on the topics of aging and its related disorders and stress. Stress, defined as a pressure or tension on a subject, can be in any of many forms. As related to aging, two general areas of stress have been entertained as contributing to the process: genome damage and metabolic deterioration. While neither area satisfactorily explains the aging process, each supplies useful data. Indeed, the metrics used to monitor the aging process are unsatisfactory; a simple concise definition of aging has yet to be established. Moreover, the concept of genetic control of aging presents a dilemma; if there is none, why does a dog live only seven or eight years with a metabolism not radically different from a human? Yet if genetic programming controls the process exclusively, what are the master genes? We are left to hypothesize on bases for aging involving both environmental and genetic elements [1].

There are several keystone observations relating to aging which give opportunity for study [1]. First, the observation that calorie restriction increases life span in rodents [2] has been repeated for over seventy years and is robust [1, 3]. While studies on primates are inconclusive, in rodents, *S. cerevisiae*, *C. elegans*, and *Drosophila* results appear unambiguous. Secondary to this, in yeast and worm, later in the fly, a set of genes regulated by calorie deprivation was identified. These observations led to the identification of the SIRT genes in higher organisms, a highly conserved family involved in regulation of cellular NAD<sup>+</sup> levels and energy expenditure.

It has been possible to construct long-lived mutants for the worm, establishing genetic components to longevity [4].

A second area of seminal observations relates to oxidative damage to the cell. These studies relied on the recognition of accumulation of genome errors (mutations) and demonstration of degraded protein synthesis in aged systems compared to young systems. This has led to the school of thought that free radicals such as reactive oxygen species (ROS) can produce accumulated damage and lead to cellular senescence [5]. This has led to focus on the state and function of mitochondria and relation to energy metabolism in the face of free radical insult. That said, there is no evidence supporting rescue of senescence by use of reducing agents or antioxidants, despite extensive trials. However, schemes linking calorie restriction and mitochondrial function are plausible.

It is in this line of thought that we have included an emphasis on mitochondrial function and oxidative stress in this special issue. Hypotheses remain at the interface of reduced calorie consumption and its metabolic signaling and the response of a handful of genes which can prolong lifespan.

Xiaotao Li  
Robb E. Moses  
Jianping Jin  
Weiguo Cao  
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## Review Article

# Oxidative Stress Associated with Neuronal Apoptosis in Experimental Models of Epilepsy

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Epilepsy is considered one of the most common neurological disorders worldwide. Oxidative stress produced by free radicals may play a role in the initiation and progression of epilepsy; the changes in the mitochondrial and the oxidative stress state can lead mechanism associated with neuronal death pathway. Bioenergetics state failure and impaired mitochondrial function include excessive free radical production with impaired synthesis of antioxidants. This review summarizes evidence that suggest what is the role of oxidative stress on induction of apoptosis in experimental models of epilepsy.

## 1. Introduction

Epilepsy is a chronic neurological disease characterized by recurrent and spontaneous seizures with diverse etiology that affects up to 1% of the world population. The median prevalence of lifetime epilepsy for developed countries is 5.8 per 1,000 and 10.3 per 1,000 for developing countries [1]. Epilepsy is the most frequent neurodegenerative disease after stroke, and according to epidemiological studies, approximately 70–80% of epilepsy patients achieve remission and approximately 30% of this patients present resistance to pharmacological treatment [2]. Status epilepticus, or the condition of prolonged epileptic seizures, is a major neurological and medical emergency that is associated with significant morbidity and mortality [3]. Epilepsy comprises a large number of syndromes, which vary greatly with respect to their clinical features, treatment, and prognosis; several classifications of the seizures (symptoms) and the epilepsy syndromes have been refined with time. Several causes are associated with

epileptic seizures, between others, central nervous system (CNS) tumors, neurodevelopmental abnormalities, CNS trauma, and/or inflammation; likewise, a large group of epilepsies have unknown etiology [4]. Temporal lobe epilepsy (TLE) is the most prominent example of acquired and frequent epilepsy; the seizure origin typically involves the hippocampal formation, a structure located in the mesial temporal lobe. Two main types of TLE are generally recognized, mesial temporal lobe epilepsy, which arises in the hippocampus, parahippocampal gyrus, and amygdala, and lateral temporal lobe epilepsy, which arises in the neocortex [5–7]. In TLE associated with mesial sclerosis (MTLE), the hippocampus represents the epileptic focus, while the temporal neocortex is involved in propagation of epileptic seizures in other brain areas [8].

The brain is particularly vulnerable to oxidative damage because of its high oxygen utilization, its high content of oxidisable polyunsaturated fatty acids, and the presence of redox-active metals (Cu, Fe) [9, 10]. Neuronal cells in



the brain are highly sensitive to oxidative stress; therefore, the prolonged excitation of neurons during seizures can lead to injury resulting from biochemical alterations and specifically to the role played by the oxidation state. Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS), reactive nitrogen oxygen (RNS), and the ability to readily detoxify the reactive intermediates in a biological system [7, 11, 12]. Excessive ROS generation can cause damage of neuronal cells inducing cell death via either an apoptotic or a necrotic pathway [13]. Recent evidence has suggested an intimate link between oxidative stress and mitochondrial dysfunction with the development of neuronal death in diverse neurological disorders including epilepsy. Mitochondrial dysfunction includes bioenergetic failure and increased cytosolic calcium, oxidative stress (excessive free radical production and impaired synthesis of antioxidants, especially glutathione), mitochondrial permeability transition pore opening, and the release of key proteins into the cytosol triggering cell death pathways such as apoptosis [14].

Experimental epilepsy models have been developed to assess the pathophysiology of epileptic seizures and have played a fundamental role in our understanding of the basic molecular mechanism. Experimental animal models can be divided into three categories mainly: (1) experimental seizures induced by chemical convulsants or by electrical stimulation, (2) reflex epilepsies, and (3) idiopathic epilepsies [15]. The most well known and most frequently used are multiple spontaneous recurrent seizures TLE (kainic acid) a glutamatergic agonist, cholinergic agonist pilocarpine (PILO), or model for induction of epilepsy Pentylentetrazol (PTZ) a tetrazole that is an antagonist of gamma-aminobutyric acid receptors [15, 16]. Many experimental reports have demonstrated the involvement of oxidative stress in seizures associated with brain damage and the mechanisms associated with epilepsy. The aim of this review is to present recent evidence on its role of mitochondrial dysfunction and oxidative stress in the apoptosis induction in experimental epilepsy models.

## 2. Reactive Oxidative Species and Reactive Nitrogen Species

**2.1. Free Radicals.** Oxygen free radicals or, more generally, reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) are products of normal cellular metabolism [9, 17]. Increased oxidative/nitrosative stress generally describes a condition in which cellular antioxidant defenses are unable to inactivate the ROS and RNS; the amount of free radicals is determined in the mitochondrial oxidative phosphorylation chains [18]. It is now well established that mitochondria is the main site of the generation of oxygen radicals; there are many different varieties of partially reduced ROS including superoxide ( $O^{\bullet-2}$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical ( $OH^{\bullet}$ ) [19]. The modern use of the term ROS includes both oxygen radicals and nonradicals that easily converted into free radicals ( $O_3$ ,  $H_2O_2$ , and  $^1O_2$ ). RNS refer to nitric oxide (NO) and molecules derived from NO, such as peroxynitrite ( $ONOO^-$ ), nitrosyl ( $ON^+$ ), and nitrogen

dioxide ( $NO_2$ ) [17, 18]. Ubiquinone, a component of the mitochondrial respiratory chain connecting Complex I with III and Complex II with III, is regarded as a major participant in the formation of  $O^{\bullet-2}$  by Complex III [9, 20, 21]. The dismutation of superoxide anions by superoxide dismutase (SODs, which are present in both cytosol, copper/zinc-associated isoform and mitochondria manganese-associated isoform) results in  $H_2O_2$  production [7, 22, 23].

**2.2. Nitric Oxide.** Nitric oxide ( $NO^{\bullet}$ ) is an abundant reactive radical that acts as an important oxidative biological signalling molecule in biological activities in several physiological processes including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation, and immune regulation [9, 17]. The nitric oxide, or nitrogen monoxide, radical ( $NO^{\bullet}$ ) produced by the stoichiometric conversion of L-arginine to L-citrulline via different isoforms of nitric oxide synthesis (NOS) [24]. Three isoforms account for  $NO^{\bullet}$  production and include neuronal NO synthase (nNOS; type I), inducible NO synthase (iNOS; type II) which is produced in very large amounts by activating microglia (macrophages), and endothelial NO synthase (eNOS; type III) [25–27].

Excessive superoxide rapidly reacts with NO and forms peroxynitrite ( $ONOO^-$ ) which protonated at relevant pH to form peroxynitrous acid ( $ONOOH$ ); this reaction is much faster than dismutation of superoxide by SOD and would result in decreased NO bioavailability; both  $ONOO^-$  and  $ONOOH$  are potent oxidizers;  $ONOOH$  exhibits hydroxyl radical ( $OH^{\bullet}$ )-like activity [28]. Peroxynitrite is a potent oxidant that can nitrate tyrosine residues of structural proteins; under physiological conditions,  $ONOOH$  can react with other components present in high concentrations, such as  $H_2O_2$  or  $CO_2$ , and function as NADPH oxidase [28, 29]. Nitric oxide may take part in nitrosylation of proteins; however, peroxynitrite is a highly reactive nitrogen species, which induces tyrosine nitration, lipid peroxidation, and cytotoxicity, including cellular death [26, 30].

An excessive generation of free radicals (ROS and RNS) and decrease of enzymatic antioxidant activity are considered as the main causes of oxidative stress that can result in cellular injury in the form of lipid peroxidation, DNA damage, protein oxidation and disruption of the cell functions, and/or inducing cell death on the CNS. ROS and RNS are involved in both apoptosis and/or necrosis mechanisms for neuronal death.

**2.3. Mitochondrial Dysfunction.** As described above, the mitochondrial electron transport chain contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of ROS production, which function as second messengers in signal transduction but are also mediators of oxidative damage and inflammation [7, 20, 31]. A neuron uses much of  $O_2$  it takes up to make, via mitochondria, ATP needed to maintain low gradients (high intracellular  $K^+$ , low  $Na^+$ , very low, and “free”  $Ca^{+}$ ) adequate energy supply by mitochondria is essential for neuronal excitability and neuronal survival [25, 32, 33].

Dysfunctional mitochondria may contribute to increased ROS production and would be unable to maintain optimal mitochondrial calcium ( $\text{Ca}^{2+}$ ) levels which consequently can lead to depolarization of the inner mitochondrial membrane potential [21]. The generation of ROS and the release of proapoptotic molecules to the cytoplasm, mitochondrial swelling, and mitochondrial membrane rupture lead to the activation of different modes of cell death. Those changes that affect neuronal calcium homeostasis may be factors that contribute to increase of susceptibility to epileptic seizures associated with mitochondrial dysfunction [7, 34].

**2.4. Role of Calcium and Mitochondria.** Calcium signaling plays an important role in regulating and maintaining normal neuronal function, including neurotransmitter release, excitability, neurite outgrowth, synaptic plasticity, gene transcription, and cell survival. The mitochondria sequester free intracellular  $\text{Ca}^{2+}$  through several transport systems maintains cell  $\text{Ca}^{2+}$  homeostasis and serves as  $\text{Ca}^{2+}$  buffer which regulates the intracellular  $\text{Ca}^{2+}$  homeostasis; when  $\text{Ca}^{2+}$  accumulates in the mitochondria, it released in the matrix along with other solutes and this process also accompanied by oxidative stress and depletion of adenine nucleotides [20]. Neuronal increases in calcium can activate a series of enzymes including protein kinase C, proteases, phosphatases, phospholipases, and xanthine oxidase; the last three (phospholipase A2) produce ROS and RNS by triggering an acid arachidonic cascade [25, 35].

Mitochondrial  $\text{Ca}^{2+}$  increase results in enhanced ROS production; between others the potential deleterious effect of ROS production in mitochondria is the facilitation of  $\text{Ca}^{2+}$ -dependent mitochondrial permeability transition pore (MPTP), which can be stimulated to open by excessive concentrations of  $\text{Ca}^{2+}$  and can also extrude  $\text{Ca}^{2+}$  [7, 36]. Moreover,  $\text{Ca}^{2+}$  can active nitric oxide synthase (NOS) and generate NO and peroxynitrite ( $\text{ONOO}^-$ ), increasing also RNS production [23]. Mitochondrial  $\text{Ca}^{2+}$  overload triggers the opening MPTP, which can lead to necrosis owing to ATP depletion or to caspase-dependent apoptosis; this confirms the complex interdependence between mitochondria,  $\text{Ca}^{2+}$ , and ROS generation [23]. The release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum and the activation of the caspase-dependent apoptosis pathway through changes in mitochondrial membrane permeability induce cellular damage (Figure 1) [36, 37].

**2.5. Cellular Antioxidant Defense.** The physiological production of ROS in aerobic organisms requires the presence of a defense system against the effects of these oxidative species. Antioxidants can be divided into two groups: endogenous and exogenous; the mitochondria possess multiple antioxidant defense systems including glutathione, glutathione peroxidase (GPx), superoxide dismutase, catalase, and vitamins E and C [38, 39]. The brain contains reduced levels of glutathione, almost no catalase, and has low concentrations of glutathione peroxidase and vitamin E [11, 40, 41].

Glutathione (GSH) is one of the most important antioxidant defenses against oxidative stress and exists in both the

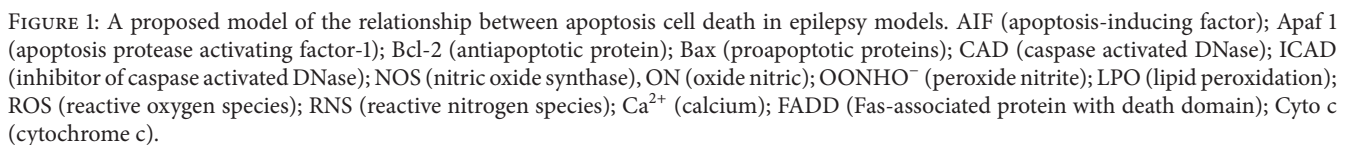
reduced (GSH) and oxidized state (GSSG; glutathione disulphide); oxidised glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism [9, 39, 42, 43]. The main role of glutathione is as cofactors of several detoxifying enzymes, participates in amino acid transport through the plasma membrane, scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase, and is able to regenerate the most important antioxidants, vitamins C and E [9, 44].

Superoxide dismutase (SOD) is an endogenous enzymatic antioxidant that has shown to protect against programmed cell death. Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified by Cu, Zn-SOD (SOD1, localized in the intermembrane space), and Mn-SOD (SOD2, localized in the matrix) [43, 45, 46]. In mitochondria and peroxisomes, finding catalase (CAT), catalyzes the dismutation of  $\text{H}_2\text{O}_2$  to water and oxygen [43].

Vitamin C is an antioxidant hydrophilic of low molecular weight and vitamin E is lipophilic; from the diet obtained both vitamin C and vitamin E. A major lipid soluble antioxidant reported is vitamin E (alpha-tocopherol), effective at protecting against membrane LPO, whereas that ascorbate can act as an efficient antioxidant and scavenge a variety of ROS in vitro [12, 28].

**2.6. Excitotoxicity.** Excessive glutamate receptor activation can induce oxidative stress increase, described by the term excitotoxicity, and play a critical role in epileptic brain damage [47]. Glutamate is the principal excitatory neurotransmitter and its interaction with specific membrane receptors is responsible for many neurological functions; these receptors divided into three major types based on their selective agonist: N-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5methyl-4-isoxalopropionate (AMPA), and kainate. In the brain ionotropic and metabotropic, receptors mediate the action of glutamate via activation of the NMDAr and play a central role in learning and memory [48]. The NMDAr, mediated by  $\text{Ca}^{2+}$ , activates protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and calcium/calmodulin-dependent protein kinase (CAMK) pathways, which converge at the cyclic-AMP-response element-binding protein (CREB) [27]. The phospholipase A2-dependent activity of  $\text{Ca}^{2+}$  mediated by glutamatergic receptors liberates arachidonic acid (AA), which generates  $\text{O}_2^-$  through its metabolism by lipoxygenases and cyclooxygenases for eicosanoid formation [49]. In various neurodegenerative disorders, excessive activation of glutamate receptors may induce neuronal injury or death predominantly mediated by excessive influx of calcium into neurons through ionic channels triggered by the activation of glutamate ionotropic receptors [47].

**2.7. DNA Damage.** Endogenous DNA damage, which is incidental to normal cellular metabolism, consists of DNA lesions continually generated by spontaneous decay, depurination, depyrimidination, and deamination; free radicals mediated oxidation and strand breaks and other DNA transactions



Oxidative damage to DNA leads to the formation of lesions such as 8-hydroxy-2-deoxyguanosine (8-OHdG) is a hydroxyl radical-damaged guanine nucleotide, excised from DNA by endonuclease repair enzymes, and is the most used biomarker of oxidative DNA alteration [55]. One important target of ROS is the mtDNA due to the close proximity to the electron transport chain and the lack of protective histones [56, 57]. The failure of adaptive responses to ongoing oxidative stress in the brain during epileptogenesis, such as mtDNA repair, could lead to an increase in seizure susceptibility. An increase in mitochondrial oxidative stress is able to impair the mtBER, which involves a highly coordinated process catalyzed by the sequential actions of the different DNA repair enzymes. The mRNA levels of some of these proteins



have been elevated following KA-induced status epilepticus but decreased during chronic epilepsy [58].

**2.8. Apoptosis.** Apoptosis is a physiological process for removing unwanted cells during development and for maintaining tissue homeostasis. Specific morphological and biochemical changes may be characterized as an apoptotic cells, including cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA [59, 60]. The extrinsic pathway is a common phenomenon induced either by specific insults mediated through death receptors, whereas, in the intrinsic pathway, death signals act directly or indirectly on the mitochondria, resulting in the release of cytochrome c and formation of the apoptosome complex [61, 62].

A combination of ROS production and mitochondrial  $\text{Ca}^{2+}$  initiates opening of the MPTP, which allows translocation of proapoptotic molecules from the mitochondria to the cytosol, in order to trigger apoptotic cell death. The activation of MPTP creates an open channel across the mitochondrial inner and outer membranes, which permits the free diffusion of cytochrome c release from mitochondria to cytoplasm where it activates caspase-9, which can then activate caspase-3 [19, 25, 63]. Another family of mitochondrial-associated proteins are the Bcl-2; this family of proteins consists of both proapoptotic (Bad, Bax, and Bim) and antiapoptotic (Bcl-2, Bcl-xL, and Bcl-w) members and it is hypothesized that they exert their effects by interacting with or controlling the inner of MPTP opening [61, 64, 65]. Apoptosis-inducing factor (AIF) is another mitochondrial-associated protein that is normally located in the intermembrane space of mitochondria and upon a proapoptotic signal AIF is released from the mitochondria. AIF subsequently migrates to the nucleus and triggers DNA damage and, on the other hand, also participates in the activation of caspase-9 in the cytoplasm [3, 61]. The alternative apoptotic pathway is the external pathway with death receptors and caspase-8 as main players. Both internal and external apoptotic pathways meet at the level of caspase-3, which activates CAD (caspase activated DNase) or DFF40, thereby inducing specific DNA fragmentation and apoptotic cell death [65, 66]. Experimental evidence has demonstrated that apoptosis is associated with signaling pathways and contributes to seizure-induced neuronal death in brain of animal models of epilepsy (Figure 1) [3, 25, 65].

### 3. Oxidative Stress in Epilepsy

Generalized epilepsy is a chronic disorder characterized by recurrent seizures, which can increase the content of ROS and RNS generation in the brain; several human and experimental studies have shown the relationship between epilepsy and oxidative stress. Despite the fact that it is still not known if oxidative stress is a cause or consequence of this pathology, it has been widely mentioned that an increase in free radical generation can lead prolonged seizures which may result in mitochondrial dysfunction in the hippocampus that precede neuronal cell death and cause subsequent epileptogenesis [67]. Animal models of epilepsy have provided inconsistent results concerning alterations in redox status.

While no changes in GSH levels were found to increase at 4 h post-SE in the cortex, suggesting that GSH may play a disproportionate role in the cortex but not in the hippocampus during epileptogenesis [68], several studies provide evidence of a decrease in hippocampal redox status following SE [69, 70]. A time-dependent decrease in the GSH/GSSG ratio accompanied by a moderate increase in GPx activity and a decrease in GR activity in hippocampal homogenates and mitochondria, following KA-induced SE, have been also reported [71]. Extensive neuronal death in the CA3 subfield occurs from 2–7 days following KA treatment after the early onset of reported redox changes, suggesting the altered redox status may contribute to seizure-induced neuronal death [72, 73].

Lipid peroxidation (LPO) is a central feature of oxidative stress and occurs through a radical-mediated abstraction of a bisallylic hydrogen atom from either the polyunsaturated  $\omega$ -3 or  $\omega$ -6 fatty acids; the delocalized radical reacts then with  $\text{O}_2$  through radical coupling leading to the formation of lipid peroxyl radicals ( $\text{LOO}^\bullet$ ).  $\text{LOO}^\bullet$  generates a number of lipid hydroperoxide products such as malondialdehyde (MDA), 4-hydroperoxy-2-nonenal (HPNE), 4-oxo-2-nonenal (ONE), and 4-hydroxy-2-nonenal (HNE) [19, 55, 56]. The studies of mitochondrial dysfunction or oxidative stress in the human brain are limited due to the low tissue availability. However, lipid peroxidation has been used as peripheral markers in experimental animals, since results demonstrated that KA-induced increased seizure susceptibility associated with mitochondrial oxidative stress in the hippocampus due to increased mitochondrial LPO and loss of glutathione homeostasis [74].

Several clinical studies have found a decrease of antioxidant (GPx, CAT, and Cu-Zn-SOD) levels and activity in blood of patients with progressive myoclonic epilepsies, showing that the activity of Cu-Zn-SOD in patients was lower than in controls. [75]. Likewise, another study showed that the erythrocyte GSH, GSH-Px, plasma total antioxidant status (TAS), and vitamin E concentration were lower than in control of refractory epilepsy group [76]. Similar results have been observed in drug-resistant epileptic patients [77] and elevated levels of MDA as markers of oxidative damage in women with epilepsy and also have been reported [78]. Lipid peroxidation and percentage hemolysis have shown that the antioxidant status was low in the blood of epileptic patients compared to controls, improved after treatment, suggesting that free radicals may be implicated in epilepsy [79].

Diverse reports have shown that prolonged seizure activity (status epilepticus; SE) results in oxidative damage involving calcium overload and induction of apoptosis. Differences in the expression of many caspases (2, 3, 6, 7, and 9) have been detected by immunohistochemistry method in human TLE brain samples; the caspases appear to localize within both the cell soma and dendrites, supporting caspase-mediated cleavage of intracellular structural or synaptic proteins [80, 81]. The Bcl-2 and caspase families analyzed in neocortex samples surgically removed from TLE patients with intractable seizures found significantly higher levels of antiapoptotic Bcl-2 and Bcl-xL compared to autopsy controls. The levels of Bcl-xL positively correlated with patient seizure frequency,

suggesting that in human TLE has been modulated both by pro- and anti-apoptotic pathways [82]. Similar studies have shown altered expression of Bcl-2, procaspases (2, 6, 7, 8, and 9), and caspases (3, 7, 8, and 9) family genes on hippocampus of patients with intractable TLE [65]. Likewise, correlative analysis with detection of expression of apoptosis-associated genes including bcl-2, p53, bax, fas, and caspase-3 showed that neuronal apoptosis occurs in mesial temporal sclerosis patients with intractable TLE [83]. Moreover, occasional TUNEL positive cells with apoptotic cells were observed in hippocampus of these TLE patients [65].

#### 4. Experimental Models

Animal models of seizures and epilepsy have proven useful as a complementary strategy in advancing our understanding of this disease. The experimental models are divided in two main categories: models of seizures and models of epilepsy. The difference between these two groups is that those models of epilepsy are characterized by multiple spontaneous recurrent seizures (TLE, evoked by pilocarpine or kainic acid), whereas models of seizures are characterized by generalized seizures in response to a single exposure to a potent neurotoxin [16]. An ideal model of epilepsy should have the following characteristics: (1) seizures should be as the spontaneous recurrent seizures, (2) seizures should be similar to seizures in humans, (3) the EEG pattern should be similar to related types of epilepsy, and (4) the frequency of seizures should be sufficient to test acute and chronic effects of drugs [15]. Therefore, the experimental model of epilepsy should be analogous to the human seizure state and it should share very similar neuropathological mechanisms.

**4.1. Kainic Acid.** Kainic acid (KA) is a rigid analog of the putative excitatory neurotransmitter glutamate and potent agonist of the AMPA/kainate class of glutamate receptors. KA model of status epilepticus (SE) is one of the most extensively studied seizure models. Systemic or intracerebral injection of KA, which stimulates a subtype of the ionotropic receptor of the neurotransmitter glutamate, can result in sustained epileptic activity in the hippocampus that lasts for hours, followed by a latent seizure-free period of weeks. Preceding the development of spontaneous recurrent focal seizures that begin between 3 and 4 weeks followed by a selective pattern of brain damage similar neuropathological (cytotoxic brain edema, neuronal degeneration and loss, microgliosis and astrogliosis) to human TLE [3, 15, 84, 85].

KA increase ROS production, mitochondrial dysfunction particularly in hippocampus. Oxidative stress and excessive glutamate receptor activation and the ensuing LPO are extensively associated with seizure activity [3, 72, 75, 86, 87]. The pyramidal neurons of the hippocampus are particularly vulnerable to the neuroexcitatory actions of KA due to the activates ionotropic glutamate receptors, which selectively induces excitotoxic cell death in the CA3 and CA1 hippocampal subfields and within the dentate gyrus showing that the vulnerability of neurons to oxidative stress varies from one brain region to another [72, 85, 88]. On the other hand,

CA2 pyramidal neurons and dentate granule cells appear to be resistant to damage induced by KA [85]. Likewise, in the hippocampus, astrocytes in the CA1 region under stress conditions, display selective loss of glutamate transport activity, increased mitochondrial ROS generation, and reduced mitochondrial membrane potential [87, 89].

Evidence from animal studies suggests that both brief and prolonged seizures treated by KA can induce activation of caspases and neuronal apoptosis within the hippocampus. DNA fragmentation and chromatin condensation in cerebellar neurons following exposure to KA were reported earlier, demonstrating that KA can induce apoptosis [90]. Similar results (DNA fragmentation) were observed in CA3 pyramidal neurons in hippocampus of rats after focal-onset status epilepticus at 24 h; thus, prolonged seizures can cause apoptosis in hippocampal subfields in addition to the dentate granule cell layer, regardless of model, age, species, and/or strain [91]. Induction of apoptosis also was observed in dentate gyrus neurons of rats with single and intermittent brief seizures induced by KA, suggesting that this process occurring early during epileptogenesis, how primary events in the development of hippocampal pathology [92]. The marked release of cytochrome c from mitochondria into the cytosol and a higher level of caspase-3 cleavage were observed in KA-treated SAM-P8 mice [74]. The cytochrome c release following intrahippocampal KA injection [93], upregulating both caspases 2 and 3 in the rat hippocampus, has been associated with status epilepticus during the period of epileptogenesis. In our works (data not shown before), we detected by immunohistochemical methods caspase-9 -3 and TUNEL (a marker of irreversible DNA fragmentation) positive cells in hippocampus of rats injected with KA (Figure 2). Related studies have corroborated these results using histochemical (TUNEL or activated caspase-3 staining) or ultrastructural analysis found features of apoptotic cell death present bilaterally in the hippocampus 1–7 days after the elicitation of sustained hippocampal seizure activity by microinjection of KA [65, 93]. Increases of oxidative stress induced by the mitochondrial production of superoxide radicals, increase in LPO, and decreases in GSH resulting from KA administration have been reported; these evidences have shown to play a critical role of oxidative stress on induction of apoptosis neurons in many regions of the brain particularly in the hippocampal regions of CA1 and CA3 [79, 90].

**4.2. Pentylenetetrazol Model (PTZ).** The Pentylenetetrazol (PTZ) model for induction of epilepsy is considered similar to primary tonic-clonic generalized epilepsy in humans. PTZ is tetrazole derivative with consistent convulsive actions in mice, rats, cats, and primates, when given by the parenteral route and it is considered a GABA selective agonist [94, 95]. It has also been reported that one of the mechanisms that underlie epilepsy produced by PTZ is the increase of voltage at the voltage-gated potassium channel [96, 97]. There is also a known relationship between the imbalance of the inhibitory and excitatory neurotransmission systems, and in the long run, a loss of inhibition mediated by GABA [98]. Specifically, PTZ blocks the GABA<sub>A</sub> receptor [95, 97] and both GABA<sub>A</sub>



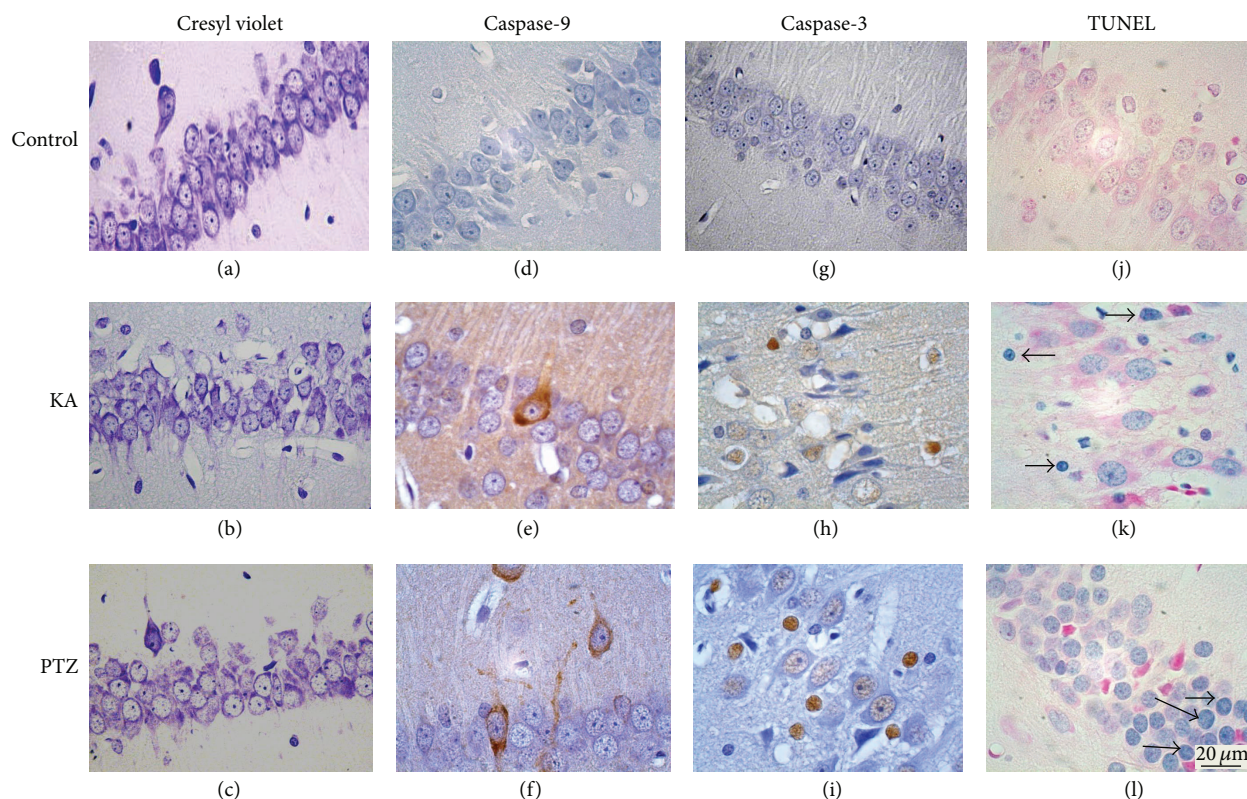


FIGURE 2: Representative photomicrographs of hippocampal fields of rats at several times after injection of KA or PTZ. Sections stained with cresyl violet, showing neuronal cells in the hippocampus CA1 field (a, b, and c). Hippocampus showing immunoreactive pyramidal cells to caspase-9 (d, e, and f). Immunoreactive cells to caspase-3. The caspase-3 staining was observed in the cytoplasm and nucleus (g, h, and i). Some pyramidal cells (j and k) and granular cells (l) of dentate gyrus were stained positively for TUNEL (↑).

and GABA<sub>B</sub><sub>Rs</sub> are involved in the control of neuronal excitability and epileptogenesis [99]. PTZ initially produces myoclonic jerks, which become sustained, and may lead to waves or polyspikes. On the other hand, the PTZ treatment needs repeated injections to result in cell loss in the hippocampus, which might be a result of enhanced activity of glutamatergic systems [98]. The PTZ treatment leads to hippocampal atrophy in rats shown a selective neuronal loss and astrogliosis [15, 100].

After PTZ induced seizures, significant decreases in GSH, GSSG were reported [101], with reductions in total SOD activity and lipid antioxidant (α-tocopherol) content [102]. MDA, NOS, and lactate dehydrogenase (LDH) had lower levels of SOD [103, 104] and increases of HO<sup>•</sup> [105] also were observed in several brain regions of PTZ-kindled rats. These results suggest that oxidative stress is implicated in PTZ-induced kindling and that antioxidants could play a role in controlling the accompanying changes [103].

On the other hand, Nasser [99] reports for the first time that PTZ-induced seizures triggered activation of caspases-3 to induce widespread apoptotic neuronal death in prenatal rat hippocampal neurons, providing a possible mechanistic link between maternal epilepsy induced neurodegeneration. Likewise, expression caspase-3 and induction of neuronal apoptosis were observed in adult rats induced epileptic seizures with PTZ [106]. We have detected mediated immunohistochemical method of some caspase-9 and caspase-3 positive cells

in hippocampus and dentate gyrus in rats exposed to PTZ (Figure 2) and also found occasional TUNEL positive cells in the dentate gyrus in rats treated with PTZ (Figure 2).

**4.3. Pilocarpine.** Systemic administration of the cholinergic muscarinic agonist, pilocarpine, in rats is widely used as an experimental model of status epilepticus because it reproduces many of its features, including refractory seizures, selective interneuron loss, and poor control of seizures by anticonvulsants [107, 108]. Some important features of the pilocarpine model are (i) the induction of acute SE more rapidly than with intraperitoneal (i.p.) KA; (ii) the presence of a latent period followed by the appearance of spontaneous recurrent seizures (SRSs, chronic phase); (iii) the occurrence of widespread lesions some of them localized in the same brain areas affected in TLE patients and associated with neuronal network reorganization in hippocampal and parahippocampal regions; (iv) the fact that seizures are poorly controlled by AEDs in patients and pilocarpine-treated epileptic rodents [95, 109].

The initiation of SE by pilocarpine is due to activation of the cholinergic system, the histopathology, cell loss in the hilus, CA3, and CA1 that leads to a reduction in the Schaffer collateral input, and spontaneous seizure activity is thought to be a result of seizure-induced glutamate release [107]. Experimental evidence has demonstrated that pilocarpine acting

through M1 muscarinic receptor subtype, which causes an imbalance between excitatory and inhibitory transmission, results in the generation of SE [110]. Associated with this, an elevation in glutamate levels in the hippocampus maintained the seizures by NMDA receptor activation [111, 112].

Pilocarpine epilepsy model can be mediated by increases in oxidative stress, which could have a role in the hippocampal neurodegeneration. Several reports demonstrated that LPO levels and nitrite content in the brain of adult rats were increased after the acute phase of seizures induced by pilocarpine [113–115]. Agreeing with these results other works have shown in rat hippocampus a significant increase in CAT activity, glutamate content, and a decrease in taurine level [116], as well as the increase of ROS generation in CA1, CA3, and the dentate gyrus [117]. The reduction of the functions of these systems during SE produced by Pilocarpine suggests an involvement of oxidative stress in neuronal death in this experimental epilepsy model.

As discussed above, oxidative stress is a major factor apoptosis induction. The model of epilepsy induced by pilocarpine has shown that it is able to increase the oxidative stress; therefore, the cellular damage observed probably may be due to this mechanism of cell death. However, there is no convincing evidence to show the presence of apoptosis and caspase activation in this model. The necrotic neurons show nuclear pyknosis, chromatin condensation, and internucleosomal DNA fragmentation without nonspecificity of these nuclear changes; these results indicate that, in adult rats, exposed lithium-pilocarpine produces neuronal injury with the appearance of necrosis rather than apoptosis [118]. On the other hand, expression of caspase-3 followed SE showed a significant increase in the number of caspase-3 positive cells in CA1/CA3 area and DG of treated pilocarpine rats [119]. Besides, the activation of caspase-9 and caspase-3 occurred at 4 h, increased into peak levels at 12 h–3 d, and then gradually went down at 7 d–14 d after onset of SE in a mouse pilocarpine model of chronic epilepsy [120]; in another study, the induction of expression of the same caspase-3 appeared at 7 days after lithium-pilocarpine administration [121].

## 5. Conclusions

This review provides an overview of evidence from experimental models that suggests the role of oxidative stress and mitochondrial dysfunction on apoptosis induction in seizure-induced neuronal damage. Oxidative stress enzymes induce a variety of cellular problems that can lead to mitochondrial dysfunction, and accumulation of ROS/RNS not only contributes to the injury of macromolecules such as lipids, proteins, but also affects bioenergetics, glutamate excitotoxicity, and the DNA, with induction of apoptotic signals (Figure 1). A wide variety of models have been developed in order to explore the principal mechanisms of epilepsies. Animal studies have demonstrated that both status epilepticus and recurrent seizures can alter the brain, and the evidence supports that prolonged seizures invoke mitochondrial dysfunction and oxidative stress, leading to caspases activation and induction to apoptosis; therefore,

both mitochondrial alteration and oxidative stress are a component of epileptogenesis. Continued research from a complete comprehension of the basic mechanisms of epilepsy, more effective drugs and treatments will be developed for the type of epileptic seizures.

## Conflict of Interests

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

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## Review Article

# Diabetes and the Brain: Oxidative Stress, Inflammation, and Autophagy

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Diabetes mellitus is a common metabolic disorder associated with chronic complications including a state of mild to moderate cognitive impairment, in particular psychomotor slowing and reduced mental flexibility, not attributable to other causes, and shares many symptoms that are best described as accelerated brain ageing. A common theory for aging and for the pathogenesis of this cerebral dysfunctioning in diabetes relates cell death to oxidative stress in strong association to inflammation, and in fact nuclear factor  $\kappa$ B (NF $\kappa$ B), a master regulator of inflammation and also a sensor of oxidative stress, has a strategic position at the crossroad between oxidative stress and inflammation. Moreover, metabolic inflammation is, in turn, related to the induction of various intracellular stresses such as mitochondrial oxidative stress, endoplasmic reticulum (ER) stress, and autophagy defect. In parallel, blockade of autophagy can relate to proinflammatory signaling via oxidative stress pathway and NF $\kappa$ B-mediated inflammation.

## 1. Introduction

Diabetes mellitus is a common metabolic disorder which is associated with chronic complications such as nephropathy, angiopathy, retinopathy, and peripheral neuropathy. However, as early as 1922 it was recognised that diabetes also can lead to cognitive dysfunction [1]. Since then, studies in experimental models and in patients observed alterations in neurotransmission, electrophysiological and structural abnormalities, and neurobehavioral alterations, in particular cognitive dysfunction and increased risk of depression [2]. Moreover, the observed cerebral manifestations of diabetes appear to develop insidiously, largely independent of diabetes-associated acute metabolic and vascular disturbances (such as severe hypo- and hyperglycemic episodes and stroke). Although the magnitude of these cognitive deficits appears to be mild to moderate, they can significantly hamper daily functioning, adversely affecting quality of life [3].

In spite of this, the concept of central neuropathy has been controversial for more than 80 years now, but while trying to describe cognitive impairment in diabetes as a complication of the disease, the term “diabetic encephalopathy” was

introduced in 1950 [4]. However, this term “encephalopathy” has not been widely accepted, probably among other reasons, because it does not seem to match with the mild cognitive problems usually seen in (nondemented) diabetic patients. More recently it has been suggested that the term “diabetes-associated cognitive decline” (DACD) describes a state of mild to moderate cognitive impairment, in particular psychomotor slowing and reduced mental flexibility, not attributable to other causes [5]. In addition, it is now clear that diabetes increases the risk of Alzheimer’s disease, vascular dementia, and any other type of dementia [6, 7].

## 2. Pathophysiological Mechanisms Involved in Brain Damage in Diabetes

Long-term effects of diabetes on the brain are manifested at structural, neurophysiological, and neuropsychological level, and multiple pathogenic factors appear to be involved in the pathogenesis of the cerebral dysfunctioning in diabetes, such as the hypoglycemic episodes, cerebrovascular alterations, the role of insulin in the brain, and the mechanisms of hyperglycemia induced damage [8]. Moreover, the emerging

view is that the diabetic brain features many symptoms that are best described as accelerated brain ageing [9].

A common theory, for aging and for the pathogenesis of this cerebral dysfunctioning in diabetes, relates cell death to oxidative stress mediated by free radicals [10]. Thus, hyperglycemia reduces antioxidant levels and concomitantly increases the production of free radicals. These effects contribute to tissue damage in diabetes mellitus, leading to alterations in the redox potential of the cell with subsequent activation of redox-sensitive genes [11].

The brain is especially vulnerable to oxidative damage as a result of its high oxygen consumption rate, abundant lipid content, and relative paucity of antioxidant enzymes as compared to other tissues. Neuronal cells are particularly sensitive to oxidative insults, and therefore reactive oxygen species (ROS) are involved in many neurodegenerative processes such as diabetes [12–14]. Although under normal physiological conditions a balance exists between the production of ROS and the antioxidant mechanisms, it has been shown that in aging tissues oxidative stress increases due to, among others, decreased activity of antioxidant enzymes [15]. Earlier work and ample evidence have shown that peroxidative damage to lipid and protein occurs with the aging process and the products of these reactions accumulate in the brain with age [16–19].

Similarly, the activities of superoxide dismutase and catalase or glutathione peroxidase enzymes, involved in the antioxidant defense of the diabetic brain, are decreased [20–23]. However, the possible source of oxidative stress in brain injury also includes autooxidation of glucose, lipid peroxidation, and decreased tissue concentrations of low molecular weight antioxidants such as reduced glutathione (GSH) [24–27]. This alteration of glutathione levels may be related to an increased polyol pathway [28] activity as this leads to a depletion of NADPH which is necessary for the enzymatic reduction of oxidized glutathione.

Moreover, in these pathological conditions, cellular stress triggers mitochondrial oxidative damage, which may result in apoptosis and/or necrosis [29], and apoptosis induced by oxidative stress has been related to neurogenesis inhibition [30]. Thus, it has been described that DM leads to alterations in the mitochondrial electron transport chain; ROS formation, mitochondrial energy metabolism dysfunction, and oxidative stress are thus being recognized as the main players in diabetes-related complications [31]. In this sense, Cardoso et al. have shown that hippocampal mitochondria of streptozotocin (STZ)-induced diabetic rats presented higher levels of MDA together with an increased glutathione disulfide reductase activity and lower manganese superoxide dismutase (MnSOD) activity and glutathione-to-glutathione disulfide (GSH/GSSG) ratio. It also showed impaired oxidative phosphorylation system characterized by a decreased mitochondrial energization potential and ATP levels and higher repolarization lag phase [32]. On the other hand, although insulin is best known for its involvement in the regulation of glucose metabolism in peripheral tissues, this hormone also affects numerous brain functions including cognition, memory, and synaptic plasticity through complex insulin/insulin receptor (IR) signaling pathways [33].

Therefore, considering the important role of insulin in many aspects of neuronal function in both the peripheral nervous system and the central nervous system, it is possible that perturbation of insulin signaling (both insulin deficiency in T1 diabetes and hyperinsulinemia in T2 diabetes) is in the pathogenesis of neurological diseases [34] and results in neurodegeneration.

Until recently, the study of insulin resistance was mainly focused on metabolic tissues such as muscle and adipose tissue; recent data, however, suggest that insulin resistance also develops in the nervous system. Although neurons are not insulin-dependent, they are insulin-responsive [35]. Insulin receptors are widely expressed in the brain, including the olfactory bulb, cerebral cortex, hippocampus, hypothalamus, and amygdala. Insulin resistance in sensory neurons makes cells respond inappropriately to growth factor signals, and this impairment may contribute to the development of neurodegeneration and subsequent diabetic neuropathy. Moreover, insulin regulates mitochondrial metabolism and oxidative capacity through PI3K/Akt signaling [36, 37]; therefore, decreased Akt signaling by hyperinsulinemia-mediated IR may have profound effects on mitochondrial function in neurons and result in subsequent increased oxidative stress [38]. In fact, two of the leading theories that have emerged to explain insulin resistance center on mitochondrial function/dysfunction, although interestingly with opposite views. In one theory, inherited or acquired mitochondrial dysfunction is thought to cause an accumulation of intramyocellular lipids that lead to insulin resistance and implies that strategies to accelerate flux through  $\beta$ -oxidation should improve insulin sensitivity [39]. In the second theory, the impact of cellular metabolic imbalance is viewed in the context of cellular and mitochondrial bioenergetics, positing that excess fuel relative to demand increases mitochondrial oxidant production and emission, ultimately leading to the development of insulin resistance. In this case, elevated flux via  $\beta$ -oxidation in the absence of added demand is viewed as an underlying cause of the disease. Therefore, mitochondrial-derived oxidative stress is fairly well established as an underlying mechanism responsible for the pathological complications associated with diabetes [40], but it also has a role as a primary factor in the development of insulin resistance (and subsequent overt diabetes), since strong experimental evidence from various animal models utilizing mitochondrial targeted approaches has established a link between mitochondrial-derived ROS and insulin resistance in vivo [41, 42].

In conclusion, convincing evidence is now available from previous studies to prove the role of oxidative stress in the development of neuronal injury in the diabetic brain and the beneficial effects of antioxidants. More concretely, the beneficial effect of lutein and DHA in the brain of diabetic animals and the way that these substances were able to ameliorate the oxidative stress present in diabetes has been studied by our group [27, 43]. However, we must take into account, that there are also studies which report the lack of effect of antioxidants in diabetic complications. Thus, Je et al. [44] reported that vitamin C supplementation alone shows limited therapeutic benefit in type 1 diabetes and is more commonly used in combination with vitamin E or

other agents [44]. Moreover, most of the evidences favoring the increased oxidative stress in diabetes come from studies in experimental models of diabetes in which the degree of hyperglycemia is excessive. Supportive evidence is also available in studies of human subjects with diabetes; however interventional studies using select antioxidant supplements have failed to show significant benefits of supplementation, as reviewed by Hasanain and Mooradian [45]. The completion of some of the ongoing large clinical trials will shed additional light on the clinical merit of antioxidant supplementation.

### 3. Inflammation in Diabetes

Inflammation represents a fundamental biological process which stands as the foreground of a large number of acute and chronic pathological conditions, and this occurs in response to any alteration of tissue integrity in order to restore tissue homeostasis through the induction of various repair mechanisms. Proper regulation of these mechanisms is essential to prevent uncontrolled amplification of the initial inflammatory response and shift from tissue repair towards collateral damage and disease development [46].

The appropriate recognition of the danger by the host is primordial for the elaboration of proper adaptive responses. Sensing of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) is ensured by a complex set-up of pattern-recognition receptors (PRRs), which include, among others, the receptor for advanced glycation end-products (RAGE). PRR activation triggers a wealth of intracellular signaling pathways, including kinases (e.g., MAP kinases, PI3 kinase), adaptors, transcription factors (mainly nuclear factor- $\kappa$ B (NF $\kappa$ B)), and activator protein-1. Such signaling cascades foster the expression of cytokines, chemokines, enzymes, growth factors, and additional molecules that are required for tissue repair [47] and homeostasis restoration. However, there are situations in which such restoration may not adequately occur, resulting in persistent cellular stress, perpetuating and amplifying the inflammatory response. In these conditions, the process leads to significant alterations of tissue functions, with systemic and persistent derangements of homeostasis [48]. Diabetes and neurodegenerative diseases are typical examples of these pathological processes associated with such chronic inflammatory changes [49].

The release of reactive oxygen species has long been recognized as a typical consequence of immune cell stimulation [50, 51], and both acute and chronic inflammatory states are coupled with significant alterations of redox equilibrium, due to the associated enhancement of oxidant generation [49, 52–54]. Accordingly, mitigating oxidative stress by the use of antioxidants has been evaluated as a potentially useful anti-inflammatory strategy in such conditions, as recently reviewed [55]. Overall, the results of innumerable studies have clearly pointed out the strong association between oxidative stress and inflammation. Since responses triggered by Toll-like receptors (TLRs) are conveyed primarily by the activation of NF $\kappa$ B, which is a master regulator of inflammation, controlling the expression of hundreds of

genes implicated in innate immune responses, and also a redox sensitive nuclear factor involved in the control of a large number of normal cellular and tissue processes, NF $\kappa$ B has a strategic position at the crossroad between oxidative stress and inflammation.

NF $\kappa$ B transcription factors are ubiquitously expressed in mammalian cells. These proteins are highly conserved across species, and in mammals the NF $\kappa$ B family (also known as the Rel family) consists of five members: p50, p52, p65 (also known as RelA), c-Rel, and RelB. Rel family members function as dimers and the five subunits can homodimerize or heterodimerize. All family members share a Rel homology domain, which contains the crucial functional regions for DNA binding, dimerization, nuclear localization, and interactions with the I $\kappa$ B inhibitory proteins. NF $\kappa$ B dimers exist in a latent form in the cytoplasm bound by the I $\kappa$ B inhibitory proteins, and when NF $\kappa$ B-inducing stimuli activate the I $\kappa$ B kinase complex that phosphorylates I $\kappa$ B, this leads to its ubiquitination and subsequent degradation in the canonical NF $\kappa$ B activation pathway. I $\kappa$ B degradation exposes the DNA-binding domain and nuclear localization sequence of NF $\kappa$ B and permits its stable translocation to the nucleus and the regulation of target genes [56]. Thus, activated NF $\kappa$ B enters the nucleus to induce transcription of a myriad of genes that mediate diverse cellular processes such as immunity, inflammation, proliferation, apoptosis, and cellular senescence [57].

Together with the evidences that relate oxidative stress and inflammation to the pathophysiology of diabetes, studies performed in a variety of cell and animal based experimental systems also suggest that NF $\kappa$ B activation is a key event early in the pathobiology of this disease and its complications [27, 58, 59]. In fact, several studies have highlighted the activation of NF $\kappa$ B by hyperglycemia and its relationship with diabetic complications, as reviewed by Patel and Santani in 2009 [59]; thus, hyperglycemia triggers a number of mechanisms that are thought to underlie diabetic neuropathy. Studies in different experimental models have established that neuronal dysfunction is closely associated with the activation of NF $\kappa$ B and the expression of proinflammatory cytokines [60, 61]. Moreover, NF $\kappa$ B pathway has been revealed as a key molecular system involved in pathological brain inflammation [62], and also experimental studies [52] have suggested that neuronal apoptosis, which is related to NF $\kappa$ B activation, may play an important role in neuronal loss and impaired cognitive function. Additionally, in the hippocampus of streptozotocin-treated rats, not only a strong increase in oxygen reactive species is observed but also a persistent activation of NF $\kappa$ B is observed [23, 27]. Activated NF $\kappa$ B can induce cytotoxic products that exacerbate inflammation and oxidative stress and promote apoptosis [63], leading to oxidative stress induced cell dysfunction or cell death, respectively [64]. However, it should not be forgotten that although NF $\kappa$ B is widely known for its ubiquitous roles in inflammation and immune responses and in control of cell division and apoptosis (and these roles are apparent in the nervous system), neurons and their neighboring cells employ the NF $\kappa$ B pathway for distinctive functions as well, ranging from the development to the coordination of cellular



responses to injury of the nervous system and to brain-specific processes such as the synaptic signaling that underlies learning and memory [60]. Therefore, understanding the function of NF $\kappa$ B transcription factors in the nervous system is now a new frontier for the general field of NF $\kappa$ B research, for the investigation of transcriptional regulation in complex neuronal systems, and for the understanding of pathological mechanisms of neurodegenerative diseases.

On the other hand, we cannot forget that type 2 (T2D) diabetes is an overnutrition related disease which usually is preceded by the metabolic syndrome, a common metabolic disorder that results from the increasing prevalence of obesity which includes several interconnected abnormalities such as insulin resistance, impaired glucose tolerance, dyslipidemia, and high blood pressure [65]. Moreover, overnutrition is considered as an independent environmental factor that is targeted by innate immune system to trigger an atypical form of inflammation, which leads to metabolic dysfunctions among others, in the central nervous system (CNS) and particularly in the hypothalamus [62, 66–69], which indeed is known to govern several metabolic functions of the body including appetite control, energy expenditure, carbohydrate and lipid metabolism, and blood pressure homeostasis [70, 71].

Deeping into the mechanisms that lead to this metabolic dysfunction, which also affects the CNS, it has been recently demonstrated that the activation of IKK $\beta$ /NF $\kappa$ B and consequently the proinflammatory pathway are a relevant feature in different metabolic disorders related to overnutrition [72–74]. The effects of NF $\kappa$ B-mediated metabolic inflammation are deleterious and can give rise to impairments of normal intracellular signaling and disruptions of metabolic physiology [62] that have been reported also in the CNS—particularly in the hypothalamus—which primarily could account for the development of overnutrition-induced metabolic syndrome and related disorders such as obesity, insulin resistance, T2D, and obesity-related hypertension [68, 75, 76]. Moreover, intracellular oxidative stress and mitochondrial dysfunction seem to be upstream events that mediate hypothalamic NF $\kappa$ B activation under overnutrition, and in turn such metabolic inflammation is reciprocally related to the induction of various intracellular stresses such as mitochondrial oxidative stress and endoplasmic reticulum (ER) stress [62]. Thus, intracellular oxidative stress seems to contribute to metabolic syndrome and related diseases, including T2D [39, 77, 78], and also to neurodegenerative diseases [79, 80]. In fact, when ROS homeostasis is disrupted, excessive ROS are accumulated in the mitochondria and cytoplasm and can cause oxidative damage to cells [81]. Regarding the ER, existing evidence also suggests that ER stress is a key link to obesity, insulin resistance, and type 2 diabetes [82], since this ER stress can also activate cellular inflammatory pathways which, in turn, impair cellular functions and lead to metabolic disorders [83] and neurodegenerative diseases [84, 85]. Indeed, unresolved ER stress can induce mitochondrial changes and finally cell apoptosis [86]. Moreover, brain ER stress is known to promote NF- $\kappa$ B activation in the development of central metabolic dysregulations associated to inflammatory pathways, since intraventricular infusion of an ER stress

inhibitor suppressed the activation of hypothalamic NF $\kappa$ B by high-fat diet feeding [68]. In addition, ER stress also appears to depend on IKK $\beta$ /NF $\kappa$ B pathway activity, because neither high-fat diet feeding nor central administration of chemical ER stress inducer is able to induce hypothalamic ER stress in mice with central inhibition of IKK $\beta$ /NF $\kappa$ B pathway [68, 87]. Finally, ER stress also causes cellular accumulation of ROS associated to oxidative stress [88], which in turn reciprocally can promote ER stress (see Figure 1).

In the case of ER stress, exposure to high glucose could induce ER stress by the generation of free radicals, aberrant protein glycosylation, or increased membrane and protein turnover. Zhang et al. have also reported that the expression of C/EBP homology protein (CHOP), the prominent mediator of the ER stress-induced apoptosis, was markedly increased in the hippocampus of diabetic rats and have suggested that this CHOP-ER stress-mediated apoptosis may be involved in hyperglycemia-induced hippocampal synapses and neuronal impairment and promote the diabetic cognitive impairment [89].

#### 4. Autophagy and Diabetes

Autophagy plays a role in the maintenance of function of organelles such as mitochondria or ER [90, 91], in order to maintain a healthy and functional intracellular environment, cells must constantly clean up defective proteins (e.g., misfolded proteins overflowing from ER stress) or damaged organelles (e.g., dysfunctional mitochondria or ER from prolonged oxidative stress). Although, autophagy is known primarily as a prosurvival mechanism for cells facing stress conditions, accumulating evidence indicates that autophagy can contribute to cell death processes under pathological conditions [92, 93]. Thus, among others, autophagy defect has been linked to the development of metabolic syndrome, diabetes, alcoholism, and lipid abnormalities [94–96], and in the majority of these cases, the underlying pathogenesis is related to the failure of autophagy machinery to efficiently remove defective proteins or damaged organelles from the cytosol. In fact, chronic intracellular stress such as mitochondria or ER stress seems to be the critical upstream events, since animal studies have shown that in early stages ER stress or oxidative stress induce adaptive autophagy upregulation, helping to restore intracellular homeostasis by disposing a number of harmful molecules such as unfolded or misfolded proteins in ER lumen, cytosolic proteins damaged by ROS, or even dysfunctional ERs and mitochondria [97, 98]. However, when intracellular stresses remain unresolved, prolonged autophagy upregulation progresses into autophagy defect [62] and, in fact, the decreased efficiency of the autophagic system with age has gained renewed attention as a result of the increasing number of reports supporting a role for defective autophagy in the pathogenesis of different age-related diseases including diabetes among others [99]. In parallel, autophagy pathway can relate to proinflammatory signaling via oxidative stress pathway [100], since mitophagy/autophagy blockade leads to the accumulation of damaged, ROS-generating mitochondria, and this in turn



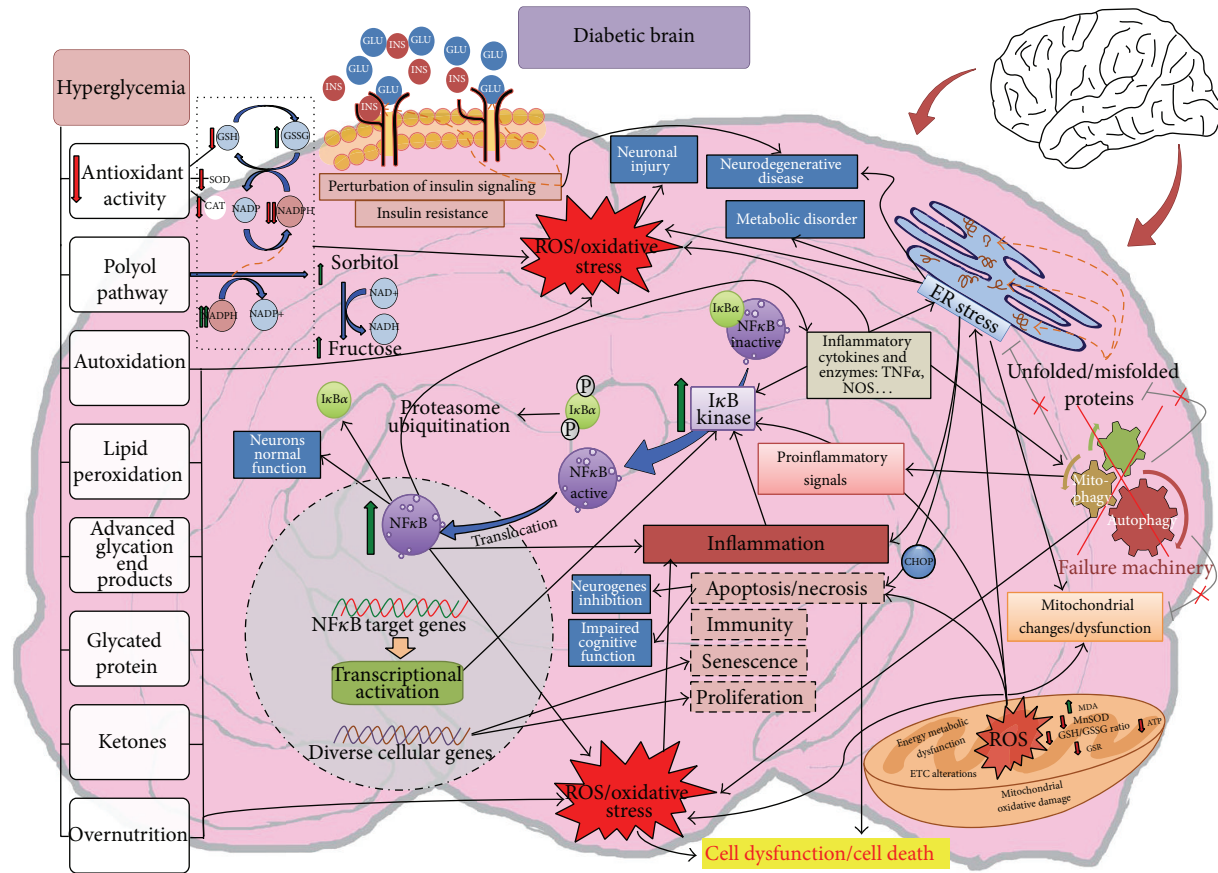


FIGURE 1: Scheme summarizing the involvement of oxidative stress (mitochondrial dysfunction and ER stress), inflammation, and autophagy in the diabetic brain. GSH: reduced glutathione; GSSG: glutathione disulfide; SOD: superoxide dismutase; NADP<sup>+</sup>: nicotinamide adenine dinucleotide phosphate oxidized; NADPH: nicotinamide adenine dinucleotide phosphate reduced; NAD<sup>+</sup>: nicotinamide adenine dinucleotide oxidized; NADH: nicotinamide adenine dinucleotide reduced; CAT: catalase; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; ER: endoplasmic reticulum; GLU: glucose; INS: insulin; P: phosphate; MDA: malondialdehyde; ATP: adenosine triphosphate; ETC: electron transport chain; ROS: reactive oxygen species; MnSOD: manganese superoxide dismutase; GSR: glutathione reductase; CHOP: C/EBP Homology Protein; TNFα: tumor necrosis factor alpha; NOS: nitric oxide synthases.

activates the NLRP3 inflammasome (a molecular platform activated upon signs of cellular “danger” to trigger innate immune defenses through the maturation of proinflammatory cytokines). Moreover, autophagy defect can induce NFκB-mediated inflammation [101, 102], even in the CNS, since Meng and Cai reported that defective hypothalamic autophagy led to hypothalamic inflammation, including the activation of proinflammatory IκB kinase β pathway [103].

Although it is clear that diabetes affects both mitochondria and ER, the role of autophagy in diabetes or metabolism is yet far from clear, and therefore the role of autophagy in the pathogenesis of diabetic complications is currently under intensive investigation.

As described by Hoffman et al., [104] specific candidates for induction and stimulation of autophagy include insulin deficiency/resistance [105, 106]; deficiency of insulin growth factor-1 (IGF-1) and insulin growth factor-1 receptor (IGF-1R) [104, 107]; hyperglucagonemia [106]; and hyperglycemia

[107]. Other candidates for perturbation of autophagy include alteration of protein synthesis and degradation [108] due to the oxidative stress of RNA [109, 110], protein damage, and altered lipid metabolism [94, 111]; increased production of ketones and aldehydes [112, 113]; and lipid peroxidation [110, 114]. Furthermore, accumulation of oxidized and glycated proteins, common protein modifications associated with diabetes, could be in part attributed to defective autophagy [115].

It is noteworthy that Hoffman et al. have reported that autophagy is increased in the brains of young T1D patients with chronic poor metabolic control and increased oxidative stress [116]. Moreover, the finding of significant expression of autophagic markers in both white and gray matter is in keeping with the structural deficits in young patients with T1D [117, 118] and the white matter atrophy in the frontal and temporal regions in these diabetic ketoacidosis cases [104]. However there are still few studies focusing on the role of

autophagy in the brains of T1D patients, and therefore further research is needed on the relationship between autophagy and pathogenesis of early onset diabetic encephalopathy in T1D.

## Conflict of Interests

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

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## Research Article

# The Effects of Sesquiterpenes-Rich Extract of *Alpinia oxyphylla* Miq. on Amyloid- $\beta$ -Induced Cognitive Impairment and Neuronal Abnormalities in the Cortex and Hippocampus of Mice

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As a kind of medicine which can also be used as food, *Alpinia oxyphylla* Miq. has a long clinical history in China. A variety of studies demonstrated the significant neuroprotective activity effects of chloroform (CF) extract from the fruits of *Alpinia oxyphylla*. In order to further elucidate the possible mechanisms of CF extract which mainly contains sesquiterpenes with neuroprotection on the cognitive ability, mice were injected with A $\beta_{1-42}$  and later with CF in this study. The results showed that the long-term treatment of CF enhanced the cognitive performances in behavior tests, increased activities of glutathione peroxidase (GSH-px) and decreased the level of malondialdehyde (MDA), acetylcholinesterase (AChE), and amyloid- $\beta$  (A $\beta$ ), and reversed the activation of microglia, degeneration of neuronal acidophilia, and nuclear condensation in the cortex and hippocampus. These results demonstrate that CF ameliorates learning and memory deficits by attenuating oxidative stress and regulating the activation of microglia and degeneration of neuronal acidophilia to reinforce cholinergic functions.

## 1. Introduction

As the global population ages, Alzheimer's disease (AD) is rapidly becoming an urgent public health challenge. It has been estimated that the number of people with dementia in the worldwide was 35.6 million in 2010, and this number will almost double every 20 years, to 65.7 million in 2030 and 115.4 million in 2050 [1]. Therefore, searching for safe, better tolerated, and effective drugs is necessary.

AD is a progressive degenerative disease of the brain which is characterized by deterioration of memory and cognitive functions with formation of senile plaques and neurofibrillary tangles and the loss of synapses in the selected regions of the brain. Several hypotheses of AD, including the  $\beta$ -amyloid peptide (A $\beta$ ) cascade hypothesis and the

cholinergic hypothesis, have been applied to investigate the etiology of AD [2]. Scientists have proposed the cholinergic hypothesis; namely, the alternations of cholinergic system are closely related to the damage of cognitive function and AD. Based on this theory, a number of studies have been launched on acetyl cholinesterase (AChE) inhibitor, and then AChE inhibitors have been successfully developed for clinical treatment of AD [3–5]. To eliminate the deposition of A $\beta$ , the main research strategy includes the following two pathways: one is to search A $\beta$  secretase inhibitors, mainly including  $\beta$ -secretase inhibitors; the other one is to prepare antibodies of A $\beta$  by using the immunological method in order to reduce the deposition of A $\beta$ .

A $\beta$  is a 40–42 amino acid proteolytic fragment of amyloid precursor protein (APP). The cascade begins with the



cleavage of the APP sequentially by  $\beta$ - and  $\gamma$ -secretase. The 42 amino acid  $A\beta$  fragment self-assembles into oligomers. Its oligomers have been considered as the principal toxic substances which induce oxidative stress, neuronal apoptosis, and increase of neuronal loss [6].  $A\beta_{1-42}$  also has direct pharmacologic effects on synaptic function, impairing memory, and long-term potentiation in animal models [7]. Neurofibrillary tangle formation, oxidation, excitotoxicity, inflammation, synaptic compromise, demyelination, mitochondrial dysfunction, and neurodegeneration follow the interaction with oligomeric  $A\beta$  [8].  $A\beta$  may also fibrillize to form insoluble aggregates that compose the neuritic plaques characteristic of AD. In rats treated with  $A\beta$ , the release of acetylcholine (ACh) and dopamine stimulated by nicotine decreased in the brain, which demonstrated the learning deficits observed in the  $A\beta$  protein-infused rats, is partly due to the impairment of neurotransmitter [9]. On the basis of the accumulating evidences on pathological roles of  $A\beta$  in the progress of AD,  $A\beta$ -injected animals have become a useful model for understanding the pathogenesis and progression of AD.

*Alpinia oxyphylla* Miq. is regarded as a precious drug and also a kind of condiment in Hainan district in southern China. Sesquiterpenes, diterpenes, flavonoids, and diarylheptanoids have been found in *Alpinia oxyphylla* Miq. previously and some of which showed inhibitory effect on nitric oxide (NO) production in lipopolysaccharide- (LPS-) activated mouse peritoneal macrophages [10–13]. There have been growing evidences showing that chloroform extract from the fruits of *Alpinia oxyphylla* possesses significant neuroprotective activity [14]. There are a few studies showed that *Alpinia oxyphylla* had therapeutic efficacy for senile dementia by reducing the apoptosis and free radical. However, the exact mechanism or components are not explicit [15]. On the basis of the findings above mentioned, we hypothesized that chloroform extract of *Alpinia oxyphylla* could ameliorate aging through inhibiting oxidative stress, improving the cholinergic system, and reducing  $A\beta$  levels in the brain. Therefore, in the present study, we investigated the actions of chloroform extract of *Alpinia oxyphylla* on cognitive ability, oxidative stress biomarkers, and  $A\beta$  deposition in the hippocampus and cortex of aging mice induced by  $A\beta_{1-42}$  to elucidate the underlying molecular mechanisms. Furthermore, compounds of the active fraction of the extract have been identified.

## 2. Materials and Methods

**2.1. Material.** *Alpinia oxyphylla* Miq. was purchased from Shenyang Tongrentang Drug Co., Ltd. (Shenyang, China). The crude drugs were of high quality and authenticated by Professor Ying Jia of Pharmacognosy Department, Shenyang Pharmaceutical University. Donepezil was supplied by Wambang Pharmaceutical Company (Zhejiang, China).  $A\beta_{1-42}$  was obtained from Sigma-Aldrich (St Louis, MO, USA) and dissolved in sterile physiological saline ( $1.0 \mu\text{g}/\mu\text{L}$ ) in the tube, which was then sealed and incubated for 120 h at  $37^\circ\text{C}$  to cause the peptide to aggregate. Commercial kits used

for determination of AChE, GSH-px, MDA,  $\beta$ -secretase, and  $A\beta_{1-42}$  were purchased from Jiancheng Institute of Biotechnology (Nanjing, China) and Qiming Biotechnology Company (Shanghai, China).

**2.2. Sample Extraction and Fractionation.** The air-dried fruits of *Alpinia oxyphylla* Miq. (10.0 kg) were extracted three times for 2 h each time by refluxing in 95% ethanol (1 : 10, w/v). The filtrates were concentrated and dried in vacuum at  $60^\circ\text{C}$ . The crude extract was dissolved in distilled water and then partitioned sequentially in different solvents, namely, petroleum ether, chloroform (CF), ethyl acetate, and *n*-butanol, to fractionate the polar and nonpolar compounds in the crude extract. The resulting solvent fractions were concentrated by rotary evaporator and dried by a vacuum oven at  $45^\circ\text{C}$ . The doses of chloroform extract were expressed as gram of the original dry materials per kilogram body weight.

**2.3. UPLC-ESI/MS Analysis for CF.** The chemical composition of CF was analysed by using a Waters-UPLC-Q-TOF/MS with an ultraviolet/visible detector (UV/Vis) coupled to an ion trap mass spectrometer with an ESI interface. The chromatogram was recorded at 255 nm. An HSS T3 Column ( $100 \text{ mm} \times 2.1 \text{ mm}$ ,  $1.8 \mu\text{m}$ ) with the column temperature set at  $25^\circ\text{C}$  was used for separation. The injection volume was  $5 \mu\text{L}$ , and elution was performed at a flow rate of  $0.6 \text{ mL}/\text{min}$  using a mixture consisting of acetonitrile (A) and 0.1% (v/v) formic acid (B). A gradient program was used as follows: 0–3 min, 20% A; 3–20 min, 20%–50% A; 20–25 min, 50%–90% A; 25–26 min, 90%–20% A; 26–28 min, 20% A.

Mass analyses were performed using an ESI interface in the positive ion mode. The data were acquired in the full scan and  $\text{MS}/\text{MS}^2$  scanning mode. The optimized instrumental parameters were set as follows: positive mode: desolvation temperature,  $250^\circ\text{C}$ ; source temperature,  $120^\circ\text{C}$ ; capillary, 3.0 kV; sampling cone 30.0; extraction cone 4.0; source temperature 130; desolvation temperature 450; desolvation gas flow (L/Hr) 800.0; collision energy 6.0 eV; and scan range,  $m/z$  100–1000 amu.

**2.4. Animals and Administration.** Seventy-two male ICR mice weighing 18–22 g were provided by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). They were maintained on standard laboratory conditions of temperature  $25 \pm 1^\circ\text{C}$  and a 12 h light/12 h dark cycle with food and water available ad libitum for the duration of the study. After 1 week of acclimatization, all mice were randomly divided into 6 groups ( $n = 12/\text{group}$ ): vehicle control group, sham-operated group, model group, donepezil group, chloroform group 1 (CF1), and chloroform group 2 (CF2). All the mice were anesthetized with 3.5% chloral hydrate ( $0.1 \text{ mL}/10 \text{ g}$ ). Then, model group, donepezil group, CF1 group ( $180 \text{ mg}/\text{kg}$ ), and CF2 ( $360 \text{ mg}/\text{kg}$ ) were injected with aggregated  $A\beta_{1-42}$  peptide ( $3 \mu\text{L}$ ) into the right lateral ventricle within 3 min by means of a stereotaxic apparatus (AP,  $-0.5 \text{ mm}$ , ML,  $\pm 1.1 \text{ mm}$ , DV,  $-3.0 \text{ mm}$ ). The needle was removed with 1 min delay to allow diffusion. Mice in the sham-operated group were injected in an identical manner

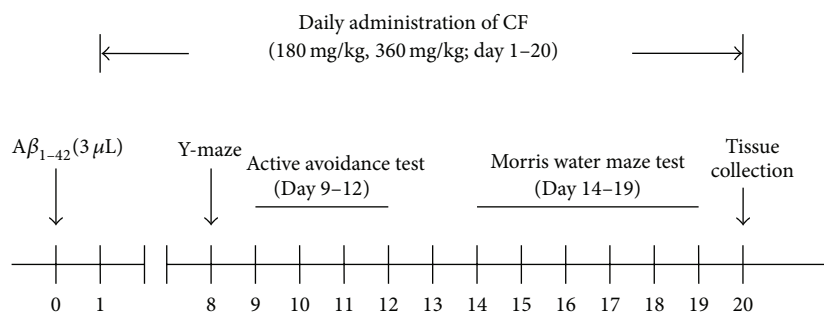


FIGURE 1: The experiment schedule.

with the same amount of physiological saline (3  $\mu$ L). From the next day, mice in the CF1 and CF2 were administered with CF of 180 mg/kg and 360 mg/kg in distilled water containing 1.0% DMSO (20 mL/kg, i.g.) daily for 20 consecutive days by intragastric infusion (i.g.). Mice in donepezil group were administered with DPZ (0.65 mg/kg, i.g.). Mice in vehicle control group, sham-operated group, and model group were treated with distilled water containing 1.0% DMSO (20 mL/kg, i.g.) in the same period. The experiment schedule is shown in Figure 1. Animal care was in accordance with the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University and the protocol was approved by the Animal Ethics Committee of the institution.

## 2.5. Behavioral Experiments

**2.5.1. Y-Maze Test.** Y-maze test was used as a measure of immediate spatial working memory which was a form of short-term memory [16]. Y-maze is a three-arm maze with equal angles between all arms. Mice were initially placed within one arm, and the sequence and number of arm entries were recorded manually for each mouse over an 8 min period. The alternation score (%) for each mouse was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100 as shown by the following equation:  $\text{Alternation\%} = [(\text{Number of alternations}) / (\text{Total arm entries} - 2)] \times 100\%$ . The number of arm entries was used as an indicator of locomotor activity.

**2.5.2. Active Avoidance Test.** Learning and memory ability were detected by the active avoidance as described previously [17]. During the training session each trial began when the animal was introduced into any of the compartments with its head oriented toward the wall opposite to the mouse hole. After a variable period ( $\pm 60$  s), a conditioned stimulus was delivered. If the mouse crossed to the opposite compartment during the presence of the conditioned stimulus, an avoidance response was scored. If the mouse did not cross during the presence of the conditioned stimulus the unconditioned stimulus was delivered and remained on for 10 s or until the animal escaped to the opposite compartment. If the animals crossed to the opposite compartment within 10 s, an escape response was scored and a new trial began. The session

consisted of 30 trials and it ended when 30 trials finished. The percent of conditioned avoidance response and total time of test were recorded.

**2.5.3. Morris Water Maze Test.** Learning and memory ability were detected by Morris water maze test as described previously [18]. The experimental apparatus consisted of a circular water basin (150 cm in diameter, 60 cm in height), containing water ( $25 \pm 2^\circ\text{C}$ ) to a depth of 40 cm, which was rendered opaque by adding black nontoxic carbon ink. A platform (9 cm in diameter, 38.5 cm in height) was submerged below the water surface and placed at the midpoint of one quadrant. It was given 90 s to find the platform and was allowed to rest on it for 15 s. The animals which failed to find the location within the given time were gently guided to the platform and were allowed to stay on it for 15 s; each mouse was given two trial sessions each day for five consecutive days, with an intertrial interval of about 15 min. To determine whether the animal would take a spatial learning strategy to locate the platform, a single spatial probe trial was assessed on day six, the platform was removed from the water basin, and the mice were allowed to swim freely for 60 s. All data were recorded and analyzed by a computerized video imaging analysis system (Huaibei Zhenghua biology apparatus Co., Ltd, Anhui, China).

**2.6. Sample Preparation.** After probe trial sessions of Morris water maze test, 72 mice were sacrificed by cervical dislocation and the brain was immediately removed. The cerebral cortex and hippocampus of 6 mice in each group were each dissected out [19]. The brain (except for the cerebellum) of the rest of the 6 mice in each group was also dissected out. Each part of the brain tissue was stored at  $-80^\circ\text{C}$  until the biochemical studies [20]. Before detection, each part of the brain tissue was rapidly homogenized in ice-cold saline and the homogenates were centrifuged at 3500 rpm at  $4^\circ\text{C}$  for 15 min. The supernatant was collected for assay.

**2.7. Assay of GSH-px and Lipid Peroxidation within the Brain of Mice.** The left cerebral cortex tissue was homogenized in ice-cold saline and centrifuged at 2000 rpm for 10 min, and the supernatant was collected. The activities of GSH-px and the amount of MDA in the supernatant were measured using commercial assay kits.

**2.8. Determination of AChE Activity in the Cerebral Cortex and Hippocampus of Mice.** The activities of AChE were measured using colorimetric methods [21]. Briefly, the reaction mixture containing samples, DTNB, and sodium phosphate (1 mmol/L, pH 8.0) was preincubated for 10 min at 37°C, and then acetylthiocholine iodide was added to the reaction mixture to incubate for 5 min at 37°C. The absorbance was measured at 412 nm at room temperature. AChE activity was expressed as nmol/mg of protein.

**2.9. Assay of  $\beta$ -Secretase in the Cerebral Cortex of Mice.** The activities of  $\beta$ -secretase in cortical of mouse brain were measured using a specific  $\beta$ -secretase ELISA kit according to the manufacturers' protocols. This formation of fluorescence was read using a fluorescence plate reader with excitation at 335–355 nm and emission at 450 nm.

**2.10. Determination of  $A\beta_{1-42}$  in Hippocampus of Mice.** Each brain was homogenized in 8 vol. (w/v) of cold 5 mol/L guanidine HCl/50 mmol/L Tris-HCl and mixed at room temperature for 4 h. Dilutions of the extracts were made in Dulbecco's phosphate-buffered saline containing 5% BSA and 0.03% Tween 20 (pH 7.4) supplemented with 1x protease inhibitor cocktail (Roche, Germany). Following centrifugation at 16,000  $\times g$  for 20 min at 4°C, aliquots were diluted with sample buffer provided by the manufacturer and used for the measurement of  $A\beta_{1-42}$  levels by enzyme-linked immunosorbent assay (ELISA) (IBL, Germany).

**2.11. Histology.** For the histological examination of tissue sections, hematoxylin-eosin and Congo red were used as described previously [22]. The brain was removed and kept overnight in the last fixative solution for dehydration. Then cut it into transparent slices and imbedded it in paraffin. Brain samples were cut into coronal sections. Serial sections were selected around the needle trace. Sections were stained with hematoxylin-eosin reagent and then dehydrated with graded alcohol and mounted with neutral balsam medium to observe changes in the cortical and hippocampal neurons.

**2.12. Statistical Analysis.** All values were expressed as the mean  $\pm$  SD. Statistical differences in all groups were analyzed using one-way ANOVA. Student's *t*-test was used to determine significant differences between groups. Differences were considered statistically significant at a value of  $P < 0.05$ .

### 3. Results and Analysis

**3.1. UPLC-ESI/MS Analysis for Chloroform Extract of *Alpinia oxyphylla*.** The chemical compositions in CF were analysed via UPLC-ESI/MS. The chromatogram of CF was obtained at 255 nm (Figure 2). The results of UPLC-ESI/MS and tentative identification are shown in Table 1. Among these compounds, some showed typical fragmentation patterns as previously reported, while the others were identified based on the retention times and the UV spectra of the reference standards [23, 24].

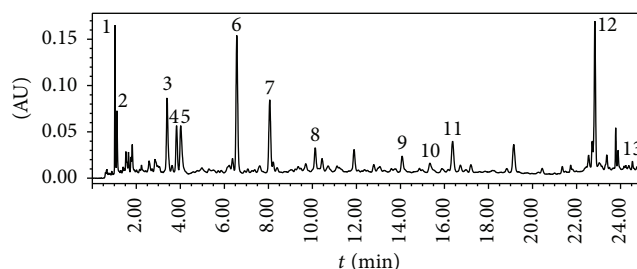


FIGURE 2: UPLC chromatogram of chloroform extract of *Alpinia oxyphylla* at 255 nm. 1, oxyphyllanene A; 2, protocatechuic acid; 4, 11S-nootkatone-11,12-diol; 5, 11R-nootkatone-11,12-diol; 6, teuhetenone A; 7, teuhetenone B; 11, oxyphyllol B; 12, Nootkatone; 13, dibutyl phthalate.

**3.2. Y-Maze Test.** The effect of CF on short-term or working memory was investigated in the spontaneous alternation behavior Y-maze test. As presented in Figure 3(a), spontaneous alternation was significantly different between groups ( $F(5, 54) = 3.338$ ,  $P < 0.05$ ). The spontaneous alternation of model mice was significantly lower than that of sham-operated mice by 18% ( $P < 0.05$ ), and the lowered spontaneous alternation induced by  $A\beta_{1-42}$  was significantly reversed by CF2 (360 mg/kg) by 17% ( $P < 0.01$ ). Moreover, the effect of CF (360 mg/kg) on the spontaneous alternation behavior was similar to that of donepezil ( $P < 0.05$ , Figure 3(a)). However, numbers of arm entries were similar in all experimental groups, demonstrating that general locomotor activity was not affected by CF (Figure 3(b)).

**3.3. Active Avoidance Test.** We assessed the effects of CF on learning and memory ability in mice exposed to  $A\beta_{1-42}$  (3.0  $\mu g$ /mouse) using active avoidance test described above. Table 2 showed that condition avoidance response percent every day and total time in four days but had no marked difference between the control and sham-operated groups. Compared with control group, the condition avoidance response (CR) was significantly decreased (day 2–4) in model group (d2,  $F(5, 54) = 4.289$ ,  $P < 0.05$ ; d3,  $F(5, 54) = 3.617$ ,  $P < 0.01$ ; d4,  $F(5, 54) = 4.471$ ,  $P < 0.001$ ) and total time was significantly increased ( $F(5, 54) = 4.490$ ,  $P < 0.01$ ). These results revealed that the  $A\beta_{1-42}$ -treated mice had obvious cognitive impairment. Moreover, the decrease of CR was reversed, respectively, by CF (180 mg/kg and 360 mg/kg) from second day to fourth day ( $P < 0.001$  and  $P < 0.01$ ;  $P < 0.05$ ;  $P < 0.01$  and  $P < 0.05$  versus the model) and by donepezil treatment from the third to fourth day ( $P < 0.05$ ,  $P < 0.05$  versus the model). Besides, the increase of total time was shortened, respectively, by CF (180 mg/kg and 360 mg/kg) ( $P < 0.01$  and  $P < 0.05$  versus the model) and by donepezil treatment ( $P < 0.05$  versus the model). In addition, the memory enhancing activity of CF was shown to be more potent than donepezil-treated group (0.65 mg/kg body weight, p.o.).

**3.4. Morris Water Maze Test.** Morris water maze test was used to assess the spatial learning and memory ability of

TABLE 1: Retention times, MS, and MS/MS<sup>2</sup> fragmentation patterns of the substances in chloroform extract of *Alpinia oxyphylla*.

Peak	$T_R$ (min)	MS <sup>+</sup> ( $m/z$ )	Major fragments (positive ion mode) ( $m/z$ )	Tentative identification
1	1.08	192.1234	175.1[M+H-H <sub>2</sub> O] <sup>+</sup> → 151.1[M+H-CH <sub>2</sub> CO] <sup>+</sup> → 133.1[M+H-CH <sub>2</sub> CO-H <sub>2</sub> O] <sup>+</sup>	Oxyphyllanene A
2	1.22	153.8901	110.8[M+H-CO <sub>2</sub> ] <sup>+</sup>	Protocatechuic acid
3	3.47	220.1544	177.1 → 133.1	Unknown
4	3.96	252.1821	235.1[M+H-H <sub>2</sub> O] <sup>+</sup> → 206.1[M-3CH <sub>3</sub> ] <sup>+</sup> → 189.1[M+H-3CH <sub>3</sub> -H <sub>2</sub> O] <sup>+</sup> → 177.1 [M-C <sub>3</sub> H <sub>5</sub> -2OH] <sup>+</sup> → 107.1	11S-nootkatone-11,12-diol
5	4.06	252.1797	235.1[M+H-H <sub>2</sub> O] <sup>+</sup> → 206.1[M-3CH <sub>3</sub> ] <sup>+</sup> → 189.1[M+H-3CH <sub>3</sub> -H <sub>2</sub> O] <sup>+</sup> → 177.1 [M-C <sub>3</sub> H <sub>5</sub> -2OH] <sup>+</sup> → 107.1	11R-nootkatone-11,12-diol
6	6.62	194.1307	177.1[M+H-H <sub>2</sub> O] <sup>+</sup> → 149.1[M+H-H <sub>2</sub> O-CO] <sup>+</sup> → 135.1 [M+H-H <sub>2</sub> O-CO-CH <sub>3</sub> ] <sup>+</sup> → 120.1[M+H-H <sub>2</sub> O-CO-2CH <sub>3</sub> ] <sup>+</sup>	Teuhenone A
7	8.10	194.1389	177.1[M+H-H <sub>2</sub> O] <sup>+</sup> → 161.1[M+H-H <sub>2</sub> O-CH <sub>2</sub> ] <sup>+</sup> → 137.1[M+H-H <sub>2</sub> O-CO-CH <sub>2</sub> ] <sup>+</sup>	Teuhenone B
8	10.18	220.1547	203.1 → 159.1	Unknown
9	14.21	222.1705	180.1 → 138.1	Unknown
10	15.82	216.1595	179.1 → 137.1	Unknown
11	16.48	234.1704	217.2[M+H-H <sub>2</sub> O] <sup>+</sup> → 207.2[M+H-CO] <sup>+</sup> → 203.2[M+H-H <sub>2</sub> O-CH <sub>2</sub> ] <sup>+</sup> → 189.2[M+H-H <sub>2</sub> O-2CH <sub>2</sub> ] <sup>+</sup>	Oxyphyllol B
12	20.47	218.1671	176.1[M+H-C <sub>3</sub> H <sub>6</sub> ] <sup>+</sup> → 163.1[M+H-C <sub>3</sub> H <sub>5</sub> -CH] <sup>+</sup> → 149.1[M+H-C <sub>3</sub> H <sub>5</sub> -CH-CH <sub>2</sub> ] <sup>+</sup> → 135.1[M+H-C <sub>3</sub> H <sub>5</sub> -CH-CO] <sup>+</sup>	Nootkatone
13	24.53			Dibutyl phthalate

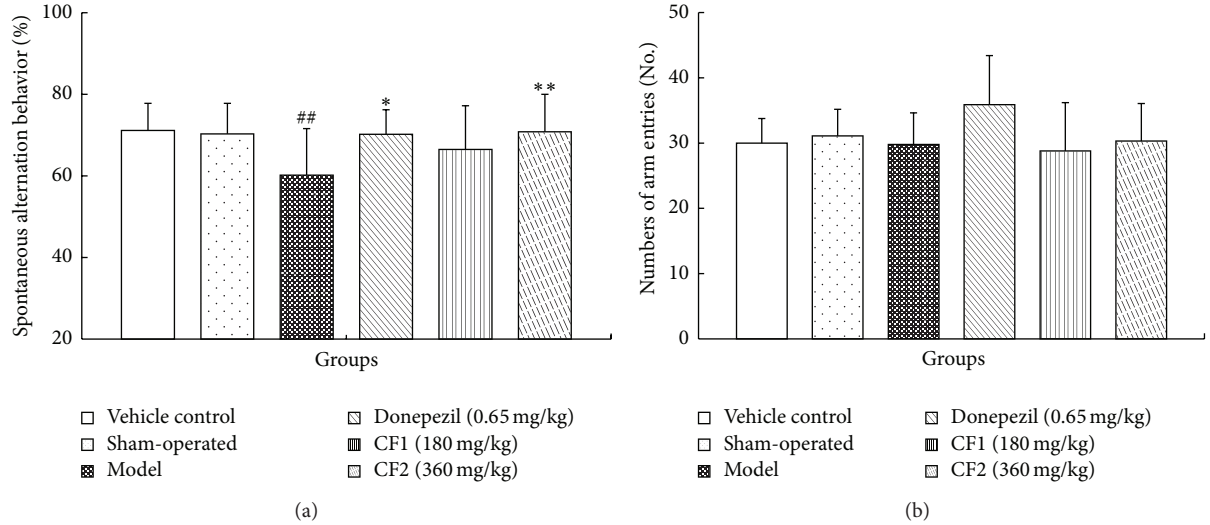


FIGURE 3: Effect of CF on Aβ<sub>1-42</sub>-induced memory deficits in the Y-maze test. Spontaneous alternation behavior (a) and the number of arm entries (b) during an 8 min session were measured. Data represent means ± SD ( $n = 10$ ). # $P < 0.05$ , compared with sham-operated group. \* $P < 0.05$ , \*\* $P < 0.01$  compared with model group.

animals. As shown in Figure 4(a), the mean latency to find the platform declined progressively during the five training days. The model group mice markedly spent longer time in finding the platform than the vehicle control mice in all training days (d3,  $F(5, 234) = 2.707$ ,  $P < 0.05$ ; d4,  $F(5, 234) = 4.259$ ,  $P < 0.01$ ; d5,  $F(5, 234) = 3.232$ ,  $P < 0.05$ ). These results revealed that the model group mice had significant cognitive

impairment. Moreover, CF1 and CF2 group mice significantly shortened the escape latency compared with the model group mice from the third to fifth day ( $42.92 \pm 17.31$  s versus  $60.66 \pm 15.75$  s,  $P < 0.05$ ;  $29.32 \pm 11.25$  s versus  $46.29 \pm 13.48$  s,  $P < 0.01$ ; and  $28.87 \pm 14.56$  s versus  $44.36 \pm 13.52$  s,  $P < 0.01$ , resp.) and from the third day backwards ( $39.01 \pm 19.92$  versus  $60.66 \pm 15.75$  s,  $P < 0.01$ ;  $27.78 \pm 9.70$  s versus  $46.29 \pm 13.48$  s,  $P < 0.01$ ;



TABLE 2: Effect of CF on condition avoidance response and total time induced by  $A\beta_{1-42}$  in mice.

Group	Condition avoidance response/%				Total time/s
	1	2	3	4 (d)	
Control	46.0 $\pm$ 29.3	59.3 $\pm$ 20.0	86.0 $\pm$ 9.7	86.0 $\pm$ 9.7	1542 $\pm$ 102
Sham-operated	46.7 $\pm$ 30.5	74.0 $\pm$ 21.4	85.3 $\pm$ 14.3	85.3 $\pm$ 15.6	1482 $\pm$ 104
Model	34.0 $\pm$ 18.9	37.3 $\pm$ 20.4 <sup>#</sup>	49.3 $\pm$ 28.5 <sup>###</sup>	51.4 $\pm$ 23.3 <sup>###</sup>	1683 $\pm$ 70 <sup>##</sup>
Donepezil 0.65 mg/kg	42.7 $\pm$ 34.6	48.0 $\pm$ 26.9	70.0 $\pm$ 29.2 <sup>*</sup>	68.7 $\pm$ 23.5 <sup>*</sup>	1586 $\pm$ 115 <sup>*</sup>
CF1 180 mg/kg	48.7 $\pm$ 32.9	71.3 $\pm$ 18.6 <sup>***</sup>	70.0 $\pm$ 24.0 <sup>*</sup>	74.7 $\pm$ 16.9 <sup>**</sup>	1544 $\pm$ 121 <sup>**</sup>
CF2 360 mg/kg	42.6 $\pm$ 27.5	67.3 $\pm$ 23.6 <sup>**</sup>	68.0 $\pm$ 22.0	68.7 $\pm$ 22.4 <sup>*</sup>	1585 $\pm$ 77 <sup>*</sup>

Results are expressed as mean  $\pm$  SD,  $n = 60$  (10 mice each group), <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$ , and <sup>###</sup> $P < 0.001$  versus the control mice; <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ , and <sup>\*\*\*</sup> $P < 0.001$  versus the model group mice.

TABLE 3: Effect of CF on brain tissue GSH-px activities and MDA contents.

Group	GSH-px	MDA
	(U/mg protein)	(nmol/mg protein)
Control	4.33 $\pm$ 0.49	1.42 $\pm$ 0.30
Model	3.44 $\pm$ 0.39 <sup>#</sup>	1.93 $\pm$ 0.47 <sup>##</sup>
Donepezil 0.65 mg/kg	4.34 $\pm$ 0.94 <sup>*</sup>	1.56 $\pm$ 0.40 <sup>*</sup>
CF 180 mg/kg	3.77 $\pm$ 0.77	1.31 $\pm$ 0.23 <sup>**</sup>
CF 360 mg/kg	4.54 $\pm$ 0.98 <sup>*</sup>	1.25 $\pm$ 0.21 <sup>**</sup>

Results are expressed as mean  $\pm$  SD,  $n = 6$ , <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$  versus the control mice; <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$  versus the model group mice.

27.55  $\pm$  11.42 s versus 44.36  $\pm$  13.52 s,  $P < 0.01$ ), respectively. Meanwhile, donepezil (0.65 mg/kg) treatment reached the similar effect from the fourth to fifth day of training trials (28.77  $\pm$  10.75 s versus 46.29  $\pm$  13.48 s,  $P < 0.01$ ; 29.83  $\pm$  14.33 s versus 44.36  $\pm$  13.52,  $P < 0.05$ , resp.). Figure 4(b) illustrated the swim paths of mice in the second trial of the second and fifth day in this test. Mice tended to explore all four quadrants of the pool in the second day. Thereafter, they changed this search strategy. On the fifth day, the vehicle control mice swam in the direction of the platform; however, the model group mice took longer swimming paths. In the probe test (Figure 4(c)), the vehicle control mice spent longer time in the target quadrant than the model group mice ( $F(5, 54) = 3.376$ ,  $P < 0.01$ ). CF (180 and 360 mg/kg) and donepezil (0.65 mg/kg) treatment mice also took longer time in the target quadrant than the model group mice ( $P < 0.05$ ,  $P < 0.001$  and  $P < 0.05$ , resp.). It is more accurate to evaluate the effects of drug on impairment of spatial learning and memory through comparison of the number of crossing, as shown in Figure 4(d). The model group made fewer platform crossings than the vehicle control group ( $F(5, 54) = 2.628$ ,  $P < 0.05$ ) and the CF (180 and 360 mg/kg) and donepezil (0.65 mg/kg) treatments could increase the number of times of crossing over the platform site than the model group ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.05$ ); however, CF2 had greater effect to reverse the deficit of spatial learning and memory induced by  $A\beta_{1-42}$  than donepezil treatments.

**3.5. Effect of CF on Brain GSH-px Activities and MDA Contents.** Table 3 summarized the antioxidant effect of CF in the

$A\beta_{1-42}$ -injected mice. GSH-px is an important antioxidant enzyme involved in cellular protection against damage caused by oxygen-derived free radicals, by means of removing harmful peroxide metabolites and blocking lipid peroxidation chain reaction.  $A\beta_{1-42}$  suppressed GSH-px activity in the brain ( $F(4, 25) = 3.178$ ,  $P < 0.05$ ). However, CF2 (360 mg/kg) and donepezil groups displayed a significant elevation of GSH-px activity ( $P < 0.05$  and  $P < 0.05$  versus the model group, resp.). The MDA level in the brain of  $A\beta_{1-42}$ -injected mice was higher than that of the vehicle control group ( $F(4, 25) = 4.439$ ,  $P < 0.01$ ). The increase was ameliorated by treatment of CF at doses of 180 or 360 mg/kg or donepezil ( $P < 0.01$ ,  $P < 0.01$  and  $P < 0.05$  versus the  $A\beta_{1-42}$ -treated group, resp.).

**3.6. Inhibitory Effect of CF on  $\beta$ -Secretase Activity in the Frontal Cortex and Accumulation of  $A\beta_{1-42}$  in Hippocampus.** The activity of  $\beta$ -secretase in the cortex of the model group was increased compared with vehicle control (Figure 4(e)). The administration of donepezil and CF lowered the increased activity of  $\beta$ -secretase induced by  $A\beta_{1-42}$  injection in cortex of mouse brain. However, it did not show any significant differences ( $F(4, 25) = 1.690$ ,  $P > 0.05$ ).

We further measured the  $A\beta_{1-42}$ -levels in brain homogenate by ELISA. The ELISA results revealed that the  $A\beta_{1-42}$  levels were increased in  $A\beta_{1-42}$ -treated group ( $F(4, 25) = 3.151$ ,  $P < 0.05$ ). Compared with the model group, the  $A\beta_{1-42}$  levels were significantly decreased in donepezil and CF2 treatment groups ( $P < 0.05$ , Figure 4(f)).

**3.7. Inhibitory Effect of CF on AChE Activity in the Frontal Cortex and Hippocampus.** As shown in Figure 4(g), the administration of CF or donepezil to  $A\beta_{1-42}$ -injected mice produced no significant change on the activity of AChE in cortex ( $F(4, 25) = 1.533$ ,  $P > 0.05$ ). The activity of AChE in hippocampus was increased in  $A\beta_{1-42}$ -injected mice and greater than those in vehicle control ( $F(5, 54) = 3.151$ ,  $P < 0.05$ ). However, the increased activity of AChE in hippocampus was significantly inhibited by the treatment with CF1 and CF2 ( $P < 0.01$  and  $P < 0.01$ ) (Figure 4(h)).

**3.8. Effects of CF on Neurodegenerative Changes in the Frontal Cortex and Hippocampus.** As shown in Figure 5, (a) and (b) showed normal cells which are intact and there are less

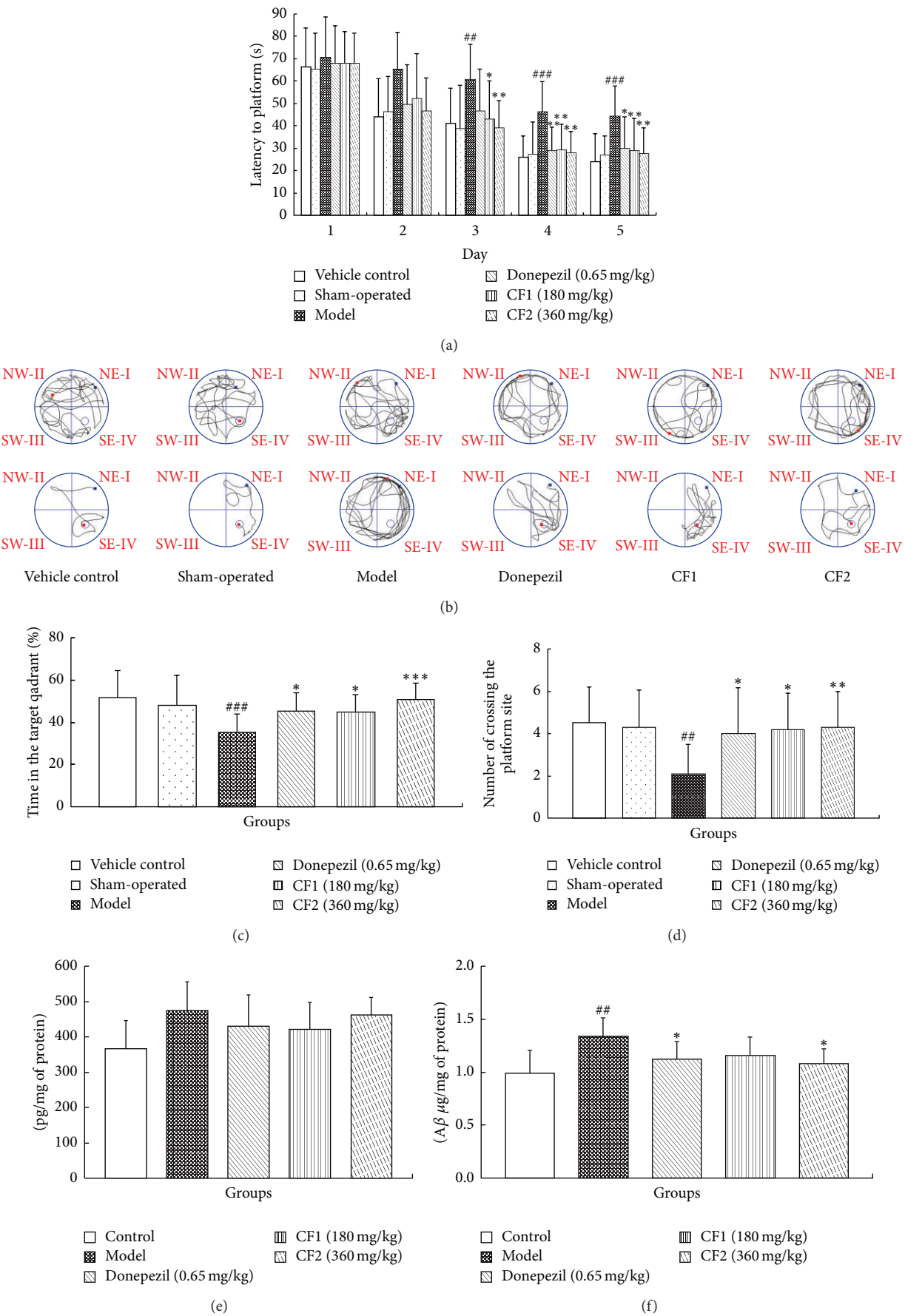


FIGURE 4: Continued.

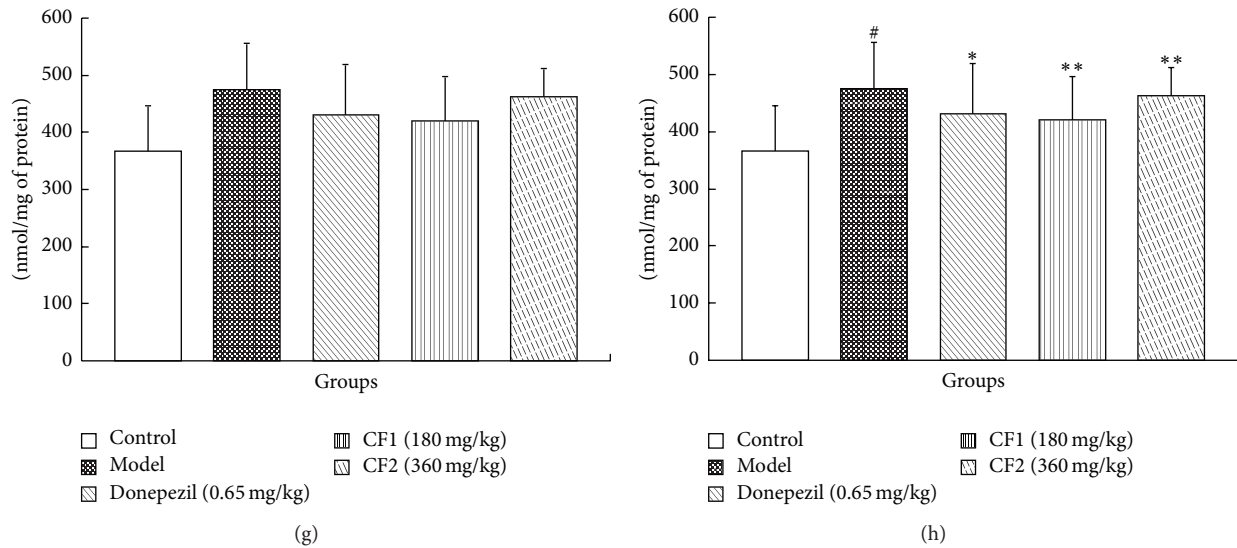


FIGURE 4: The Morris water maze test of mice treated with vehicle,  $A\beta_{1-42}$ ,  $A\beta_{1-42}$  + donepezil (0.65 mg/kg),  $A\beta_{1-42}$  + CF (180 mg/kg), and  $A\beta_{1-42}$  + CF (360 mg/kg), respectively. (a) Latencies to find a hidden platform in the water maze during five consecutive training days. (b) Search strategy of mice in the second trial on the second and fifth day. Traces show the swim path of all groups of mice. (c) The time spent in the quadrant where the platform was once placed within 60 s. (d) Number of crossings over the former platform location in the probe trial. (e) and (f) The effect of CF on  $\beta$ -secretase activity in the frontal cortex and accumulation of  $A\beta_{1-42}$  in hippocampus. (g) and (h) The effect of CF on acetylcholinesterase activity in the cortex and hippocampus of  $A\beta_{1-42}$ -injected mice. Data are expressed as mean  $\pm$  SD. #  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$  versus the control mice; \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$  versus the model group mice.

degeneration in normal cortex and hippocampus of mice. (c)  $A\beta_{1-42}$ -induced neuron death was observed consistently in the cortex and hippocampus, as indicated by the appearance of pyknotic black neurons, karyorrhexis, and karyolysis with condensed nucleus. Figure 5(d) showed moderate damage to the cortex and hippocampus area pretreated with donepezil 0.65 mg/kg, indicated by presence of less number of degenerative cells compared to the model group. Figures 5(e) and 5(f) showed mild damage to the cortex and hippocampus area pretreated with CF (180 mg/kg, 360 mg/kg) indicated by presence of medium number of degenerative cells compared to the model group.

#### 4. Discussion and Conclusion

In common parlance, *Alpinia oxyphylla* Miq. has been often touted as a drug hid in kitchen; therefore, it is not necessary to worry about the side effect of taking it. In terms of neuroprotection, it has been reported that ethanol extract of *Alpinia oxyphylla fructus* shows inhibition of tau protein phosphorylation in cell culture and the mechanism of sharp leaf galangal fruit extract can improve the spatial learning ability having business with its inhibiting ability on serum levels of cytokines [25]. For the preparation of the bioactive fraction of *Alpinia oxyphylla* which includes major sesquiterpenes with neuroprotective, antioxidant, or cognitive-enhancing activities, we chose chloroform fraction of ethanol extract of *Alpinia oxyphylla* Miq. However, screening of activities in vitro always lacks details of facts. To verify the potential of *Alpinia oxyphylla*, which was rich with neuroprotective or

cognitive enhancing, we examined the protective effects of CF on cognitive impairment and neurotoxicity within the cortex and hippocampus in  $A\beta_{1-42}$ -injected mice.

However,  $A\beta$  is transported bidirectionally across the blood-brain barrier (BBB), that is, both in brain-to-blood (efflux) and the blood-to-brain (influx) directions. Therefore, how to remove the toxic  $A\beta_{42}$  peptide through BBB safely and effectively is considered to be an effective method of the prevention and treatment of AD. Previous studies have shown the influence of traditional Chinese aromatic medicine between the structure and function of BBB. Modern pharmacological studies indicate that the permeability of BBB could be changed by traditional Chinese aromatic medicine in three ways. Firstly, it can develop potency by permeating the BBB reinlessly, secondly, it can protect the brain tissue by reducing the permeability of BBB, and thirdly, it can promote the opening of BBB, while its mechanism of action may be concerned with restraining the expression and function of P-glycoprotein. We supposed that sesquiterpenoids contained in CF may play the same role.

At present, there is no animal model can mimic all the cognitive, behavioral, biochemical, and histopathological abnormalities observed in patients with AD. Mice intracerebroventricularly injected with  $A\beta_{1-42}$  can mimic some cognitive deficits in AD, which is an economic and reliable model. Therefore, we first tested the memory enhancing effect of CF in mice treated with  $A\beta_{1-42}$ . The cognitive-enhancing activity of CF was evaluated by Y-maze test, active avoidance test, and Morris water maze test. CF mitigated the impairment of recognition induced by  $A\beta$  in mice. The



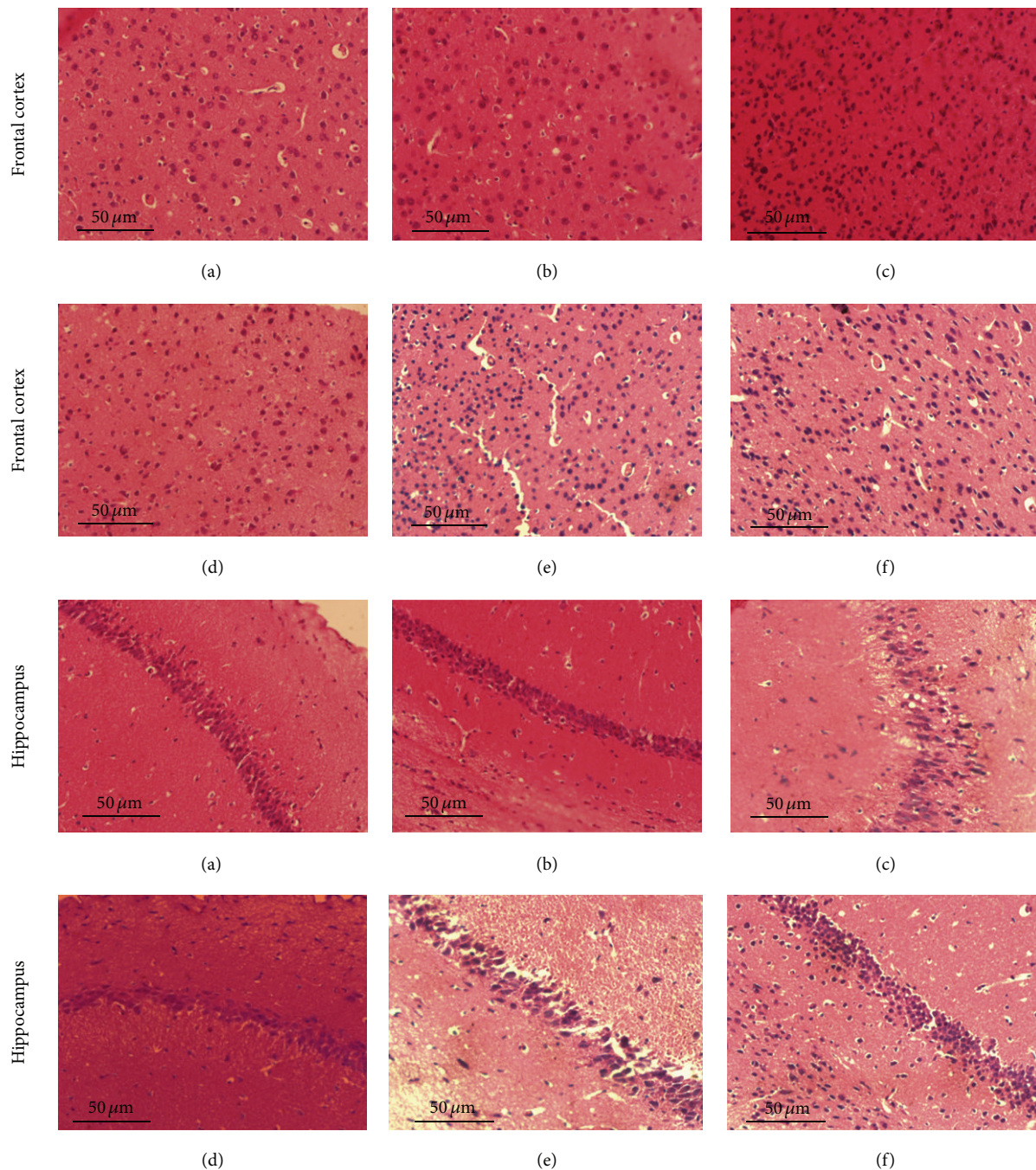


FIGURE 5: Effect of CF on neuronal degeneration in frontal cortex and hippocampus induced by  $A\beta_{1-42}$  treatment in mice (H&E staining, 400x). (a) Vehicle control group; (b) sham-operated group; (c) model group; (d) donepezil group (0.65 mg/kg); (e) CF1 group (180 mg/kg); (f) CF2 group (360 mg/kg).

treatment of CF more effectively improved the deficit in long term memory than in short term memory. Oral treatment with CF also exhibited a mitigation of memory deficit in mice induced by i.c.v. injection of  $A\beta$  on escape latency, time spent in target quadrant, and cross-platform times in Morris water maze test. The treatment with CF decreased the latency to a shorter level than that of nontreated normal control. In addition, cognitive-enhancing effect of CF was found to

be much more effective than that of donepezil. Besides, the same effect was observed in active avoidance test; the data showed that CF exhibited significant influence on condition avoidance response (CR) and total time (TT). The results indicate that CF has the potential to ameliorate cognitive deficits induced by  $A\beta_{1-42}$ .

In spite of conflicting hypothesis on the pathological progress in AD patients, it is now generally recognized that



the accumulated amyloid plaque in the brain is characteristic and diagnostic features of AD.  $\beta$ -secretase initiates the cleavage of  $\beta$ -amyloid precursor protein (APP) leading to the production of amyloid- $\beta$  ( $A\beta$ ); the gathering of  $A\beta$  is the main cause of senile plaque in the brain of AD patient, causes neuron damage and the hypofunction of cognition, and works as the center and cogallery in the pathogenesis of AD. The suppressant's partial inhibition of  $\beta$ -secretase can decrease the production of  $A\beta$  and get a manifesting therapeutic effect. Further study on the way BACE inhibitors work has favourable perspective in the treatment of AD. Our data showed that the activities of BACE1 increased by  $A\beta$  injection in the cortex of mouse brain were significantly inhibited by CF. Accumulation of deposits of  $A\beta$  is one of the classical neuropathological hallmarks in AD and may be the primary event in the pathogenesis of AD. In accordance with the reduction in BACE1 activity by CF, the aggregation of  $A\beta$  in the hippocampus of  $A\beta$ -injected mouse brain was found to be markedly reduced by CF by enzyme-linked immunosorbent assay (ELISA).

Growing data from experimental models and human brain studies suggest that oxidative stress, inflammation, and apoptotic cell death induced by  $A\beta$  play important roles in neuronal degeneration in AD [26]. Each of these factors can act independently or collectively damage neurons and disturb cognitive processes.

The oxidative stress hypothesis of AD suggests that the aggregation of  $A\beta$  on the membrane generates reactive oxygen species (ROS), resulting in protein oxidation, lipid peroxidation, cellular dysfunction, and subsequent neuronal death [27]. It may be one of the first pathogenic events during disease progression. In the  $A\beta_{1-42}$ -induced model mice, the level of MDA (a well-known indicator of lipid peroxidation) increased, and activities of GSH-px (the line of the antioxidant defense systems) decreased. GSH-px scavenges ROS by directly reacting with it and prevents  $H_2O_2$ -induced hydroxyl radical formation. GSH-px level parallels the antioxidant defense capacity and is a first indicator for oxidative stress in the brain [28]. Thus, the increase in activities of antioxidant defense systems is considered to be beneficial in the event of oxidative stress. The results indicated that an increase of oxidative stress occurred in the brain of  $A\beta_{1-42}$ -treated mice. After the administration of CF, the decreased activities of GSH-px, as well as the increased level of MDA, were restored in the brain. The possible mechanisms of CF are largely related to its antioxidant and other scavenging properties, for example, over nitric oxide (NO), possibly, through downregulation of NOS activity in brain. Previous phytochemical investigation on this plant revealed oxyphyllanene A, teuhetenone A, oxyphyllol B, and nootkatone with inhibitory activity against NO production in LPS and IFN- $\gamma$ -induced RAW 264.7 macrophages, which showed that these compounds exhibited potent activities of NO inhibitory and neuroprotection [12]. Besides, it was reported that *Alpinia protocatechuic acid* protects against oxidative damage in vitro and reduces oxidative stress in vivo [14]. Therefore, it is undoubtedly that CF could effectively attenuate oxidative stress induced by  $A\beta$ .

Microglia, the resident macrophages in the central nervous system, can be activated by  $A\beta$ . Oligo- $A\beta_{1-42}$  is always likely to damage neurons through the microglia-inflammation response. Activated microglia not only can eliminate  $A\beta$  but also can release inflammatory factors, which play a critical role in AD pathology. Recently reports strongly suggest that regulating microglia function may be a promising therapeutic approach to AD. From histological evidence of tissue sections, we observed that the model group mice showed significant microglial cell hyperplasia, neuronal disorder, and degeneration in the frontal cortex and hippocampus, while in CF (180 mg/kg and 360 mg/kg) treated group the microglia activation, neuronal acidophilia degeneration, and nuclear condensation decreased, resulting in homogeneous morphology of neuronal cellular.

It has been demonstrated that impairments in learning, memory, and behavior observed in AD patients are associated with the cholinergic hypofunction. Neuronal loss in the basal forebrain particularly within the septohippocampal acetylcholinergic systems involved in learning and memory processes constitutes a pathological hallmark of AD. The mechanism of aggregated  $A\beta_{1-42}$  peptide-induced increase of AChE activity was related with two pathways: (1) indirect action-increase intracellular  $Ca^{2+}$  and free radical, which are symbols of cellular damage and (2) direct action-change conformation of AChE. Recently, it has been reported that ethanolic extracts of *Alpinia oxyphylla* Miq. showed most potent AChE inhibitory activities at the concentration of 0.1 mg/mL, with 44.49% inhibition among 48 traditional Chinese medicinal herbs [29]. In our research, administration of CF significantly showed greater inhibition activity of AChE than donepezil known as the most common prescribed AChE inhibitor in hippocampus of  $A\beta$ -injected mouse. It may be related to acetylcholinesterase inhibition of nootkatone [30].

From the above results, it could be deduced that cognitive-enhancing activity of CF might result in part from the inhibition on the accumulation of  $A\beta$  and the BACE1 and AChE and from the reduction in ROS by recovering the antioxidative defense system. These results suggested that chloroform extract of *Alpinia oxyphylla* containing bioactive sesquiterpenes, such as oxyphyllanene A, teuhetenone A, oxyphyllol B, and nootkatone, might offer a useful therapeutic choice in either the prevention or the treatment of Alzheimer's disease.

## Conflict of Interests

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

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## Review Article

# Multimarker Screening of Oxidative Stress in Aging

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Aging is a complex process of organism decline in physiological functions. There is no clear theory explaining this phenomenon, but the most accepted one is the oxidative stress theory of aging. Biomarkers of oxidative stress, substances, which are formed during oxidative damage of phospholipids, proteins, and nucleic acids, are present in body fluids of diseased people as well as the healthy ones (in a physiological concentration). 8-*iso* prostaglandin  $F_{2\alpha}$  is the most prominent biomarker of phospholipid oxidative damage, *o*-tyrosine, 3-chlorotyrosine, and 3-nitrotyrosine are biomarkers of protein oxidative damage, and 8-hydroxy-2'-deoxyguanosine and 8-hydroxyguanosine are biomarkers of oxidative damage of nucleic acids. It is thought that the concentration of biomarkers increases as the age of people increases. However, the concentration of biomarkers in body fluids is very low and, therefore, it is necessary to use a sensitive analytical method. A combination of HPLC and MS was chosen to determine biomarker concentration in three groups of healthy people of a different age (twenty, forty, and sixty years) in order to find a difference among the groups.

## 1. Introduction

Aging is a multifactorial process of time-dependent decline in physiological function [1]. It is manifested by the decrease of the efficiency of the organism functions, the accumulation of various defects and declining ability to repair them, increased susceptibility to various diseases, and eventually increased mortality [2, 3].

Many theories explain the phenomenon of aging. The most popular one is the free radical theory which was proposed by Harman in 1956 [4]. Harman suggested that OH and OH<sub>2</sub> radicals are produced endogenously in living organisms during oxygen-utilizing processes (such as respiration). Later on, it was found that there are other oxygen compounds such as hydrogen peroxide or hypochlorous acid which react with biomolecules in the same way. These are, together with oxygen radicals, called reactive oxygen species (ROS). Considering this, the free radical theory was modified to oxidative stress theory of aging [5, 6].

Oxidative stress is defined as an imbalance between oxidants (ROS) and the antioxidant defense in the organism in favor of oxidants [7]. The oxidants interact with

biomolecules in cells such as phospholipids, proteins, and nucleic acids. This leads to cell dysfunctions and consequently cell death. The molecules formed during oxidation may serve as biomarkers as their analysis in various biological matrices is used for the quantification of oxidative stress in humans. The most significant biomarker of oxidative stress is 8-*iso* prostaglandin  $F_{2\alpha}$  (8-isoprostane). 8-Isoprostane is formed by nonenzymatic oxidation of arachidonic acid. Oxidation of proteins and amino acids gives rise to *o*-tyrosine, 3-chlorotyrosine, and 3-nitrotyrosine. FENO (fractional exhaled nitric oxide) present in EBC is also formed from amino acid, L-arginine, by its oxidation; elevated or even depressed level of FENO is linked with asthma, upper airway infections, and other lung diseases [8, 9]. Biomarkers of nucleic acid oxidation are 8-hydroxyguanosine and 8-hydroxy-2'-deoxyguanosine. High concentrations of the biomarkers were determined not only in body fluids or tissues of patients with age-related and/or degenerative diseases such as Alzheimer's disease, hypertension, type II diabetes, or several types of cancer (see Table 1 for the summary of diseases and detected biomarkers) but also in relation to

TABLE 1: Age-related diseases and detected biomarkers.

Disease	Biomarkers	Body fluid/tissue
Alzheimer's disease	3-Chlorotyrosine	Hippocampus proteins [14]
	3-Nitrotyrosine	Brain [15]
		Cerebrospinal fluid [16, 17]
	8-Hydroxy-2'-deoxyguanosine	Brain [18]
	8-Hydroxyguanosine	Brain [18]
Arthritis		Blood serum [19]
		Cerebrospinal fluid [19]
	8-Isoprostane	Blood plasma [20]
		Urine [20]
	3-Nitrotyrosine	Blood serum [21, 22]
Atherosclerosis		Synovial fluid [22]
		Blood plasma [23]
		Synovial fluid [23]
		Urine [24]
	8-Hydroxy-2'-deoxyguanosine	
Cataracts	3-Chlorotyrosine	Human aortic tissue [25]
	3-Nitrotyrosine	Atherosclerotic blood vessels [26]
	8-Hydroxy-2'-deoxyguanosine	Urine [27]
	8-Isoprostane	Blood plasma [28]
	<i>o</i> -Tyrosine	Cataractous lenses [29]
Hypertension	<i>m</i> -Tyrosine	Cataractous lenses [29]
	8-Hydroxy-2'-deoxyguanosine	Blood plasma [30]
	8-Isoprostane	Blood plasma [31, 32]
	3-Nitrotyrosine	Lung tissue [33]
	8-Hydroxyguanosine	Lung tissue [33]
Osteoporosis	8-Hydroxy-2'-deoxyguanosine	Urine [34]
	8-Hydroxy-2'-deoxyguanosine	Blood serum [35]
	8-Isoprostane	Blood plasma [36, 37]
		Urine [38]
	<i>o</i> -Tyrosine	Blood plasma [39]
Type II diabetes		Urine [39]
	8-Hydroxy-2'-deoxyguanosine	Blood serum [40]
		Urine [38]

chronic obstructive pulmonary disease [10], smoking [11, 12], and air pollution [13].

## 2. Biomarkers of Oxidative Stress

**2.1. 8-Isoprostane.** 8-Isoprostane is formed by nonenzymatic oxidation of arachidonic acid (Figure 1) which is present in phospholipid membranes [40]. A similar metabolic pathway, the enzymatic  $\omega$ -hydroxylation of arachidonic acid in the

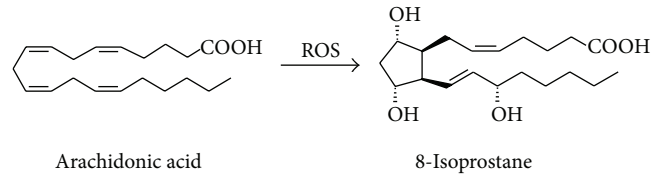


FIGURE 1: The formation of 8-isoprostane from arachidonic acid.

presence of an increased cytochrome P450 4A, owing to organism aging, leads to a similar compound, that is, 20-hydroxyeicosatetraenoic acid (20-HETE), as a very potent vasoconstriction agent [41].

Although it was thought that 8-isoprostane acts only through thromboxane (TP) receptors, the biological activity of 8-isoprostane is slightly different which suggests the existence of a specific isoprostane receptor. Incubation of 8-isoprostane with platelets causes only shape changes of platelets and in very high concentrations a reversible aggregation, while thromboxane A<sub>2</sub> (TXA<sub>2</sub>) causes an irreversible aggregation of platelets [43]. Isoprostanes have a strong vasoconstriction effect also partly by influencing TP receptors but have stronger influence on renal vasoconstriction and weaker influence on bovine coronary arteries than TXA<sub>2</sub> agonists [44, 45]. According to these findings, a hypothesis for existence of specific isoprostane receptor on smooth muscle cells in vascular system has been proposed. Other studies showed that there are high-affinity and low-affinity binding sites for 8-isoprostane on smooth muscle cells in vascular system and on endothelium cells. Low-affinity binding sites could represent TP receptors and high-affinity binding sites specific isoprostane receptors [46]. In conclusion, 8-isoprostane causes vasoconstriction of blood vessels and bronchi, lowers blood flow in kidneys, influences aggregation of platelets, and, thus, participates in pathology of several diseases (Table 2).

Concentration of 8-isoprostane in body fluids is used for the monitoring of oxidative stress. Higher concentration levels were observed, for example, in smokers ( $24 \pm 8$  pg/mL) and patients with cystic fibrosis ( $43 \pm 7$  pg/mL) compared to healthy nonsmokers ( $11 \pm 4$  pg/mL) [47] and also in arthritis [20], age-related cataracts [28], hypertension [31, 32], asthma [48, 49], and type II diabetes [36, 37].

**2.2. *o*-Tyrosine and *m*-Tyrosine.** In the organism, tyrosine is formed from phenylalanine. Physiological *p*-tyrosine occurs by enzymatic oxidation of phenylalanine by phenylalanine hydroxylase. Important derivatives of tyrosine are catecholamines (dopamine, adrenaline, and noradrenaline) or thyroid hormones.

*o*-Tyrosine (*o*-Tyr) and *m*-tyrosine (*m*-Tyr) are formed by the attack of ROS on phenylalanine (Figure 2). Unlike *p*-tyrosine, *o*-Tyr and *m*-Tyr are not natural amino acids and are considered to be oxidative stress biomarkers.

Estrogen receptor  $\alpha$  (ER $\alpha$ ) is a nuclear protein which is overexpressed in breast cancer cells [50]. The anticancer property of chlorambucil linked to estradiol was observed [51]. Nevertheless, estrogenic drug can not only target



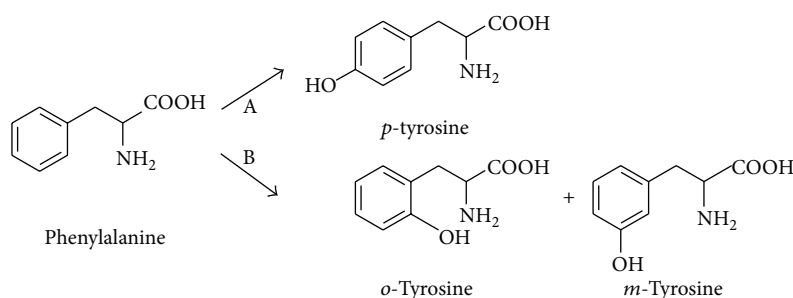


FIGURE 2: The formation of tyrosine from phenylalanine. A: enzymatic oxidation; B: oxidation by hydroxyl radicals.

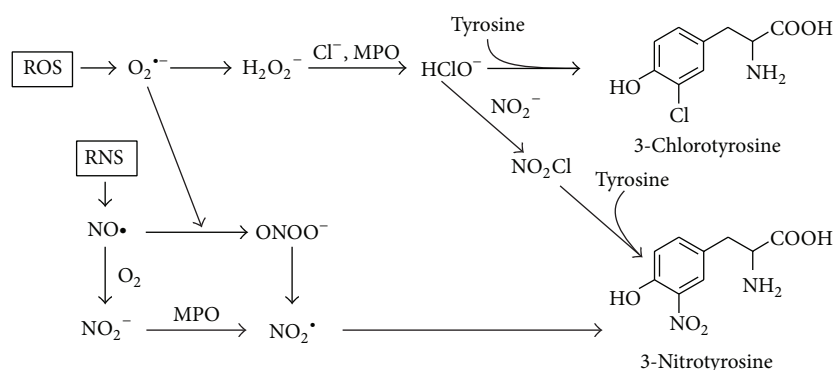


FIGURE 3: The formation of 3-chlorotyrosine and 3-nitrotyrosine by myeloperoxidase (MPO), adapted from [42].

TABLE 2: Potential pathogenic role of 8-isoprostane in several diseases (reviewed in [46]).

Disease	Potential pathogenic role of 8-isoprostane
Atherosclerosis	(i) Vasoconstriction in blood vessels
	(ii) Influencing the aggregation of platelets
	(iii) Inducement of proliferation of smooth muscle cells
	(iv) Stimulation of proliferation of calcification blood vessel cells
	(v) Inhibition of differentiation of preosteoblasts
Diabetes mellitus	(i) Increase in DNA synthesis in smooth muscle cells
	(ii) Inducement of proliferation of smooth muscle cells
	(iii) Influencing membrane fluidity and permeability
	(iv) Renal vasoconstriction can cause systemic hypertension
Hepatorenal syndrome	(i) Renal vasoconstriction (ii) Increasing the release of endothelin-1
Preeclampsia	Renal vasoconstriction
Lung diseases	(i) Bronchoconstriction
	(ii) Vasoconstriction of lung artery

the cancer cells but also induce transcriptional activity [52]. Such estrogenic activity could be avoided by choosing non-steroidal drugs with structural similarities. Tyrosine shows some structural similarities with estradiol and the phenol group was found important for binding to ER $\alpha$ . The affinity of

tyrosine-chlorambucil derivate to ER $\alpha$  was tested in order to investigate the role of the position of the hydroxyl group (use of *o*-tyrosine, *m*-tyrosine, and *p*-tyrosine). All compounds with tyrosine showed higher cytotoxicity than pure chlorambucil. Compared to the other regioisomers, the *m*-tyrosine-chlorambucil compound showed greater cytotoxicity and it was also slightly more specific for hormone-dependent cancer cells, probably due to closer similarity to estradiol [53].

Higher concentration of *o*-Tyr was found, for example, in lenses of patients with cataracts [29] and in blood plasma and urine of patients with type II diabetes [39]. Elevated levels of *m*-Tyr were confirmed in lenses of patients with cataracts [29].

**2.3. 3-Chlorotyrosine and 3-Nitrotyrosine.** 3-Chlorotyrosine (3-ClTyr) is formed by the reaction of hypochlorous acid (HClO) and *p*-tyrosine (Figure 3). Hypochlorous acid is formed from hydrogen peroxide and chloride anion by myeloperoxidase (MPO) as a catalyst. MPO is a phagocyte heme protein, catalyses the transformation of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and chloride anion ( $\text{Cl}^-$ ) into highly reactive hypochlorous acid, and plays an important role in the microbicidal activity of phagocytes [54, 55].

MPO causes also nitration of tyrosine (the formation of 3-nitrotyrosine). 3-Nitrotyrosine (=3 NOTyr) can also be formed by the reaction of peroxynitrite ( $\text{ONOO}^-$ ) and *p*-tyrosine in proteins (Figure 3).

The formation of 3-ClTyr in proteins plays an important role in cardiovascular system. HDL (high-density lipoprotein) and its major protein, apolipoprotein A-I (apoA-I),

are thought to protect the organism against atherosclerosis. One of the mechanisms is the removal of excess intracellular cholesterol from macrophages [56]. The removal is controlled by ATP-binding cassette transporter (ABCA1) [57], a membrane protein that exports cholesterol from cells to apoA-I. ABCA1 is induced by intracellular cholesterol and is highly expressed in cholesterol-loaded cells (such as foam cells in early atherosclerosis lesions) [58]. The cholesterol removal requires (1) direct binding of apoA-I to ABCA1 [59, 60], (2) solution of lipid domains formed by ABCA1 in cell membrane by apoA-I [61–64], and (3) activation of several signaling pathways. It was shown that MPO-mediated chlorination of apoA-I impairs the direct binding of apoA-I to ABCA1 and, thus, contributes to atherogenesis by impairing cholesterol efflux from macrophages [65].

It was also found that free 3-ClTyr promotes the migration of human aortic smooth muscle cells (the major mechanism of the vascular lesion formation) and that increased levels of 3-ClTyr under inflammation conditions may contribute to vascular diseases [66].

Also nitration of proteins changes the function of proteins. Nitration of tyrosine lowers the  $pK_a$  from 10.0–10.3 to 7.2–7.5 [67] and, thus, changes the pI of a protein; 3-NOTyr containing proteins are more hydrophobic [68]; and the nitrogroup is a relatively bulky substituent, which may add steric restrictions to the molecule of protein [69]. However, only a limited number of proteins constitute preferential target to nitration and only few tyrosines can be nitrated within a protein [70], but several common features of tyrosine nitration have been revealed: (1) the presence of one or more acidic residues in the vicinity of the target tyrosine (glutamic or aspartic residues), (2) the small number of cysteine or methionine residues adjacent to the nitrated tyrosine residue, and (3) the presence of turn-inducing amino acids such as proline and glycine [70, 71]. In the organism, posttranslational modification such as nitration can cause (1) no change in protein function, (2) loss of function, or (3) gain of function. The loss of function was demonstrated, for example, on MnSOD (manganese superoxide dismutase, a mitochondrial enzyme) [72] or PGI<sub>2</sub> (prostacyclin (prostaglandin I<sub>2</sub>) synthase, a vascular enzyme) [73]. The gain of function was demonstrated, for example, on cytochrome *c*, which gains peroxidase activity [74, 75]; on fibrinogen (higher aggregation in coagulation) [76]; or on protein kinase C $\epsilon$  [77] (summary in Table 3, adapted from [71]).

Higher concentration of 3-ClTyr was found in patients with Alzheimer's disease. These patients have higher activity of MPO, increased formation of hypochlorous acid, and therefore higher concentration of 3-ClTyr [14]. In the blood plasma, 3-ClTyr can bind to HDL and LDL (high- and low-density lipoprotein) and thus cause the progress of atherosclerosis. The concentration of 3-ClTyr in LDL of patients with atherosclerosis was 30 times higher compared to healthy people [25]. 3-ClTyr serves also as a biomarker of MPO increased activity because it is not formed by other mechanisms and is stable at elevated temperature [42].

Higher concentrations of 3-NOTyr were found in cerebrospinal fluid [20, 21] of Alzheimer's disease patients. The concentration of 3-NOTyr was  $11.4 \pm 5.4$  nM in patients

TABLE 3: Functional changes of nitrated proteins, adapted from [71] (shortened version).

Protein	Normal activity	Activity after nitration
Cytochrome <i>c</i>	Electron transfer and apoptosis	Higher peroxidatic activity [74, 75] Decreased apoptosome activation [78]
Fibrinogen	Coagulation	Higher aggregation [76]
Protein kinase C $\epsilon$	Serine/threonine kinase	Translocation and activation [77]
$\alpha$ -Synuclein	Presynaptic protein	Higher aggregation [79]
Nerve growth factor	Neurotrophic factor	Neuronal apoptosis [80]
MnSOD	Superoxide dismutation	Decreased activity [72]
Prostacyclin synthase	Synthesis of prostacyclin	Decreased activity [73]
Tyrosine hydroxylase	Synthesis of L-DOPA	Decreased activity [81]
Protein kinase C	Serine/threonine kinase	Decreased activity [82]

and  $1.6 \pm 0.4$  nM in the group of healthy volunteers [16]. Besides, elevated 3-NOTyr levels can be found in patients with arthritis [21, 22], atherosclerosis [26], and hypertension [33].

**2.4. Advanced Oxidation Protein Products.** Extracellular fluids contain only minor amounts of antioxidant enzymes and thus plasma proteins (e.g., albumin) are prone to oxidation by ROS. Elevated levels of oxidized protein products are termed “advanced oxidation protein products” (AOPP). AOPP are produced by the myeloperoxidase- (MPO)-H<sub>2</sub>O<sub>2</sub>-halide system of activated phagocytes. First step of this reaction is oxidation of coenzyme NADPH by hydrogen peroxide. During this reaction hypochlorous acid (HOCl) is produced. The Cl<sup>−</sup> ion is used as a substrate by the MPO enzyme. Myeloperoxidase is produced from hydrogen peroxide activated leukocytes. The generation of cytotoxic HOCl also causes the formation of advanced oxidation protein products (AOPP) by attacking normal tissue with consequent protein oxidation.

Higher concentrations of AOPP were found in plasma or urine of patients with acute coronary syndrome or active ulcerative colitis. The concentration of AOPP in plasma was determined by 140–180  $\mu$ M for patients and 60–70  $\mu$ M for the group of healthy volunteers [83, 84].

**2.5. 8-Hydroxy-2'-deoxyguanosine and 8-Hydroxyguanosine.** 8-Hydroxy-2'-deoxyguanosine (8-OHdG; Figure 4) is the main product of DNA oxidation. 8-Hydroxyguanosine (8-OHG; Figure 5) is formed by oxidation of RNA.

Two mechanisms are possible for the release of 8-OHdG to urine and blood plasma. First, 2'-deoxyguanosine triphosphate and hydroxyl radical form 8-hydroxy-2'-deoxyguanosine triphosphate which is enzymatically transformed to 8-hydroxy-2'-deoxyguanosine monophosphate (8-OHdGMP). 8-OHdG is released by digestion of 8-OHdGMP.

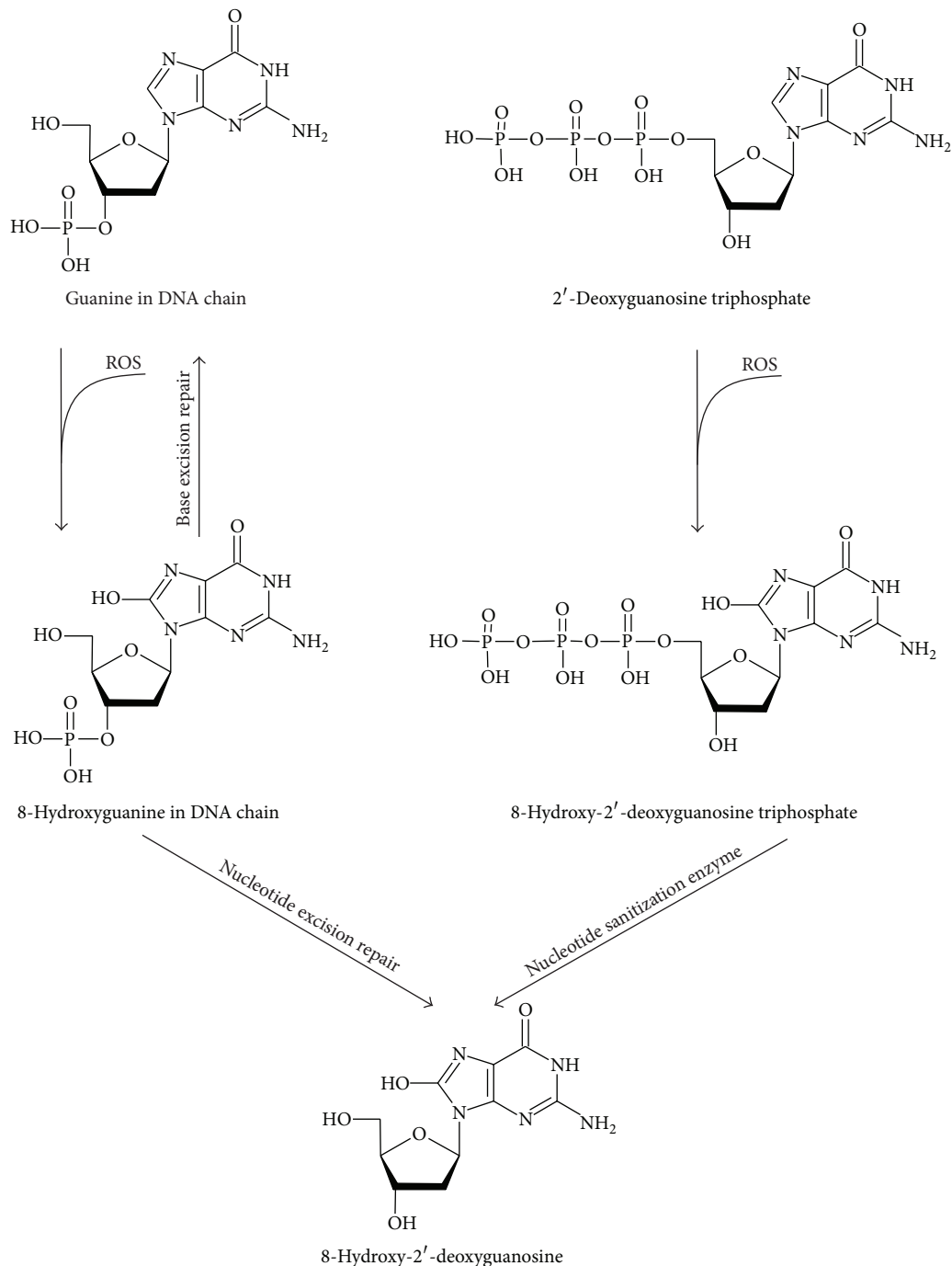


FIGURE 4: The formation of 8-hydroxy-2'-deoxyguanosine.

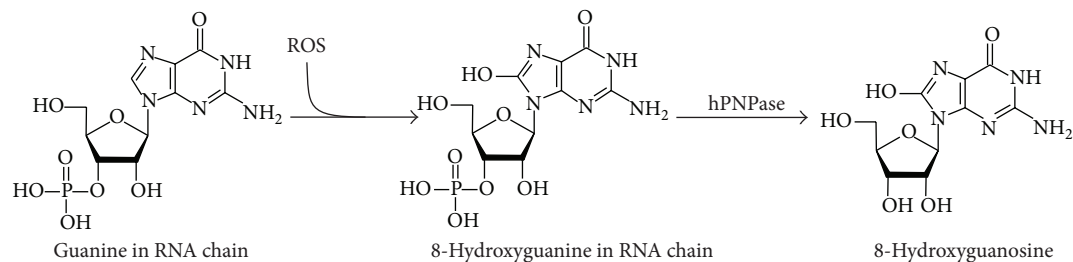


FIGURE 5: The formation of 8-hydroxydeoxyguanosine.

Second, 8-OHdG is formed by nucleotide excision repair (NER) mechanism. The whole sequence containing damaged nucleic base (oligonucleotide) is removed from DNA and the missing part of the strand is synthesized according to the other complementary strand [85].

8-OHG is formed by the reaction of RNA and hydroxyl radical [86]. Human polynucleotide phosphorylase (ribonuclease hPNPase) is assumed to remove 8-OHG from RNA [87]. Oxidation of mRNA lowers the effectiveness of translation (synthesis of primary protein structure according to the genetic information in mRNA), induces formation of abnormal proteins [88], and is one of the primary factors causing cell death [89].

In rat model, 8-OHdG was found to have anti-inflammatory effect [90]. Rats treated with lipopolysaccharide (LPS) exhibited inflammatory lung injury dependent on neutrophils with increase in proinflammatory cytokines such as interleukins 6 and 18 (IL-6, IL-18) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). Rats pretreated with 8-OHdG prior to LPS treatment showed inhibited LPS-induced inflammatory responses. 8-OHdG anti-inflammatory action was found to be higher than that for aspirin and other nucleosides (8-OHG, deoxyguanosine, guanosine, and adenosine). 8-OHG and adenosine also exhibited anti-inflammatory activity, but it was much lower than that for 8-OHdG. Deoxyguanosine was found to be almost ineffective. Compared to aspirin, which acts through cyclooxygenase (COX) inhibition, 8-OHdG seems to be more versatile and, therefore, more effective as it was found that 8-OHdG suppresses ROS formation in human neutrophils. However, in humans, 8-OHdG is excreted in much lower concentrations than in rats and, therefore, only exogenously administered 8-OHdG could have a therapeutic potential as anti-inflammatory agent [90].

Higher concentration of 8-OHdG was found, for example, in patients with Alzheimer's disease [18], arthritis [23, 24], atherosclerosis [27], cataracts [30], hypertension [34], osteoporosis [35], or type II diabetes [38, 40]. 8-OHdG is also considered to be a potential biomarker of cancers related to smoking (e.g., lung cancer). The concentration was  $1.57 \pm 0.86$  nM in patients with cancer and  $1.09 \pm 0.52$  nM in the control group of healthy volunteers [91].

8-OHG can be found in patients with Alzheimer's disease and it has been shown that oxidative damage of RNA is higher than damage of DNA [18, 19]. The concentrations in cerebrospinal fluid for Alzheimer's disease were  $500 \pm 213$  pM in the patients and  $97 \pm 32$  pM in the control group. The difference of the concentration in blood serum was not significant [19].

### 3. Methods for Determination of Oxidative Stress Biomarkers

The complexity of biological matrices, different molecular structures of biomarkers, and variety of existing analytical methods gives us a lot of possibilities to determine the biomarkers. The most common analytical methods are summarized in Table 4.

TABLE 4: Analytical methods used for determination of age-related diseases.

Detected biomarker	Analytical method
8-Isoprostane	EIA [28, 32, 37, 38], ELISA [20, 31, 36], RIA [92–94], GC/MS [95], HPLC-MS [96], and LC-ESI-MS/MS [97]
<i>o</i> -Tyrosine	GC-MS [98–100], GC-ECD [101], HPLC-UV [29, 39], HPLC-APCI/MS/MS [101], and HPLC-MS/MS [102]
<i>m</i> -Tyrosine	GC-MS [99, 100], GC-ECD [101], HPLC-UV [39], and HPLC-APCI/MS/MS [101]
3-Chlorotyrosine	GC-MS [14, 77], GC-ECD [101], HPLC-APCI/MS/MS [101], and HPLC-ECD [103]
3-Nitrotyrosine	Immune histochemistry [33], ELISA [21], HPLC-ECD [15, 16], LC-MS/MS [17], HPLC-UV [22, 26], and HPLC-MS [22]
8-Hydroxy-2'-deoxyguanosine	Immunostaining [18], ELISA [23, 24, 34, 35, 38, 40], LC-MS/MS [27], HPLC-UV [27], and HPLC-ECD [27]
8-Hydroxyguanosine	Immunostaining [18], immune histochemistry [33], and HPLC-ECD [19]

Biochemical methods such as ELISA (enzyme-linked immunosorbent assay) and EIA (enzyme immunoassay) allow the determination of lower concentrations than methods combining chromatographic methods and mass spectrometry (GC-MS, HPLC-MS). However, the disadvantage of biochemical methods is the possibility of cross-reactions which cause false-positive or false-negative results [104]. Currently, the limit of detection (LOD) of methods combining chromatography and mass spectrometry is pico- and femtomoles per milliliters which are concentrations of biomarkers in body fluids. Additionally, both quantitative and qualitative (structure of the substance) information are gained. Therefore MS<sup>n</sup> techniques, which have a high selectivity, are used more often.

ELISA (enzyme-linked immunosorbent assay), also called EIA (enzyme immunoassay), is one of the most frequently used methods applicable in the quantitative analysis of antigens. This method exists in a range of modifications which are all based on a highly specific interaction of antigen and antibody. One of these binding partners is covalently bound to an enzyme (usually peroxidase, acetylcholinesterase, or alkaline phosphatase) whose role is the catalytic conversion of the added substrate to a colored product. The color intensity, determined by spectrophotometric or fluorimetric methods, directly or indirectly reflects the amount of the antigen present in the sample. When the antigen is determined, the immobilization (via adsorption or a covalent bonding) of the antibody on a solid support is a common characteristic of all ELISA methods. The immobilization of antibodies (e.g., on a microtiter plate) enables the separation of antigens (biomarkers) from biological matrices (exhaled breath condensate, blood plasma, and urine).

Radioimmunoassay (RIA) works on a similar principle as ELISA. The main difference is in the use of a labeled antigen.



The enzyme on the antigen is replaced by a tyrosine moiety containing a  $\gamma$ -radioactive iodine isotope. The  $\gamma$ -radiation is monitored by the presence of the nonbonded labeled antigen in the sample. The very sensitive and specific methods based on RIA for 8-isoprostane determination in EBC have been successfully developed and validated [104]. RIA is also very suitable for determination of FENO or prostaglandins in EBC from patients suffering from asthma or cystic fibrosis [105, 106]. However, radioactive species can be operated only in specialized laboratories with appropriate equipment, which is a relevant disadvantage and explains the less frequent utilization of RIA in practice.

For detection of proteomics markers of oxidative stress (AOPP), ELISA test [107, 108] or methods with mass spectrometric detection [104, 109, 110] can be used. During MS detection can be used protein digest method (digest of proteins to smaller peptides using a protease such as trypsin) or protein nondigest method (intact proteins are ionized by ESI or MALDI ionization and then introduced into a mass analyzer. This approach is referred to as “top-down” strategy of protein analysis).

Electronic nose as a novel analytical technique for determination of volatile compounds in EBC usually comprises an array containing a number of chemical sensors. The choice of the sensors represents difficult task due to their specificity, response and recovery time, range of compounds detected, sensitivity, operating temperature, physical size, temperature and humidity effect on sensor functioning, portability, and cost and circuitry complexity. The molecules of analyte are adsorbed on the sensor surface providing the signal that fades with desorption. The similarity to biochemical methods mentioned above and detection limits as low as tens of ppb make this innovative technique really promising [111, 112].

Nuclear magnetic resonance (NMR) is primarily intended for qualitative structural analysis although it has been proven to be a valuable tool for comparison of different groups of individuals and statistical evaluation of collected data using methods such as PCA (principle component analysis) for key biomarkers present in EBC [113, 114]. This method uses the interaction of strong magnetic field with atomic nuclei possessing nonzero spin. The signal is created via absorption of high frequency radiation causing specific spin energy distribution. NMR technique can be used for both proteomics and metabolomics [115, 116].

Gas chromatography coupled with mass spectrometry (GC-MS) can be used for the analysis of analytes giving information about both their structures and their quantities. This analytical method takes advantage of its (1) high separation selectivity determined by the type of capillary columns used and (2) high specificity and sensitivity enabled by the integration of the mass spectrometric detector. Therefore, the GC-MS method allows the quantification of substances in biological matrices or tissue on nanogram per milliliter or gram level. The most significant disadvantage is the need for a sufficient volatility and thermal stability of analytes in the sample. To resolve it, pretreatment procedures (extraction and derivatization) are necessary to be included in this particular case prior to quantitative and qualitative analysis. Derivatization is a chemical reaction of an analyte with a suitable derivatization

reagent which changes its physical and chemical properties (in this case mainly volatility and thermal stability). Additionally, derivatization prior to a GC-MS analysis is carried out to improve the sensitivity of the MS detection by enabling a better fragmentation in the detector. For example, 3-NOTyr is measured as methyl ester-diheptafluorobutyl amide-methyl ether (Me-HFB-Me) derivative [117], di-*O*-methyl-di-*N*-heptafluorobutyl derivative [117], *n*-propyl-PFP-TMS derivative [118, 119], and pentafluorobenzyl derivative [120]. 3-ClTyr is measured as *N*(*O*)-ethoxycarbonyl trifluoroethyl amino acid ester [121].

High performance liquid chromatography (HPLC) in combination with mass spectrometry (MS) is generally used for the analysis of low volatile and thermally labile substances. The high selectivity of separation is achieved by a suitable choice of chromatographic phase systems, that is, the liquid and stationary phase. Reversed-phase HPLC is the most commonly used with the stationary phase consisting of silica gel modifiable by nonpolar octadecyl groups and the polar liquid phase usually consisting of water, acetonitrile, or methanol, optionally with addition of buffers. For the detection, usually UV, fluorescence, electrochemical, or MS methods are used. Nowadays, the combination of HPLC and MS allows facile separation and parallel detection of even very low analyte concentrations present in complex matrices. Since the remaining detectors mentioned above do not allow the quantification of analytes and lack the high specific structural information, HPLC-MS is becoming the first choice method for the analysis of substances in biological matrices. Therefore, the analysis of complex body fluids on a picogram scale is viable using HPLC-MS and also suitable for future routine practice. In order to increase the detector precision and sensitivity, the following is advisable prior to the HPLC-MS analysis: (1) the addition of an isotopically labeled internal standard and (2) the use of a pretreatment method (immunoextraction, solid phase extraction, and lyophilisation) to remove undesired species and concentrate the sample. When MS detection is utilized, the analytes need to be evaporated and ionized. As this can be carried out at atmospheric pressure (API: atmospheric pressure ionization), it is also feasible with thermally labile substances. Electrospray ionization (ESI) is one of the most frequently used API techniques. It is a soft ionization technique characterized by the preservation of a molecular ion peak with minimal fragmentation of the analyzed molecule. Depending on the molecule charge of a measured analyte, two measurement modes can be distinguished, that is, positive electrospray ionization (ESI<sup>+</sup>) in which protonated molecular ion  $[M+H]^+$  is produced and negative electrospray ionization (ESI<sup>-</sup>), where the molecule is deprotonated  $[M-H]^-$ . The molecule ion ( $[M+H]^+$  or  $[M-H]^-$  ion (given chemical structure of the detected biomarkers)) is preferred for all determined biomarkers. The combination of ESI ionization and a triple-stage quadrupole analyzer (TSQ) is a suitable detection technique for the quantification of the analytes. The first and the third quadrupole (Q1 and Q3) are identical and capable of using the same scan modes. On the contrary, the second quadrupole (Q2) is different in both its construction and function, allowing the fragmentation of the analyte upon

TABLE 5: SRM transitions for the quantification of biomarkers.

Biomarker	Molecular ion [Da]	Product ion [Da]	Collision energy [eV]
8-iso PGF <sub>2α</sub>	352.9	193.2	27
<i>o</i> -Tyr	180.1	119.1	20
3-ClTyr	214.2	153.1	17
3-NO <sub>2</sub> Tyr	225.2	164.1	15
8-OHdG	282.2	192.1	21
8-OHG	298.2	208.1	20

elastic collision with an inert gas (argon). Therefore, it is often referred to as the collision cell. A mass spectrometer equipped with a triple quadrupole uses a highly selective single reaction monitoring mode (SRM) for the quantification and structural identification of substances. In the case of oxidative stress biomarkers, Q1 isolates the deprotonated  $[M-H]^-$  molecular ions, which are further used as precursor ions for the subsequent collision-induced dissociation (CID) in Q2. In the collision cell, the molecule selectively degrades and yields product ions which are analyzed on quadrupole Q3 giving MS/MS spectra (Figure 6). Methods used for the quantification of the biomarkers are in Table 5. For HPLC, a gradient elution with flow rate of 200  $\mu$ L/min was used (Table 6) [97], mobile phase A was a water solution of ammonium hydroxide (pH = 10.5), and mobile phase B was a mixture of MeOH/ACN (60 : 40 v/v) with 0.1% ammonium hydroxide. The retention times were as follows: dead time of the column = 0.8 min;  $R_t$  (8-OHdG) = 1.9 min;  $R_t$  (8-OHG) = 3.1 min;  $R_t$  (3-ClTyr) = 14.4 min;  $R_t$  (3-NO<sub>2</sub>Tyr) = 17.0 min;  $R_t$  (*o*-Tyr) = 20.6 min; and  $R_t$  (8-iso) = 29.5 min (Figure 7).

#### 4. Clinical Study

It is generally accepted that concentrations of oxidative stress biomarkers are increasing with increasing age. Although it has not been proven for every single known biomarker, several studies confirm initial statement [122]. The studies that have been published so far are generally not focused on relation between levels of biomarkers in healthy subjects and their age, but they are focused on monitoring of levels of oxidative stress biomarkers linked to particular disease (e.g., Alzheimer's disease and Parkinson's disease; see Table 1). Some authors [123, 124] have observed the elevation of specific oxidative stress biomarker in biological matrix, but so far there has not been performed wider metabolomic screening of oxidative stress biomarkers in relation to the age of healthy individuals.

We compared three groups of people with similar age. The first, labelled "20," consisted of 30 people of an average age  $21 \pm 4.3$  years. The second, labelled "40," consisted of 30 people of an average age  $39 \pm 8.4$  years and the third, labelled "60," consisted of 30 people of an average age  $62 \pm 9.1$  years. All subjects were healthy nonsmokers. As a biological matrix, we have chosen exhaled breath condensate (EBC). The EBC sampling is noninvasive and can be used as a tool for diagnosis of lung diseases [97, 102]. The most significant

TABLE 6: HPLC elution program. Solvent A: water solution of ammonium hydroxide (pH 10.5); solvent B: solution of methanol:acetonitrile (60:40, v/v) with 0.1% of ammonium hydroxide.

Time [min]	Solvent A [%]	Solvent B [%]
0:00	70	30
10:00	70	30
25:00	5	95
30:00	5	95
32:00	70	30
40:00	70	30

difference in biomarker concentration can be observed for 8-iso PGF<sub>2α</sub>, but all biomarkers show a trend of an increasing concentration with increasing age. The levels of oxidative stress biomarkers in first group were 8-iso PGF<sub>2α</sub> ( $15.0 \pm 1.9$  pg/mL EBC); *o*-Tyr ( $33.3 \pm 3.4$  pg/mL EBC); 3-ClTyr ( $14.5 \pm 1.9$  pg/mL EBC); 3-NO<sub>2</sub>Tyr ( $25.4 \pm 4.8$  pg/mL EBC); 8-OHdG ( $11.4 \pm 2.1$  pg/mL EBC); and 8-OHG ( $10.4 \pm 2.7$  pg/mL EBC). The second group exhibited increased values of monitored biomarkers: 8-iso PGF<sub>2α</sub> ( $26.8 \pm 1.9$  pg/mL EBC); *o*-Tyr ( $41.4 \pm 4.1$  pg/mL EBC); 3-ClTyr ( $17.1 \pm 2.4$  pg/mL EBC); 3-NO<sub>2</sub>Tyr ( $31.3 \pm 4.6$  pg/mL EBC); 8-OHdG ( $14.9 \pm 2.2$  pg/mL EBC); and 8-OHG ( $15.7 \pm 3.2$  pg/mL EBC). The highest levels were confirmed in the third group: 8-iso PGF<sub>2α</sub> ( $44.5 \pm 5.3$  pg/mL EBC); *o*-Tyr ( $55.6 \pm 4.7$  pg/mL EBC); 3-ClTyr ( $27.1 \pm 3.2$  pg/mL EBC); 3-NO<sub>2</sub>Tyr ( $43.4 \pm 3.0$  pg/mL EBC); 8-OHdG ( $24.6 \pm 2.4$  pg/mL EBC); and 8-OHG ( $32.4 \pm 4.1$  pg/mL EBC) (Syslova et al., unpublished results).

The study was carried out according to the Helsinki Declaration. The Ethics Committee of the 1st Faculty of Medicine, Charles University, approved all examinations and tests, and all of the study subjects gave their written informed consent for all tests and examinations.

#### 5. Conclusion

Oxidative stress plays an important role in many pathological processes including age-related diseases such as atherosclerosis, hypertension, and type II diabetes. The level of oxidative damage can be measured through specific molecules, which are formed in the organism via oxidative stress. Subsequently, these substances, biomarkers of oxidative stress, not only can be monitored in body fluids and tissues of patients but also are present in healthy people in a physiological concentration. Regarding low concentrations of biomarkers in body fluids, it is necessary to choose a sensitive analytical method for the detection. A combination of separation by HPLC and detection by MS enables determination of picogram concentrations of analytes in complex biological matrices. By comparing three groups of healthy people with a different age, we found that the concentration of oxidative stress biomarkers (8-isoprostane, *o*-tyrosine, 3-chlorotyrosine, 3-nitrotyrosine, 8-hydroxy-2'-deoxyguanosine, and 8-hydroxyguanosine) is

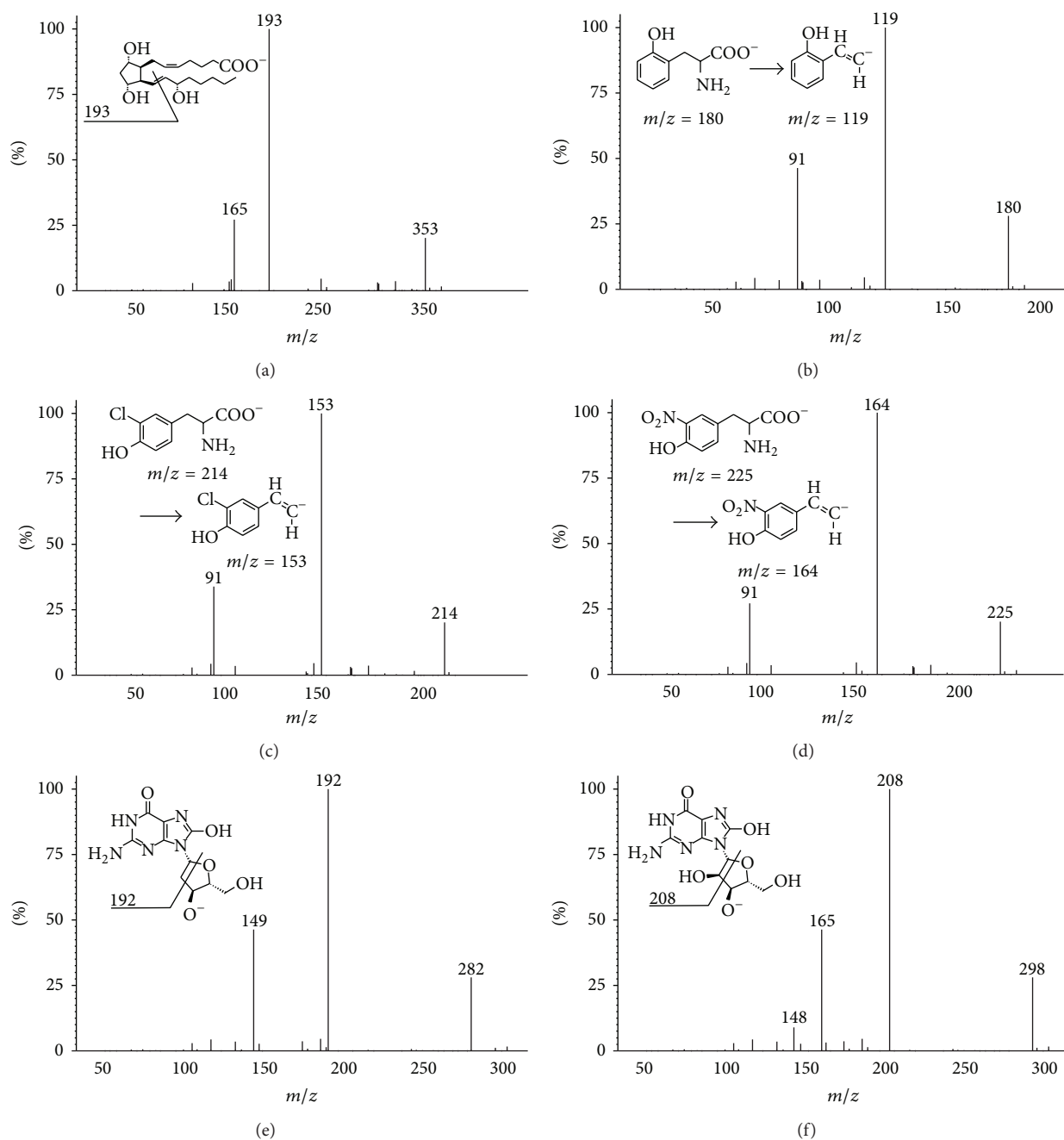


FIGURE 6: MS/MS spectra for (a) 8-isoprostane, (b) *o*-tyrosine, (c) 3-chlorotyrosine, (d) 3-nitrotyrosine, (e) 8-hydroxy-2'-deoxyguanosine, and (f) 8-hydroxyguanosine.

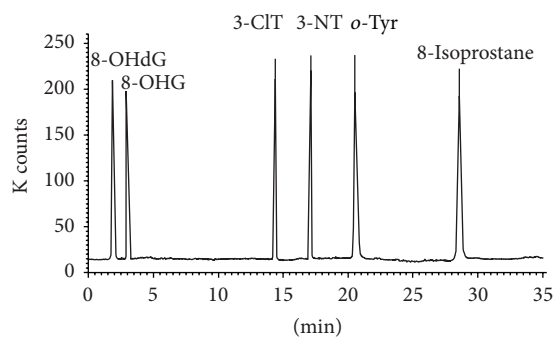


FIGURE 7: HPLC-ESI-MS/MS chromatogram.

increasing with the increasing age of people. This study confirms the hypothesis that the physiological level of biomarkers depends on the age of people.

## Conflict of Interests

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

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## Review Article

# Oxidative Stresses and Mitochondrial Dysfunction in Age-Related Hearing Loss

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Age-related hearing loss (ARHL), the progressive loss of hearing associated with aging, is the most common sensory disorder in the elderly population. The pathology of ARHL includes the hair cells of the organ of Corti, stria vascularis, and afferent spiral ganglion neurons as well as the central auditory pathways. Many studies have suggested that the accumulation of mitochondrial DNA damage, the production of reactive oxygen species, and decreased antioxidant function are associated with subsequent cochlear senescence in response to aging stress. Mitochondria play a crucial role in the induction of intrinsic apoptosis in cochlear cells. ARHL can be prevented in laboratory animals by certain interventions, such as caloric restriction and supplementation with antioxidants. In this review, we will focus on previous research concerning the role of the oxidative stress and mitochondrial dysfunction in the pathology of ARHL in both animal models and humans and introduce concepts that have recently emerged regarding the mechanisms of the development of ARHL.

## 1. Introduction

Oxidative stress represents an imbalance between the production of reactive oxygen species (ROS) and the detoxification of their reactive intermediates. ROS, such as hydroxyl radicals, superoxide anions, hydrogen peroxide, and singlet oxygen, are primarily generated by mitochondria in most mammalian cells and are generally regarded as the toxic side-products of cellular metabolism [1–3]. ROS are normally detoxified by a variety of antioxidant enzymatic scavengers, including superoxide dismutase (SOD), catalase, glutathione S-transferase (GST), and glutathione peroxidase (GPX) [4].

Mitochondria are a major site of ROS-induced oxidative damage [5, 6]. ROS generated by mitochondria are hypothesized to damage key mitochondrial components such as mitochondrial DNA (mtDNA), mitochondrial membranes, and respiratory chain proteins and nuclear DNA that affect mitochondrial function. mtDNA is a circular, closed, double-stranded molecule and is not protected by histones. Therefore, mtDNA is more susceptible to DNA insults in comparison with nuclear DNA. Most of mtDNA mutations are characterized by heteroplasmy, which is defined as the presence

of more than one an organellar genome within a cell or tissue from a single individual. As the percentage of mutant alleles increases, the mitochondrial bioenergetic defect becomes more severe. The expression of disease depends on the percentage of mutant alleles.

It has been widely considered that aging is the process of accumulated oxidative damage caused by ROS [7, 8]. This damage accumulates over time, causing mitochondrial dysfunction and an associated decrease of energy production, and results in tissue dysfunction. ROS production increases with age and it is known that oxidative stress and associated mitochondrial dysfunction play an important role in aging and age-related diseases [1, 2].

Age-related hearing loss (ARHL), which is also called presbycusis, is the progressive loss of hearing associated with aging and is the most common sensory disorder in the elderly population [9–11]. ARHL afflicts approximately half of the people over 65 years of age in the United States [12]. The prevalence of the ARHL is expected to increase as the elderly population grows [9, 13, 14]. It has been proposed that ARHL is associated with many factors, including environmental,

medical, and hereditary factors [12, 15]. So far, no effective treatment has been found for this age-related disorder.

Many studies have been conducted based on the assumption that age-related oxidative stress and mitochondrial dysfunction could be an underlying pathology of ARHL as well as other age-related diseases. In this review, we will focus on previous research concerning the role of the oxidative stress and mitochondrial dysfunction in the pathology of ARHL in both animal models and humans and introduce concepts that have recently emerged as potential mechanisms for the development of ARHL.

## 2. Pathological Findings in ARHL

Sound waves travel down the external ear canal and cause the tympanic membrane to vibrate. The ossicles in the middle ear link the vibrating tympanic membrane to the cochlea, the auditory end organ of the inner ear. The cochlea is filled with fluid that vibrates in response to the movement of the ossicles. The inner and outer sensory hair cells are located within a core component of the cochlea, the organ of Corti. When a sound pressure wave travels from the basal turn to the apical turn of the cochlea, the basilar membrane vibrates [16]. Displacement of stereocilia, the mechanosensing organelles of the hair cell, in association with the vibration of the basilar membrane, opens transduction ion channels, allowing entry of potassium ions from the endolymph produced by the stria vascularis. This transduction current then activates voltage-dependent calcium channels along the hair cell lateral wall and base [17]. The inner hair cells release the neurotransmitter glutamate to encode acoustic signals for the adjacent spiral ganglion neurons (SGNs), which are the primary auditory neurons [18].

Based on postmortem pathological analysis, ARHL in humans is generally classified into 3 types: sensory hearing loss (loss of sensory hair cells), neuronal hearing loss (loss of SGNs), and metabolic hearing loss (atrophy of the stria vascularis) [9, 19], although it is now well established that most cases of ARHL exhibit mixed pathological changes [9]. This idea is supported by the observation that the progressive loss of hair cells and SGNs leads to ARHL because these two cell types do not regenerate in mammals.

## 3. Candidate Genes for ARHL Associated with Oxidative Stress and Mitochondrial Dysfunction

Many genetic investigations of ARHL, such as genome-wide association studies and candidate-gene-based association studies, have been performed recently [20]. With regard to oxidative stress and mitochondrial function, several genes and loci have been proposed as a result of candidate-gene-based association studies, which are based on hypotheses about the relationship between specific known loci and phenotypes.

The superoxide dismutases (SODs), which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, are an important part of the antioxidant defense system

against ROS. Recently, evidence from the London ARHL cohort suggested an effect of common superoxide dismutase 2 (SOD2, also known as manganese SOD or mitochondrial SOD) promoter variation,  $-38\text{C} > \text{G}$ , on SOD2 promoter regulation and linked it to ARHL risk in men; however, this association was only suggestive due to a lack of replication [21].

The glutathione S-transferases (GSTs) catalyze the detoxification of electrophilic substrates by conjugation with reduced glutathione and participate in intracellular binding and transport of lipophilic substances. Decreased glutathione and GST activity levels cause an increase in susceptibility to cell damage. A previous study investigated the association between ARHL and genes related to oxidative stress using a large set of samples from two population groups, a general European group and a Finnish group [22]. Although an association between the polymorphisms of glutathione S-transferase, mu 1 (*GSTM1*) or glutathione S-transferase, theta 1 (*GSTT1*), and ARHL was not detected in the former population, there were significant associations between both genes and ARHL in the latter population.

Mitochondrial uncoupling proteins (UCPs), which are members of the larger family of mitochondrial anion carrier proteins, facilitate the transfer of anions from the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner mitochondrial membrane. UCPs reduce the mitochondrial membrane potential in mammalian cells. The main function of uncoupling protein 2 (UCP2) is the control of mitochondria-derived ROS [23]. *UCP2* Ala55Val polymorphisms exhibited a significant association with ARHL in a Japanese population [24].

## 4. Deletions and Mutations of mtDNA in the Peripheral Auditory System of ARHL Patients

Acquired mtDNA defects have been proposed as important factors in aging. Increases in deletions, mutations, or both, in mtDNA have been reported in human temporal bone studies from ARHL patients in comparison with normal-hearing control tissues. A 4977-base pair deletion of mtDNA from celloidin-embedded temporal bone sections was significantly more frequent in cochlear tissue from ARHL patients in comparison to those with normal hearing [25]. Another study reported that quantitative analysis of the mtDNA in archival cochlear tissue samples revealed a mean common deletion level of  $32 \pm 14\%$  in ARHL patients, in comparison with a level of  $12 \pm 2\%$  in age-matched controls with normal hearing, and showed a significant correlation between the common deletion level and the severity of hearing loss [26]. Cytochrome c oxidase subunit 3 (*COX3*) expression was significantly diminished in SGNs from ARHL patients in comparison with age-matched normal-hearing individuals. In addition to the mtDNA common deletion, other deletions involving the mtDNA major arc contributed to the observed deficit in *COX3* expression [27]. Mutations within the cytochrome c oxidase subunit 2 (*COX2*) gene in the spiral ganglion and

membranous labyrinth from archival temporal bones occur more commonly in ARHL patients relative to controls [28].

## 5. Basic Research in Animals on the Role of Oxidative Stresses and Mitochondrial Dysfunction in ARHL

Although details of the aging process differ in various organisms, there is a common understanding that oxidative stress and mitochondrial dysfunction play a major part in aging. The auditory system is no exception and it is thought that oxidative damage caused by ROS and mitochondrial dysfunction plays a causal role in ARHL. The fast-aging senescence-accelerated mouse-prone 8 (SAMP8) strain that is a useful model for probing the effects of aging on biological processes displays premature hearing loss associated with strial, sensory, and neural degeneration [29]. The molecular mechanisms associated with premature ARHL in SAMP8 strain mice involve oxidative stress, altered levels of antioxidant enzymes, and decreased activity of complexes I, II, and IV, which lead to triggering of apoptotic cell death pathways.

In the organ of Corti of CBA/J mice, glutathione-conjugated proteins, markers of  $H_2O_2$ -mediated oxidation, were shown to begin to increase at 12 months, and 4-hydroxynonenal and 3-nitrotyrosine, products of hydroxyl radical and peroxynitrite action, respectively, were elevated by 18 months [30]. On the other hand, apoptosis-inducing factor and SOD2 were decreased by 18 months in the organ of Corti and SGNs [30]. Mice lacking superoxide dismutase 1 (*Sod1*) showed premature ARHL [31, 32]. Age-related cochlear hair cell loss was observed in *Sod1* knockout mice [32] and a reduced thickness of the stria vascularis and severe degeneration of SGNs were observed at middle age [31]. A previous study showed that increased GPX activity was observed in the stria vascularis and spiral ligament of the cochlea in aged Fischer 344 rats [33]. Two-month-old knockout mice with a targeted inactivating mutation of the gene coding for glutathione peroxidase 1 (*Gpx1*) showed a significant increase in hearing thresholds at high frequency [34]. Mice lacking senescence marker protein 30 (SMP30)/gluconolactonase (GNL), which are not able to synthesize vitamin C, showed a reduction of vitamin C in the inner ear, an increase of hearing thresholds, and loss of spiral ganglion cells, suggesting that depletion of vitamin C accelerates ARHL [35]. Oxidative stress induces the expression of BCL2-antagonist/killer 1 (*Bak*); the mitochondrial proapoptotic gene, in primary cochlear cells and *Bak* deficiency prevents apoptotic cell death [36]. C57BL/6J mice with a deletion of *Bak* exhibit reduced age-related apoptotic cell death of SGNs and hair cells in the cochlea and prevention of ARHL [36]. A mitochondrially targeted catalase transgene suppresses *Bak* expression in the cochlea, reduces cochlear cell death, and prevents ARHL [36]. Collectively, these findings indicate that age-related increases in ROS levels play an important role in the development of ARHL.

It has been shown that accumulation of mtDNA mutations leads to premature aging in mice expressing a proof-reading-deficient version of the mtDNA polymerase  $\gamma$  (POLG D257A mice), indicating a causal role of mtDNA mutations

in mammalian aging [37, 38]. POLG D257A mice accumulate mitochondrial mutations more rapidly than wild-type mice. At 9-10 months old, POLG D257A mice showed a variety of premature aging phenotypes, including the early onset of ARHL. Histological findings in the cochlear basal turn confirmed that POLG D257A mice at the age of 9-10 months showed a severe loss of SGNs and hair cells and significant elevation in TUNEL-positive cells and cleaved caspase-3-positive cells in the cochlea [39].

Mitochondrial biogenesis and degradation are involved in mitochondrial turnover. In the SGNs of SAMP8 strain mice, mitochondrial biogenesis, characterized by the ratio of mtDNA/nuclear DNA and the activity of citrate synthase, was increased at younger ages and decreased in old age [29]. Age-related reductions of peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 (PGC-1 $\alpha$ ), one of the key regulators of mitochondrial biogenesis, might be an important factor for mitochondrial function in age-related diseases [40]. When it comes to mitochondrial function in the cochlea, the overexpression of PGC-1 $\alpha$  with a consequent increase of nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM) caused a significant decrease in the accumulation of damaged mtDNA and the number of apoptotic cells in the strial marginal cells senescence model [41]. Autophagy is one of the major intracellular degradation pathways along with the ubiquitin-proteasome system [42]. Unnecessary cytoplasmic proteins and organelles are enclosed by the autophagosome and then delivered to the lysosome by autophagy. It has been reported that the SGNs of SAMP8 undergo autophagic stress with accumulation of lipofuscin inside these cells [29]. Downregulation of mitophagy, the selective removal of damaged and dysfunctional mitochondria by autophagosomes will cause abnormal mitochondrial morphological changes. Impairment of mitophagy might result in the formation of giant mitochondria, which have been characterized as having low ATP production, a loss of cristae structure, and a swollen morphology [43]. Accumulation of abnormally functioning and shaped mitochondria accelerates apoptosis [44], which merits further investigation in the cochlea.

## 6. Prevention and Retardation of ARHL by Supplementation or Caloric Restriction

Several studies have reported the effects of supplementation of antioxidants against ARHL. A cross-sectional and 5-year longitudinal study in Australia demonstrated that dietary vitamin A and vitamin E has a significant association with the prevalence of hearing loss, although dietary antioxidant intake did not increase the incidence of hearing loss [45]. Another cross-sectional study in Australia showed that higher carbohydrate, vitamin C, vitamin E, riboflavin, magnesium, and lycopene intakes were significantly associated with larger transiently evoked otoacoustic emission (TEOAE) amplitudes and better pure tone averages (PTAs) whereas higher cholesterol, fat, and retinol intakes were significantly associated with lower TEOAE amplitude and worse PTAs [46]. Another further cross-sectional study in the United States showed that higher intakes of beta-carotene, vitamin C,



and magnesium were associated with better PTAs at both speech and high frequencies, and high intakes of beta-carotene or vitamin C combined with high magnesium compared with low intakes of both nutrients were significantly associated with better PTAs at high frequencies [47].

In animal studies, Fischer 344 rats given vitamin C, vitamin E, melatonin, or lazaroid had better auditory sensitivities and a trend for fewer mtDNA deletions in comparison with placebo subjects [48]. Fischer 344 rats of 18–20 months old supplemented orally for 6 months with lecithin, a polyunsaturated phosphatidylcholine (PCP) which has antioxidant effects, showed significantly better hearing sensitivities, higher mitochondrial membrane potentials, and reduced frequency of the common aging mtDNA deletion in the cochlear tissues compared with controls [49]. Aged dogs fed a high antioxidant diet for the last 3 years of their life showed less degeneration of the spiral ganglion cells and stria vascularis in comparison with dogs fed a control-diet [50]. In C57BL/6 mice, supplementation with vitamin C did not increase vitamin C levels in the cochlea or slow ARHL [35], but animals fed with a diet comprising 6 antioxidant agents (L-cysteine-glutathione mixed disulfide, ribose-cysteine, NW-nitro-L-arginine methyl ester, vitamin B12, folate, and ascorbic acid) showed significantly better auditory sensitivity [51]. When C57BL/6 mice were fed with a diet containing one of 17 antioxidant agents (acetyl-L-carnitine, alpha-lipoic acid, beta-carotene, carnosine, coenzyme Q10, curcumin, d-alpha-tocopherol, epigallocatechin gallate, gallic acid, lutein, lycopene, melatonin, N-acetyl-L-cysteine, proanthocyanidin, quercetin, resveratrol, and tannic acid), ARHL was nearly completely prevented by alpha-lipoic acid and coenzyme Q10 and partially by N-acetyl-L-cysteine, but not by other agents [36]. When CBA/J mice were fed with an antioxidant-enriched diet containing vitamin A, vitamin C, vitamin E, L-carnitine, and alpha-lipoic acid from 10 months through 24 months of age, the antioxidant capacity of the inner ear tissues was significantly increased, but the loss of hair cells and spiral ganglion cells and the magnitude of ARHL were not improved [52]. These studies show that the prevention and retardation of ARHL by supplementation with antioxidants can be influenced by many factors such as the type and dosage of antioxidant compounds, the timing and duration of the treatment, and the species and strains involved.

Caloric restriction (CR) extends the lifespan of various organisms including yeast, worms, flies, rodents and non-human primates. It has been reported that CR plays an important role in reducing age-related diseases such as cancer [53], protecting age-related mitochondrial dysfunction [54] and reducing mtDNA damage [55]. It has also been reported that CR can protect neurons against degeneration in animal models of neurodegenerative diseases, as well as promote neurogenesis and enhance synaptic plasticity [56]. The ability of CR to prevent cochlear pathology and ARHL has been extensively studied using laboratory animals [57]. C57BL/6 mice with CR by 15 months of age maintained normal hearing and showed no obvious cochlear degeneration and a significant reduction in the number of TUNEL-positive and cleaved caspase-3-positive cells in the spiral ganglion cells in comparison with controls [58]. Fischer 344 rats with CR to 70% of

the control intake beginning at one month of age and then housed for 24–25 months showed significantly better hearing thresholds, reduced hair cell loss, and decreased mtDNA common deletion in the auditory nerve and stria vascularis of the cochlea than control rats [48]. Beneficial effects of CR for the prevention of ARHL has been reported in the AU/Ss, CBA/J strains of mice as well as the C57BL/6 strain, but not in the DBA/2J, WB/ReJ, or BALB/cByJ strains [57]. The effects of CR may depend on genetic background. On the other hand, a high fat diet given to Sprague Dawley rats for 12 months resulted in elevated hearing thresholds in the high-frequency region, increased ROS generation, expression of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and UCP, accumulation of mtDNA common deletion, and cleaved caspase-3 and TUNEL-positive cells in the inner ear [59]. A microarray analysis study of the cochlea revealed that CR down-regulated the expression of 24 apoptotic genes, including *Bak* and *BCL2*-like 11 (*Bim*), suggesting that CR could prevent apoptosis of cochlear cells [58]. It has been reported that the mitochondrial deacetylase Sirtuin 3 (*Sirt3*) mediates reduction of oxidative damage and prevention of ARHL under CR [60]. CR failed to reduce oxidative DNA damage or prevent ARHL in C57BL/6 mice lacking *Sirt3* [60]. In response to CR, *Sirt3* directly deacetylated and activated mitochondrial isocitrate dehydrogenase 2 (*Idh2*), leading to increased NADPH levels and an increased ratio of reduced-to-oxidized glutathione in mitochondria [60]. In cultured human kidney cells (HEK293), overexpression of *Sirt3* and/or *Idh2* increased NADPH levels and gave protection from oxidative stress-induced cell death [60].

## 7. Putative Role of Oxidative Stress and Mitochondrial Dysfunction in ARHL

The important role of oxidative stress and mitochondrial dysfunction in the development of ARHL has been established by reviewing previous studies. The severity of hearing loss is probably associated with cochlear degeneration. Accumulation of mtDNA damage, ROS production, and decreased antioxidant function are primarily involved in the process of cochlear senescence in response to aging stress. Mitochondria play a crucial role in the induction of intrinsic apoptosis in cochlear cells. ARHL in laboratory animals can be prevented by certain interventions, such as CR and supplementation with antioxidants. Further large clinical studies are needed to confirm whether ARHL can be prevented by the above-mentioned interventions in humans.

## Conflict of Interests

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

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## Research Article

# Approach to Reduction of Blood Atherogenicity

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We have earlier found that blood sera of patients with coronary heart disease (CHD) increase lipid levels in cells cultured from subendothelial intima of human aorta. We have also revealed that the ability of blood sera to raise intracellular cholesterol; that is, their atherogenicity is caused by at least modified low density lipoprotein (LDL) circulating in the blood of patients and autoantibodies to modified LDL. In the present work we have demonstrated significant impact of nonlipid factor(s) to blood atherogenicity. We have developed an approach to removal of nonlipid atherogenicity factor(s) from blood serum based on the use of immobilized LDL. This approach was used for extracorporeal perfusion of patient's blood through the column with immobilized LDL. Pilot clinical study confirmed the efficacy of this approach for prevention of coronary atherosclerosis progression.

## 1. Introduction

Accumulation of lipids in the cytoplasm of arterial cells is an early and the most prominent manifestation of atherosclerosis at the cellular level. Apparently, initial deposition of intracellular lipids transported into the vessel wall by low density lipoprotein (LDL) plays an important, if not the decisive, role in the initiation of an atherosclerotic lesion [1, 2]. It is well known that lipoprotein interacting with the elements of connective tissue matrix is accumulated within the extracellular space of the arterial intima [3, 4]. However, the mechanism of intracellular fat accumulation remains to be debated.

Earlier we have found that blood serum of patients with coronary heart disease (CHD) causes significant elevation of total cholesterol content in cultured human aortic subendothelial intimal cells [5, 6]. The term “atherogenicity” is used since 1986 [5] referring to the accumulation of intracellular lipids, which is a trigger of cellular atherogenesis [6]. We describe the ability of blood serum to cause accumulation of intracellular lipids by the term “atherogenicity,” since lipid accumulation is accompanied by other atherogenic manifestations at the cellular level, namely, the increase of proliferative activity and stimulation of extracellular matrix

synthesis [7–9]. The majority of sera from healthy subjects were not atherogenic. We then attempted to delineate the reason for this atherogenicity by investigating the role of lipid and nonlipid factors of blood serum in intracellular lipid accumulation. It was shown that, at least, modified low density lipoprotein (LDL) and autoantibodies to modified LDL are responsible for blood atherogenicity.

The aim of this work was to find approaches to reducing blood atherogenicity. It is known that lowering LDL including modified LDL in the blood of patients using drug therapy or extracorporeal LDL removal causes regression of atherosclerosis [10–12]. In the present work we have shown a significant impact of nonlipid factor(s) to blood atherogenicity. Based on these investigations, we developed a procedure for removing the nonlipid factor(s) from the blood. The effect of the multiple procedures on the clinical status of CHD patients is reported.

## 2. Materials and Methods

**2.1. Patients.** Blood was drawn from the cubital vein into plastic tubes in the morning before meals from two groups of

patients. The first group consisted of 139 apparently healthy subjects (92 males and 47 females aged 20 to 57 years) free from any signs of coronary heart disease. The second group consisted of 224 patients (171 males and 53 females aged 28 to 56 years) who had CHD of functional classes II–IV. As determined by selective coronary radiography, the extent of blockage of 1 to 3 major coronary arteries was 75% or higher. Blood was taken within the first days from patients' admission to the hospital prior to the beginning of drug therapy. The blood was incubated for 1 hour at 37°C and centrifuged for 20 min at 3,000 rpm. The sera obtained were sterilized by filtration (pore size, 0.22  $\mu$ m).

**2.2. Cellular Test for Atherogenicity.** Cells were obtained from the aorta of 40- to 60-year old males and females within 1.5 to 3 hours after sudden death occurred mainly from myocardial infarction. Subendothelial cells were isolated from grossly normal intima by digestion of aortic tissue with elastase and collagenase; these cells were cultured as described elsewhere [14]. All experiments were conducted on the seventh day of cultivation. The cells were rinsed with medium 199 and cultured in medium 199 containing 40% of the serum under study, 2 mM glutamine, 100  $\mu$ g/mL kanamycin, and 2.5  $\mu$ g/mL fungizone (all GIBCO Europe, Paisley, UK) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. After 24-hour incubation with the serum, the cultures were washed vigorously with phosphate-buffered saline (PBS), and the cultured cells were suspended with trypsin-EDTA. Control cells were cultured in the medium containing 40% lipoprotein-deficient nonatherogenic serum of a healthy subject.

Lipids from the cells were extracted with a chloroform-methanol mixture (1:1, vol/vol) as described [15]. Total cholesterol content in the lipid extracts was determined colorimetrically using enzymatic Boehringer kits (Boehringer Mannheim GmbH, Mannheim, Germany).

**2.3. Lipoproteins.** Venous blood (15 mL) was taken after overnight fasting in plastic tube containing 1 mM ethylenediaminetetraacetic acid (EDTA). Plasma was separated by centrifugation (20 min at 900 g), and lipoproteins of different density classes (VLDL, LDL, and HDL), as well as lipoprotein-deficient serum (LDS), were isolated by preparative ultracentrifugation using NaBr density gradient as described earlier [15]. Lipoprotein preparations and LDS were dialyzed against 2,000 vol phosphate buffered saline (PBS), pH 7.4, containing 1 mM EDTA overnight at 4°C, sterilized by filtration (pore size, 0.45  $\mu$ m), and stored at 4°C.

Lipid and phospholipid content of lipoproteins was determined by scanning densitometry after a thin-layer chromatography on HPTLC Kieselgel-60 plates (E. Merck, Darmstadt, Germany). Neutral lipids were separated using the solvent system of n-hexane-diethyl ether-acetic acid (80:20:1, vol/vol/vol). Phospholipids were separated using the mixture of chloroform-methanol-acetic acid-water (25:15:4:2, vol/vol/vol/vol).

TABLE 1: Immunocytochemical identification of cells in primary culture of human subendothelial intimal aortic cells.

asm-1	Positively stained cells, % of total			
	3G5	2A7	CD45	CD68
89.6 $\pm$ 6.7	45.8 $\pm$ 10.9	24.1 $\pm$ 9.9	3.6 $\pm$ 0.4	5.2 $\pm$ 1.3

Values listed are means  $\pm$  SEM.

Serum levels of total and HDL cholesterol were measured on an autoanalyzer AAII (Technicon Instrument Corporation, Tarrytown, USA). Concentrations of apo B and apo A-I were measured using ELISA technique.

LDL was coupled to CNBr-activated Sepharose CL 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) by a routine procedure.

**2.4. Statistics.** Significance of differences was evaluated using SPSS 10.1.7 statistical program package (SPSS Inc., USA).

### 3. Results

**3.1. Blood Serum Atherogenicity and Lipoproteins.** As a model for investigation of cellular lipidosis, we used primary culture of subendothelial cells isolated from human aortic intima. This culture is heterogeneous and consists of cells of smooth muscle origin (typical smooth muscle cells and modified smooth muscle cells, or pericytes-like cells), and cells of hematogenous origin. Table 1 shows that cells of smooth muscle origin represent about 90% of the population. Pericyte-like cells cross-reacted with the markers of smooth muscle cells and macrophages [9]. These cells are also the major part of lipid-loaded cells [8, 9]. Leukocytes and macrophages were minor part of cultured cells representing 4–5% of cell population (Table 1). That is why we focused our studies on cells of smooth muscle origin (smooth muscle cells and pericyte-like cells) and not macrophages or lymphocytes.

For immunocytochemical identification of cultured cells following antibodies were used smooth muscle cells, asm-1 (Boehringer Mannheim GmbH, Mannheim, Germany); leukocytes, CD45 and macrophages, CD68 (Dako North America, Inc., Carpinteria, CA, USA); pericytes-3G5 (ATCC, Rockville, MD, USA) and 2A7 (Dr. M. Verbeek, Department of Pathology, University Hospital Nijmegen, The Netherlands). Incubation parameters are 80 min, +20°C.

We have carried out the measurement of cholesterol accumulation in cultured subendothelial cell of human aorta. This assay represents the estimate of serum atherogenicity. Usually the cultured subendothelial cells isolated from grossly normal intima of human aorta for 24 hours in medium 199 containing 10 to 60% of fetal calf serum, or of sera from most of the healthy subjects, did not cause any changes in the intracellular cholesterol level [5]. At the same time, in the most cases 24-hour cultivation of cells in a medium containing 20% or more of the CHD patients' serum led to a two- to four-fold increase in intracellular cholesterol, and saturation was reached at serum concentration of 20 to 40%. Prolonged cultivation of cells for 48 and 72 hours in the medium containing 40% of CHD patients' serum brought



TABLE 2: Lipid concentration of the serum of healthy subjects and CHD patients.

	<i>n</i>	Lipid levels, mg/dL				
		Total Ch	HDL Ch	LDL Ch	apo B	apo A-I
Healthy subjects	15	175 ± 6	42 ± 2	114 ± 6	80 ± 5	122 ± 9
CHD patients	38	246 ± 11*	37 ± 2	174 ± 12*	123 ± 7*	99 ± 6*

Values listed are means ± SEM.

\*Significant difference from healthy subjects,  $P < 0.05$ . Ch, cholesterol.

TABLE 3: Correlation between atherogenicity and plasma lipid levels.

Lipid	Correlation coefficient	<i>P</i>
Total cholesterol	0.10	N.S.
Triglycerides	0.15	N.S.
LDL cholesterol	0.18	N.S.
HDL cholesterol	0.01	N.S.
apo B	0.20	N.S.
apo A-I	0.03	N.S.
apo B/apo A-I	0.37	<0.05

Total number of sera, 68; atherogenic sera, 41; nonatherogenic sera, 27.

N.S., not significant.

about a further increase in intracellular cholesterol. Within 72 hours the cholesterol level in these cells exceeded by four- to sixfold the level in cells cultured in the presence of 10% fetal calf serum (control).

Blood sera from healthy subjects and from CHD patients were analyzed for atherogenicity. It was determined that 12% of sera from healthy subjects and 85% of sera from the patients were atherogenic.

In healthy subjects, the serum levels of total cholesterol and apolipoprotein (apo) B were significantly lower than in CHD patients, whereas the apo A-I level was significantly higher (Table 2). No correlation was found between the presence of atherogenic properties of sera and serum level of total cholesterol, triglycerides, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, apo B, and apo A-I (Table 3). The serum atherogenicity correlated significantly only with one of the indices examined, namely, the apo B/apo A-I ratio (Table 3).

We believe that correlation between the sera's atherogenic properties and the apo B/apo A-I ratio indicates that atherogenicity may be related to imbalances in the concentrations of LDL and HDL. On the other hand, some sera with an elevated apo B/apo A-I ratio failed to cause the accumulation of intracellular cholesterol. Thus, other factors may be responsible for serum atherogenicity.

LDL, very low density lipoproteins (VLDL), and HDL were isolated from sera of the patients. LDL was found to be the atherogenic component. LDL isolated from atherogenic serum induced a threefold increase in intracellular cholesterol of cultured cells, whereas LDL from nonatherogenic serum possessed no atherogenicity (Table 4). HDL and VLDL isolated from either atherogenic or nonatherogenic serum did not induce cholesterol accumulation; that is, they had no atherogenicity (Table 4).

TABLE 4: Effect of whole serum and lipoprotein fractions on total cholesterol content of subendothelial intimal cells cultured from human aorta.

Sera	Serum	Cholesterol content, % control			
		LDL	VLDL	HDL <sub>2</sub>	HDL <sub>3</sub>
Nonatherogenic, <i>n</i> = 4					
	109 ± 9	106 ± 5	113 ± 11	108 ± 15	114 ± 8
Atherogenic, <i>n</i> = 4					
	260 ± 18*	292 ± 31*	158 ± 13	106 ± 11	121 ± 9

Initial control value of cholesterol was  $11.8 \pm 0.9 \mu\text{g}/10^5$  cells (15 determinations).

The serum and each lipoprotein fraction were added to culture in the concentration of 40% and 250  $\mu\text{g}$  protein/mL, respectively. Values listed are mean of 12 determinations ± S.E.M.

\*Significant differences from the control.

TABLE 5: Total cholesterol content of cells cultured in the presence of lipoprotein-deficient sera (LDS) and LDL isolated from atherogenic or nonatherogenic sera.

Nonatherogenic serum		Atherogenic serum		Intracellular total cholesterol, ( $\mu\text{g}/10^5$ cells)	<i>P</i>
LDS	LDL	LDS	LDL		
+	—	—	—	13.1 ± 0.8	
+	+	—	—	14.3 ± 1.5	
—	—	+	—	14.5 ± 0.2	
—	—	—	+	40.3 ± 2.4	<0.05
+	—	—	+	35.2 ± 1.3	<0.05
—	+	+	—	28.3 ± 0.2	<0.05
Control				14.8 ± 0.7	

**3.2. Removal of Nonlipid Factor of Atherogenicity from Blood Serum.** To explore whether some nonlipid factor of the serum might produce LDL atherogenicity, LDL from atherogenic and nonatherogenic serum was isolated by ultracentrifugation. When mixed with the lipoprotein-deficient fraction of the atherogenic serum, LDL from the nonatherogenic serum became atherogenic; that is, LDL was able to induce cholesterol accumulation in the cultured cells (Table 5).

Cells were incubated for 24 hours in a medium containing 40% lipoprotein-deficient atherogenic or nonatherogenic sera and LDL at a concentration identical to that in the initial sera. Cells cultured in the presence of 10% lipoprotein-deficient fetal calf serum were used as a control.

We then postulated that the putative nonlipid factor could be removed from the serum by using a column with immobilized LDL. After passing the atherogenic serum, which had

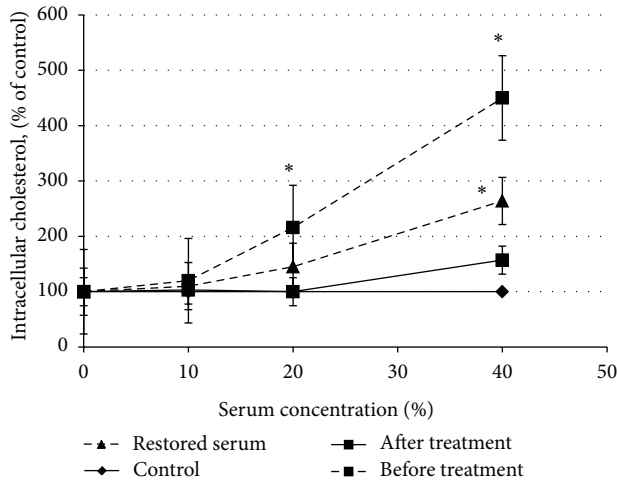


FIGURE 1: Elimination of serum atherogenicity with LDL-agarose column. Five milliliters of the serum was passed through the LDL-sepharose column at a flow rate of 1 mL/min for 30 min. The sorbent was then eluted with 2 mL glycine buffer (pH 2.7), and the eluate was dialyzed against a 2,000-fold excessive volume of medium 199 for 24 hours at 4°C. The cells were cultured in the presence of the initial or treated serum and with the proper volume of the dialyzed eluate.

previously produced nearly a five-fold increase in cholesterol content in the cultured cells, through a column with LDL covalently bound to agarose, it lost its atherogenicity. The serum that was passed through the LDL-agarose column did not induce statistically significant accumulation of cholesterol in the cells even when applied at a concentration of 40% (Figure 1). The substance absorbed on the immobilized LDL was eluted with glycine buffer (pH 2.7) and combined with the sera that were previously passed through the column, resulting in the serum's recovery of atherogenicity to almost its initial extent (Figure 1). These data suggest that serum atherogenic factor(s) may be absorbed on immobilized LDL.

**3.3. Reduction of Blood Atherogenicity in Patients.** A column with immobilized LDL was then used to remove atherogenicity from the blood of patients by extracorporeal perfusion. This procedure was applied to four patients (their clinical and angiographic characteristics are given in Table 6). All four patients were males aged 46–59 years with CHD, functional class II–III angina pectoris, and angiographically documented stenosis of 2 to 3 coronary arteries. Cholesterol level was normal in all patients. Three men were smokers, and one had mild arterial hypertension.

Extracorporeal perfusion of the plasma for 2 hours through a column with autologous LDL resulted in an abrupt decrease in atherogenicity (Figure 2(a)). The next day after the procedure, the atherogenicity disappeared completely and then it gradually reappeared, reaching a significant level within 1 week. The procedure was repeated, again resulting in an abrupt decrease in plasma atherogenicity. It is noteworthy that the second and third procedures reduced atherogenicity for a prolonged period, thus negating the need to repeat the procedure on a weekly basis. When applied once every 2

TABLE 6: Clinical and angiographic characteristics of four patients.

Characteristic	Patients			
	1	2	3	4
Sex	M	M	M	M
Age, years	59	48	47	46
Angina pectoris				
Canadian functional class	III	II	III	III
Duration of disease, months	144	12	8	8
Stenosis of coronary arteries, %				
Left anterior descending coronary artery	50	75	95	85
Circumflex artery	50	70	<50	0
Right coronary artery	85	90	>75	85
Risk factors				
Cholesterol, mg%	260	240	200	210
Smoking	–	+	+	+
Diabetes mellitus	–	–	–	–
Arterial hypertension	+	–	–	–

to 3 weeks, the procedure provides for low levels of plasma atherogenicity for long periods (Figure 2(b)).

The procedure has now been applied twice each month in one patient for 9 months and in another patient for more than 7 months. State of health, number of angina pectoris attacks, amount of medicine (nitrates) taken, and capacity for exercise have been assessed in each patient. Bicycle test, 24-hour Holter ECG monitoring, and control of hematological and biochemical parameters have been performed every 3 months. During this trial, the patients have felt better, moved from functional class III to II (according to Canadian classification), and endured higher physical loads during bicycle test (Table 7). Arterial pressure of the first patient has stabilized and reached nearly normal values. Both patients have noted heightened sexual activity and have associated this with reduced angina pectoris (Table 8).

The repeated angiograms have been assessed after 20–25 months of treatment. There were no new stenoses, 50% stenoses have progressed, 25% regressed, and 25% unchanged (Table 9). This situation is much better than the one observed in the normal course of coronary atherosclerosis [16].

## 4. Discussion

We were the first who discovered the ability of serum of atherosclerotic patients to cause accumulation of lipids in the cells of the arterial wall [5]. The term “atherogenicity” which we use to refer to this phenomenon is also used in other meanings, such as blood lipid profile characteristics or characteristics of lipoproteins [17–19]. Nevertheless, we continue to use the term “atherogenicity” because it has been shown that the accumulation of lipids in the arterial cells is a trigger of atherogenesis at the cellular level [7]. Moreover, clinical studies have shown that blood atherogenicity is associated with the presence of atherosclerosis in patients and is also associated with the dynamics of atherosclerosis [20–22]. Suppression of blood atherogenicity using the drug leads to regression of atherosclerosis [23–27].

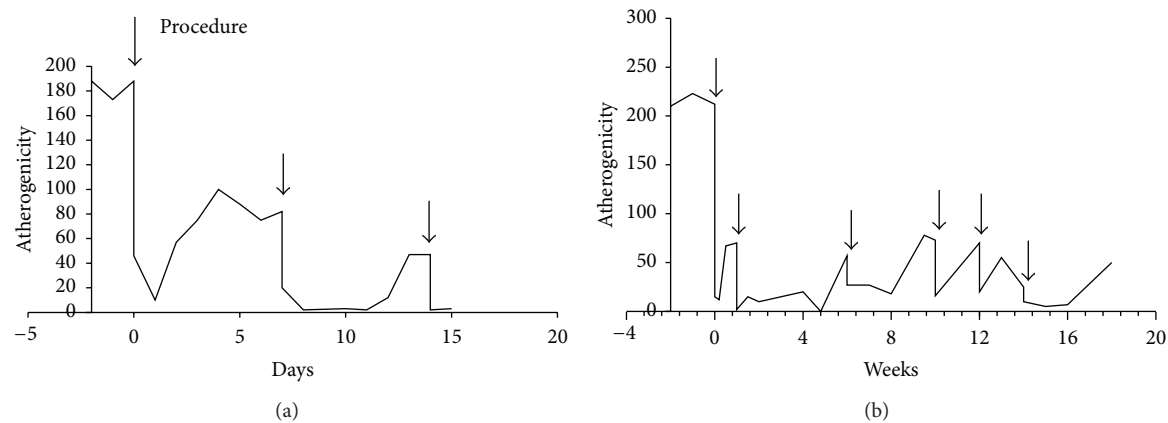


FIGURE 2: Monitoring of atherogenicity. The patient’s plasma was subjected to 2-hour extracorporeal perfusion through a column with 200 mL of the sorbent; the flow rate was 30 mL/min. The total plasma volume of 2-3 liters was perfused during the procedure. The serum atherogenicity after 3 procedures was assessed daily ((a), patient 3) and once or twice a week afterwards ((b), patient 1). Ordinate (atherogenicity), % of cholesterol accumulation in the cells cultured in the presence of the serum from the CHD patient.

TABLE 7: Effect of atherogenicity reduction on clinical status of CHD patients.

Clinical parameter	Patient 1*		Patient 3*	
	Before	After	Before	After
Age, years	59		47	
Duration of treatment, months	8		7	
Objective parameters				
Exercise bicycle test (J)	50	70–100	75–100	100
Blood pressure (mmHg)	150/90–200/110	150/90 (stable)	140/90	120/80
Subjective parameters				
Angina pectoris functional class	III	II	III	II
Attacks per week	20–35	7–14	35–50	0–3
Walking (M)	300	5,000	100–300	1,000
Sexual activity (frequency per month)	1	4–5	4	4–8

\* See Table 6.

TABLE 8: Repeated coronary angiography.

	Patient				Total
	1*	2*	3*	4*	
Total number of stenoses	5	3	4	3	15
New stenoses	0	0	0	0	0
Progression	3	1	2	1	7
Regression	1	1	0	2	4
Unchanged	1	1	2	0	4

\* See Table 6.

TABLE 9: Affinity constant of anti-LDL ( $\times 10^{-7} \text{ M}^{-1}$ ).

LDL of healthy individuals	2.4
Glycosylated LDL	2.6
Acetylated LDL	2.8
Cu <sup>2+</sup> -oxidized LDL	3.5
Patients’ LDL	11.3
MDA-LDL	10.9
Desialylated LDL	89.4

Adapted from [13].

One of factors of blood atherogenicity may be modified LDL. It is known that native LDL does not cause accumulation of intracellular lipids but chemically modified LDL is atherogenic causing accumulation of lipids in arterial cells and transforming them into foam cells [28–30]. In the blood of atherosclerotic patients 3 types of atherogenic modified LDL were found, namely, small dense [31], electronegative [32], and desialylated [33] LDLs. All types of atherogenic LDL modification are characterized by the formation of

lipoprotein self-associates [34]. It was shown that without formation of self-associates even modified LDL does not cause accumulation of intracellular lipid; that is, it is not atherogenic [34]. Therefore, lipoprotein association is an essential condition of intracellular lipid accumulation caused by modified LDL. Uptake of such large particles as LDL associates occurs bypassing the receptor-regulated pathway. This leads to an excessive intracellular lipid accumulation.

Modified LDL circulating in the blood has a high oxidizability and is an oxidized lipoprotein [35, 36]. However, oxidation is not the only modification of LDL [37]. Circulating modified LDLs multiply modified particles with disturbed physical and chemical properties compared to native LDL so that modified LDL becomes atherogenic, that is, possessing an ability to induce intracellular lipid accumulation [15, 38]. In a sequence of physical and biochemical changes occurring during atherogenic lipoprotein modifications, oxidation of particles is one of the last stages of multiple modification. Long before oxidation, lipoprotein particle acquires atherogenic properties due to modifications of lipid and protein moieties at glycoconjugates level [37]. Thus, oxidation is neither the only atherogenic modification of LDL nor a major modification.

A major argument against the oxidative modification of LDL as a cause of cellular lipidosis in the organism is the fact that oxidized LDL was not detected in the blood. On the other hand, autoantibodies against LDL modified by malondialdehyde were found in circulation [39]. LDL conjugated with malondialdehyde (MDA-LDL) is artificial formation, which cannot appear in the organism in principle. However, despite the fact that both MDA-LDL and oxidized LDL were not detected in the blood, the presence of autoantibodies against MDA-LDL is regarded as evidence that oxidized LDL exists *in vivo* [39].

Detection of autoantibodies against MDA-LDL remains the most important argument in favor of the oxidative modification of LDL *in vivo* [39]. We have also found autoantibodies against modified LDL in the blood [13, 40]. We have evaluated the affinity of the antibodies to various lipoproteins (Table 9). LDL modified by glycosylation, acetylation, and oxidation by copper ions interacted with autoantibodies with the same affinity as native LDL of healthy individuals. LDL isolated from the blood of patients with diagnosed atherosclerosis interacted with anti-LDL with an affinity of the higher order (Table 9). It has been established that anti-LDL antibodies interact with MDA-LDL with similar affinity, and the affinity of autoantibodies to MDA-LDL is higher compared to native LDL. However, autoantibodies had the highest affinity to desialylated LDL. Affinity constant of autoantibodies to desialylated LDL was much higher than to MDA-LDL and by 2 orders higher than to native LDL (Table 9).

Thus, circulating anti-LDL autoantibodies are not antibodies to oxidized LDL but rather to desialylated lipoprotein. Despite the huge amount of work on the role of oxidized LDL in atherogenesis, neither oxidized LDL nor MDA-LDL was detected in the blood. This suggested that LDL oxidation takes place not in the blood but in the vessel wall although it has been shown that circulating multiple modified LDL has some signs of oxidation but along with many changes occurring in the modified lipoprotein particle [15].

In this paper we have shown that, in addition to modified LDL, there is nonlipid factor of atherogenicity in blood plasma that can be removed from plasma by passing it through a column with immobilized LDL. We believe that one of the nonlipid atherogenic factors could be autoantibodies to modified LDL. Autoantibodies against modified

LDL were isolated from blood plasma of patients with coronary atherosclerosis by affinity chromatography on agarose covalently bound to LDL [13]. Autoantibodies were class G immunoglobulins. Antibodies interacted with the protein but not with the lipid moiety of LDL. Autoantibodies were capable of binding to the native, glycosylated, acetylated, and oxidized LDL but exhibited the greatest affinity to LDL treated with malondialdehyde, LDL of patients with coronary atherosclerosis, and desialylated lipoprotein. Interacting with native LDL autoantibodies provided its atherogenic properties, and forming complexes with multiple modified LDL significantly increased its atherogenic potential [13]. Binding to the complex formed by LDL-autoantibody complement component C1q- and fibronectin resulted in an even more pronounced accumulation of lipids in the subendothelial cells cultured from unaffected human aortic intima.

In the blood of patients with coronary atherosclerosis circulating immune complexes consisting of LDL and autoantibodies were found [41]. It has been shown that the amount of LDL-containing circulating immune complexes directly correlates with the degree of coronary atherosclerosis and atherosclerosis of other localizations [41].

We believe that anti-LDL autoantibodies as well as LDL-containing circulating immune complexes are factors of blood atherogenicity. Naturally, anti-LDL will be absorbed on immobilized LDL. On the other hand, we cannot state that anti-LDL is the only atherogenic factor adsorbed on immobilized LDL. LDL binding material should be studied in detail.

Application of column with immobilized LDL allowed not only revealing nonlipid factor of blood atherogenicity but also opened the prospect for reducing atherogenicity in patients. It should be mentioned that we did not aim to develop a new treatment for angina pectoris. While clinical results are suggestive, a controlled, blinded study with a greater number of patients is necessary in order to clarify these observations. The data obtained, however, suggest, first, that plasma atherogenicity may determine the development of coronary atherosclerosis and, second, that reducing plasma atherogenicity improves the condition of CHD patients at least in some cases.

Extracorporeal procedures are widely used in clinical practice for treatment of many diseases. The results of a successful treatment of atherosclerosis have been reported [42, 43]. We believe that these data and our own results will stimulate the search for new approaches to antiatherosclerotic therapy including the removal of atherogenic factor(s) from blood.

## 5. Conclusions

- (1) Blood sera of atherosclerotic patients capable of causing lipid accumulation in cultured arterial cells. This phenomenon we have called blood atherogenicity.
- (2) At least two factors, namely, multiply modified LDL and autoantibodies against LDL that may be responsible for blood atherogenicity.



- (3) Extracorporeal perfusion of patients' blood plasma through the column with immobilized LDL considerably reduces blood atherogenicity.
- (4) Pilot clinical study confirmed the efficacy of blood atherogenicity reduction for prevention of coronary atherosclerosis progression.

## Conflict of Interests

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

## Acknowledgment

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## Research Article

# ***Bach1* Deficiency and Accompanying Overexpression of Heme Oxygenase-1 Do Not Influence Aging or Tumorigenesis in Mice**

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Oxidative stress contributes to both aging and tumorigenesis. The transcription factor *Bach1*, a regulator of oxidative stress response, augments oxidative stress by repressing the expression of heme oxygenase-1 (HO-1) gene (*Hmox1*) and suppresses oxidative stress-induced cellular senescence by restricting the p53 transcriptional activity. Here we investigated the lifelong effects of *Bach1* deficiency on mice. *Bach1*-deficient mice showed longevity similar to wild-type mice. Although HO-1 was upregulated in the cells of *Bach1*-deficient animals, the levels of ROS in *Bach1*-deficient HSCs were comparable to those in wild-type cells. *Bach1*<sup>-/-</sup>; *p53*<sup>-/-</sup> mice succumbed to spontaneous cancers as frequently as *p53*-deficient mice. *Bach1* deficiency significantly altered transcriptome in the liver of the young mice, which surprisingly became similar to that of wild-type mice during the course of aging. The transcriptome adaptation to *Bach1* deficiency may reflect how oxidative stress response is tuned upon genetic and environmental perturbations. We concluded that *Bach1* deficiency and accompanying overexpression of HO-1 did not influence aging or p53 deficiency-driven tumorigenesis. Our results suggest that it is useful to target *Bach1* for acute injury responses without inducing any apparent deteriorative effect.

## 1. Introduction

Elimination of excessive reactive oxygen species (ROS) is pivotal to prevent malignant transformation and to maintain tissue homeostasis [1], because they modify DNA, lipids, and proteins, compromising their functions. According to the “free radical theory,” aging results from the accumulation of the cellular damage due to oxidative stress [2, 3]. Cells are equipped with many protective genes against ROS, among which heme oxygenase-1 (HO-1) is placed in the central position in that its expression is strongly induced in response to oxidative stress and is tightly associated with the progression of diseases involving oxidative stress [4–6]. The two beneficial

functions of HO-1 have been pointed out. First, it reduces the levels of free heme, which catalyzes the production of ROS by the Fenton reaction. Second, among the products of HO-1 reaction, biliverdin and carbon monoxide (CO) mitigate ROS. Since genetic ablation of HO-1 in mice causes severe anemia, disorders of iron homeostasis, and shortening of life span, HO-1 is indispensable for organismal homeostasis [7]. Reflecting its antioxidant activities, it has been shown that the overexpression of HO-1 is protective against diverse tissue damages in disease models of mice, including disorders of heart, liver, lung, and intestine [8–11]. However, overexpression of HO-1 has also been considered to result in a pathological iron deposition and mitochondrial damage in

aging-related neurodegenerative diseases [12]. Thus, whether a long-term overexpression of HO-1 would be protective or induce detrimental side effects still remains to be examined.

HO-1 is an inducible enzyme and its expression is mainly determined by the transcription level of *Hmox1* gene [13–16]. The transcription factor Bach1 forms heterodimers with small Maf oncoproteins and binds to the Maf-recognition elements (MARE) in the enhancer regions of *Hmox1* to repress its transcription [13, 17–22], whereas heterodimers composed of Nrf2 and small Maf oncoproteins bind to the same sequences to activate *Hmox1* [21, 23–25]. In *Bach1*-deficient mice, the levels of HO-1 are higher compared with control mice in many tissues, including the liver, indicating that Bach1 is a physiological repressor of *Hmox1* [18]. The protective effect of HO-1 appears to be constrained by Bach1 under several specific disease conditions, because *Bach1*-deficient mice are more resistant to tissue damage than wild-type mice in the models of lung, liver, intestine, and cardiovascular diseases [26–31]. Another function of the Bach1-HO-1 axis resides in the regulation of innate immunity [32]. Particularly, the Bach1-HO-1 axis is important for the proper function of the antigen presenting cells such as macrophages and dendritic cells [32].

Recently we have reported that Bach1 restricts the implementation of cellular senescence transcription program in mouse embryonic fibroblasts (MEFs) [33]. Cellular senescence is induced by ROS through DNA damage responses and acts as a barrier against malignant transformation of damaged cells [34, 35]. The tumor suppressor p53 induces cellular senescence in response to oxidative stress, oncogenic stress, and direct DNA damage [36]. Bach1 represses p53-mediated cellular senescence by forming a complex with p53, recruiting histone deacetylase-1 (HDAC1) and thereby repressing a subset of p53 target genes through histone deacetylation [33, 37]. Bach1 specifically inhibits oxidative stress-induced p53-dependent cellular senescence [33]. The p53-Bach1 interaction is inhibited by the tumor suppressor p19<sup>ARF</sup> [38], which is consistent with the senescence-restricting function of Bach1. Furthermore, *Bach1*-deficient MEFs are resistant to transformation by activated H-Ras oncogene, which is known to utilize increased levels of ROS for transformation [39]. Therefore, a reduction in the Bach1 activity may lead to an enhancement in the tumor-resistant phenotype. However, the roles of Bach1 in tumorigenesis are controversial. There are several reports suggesting that increased levels of HO-1 are associated with tumorigenesis [40–43]. An oncogenic microRNA, miR-155, targets Bach1 and their interaction has been discussed in the context of leukemogenesis [44].

Despite the growing number of evidences that inhibition of Bach1 may be beneficial in certain clinical situations [1, 26, 28, 29, 31, 45], the long-term, organismal response to *Bach1* ablation has not yet been examined. Considering its distinct effects on ROS homeostasis and cell proliferation, *Bach1* deficiency may affect the aging and/or life span in mice under normal conditions; ROS levels would decrease via the derepression of HO-1, and/or the p53-dependent cellular senescence would be increased by the enhanced activity of p53. The purpose of this study was to determine the lifelong effects of *Bach1* deficiency on mice. We monitored cohorts of

mice under typical laboratory conditions. We also generated *Bach1*; p53-double deficient mice to examine whether the *Bach1* deficiency and accompanying overexpression of HO-1 would affect tumor incidence in the absence of the main tumor suppressor p53. To investigate the possibility that some of the effects of *Bach1* deficiency might be compensated for, we carried out an expression profiling of the liver, a major organ of the iron/heme metabolism, during aging. The overall results indicated that Bach1 was not necessary for the normal life course of mice, including longevity and tumorigenesis under the laboratory conditions. The apparent normal phenotypes of *Bach1*-deficient mice involved a transcriptome-wide adaptation in the liver induced upon aging, which shows a novel gene regulatory mechanism compensating for the loss of Bach1. Based on our results, we discuss possible application of Bach1 inhibition for a clinical treatment.

## 2. Material and Methods

**2.1. Mice.** *Bach1*-deficient mice were previously reported [18] and back-crossed to C57B6J at least 12 times. p53-deficient mice with C57B6J background were provided by Dr. Motoya Katsuki. Mice were fed *ad libitum*.

**2.2. Genotyping.** Four-week-old mice were used for genotype. Mouse DNA was extracted as previously reported [18]. PCRs were carried out using the following primers: Bach1-F 5'-CATGTGTGTTTGCAGGTCGA-3', Bach1 mutant-F 5'-AGTAGGTGTCATTCTATTCTGGG-3', Bach1-R 5'-GTGGAAGTAGCTGCTGCACG-3', p53-F 5'-CACCTGCACAAAGCGCCTCTC-3', and p53-R 5'-GCTGTCTCCAGACTCCTCTG-3'. PCR product of p53 amplicon was digested by EcoRI to discriminate the genotypes.

**2.3. Antibodies for Flow Cytometry.** The following fluorescent dye-conjugated monoclonal antibodies were purchased from BD Bioscience (Franklin Lakes, NJ) and used for flow cytometry and cell sorting (FACSCantoII or FACSARIAII): Sca-1 (D7), c-kit (2B8), Flt-3 (A2F10), and CD150 (TC15-12F12.2). Biotinylated lineage antibodies CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), B220 (RA3-6B2), Gr-1 (RB6-8C5), NK1.1 (PK136), and Ter119 were revealed with streptavidin-PerCP or streptavidin-PerCP5.5.

**2.4. Analysis of Intercellular ROS in HSCs.** Bone marrow cells were collected by aspiration and  $5 \times 10^6$  cells were stained with PE-Flt3, APC-Sca-1, APC-Cy7-c-kit, and PerCP-lineage antibodies. The cells were incubated with 5  $\mu$ M DCF-DA (Sigma) in 10% FBS IMDM (Gibco) at 37°C for 30 min and then analyzed by FACSCantoII (BD Bioscience) (52).

**2.5. Gene Expression Profiling and Real-Time qPCR.** All equipment and reagents used for the gene expression profiling were purchased from Agilent Technologies (Santa Clara, CA). Total RNA samples were isolated, amplified, labeled with cyanine-3 dye, and hybridized with Whole Mouse Genome Array (4  $\times$  44 K) slides exactly following the manufacturer's protocol. The analyses were carried out in



triplicate. Genes expression data were analyzed using GeneSpring GX 12 (Agilent Technologies) and IPA 8 (Ingenuity Systems, Redwood City, CA) softwares. The difference in the genes expression was assessed by Volcano plots using moderated *t*-test with Benjamini-Hochberg FDR correction ( $\alpha = 0.05$ ), and 1.5-fold difference with corrected *P* value  $< 0.05$  was considered significant. For pathway analysis and GO semantic analysis, enrichment with corrected *P* value 0.0001 (Fisher's exact test, Bonferroni FDR correction) was considered significant. qPCRs were performed using a Light Cycler 2.0 instrument in the SYBR green format (Roche Diagnostics, Mannheim, Germany). Expression of  $\beta$ -actin was used as an internal control. The primer sequences for  $\beta$ -actin and *Alas1* were published previously [46, 47].

**2.6. Histological Analysis.** The sections of formalin fixed paraffin embedded tissue samples were stained with hematoxylin and eosin or Prussian blue dye. The specimens were examined by an experienced pathologist.

**2.7. Bone Marrow Transplantation.** The 8-week-old CD45.1 congenic C57/B6 mice were subjected to lethal  $\gamma$ -irradiation in two doses of 500 rads each (for a total of 1000 rads) with 3 hours blank time and were injected with 100 sorted LT-HSCs ( $\text{Lin}^-$ ,  $\text{c-Kit}^+$ ,  $\text{Sca-1}^+$ ,  $\text{Flt3}^-$ , and  $\text{CD150}^+$ ) from wild-type or *Bach1*-deficient CD45.2 mice, in competition with  $2 \times 10^5$  bone marrow mononuclear cells from CD45.1 and CD45.2 heteromice via a tail vein. Mice were treated with ampicillin (30 mg/mL) in the drinking water for 4 weeks. Peripheral blood cells (PBs) reconstitution by donor cells was monitored every 4 weeks. PBs were stained with fluorescent-conjugated antibodies specific for CD45.1, CD45.2, CD4, CD8, CD11b, Gr-1, and B220 and analyzed by a flow cytometry.

**2.8. Statistical Analysis.** Quantitative data except for the genes expression analysis were evaluated using JMP 10 (SAS Institute, Inc., Cary, NC). Log-rank test was used for analysis of survival curves. Student's *t*-test with Welch's correction was used for analysis of measurement of ROS level and bone marrow transplantation experiments. Fisher's exact test was used for comparison of death rates among *Bach1*; *p53* mutant mice. *P* values less than 0.05 were considered statistically significant in all tests.

### 3. Results and Discussion

**3.1. Life Span of *Bach1*-Deficient Mice.** As reported previously [18], *Bach1*-deficient mice were born according to Mendelian ratio with normal fertility being indistinguishable from wild-type mice. To examine the effect of *Bach1* deficiency during the life span, cohorts of *Bach1*-deficient and wild-type mice were followed up under pathogen-free condition. Life span was not affected (log-rank test,  $P = 0.93$ ) by *Bach1* deficiency (Figure 1(a)). We used body weight as a basic indicator of mice health and general condition. Logistic regression model indicated that age ( $P = 0.045$ ) and sex ( $P < 0.0001$ ) but not genotype ( $P = 0.45$ ) were significantly associated with body weight (Figure 1(b)). No difference was found

by the histological analysis of major organs such as kidney and spleen in around 100-week-old mice (Figure 1(c)). The thickness of skin from ear and villi of small intestine in aging *Bach1*-deficient mice were similar to those of wild-type mice (Figure 1(c)). Because HO-1 liberates iron from heme, we expected that *Bach1*-deficient mice would show enhanced iron deposition. However, there was no apparent change in morphology, iron deposits, and concentration of heme in the liver of aging *Bach1*-deficient mice compared with those in wild-type mice (Figure 1(d)). Our results demonstrated that *Bach1* deficiency did not affect the phenotypes of aging and life span.

#### 3.2. Effect of *Bach1* Deficiency on Hematopoietic Stem Cells.

We evaluated *Bach1*-deficient HSCs since these cells are sensitive to increased levels of ROS and become senescent under such conditions [48, 49]. HSCs isolated from *Bach1*-deficient and wild-type mice (around 10 and 100 weeks) were stained with DCFDA, which was converted to fluorescent DCF within cells by ROS including hydrogen peroxide (Figure 2(a)). To quantify levels of fluorescence, we showed the averages of median in the three independent experiments (Figure 2(b)). The levels of ROS were similar irrespective of the genotype and age of the mice, indicating that *Bach1* deficiency did not apparently influence the metabolism of ROS in the HSCs. While those in young *Bach1*-deficient mice were comparable to wild-type mice, the numbers of HSCs in aged *Bach1*-deficient mice increased (Figure 2(c)). Therefore, it is speculated that *Bach1* would affect proliferation and/or maintenance of HSCs during aging. To more directly assess the function of HSCs, transplantations of young wild-type or *Bach1*-deficient LT-HSCs to lethally irradiated wild-type mice were performed (Figure 2(b)). No obvious difference in the chimerism was observed irrespective of the genotypes. These data demonstrated that *Bach1* deficiency did not apparently affect ROS metabolism and the reconstitution activity of HSCs under the normal physiological condition.

We conclude that cellular senescence and its associated phenotype were not accelerated to a degree affecting organismal soundness in *Bach1*-deficient mice kept under the nonstressed condition. We need to examine whether *Bach1* deficiency modulates ROS metabolism when mice are challenged with oxidative stress. The results suggest that a targeting of *Bach1* in medical treatment is acceptable for acute and chronic injuries without any long-term side effect as shown in several papers [1, 26, 28, 29, 31, 45].

**3.3. Effect of *Bach1* Deficiency on Tumor Formation in the *p53*-Null Mice.** *Bach1* deficiency in fibroblasts leads to the resistance to transformation by the H-Ras<sup>V12</sup> oncogene, possibly by means of mitigating ROS accumulation induced by H-Ras<sup>V12</sup>. Also *Bach1*-deficient mice are less susceptible to 4-nitroquinoline-1-oxide- (4-NQO-) induced tongue carcinoma than wild-type mice [39]. These observations suggest that higher HO-1 levels may be causal for the tumor-suppressive phenotype of *Bach1* deficiency in this experimental model. On the other hand, it has recently been reported that an overexpression of HO-1 is associated

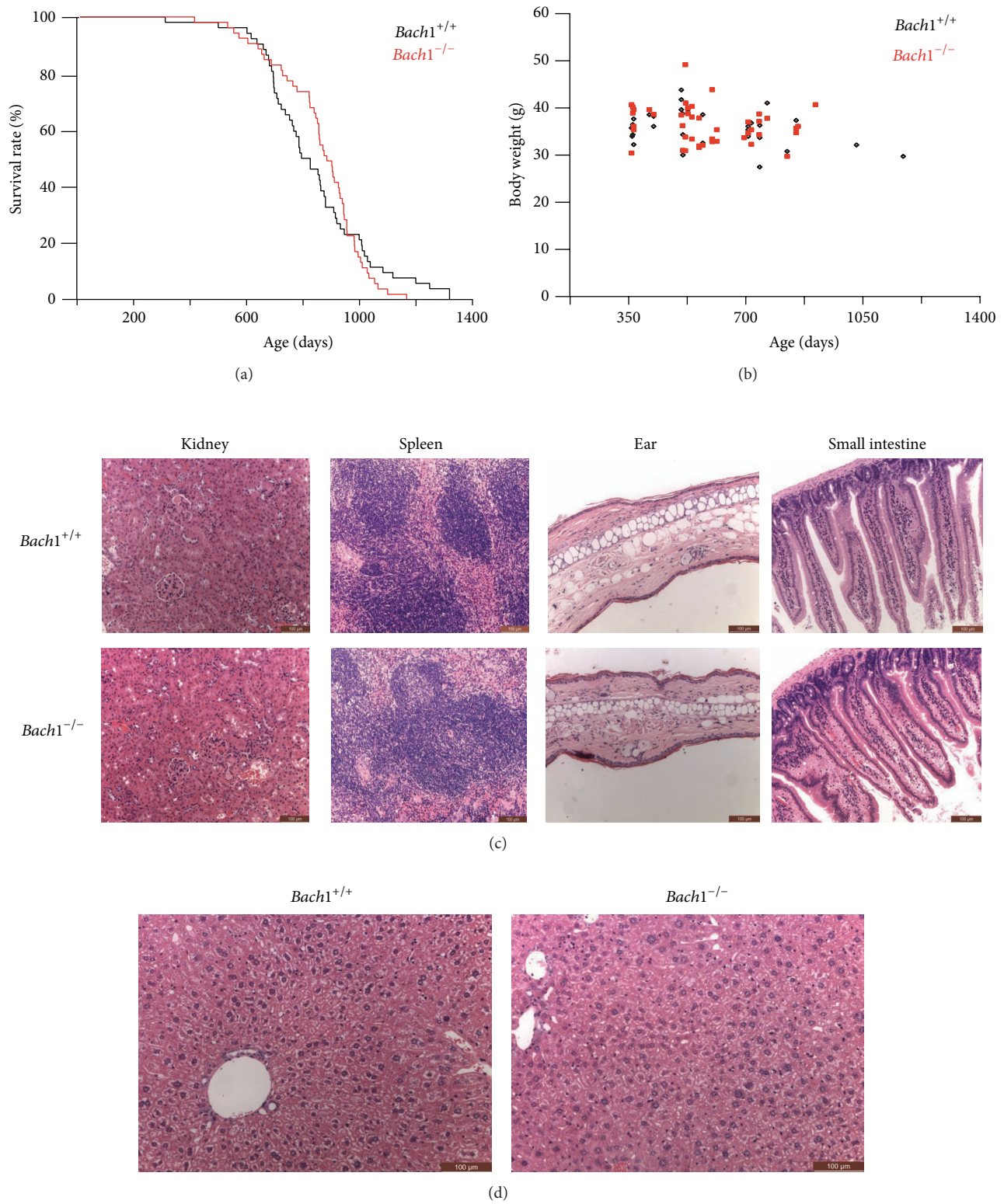
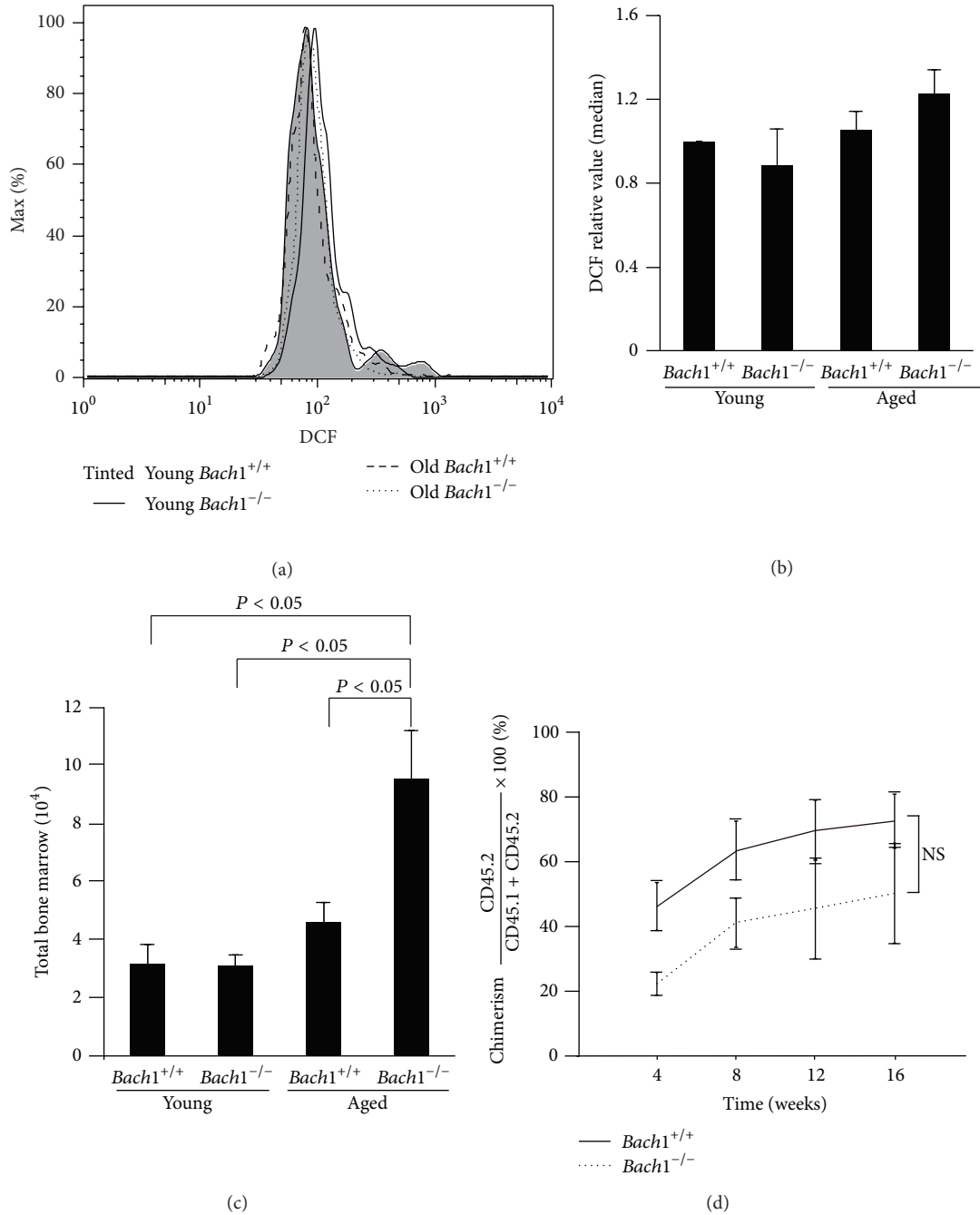


FIGURE 1: Effects of *Bach1* deficiency on life span. (a) 52 wild-type and 53 *Bach1*-deficient mice were monitored for their lives. The average life span was  $846.1 \pm 199.3$  days and  $867.5 \pm 154.0$  days, respectively. (b) The body weight of wild-type and *Bach1*-deficient mice is plotted against the age. (c) The histology of kidney, spleen, skin of ear, and small intestine in wild-type and *Bach1*-deficient mice. Representative images are shown among three mice of each genotype. Scale bars are 100  $\mu\text{m}$ . (d) The histology of liver in wild-type and *Bach1*-deficient mice. Representative images are shown among three mice of each genotype. Scale bars are 100  $\mu\text{m}$ .



**FIGURE 2: Effects of *Bach1* deficiency in HSCs.** (a) The levels of oxidized DCFDA in HSCs in young and aged, wild-type and *Bach1*-deficient mice. A representative result from three independent experiments is shown. (b) The averages of median of the experiments in the three experiments of (a). The results are expressed as mean  $\pm$  S.D ( $n = 3$ ); relative values to young wild-type HSCs in each experiment. (c) The numbers of HSCs in young and aged, wild-type and *Bach1*-deficient mice. The results are expressed as mean  $\pm$  S.D ( $n = 3$ ). \* $P < 0.05$ . (d) Transplantations of bone marrow LT-HSCs between wild-type and *Bach1*-deficient mice. The chimerism data from five wild-type and four *Bach1*-deficient mice are presented as means  $\pm$  S.E.M. The chimerism was calculated by a formula,  $CD45.2 \times 100 / (CD45.1 + CD45.2)$  (%).

with certain types of cancers [40–43, 50]. Therefore, we examined whether *Bach1* or its absence would contribute to spontaneous tumorigenesis. We generated cohorts of *Bach1*-deficient mice and *Bach1*- and *p53*-double deficient (*Bach1*<sup>-/-</sup>; *p53*<sup>-/-</sup>) mice because *p53* deficiency induces intrinsic onset of tumorigenesis [51, 52]. All of the combinatorial genotypes

were viable and indistinguishable to one another. The absence of obvious genetic interaction between *Bach1* and *p53* during embryonic development and neonatal period was not contradictory to our previous report that *Bach1* inhibited *p53* [33] and showed that their combined effects could be assessed in the tumorigenesis at adult stages.

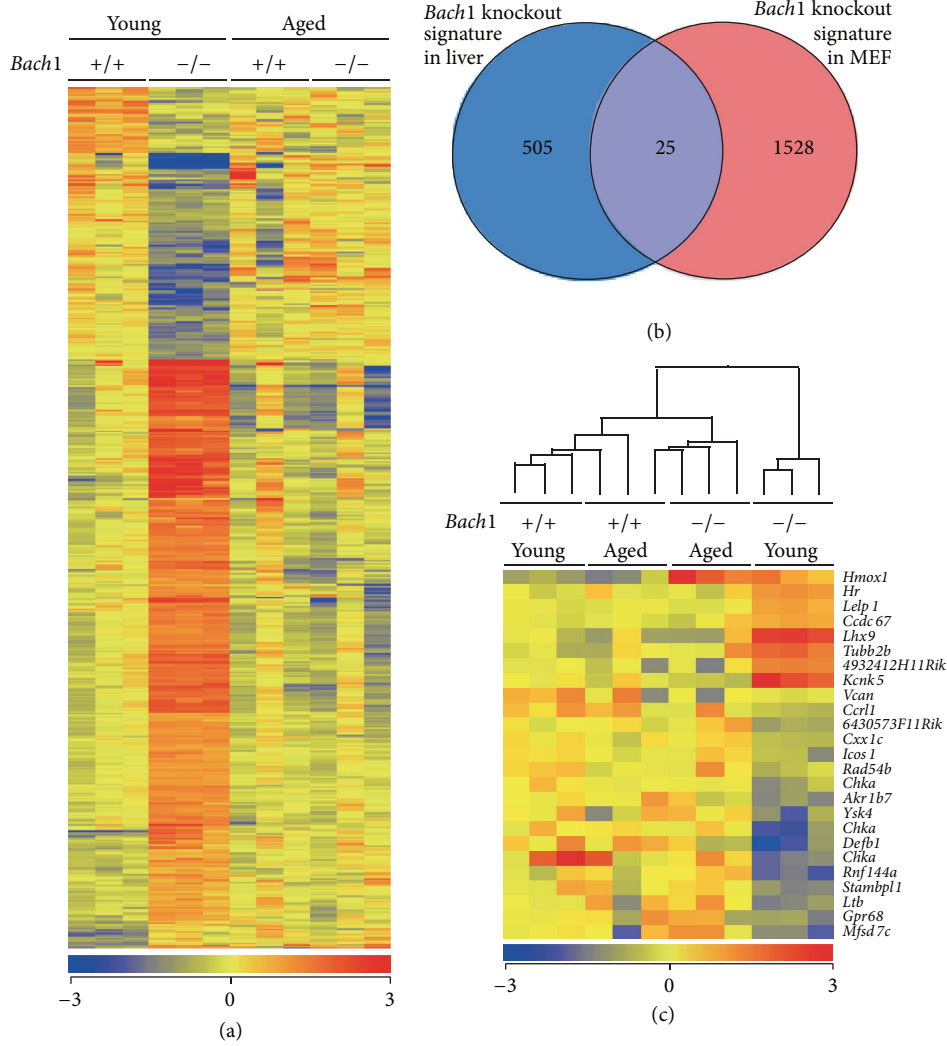


FIGURE 3: Effects of age and *Bach1* deficiency on gene expressions in mice. (a) Heat map visualizations of the 530 entities, differentially expressed in the livers of young *Bach1*-deficient compared with young wild-type mice. Three mice were used for each group. Here and elsewhere the data for heat maps are normalized and represented as median-centered log-transformed values, using average linkage clustering on entities. Red and blue correspond to high and low expression, respectively, compared with the experiment-wide median. (b) Venn diagram shows overlap between the *Bach1* knockout gene expression signature in liver and the previously published [39] *Bach1* knockout signature in MEF. (c) Heat map visualizations of the 25 entities common for both signatures. Hierarchical clustering on entities and conditions.

*p53*-deficient mice die earlier than wild-type ones due to frequent tumor formation [51, 52]. Mice of various genotypes including *Bach1*<sup>-/-</sup>; *p53*<sup>-/-</sup> were followed up for thirty weeks during which most of *p53*-deficient mice succumbed to cancers (Table 1). While all of the wild-type mice were alive by the end of observation, many of the *p53*-deficient mice died due to spontaneously developed tumors. *Bach1* deficiency did not apparently reduce the death rate in *p53*-deficient mice (Fisher's exact test;  $P = 0.6$ ).

Considering functions of *Bach1*, we need to clarify the possible involvement of *Bach1* and HO-1 in specific tumors by using of different tumor initiation models. Since cellular senescence would not occur in the absence of *p53*, *Bach1* may not affect transformation under the experimental conditions we used. Likewise, the involvement of HO-1 in cancer may

TABLE 1: Death rate before age of thirty weeks<sup>a</sup>.

Genotype	<i>p53</i>		
	+/+	+/-	-/-
<i>Bach1</i>			
+/+	0/2	0/4	7/10
+/-	0/11	0/19	6/11
-/-	0/9	1/9	2/5

<sup>a</sup>The denominator is the number of observed littermates and the numerator is the number of dead littermates born from *Bach1*<sup>+/-</sup>; *p53*<sup>+/-</sup> parents ( $n = 80$ ).

depend on genetic changes underlying the transformation process [40–43, 50]. It should be noted that HO-1 can accelerate tumor growth and affect malignancy of the tumor [53].



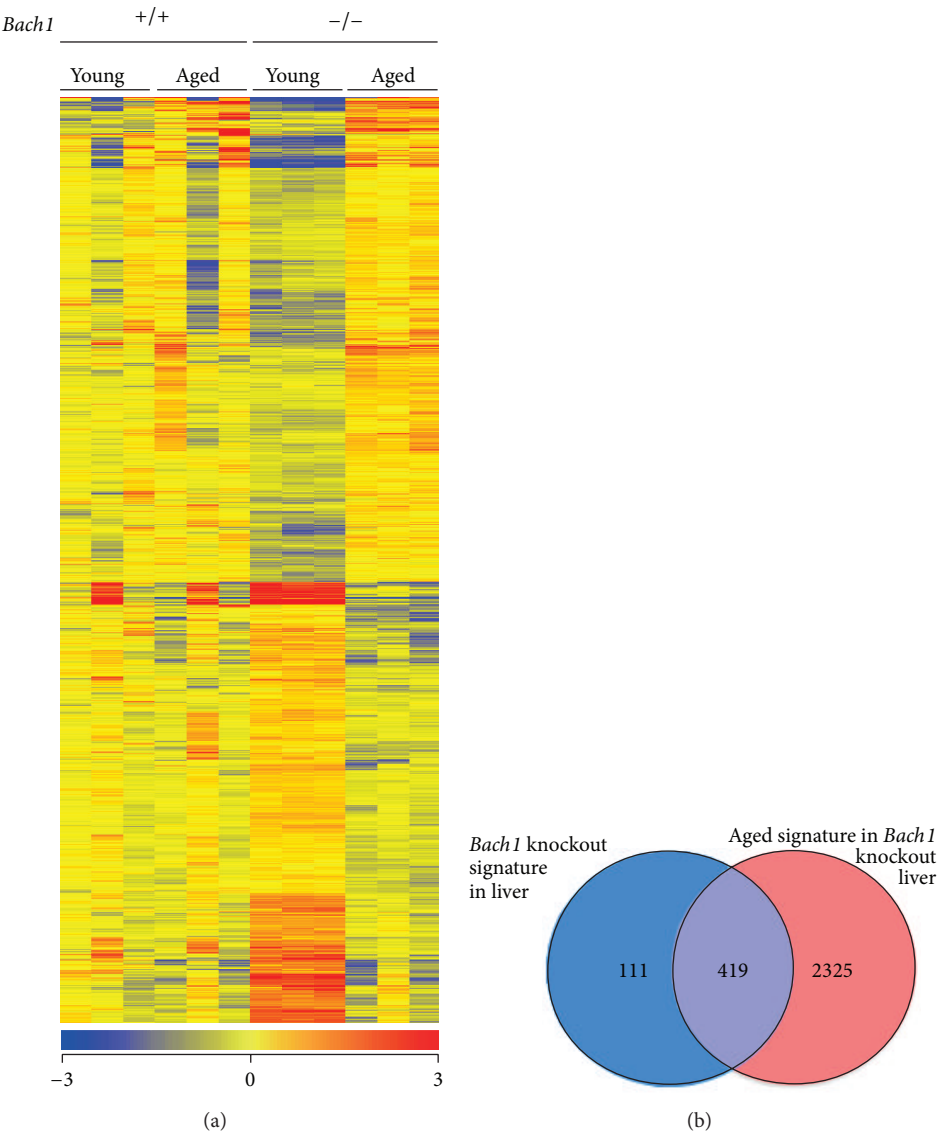


FIGURE 4: The effect of aging on genes expression profile in *Bach1*-deficient mice liver. (a) Heat map visualizations of the 2744 entities, differentially expressed in the livers of young *Bach1*-deficient mice compared with aged ones. (b) Venn diagram shows overlap between the *Bach1* knockout genes expression signatures (Figure ??) and aging signature in *Bach1*-deficient liver.

Hence, we need to assess impacts of *Bach1* deficiency in terms of tumor incidence, spread, and progression as well. Notwithstanding these remaining issues, our current observations indicate that *Bach1* does not play a critical modifying role in incidence of death due to tumor in the context of *p53*-null condition.

**3.4. Transcriptome-Wide Adaptation in *Bach1*-Deficient Mice.** One possible explanation for the above observations was that the loss of *Bach1* was compensated by other genes and/or pathways. To address this possibility, we focused on gene expression profiles as an intermediary trait. Liver samples were used for this analysis since it is one of the major organs for the metabolism of heme, which is a ligand of *Bach1* [19, 54]. We compared the gene expression profiles of the livers of

wild-type and *Bach1*-deficient mice along the course of aging. We identified a *Bach1* knockout signature comprised of 530 entities (1.7% of 31433 examined) which were differentially expressed in the livers of 8-week-old *Bach1*-deficient mice compared with wild-type mice of the same age (Figure ?? and see Supplementary Table 1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2014/757901>). Interestingly, the genes expression signature of the *Bach1*-deficient livers disappeared when examined at 100 weeks of age (Figure ??). The 530 gene sets showed overlap with previously published *Bach1* knockout signature in MEFs (25 gene entities, Figure ??) [39]. To our surprise, hierarchical clustering of the 25 entities common for both signatures demonstrated that all genes except for *Hmox1* returned to the normal levels similar to those in wild-type livers (Figure ??).

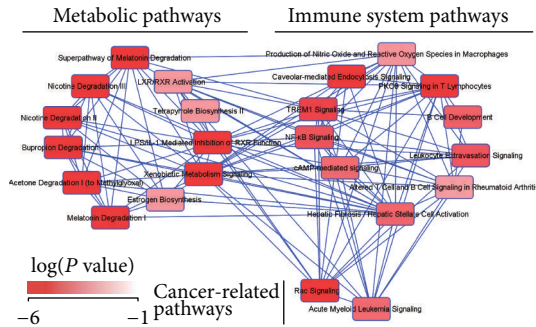


FIGURE 5: Pathway analysis of the genes which were affected by aging in the livers of *Bach1*-deficient mice. Network presentation of the canonical pathways, components of which were significantly enriched in the aging signature of *Bach1*-deficient mice liver. The intensity of red nodes color indicates the degree of significance (Fisher's exact test). The lines indicate overlap between pathways.

While the expression of *Bach1*-regulated genes is deregulated in the absence of *Bach1* in the liver, their expression can be modulated to a normal pattern upon aging.

To understand which part of the transcriptome was affected in young *Bach1*-deficient mice and resolved with age, we analyzed the aging signature in wild-type and *Bach1*-deficient animals. A relatively small number of genes are known to be affected by aging in mouse liver [55]. There was no statistically significant difference in genes expression in the livers of the aged wild-type mice compared with those of young wild-type mice. In contrast, expression of 2744 entities (8.7% of 31433 examined) was significantly altered by aging in *Bach1*-deficient livers (Figure 4(a)). Roughly half of them were upregulated with aging, whereas the other half was repressed upon aging (Figure 4(a) and Supplementary Table 2). The set of genes repressed in the aged *Bach1*-deficient livers could include direct transcriptional target of *Bach1*. Their reductions in the expression levels upon aging suggest that the activities of transcription factors responsible for their expression may recede upon aging. On the other hand, the genes which were induced upon aging in *Bach1*-deficient livers may represent indirect target genes of *Bach1* and may require specific activating signals that set off upon aging. Approximately 80% of the genes which were differentially expressed in young wild-type and *Bach1*-deficient mice and then reverted to the normal levels (Figure ??) belonged to the genes altered upon aging in *Bach1*-deficient mice (Figure 4(b)), indicating that the genes induced or reduced during aging in *Bach1*-deficient mice may compensate for the *Bach1* deficiency and may contribute to the apparent lack of any phenotypic alteration in the long term.

To understand the relationship between the transcriptome alterations in *Bach1*-deficient livers and organism functions, we performed pathway analysis (IPA software, Ingenuity Systems) on the list of entities expression of which was significantly altered by aging in *Bach1*-deficient mice (Figure 4(a) and Supplementary Table 2). We identified two clearly distinguished but interconnected modules in the gene regulation network: metabolic pathways module and

immune system signaling module, the latter of which also included two tumor-related pathways (Figure 5). Among the significantly enriched pathways, the highest enrichment scores were associated with metabolic pathways (Figure 6(a); genes are listed in Figure 6(b)). These genes showed little change in wild-type mice but showed either increases or decreases in *Bach1*-deficient mice upon aging (Figure 6(b)). Semantic analysis of these genes and their functions revealed significant enrichment of the genes responsible for catalytic activity of oxidoreductases including cytochrome P450 monooxygenase system (Figure 6(c)). A significant portion of oxidoreductases genes were downregulated in the liver of young *Bach1*-deficient mice compared with those of young wild-type mice, and this alteration disappeared upon aging (Figure 6(d)). Many of the oxidoreductases genes encode hemoproteins. Remarkably, we found that expression of *Alas1*, the rate-limiting enzyme in the heme synthetic pathway, was upregulated in the liver tissue of the young *Bach1*-deficient mice (Figure 6(e)). The heme biosynthesis pathway may be activated in *Bach1*-deficient mice when they are young, compensating for the higher expression of HO-1 and therefore enhanced degradation of heme. It has been reported previously that the enzymatic activity of *Alas1* decreases upon aging in several tissues including the liver in rats [56]. While this particular paper assessed the enzymatic activity of *Alas* using aging rats, their results suggest that the expression of *Alas1* mRNA would decrease upon aging [56]. While we did not observe such an alteration in the wild-type mice, *Alas1* mRNA indeed decreased upon aging in *Bach1*-deficient mice. Genes involved in aging may affect the expression of *Alas1* and such a response may have been easily observed in *Bach1*-deficient liver cells due to the higher expression of *Alas1* mRNA in the young *Bach1*-deficient mice compared with corresponding wild-type mice. The reduction of *Alas1* mRNA may indirectly facilitate the transcriptome adaptation in *Bach1*-deficient mice by altering the heme synthesis.

The observed transcriptome-wide adaptation in the gene regulatory network upon aging may be one possible mechanism to suppress a long-term effect of *Bach1* deficiency and HO-1 overexpression. Any side effect of HO-1 overexpression in the absence of *Bach1* could be avoided by the transcriptome adaptation. Considering that *Bach1* is primarily a repressor of gene expression, the activities of activating transcription factors such as Nrf2 may affect the expression levels of downstream genes in the absence of *Bach1*. Upon aging, such activators may be inhibited, resulting in a stable and harmless transcriptome. This hypothesis posits that ROS levels would decrease in the liver upon aging. However, we did not see any significant change in the levels of ROS in HSCs upon aging. Since the metabolism of ROS may be differentially regulated upon aging depending on tissues, we need to compare ROS levels in the liver as well.

## 4. Conclusions

Here we showed that *Bach1* did not affect phenotypes of aging or life span of mice. At the transcriptome level, *Bach1* deficiency itself affected expression of many genes, including

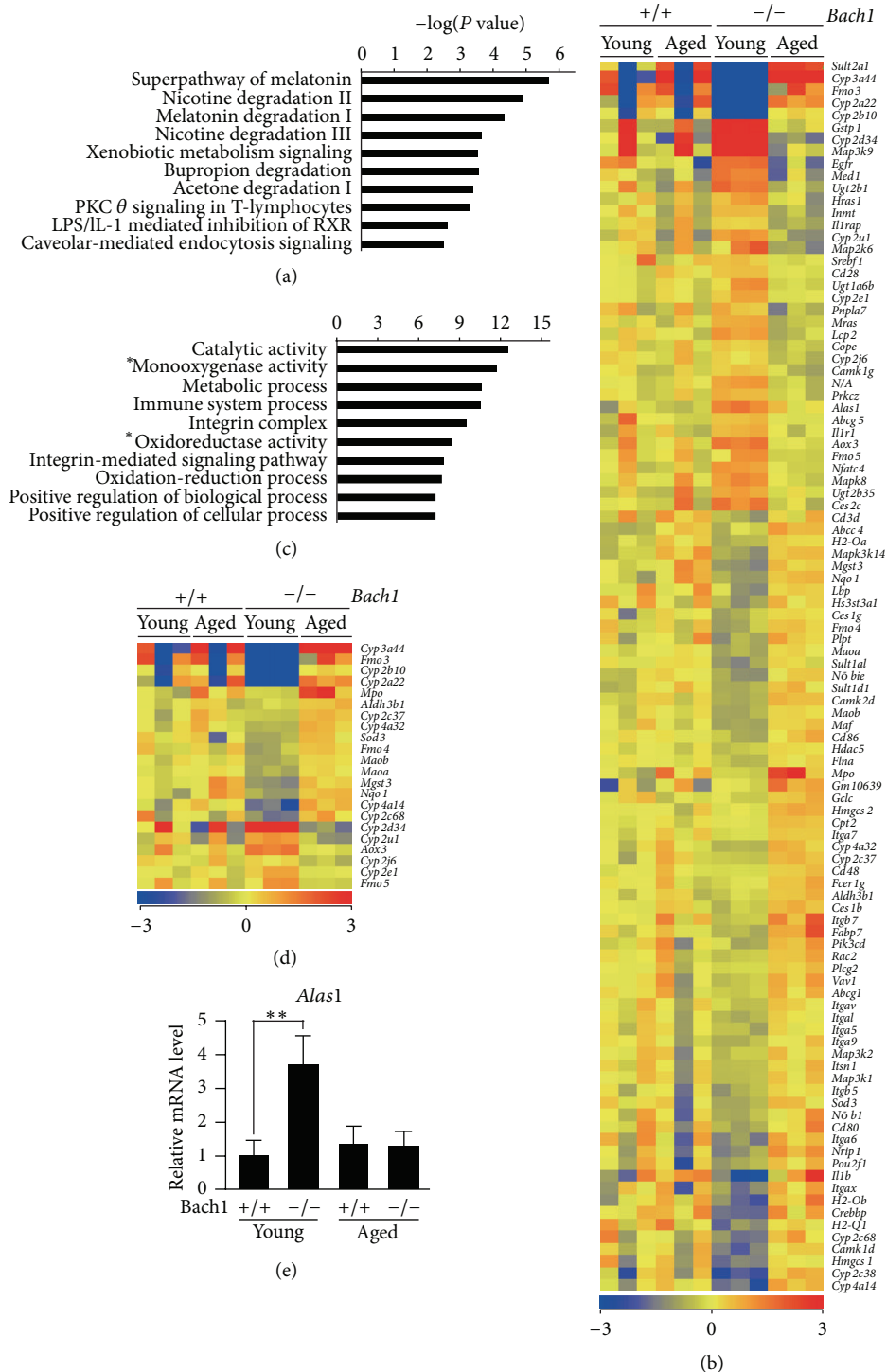


FIGURE 6: Semantic analysis of the genes which were affected by aging in the livers of *Bach1*-deficient mice. (a) Ten most significantly enriched canonical pathways are listed in significance order (Fisher's exact test). (b) Heat map visualization of the genes from ten most significantly enriched canonical pathways. (c) GO analysis of the genes from ten most significantly enriched canonical pathways. The GO terms are listed in enrichment significance order (Fisher's exact test). (d) Heat map visualizations of the genes corresponding to the GO terms \*monooxygenase activity and \*oxidoreductase activity in the panel (c). (e) mRNA levels of *Alas1* normalized with *beta-actin*. The results are expressed as mean  $\pm$  S.D ( $n = 3$ ). \*\*  $P < 0.05$ .

*Hmox1*, cytochrome P450 monooxygenase system, and *Alas1*. However, the difference in the expression of all these genes except for *Hmox1* disappeared when the mice aged. Our findings suggest that long-term targeting of *Bach1*, retaining high expression levels of *Hmox1* during the life span, is safe and free from adverse effects owing to compensation at the level of transcriptome. However, although we did not observe any detrimental effect of *Bach1* deficiency and the long-term overexpression of HO-1 under the normal conditions, it remains possible that *Bach1* becomes critical under specific conditions. This is a rational inference considering its evolutionary conservation among vertebrates [22].

## Abbreviations

4-NQO:	4-Nitroquinoline-1-oxide
Bach1:	BTB and CNC homology 1
CO:	Carbon monoxide
DCFDA:	Dichlorodihydrofluorescein diacetate
HDAC1:	Histone deacetylase 1
HSCs:	Hematopoietic stem cells
HO-1:	Heme oxygenase-1
MARE:	Maf-recognition element
MEFs:	Mouse embryonic fibroblasts
PBs:	Peripheral blood cells
ROS:	Reactive oxygen species.

## Conflict of Interests

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

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## Review Article

# Protein Redox Modification as a Cellular Defense Mechanism against Tissue Ischemic Injury

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Protein oxidative or redox modifications induced by reactive oxygen species (ROS) or reactive nitrogen species (RNS) not only can impair protein function, but also can regulate and expand protein function under a variety of stressful conditions. Protein oxidative modifications can generally be classified into two categories: irreversible oxidation and reversible oxidation. While irreversible oxidation usually leads to protein aggregation and degradation, reversible oxidation that usually occurs on protein cysteine residues can often serve as an “on and off” switch that regulates protein function and redox signaling pathways upon stress challenges. In the context of ischemic tolerance, including preconditioning and postconditioning, increasing evidence has indicated that reversible cysteine redox modifications such as S-sulfonation, S-nitrosylation, S-glutathionylation, and disulfide bond formation can serve as a cellular defense mechanism against tissue ischemic injury. In this review, I highlight evidence of cysteine redox modifications as protective measures in ischemic injury, demonstrating that protein redox modifications can serve as a therapeutic target for attenuating tissue ischemic injury. Prospectively, more oxidatively modified proteins will need to be identified that can play protective roles in tissue ischemic injury, in particular, when the oxidative modifications of such identified proteins can be enhanced by pharmacological agents or drugs that are available or to be developed.

## 1. Introduction

Increasing evidence continues to support the concept that reactive oxygen species (ROS) and reactive nitrogen species (RNS) can exert great beneficial effects on cellular adaptation to stress challenges and cell survival [1–5]. This is particularly true in the context of ischemic tolerance that includes preconditioning and postconditioning; both of which are used to prepare tissues to tolerate injuries against lethal ischemic occurrence by triggering endogenous adaptive and defensive responses [5–13]. Evidence supporting the involvement of ROS and RNS in ischemic tolerance comes directly from the observations that administration of antioxidants before or during the induction of ischemic tolerance can abolish the protective effects of either preconditioning or postconditioning [14–20]. As one of the means that ROS/RNS work is via modifying proteins, protein redox modifications can thus execute the beneficial effects of ROS/RNS [21–26]. In this review, I will summarize evidence that protein redox

modifications, in particular, reversible modifications on protein cysteine residues when induced by preconditioning or postconditioning, can serve as a cellular defense mechanism against tissue ischemic injury. Evidence presented indicates that protein redox modifications can serve as therapeutic targets in tissue ischemic injury.

## 2. Protein Redox Modifications

Under stress conditions, cells can produce an elevated level of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [27, 28], which, in turn, can oxidize or modify proteins [29–32]. As shown in Figure 1, protein oxidation can be classified into two general categories. One is irreversible and the other is reversible. Irreversible oxidation usually leads to protein aggregation and degradation. This type of oxidation includes formation of protein carbonyls [33], nitrotyrosine [34], and sulfonic acids [35]. On the other hand, reversible

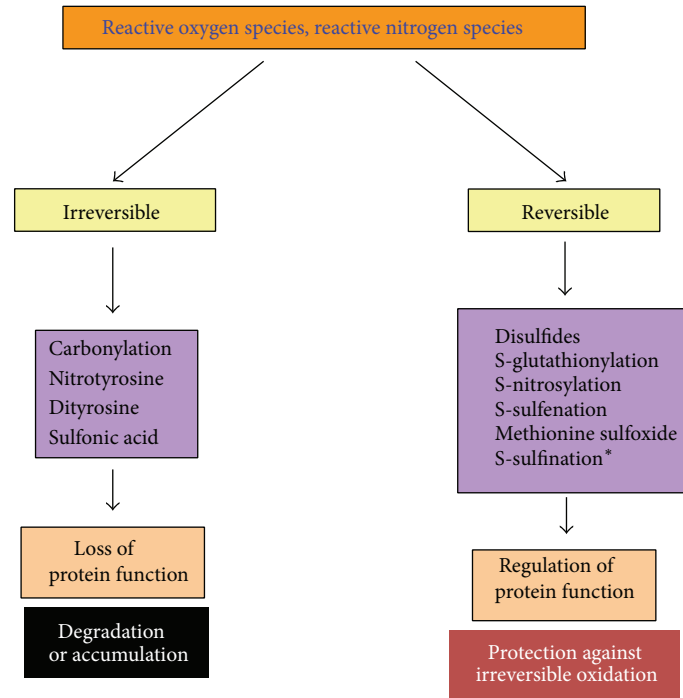


FIGURE 1: Classification of protein oxidative modifications into two categories: irreversible oxidation and reversible oxidation. \* Note: only a few studies so far have reported that sulfinic acid (S-sulfinitation) formation could be reversible [180, 181].

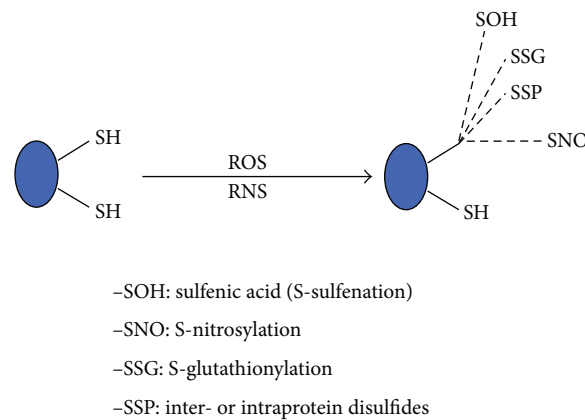


FIGURE 2: Reversible cysteine modification products that are widely studied. These products include S-sulfenation (sulfenic acid, -SOH), S-nitrosylation (-SNO), S-glutathionylation (-P-S-S-G), and either intra- or interdisulfides.

protein oxidation is usually involved in redox signaling pathways and regulation of protein structure and function [36–38]. This type of oxidation often occurs on protein cysteine residues leading to formation of S-sulfenation, S-nitrosylation, disulfides, and S-glutathionylation [35, 39, 40] (Figure 2). Additionally, formation of methionine sulfoxide, involving methionine sulfoxide reductase [41–44], is also a reversible process and has been shown to be involved in protection against ischemic injury [45–47]. It should be pointed out that, strictly speaking, disulfide formation (P-S-S-P) and S-glutathionylation (P-S-S-G) are not oxidative modifications as the end products do not contain an oxygen atom like those found in S-nitrosylation (-SNO)

and S-sulfenation (-SOH). Nonetheless, formation of both disulfides and glutathionylation requires the presence of reactive oxygen species such as hydrogen peroxide [48–56]. Therefore, it would be more appropriate to name these two modifications as redox modifications.

### 3. Production of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

While all endogenous RNS originate from nitric oxide synthases, ROS can be produced by many cellular systems. Among which, mitochondria remain as a major cellular site



for ROS production [28, 57–59]. It has been established that mitochondrial complexes I and III are the major two sites for mitochondrial ROS production [57, 58]. Other enzyme systems in mitochondria that can generate ROS include complex II [60],  $\alpha$ -keto acid dehydrogenase complexes that contain dihydrolipoamide dehydrogenase [61–65]. Outside mitochondria, NADPH oxidase [66, 67], xanthine oxidase [68, 69], and cytochrome P-450 enzymes [70] can also generate ROS. It has been recently reported that the  $\alpha$ -keto acid dehydrogenase complexes can generate more ROS than complex I [71]. The initial species is always a superoxide anion, which can be dismutated to  $H_2O_2$  [72–74]. The latter can decay to form hydroxyl radical in the presence of metal ions [75]. Additionally, when superoxide meets nitric oxide, peroxynitrite is formed [76, 77]. Both hydroxyl radical and peroxynitrite are known to be highly reactive towards proteins [78, 79].

#### 4. Balance and Imbalance between Oxidants and Antioxidants

Production of ROS and RNS is a well-controlled process under normal conditions [80, 81]. This is because cells have a variety of antioxidant defense systems. These include but are not limited to superoxide dismutase, catalase, glutathione peroxidase, thioredoxin, and peroxiredoxin [27]. Moreover, there are also small antioxidant molecules such as vitamin C, vitamin E, glutathione, and coenzyme Q [27]. Under normal physiological conditions, a balance between ROS production and antioxidant defense is well maintained [53, 82]. However, under stress or pathophysiological conditions, more ROS and RNS can be produced that can overwhelm the cellular antioxidant defense system, leading to severe oxidative stress and oxidative damage [80, 83]. On the other hand, intentionally induced oxidative stress can serve as a defense mechanism against further oxidative challenges [80, 83–87]. This is known as hormetic effect [88–90] or “positive oxidative stress” and are often explored as a protective approach in ischemic tissue injuries [26], a phenomenon often termed as ischemic tolerance that includes both preconditioning and postconditioning [7, 17, 91].

#### 5. Ischemic Tolerance: Preconditioning and Postconditioning

Preconditioning is a prophylactic approach, which often involves noninjurious stimulation of the tissues that are of interest [7, 17, 91]. Such stimulation can prepare the tissues to resist further challenges that are lethal [7, 17, 91]. Induction of preconditioning can be achieved by many ways, including short episodes of ischemia reperfusion [92], treatment with chemicals or drugs that are often inhibitors of mitochondrial electron transporter complexes [26, 93], hyperoxia [94, 95], and hypoxia [96], as well as remote preconditioning [97]. Remote preconditioning means that the tissues that receive preconditioning can defend other tissues against ischemic injuries. Therefore, the target to be preconditioned and the target to be protected are not the

same in the settings of remote preconditioning. As opposed to that of preconditioning, postconditioning is the interruption or intervention at the onset of reperfusion after an ischemia has occurred [98–102]. Therefore, postconditioning may be more clinically relevant as ischemic occurrence is generally not a predictable event. Nonetheless, preconditioning is still intensively studied because investigating how tissues respond to preconditioning may identify endogenous therapeutic targets for treatment of ischemic injury [103, 104]. Moreover, both preconditioning and postconditioning have been shown to involve similar signaling pathways or trigger similar defense mechanisms [100, 105–107].

#### 6. Reversible Protein Cysteine Modifications and Ischemic Tolerance

In the context of ischemic tolerance including preconditioning and postconditioning, cysteine redox modifications have been explored extensively. This is because cysteine oxidation is closely associated with cellular redox potential reflected by the ratio between GSH/GSSG and  $NADH/NAD^+$  [108, 109]. Moreover, cysteine residues can undergo reversible modifications that are involved in an “on and off” switch during stress conditions [36, 110–112]. Therefore, reversible cysteine modifications are often involved in regulating redox signaling pathways and protein function [37, 113, 114]. Accordingly, I will cover only reversible cysteine modifications and their protective roles in ischemic injury in this review. These include S-sulfenation, S-nitrosylation, S-glutathionylation, and disulfide formation. But, before discussing each of the four modifications, I would like to briefly introduce a general method for analysis of reversible cysteine modifications as the method has contributed significantly in the paradigms to be presented below in this review.

#### 7. General Detection Method for Reversible Cysteine Modifications

As cysteine oxidation does not involve a change in optical density of the modified proteins, a probe is always needed for the detection of cysteine modifications [115]. In fact the approaches are quite similar for S-sulfenation, S-nitrosylation, and S-glutathionylation. Figure 3 shows one of the general procedures for detection of cysteine oxidation products. This widely used method is often called biotin switch assay [116]. The steps involve blocking unmodified cysteine residues with alkylating reagents such as N-ethylmaleimide (NEM), reducing the modified cysteine residues using a specific reducing reagent for each modified species [38, 117]. For example, ascorbic acid is used for the reduction of S-nitrosylation [118], arsenite is used for the reduction of S-sulfenation [119], and glutaredoxin is used for the reduction of S-glutathionylation [120, 121]. This is followed by relabeling of the reduced cysteine residues using biotin conjugated with an alkylating reagent such as NEM. This approach not only facilitates gel-based detection as biotin can be readily recognized by streptavidin, but also can be conducive to affinity purification of the modified

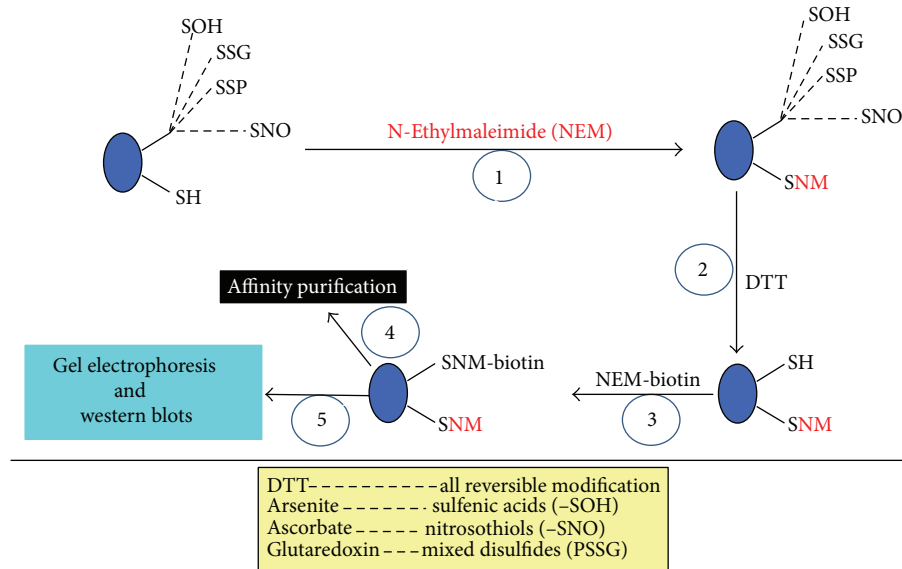


FIGURE 3: Analysis of reversible cysteine redox modifications. What is shown is one of the popular methods generally called a “biotin switch” assay, which involves alkylating the free thiol groups (step 1), reducing the modified cysteine residues using specific reductant for each oxidation product (step 2), and relabeling of the newly generated free thiol groups using biotin conjugated probes (step 3). Following biotinylation, the samples can be further analyzed by either western blot (step 5) or affinity purification (step 4). Note that protein sulfenic acids can be directly labeled by dimedone conjugated biotin probes as described in the text.

proteins. Additionally, NEM-biotin labeling can also pinpoint the site of modifications when used in conjunction with mass spectrometric peptide sequencing. It should be noted that, for the detection of protein sulfenic acids, biotin conjugated dimedone probes have been developed that only reacts with  $-SOH$  [122, 123]; therefore no blocking and reducing steps are needed. For the detection and quantification of S-glutathionylation, the enzyme glutaredoxin is needed in the presence of GSH. DTT and 2-mercaptoethanol are nonspecific reducing reagents; hence they are not good for a specific modifying species.

## 8. Paradigms of Reversible Protein Cysteine Modifications as a Defense Mechanism in Ischemic Injury

**8.1. S-Sulfenation ( $-SOH$ ).** S-Sulfenation or protein sulfenic acid ( $-SOH$ ) is now attracting increasing attention because this cysteine redox modification product can now be readily trapped and quantified [115, 123]. Moreover, although once considered a transient product of cysteine oxidation adduct, stable  $-SOH$  has been found to exist that plays an “on and off” switch in regulating protein function and redox signaling [40]. An elegant model of protein sulfenic acid formation in protecting ischemic tissue injury is the enzyme aldose reductase that has been studied thoroughly by Dr. Bhathagar’s group at University of Louisville. This group initially found that AR could be activated by ischemic reperfusion in the heart, and this activation was due to the formation of a sulfenic acid on cysteine residue 298 [124]. Furthermore, this sulfenation process of cysteine 298 was found to be achieved by peroxynitrite [125], a highly reactive species formed

between superoxide anion and nitric oxide [126]. The group next found that this activation of AR via cysteine sulfenic acid formation was regulated by the PI3K/AKT/eNOS signaling pathway [125]. As this pathway is known to be involved in protection against ischemic injury [127, 128], AR activation by sulfenic acid formation on cysteine 298 thus is suggested to be involved in cardioprotection against cardiac injury, which is further supported by the observation that AR inhibitors such as sorbitol or tolrestat, when applied before ischemia or at the onset of reperfusion, hindered postischemic recovery in the heart [125]. Interestingly, as this seems to be the end of the story, this laboratory went further and demonstrated that formation of AR- $-SOH$  on cysteine 298 during cardiac ischemia reperfusion could be reversed back to AR-SH [129], which involved two enzymes, glutathione S-transferase converting AR- $-SOH$  to AR-SSG, and glutaredoxin converting AR-SSG to AR-SH [129]. Therefore, both enzymes may be involved in regulation of AR- $-SOH$  reduction when tissue oxygen and nutrient supply is resumed after an ischemic incident.

**8.2. S-Nitrosylation ( $-SNO$ ).** Protein cysteine nitrosylation (P-SNO), another form of reversible modification, has been studied by numerous investigators. The role of this modification has been thought to be equivalent to that of protein phosphorylation [130, 131]. It not only has detrimental effects on protein function and cell survival [132, 133], but also can exert beneficial effects under a variety of pathophysiological conditions [134, 135]. In the context of tissue ischemic injury, it has been found that overall protein  $-SNO$ , in connection with the activation of the PI3K/AKT signaling pathway, increases after postconditioning in the heart [136], indicating that nitrosylation of individual proteins play a protective role

in ischemic injury. This is indeed the case as presented in the following two examples.

**8.2.1. S-Nitrosylation of TRIM72 at Cysteine-144 Is Cardioprotective.** Tripartite motif-containing protein 72 (TRIM72) is a membrane repair protein that can undergo posttranslational modifications leading to its either activation or degradation. Using the biotin switch assay shown in Figure 3, Kohr et al. reported that TRIM72 exhibited an elevated level of SNO at cysteine-144 upon ischemic preconditioning [137]. As ischemic preconditioning is an established approach for cardioprotection against ischemic injury [138], the authors hypothesized that increase in TRIM72's cysteine-144 nitrosylation protects against cardiac ischemic injury. The authors tested the hypothesis by mutating C144 to a serine residue (C144S) in a tissue culture system using HEK-293 cells that lack TRIM72. This mutation would abolish the protein's S-nitrosylation at C144, hence changing the protein's property and function. Indeed, the authors found that after the mutation, protein levels of TRIM72 (wildtype) but not TRIM72-C144S (mutant) were decreased upon  $H_2O_2$  treatment, and this decrease correlated with enhanced  $H_2O_2$ -induced cell death in the wild type cells. Moreover, treatments of the cells with an S-nitrosylating agent S-nitrosoglutathione (GSNO) [139] could maintain TRIM72's protein level and reduce cell death. The authors further demonstrated that GSNO induced TRIM72 nitrosylation stopped ischemia reperfusion triggered decrease in TRIM72 levels and decreased infarct size in heart ischemia reperfusion. Thus, cys144-SNO of TRIM72 prevents degradation of TRIM72 upon ischemic challenge and thus preserves its membrane repair capacity.

**8.2.2. S-Nitrosylation of Mitochondrial Complex I ND3 Subunit Participates in Cardioprotection against Ischemic Injury.** Complex I is the electron entry point in the mitochondrial electron transport chain. It has at least 45 subunits in the mammalian systems and many of them are redox sensitive [140–142]. Dysfunction of complex I is thought to be a causal factor in the pathogenesis of many mitochondrial diseases including ischemic injury [143–145]. Recently, Chouchani et al. reported that S-nitrosylation of the complex I subunit ND3 is involved in cardioprotection against ischemic insult [146]. The authors reported that S-nitrosylation of ND3-cysteine-39 inhibited complex I activity and slowed mitochondrial recovery at the initial minutes of reperfusion, hence attenuating ROS generation upon sudden oxygen resupply, leading to less oxidative damage and tissue necrosis. Interestingly, ND3 only became accessible to nitrosylation after an ischemic insult as mitoSNO, a membrane permeable nitrosylating agent, could only provide the protective effect at the onset of reperfusion via ND3 cysteine-39 nitrosylation. As mitoSNO was applied during reperfusion and its protective effect could only be observed when administered at the onset of reperfusion, this study provides an elegant postconditioning paradigm whereby S-nitrosylation could serve as one mechanism contributing to postconditioning-induced ischemic tolerance.

**8.3. S-Glutathionylation.** Well-defined roles of protein S-glutathionylation in ischemic tolerance have not been clearly reported in the literature. Nonetheless, there are direct link that protein S-glutathionylation induced by preconditioning prevents cell death and enhances cell survival. The results of two studies will be summarized here. The first one is S-glutathionylation of mitochondrial adenine nucleotide translocase (ANT) induced by carbon monoxide preconditioning [147]; and the second one is S-glutathionylation of ryanodine receptor 2 induced by tachycardia preconditioning via elevation of NADPH oxidase activity. In the first study, Queiroga et al. reported that carbon monoxide prevents mitochondrial permeability transition pore opening and cell death via S-glutathionylation of ANT [147]. In particular, using nonsynaptic mitochondria isolated from rat brain and primary astrocytes prepared from the cortex of neonatal rats, the authors found that carbon monoxide could partially inhibit loss of mitochondrial membrane potential, the opening of mitochondrial membrane permeability transition pore, mitochondrial swelling, and cytochrome c release. To understand the underlying mechanisms, the authors further found that carbon monoxide could modulate ANT activity as ADP/ATP exchange rate was enhanced. As ANT is part of the mitochondrial membrane permeability transition pore [148], this enhancement of ANT activity thus also prevented pore opening. Moreover, it was further found that the modulation of ANT activity was due to ANT glutathionylation caused by carbon monoxide-induced ROS production. It should be noted that the site of glutathionylation on the ANT molecule was not identified in this study.

In the second study, Sánchez et al. reported that while electrically induced tachycardia can effectively create myocardial preconditioning, the mechanisms remain elusive [149]. Therefore, the authors set out to elucidate the underlying mechanisms. Focusing on sarcoplasmic reticulum (SR) isolated from dog cardiac ventricular muscle, they found that preconditioning tachycardia increased NADPH oxidase activity by nearly 200% as measured by NADPH dependent superoxide production. This increase in enzymatic activity was due to the enhanced association of rac1 with the NADPH oxidase cytosolic subunit p47 (phox) to the microsomal fraction without altering the content of the enzyme's membrane subunit gp91 (phox). As an elevated level of superoxide can induce protein S-glutathionylation, the author further found that cardiac ryanodine receptor 2 (RyR2) was S-glutathionylated under their experimental conditions. Conversely, when catalase, superoxide dismutase, and NADPH oxidase inhibitors were added in the experimental system, RyR2 S-glutathionylation was greatly attenuated, indicating a potential link between RyR2 glutathionylation and tachycardia preconditioning. Interestingly, this same laboratory further reported that exercise could also produce a preconditioning effect by increasing NADPH oxidase activity and RyR2 S-glutathionylation [150]. Similar to the ANT studies presented above, the site of modification on RyR2 was also not pinpointed in this study.

**8.4. Disulfides.** Numerous studies have demonstrated that oxidative stress-induced disulfide formation can be beneficial



to cell survival [151–155]. An excellent study by Fourquet et al. [156] presented a well-delineated role of protein disulfide formation in activation of the Nrf2 signaling pathway that regulates the expression of the second phase defensive enzymes such as hemoxygenase-1 (HO-1) and NAD(P)H dehydrogenase quinone-1 (NQO-1) [157]. Using HeLa cells treated with  $H_2O_2$ , nitric oxide, and hypochlorite, the authors found that Keap1, a protein that controls the fate of Nrf2, can form intramolecular disulfides, leading to release and nuclear translocation of Nrf2. The authors further found that cysteine-151 of Keap1 was involved in disulfide bond formation between two molecules of Keap1, forming a Keap1 homodimer. This formation of Keap1 homodimer is important for Nrf2 release from the Keap1-Nrf2 complex as mutation of cysteine-151 led to an unstabilized form of Nrf2. Additionally, the authors also found that, when the thioredoxin and glutathione pathways were inactivated, Keap1 intramolecular disulfide bond formation was constitutive, leading to a stable Nrf2 molecule in the cell. Therefore, this study further demonstrates that Keap1 cysteine-151 disulfide bond formation is at least one of the mechanism by which cells utilize to resist ischemic injury by upregulating the second phase antioxidative proteins [158–166], which include thioredoxin reductase, glutamate-cysteine ligase (GCL), glutathione S-transferase, HO-1, and NQO-1, [157, 167–171].

## 9. Summary and Perspective

Protein redox modification is a double-edged sword. While there is no doubt that protein redox modifications can have detrimental effects on cell survival [172–179], there is also increasing evidence, as summarized in this review, that redox modification of certain proteins, when induced purposely by approaches that trigger positive oxidative stress [26], can play a protective role in tissue ischemic injury. Studying how proteins respond to oxidative modifications in the settings of preconditioning and postconditioning, may identify novel proteins as potential therapeutic targets for treatment of ischemia-related diseases, in particular, when such modifications can be enhanced by pharmacological agents.

## Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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