Oxidative Medicine and Cellular Longevity

New Therapeutic Approaches Against Inflammation and Oxidative Stress in Neurodegeneration

Lead Guest Editor: Fabrizio Biundo
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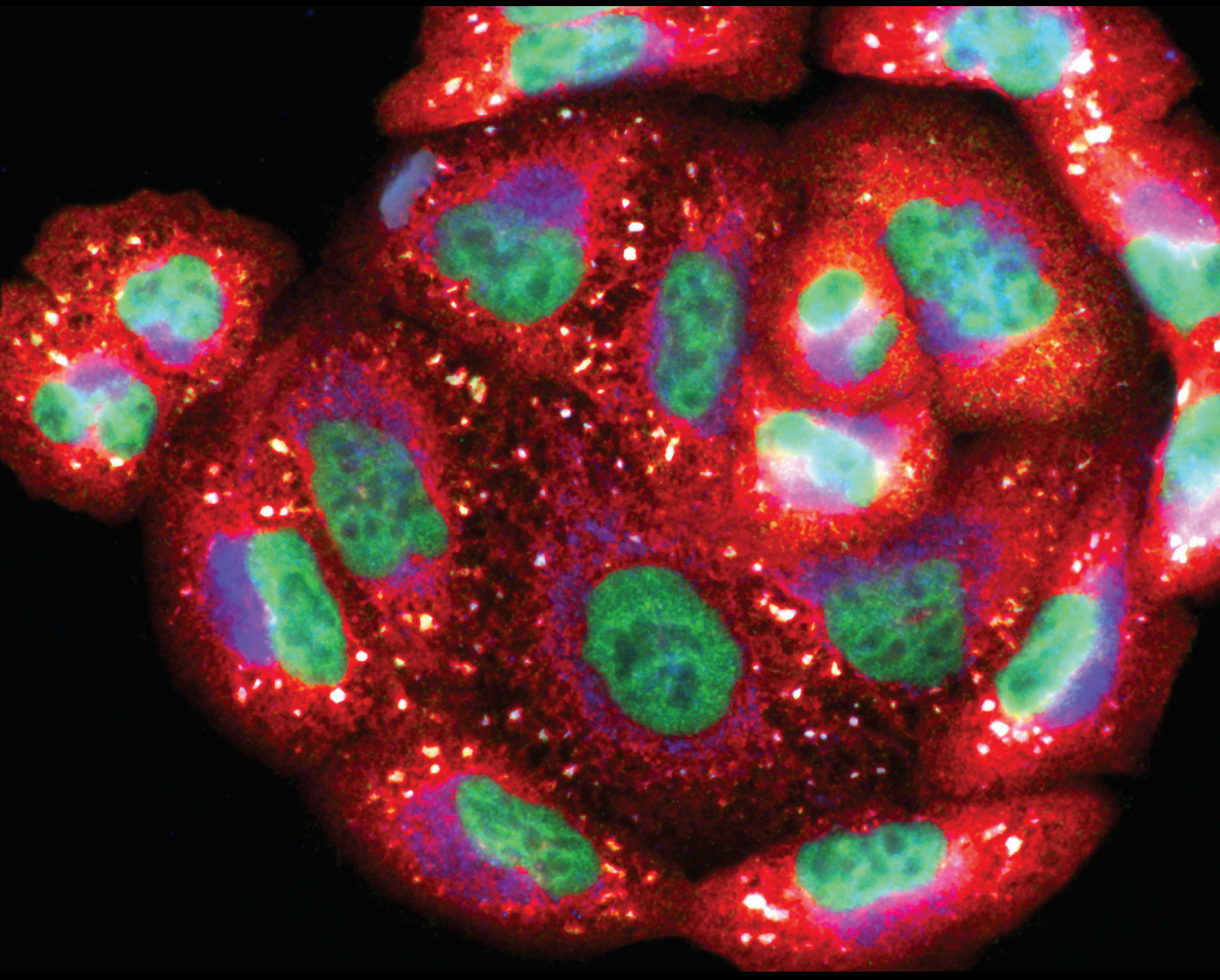

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Editorial

New Therapeutic Approaches against Inflammation and Oxidative Stress in Neurodegeneration

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Due to the increase of life expectancy, the world aging population has been increasing significantly and is expected to triple by 2050. Aging is a physiological change characterized by a progressive decline of biological functions and of the organism's ability to adapt to metabolic stress, and it is considered one of the main factors for neurodegenerative diseases [1]. Among these, Alzheimer's disease (AD) is the most common neurodegenerative pathology characterized by memory decline and dementia [2]. Markers of oxidative stress have been observed in brains of patients affected by amnestic Mild Cognitive Impairment (aMCI), and of late AD patients [3, 4]. Accumulation of oxidized molecules is caused by an imbalance between the production of reactive oxygen/nitrogen species (ROS/RNS) and the antioxidative cell systems, and has been indicated as one of the molecular events that characterize the pathogenesis of multiple neurodegenerative diseases [5]. In addition to oxidative stress, dysregulated chronic neuroinflammation has been described to be a driving force of decade-long neurodegenerative processes [6]. A large body of studies in the last decades detected an association between these two pathogenic events in brains of patients affected by neurodegeneration and in mouse models of various neurodegenerative diseases [7–9]. Hence, limiting these two intertwined pathologic factors may have greater efficacy for the treatment of neurodegenerative diseases [10].

This special issue collected a set of 7 multidisciplinary studies addressing the function of molecular mechanisms underlying the production of reactive oxidation and inflammation in different *in vitro* and *in vivo* models, and the potential therapeutic role of natural antioxidants.

The study conducted by Kent et al. focused on the role of the mitochondrial permeability transition pore (mPTP) which is a protein involved in ROS expansion [11]. mPTP opening is also associated with chronic inflammation and can be controlled by nicotinamide adenine dinucleotide (NAD+), an antioxidant agent declining with age [12]. The authors covered a large part of literature describing the role of mPTP in neurodegenerative diseases, particularly on Parkinson's disease (PD) and Alzheimer's disease (AD). Both AD and PD are associated with an increased oxidative damage of DNA, both of which are linked to mPTP opening and consequent ROS release [13]. Targeted therapies aiming at reducing the frequency and duration of mPTP opening may therefore be a promising path for the development of specific drugs against age-related declines of the central nervous system.

Lin and colleagues dissected the pathway leading to carbon monoxide releasing molecule-3 (CORM-3)-induced upregulation of heme oxygenases-1 (HO-1), a key enzyme that plays an important role in maintaining cellular homeostasis, in rat brain astrocytes (RBA-1) [14]. They observed that CORM-3-induced HO-1 expression was mediated through ROS generation by NADPH oxidase (Nox), and a mitochondria/ROS-dependent PI3K/Akt/mTOR cascade triggering FoxO1. The authors concluded that in RBA-1 cells, CORM-3-induced HO-1 expression is, at least partially, mediated

through Nox, and their findings strengthened the previous observations indicating HO-1 potentiation as a potential therapeutic target [15].

In the experimental study conducted by Moon et al., calcineurin, a calcium-related protein phosphatase of type 2B expressed in the brain, was found to act as a critical checkpoint in the prion-dependent control of different cellular functions [16]. PrPsc can accumulate in the brain in pathological conditions and induce mitochondrial dysfunctions and reactive oxygen species (ROS) generation in neurons [17]. The authors observed that the human prion peptide increases mitochondrial ROS by activating calcineurin, and that calcineurin inhibition prevented the mitochondrial dysfunction and neuronal apoptosis induced by PrPsc. These results suggest that calcineurin plays a role in PrPsc-induced ROS release and neuronal apoptosis, and indicate it as another potential pharmacological target.

The systematic review of literature conducted by Adeyemi et al. focused on implications and therapeutic prospects of hypoxia and the kynurenine pathway in Alzheimer's disease (AD) [18]. Although the underlying molecular events or mechanisms connecting hypoxia to neurodegeneration are not well-understood, hypoxia-inducible factor 1-alpha (HIF-1α) is a master regulator of the cellular/tissue response to hypoxia and seems to be correlated with the pathogenesis of different neurodegenerative diseases, including AD [19]. The authors focused on HIF-1α role and hypoxia in the progression of AD underlining in a simple and schematic approach HIF-1α and kynurenine pathway and the possible connection between these two signaling cascades. This work indicated that hypoxia is related to oxidative stress and inflammation, which in turn affect tryptophane catabolism through indoleamine 2,3-dioxigenase (IDO) enzyme, resulting in neurotoxic metabolites that contribute to neurodegeneration. Despite the significant achievements in this field, the authors suggest that further *in vitro* and *in vivo* experiments are necessary to fully understand hypoxia and IDO roles in the kynurenine pathway in order to identify novel therapeutic targets.

The study conducted by Alvi et al. in pentylenetetrazole (PTZ)-kindled epileptic rats focused on carveol, a natural compound that possesses robust antioxidant, anti-inflammatory, and protective properties in various degenerative models [20]. It has been reported that oxidative stress can exacerbate epilepsy and the degree of oxidative damage is proportional to epileptic episodes [21]. Hence, the aim of this work was to investigate the effective dose of carveol, its mechanism of action in regulating Nrf2, and ultimately its neuroprotective effects. The authors reported that PTZ-kindled animals experienced oxidative stress and revealed diminished levels of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and glutathione (GSH) associated with elevated lipid peroxidation (LPO) and inflammatory cytokines level such as tumor necrosis factor-alpha (TNF-α), and mediators like cyclooxygenase (COX-2), and nuclear factor kappa B (NFκB). Carveol was demonstrated to increase these antioxidants and reduced LPO levels together with a positive modulation of the antioxidant gene Nrf2 and its downstream target HO-1. Moreover, Nrf2 pathway activation inhibited proinflammatory cytokine release and downregulated the p-NF-κB pathway, highlighting the anti-inflammatory potential of carveol. The strong involvement of Nrf2 pathway in the cytoprotective nature of carveol was confirmed by all-trans retinoic acid (ATRA) treatment, which abolished carveol effects and exacerbated PTZ toxicity. Furthermore, different studies have suggested that local immune response and inflammation are associated with the upregulation of hippocampal acetylcholinesterase (AChE) levels, resulting in cholinergic imbalance and epileptogenesis. In this context, the authors also reported that increased brain AChE level was significantly inhibited by carveol treatment, indicating a modulating effect of carveol on cholinergic transmission that is further linked to attenuated neuroinflammatory cascade. Additionally, carveol treatment was also found to ameliorate VEGF expression, indicating an improvement in PTZ-mediated angiogenesis. Altogether, these findings suggest that carveol, acting as a Nrf2 activator, attenuates seizure severity and neuroinflammation in PTZ-kindled epileptic rats.

Extensive research over the last few years has demonstrated the potential neuroprotective role of phytochemicals and their beneficial effects on the prevention of neurodegenerative diseases including Parkinson's disease. Epidemiological studies have shown that a diet based on the consumption of beverages such as tea, coffee, fruit, and vegetables is associated with a reduced risk of neurological damage or pathologically related diseases [22].

Balakrishnan et al. performed a comprehensive evaluation of various phytochemicals present in foods such as chrysin, vanillin, ferulic acid, thymoquinone, ellagic acid, caffeic acid, epigallocatechin-3-gallate, theaflavin, and other plant-derived antioxidant phytochemicals highlighting their beneficial and neuroprotective effects in different experimental models. In light of this, dietary antioxidant and anti-inflammatory phytochemicals or extracts from waste products of the food industry could be a potential new therapeutic strategy against the symptoms or progression of neurodegenerative diseases [23].

In line with this topic, Angeloni et al. provided an experimental study detecting the phenolic profile and the antioxidant and anti-inflammatory activity of spent coffee ground (SCG) extracts in cellular models of neuroinflammation. The main SCG components—caffeine, 5-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, and 3,5-O-dicaffeoylquinic acid—proved to be efficient in counteracting oxidative stress and neuroinflammation *in vitro* by upregulating endogenous antioxidant enzymes such as thioredoxin reductase, heme oxygenase 1, NADPH quinone oxidoreductase, and glutathione reductase. Based on these findings, SCG extracts could represent a valuable source of potential neuroprotective bioactive molecules for the treatment of neurodegeneration [24].

Conflicts of Interest

The editors of this special issue declare that there is no conflict of interest with any of the authors that submitted their manuscripts.

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Erica Buoso
Fabrizio Biundo
Alessandro Attanzio

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Review Article

Hypoxia and the Kynurenine Pathway: Implications and Therapeutic Prospects in Alzheimer's Disease

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Neurodegenerative diseases (NDs) like Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, and Huntington's disease predominantly pose a significant socioeconomic burden. Characterized by progressive neural dysfunction coupled with motor or intellectual impairment, the pathogenesis of ND may result from contributions of certain environmental and molecular factors. One such condition is hypoxia, characterized by reduced organ/tissue exposure to oxygen. Reduced oxygen supply often occurs during the pathogenesis of ND and the aging process. Despite the well-established relationship between these two conditions (i.e., hypoxia and ND), the underlying molecular events or mechanisms connecting hypoxia to ND remain ill-defined. However, the relatedness may stem from the protective or deleterious effects of the transcription factor, hypoxia-inducible factor 1-alpha (HIF-1α). The upregulation of HIF-1α occurs in the pathogenesis of most NDs. The dual function of HIF-1α in acting as a "killer factor" or a "protective factor" depends on the prevailing local cellular condition. The kynurenine pathway is a metabolic pathway involved in the oxidative breakdown of tryptophan. It is essential in neurotransmission and immune function and, like hypoxia, associated with ND. Thus, a good understanding of factors, including hypoxia (i.e., the biochemical implication of HIF-1α) and kynurenine pathway activation in NDs, focusing on Alzheimer's disease could prove beneficial to new therapeutic approaches for this disease, thus the aim of this review.

1. Introduction

Neurodegeneration is the gradual loss of neuronal function coupled with the partial or total loss of cerebral and body coordination. Diseases associated with this disorder include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) [1]. In most cases, the underlying mechanisms behind these neurodegenerative diseases (NDs) remain elusive. Several morphological and pathological studies suggest that NDs may arise due to mitochondrial dysfunction, genetic predisposition, and environmental factors [2, 3].

There is a consensus that hypoxia (characterized by diminished tissue or cellular oxygen) correlates positively

with the development of NDs [4]. However, the direct relationship between hypoxia and ND is not well understood. Evidence suggests that the presence of the transcription factor subunit, hypoxia-inducible factor 1-alpha (HIF-1α), could be the link between hypoxia and ND [5]. HIF-1α, a master regulator of the cellular/tissue response to hypoxia, plays dual roles by acting as a "killer factor" and "protective transcription factor" depending on the severity of the disease(s) causing the hypoxic condition [6]. Besides hypoxia, hyperactivity of the kynurenine pathway correlates positively with ND pathogenesis [2, 7–9]. The kynurenine pathway (KP) is a metabolic pathway involved in converting tryptophan to kynurenine. Apart from kynurenine synthesis, the KP also functions in immune and neurotransmission functions [10]. The KP's rate-limiting step consists of the indoleamine 2,3-dioxygenase (IDO) enzymatic catalysis of tryptophan breakdown to N-formylkynurenine (NFK). The further conversion of NFK to neurotoxic metabolites (like 3-hydroxykynurenine and quinolinic acid (QA)) aids ND development.

To date, there is no definitive cure for NDs despite the enormous efforts made by researchers in this field. Thus, this review discusses the factors that influence NDs, with a specific focus on AD, and highlights the roles of HIF-1α and the kynurenine pathway as potential therapeutic targets toward the discovery of novel treatments.

2. Factors Influencing Neurodegeneration

Several factors that trigger neurodegeneration, such as genetic risk, aging, and environmental factors, may ultimately lead to neuronal death (Figure 1). Additionally, systemic inflammation can result in microglial activation linked to chronic neurodegeneration [11]. For instance, high proinflammatory immunoregulatory proteins are observed in the cerebrospinal fluid of most patients with ND [12]. Also, the imbalance in reactive oxygen species (ROS) produced results in oxidative stress and dysfunction in axoplasmic transport and, eventually, neuronal cell death [13]. Furthermore, compromised redox homeostasis resulting from hypoxia and kynurenine pathway activation [14, 15] plays a role in neurodegeneration [16, 17]. Discussed below are the major factors or conditions contributing to neurodegeneration.

2.1. Hereditary and Genetics. Nearly 70% of ND cases are related to genetic factors with the involvement of many specific genes, for example, in AD, amyloid precursor protein (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2) genes. The mutations in any of these three genes may cause an early familial onset of AD. Also, mutations in more than 20 genes (PRKN, UCHL1, PARK7, LRRK2, PINK1, POLG, HTRA2, SYNJ1, DNAJC13, TMEM230, VPS13C, LRP10, ATP13A2, FBXO7, GIGYF2, GBA, PLA2G6, EIF4G1, VPS35, and DNAJC6) are associated with this disease, most of which are highly penetrant and often cause early-onset or atypical symptoms [18]. Gene mutation is also known to directly or indirectly affect oxidative stress via modulation of other influencing factors such as the impairment in mitochondrial function, protein misfolding, and microglial activation [19].

2.2. Mitochondrial Dysfunction. Mitochondrial dysfunction occurs in most neurodegenerative diseases. Several essential genes, including PARK7 (encoding DJ-1), α-synuclein, parkin, PINK1, or LRRK2, have pathogenic mutations in PD, which cause defects in mitochondrial dynamics and function. Meanwhile, PINK1 deletion results in increased oxidative stress within mitochondria [18]. AD defining the appearance of amyloid-β (Aβ) aggregates and tau pathologies correlates with mitochondrial dysfunction in neurons. Elevated Ca^{2+} and ROS levels during mitochondrial dysfunction contribute to the accumulation of tau protein aggregates [20].

2.3. Oxidative Stress. Although there is a strong association of oxidative stress resulting from highly reactive oxygen species (ROS) with neuronal death [21], it is difficult to establish if oxidative stress is solely responsible for neuronal death in neurodegenerative disorders. The cascade of unstable reactions involving ROS includes DNA oxidation, lipid peroxidation, and protein oxidation. Furthermore, these reactions lead to electron loss in the DNA and protein structures and ultimately damage the mitochondrial protein and DNA. This damage may create a pathway to neurodegeneration [22]. Oxidative stress, characterized by the overproduction of reactive oxygen species, induces mitochondrial DNA mutations, damages the mitochondrial respiratory chain, alters membrane permeability, and influences Ca^{2+} homeostasis and mitochondrial defense systems. ROS generated via exogenous and endogenous sources are superoxide ions, hydrogen peroxide, hydroxyl ions, and singlet oxygen [23]. Environmental toxicants such as pesticides are exogenous sources of ROS, while endogenous sources result from the endoplasmic reticulum and mitochondrial enzymes. A balanced cellular ROS level plays a vital role in regulating cellular signaling necessary for cell survival. Therefore, an imbalance in ROS homeostasis results in protein misfolding and DNA damage. Hence, imbalanced redox homeostasis amplifies neuronal dysfunction and triggers neurodegeneration, leading to the development of these neurodegenerative diseases [23].

2.4. Heavy Metals. Heavy metals (e.g., mercury and lead) may play a role in the pathogenesis of AD. These metals extend Aβ deposition and tau protein phosphorylation that characterizes AD [24]. Manganese and toxic solvents are also associated with PD features, including the accumulation of α-synuclein and impaired mitochondrial function, although metals are crucial in biological reactions as cofactors; however, dysregulation in homeostasis leads to ROS generation. For example, the increased cellular iron concentration may lead to an elevated oxidative stress state. The neurotoxin, 6-hydroxydopamine (6-OHDA), exemplifies this phenomenon. 6-Hydroxydopamine (6-OHDA) releases iron from ferritin, which leads to increased lipid peroxidation. Deferoxamine (DFO), an iron chelator, inhibits this reaction [25] and also upregulates HIF-1α [26]. HIF-1α is a crucial regulator of hypoxia; overexpression of HIF-1α is an essential factor to show aggressive phenotypes under hypoxic conditions [27].

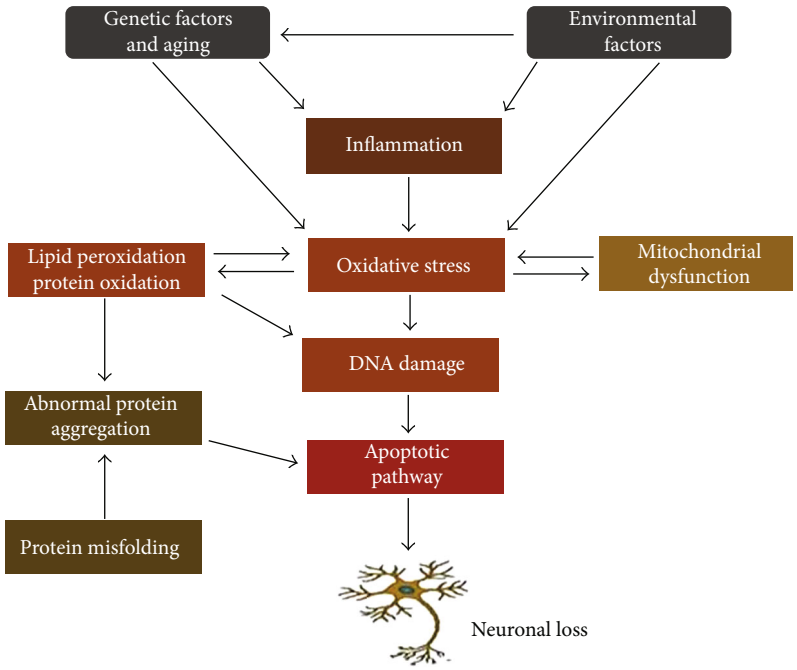

Figure 1: A schematic depiction of factors and cellular events involved in neurodegeneration. Factors such as aging, genetics, and environmental factors trigger neuronal loss via inflammation, oxidative stress, mitochondrial dysfunction, and abnormal protein aggregation.

2.5. Protein Misfolding and Aggregation. A hallmark of NDs is the accumulation of misfolded or aggregated proteins [28]. Protein functionality rests on the 2D amino acid sequence's proper folding to an energy-favorable 3-dimensional structural conformation of that protein [29]. In other words, protein misfolding arising from external factors, including aging and oxidative stress, results in the formation of protein aggregates and, ultimately, protein dysfunction [30].

2.6. Inflammation. Neuroinflammation triggers the onset of several neurodegenerative disorders. Several damage signals appear to induce neuroinflammation, such as trauma, infection, oxidative agents, redox iron, oligomers of tau, and Aβ [31]. In effect, neuroinflammation causes abnormal secretion of proinflammatory cytokines that trigger signaling pathways that activate tau hyperphosphorylation in residues not modified under normal physiological conditions [32].

3. Pathophysiology of Alzheimer's Disease

Alzheimer's disease (AD), named after a German Physician, is marked by the development of multiple cognitive deficits such as impaired memory, inability to initiate and plan complex behaviors, and aphasia [33]. AD is the most common form of dementia. The estimated global prevalence rate for AD will surpass 100 million patients by 2050 [34]. This situation will not only create a social burden but also increase the economic burden worldwide. In 2010, there was a worldwide estimated burden of 46.8 million people having dementia with care costs of about US$818 billion. By 2030, the number of people with dementia should exceed 74.7 million, with a caring cost of US$2 trillion [35]. Despite the vast number of scientific reports on AD, pharmacological prevention remains a challenge, although lifestyle changes (e.g., exercise, social and mental stimulation) could be effective preventive measures.

4. Key Players in the Pathophysiology of Alzheimer's Disease

4.1. Amyloid Precursor Protein. Amyloid precursor protein (APP) is an abundant type 1 integral transmembrane protein in the central nervous system. It is ubiquitously expressed in human tissues and localizes at the plasma membrane and organelles, such as the mitochondria, Golgi apparatus, and endoplasmic reticulum [36, 37]. Proteolytic processing of the synaptic protein APP produces a 40- or 42-amino acid protein fragment, Aβ, the chief component of amyloid plaques [38]. The most abundant amyloid-β protein among others is Aβ40 and Aβ42 in the brain; the only difference between Aβ40 and Aβ42 is the presence of isoleucine and alanine amino acid residues at the C-terminus of Aβ42 [39]. There is a preferential production of Aβ in the aforementioned cellular organelles based on the protein needs by both the amyloidogenic and nonamyloidogenic pathways (the secretory pathway) [40].

The formation of Aβ oligomers results from the release of Aβ species in monomers, which then aggregates to form the amyloid plaques [41]. The Aβ oligomers trigger proinflammatory cascades, oxidative stress and mitochondrial impairment, induction of neuronal apoptosis, increased phosphorylation of tau proteins, deregulation of calcium metabolism, and cell death by interacting with neurons and glial cells (Figure 2) [42], making them the most toxic of all known amyloid derivatives. These have led to an impairment in APP metabolism due to a feedback loop that causes damage to neuronal cells.

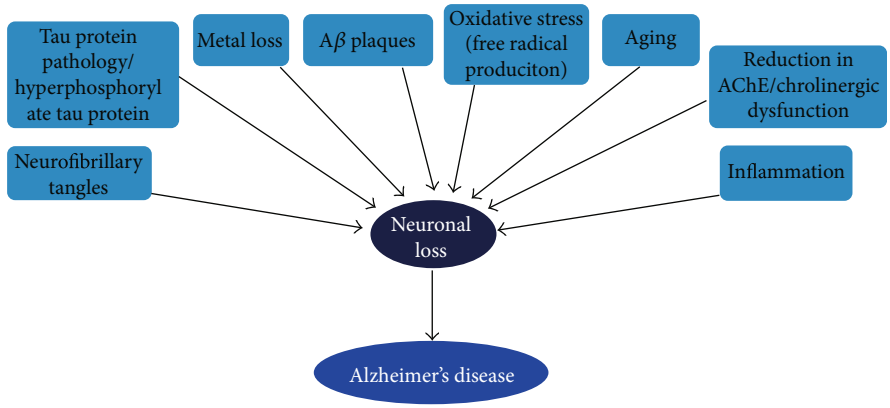

Figure 2: Schematic diagram showing some contributory factors to the pathology of AD. The mechanism by which amyloid-beta and neurofibrillary tangles are deposited in the brain to cause neuronal loss is unknown. Several hypotheses postulate neuronal loss in AD; however, amyloid-beta accumulation in the brain triggers a series of complex reactions that result in neuronal loss. Affected regions of the brain demonstrate inflammation, amyloid plaques, and neurofibrillary tangles.

4.2. Tau Protein. Tau protein belongs to a group of proteins referred to as microtubule-associated proteins (MAPs) and mainly expressed in neurons and plays a crucial role in neuronal cytoskeleton stabilization [43]. Phosphorylated tau proteins and isoforms interact with tubulins and stabilize microtubulin polymers. It also helps recruit signaling proteins and regulate microtubule-mediated axoplasmic transport or flow [44]. Furthermore, neuronal polarity depends on the microtubular properties of the dendrite and axon [45]. The mechanism of tau phosphorylation brings about synapse plasticity through cytoskeleton remodeling. Although the tau protein phosphorylation is essential, it requires tight control. Overphosphorylation will lead to neuronal death and disruption of the microtubular cytoskeleton. Therefore, the hyperphosphorylation of tau proteins causes axoplasmic transport dysfunction and impaired synaptic metabolism.

Tau phosphorylation and dephosphorylation thus serve as the regulatory point for neural homeostasis of serine-threonine phosphoepitopes by serine/threonine-protein kinase N1 (PKN). Here, phosphatase binds to guanine protein-coupled receptors (GPCRs) and ion channels, thus reversing protein phosphorylation [43]. Tau protein can be in insoluble and soluble forms. The insoluble form found in PHF (paired helical filaments) is the primary constituent of neurofibrillary tangles (NFTs), while the soluble form may devastate structural plasticity. There are six isoforms of tau in humans based on the number of tubulin-binding domains and differences in the N-terminus of the protein [45]. The dynamic polymerization of tau proteins occurs via tau and tubulin interaction, controlled by the phosphorylation and dephosphorylation of PKN. The longest tau protein isomer is said to have about 79 potential phosphorylation sites at threonine (Thr) and serine (Ser) residues, with an average of 30 in a normal isomer [36]. Phosphorylation and dephosphorylation increase conformational changes that affect tau interaction with α- and β-tubulins and stabilize microtubules [46]. The phosphorylation of tau proteins occurs with numerous proteases and protein kinases with an essential tau kinase in the neurons, glycogen synthase kinase-3β (GSK3β). The expression of protein phosphatases (PP1, PP2A, and PP5) is limited in the cerebral tissues of patients who have AD [47]. Hyperphosphorylation of tau protein by phosphoepitopes in PHF occurs in AD patients' brains. Some proline-directed kinases like casein kinases I and II, protein kinases A and C, calcium/calmodulin kinase II are present in NFTs and are central in regulating the action of neurofibers [47, 48]. During the embryonic developmental stage of the CNS, the neuronal tau protein is mainly in a hyperphosphorylated state because there is a high demand for neuroplastic changes in neurons and synapses [48].

Contrary to the developmental stage of the CNS, there is maintenance in the stability of neuronal homeostasis in the matured stage because tau phosphoepitopes are predominantly in a dephosphorylated state [49]. However, the neuronal process outgrowth and synaptic plasticity are maintained continuously by the changes in tau phosphorylation. There is an abnormality in tau hyperphosphorylation in some pathologic conditions like AD, causing impairment in its binding to tubulin, destabilizing the microtubular structure. This impairment also leads to synaptic metabolism and axoplasmic transport dysfunction, resulting in cytoskeleton collapse and neural death [50].

4.3. The Amyloid Cascade. The amyloid cascade hypothesis of AD was initially reported in the year 1992 [51]. This theory postulated that the enhanced aggregation of Aβ peptides into neuritic and senile plaques in the brain triggers neuronal degradation involving impaired mitochondrial function, decreased neurogenesis and synaptic plasticity, free radical generation, tau protein hyperphosphorylation, and impaired calcium metabolism in AD [52]. However, recent studies have shown that Aβ peptides act to trigger the amyloid cascade and promote fibrillary and oligomeric forms (the most toxic forms of Aβ peptides) [53].

The genetic mutations in early-onset familial AD strengthen the *in vivo* and *in vitro* findings on the amyloid cascade hypothesis. Consequently, harnessing the tool of gene engineering created AD animal models bearing these mutations [54]. The amyloid hypothesis of AD came with several cautions, such as the fact that no significant

correlation exists between chronic dementia and the density of amyloid plaques in the brain. Neuritic plaques, also called senile plaques, are extracellular deposits of Aβ peptides enclosed by reactive astrocytes and activated microglia in the brain's gray matter associated with neurodegeneration [55]. In contrast, AD is considered the sole neurodegenerative disease in which the Aβ peptide is the pathological cause, as revealed by the statistics of nondemented elderly individuals who have amyloid plaques in their brain following necropsy examination. Also, there are cases of plaque counts in nondemented subjects corresponding to those detected in AD patients [56]. Secondly, clinical trials showed that anti-amyloid-based therapeutic drugs and strategies failed to combat dementia progression or improve cognitive processing [56]. Finally, AD's early onset has been AD based on the genetic mutations proven by AD cellular and animal models. However, early-onset AD records a smaller percentage of cases of dementia, while late-onset AD is far more frequent and has no relationship with genetic mutations. Contrary to that, sporadic AD has a multifactorial origin involving numerous genetic polymorphisms with fewer risk effects, pathogenic amyloid, and other pathological mechanisms [57].

4.4. The Cholinergic Hypothesis. Over the years, the cholinergic hypothesis has been the main postulation for neurodegenerative disorders. All cholinergic neurons use the neurotransmitter acetylcholine (AChE). AChE is an α/β-fold protein produced in the cell from acetyl-CoA and choline. Transported into the synapse through microtubules, AChE binds to a fast nicotinic receptor and a slow muscarinic receptor. AChE is involved in the consolidation/reconsolidation and retrieval of memory. This role of AChE lends credence to studies showing that acetylcholine diminishes in individuals with neurodegenerative disorders such as AD [58, 59]. The cholinergic hypothesis proposed that acetylcholine changes the conformation of NFTs in the brain of AD patients through noncholinergic function by amyloid-beta deposition [60]. Furthermore, the literature suggests that the cholinergic neurons' degeneration from the nucleus basalis of Meynert plays a crucial role in the memory loss experienced by AD patients [59].

5. The Role of Hypoxia in the Progression of Alzheimer's Disease

A limited supply of oxygen to the tissues results in hypoxia. The heart pumps oxygenated blood to the periphery and is crucial for cellular/tissue/organ function and oxidative phosphorylation performance. Hypoxia occurs by several mechanisms, including respiratory system failure, inadequate hemoglobin production, chemical induction of hypoxia, or inadequate blood flow to an organ [61]. The stabilization of the HIFs (hypoxia-inducible factors) controls the hypoxia signaling pathway, which is activated by hypoxia. HIF protein is degraded by von Hippel-Lindau protein (pVHL), an E3 ubiquitin ligase, when it binds to the hydroxylated HIF-α acting as a substrate recognition element of the E3 ubiquitin ligase complex. On the other hand, factors inhibiting HIF-α (FIHs) hydroxylate the asparagine residues of HIF-α subunits, which then inhibits the binding of HIF to the coactivator's p300/CREB-binding protein [62]. The activity of prolyl hydroxylase domains (PHDs) and FIHs is suppressed under hypoxic conditions. The heterodimeric HIF-α:HIF-1β transcription factor complex then translocates to the hypoxia-responsive elements (HREs) of its target genes, resulting in their transcriptional upregulation [63].

5.1. Brain Hypoxia and HIF-1α. The brain is a great energy consumer; therefore, it is particularly susceptible to hypoxia. Consequently, severe and prolonged oxygen deprivation can contribute to brain damage by inducing cell death and neurodegeneration. However, physiological responses to hypoxia are activated and mediated by HIF-1α for the cell to adapt to the microenvironment [64]. Transcriptional complex HIF-1α/β plays a crucial role in cellular and systemic oxygen homeostasis. This complex translocates into the nucleus, becoming a transcriptional activator of over 100 genes [65]. HIF-1α induces the transcription of vascular endothelial growth factor (VEGF), erythropoietin (EPO), and corresponding receptors (i.e., VEGF-R and EPO-R), promoting erythropoiesis and angiogenesis, thus increasing oxygen availability.

Furthermore, HIF-1α may also help in the activation of genes involved in glucose transportation and metabolism. Similarly, HIF-1α plays a vital role in maintaining homeostasis when oxygen deprivation occurs [5, 66].

5.2. Hypoxia Modulates the Accumulation of Aβ Peptides. Many studies have highlighted amyloid precursor protein (APP) and cleavage product Aβ in AD. APP is a single transmembrane protein expressed at high levels in the brain and is rapidly metabolized in a highly complex fashion by a series of sequential proteases [67]. The intramembranous γ-secretase complex also processes other key regulatory molecules [67]. Evidence supporting APP processing regulation spans the differentiation stages of cortical neurons, and amyloidogenic APP processing, as reflected by Aβ1-40/42, is associated with mature neuronal phenotypes [68]. Furthermore, genetic, biochemical, and behavioral investigations have also proved that physiologic generation of the neurotoxic Aβ peptide from sequential APP proteolysis is the crucial step in the development of AD [67]. Though the reason for the accumulation of Aβ in the brains of elderly individuals remains unclear, understanding the APP processing may be crucial to the development of therapeutic targets to treat AD. Hypoxia drives the metabolism of APP, leading to the amyloidogenic pathway, with Aβ protein as the end product [69]. Mattson [70] reported that this pathway could be a defense mechanism by increasing soluble neuroprotective APPα production. However, hypoxia favors APP metabolism through the amyloidogenic pathway, causing an increase in Aβ levels and not APPα levels [71]. Chronic hypoxia is shown to decrease the expression of disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), a presumed α-secretase. Proteolytic processing of the APP by the β- and γ-secretases releases the Aβ peptide, which deposits in senile plaques and contributes to the etiology of AD. It also decreases APP cleavage through the nonamyloidogenic

pathway [72, 73]. Hypoxia, respectively, decreases and increases the mature and immature forms of ADAM10 and reduces α-secretase processing of APP, which may represent a posttranslation effect [74]. However, studies have shown the decreased expression of ADAM17, an affiliated sheddase that also processes APP and TNF-α, after three days of chronic hypoxia [75]. *In vitro* studies of chronic hypoxia using cell lines and animal stroke models have shown increased β-secretase (BACE1) expression, an enzyme that increases the amyloidogenic pathway [76]. The positive feedback loop increases BACE1 levels and HIF-1α-induced genes, increasing amyloid-β protein production. This effect may occur because of the stabilization of HIF-1α, which Aβ also upregulates. Moreover, the fact that HIF-1α-deficient mice reduce BACE1 expression shows that HIF-1α is an essential mediator in BACE1 induction in hypoxic conditions [77].

5.3. Hypoxia, Aβ Accumulation, and Ca^{2+} Homeostasis. Hypoxia can cause a significant ionic disturbance because the ion channels are the first detector of a low oxygen level. During hypoxia, the intracellular ATP/ADP level ratio decreases, decreasing Na^+/K^+-ATPase activity and the influx of Ca^{2+}, leading to membrane depolarization and increased intracellular Ca^{2+} [78]. As an intracellular ion, calcium ion is involved in several physiological processes, including neural excitability, second messenger signaling, and neurotransmitter release. However, excessive intracellular Ca^{2+} can cause changes to mitochondrial metabolism, activation of endonucleases, generation of ROS, and subsequent neurotoxicity [79]. Na^{2+}-Ca^{2+} exchanger efflux pumps and buffering of the mitochondria and endoplasmic reticulum (ER) help maintain the homeostasis of intracellular Ca^{2+} in a healthy neuron [80]. However, excessive Ca^{2+} in neurons could result in the accumulation of amyloid-β protein. A possible mechanism for this accumulation is calcium-conducting pores formed by Aβ in the plasma membrane to regulate calcium ions in the neuron [81].

6. Kynurenine and Alzheimer's Disease: The Role of Indoleamine 2,3-Dioxygenase (IDO)

The kynurenine pathway involves the breakdown of tryptophan to nicotinamide adenine dinucleotide (NAD^+) and other active metabolites. Tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) are the main enzymes involved in this pathway; they catalyze the rate-limiting step, which is the conversion of tryptophan to N-formylkynurenine (Figure 3) [82]. The cytosolic enzyme IDO is an endocellular, monomeric hemoprotein with a molecular mass of 45 kDa. Although first discovered in rabbit intestines, IDO expression occurs in the brain, kidney, lungs, spleen, and liver [83]. There are two isoforms of IDO: IDO1 and IDO2, which are widely expressed in various tissues. Although the gene coding for these isoforms is adjacent, they possess different biochemical properties and functions [83]. For instance, IDO2 expressed in the reproductive tract, kidney, colon, and liver has lower substrate specificity and expression levels than IDO1 [84]. The superior specificity of IDO1 marks it as a potential therapeutic target. The phosphorylation of IDO1 at tyrosine residues Y115 and Y253 helps to modulate its activity by changing IDO1 protein conformation, thus rendering it inactive [85].

Quinolinic acid (QA) and 3-hydroxykynurenine (3-HK) are also metabolites generated by the kynurenine pathway. These neurotoxic metabolites cause excitotoxicity and oxidative stress, respectively (Figure 3) [86, 87]. Furthermore, 3-HK aggravates neurodegeneration and contributes to AD development, while QA produced in the brain acts as an agonist and can induce oxidative stress [87, 88]. QA is also shown to be involved in tau protein phosphorylation by increasing synaptic and neuronal dysfunction [89]. The neuroprotective activity of kynurenic acid (KA) results from its antagonist effect on NMDA receptors [90]; hence, KA decreases QA-induced excitotoxicity. KA is an antagonist of the alpha-7 (α7) nicotinic receptors, reducing Aβ42 endocytosis, although the amount of KA produced is significantly lower than that of QA and 3-HK [91]. Interferon-gamma (INF-γ) and Aβ42 help stimulate the expression of IDO enzymes [92]. The overexpression of IDO enzymes can initiate different mechanisms in AD development, which may cause loss of neuronal activity and behavioral failure. More so, high levels of IDO are observed in the hippocampus of AD patients [7].

Besides, in experiments using a standard AD model, triple-transgenic AD (3xTg-AD) mice show high levels of INF-γ and IDO in their cerebrum [82]. The impairment caused by these high levels of INF-γ and IDO includes oxidative stress, increased levels of tau phosphorylation, impairment in the immune system, and increased Aβ42 levels. However, the role of TDO in the development of AD remains unclear. The TDO level is abundant in the liver despite being measured in the frontal cortex of patients with schizophrenia and at different levels in the mouse brain during their developmental phase [92, 93]. Wu et al. [94] showed that the hippocampi of patients with AD and 3xTg-AD mice presented with significantly elevated TDO levels in the cerebellum but not in the cerebrum. Thus, the kynurenine pathway might be a good target for AD treatment, as suggested by the increased levels of 3-HK and QA in the hippocampus and serum of patients with AD, respectively. A comparable elevation in QA was also noticed in 3xTg-AD mice [82, 95]. Evidence suggests that reducing the activity of the kynurenine pathway can mitigate some of the symptoms seen in experimental animal models of AD [96].

6.1. HIF-1α and the Kynurenine Pathway as Therapeutic Targets in Combating Neurodegenerative Disorders. The catabolism of tryptophan in the kynurenine pathway involves several enzymes that lead to the production of bioactive metabolites, including kynurenine. Kynurenine is involved in the modulation of the central nervous system and the immune system [97]. Due to this modulatory effect, kynurenine and some other metabolites from the kynurenine pathway are extensively studied for their usefulness in psychiatric, cancer, neuroinflammatory, and neurodegenerative diseases [98]. The kynurenine pathway is a shift to control inflammatory responses when there is a high level of inflammatory cytokines to produce KA, QA, anthranilic

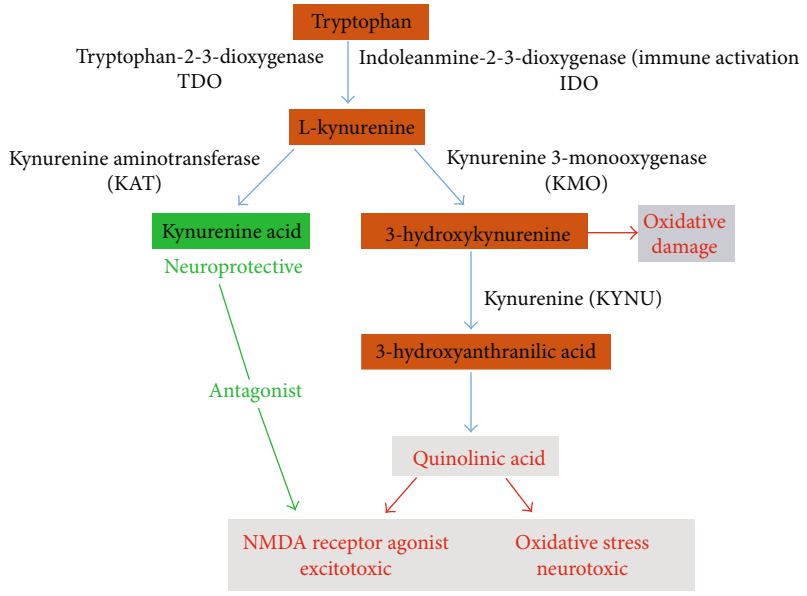

Figure 3: The kynurenine pathway. Breakdown of the kynurenine pathway in the brain as related to neurodegeneration. The four primary metabolites in the kynurenine pathway that readily cross the BBB are tryptophan, L-kynurenine, 3-hydroxykynurenine, and anthranilic acid. The metabolism of kynurenine metabolites in the brain occurs in two separate cells, microglial cells and astrocytes. In microglial cells, kynurenine is converted to 3-hydroxykynurenine, which causes oxidative damage and serves as the entry point of QA. QA is excitotoxic and neurotoxic. It acts as an agonist to the N-methyl-D-aspartate receptor and causes oxidative stress in the central nervous system. In astrocytes, L-kynurenine is converted to KA, an antagonist to the N-methyl-D-aspartate and alpha-7 nicotinic acetylcholine receptor. It also acts as a neuroprotective agent by blocking QA-induced neurodegeneration.

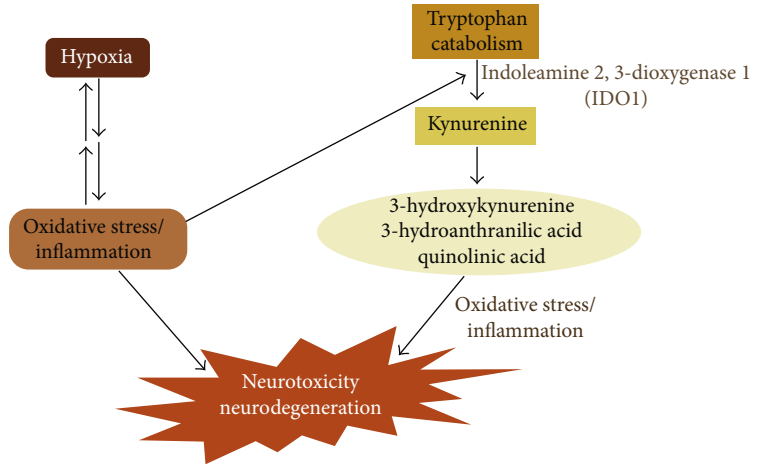

Figure 4: Diagram showing the effect of hypoxia and the kynurenine pathway on neurodegeneration.

acid (AA), 3-hydroxyanthranilic acid (HAA), and 3-hydroxykynurenine (3-HK) [99, 100]. Among these metabolites, QA, 3-HK, and HAA are the neurotoxic metabolites generated. The level of 3-HK is excessively high in degenerative neural diseases like AD and Huntington's disease [97]. 3-Hydroxykynurenine is an oxidative stress generator, and its catabolism to HAA is an antitumor immunity highly beneficial for cancer treatment.

QA exhibits not only neurotoxicity but also neuroexcitatory effect [101]. Excess QA is produced during inflammation, and instead of being converted to NAD^+ to protect the neurons, it gets saturated, resulting in lipid peroxidation and eventually ND. Moreover, AA shows immunomodulatory and anti-inflammatory activities [102], and together with HAA, 3-HK, and 3-HAA, they are all potent apoptotic agents [103]. A decrease in the plasma 3-HAA/AA ratio is seen in different NDs, which might be due to an increase in AA or a reduction in HAA [104]. According to Badawy [98], this ratio change might be an anti-inflammatory response or the presence and progression of inflammatory diseases. Although HIF-1α inhibits IDO, a key enzyme involved in the kynurenine pathway [105, 106], HIF-1α has

implications in neuronal inflammatory disease pathology. It is responsible for the body's adaptation during hypoxia, and an altered HIF-1 α expression is reported in NDs [62, 107]. The accumulation and increase in HIF-1α attenuate the apoptosis caused via rotenone in PD by rescuing injured neurons [108].

Furthermore, energy or oxygen supply imbalance may activate various signaling mechanisms, including glutamatergic synapse formation, MAPK/PI3K-Akt signaling, and phosphatidylserine translocation, which play vital roles in oxidative stress and NDs. Indeed, HIF-1α plays a twofold role through gene activation, in the sense that this factor has to "choose" whether to protect or to kill the affected cells [109]. The regulation of HIF-1α, the exploration and internal control of the kynurenine pathway via the blockage or the expression of some critical metabolites, is a beneficial and potential medical target in neuroinflammatory and ND treatment. Taken together, the activation or inhibition of hypoxic intermediates with or without the kynurenine pathway metabolites could serve as novel therapeutic strategies for neurodegenerative disorders (Figure 4).

7. Conclusion

Hypoxia promotes the formation and accumulation of Aβ, which dysregulates calcium homeostasis in the neurons and astrocytes of the brain leading to neuronal loss or death and microglial activation. There is some evidence suggesting that APP cleavage alters the relationship between AD and hypoxia. This cleavage of APP leads to Aβ accumulation, an initial trigger of AD. Therefore, compounds with inhibitory potential against hypoxia and, in particular, HIF-1α may hold prospects in the development of neurodegeneration therapy. In addition, the rate-limiting enzyme in the kynurenine pathway, indoleamine 2,3-dioxygenase (IDO), produces two neurotoxic metabolites, 3-HK and QA, as end products of this pathway. When released locally in the brain, these metabolites can cause excitotoxic death to neurons and oligodendrocytes through their agonist effect on N-methyl-D-aspartic acid (NMDA) receptors. Therefore, compounds that restrict the kynurenine pathway activation may hold therapeutic prospects in neurodegenerative diseases such as AD. The typical therapies for AD, donepezil and galantamine, can only suppress or reduce AD symptoms but not as a cure to AD. Therefore, further *in vitro* and *in vivo* experiments are warranted to fully understand hypoxia and IDO enzyme roles on the kynurenine pathway to design novel therapeutic agents against ND, like AD.

Data Availability

All data used have been reported in the article.

Conflicts of Interest

The authors report no competing interests.

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Research Article

Carveol Attenuates Seizure Severity and Neuroinflammation in Pentylenetetrazole-Kindled Epileptic Rats by Regulating the *Nrf2* Signaling Pathway

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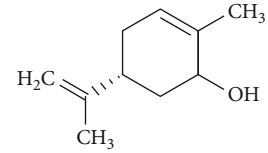
Epilepsy is a neurodegenerative brain disorder characterized by recurrent seizure attacks. Numerous studies have suggested a strong correlation between oxidative stress and neuroinflammation in several neurodegenerative disorders including epilepsy. This study is aimed at investigating the neuroprotective effects of the natural compound carveol against pentylenetetrazole- (PTZ-) induced kindling and seizure model. Two different doses of carveol (10 mg/kg and 20 mg/kg) were administered to male rats to determine the effects and the effective dose of carveol and to further demonstrate the mechanism of action of nuclear factor E2-related factor (*Nrf2*) in PTZ-induced kindling model. Our results demonstrated reduced levels of innate antioxidants such as superoxide dismutase (SOD), catalase, glutathione-S-transferase (GST), and glutathione (GSH), associated with elevated lipid peroxidation (LPO) and inflammatory cytokines level such as tumor necrosis factor-alpha (TNF-α), and mediators like cyclooxygenase (COX-2) and nuclear factor kappa B (NFκB). These detrimental effects exacerbated oxidative stress and provoked a marked neuronal alteration in the cortex and hippocampus of PTZ-intoxicated animals that were associated with upregulated *Nrf2* gene expression. Furthermore, carveol treatment positively modulated the antioxidant gene *Nrf2* and its downstream target *HO-1*. To further investigate the role of *Nrf2*, an inhibitor of *Nrf2* called all-trans retinoic acid (ATRA) was used, which further exacerbated PTZ toxicity. Moreover, carveol treatment induced cholinergic system activation by mitigating acetylcholinesterase level which is further linked to attenuated neuroinflammatory cascade. The extent of blood-brain barrier disruption was evaluated based on vascular endothelial growth factor (VEGF) expression. Taken together, our findings suggest that carveol acts as an *Nrf2* activator and therefore induces downstream antioxidants and mitigates inflammatory insults through multiple pathways. This eventually alleviates PTZ-induced neuroinflammation and neurodegeneration.

1. Introduction

Epilepsy, which affects nearly 65 million people worldwide, is among the most prevalent neurodegenerative disease after stroke [1]. It is a syndrome characterized by various neurological conditions, including recurrent epileptic seizures, cognitive deficits, behavioral impairments, and electroencephalographic changes. Epilepsy is most often aggravated by psychiatric comorbidities, like memory and learning deficits, which affect about 30% of the population [2]. Although transient abnormal cortical nerve stimulation can lead to seizure formation, there are numerous other contributing factors having a role in seizure generation, such as excitotoxicity, mitochondrial dysfunction, altered cytokine levels, oxidative and endoplasmic reticulum stress, and genetic factors [3–5]. The clinical use of existing antiepileptic drugs (AEDs) is compromised due to frequent onset of adverse effects along with chronic toxicities of the vital organs [6–8]. Therefore, an in-depth understanding of the mechanisms underlying this disease is required to develop alternative better treatment choices.

Multiple studies have proposed that oxidative stress and inflammation can exacerbate the severity of epilepsy [9, 10] as a consistently high level of inflammatory cytokines is found in seizure pathophysiology [11]. These anomalies can lead to numerous functional and biochemical alterations, including lipid peroxidation (LPO), BBB disruption, and angiogenesis [12, 13]. Moreover, the surge in cytokines is directly related to the permeation of leukocytes and granulocytes to the brain from the surrounding vasculature [14]. Furthermore, resident glial cells are rapidly activated and trigger the release of proinflammatory cytokines such as interleukin-1 (IL-1β), tumor necrotic factor-alpha (TNF-α), and interleukin-6 (IL-6), which clinically compromise the prognosis of epilepsy [15, 16]. Pentylenetetrazole (PTZ), which is a GABA receptor antagonist, is a widely accepted and established model for inducing epileptic seizures via blockade of the gamma-aminobutyric acid (GABA) receptor, a major inhibitory neurotransmitter in the brain [17]. PTZ exerts a convulsant effect similar to that of human absence seizure and can be used for generating rodent epileptic model [18–20]. Multiple studies have suggested elevated NO levels and reduced antioxidant activity in the rat brain after PTZ treatment [21, 22]. Therefore, maintaining low ROS/RNS levels in the brain is crucial for normal cellular function as it facilitates ablation of subsequent neuroinflammation [23].

Nuclear factor erythroid 2-related factor 2 (*Nrf2*, or NFE2L2) is critically involved in the natural cellular defense system, as it governs the gene expression of numerous antioxidant proteins and ROS-eliminating enzymes, thereby preventing ROS-induced neuronal and cellular damage [24]. Upon activation, *Nrf2* activates the innate antioxidant cellular machinery and upregulates multiple inducible antioxidant enzymes, including heme-oxygenase-1 (*HO-1*), NAD(P)H quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) [25]. There is evidence suggesting the crosstalk between *Nrf2* and NF-κB, which reveals the mechanism through which activated *Nrf2* exerts an anti-inflammatory effect [26–29]. Moreover, previous studies demonstrated the neuroprotective role of *Nrf2* not only in laboratory animals but also in human brain samples [30–32]. We have previously shown that activation of *Nrf2* signaling attenuated infarction area and inflammatory-related pathologies not only in stroke but also in the depression model [33–35]. Therefore, *Nrf2* may be a suitable therapeutic target for managing epilepsy and seizures.



(2-Methyl-5-(1-methylethenyl)-2-cyclohexen-1-ol

Figure 1: Structure of carveol.

Natural moieties are an attractive source of new drugs, owing to their rich antioxidant potential. Several natural drugs have shown protective potential against a variety of mediators, including free radicals and inflammatory factors [36, 37]. Carveol is a natural monocyclic monoterpenoid antioxidant compound (Figure 1) that is abundant in caraway seeds, mandarin, black tea, dill, and essential oils of orange peel [38, 39]. Carveol has been reported in traditional Chinese medicine as an antispasmodic, carminative, astringent, and further used for indigestion and dyspepsia [40]. We previously demonstrated the neuroprotective potential of carveol in ischemic brain injury by attenuating infarction area [33]. In another study, carveol mitigated hepatocellular necrosis by showing antioxidant, antihyperlipidemic, and anti-inflammatory activities [41]. Furthermore, carveol exhibited promising results in the management of diabetes [42]. Given the strong antioxidant potential of carveol and its promising properties, this study is aimed at evaluating whether carveol administration can ameliorate PTZ-induced epileptogenesis in a rat model by modulating the *Nrf2* pathway.

2. Materials and Methods

2.1. Chemicals and Reagents. Carveol (#192384, PubChem ID:24851543), a mixture of isomers, with 97% purity, and 3,3-diaminobenzidine tetrahydrochloride hydrate (#D5637, PubChem ID:57654109) were purchased from Sigma-Aldrich (USA). Mouse monoclonal anti-p-NF-κB (SC-271908), mouse monoclonal anti-TNF-α (SC-52B83), mouse monoclonal anti-*HO-1* (SC-136960), rabbit polyclonal anti-Nrf2 (SC-722), mouse monoclonal anti-VEGF (SC-7269), and ABC Elite kit (SC-516216) were purchased from Santa Cruz Biotechnology, Dallas, TX, USA). Rat ELISA kits p-NF-κB (SU-B28069) and TNF-α ELISA kit (SU-B3098) were procured from Shanghai Yuchun Biotechnology, Shanghai, China, while rat COX-2 (E-EL-M0959) was purchased from Elabscience Biotechnology Inc., Houston, TX, USA. The horseradish peroxidase-conjugated secondary antibody (ab-6789) was obtained from Abcam (Cambridge, UK). Proteinase K (#02193981-CF) was obtained from MP Bio USA. All other solvents and reagents as DPX Mounting media (#06522), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, #D8130, PubChem

ID:24894189), trichloroacetic acid (TCA, #T6399, PubChem ID:24900373), and N-(1-naphthyl) ethylenediamine dihydrochloride (#222488, PubChem ID:24853334) were procured from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals and Ethical Approval. Adult male Sprague-Dawley rats (weight 250–300 g) were habituated under laboratory conditions at 25°C for 7 days, with 12 h alternating light and dark cycles; moreover, they received standard commercial diet and water ad libitum. All experimental procedures were conducted following the ARRIVE guidelines and approved by the Research and Ethical Committee (REC) of the Riphah Institute of Pharmaceutical Sciences (Approval ID: Ref. No. REC/RIPS/2018/14; date of approval: November 15, 2018).

2.3. Acute Toxicity Testing. To determine the acute toxicity of the selected natural compound, we included 10 nonpregnant nulliparous female rats and divided them into the control and treatment groups (each $n = 5$). After being deprived of food and water overnight, one rat was administered a limited oral dose of 2000 mg/kg per OECD guidelines 425 on the next day [43, 44]. After being observed for 24 h and survival being confirmed, the same protocol was followed for the remaining rats in the treatment group. They were initially observed for 48 h for any signs of distress and mortality; subsequently, they were observed daily for 14 days for other toxicity signs, including squinted eyes, writhing, salivation, tremors, convulsions, loss of fur, change in overall behavior, stress, and mortality. On the 15th day, blood samples were obtained from animals via cardiac puncture for various biochemical analyses, including wet organ weight, antioxidant profile, liver function tests, renal function tests, and hematological profile. Subsequently, the animals were sacrificed under anesthesia, and vital organs were collected for histopathological examination.

2.4. Seizure Induction Using PTZ. Seizures were induced as previously described, with slight modifications [45, 46]. Briefly, PTZ was dissolved in normal saline and intraperitoneally (IP) injected into the PTZ-kindled group at a subconvulsive dose of 40 mg/kg at 48 h intervals for 15 days until they were fully kindled and showed stage 5 or 6 on three consecutive injections. Only successfully kindled animals were included in the study.

2.5. Study Design and Animal Treatment. Animals were randomly divided into seven groups ($n = 10$, each group) as follows: group 1 (control group): saline injection containing 5% DMSO were administered at 48 h intervals for 15 days; group 2 (PTZ control group): 40 mg/kg PTZ administered until stage 5 convulsions, with eight injections being administered; group 3/4 (treated group): rats received protective doses of carveol 10 (Car-10) and 20 mg/kg (Car-20) and were administered 30 min before PTZ; group 5 (ATRA+PTZ group): rats were treated with 5 mg/kg all-trans retinoic acid (ATRA) and were administered 30 min before PTZ; group 6 (ATRA+PTZ+Car): rats were treated with ATRA 30 mins before giving carveol and PTZ was administered 30 mins after carveol treatment, followed by behavior recording for 30 min; group 7 (standard group): rats were treated with 2 mg/kg diazepam at 30 min before PTZ administration. Carveol, ATRA, PTZ, and diazepam were dissolved in normal saline containing 5% DMSO and were administered for 15 days at a 48 h interval (Figure 2). Notably, the selected carveol dose was determined in a previous study using a neurodegenerative model established in our lab [33].

2.6. Evaluation of Behavioral Characteristics

2.6.1. Racine's Scale. Seizure activity was evaluated for 30 min after each PTZ administration. Behavioral characteristics, including latency, intensity, and convulsion stage, were recorded for 30 min after each PTZ dose using the modified Racine scale [47]: stage 0—no response; stage 1—hyperactivity, restlessness, and vibrissae twitching; stage 2—head nodding, head clonus, and myoclonic jerks; stage 3—unilateral or bilateral limb clonus; stage 4—forelimb clonic seizures; stage 5—generalized clonic seizures with falling; stage 6—hind limb extensor; and stage 7—death (Table 1). We calculated the mean seizure intensity by taking the mean of all individual seizure scores and dividing them by the number of animals, followed by plotting against the treatment duration. Seizure latency was measured as the duration between PTZ administration and the appearance of the first clonic seizure, jerky body movement, or sudden twitch. Seizure frequency was calculated as the number of seizures experienced by the animal within 30 min of PTZ administration, regardless of the seizure stage. Animals were considered kindled when they reached stage 5 (clonic-tonic seizures) or 6, after three consecutive PTZ injections at 48 h intervals. The investigator who performed behavioral trials was blinded from the group allocation to avoid any bias.

2.6.2. Morris Water Maze (MWM) Test. The MWM test was performed to assess the cognitive deficits and spatial learning ability of the rats as previously discussed [47]. The MWM is comprised of a circular pool with a height and diameter of 50 cm and 120 cm, respectively. The pool was hypothetically divided into four quadrants with reference to the target quadrant. The quadrant where the probe or the elevated platform (placed 1 cm beneath the water) was placed was tagged as the target quadrant and then the right-left and opposite quadrant. The water temperature was maintained at 25°C ± 1°C, the position of the platform was fixed, and each time, rats were dropped from different quadrants. A blind observer recorded the escape latency period, i.e., the time taken by the rat to locate and climb the raised platform. The experiment lasted for four days. In the training sessions, the rats were trained to locate and climb the raised platform with a staying time of 5-7 s. The observer recorded the time when the animal was dropped into the water, and if the rat failed to locate the platform within 90 s, the observer manually guided the rat to the platform. The training session was conducted twice a day at 25 min intervals. Similarly, the escape latency interval for each rat was observed and recorded in each training session during the three days of the testing sessions. A decrease in escape latency was considered to indicate neurodegeneration.

On the last day of behavior analysis, we performed a probe test to check the spatial memory. The probe was removed

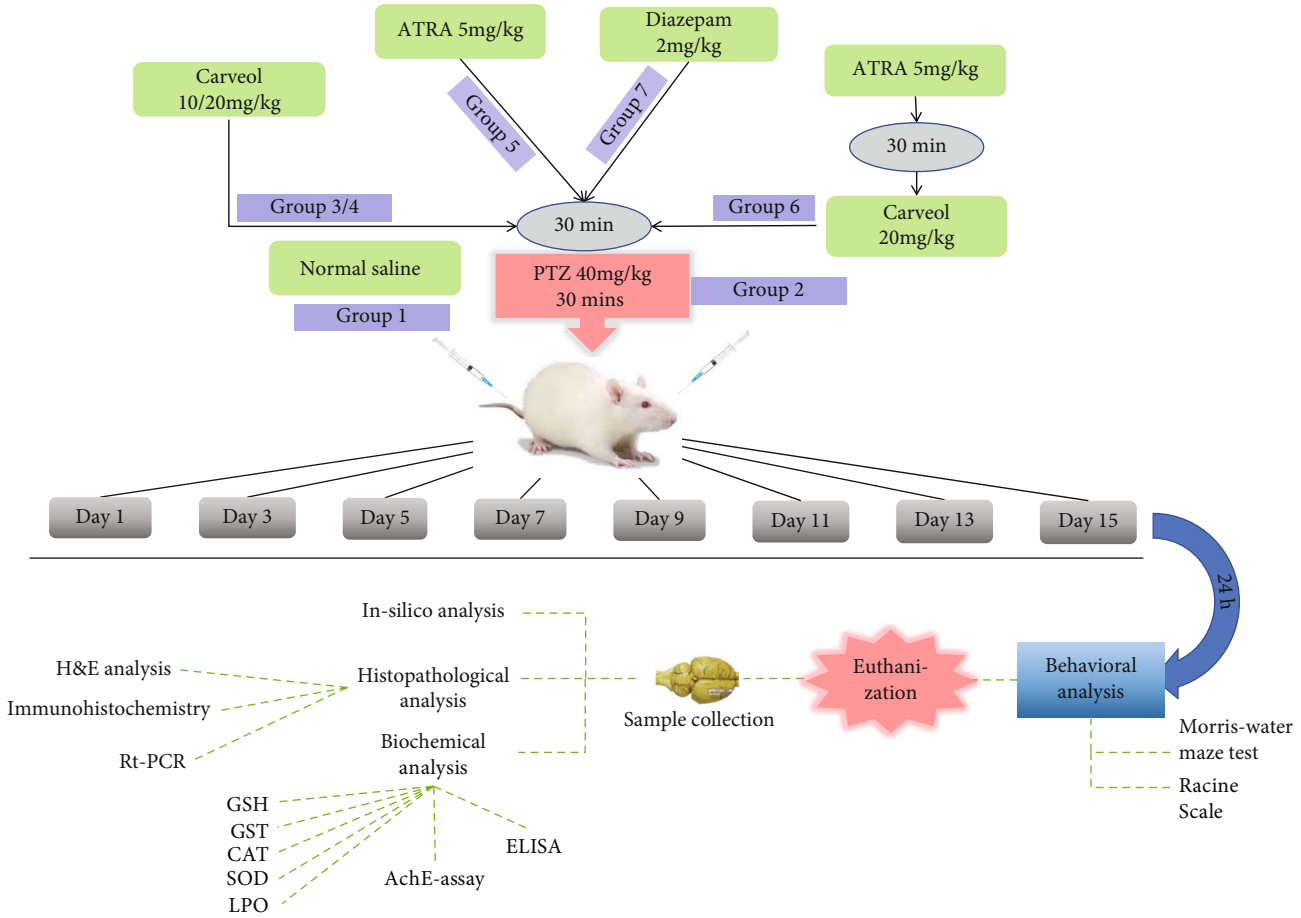


Figure 2: Diagrammatic illustration of the experimental protocol. The treatment protocol was performed for 15 days. In all these groups, a loading dose of PTZ (40 mg/kg, IP) was injected 30 mins after drug treatment (ATRA, carveol, or diazepam), except in the saline group. Brain tissues were collected after 24 h of the last dose for further analysis.

Table 1: Modified Racine's scale.

Stages	Seizure intensity
0	No response
1	Hyperactivity, restlessness, and vibrissae twitching
2	Head nodding, head clonus, and myoclonic jerks
3	Unilateral or bilateral limb clonus
4	Forelimb clonic seizures
5	Generalized clonic seizures with falling
6	Hind limb extensor
7	Death

from the target quadrant, and rats were dropped opposite to the target quadrant, with the time spent in each quadrant being recorded for 60 s. The percentage of time spent in the target quadrant was considered a measure of the extent of neurodegenerative potential and memory function.

2.7. Tissue Collection and Histological Preparation. At 24 h after the last PTZ administration, the rats were quickly decapitated, and their brains were removed on an ice-cold glass plate. The hippocampus and cortex were separated; subsequently, half of the samples were stored at -80°C for biochemical processing while the other half was kept in 4% formalin solution for histopathological and immunohistochemical analysis. Brain tissue samples were homogenized using 0.1 M sodium phosphate buffer (pH 7.4) containing phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. Subsequently, samples were centrifuged at 4000×g for 10 min at 4°C, and the supernatant was used for various biochemical assays.

2.8. Estimation of Brain Acetylcholinesterase (AChE) Activity. Brain AChE activity was determined as previously described by Ellman et al. (1961), with slight modifications [48]. Briefly, 100 μL of DTNB reagent (0.1 mM) was added to 2.6 mL of phosphate buffer (pH 7.4); subsequently, 0.4 mL of brain tissue homogenate was added to this mixture. The initial reading of this reaction mixture was taken at 412 nm before substrate addition. Next, 20 μL of the substrate (acetylcholine iodide, 1 mM) was added to this mixture, and the absorbance was recorded every 10 min for 20 min. The mean change in the absorbance was calculated as follows:

$$R = 5.74 \times 10 - 4 \times \frac{A}{C_o}, \quad (1)$$

A = change in absorbance per minute.

R = rate of moles of acetylthiocholine iodide hydrolyzed per min/g of brain tissue.

C_o = original concentration.

Enzyme activity was expressed as μmoles of acetylcholine hydrolyzed per milligram of protein.

2.9. Antioxidant Assays

2.9.1. Reduced Glutathione (GSH) Activity. GSH was determined to estimate the degree of PTZ-induced oxidative damage and the resulting effect of carveol as previously discussed [34]. We mixed 0.2 mL of the tissue supernatant with 2 mL of DTNB mixture, followed by the addition of 0.2 M phosphate buffer to yield a final volume of 3 mL. The absorbance was measured after 10 min using a spectrophotometer at 412 nm, where phosphate buffer and DTNB solution were used as a blank and control, respectively. The real absorbance value was obtained by subtracting the absorbance of the control from that of the tissue lysate. The final GSH activity was expressed in units of μmol/mg of protein.

2.9.2. Glutathione-S-Transferase (GST) Activity. To calculate GST activity, we freshly prepared 1 mM CDNB and 5 mM GSH solutions in 0.1 M phosphate buffer. Three replicates of the 1.2 mL reaction mixture were kept in glass vials, followed by the addition of 60 μL of tissue homogenate to each of these mixtures. The blank contained water rather than tissue lysate. Next, 210 μL aliquots from the reaction mixture were pipetted out in a microtiter plate; further, absorbance was measured at 340 nm for 5 min at 23°C using an ELISA plate reader (BioTek ELx808, Winooski, VT, USA). GST activity was expressed in units of μmol of CDNB conjugate/min/mg of protein [49, 50].

2.9.3. Superoxide Dismutase (SOD) Activity. We mixed 0.1 mL of tissue homogenate with 0.1 mL of pyrogallol solution (1 M) and 2.8 mL of 0.1 M potassium phosphate buffer (pH 7.4), which yielded a reaction mixture of 3 mL. The absorbance was measured at 312 nm [51]. SOD activity was expressed in U/mg of protein.

2.9.4. Catalase (CAT) Activity. We added 0.05 mL of tissue homogenate to 1.95 mL of phosphate buffer (50 mM, pH 7) and 1 mL of H_2O_2 solution (30 mM). The absorbance of the final mixture was measured at a wavelength of 240 nm. The catalase activity was calculated using the following formula:

$$\text{CAT} = \delta\text{O.D} \div E \times \text{Volume of sample(mL)} \times \text{protein(mg)}, \quad (2)$$

where δO.D represents the change in absorbance per minute and E represents the extinction coefficient of H_2O_2 with a value of 0.071 mmol cm^{-1} [51]. The Lowery method was used to measure protein levels. Catalase activity was expressed as μmol of H_2O_2/min/mg of protein.

2.9.5. Determination of Lipid Peroxidation (LPO). The extent of LPO was estimated by detecting thiobarbituric acid reactive substances (TBARS), as previously described with slight modifications [52]. The assay mixture contained 580 μL of phosphate buffer (0.1 M, pH 7.4), 200 μL of supernatant, 20 μL of ferric chloride, and 200 μL of ascorbic acid (100 mM). The mixture was incubated in a water bath at 37°C for 60 min. Next, the reaction was stopped by adding 1000 μL of trichloroacetic acid (10% TCA) and 1000 μL of thiobarbituric acid (0.66% TBA) to the samples. The tubes were kept in a water bath for 20 min, cooled in an ice bath, and centrifuged at 3000×g for 10 min. The absorbance of the supernatant and blank containing all reagents except the test drug was measured at 535 nm and expressed as TBARS- nmol/mg protein.

2.10. Histological Preparation. Following brain extraction, the tissue was stored in 4% paraformaldehyde solution, washed, and cut into 3 mm thick coronal sections using a sharp blade. Subsequently, these sections were fixed in paraffin blocks and sliced into 4 μm thin coronal sections using a microtome [53]. These sections were processed using the following staining techniques.

2.11. Hematoxylin and Eosin Staining (H&E Staining). Our previous lab protocols were used for H&E staining [54]. Briefly, tissue-coated slides were deparaffinized using absolute xylene followed by a graded alcohol solution. Next, the slides were stained by immersion in hematoxylin solution until the stain was retained in the nucleus. After treatment with 1% HCl and 1% ammonia water, the slides were treated with eosin solution for a few minutes and then air-dried. After dehydration with graded ethanol and xylene, as well as coverslipping, five images per slide were captured under an Olympus light microscope (Olympus, Japan) and analyzed using the ImageJ software. Histopathological changes in cellular morphology, shape, number, and edema were determined using light microscopy.

2.12. Immunohistochemical Analysis. Immunostaining was performed as previously discussed [55]. First, the tissue was rehydrated using xylene, graded alcohol series, and distilled water, followed by washing three times with PBS for 5 min. Proteinase K was used as the antigen recovery step. After washing, the tissue was treated with 3% H^2O^2 solution for 5 min to prevent endogenous peroxidase activity. Next, blocking serum was applied at room temperature for a minimum of 1 h to ensure blocking of areas outside the antigenic areas. The slides were then treated with anti-rabbit *Nrf2* antibody, anti-mouse VEGF antibody, anti-mouse *HO-1* antibody, anti-mouse p-NF-κB antibody, and anti-mouse TNF-α antibody (dilution 1 : 100, Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. The next day, the slides were initially treated with a secondary antibody for 2 h after washing with PBS. Next, the slides were treated with an ABC staining kit and left for 1 h. Finally, the slides were stained with DAB solution for 5 min, washed with water, dipped in xylene and 100% ethanol, and covered using mounting media. The slides were air-dried for a minimum of one day with images being obtained using an Olympus microscope and evaluated using ImageJ software. The slides were observed at 10x and 40x magnification; additionally, five random overlapping sections were chosen to calculate the number of stained neurons in the cortex and hippocampal CA1,

CA2, and DG granule cells. The means were plotted against the groups.

2.13. ELISA (Enzyme-Linked Immunosorbent Assay). COX-2, p-NF-κB, and TNF-α expression were quantified using rat ELISA kits following the manufacturer's instructions. Briefly, an appropriate quantity of brain tissue (50 mg) was homogenized using a Heidolph crusher at 15,000 rpm in 2500 μL PBS containing PMSF as the protease inhibitor [56]. Next, the tissue homogenate was centrifuged at 4000×g for 10 min, and the supernatant was collected. Total protein concentration in the supernatant of each group was calculated using the BCA method (Elabscience); moreover, an equivalent protein quantity was used to quantify the protein concentration of COX-2, p-NF-κB, and TNF-α using an ELISA microplate reader (BioTek EL×808). Finally, the protein concentration (pg/mL) was normalized to the total protein content (pg/mg total protein).

2.14. Real-Time Polymerase Chain Reaction (RT-PCR). TRIzol was used to extract the total RNA amount in freshly isolated brain tissues as previously discussed [57]. RNA quality and quantity were assessed using a NanoDrop plate (Skanit RE 4.1, Thermo Scientific). To convert RNA to cDNA, we used a viva cDNA synthesis kit (Vivantis cDSK01-050). Polymerase chain reactions were performed on a Galaxy XP Thermal Cycler (BIOER, PRC) and 2X Amplifyme Universal qPCR mix (Blirt, Germany), following the manufacturer's specifications. The sequences of forwarding and reverse primers were as follows: Rat_*Nrf2*_Forward: CACATCCAG ACAGACACCAGT and Rat_*Nrf2*_Reverse: CTACAAATG GGAATGTCTCTGC; Rat_*HO-1*-Forward: CGTGCAGAG AATTCTGAGTTC and Rat_*HO-1*-Reverse: AGACGCTTT ACGTAGTGCTG; Rat_GAPDH-Forward: CGTGCAGAG AATTCTGAGTTC and Rat_GAPDH-Reverse: TTCAGG TGAGCCCCAGCCTT. The relative gene expression of *Nrf2* was determined using the $2^{-\Delta\Delta CT}$ method for real-time quantitative PCR.

2.15. Statistical Analysis. Statistical analysis was performed using the GraphPad prism-8 software. Neurobehavior and oxidative data were analyzed using one-way analysis of variance (ANOVA) followed by a post hoc Bonferroni multiple comparison test. The other data were interpreted using two-way ANOVA followed by post hoc Bonferroni multiple comparison tests. ImageJ software was used to analyze morphological data. Statistical significance was set at $p < 0.05$. Symbol $*$ or $^{\#}$ represents $p < 0.05$; ** or $^{\#\#}$ represents $p < 0.01$; and *** or $^{\#\#\#}$ represents $p < 0.001$. Finally, * and $^{\#}$ represent significant differences relative to saline and disease, respectively. All data are expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Acute Oral Toxicity Testing of Carveol. To assess the safety profile of carveol, OECD guidelines 425 were followed. Fur and skin, fecal consistency, urine color, respiration, and sleep patterns were found to be normal after administration of 2000 mg/kg of carveol. All animals in both groups survived, with none showing signs of convulsions or distress. Both groups showed normal weight progression during the 14-day protocol. There were no alterations in antioxidant profile, liver function tests, kidney function tests, and hematological indices (Suplementary Figures S1and S2). Histopathological screening of vital organs, including the brain, liver, kidney, and heart, revealed no signs of vacuolation, dystrophy, and/or atrophy (Figure 3). A detailed toxicity profile of carveol indicated that it was safe up to a dose of 2000 mg/kg.

3.2. Anticonvulsant Effect of Carveol on PTZ-Induced Seizure-Like Behavior. PTZ-treated animals presented significant generalized tonic-clonic convulsions also called epileptogenesis, and it corresponds to stage 6 and/or 7 of Racine's scale, as indicated by the significant mean seizure intensity score relative to the saline group (Figure 4(a), $^{***}p < 0.001$). Similarly, PTZ significantly increased the seizure frequency on the 15th day of administration (Figure 4(b), $^{***}p < 0.001$) with a corresponding very short latency time, which depicts rapid seizure initiation on successive PTZ administration (Figure 4(c)). Overall, animals subjected to PTZ kindling exhibited a percentage survival of 71.4% (Figure 4(d)). Carveol treatment (10 mg/kg) reversed the PTZ-induced behavioral deficits, as indicated by the significantly low mean seizure intensity score (Figure 4(a), $^{\#\#}p < 0.01$). Moreover, carveol significantly reduced seizure frequency (Figure 4(b), $^{\#\#}p < 0.01$) and was associated with an extended latency time (Figure 4(c), $^{\#\#}p < 0.01$). Additionally, the percentage survival improved to 85% compared with PTZ (Figure 4(d), $^{\#\#}p < 0.01$). Similarly, carveol at a dose of 20 mg/kg showed similar protection given that none of the animals exhibited a seizure score of 4–5 (Figure 4(a), $^{\#\#\#}p < 0.001$) during the whole kindling period; moreover, there was a significantly reduced seizure frequency (Figure 4(b), $^{\#\#}p < 0.01$) and an extended latency period (Figure 4(c), $^{\#\#\#}p < 0.001$). Diazepam showed similar protection to Car-20 ($^{\#\#\#}p < 0.001$). Moreover, cotreatment with PTZ and ATRA further aggravated seizure-like behavioral deficits, which could not be mitigated by carveol treatment, indicating the termination of carveol activity by ATRA administration.

3.3. Carveol Attenuated Cognitive Impairment and Memory Dysfunction in Epileptic Rats. MWM test was used to assess the effect of carveol on memory and cognition in PTZ-induced epileptic rats. In the hidden-platform swimming test, PTZ-treated rats exhibited a higher latency time compared with saline-treated rats, which indicated severe memory deficits (Figure 5(a), $^{***}p < 0.001$). Carveol treatment with 10 and 20 mg/kg doses significantly improved memory deficits and improved the latency time to reach the hidden platform (Figure 5(a), $^{\#\#\#}p < 0.001$). To assess reference memory, a probe trial was conducted 24 h after the last acquisition period. Figure 5(b) shows the time spent by each group of rats in specific quadrants. Increased time spent in quadrants other than the target quadrant is indicative of impaired spatial learning, as observed in the PTZ-kindled group (Figures 5(b) and 5(c), $^{***}p < 0.001$, $^{**}p < 0.01$). Upon treatment with 10 mg/kg carveol, the animals displayed

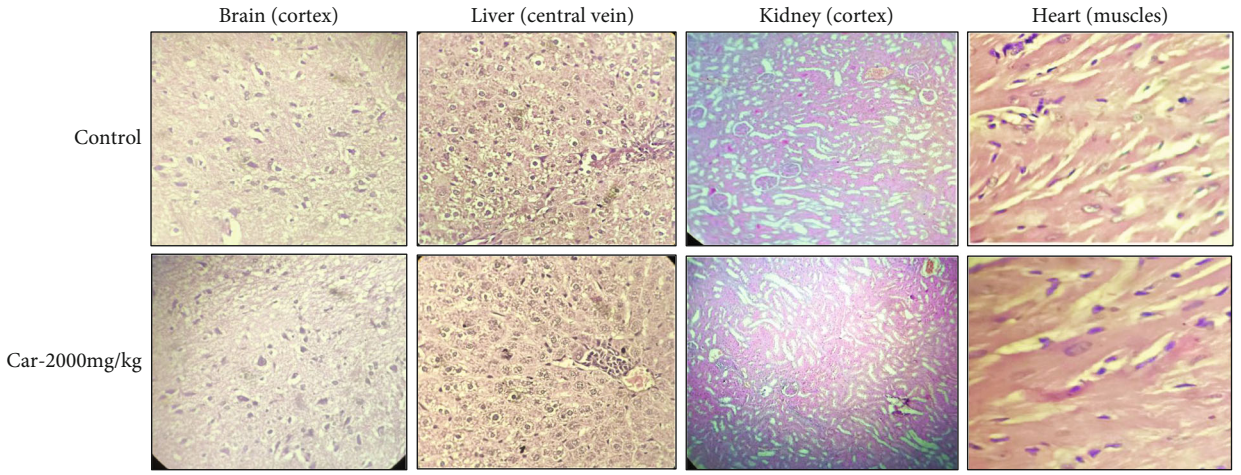


Figure 3: Data regarding acute oral toxicity showing histopathology of the control and carveol-treated groups at a limited dose (2000 mg/kg).

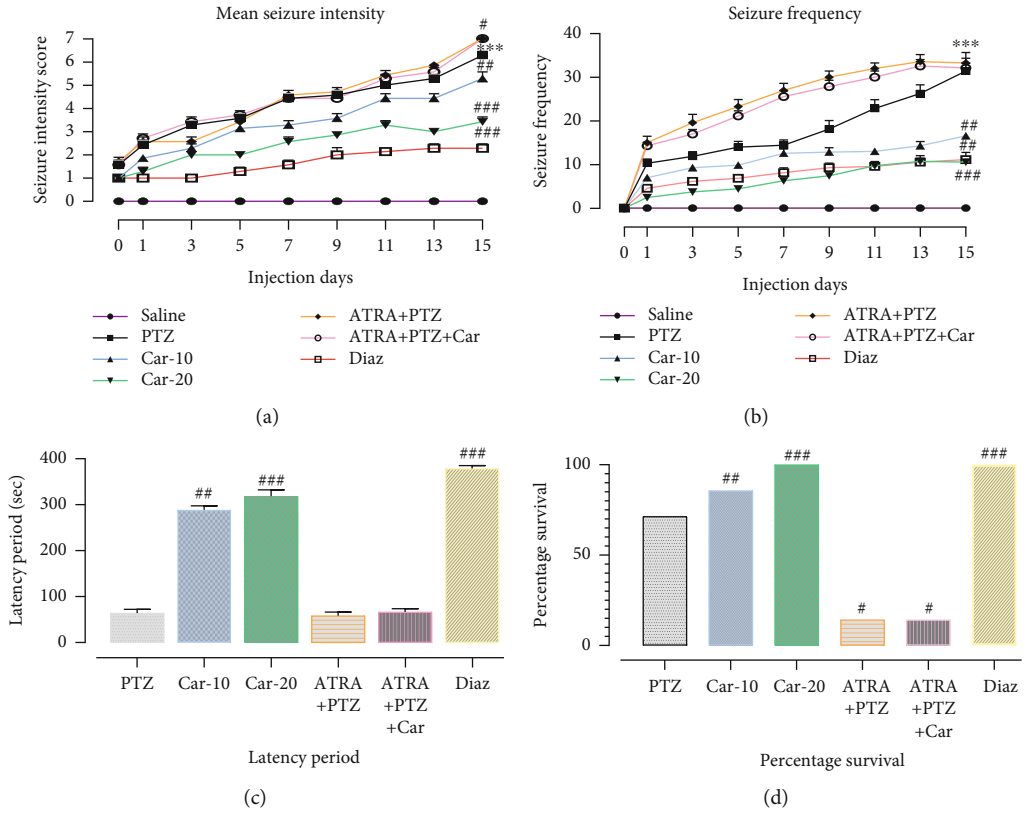


Figure 4: Effect of carveol on PTZ-induced seizure-like behavior. (a) The mean seizure intensity score was recorded after each PTZ injection. Each criterion was scored from 1 to 7. Carveol significantly reduced the mean seizure score as compared with PTZ-kindled animals. (b) Effect of carveol on seizure frequency. Seizure frequency is calculated as the number of tonic-clonic seizures within 30 min after PTZ injection. Car-20 displayed a significantly lower seizure frequency compared with kindled animals. (c) The latency period was measured as the duration between PTZ administration and the appearance of the first clonic seizure. Carveol displayed a delayed latency period compared with PTZ. (d) Compared with the saline group, the PTZ-kindled group showed a lower percentage of survival; moreover, Car-20 displayed an improved survival of the treated animals. All data were expressed as mean ± SEM ($n = 10$/group). # and * denotes a significant difference compared with the PTZ-kindled and saline groups, respectively.

significantly improved spatial memory and learning (Figures 5(b) and 5(c), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$). Similarly, 20 mg/kg carveol significantly improved spatial memory and learning compared with the PTZ-treated animals (Figures 5(b) and 5(c), $^{\#\#\#}p < 0.001$). Additionally, carveol treatment did not improve the ATRA-treated group, which indicated cessation of carveol activity by ATRA administration.

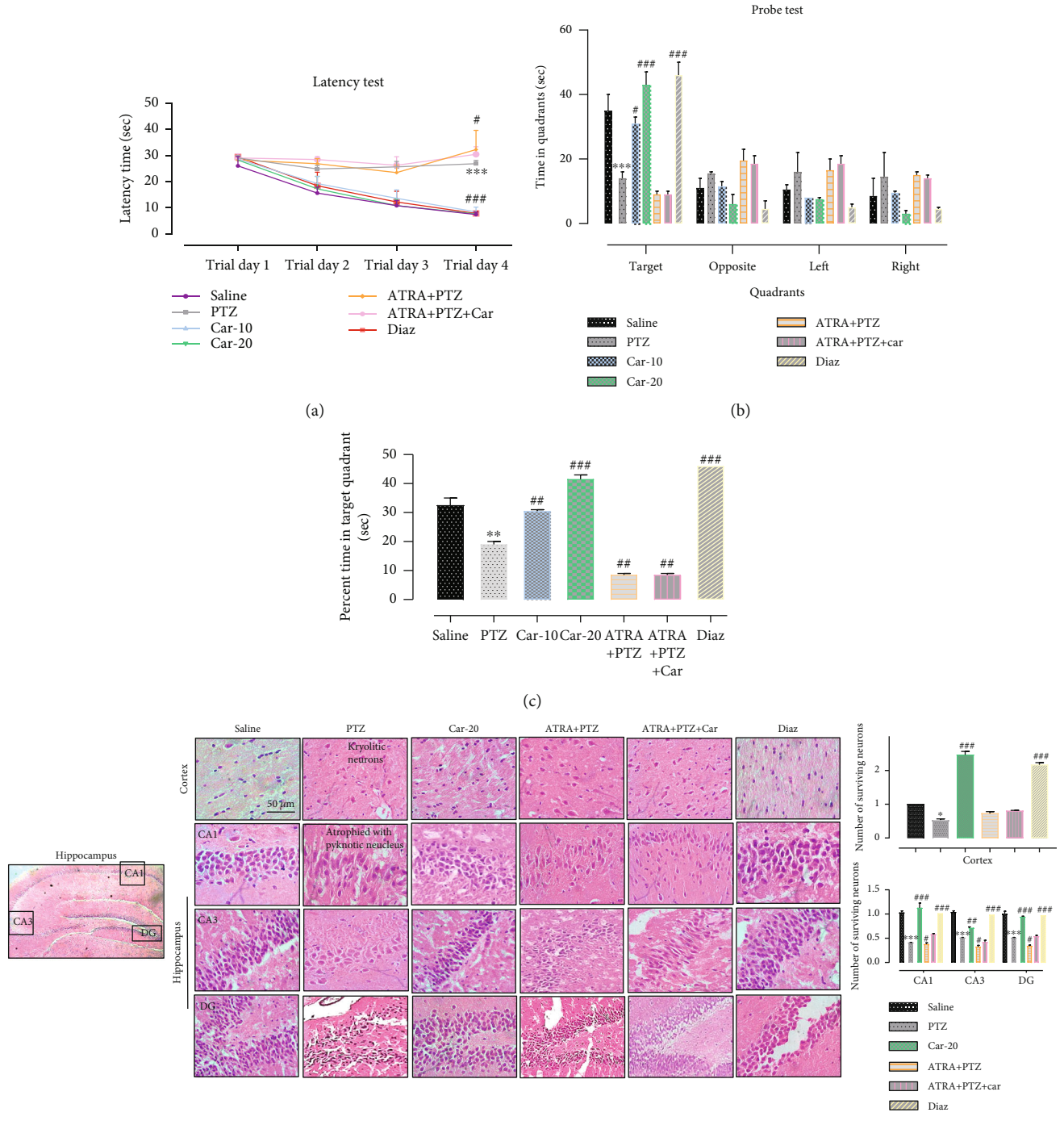
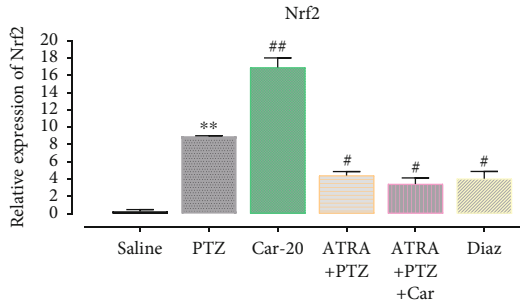
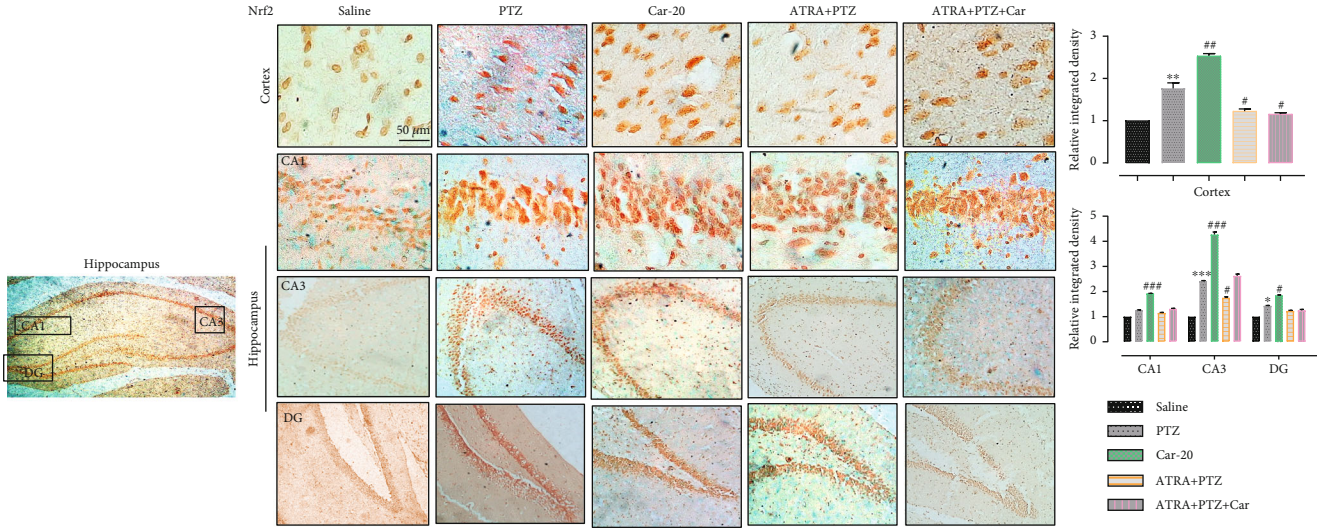


FIGURE 5: Effect of carveol on PTZ-induced memory impairment and neuronal survival. (a) The latency time of rats on the hidden platform. (b) Time spent by PTZ-treated rats in each quadrant in the probe test on the 5th day. (c) The percentage time spent by animals in the target quadrant, with $n = 10$/group. (d) Representative photomicrographs of the H&E-stained cortex and hippocampal tissue revealing the presence of kryolitic and atrophied nuclei in PTZ-kindled animals while the Car-20 group showed only a few cells with degenerative signs (40x, scale bar 50 μm). All data were expressed as mean ± SEM ($n = 5$/group). $^{***}p < 0.001$ denotes a significant difference compared with the saline group. $^{###}p < 0.001$ denotes a significant difference compared with the PTZ-kindled group.

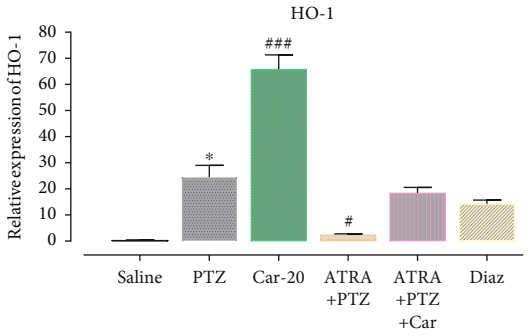
To further validate our hypothesis, we examined morphological changes in the cortical and hippocampal regions using H&E staining. The saline group showed round, well-demarcated intact cells without nuclear condensation or distortion with a basophilic cytoplasm (Figure 5(d)). The PTZ-treated group showed significant histopathological alterations, including altered neuronal shape and size, as well as other atypical features, including swollen, flattened, atrophied, and kryolitic neurons with pyknotic nuclei (Figure 5(d)). Examination of cortical and hippocampal areas



(a)

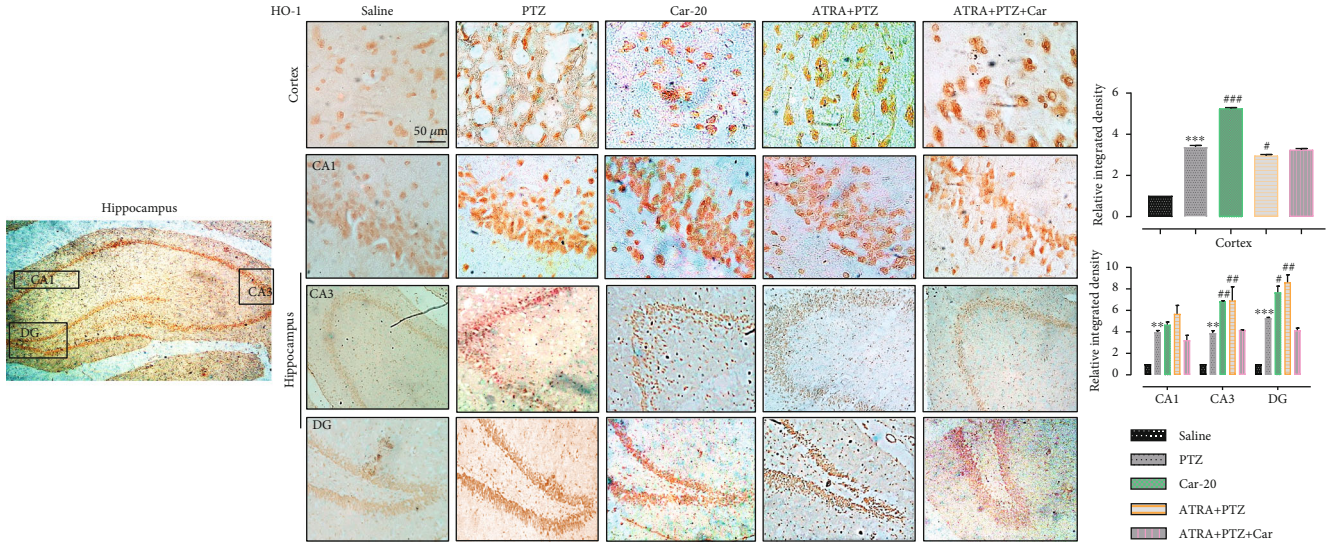


(b)



(c)

Figure 6: Continued.



(d)

Figure 6: Carveol augments the antioxidant capacity of the brain via the *Nrf2* signaling pathway. (a) *Nrf2* gene expression as quantified by RT-PCR with ($n = 5$/group). (b) Immunohistochemistry results for *Nrf2* in the cortical and hippocampal tissues. Histograms exhibit higher *Nrf2* nuclear localization in treated brain tissues. Scale bar 50 μm, magnification 40x ($n = 5$/group. (c) *HO-1* expression as quantified by RT-PCR with ($n = 5$/group). (d) Immunohistochemistry results for *HO-1* in the cortical and hippocampal tissues. Histograms exhibit higher *HO-1* nuclear localization in treated brain tissues. Scale bar 50 μm, magnification 40x ($n = 5$/group). $^{***}p < 0.001$ indicates significant difference relative to saline, while $^{###}p < 0.001$ shows significant difference compared with the PTZ group. All data are presented as means ± SEM.

confirmed that carveol (20 mg/kg) significantly ameliorated these morphological damages, as indicated by an increase in the number of intact neurons and cell count (Figure 5(d), cortex: $^{###}p < 0.001$, CA1 and DG: $^{###}p < 0.001$, CA3: $^{##}p < 0.01$). Additionally, carveol pretreatment in the ATRA-treated group did not improve PTZ-induced histopathological damage.

3.4. Carveol Augments the Antioxidant Capacity of the Brain through Nrf2 and Nrf2-Dependant Downstream Antioxidant HO-1. *Nrf2* combines with free radicals and executes vital antioxidative functions. To further investigate the antioxidant potential of carveol, we analyzed the expression of the *Nrf2* gene and the downstream *HO-1*. RT-PCR analysis indicated upregulated *Nrf2* expression in the PTZ-treated group given that PTZ kindling exerted enough oxidative stress in the brain to activate the body's innate antioxidant *Nrf2* (Figure 6(a), $^{**}p < 0.01$). To further validate this, immunohistochemistry revealed a notable upregulation (Figure 6(b)). Consistent with the upregulated *Nrf2* expression, there was a significant increase in the expression of the downstream antioxidant *HO-1* (Figure 6(c), $^{*}p < 0.05$), also validated by immunohistochemistry (Figure 6(d)). Compared with PTZ, Carveol significantly upregulated hippocampal and cortical expression of *Nrf2* and *HO-1* (Figures 6(a)–6(d)). However, ATRA-treated groups exhibited blockade of carveol-mediated upregulation of the innate antioxidants *Nrf2* and *HO-1*, which suggested that the *Nrf2*/*HO-1* signaling pathway is involved in the antioxidant potential of carveol.

3.5. Carveol Ameliorates Inflammatory Mediators via the Nrf2 Signaling Pathway. Numerous studies have shown that PTZ-kindling is associated with an intensified inflammatory cascade. Therefore, we proceeded to determine whether carveol treatment could affect neuroinflammation. TNF-α, an inflammatory cytokine, was highly expressed in the kindled model as evaluated both by ELISA (Figure 7(a), cortex: $^{*}p < 0.05$, hippocampus: $^{*}p < 0.05$) and by immunohistochemistry (Figure 7(b), cortex, CA1: $^{*}p < 0.05$; CA3: $^{**}p < 0.01$; DG: $^{***}p < 0.001$). As part of downstream targets, we evaluated p-NF-κB and COX-2 expression. The PTZ-treated group showed elevated p-NF-κB expression as shown by ELISA (Figure 7(c), cortex: $^{*}p < 0.05$, hippocampus: $^{***}p < 0.001$) and by immunohistochemistry (Figure 7(d), $^{***}p < 0.001$). A similar expression pattern was also observed for COX2 by ELISA (Figure 7(e), cortex: $^{**}p < 0.01$, hippocampus: $^{***}p < 0.001$).

Additionally, AChE levels were measured given the involvement of the cholinergic system in neuroinflammation. Compared with the saline group, the PTZ-treated group showed a significant upregulation of AChE levels (Figure 7(f), cortex: $^{**}p < 0.01$, hippocampus: $^{***}p < 0.001$). Carveol pretreatment remarkably attenuated expression of TNF-α (Figures 7(a) and 7(b)), p-NF-κB (Figures 7(c) and 7(d)), COX-2 (Figure 7(e)), and AChE (Figure 7(f)) in both the cortex and hippocampus. When cotreatment with ATRA and PTZ exaggerated the neuroinflammatory markers, carveol treatment could not reverse the deleterious effects of PTZ in the ATRA-treated groups.

3.6. Carveol Improves BBB Disruption through Growth Factors. Previous studies have demonstrated the induction of growth factors such as vascular endothelial growth factor (VEGF) in seizures due to BBB disruption. Consistently,

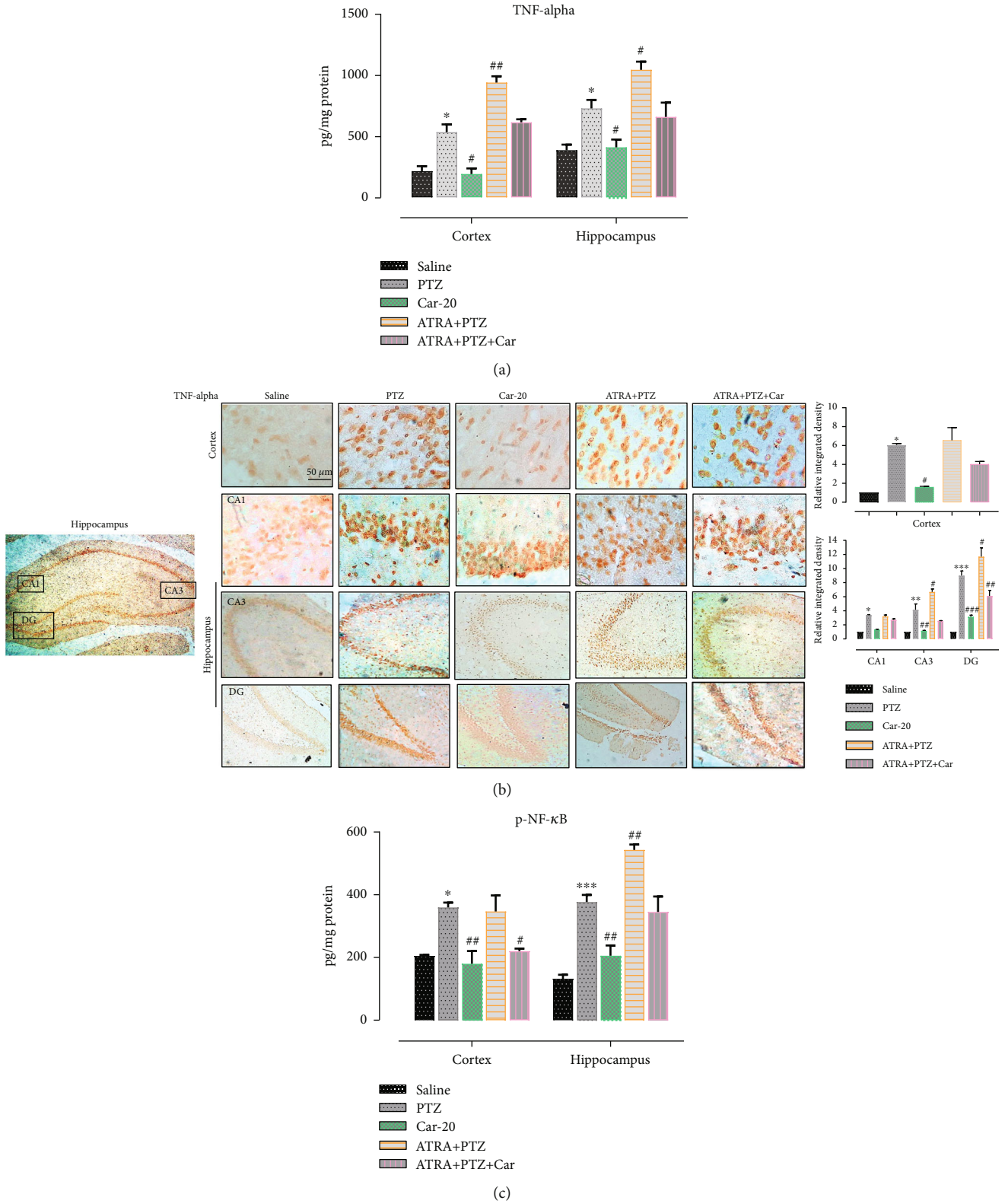


Figure 7: Continued.

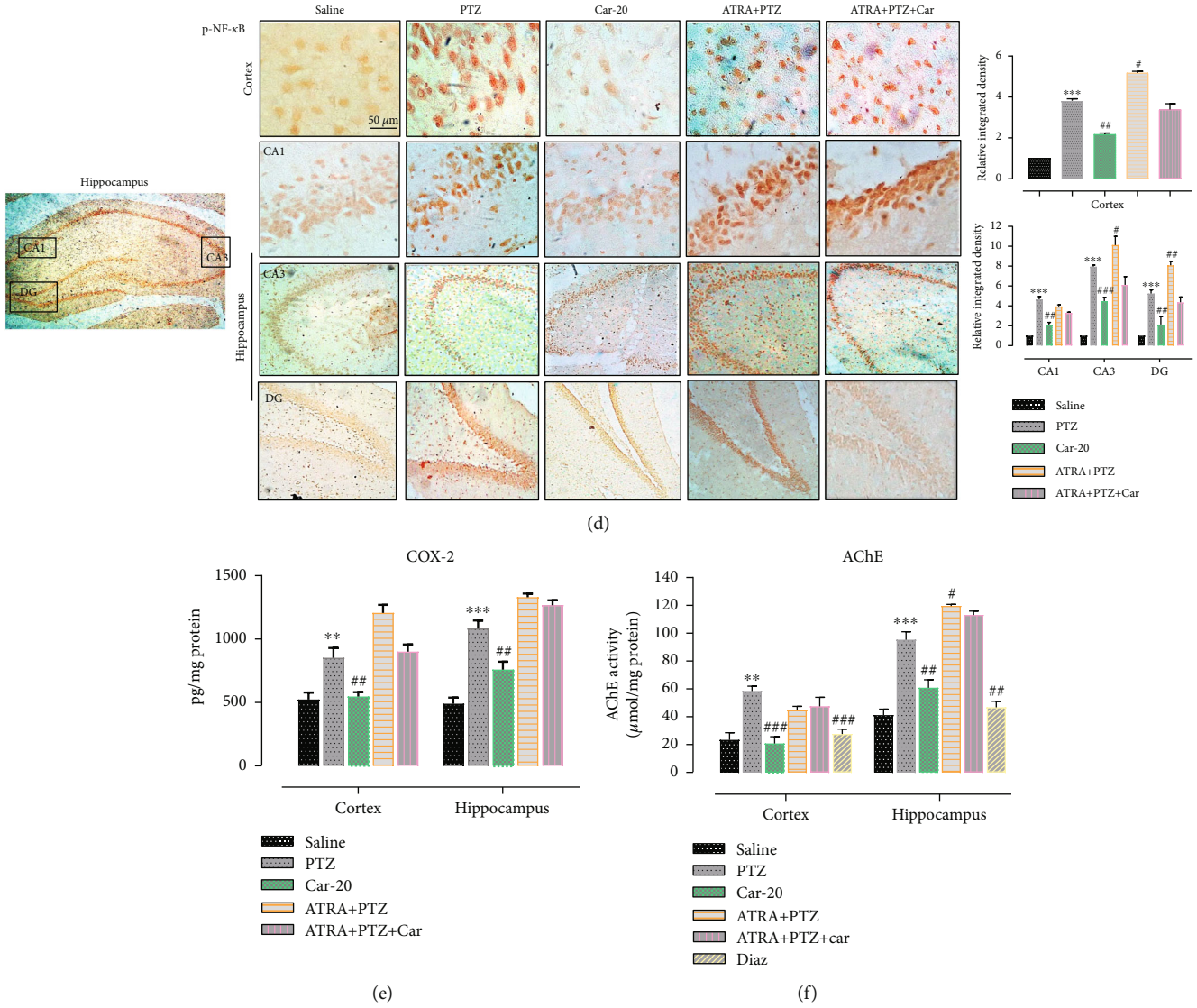


Figure 7: Effect of carveol on outcomes of PTZ-induced inflammatory mediators. (a) TNF-alpha protein expression as quantified by ELISA. (b) Immunohistochemistry results for TNF-alpha in the cortical and hippocampal tissues. TNF-α exhibited cytoplasmic localization in treated brain tissues. (c) p-NF-$_K$B protein expression as quantified by ELISA. (d) Immunohistochemistry results for p-NF-$_K$B in the cortical and hippocampal tissues. p-NF-$_K$B exhibited nucleus localization in the treated tissue. (e) COX-2 protein expression as quantified by ELISA. (f) Acetylcholinesterase levels in both the cortex and hippocampal tissue. The data were expressed as the mean ± SEM, $n = 5$ /group in each experiment. $^{***}p < 0.001$ shows differences compared with saline while $^{\#\#\#\#}p < 0.001$ shows significant differences compared with PTZ. Scale bar 50 μm, magnification 40x.

our study demonstrated abrupt VEGF induction following epileptiform activity in PTZ-treated animals compared with the saline group (Figure 8, cortex: $^{***}p < 0.001$, CA1: $^{*}p < 0.05$, DG: $^{***}p < 0.001$). Carveol attenuated VEGF hyperexpression in all brain regions, which successfully restored brain permeability (Figure 8, cortex: $^{\#\#}p < 0.01$, DG: $^{\#}p < 0.05$). On the other hand, ATRA treatment significantly induced angiogenic factor VEGF, which resulted in BBB dysfunction and diminished the restorative potential of carveol in the cortex of ATRA-treated groups (Figure 8, cortex: $^{\#}p < 0.05$).

3.7. Effect of Carveol on PTZ-Induced Lipid Oxidation and Oxidative Stress Markers. To assess the neuroprotective potential of carveol against PTZ-induced oxidative stress markers, we measured the cortical and hippocampal levels of various enzymatic and nonenzymatic antioxidants, including SOD, CAT, GST, GSH, and TBARS (Figure 9). Carveol treatment significantly restored the level of these antioxidants in the cortex and hippocampus to varying degrees. Compared with the saline group, the PTZ-treated group showed significantly lower levels of CAT, SOD, GST, and GSH (Figures 9(a)–9(d), $^{***}p < 0.001$). Contrastingly, compared with the saline group, the PTZ-treated group showed a marked elevation in LPO levels (Figure 9(e), $^{***}p < 0.001$). Carveol treatment significantly increased cortical and hippocampal levels of CAT, SOD, GST, and GSH (Figure 9(a), $^{\#\#}p < 0.01$; Figure 9(b), cortex: $^{\#\#}p < 0.01$, hippocampus:

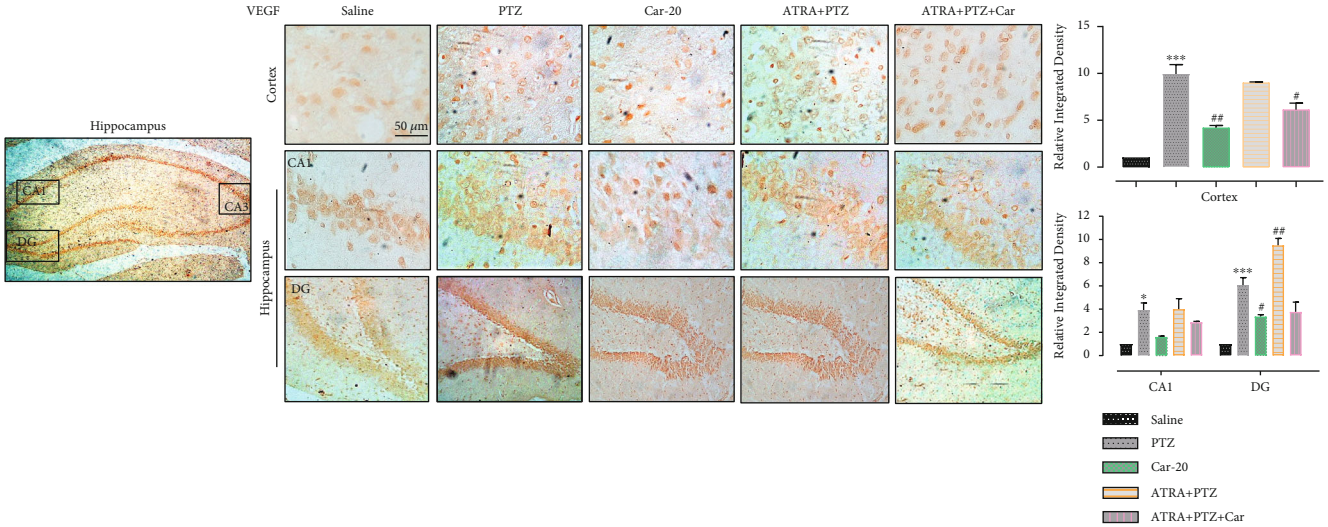


FIGURE 8: Carveol improves BBB-disruption through VEGF. Immunohistochemistry results for VEGF in the cortical and hippocampal tissues exhibited cytoplasmic localization in both brain tissues. Data are expressed as the mean ± SEM, $n = 5$. $^{***}p < 0.001$ shows difference compared with saline while $^{\#\#\#\#}p < 0.001$ shows significant difference compared with PTZ. Scale bar 50 μm, magnification 40x.

$^{\#\#\#}p < 0.001$; Figure 9(c), $^{\#}p < 0.05$; Figure 9(d), $^{\#}p < 0.05$). Conversely, carveol-treated animals demonstrated noticeably reduced TBARS levels in both the cortex and hippocampus compared with the PTZ group (Figure 9(e), $^{\#\#\#}p < 0.001$). Moreover, carveol treatment in ATRA-treated groups demonstrated minimal or no antioxidant effects (Figure 9), which is consistent with our previous findings (Figure 6).

4. Discussion

Plant-derived natural compounds are consistently employed against different pathological disorders owing to better treatment options and minimum side effects. This is due to their inherent properties of targeting multiple steps in the pathological cascade. For this purpose, extensive research must be conducted on phytochemicals before human clinical trials as neuroprotectants [58]. Previous studies have shown that carveol is a monoterpene possesses robust antioxidant, anti-inflammatory, and protective properties in various degenerative models [33, 59]. However, there have been no direct reports regarding the antiepileptic potential of carveol. This study investigated the neuroprotective potential of carveol in a PTZ-induced chronic epilepsy model by ameliorating cognitive deficits, oxidative stress, and neuroinflammation. Our findings showed that carveol had significant potential in reverting seizures by augmenting the endogenous *Nrf2* antioxidant pathway.

In this study, we used subconvulsive PTZ doses, which is a well-studied chemical inducer of epileptic seizures that exerts proconvulsant activity through GABAergic inhibition and therefore causes an imbalance in inhibitory and excitatory neurotransmission that induces seizures [19, 60, 61]. Carveol treatment attenuated these seizures by decreasing the seizure intensity and frequency, as well as delaying seizure onset. Hippocampal dysfunction and neuronal hyperexcitability in epileptic seizures are directly associated with various memory and cognitive impairments [62]. Our findings were consistent with previous findings as a decline in cognition and memory impairment in PTZ-treated animals was demonstrated by a significant increase in escape latency and the probe test [63]. However, carveol significantly improved memory deficits, as demonstrated by a shorter latency time and greater time spent in the target quadrant.

Several studies have reported the involvement of ROS in the pathophysiology of neurodegenerative diseases including epilepsy [64, 65]. Likewise, oxidative stress can exacerbate epilepsy as the brain has limited antioxidants combating capacity [66]. Moreover, the degree of oxidative damage is proportional to epileptic episodes [67]. This notion is further supported by the fact that several clinically used antiepileptic drugs (AEDs) alleviated ROS in seizure [68], while many other AEDs exacerbated oxidative damage [69, 70]. Therefore the use of adjunct antioxidants with AEDs can be useful in the management of epilepsy as demonstrated previously [71]. Our results were consistent with those of previous studies where PTZ-kindled animals experienced oxidative stress and revealed diminished levels of SOD, CAT, GST, and GSH [72, 73]. Carveol augmented these antioxidants and reduced LPO levels, which may partly account for its neuroprotective ability. The substantial oxidative stress caused by seizures activates the endogenous antioxidant response pathway, *Nrf2*, and therefore increases the expression of cytoprotective enzymes and ROS scavengers [74, 75]. The *Nrf2* pathway forms an important defense against oxidative insults in both glial cells and neurons [76–78]. Using *Nrf2*-knockout mice, Wang et al. concluded that the *Nrf2*-ARE pathway is directly involved in protecting the brain from seizure-mediated neuronal damage [79, 80]. In another study, Mazzuferi et al. used gene expression datasets and observed exaggerated *Nrf2* mRNA levels in the hippocampus of mice that initiated spontaneous recurrent seizures [81]. Additionally, Li et al. confirmed the involvement of the *Nrf2*-ARE pathway through nuclear *Nrf2* translocation and direct ARE activation [82]. Another study suggested a strong correlation between

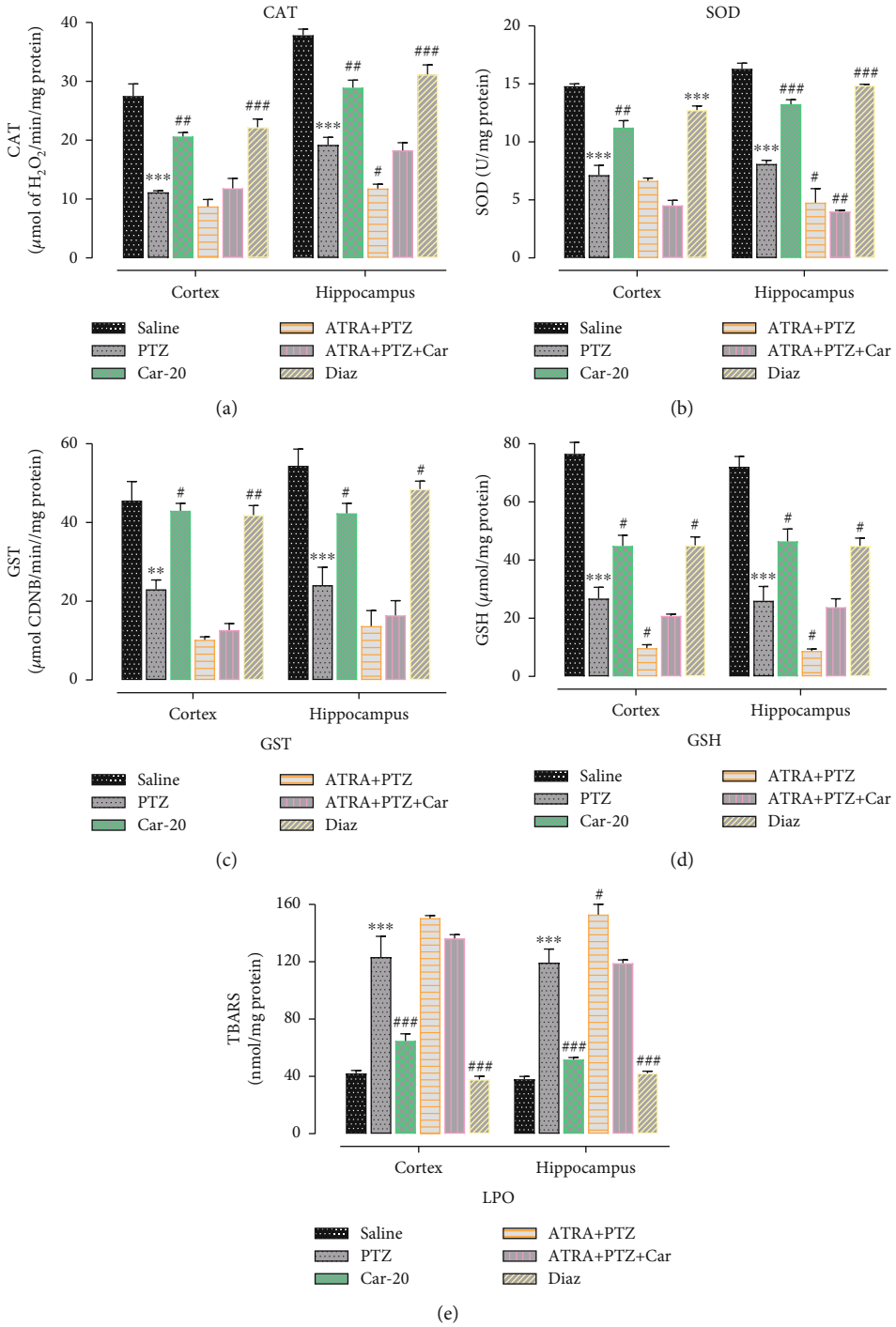


Figure 9: Effect of carveol on oxidative enzymes (Catalase, SOD, GST, GSH, and LPO) in the cortex and hippocampus. (a) CAT level. (b) SOD level. (c) GST level. (d) GSH level. (e) LPO level. $^{***}p < 0.001$ denotes a significant difference compared with the saline group. $^{###}p < 0.001$ denotes significant differences compared with the PTZ group. $n = 7$/group. Data are expressed as mean ± SEM.

ARE activation and *HO-1* expression [83]. Our findings are consistent with these previous findings and we demonstrated upregulated *Nrf2* expression along with the downstream inducible *HO-1* gene and protein in PTZ-treated animals. Moreover, carveol treatment led to further augmentation of these genes and protein levels. However, *Nrf2* inhibition through ATRA abolished these effects, indicating the strong involvement of the *Nrf2* pathway in the cytoprotective nature of carveol.

Nrf2 pathway activation inhibits proinflammatory cytokine release and downregulates the p-NF-κB pathway [26, 27]. Numerous studies have suggested the role of inflammatory mediators such as COX-2 and p-NF-κB activation along with other inflammatory cytokines including interleukin-6

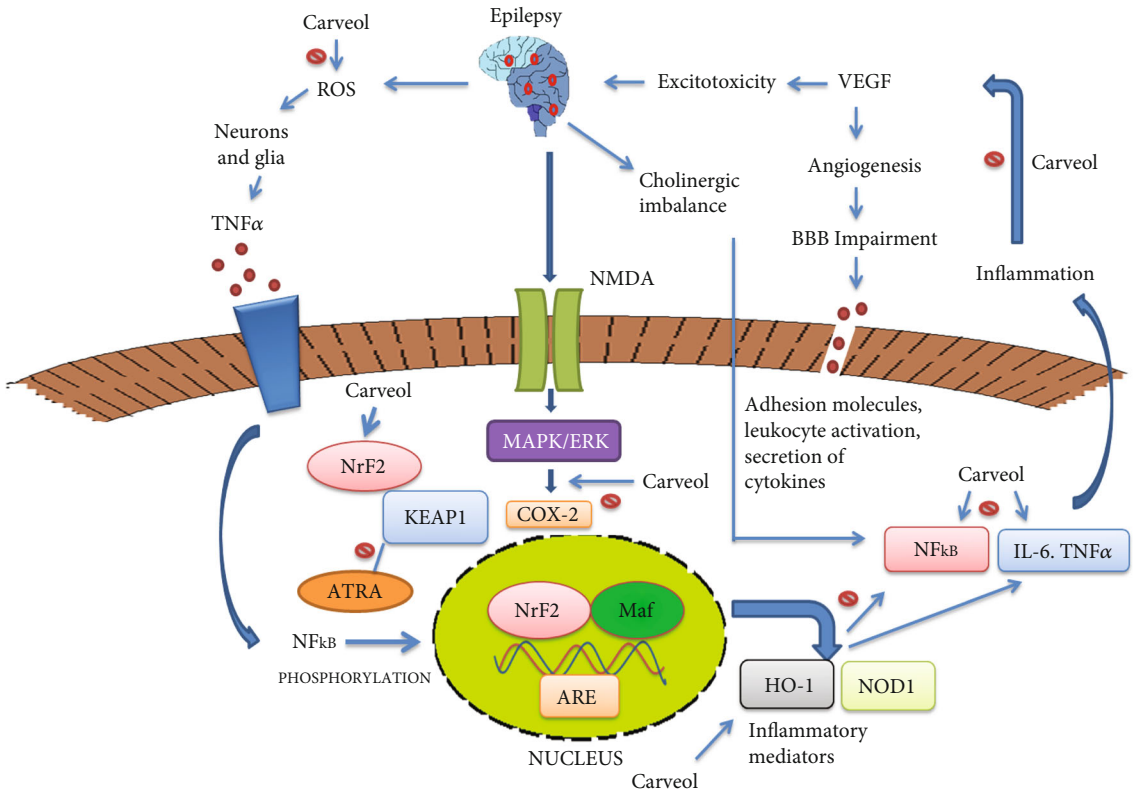


Figure 10: Diagrammatic illustration elaborating the underlying antioxidant and neuroprotective potential of carveol in a PTZ-induced epilepsy model.

(IL-6), interleukin-1 (IL-1b), and TNF-α that form the basis of neuronal injury in several neurodegenerative models including epilepsy [11, 84, 85]. Furthermore, studies have suggested increased COX-2 induction in the mouse brain after electrical kindling of hippocampal pyramidal cells [10]. Carveol significantly ameliorated the upregulated inflammatory mediators in PTZ-treated animals, which is consistent with recent findings regarding the modulatory effect of carveol on proinflammatory cytokines [33, 59]. ATRA treatment further exacerbated the expression of inflammatory markers and abolished the anti-inflammatory potential of carveol, which supports our hypothesis that carveol exerts its anti-inflammatory potential by modulating the *Nrf2* pathway.

Moreover, the cholinergic anti-inflammatory pathway is significantly involved in the modulation of immune response and inflammation in the brain [86]. This notion is further supported by the fact that several acetylcholinesterase (AChE) inhibitors promoted anti-inflammatory activities [82, 87]. Studies have suggested that the local immune response and inflammation are associated with the upregulation of hippocampal AChE levels, resulting in cholinergic imbalance and epileptogenesis [86]. Studies have shown that AChE may be an important therapeutic target for adjunct treatment in epilepsy as numerous AChE-inhibitors were tested in experimental settings for this purpose as a memory enhancer [88–90]. Moreover, our findings suggested increased brain AChE level, which was significantly inhibited by carveol treatment, and indicated a modulating effect of carveol on cholinergic transmission.

Studies have shown the association between vascular malformations and epilepsy, which suggested that subsequent BBB dysfunction could induce neuronal hyperactivity. Additionally, BBB leakage is associated with excessive angiogenesis induced by VEGF, and this expression is increased in patients with temporal lobe epilepsy [91]. Similarly, altered membrane fluidity and enhanced permeability caused by BBB disruption in epileptic tissue cause angiogenesis and dysfunctional vascular permeability [92]. Additionally, our experimental findings suggested upregulated VEGF expression in epileptic animals, which was ameliorated by carveol treatment, indicating an improvement in angiogenesis caused by PTZ.

5. Conclusions

In conclusion, our findings demonstrated that carveol could be a potent antioxidant and anti-inflammatory drug candidate that can exert neuroprotection in a PTZ-induced animal epilepsy model. We also demonstrated certain safety aspects of carveol, and it exhibited a relative safety profile as no impairment was observed in the kidneys, heart, liver, and brain further assisted by biochemical analysis. Furthermore, we demonstrated the involvement of the *Nrf2*-pathway in the neuroprotective activity of carveol (Figure 10). Additionally, we observed the potential of carveol to negatively modulate inflammatory mediators, angiogenic factors, and cholinergic imbalance; however, still, further experimentation is required to unveil its exact mechanism in epilepsy.

Data Availability

The research data used to support the findings of this study are included within the article.

Conflicts of Interest

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

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Supplementary Materials

Supplementary 1 Supplementary Figure S1: effect of carveol at 2000 mg/kg on the antioxidant enzymes of the brain. Supplementary 2. Supplementary Figure S2: effect of carveol at 2000 mg/kg on (a) liver enzymes, (b) renal function, (c) weights of organs, and (d) hematological indices. *(Supplementary Materials)*

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Research Article

Calcineurin Activation by Prion Protein Induces Neurotoxicity via Mitochondrial Reactive Oxygen Species

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Prion diseases are caused by PrPsc accumulation in the brain, which triggers dysfunctional mitochondrial injury and reactive oxygen species (ROS) generation in neurons. Recent studies on prion diseases suggest that endoplasmic reticulum (ER) stress induced by misfolding proteins such as misfolded prion protein results in activation of calcineurin. Calcineurin is a calcium-related protein phosphatase of type 2B that exists in copious quantities in the brain and acts as a critical nodal component in the control of cellular functions. To investigate the relationship between calcineurin and intracellular ROS, we assessed the alteration of CaN and ROS induced by prion peptide (PrP) 106-126. Human prion peptide increased mitochondrial ROS by activating calcineurin, and the inhibition of calcineurin activity protected mitochondrial function and neuronal apoptosis in neuronal cells. These results suggest that calcineurin plays a pivotal role in neuronal apoptosis by mediating mitochondrial injury and ROS in prion diseases.

1. Introduction

Prion diseases are fatal neurodegenerative conditions that cause protein accumulation in the brain. This accumulation is a key pathogenic mechanism of various neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease [1, 2]. In these diseases, the misfolded prion protein (PrPsc) is incorporated into the fibrillary beta-sheet-rich structures, known as amyloid plaques, that have been associated with numerous protein misfolding disorders [3]. Although there is literature that confirms that PrPsc strain induces neuronal apoptosis in the brain [4–6], the molecular mechanisms and signaling cascades that result in neuronal apoptosis remain unclear [7–9].

The synthetic human prion peptide (PrP) 106-126 originates in PrP molecules found in numerous species, preserves features of the physiological and pathogenic abilities of PrPsc, and can trigger neuronal apoptosis [10–12]. Amino acids 106-126 of the PrP sequence could replicate biological features of PrPsc *in vitro*, such as amyloidogenesis, and its neurotoxic as well as its gliotrophic effects [13–15]. Recent studies have used neurotoxic PrP peptides such as PrP 106-126 to test the neuroprotective effect of autophagy-inducing agents [16–18].

Accumulated evidence suggests that cellular damage, such as oxidative stress caused by free radicals and various proteins, is dynamically implicated in the cellular apoptosis associated with several neurodegenerative diseases [19–23]. There is also evidence that prion-mediated neuronal cell death is related to oxidative stress [24–26]. Mitochondria play an essential role in regulating apoptosis and the production of reactive oxygen species in many neurodegenerative disorders, including prion diseases [27–29]. In *in vitro* models, endoplasmic reticulum (ER) stress resulting from the accumulation of misfolded prion proteins is associated with mitochondrial dysfunction and ROS production [30–32]. Over the past decade, numerous studies have used mitochondrion-targeted probe MitoSOX for mitochondrial ROS detection [33, 34]. Dichlorodihydrofluorescein diacetate (DCFH-DA) is typically used for detecting intracellular ROS [35]. To determine the role of cellular ROS and mitochondrial ROS in prion-mediated neuronal apoptosis, we performed experiments to detect cellular ROS and mitochondrial ROS.

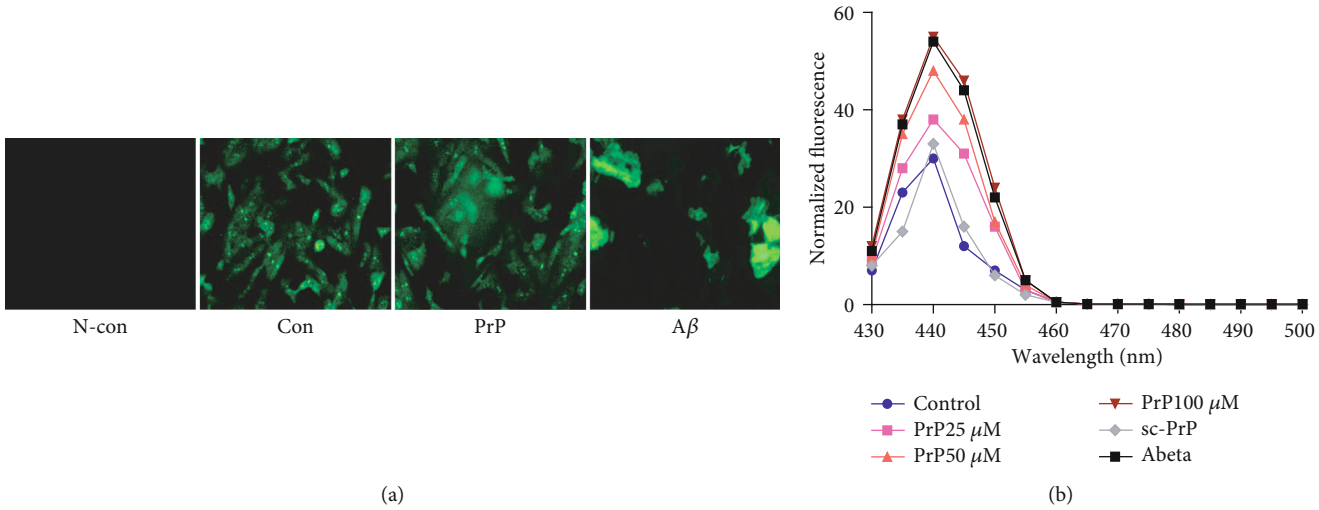


FIGURE 1: Prion peptide 106-126 aggregation status. (a) Neuroblastoma cells (SK-N-SH) were pretreated with 20 μM ThT for 30 min and then exposed to 25, 50, and 100 μM PrP 106-126, 100 μM sc-PrP, or 100 μM amyloid β for 6 h. (b) Fluorescence was evaluated by spectrum analysis and fluorescence microscopy.

Impaired calcium signaling caused by the accumulation of a misfolded prion protein triggers crosstalk between the endoplasmic reticulum and the mitochondria as a means of counteracting stress [36–38]. Our previous studies suggested that PrP 106-126 increases intracellular calcium levels and calcineurin (CaN) activity in neurons [39, 40]. Other researchers have shown that increased calcium levels are associated with the mitochondrial apoptotic pathway in neurodegenerative diseases that involve prions [31, 41]. When mitochondrial depolarization occurs with a sustained cytosolic rise in Ca^{2+}, the cytosolic phosphatase activity of the serine/threonine phosphatase CaN is activated [42]. Activated CaN dephosphorylates cytoplasmic NFAT, which exposes its nuclear localization sequence and leads to rapid nuclear import [43]. CaN is a calcium-mediated type 2B protein phosphatase and a crucial nodal factor in controlling cellular functions [44]. CaN is highly abundant in the brain and has been implicated in the regulation of synaptic plasticity, memory, and neuronal death [45]. The relationship between prion-mediated ROS and CaN activation has, until now, not been described. In this study, we investigated what kind of ROS is generated by a prion peptide and the relationship between CaN and intracellular ROS in prion *in vitro* models.

2. Materials and Methods

2.1. Cell Culture. The human neuroblastoma cell line SK-N-SH was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell culturing method has been described previously in detail [39]. The cells were cultured in Minimum Essential Medium (HyClone Laboratories, Logan, UT, USA) containing 10% FBS (Gibco, Grand Island, NY, USA) and gentamycin (0.1 mg/mL) in a humidified incubator at 37°C with 5% CO_2.

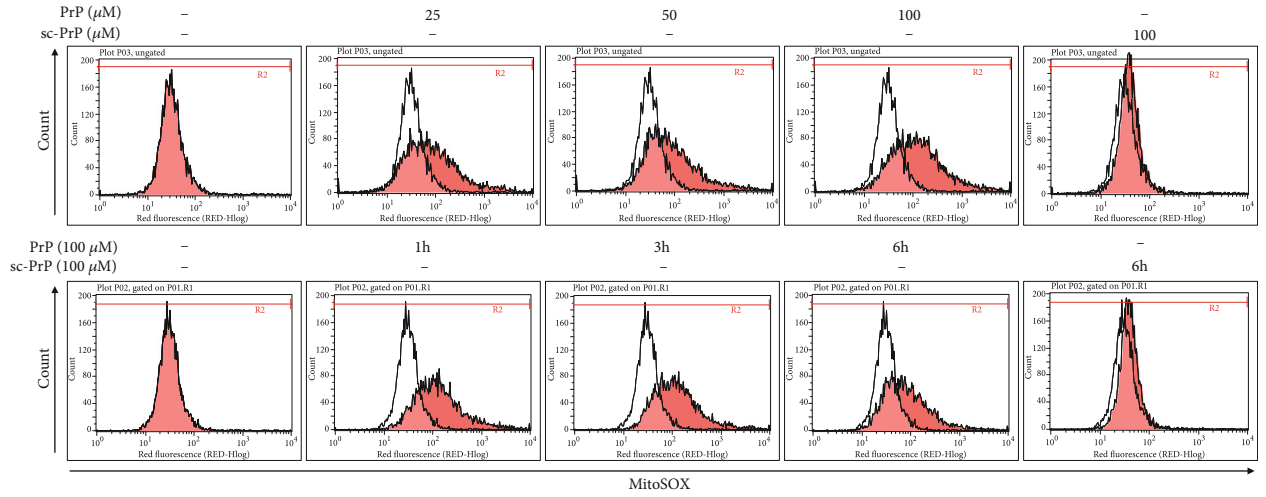
2.2. Chemical and PrP (106-126) Treatment. Synthetic prion peptides PrP (106-126) (sequence, Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly) and scrambled PrP (106-126) (sequence, Asn-Gly-Ala-Lys-Ala-Leu-Met-Gly-Gly-His-Gly-Ala-Thr-Lys-Val-Met-Val-Gly-Ala-Ala-Ala) were synthesized by Peptron (Seoul, Korea) [20]. The PrP peptides were dissolved in sterile dimethyl sulfoxide (DMSO) at a concentration of 10 mM (stock) and stored at -20°C.

The stock solution of FK506 (10 mM; F4679, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO. The stock solution of *N*-acetyl-L-cysteine (NAC, 1 M; A7250, Sigma-Aldrich), diethyldithiocarbamate (DDC, 100 mM; Sigma-Aldrich), and 3-amino-1,2,4-triazole (AT, 500 mM; Sigma-Aldrich) was dissolved in distilled water.

2.3. Thioflavin-T Binding Assay. Cells in the logarithmic phase were collected and cultured in 6-well plates at 3×10^5 cells/well. Binding of ThT to PrP 106-126 and amyloid β fibrils was assayed by adding 20 μM ThT (T3516, Sigma-Aldrich, St. Louis, MO, USA) to a solution of fibrils. Fluorescence was monitored using a SpectraMax M2 (Molecular Devices) with excitation and emission slit widths set to 5 nm, respectively. Spectra were obtained by scanning the fluorescence emission from 430 to 500 nm, with excitation at 442 nm. Fluorescence images were obtained using fluorescence microscopy (Nikon Eclipse 80i). An image was evaluated using the NIS-Elements F ver4.60 Imaging software.

2.4. Annexin V Assay. Cells in the logarithmic phase were collected and cultured in a 24-well plate at 4×10^4 cells/well. Cell survival was evaluated using an annexin V assay kit (Santa Cruz Biotechnology, CA, USA) following the manufacturer's procedure. The fluorescence was determined at 488 nm excitation and 525/30 emission using a Guava EasyCyte HT System (Millipore, Bedford, MA, USA).

2.5. Terminal Deoxynucleotidyl Transferase dUTP Nick End-Labeling (TUNEL) Assay. Cells in the logarithmic phase were collected and cultured in 6-well plates at 3×10^5 cells/well. After treatment, neuronal apoptosis was assessed by using



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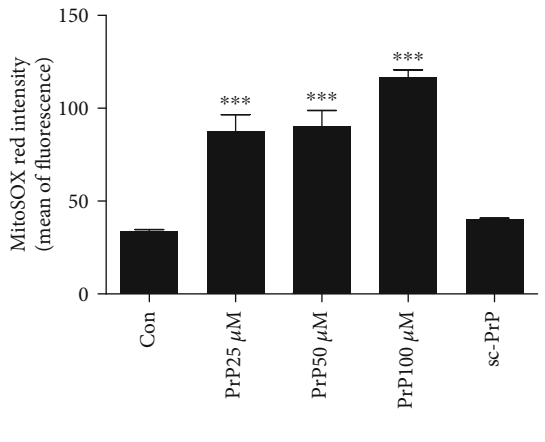

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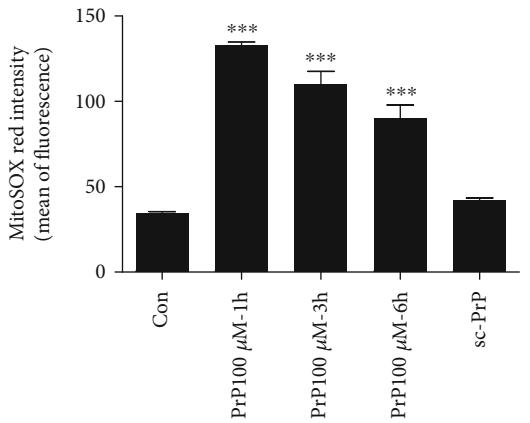

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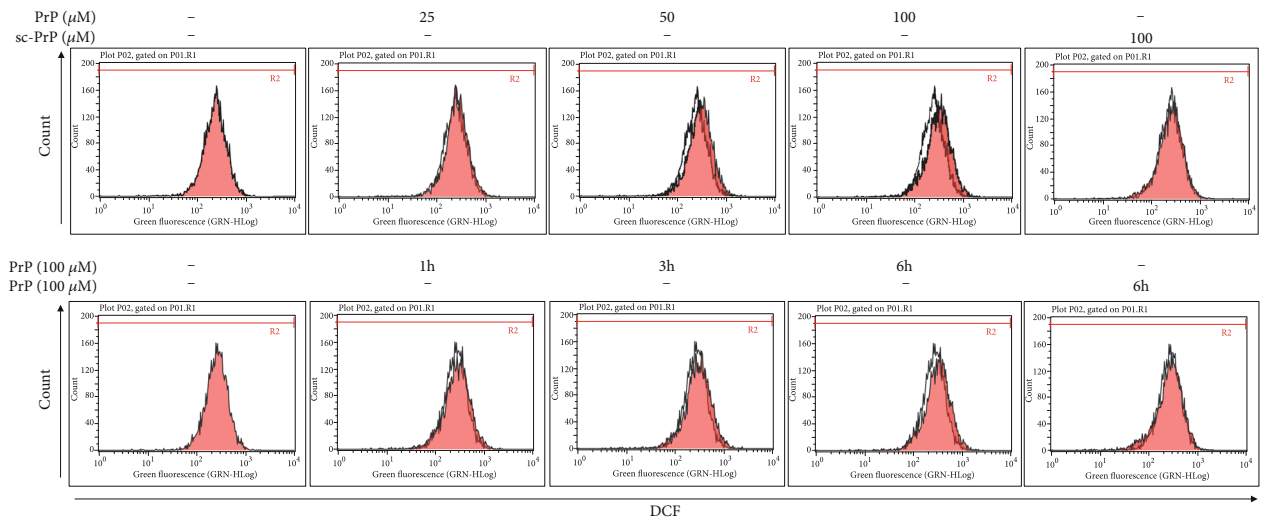

(d)

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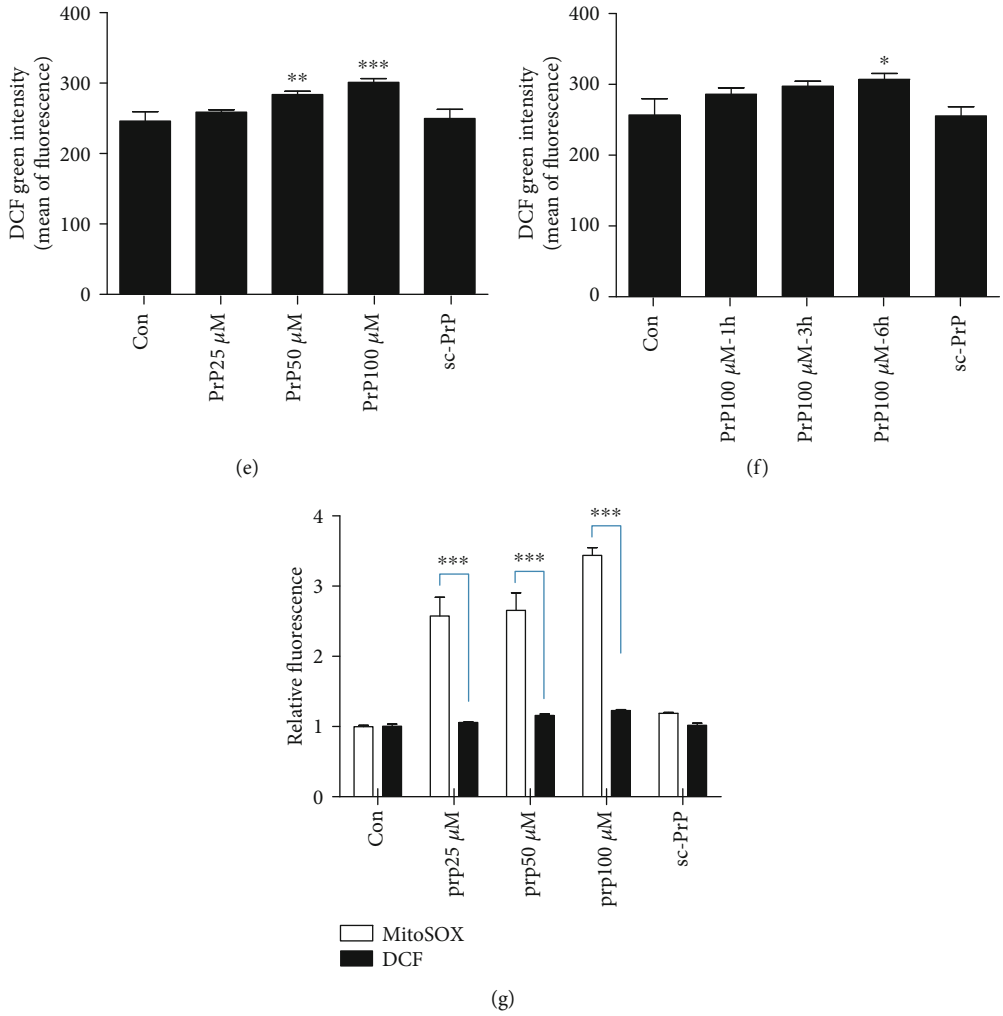


Figure 2: PrP (106-126) increased superoxide more than hydrogen peroxide. (a) Neuroblastoma cells (SK-N-SH) were exposed to PrP (106-126) or scrambled PrP in a dose- and time-dependent manner. Mitochondrial ROS was evaluated by a MitoSOX assay. (b, c) Bar graph showing the averages of the red fluorescence (MitoSOX) in a PrP dose- and time-dependent manner, respectively. Values represent the mean ± SEM ($n = 10$). $^{***}p < 0.001$ vs. control. (d) SK-N-SH cells were treated with PrP (106-126) or scrambled PrP in a dose- and time-dependent manner. Cytosol ROS was evaluated by a DCF assay. (e, f) Bar graph showing the averages of the green fluorescence (DCF) in a PrP dose- and time-dependent manner, respectively. (g) Relative MitoSOX and DCF fluorescence in a PrP dose-dependent manner. Values represent the mean ± SEM ($n = 10$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control.

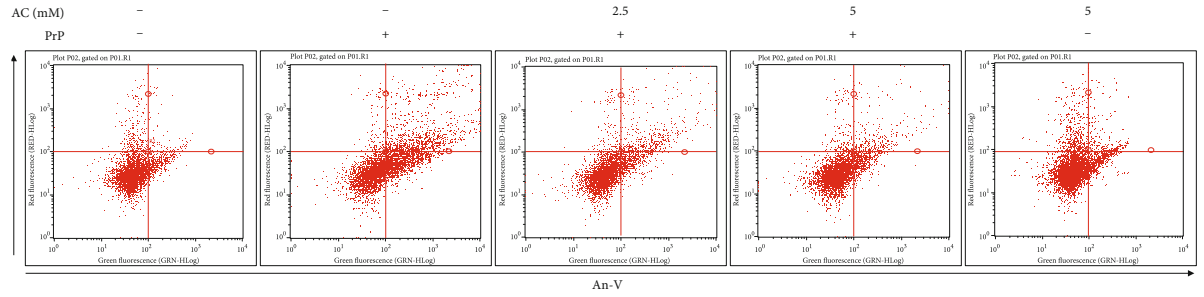
an ApoBrdU DNA Fragmentation Assay Kit (BioVision, Mountain View, CA, USA), consistent with the manufacturer's instructions. The nuclei were counterstained with PI.

2.6. Confocal Microscopy. In a confocal dish, SK-N-SH cells were incubated in HBSS medium (Gibco, Grand Island, NY, USA) containing 5 μM MitoSOX and washed three times with HBSS. The cells were imaged on a Zeiss LSM710 microscope equipped with a standard set of lasers through a 63x oil objective, installed at the Center for University Wide Research Facilities at Jeonbuk National University. The excitation wavelengths were 488, 543, and 633 nm. The bandpass filters were set at 500–550 (Alexa Fluor 488), 560–615 nm (Cy3, Alexa Fluor 568), and 650–750 nm (Alexa Fluor 647).

2.7. Cytosol and Mitochondrial ROS Assay. SK-N-SH cells were incubated in either HBSS containing 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA) at 37°C for 30 min or 5 μM MitoSOX at 37°C for 10 min. Cells were transferred to a clear 96-well plate for flow cytometry analysis using a Guava EasyCyte HT System (Millipore, Bedford, MA, USA).

2.8. JC-1 Assay. SK-N-SH cells were incubated in HBSS containing 10 μM JC-1 at 37°C for 30 min. Cells were transferred to a clear 96-well plate for flow cytometry analysis using a Guava EasyCyte HT System (Millipore, Bedford, MA, USA). JC-1-stained cells on coverslips were imaged on a fluorescence microscope (Nikon Eclipse 80i).

2.9. Calcineurin Activity Assay. The calcineurin cellular activity assay kit (Enzo Life Sciences #BML-AK816-0001, USA) was used consistent with the manufacturer's instructions to determine the phosphatase activity of calcineurin in SK-N-SH cells [39]. In brief, the cells were lysed on ice in lysis buffer containing protease inhibitors. Phosphatase activity was



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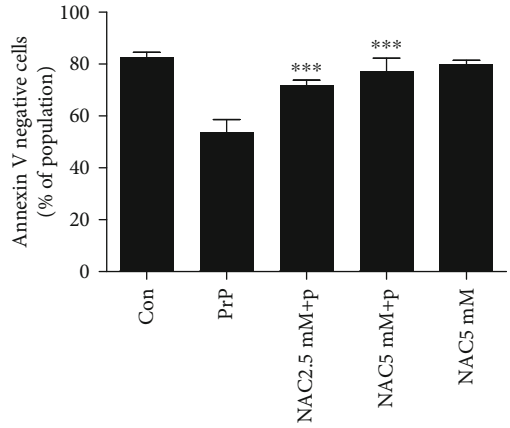

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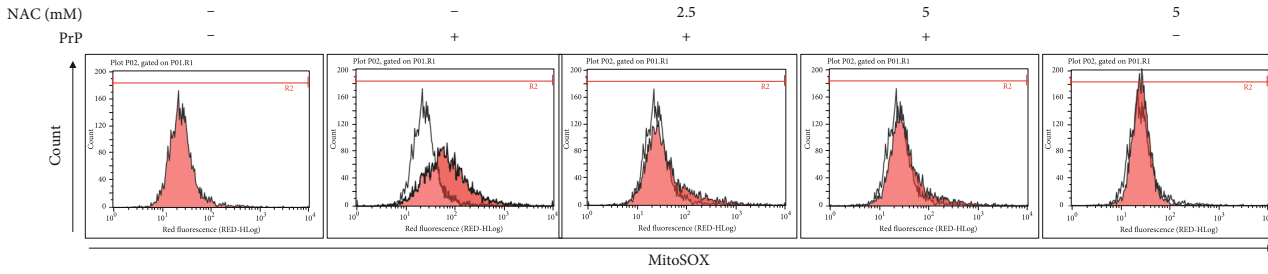

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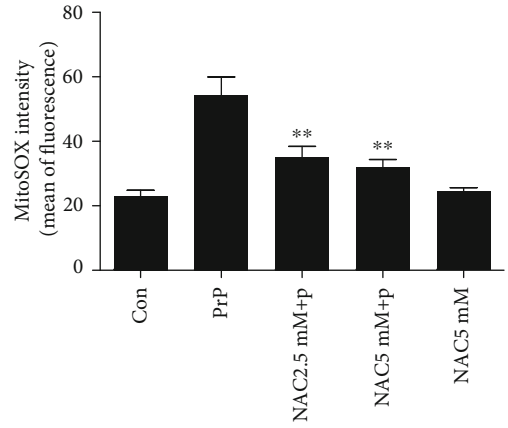


(d)

Figure 3: ROS scavenger treatment attenuated PrP (106-126)-mediated mitochondrial ROS and neurotoxicity. SK-N-SH cells were pretreated with NAC (*N*-acetyl-l-cysteine) (1 h) at 2.5 and 5 mM and then exposed to 100 μM PrP (106-126) for 6 hours. (a) Cell viability was evaluated by an annexin V assay using FITC-annexin V, which combines with phosphatidylserine on the plasma membrane during the apoptotic processes. (b) Bar graph showing the averages of the annexin V-negative cells. Values represent the mean ± SEM ($n = 10$). $^{***}p < 0.001$ vs. PrP. (c) Mitochondrial ROS was evaluated by a MitoSOX assay. (d) Bar graph showing the averages of the red fluorescence (MitoSOX). Values represent the mean ± SEM ($n = 10$). $^{**}p < 0.01$ vs. PrP.

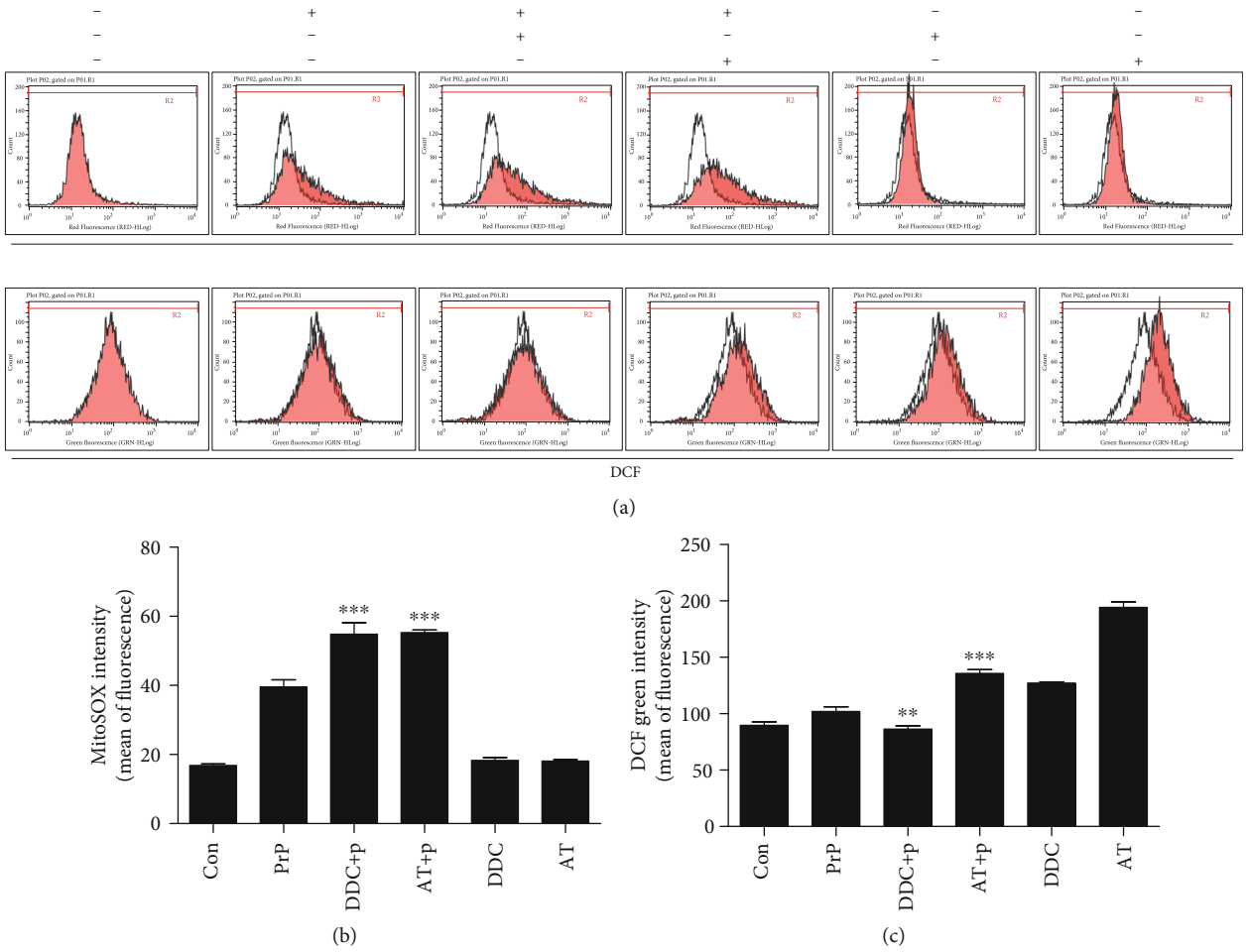


Figure 4: PrP (106-126) did not influence SOD or catalase enzyme. SK-N-SH cells were pretreated with DDC (diethyldithiocarbamate) (1 h) or AT (3-amino-1,2,4-triazole) (24 h) and then exposed to 100 μM PrP (106-126) for 6 hours. (a) Mitochondrial ROS was evaluated by a MitoSOX assay, and cytosol ROS was evaluated by a DCF assay. (b) Bar graph showing the averages of the red fluorescence (MitoSOX). (c) Bar graph showing the averages of the green fluorescence (DCF). Values represent the mean ± SEM ($n = 10$). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. PrP.

quantified by detecting free phosphate released from the reaction by measuring the absorbance of malachite green (OD 620 nm) using a SpectraMax M2 (Molecular Devices).

2.10. Western Blot Analysis. Cells in the logarithmic phase were collected and cultured in a 6-well plate at 3×10^5 cells/well. The western blot method has been described in detail previously [20]. We used a nuclear/cytosol fractionation kit (#K266, BioVision). After treatments, cells were washed with PBS and lysed in lysis buffer (25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, 100 mM NaCl, 1 mM ethylene diamine tetra acetic acid (EDTA), 5 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), and a protease inhibitor mixture). Equal quantities of proteins (more than 15 μg/μL) in the nuclear or cytosolic extracts were electrophoretically resolved on a 10% sodium dodecyl sulfate (SDS) poly-acrylamide gel and transferred to a nitrocellulose membrane. Immunoreactivity was detected through consecutive incubation with blocking solution using 5% skim milk and primary antibodies, followed by the corresponding horseradish peroxidase-conjugated secondary antibodies, and finally developed using enhanced chemiluminescence substances (i.e., west save gold detection kit (LF-QC0103, AbFrontier Inc.)). The primary antibodies (anti-calcineurin at a dilution of 1 : 1000 (ab109412, Abcam plc), anti-NFAT1 at a dilution of 1 : 1000 (#5861, Cell Signaling), anti-lamin A/C at a dilution of 1 : 10000 (ab108595, Abcam plc), and anti-β-actin at a dilution of 1 : 5000 (A5441, Sigma Aldrich)) were diluted with antibody solution (1% skim milk in TBST). Images were inspected using a Fusion FX7 imaging system (Vilber Lourmat, Torcy Z.I. Sud, France). Densitometry of the signal bands was evaluated using the Bio-1D software (Vilber Lourmat, Marne La Vallee, France).

2.11. Statistical Analysis. Results are expressed as the means ± standard error of the mean (SEM) from at least three independent replicates. All experiments were analyzed by the one-way analysis of variance (ANOVA). Comparisons of three or more groups were made using Tukey's posttests. All statistical analyses were implemented with GraphPad Prism version 5.0. p values ($^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$) were considered statistically significant.

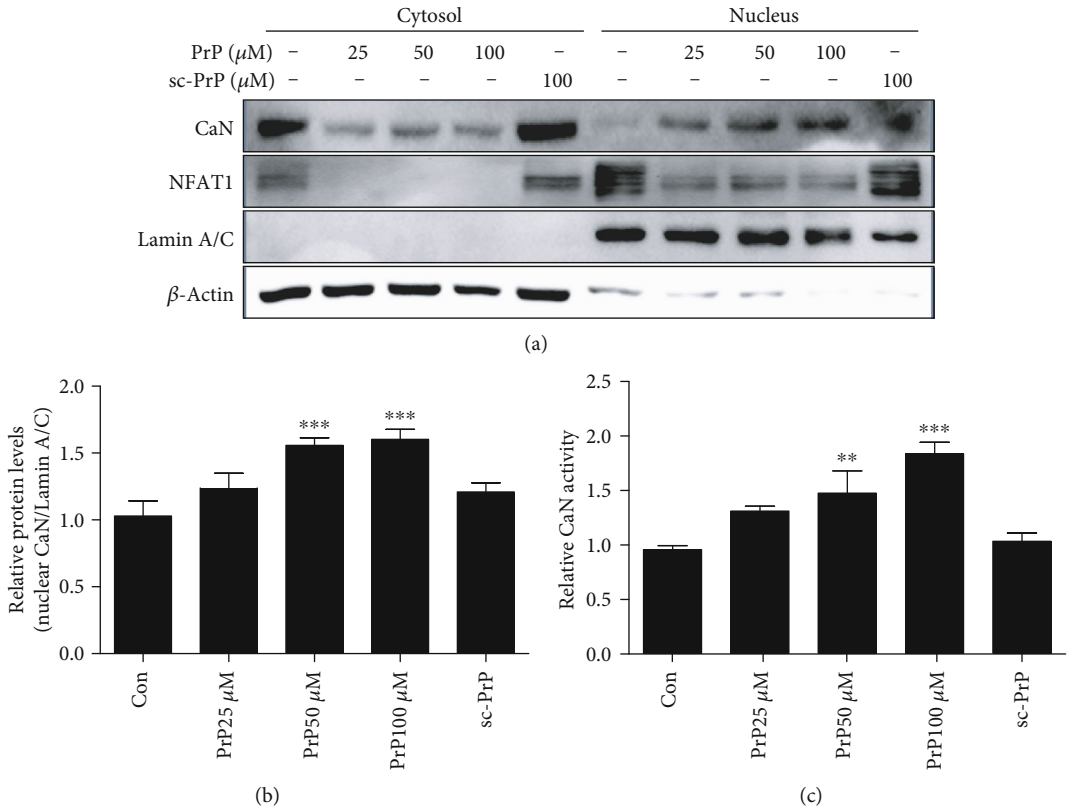


Figure 5: PrP (106-126) treatment upregulated calcineurin (CaN) activity. SK-N-SH cells were exposed to PrP (106-126) or scrambled PrP in a dose-dependent manner for 5 hours. (a) Cytosolic and nuclear fractions obtained from SK-N-SH cells induced for human calcineurin expression are analyzed by western blot with antibodies for CaN, for the cytosolic β-actin marker, and for the nuclear lamin A/C marker. (b) Bar graph representing the average nuclear CaN protein levels. The expression data were normalized to lamin A/C expression. The expression levels were evaluated by quantifying the protein bands, depicted by densitometric values beside the blot. Values represent the mean ± SEM ($n = 5$). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

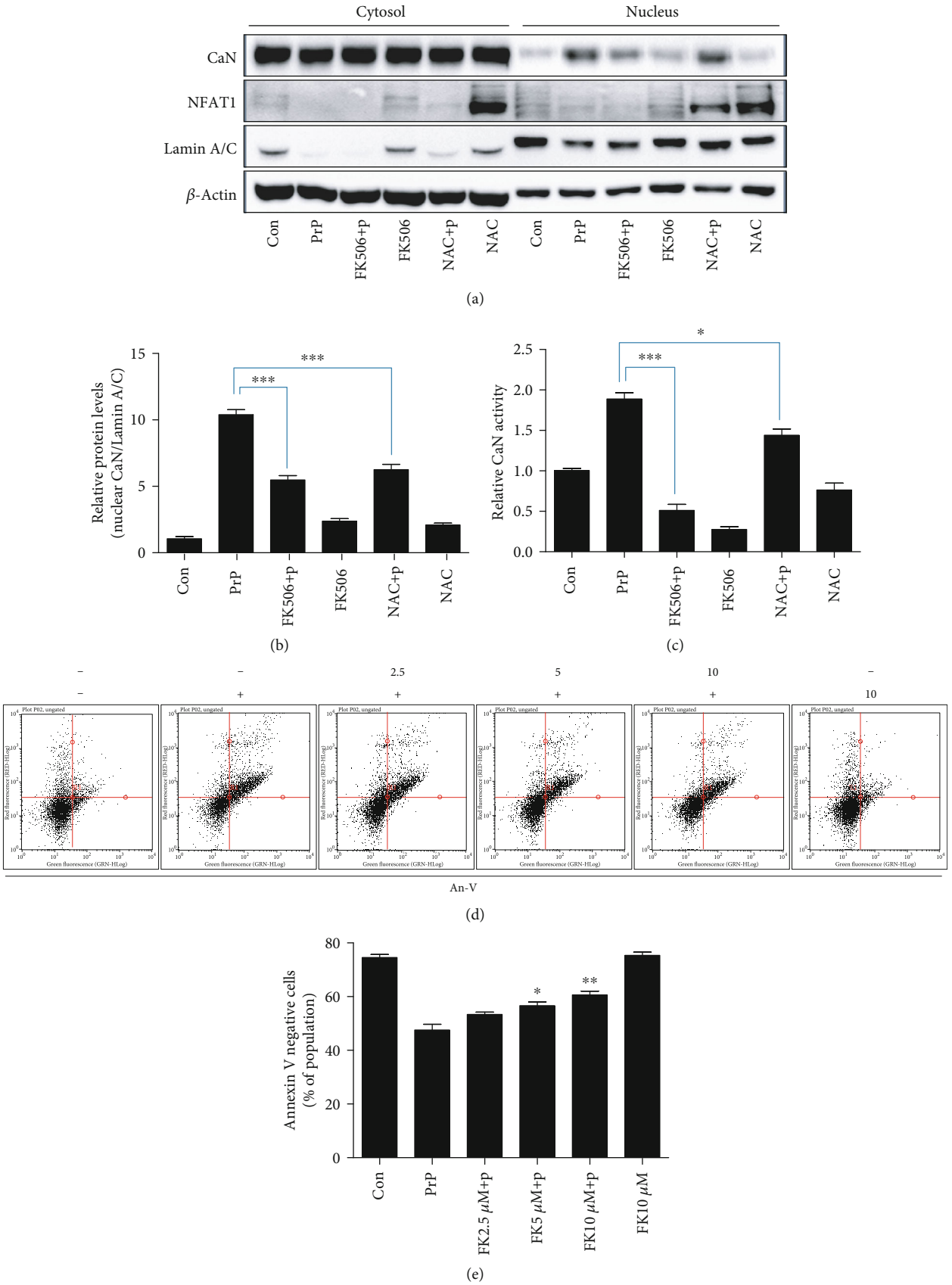
3. Results

3.1. Prion Peptide 106-126 Generated More Mitochondrial ROS than Cytosolic ROS. Prion peptide 106-126 has previously been known to induce neurotoxicity as a result of its ability to form aggregates [46]. Thioflavin-T (ThT) binding was employed to confirm the amyloid fibrils formed by our PrP 106-126 peptides. Binding of ThT to polypeptide chains is specific for the cross-β structure of amyloid fibrils. We identified binding of ThT to prion peptide and amyloid β by fluorescence microscopy (Figure 1(a)). An increase in ThT fluorescence at 430–500 nm is observed, as shown in Figure 1(b), supporting a cross-β structure for PrP 106-126 fibrils.

Although there is prior research that has suggested that prion peptide could induce cellular ROS in neurons, ROS's origin has not been examined. To determine this, we performed mitochondrial and cytosolic ROS detection experiments using both DCF and MitoSOX assays. PrP (106-126)-mediated mitochondrial ROS generation rose dose- and time-dependently in SK-N-SH neuroblastoma cells, whereas scrambled PrP did not increase mitochondrial ROS (Figures 2(a)–2(c)). PrP (106-126) slightly increased cytosolic ROS generation dose- and time-dependently (Figures 2(d)–2(f)). We determined that PrP (106-126) upregulated mitochondrial ROS more than cytosolic ROS (Figure 2(g)), suggesting that PrP (106-126) influences mitochondrial ROS and mitochondrial dysfunction in neuronal cells.

3.2. Prion Peptide 106-126 Promotes Neuronal Apoptosis through Mitochondrial ROS Generation. We examined whether PrP-induced mitochondrial ROS influenced neurotoxicity using the ROS scavenger NAC. NAC treatment attenuated PrP-mediated neuronal apoptosis dose-dependently (Figures 3(a) and 3(b)). We determined that NAC, as a reactive oxygen species (ROS) scavenger, decreased PrP-mediated mitochondrial ROS generation (Figures 3(c) and 3(d)), confirming that the prion peptide induces neuronal apoptosis through mitochondrial ROS generation.

Sinclair et al. have suggested that the prion aggravates an apoptotic pathway through mitochondrial dysfunction and mislocalisation of SOD2 to cytosolic caspases [47]. We investigated the impact of PrP (106-126) on superoxide dismutases (SODs) and a catalase enzyme using an SOD inhibitor (diethyldithiocarbamate; DDC) and a catalase inhibitor (3-amino-1,2,4-triazole; AT). We determined that DDC and AT increased mitochondrial ROS, including superoxide, which had already been raised by PrP (106-126), meaning that this prion peptide did not influence SOD function (Figures 4(a) and 4(b)). DDC decreased cytosolic ROS such



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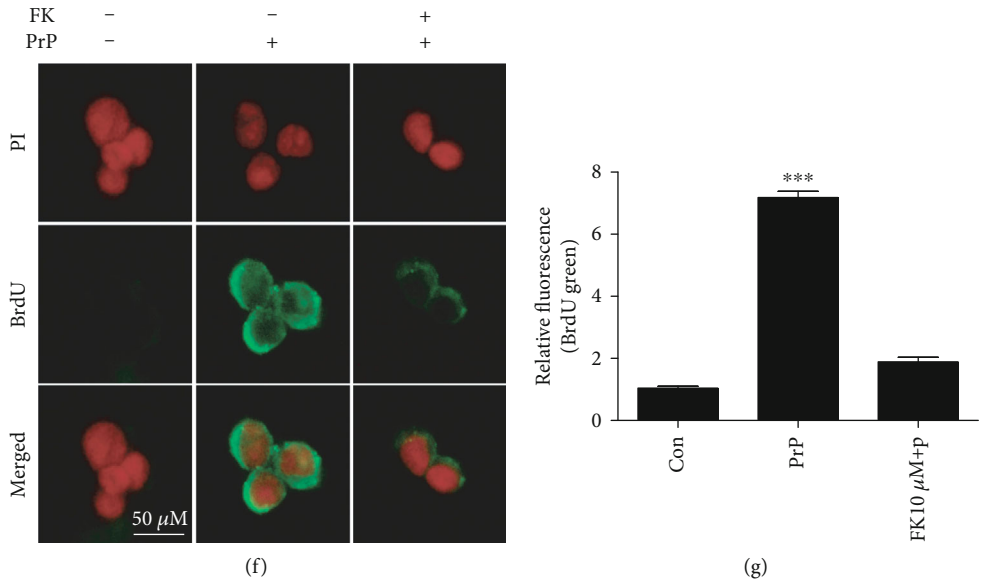


Figure 6: FK506 treatment attenuates PrP (106-126)-induced neurotoxicity via CaN inhibition. SK-N-SH cells were pretreated with FK506 (10 μM, 1 h) or NAC (5 mM, 1 h) and then exposed to 100 μM PrP (106-126) for 5 hours. (a) Cytosolic and nuclear fractions obtained from SK-N-SH cells induced for human calcineurin expression are analyzed by western blot with antibodies for CaN, for the cytosolic β-actin marker, and for the nuclear lamin A/C marker. (b) Bar graph representing the average nuclear CaN protein levels. The expression data were normalized to lamin A/C expression. (c) CaN activity was evaluated by a CaN activity assay. Values represent the mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{***}p < 0.001$ vs. PrP. (d) SK-N-SH cells were pretreated with FK506 (1 h) in a dose-dependent manner and then exposed to 100 μM PrP (106-126) for 6 hours. Cell viability was evaluated by an annexin V assay using FITC-annexin V, which combines with phosphatidylserine on the plasma membrane during apoptotic processes. (e) Bar graph showing the averages of the annexin V-negative cells. Values represent the mean ± SEM ($n = 10$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. PrP. (f) TUNEL-positive (green) immunofluorescence images were obtained after exposure to 100 μM PrP (106-126) (6 h) in the absence or presence of FK506 (10 μM, 1 h). The cell nuclei were counterstained with PI (red). (g) Bar graph showing the relative mean values of the green fluorescence (BrdU). Values represent the mean ± SEM ($n = 5$). $^{***}p < 0.001$ vs. control.

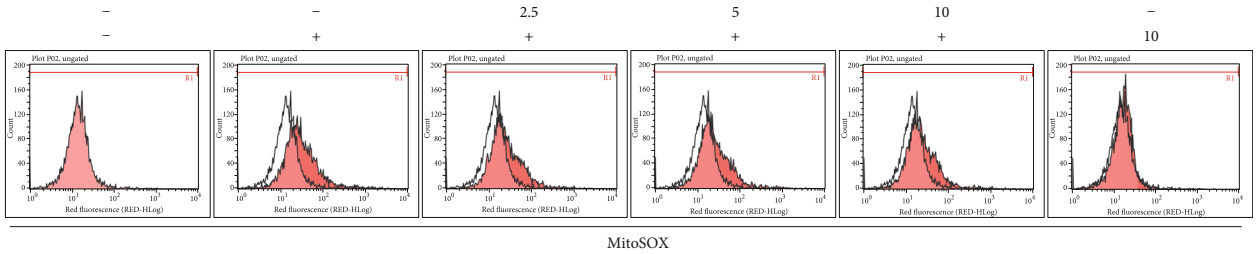
as hydrogen peroxide while AT increased cytosolic ROS that had already been raised by PrP (106-126) (Figures 4(a) and 4(c)), meaning that PrP (106-126) did not impair the SOD and the catalase. These results indicate that the prion peptide increased mitochondrial ROS production through pathways other than SOD and catalase impairment.

3.3. Prion Peptide Induced Neurotoxicity via CaN Activation. In a previous study, we demonstrated how PrP (106-126) induces neurotoxicity through calcium alteration [40]. In this study, we checked whether PrP (106-126) alters CaN in neuronal cells. PrP (106-126) increased nuclear CaN translocation dose-dependently (Figures 5(a) and 5(b)). We identified CaN activation by prion peptide treatment using a CaN phosphatase activity assay (Figure 5(c)). We also identified NFAT1 as a transcriptional factor related to CaN, and PrP (106-126) decreased NFAT1 protein expression in both the nucleus and cytosol (Figure 5(a)). This result suggests that NFAT is not dependent on CaN.

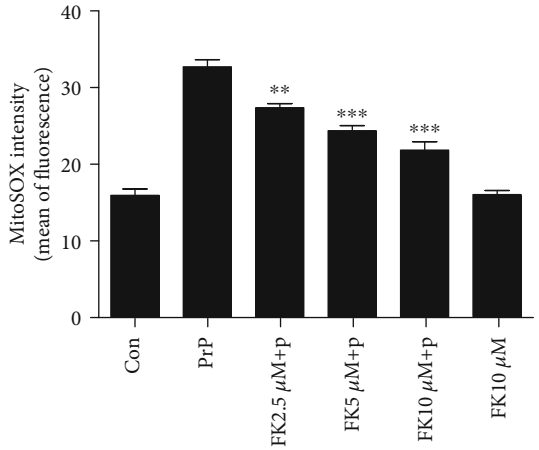
PrP (106-126)-increased CaN was reduced by the CaN inhibitor FK506 and ROS scavenger NAC (Figures 6(a) and 6(b)). CaN activity was also decreased by FK506 and NAC (Figure 6(c)). NFAT1 is decreased in PrP-treated cells, and FK506 also decreased NFAT1 protein expression (Figure 6(a)). Based on these results, we suggest that CaN and ROS influence each other through a feedback loop.

To investigate the effect of prion peptide-mediated CaN activation on neuronal apoptosis, we employed the CaN inhibitor FK506. We found that FK506 attenuated prion peptide-induced neuronal apoptosis dose-dependently using an An-V/PI assay (Figures 6(d) and 6(e)). In addition, we identified that FK506 repressed PrP-mediated DNA strand damage using a TUNEL assay (Figures 6(f) and 6(g)). We suggest that PrP (106-126) induces neuronal apoptosis through CaN activation.

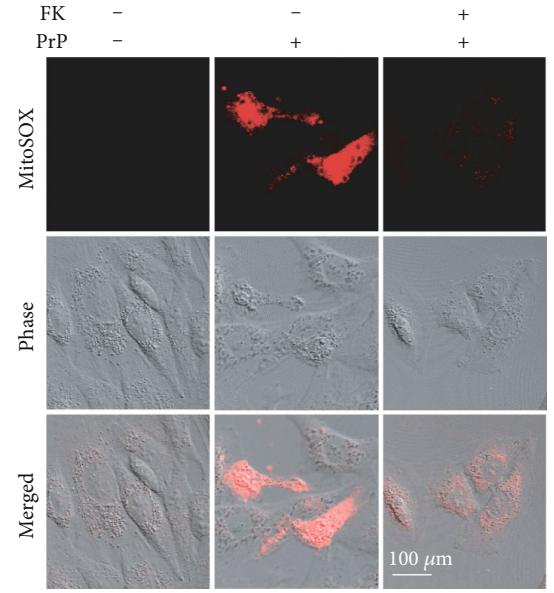
3.4. CaN Activation by Prion Peptide Promoted Mitochondrial Dysfunction. To investigate the effect of prion peptide inducing CaN activation on mitochondrial function, we ran an experiment using the MitoSOX and JC-1 assay using FK506. We used the MitoSOX assay to determine that treatment with FK506 decreased the mitochondrial ROS in PrP-treated neuronal cells (Figures 7(a) and 7(b)). We also imaged the MitoSOX using confocal microscopy (Figure 7(c)). Further, we determined that FK506 repressed the prion peptide-induced mitochondrial dysfunction using the JC-1 assay (Figures 7(d) and 7(e)) and fluorescence microscopy (Figure 7(f)). These results demonstrate that prion peptide-mediated neuronal apoptosis is dependent on CaN activation and mitochondrial ROS and that CaN is a key regulator of prion peptide-mediated ROS generation and neurotoxicity. In sum, our results suggest that prion



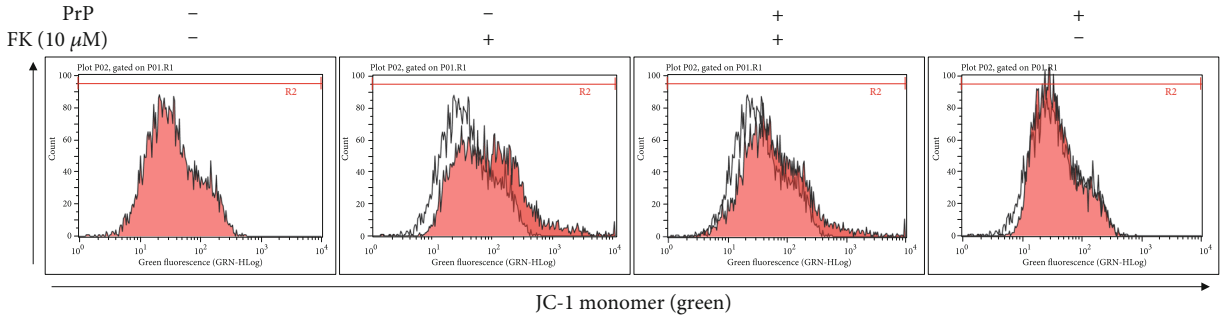
(a)



(b)



(c)



(d)

Figure 7: Continued.

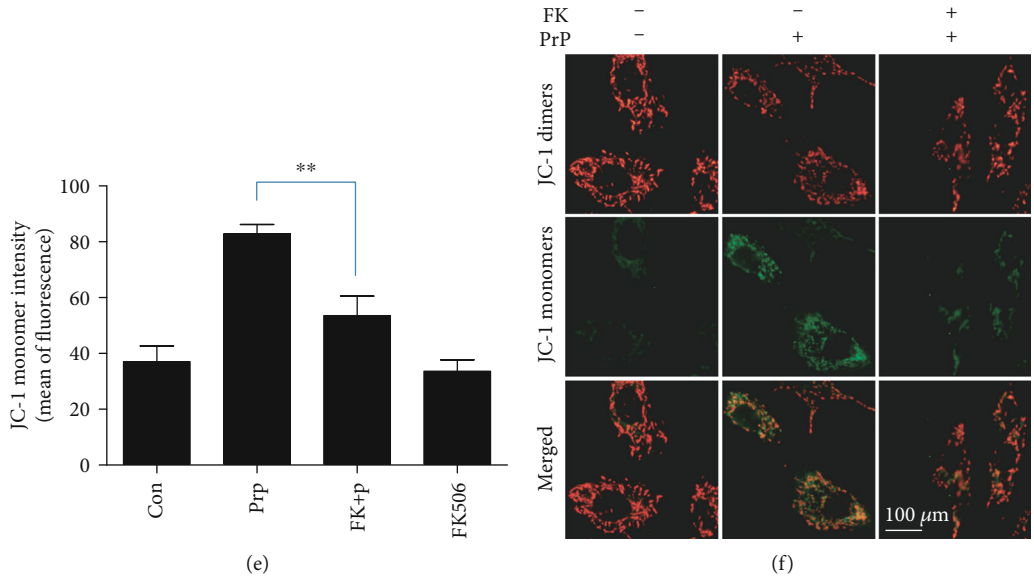


Figure 7: PrP (106-126)-mediated calcineurin activation induced neurotoxicity via mitochondrial dysfunction. SK-N-SH cells were pretreated with FK506 (1 h) and then exposed to 100 μM PrP (106-126) for 6 hours. (a) Mitochondrial ROS was evaluated by a MitoSOX assay. (b) Bar graph showing the averages of the red fluorescence (MitoSOX). Values represent the mean ± SEM ($n = 10$). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. PrP. (c) MitoSOX fluorescence images were obtained after exposure to 100 μM PrP (106-126) (6 h) in the absence or presence of FK506 (10 μM, 1 h). (d) Mitochondrial membrane potential was evaluated by a JC-1 assay using flow cytometry. In green fluorescent colors, JC-1 accumulates as green monomers in the mitochondria of cells with impaired mitochondrial membrane potential function. (e) Bar graph showing the averages of the green fluorescence (JC-1 monomers). Values represent the mean ± SEM ($n = 10$). $^{**}p < 0.01$ vs. PrP. (f) JC-1 fluorescence images were obtained after exposure to 100 μM PrP (106-126) (6 h) in the absence or presence of FK506 (10 μM, 1 h).

peptide (106-126) induces mitochondrial ROS and neuronal apoptosis through CaN activation in neuronal cells.

4. Discussion

Neurotoxic PrP 106-126 preserves biochemical substances similar to those of the abnormally folded prion protein PrPsc, together with protease resistance, β-sheet construction, and cytotoxicity [13, 48–50]. Previous literature has investigated prion pathogenesis and neurotoxic pathways using Rocky Mountain Laboratory (RML) strain and antibody-derived anti-PrP ligands in *in vivo* models [51–53]. Typical neurodegenerative fluctuations in prion diseases have been observed in the absence of detectable PrPsc, suggesting that prion disorders are caused by alternative mechanisms of neuronal damage, as well as PrPsc [54, 55]. PrP 106-126 has been suggested as one such alternative contributor to the pathogenic and molecular properties of PrPsc [56]. Although the functional and mechanical properties of prion protein remain unclear, a number of studies have suggested that prions are triggered by accumulation in the brain of amyloid plaque PrPsc, similar to amyloid-β in Alzheimer's disease [5, 57, 58].

CaN was recently suggested as an important therapeutic target for the potential treatment of neurodegenerative diseases [59, 60]. CaN is abundant in the synaptic terminals and cytosol of neurons, which suggest that it may play a critical role in the maintenance of cellular homeostasis during calcium alteration [44, 45]. Hyperactivation of CaN is implicated in a reversible neuronal apoptotic pathway involving Bcl-2 family proteins [61, 62]. Moreover, CaN plays an essential role in modulating gene expression, including cAMP-response element binding protein (CREB) and nuclear factor of activated T-cell (NFAT), as well as regulating calcium alteration [44, 63–65]. Since phosphorylated CREB and dephosphorylated NFAT4 translocate into the nucleus and induce gene expression, several studies have observed CaN protein expression in nuclear fraction [66–68]. Consistent with these studies, we identified translocation of CaN into a nucleus caused by prion peptide treatment in neuronal cells (Figure 4). Other literature has suggested that CaN is activated by cleavage of the autoinhibitory domain that transforms it into a constitutively active form [69–71]. We could not observe any meaningful cleavage form CaN in prion peptide-treated neuronal cells (not shown). Accordingly, we suggest that prion peptide may induce CaN activation by nuclear translocation. However, the association between CaN and neurodegenerative progression remains contentious [72–74].

In our previous study, we prove that prion peptide 106-126 induced neurotoxicity through the AMPK-CaN-autophagy pathway [39, 40]. AMPK has been recognized in the past few years to act as a crucial integrator of autophagy and mitochondrial homeostasis by controlling various aspects of the cellular life cycle [75]. In this study, our goal is to investigate what kind of ROS is generated in prion-mediated neurotoxic conditions and elucidate the relationship between this ROS and CaN pathway in prion diseases. In future, further studies are required to investigate the role

of AMPK in this mitochondrial ROS generation as well as apoptosis in prion diseases.

We observed that the expression of NFAT1 was decreased in the nucleus in PrP-treated condition (Figure 5(a)). CaN is well known to the phosphatase that promotes NFAT nuclear import, and its activity is regulated not only by upstream Ca^{2+} and calmodulin but also by multiple endogenous calcineurin inhibitors [76]. However, the specific role of each NFAT member in gene transcription during the cell cycle and apoptosis is not fully clear, especially in neurons [77]. In our results, NFAT1 alteration was not dependent on the CaN (Figures 5 and 6), but rather the total amount of NFAT1 of whole cell lysates was decreased in PrP-treated cells. Further studies will be needed to discover how prion peptide decreased NFAT1 protein expression in cellular pathways.

We demonstrated ROS generation using the MitoSOX and DCF assay. A MitoSOX usually indicates mitochondrial ROS, and DCF indicates cytosolic ROS. In Figure 3(c), AT alone induces DCF generation as compared to control. AT was used as a catalase inhibitor that prevents hydrogen peroxide to water (H_2O) substitution. DDC and AT affected PrP-induced DCF generation. However, we focused on PrP-induced mitochondrial ROS (MitoSOX) because DCF generation by prion peptide was insignificant.

Several studies have reported that CaN activity is regulated by Ca^{2+} as well as by oxidative stress conditions [45, 78]. Accordingly, we investigated the relationship between CaN activity and intracellular ROS. Prior literature has suggested that PrP 106-126 induces intracellular ROS [31, 79].

5. Conclusion

Our results prove that PrP 106-126 generates more mitochondrial ROS than cytosolic ROS and PrP-mediated CaN activation regulated mitochondrial ROS in neuronal cells. These results also suggest that PrP-induced mitochondrial ROS production triggers CaN activation partially as circulating feedback action, and the regulation of CaN may be a practical therapeutic treatment for prion disease.

Data Availability

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

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

J.H.M. and S.Y.P. designed and executed the study, analyzed the data, and wrote the manuscript. All authors have read and approved the final manuscript.

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Research Article

Carbon Monoxide Releasing Molecule-3 Enhances Heme Oxygenase-1 Induction via ROS-Dependent FoxO1 and Nrf2 in Brain Astrocytes

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Carbon monoxide releasing molecule-3 (CORM-3) has been shown to protect inflammatory diseases via the upregulation of heme oxygenases-1 (HO-1). However, in rat brain astrocytes (RBA-1), the mechanisms underlying CORM-3-induced HO-1 remain poorly defined. This study used western blot, real-time PCR, and promoter activity assays to determine the levels of HO-1 expression and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and dihydroethidium (DHE) to measure reactive oxygen species (ROS). We found that CORM-3-induced HO-1 expression was mediated through ROS generation by Nox or mitochondria. The signaling components were differentiated by pharmacological inhibitors and small interfering RNA (siRNA). Subcellular fractions, immunofluorescent staining, and chromatin immunoprecipitation assay were used to evaluate the nuclear translocation and promoter binding activity of Nrf2 induced by CORM-3. The roles of mTOR and FoxO1 in CORM-3-stimulated responses are still unknown in RBA-1 cells. Our results demonstrated that transfection with siRNAs or pretreatment with pharmacological inhibitors attenuated the levels of HO-1 and phosphorylation of signaling components including Akt, mTOR, FoxO1, and Nrf2 stimulated by CORM-3. Moreover, pretreatment with N-acetyl-L-cysteine, diphenyleneiodonium chloride, apocynin, or rotenone blocked nuclear translocation and promoter binding activity of Nrf2 induced by CORM-3. The present study concluded that in RBA-1 cells, CORM-3-induced HO-1 expression is, at least partially, mediated through Nox and mitochondria/ROS-dependent PI3K/Akt/mTOR cascade to activate FoxO1 or ROS leading to activation of Nrf2 activity.

1. Introduction

Heme oxygenases (HOs) catabolize rate-limiting enzymatic degradation of heme into three products: free iron which triggers the induction of ferritin, biliverdin which is converted to bilirubin by biliverdin reductase, and carbon monoxide (CO). Three isoforms of HO have been characterized, including HO-1, HO-2, and HO-3 [1]. Among this family, HO-1 is inducible by numerous stimuli such as UV irradiation, heavy metals, endotoxin, cytokines, oxidants, and CO [2–4]. HO-1 plays a vital function in maintaining cellular homeostasis besides heme degradation. In the brain, the basal level of HO-1 expression is low, its expression increases under stress stimulation. Induction of HO-1 can effectively reverse neurodegenerative diseases such as stroke [5], Alzheimer's disease [6], Parkinson's disease [7], and multiple

sclerosis [8]. However, in rat brain astrocyte- (RBA-) 1 cells, the neuroprotective effects of HO-1 induced by CO releasing molecule-3 (CORM-3) are not completely verified.

CO is a simple diatomic gas and long thought to be an environmental pollutant and a neurotoxin due to its high affinity for hemoglobin. CO arises in biological systems by the HO enzymes catabolizing heme. For many animal models of brain insults, low levels of CO administration, including CORMs and CO gas, could be a possible therapeutic strategy. For example, the low levels of inhaled CO decrease the infarct volume via an Nrf2 pathway in experimental middle cerebral artery occlusion (MCAO) models [9]. However, the clinical application of inhaled CO presents several disadvantages and limitations such as carboxyhemoglobin-related hypoxia. Thus, chemists developed prodrugs able to deliver CO, which were the first CORMs [10]. The water-soluble CORM-3 ($[Ru(CO)_3Cl(H_2NCH_2CO_2)]$) is one of the most studied prodrugs. CORM-3 could exert neuroprotection via reducing inflammatory responses [11]. Another report demonstrates that CORM-A1 diminishes the occurrence and clinical signs of the experimental allergic encephalomyelitis (EAE) and infiltrations of inflammatory cells in spinal cords [12]. However, the detailed mechanism underlying the CORM-3-mediated HO-1 expression is not completely defined in RBA-1 cells.

Several reports have demonstrated that CORMs stimulate ROS generation by NADPH oxidase (Nox) and mitochondria to induce the expression of antioxidant enzymes including HO-1 [13]. ROS exert as second messengers which trigger the HO-1 expression via modulating downstream signaling components and transcription factors [14–16]. Nox-dependent ROS generation could regulate the activities of mitogen-activated protein kinases (MAPKs) [17], phosphoinositide 3-kinase (PI3K)/Akt [18], mammalian target of rapamycin (mTOR), peroxisome proliferator-activated receptor (PPAR)γ [19], hypoxia-inducible factor 1 (HIF1) α [20], and ROS/nitric oxide (NO) production [21, 22]. Several redox-sensitive transcription factors, such as AP-1, NF-κB, forkhead box O1 (FoxO1), and NF-E2-related factor 2 (Nrf2) bind with their binding sites in the regulatory elements of HO-1 gene promoter [23]. Especially, activation of FoxO1 and Nrf2 is well known as a cellular defender against oxidative stresses through the ARE-mediated expression of antioxidant genes such as HO-1 [24, 25]. However, the roles of the mTOR/FoxO1 pathway in CORM-3-induced HO-1 expression are still unknown in RBA-1 cells. The present study is aimed at examining the mechanisms of CORM-3-induced HO-1 expression in RBA-1 cells.

2. Material and Methods

2.1. Reagents and Antibodies. Dulbecco's modified Eagle's medium (DMEM)/F-12, fetal bovine serum (FBS), Lipofectamine 2000, OPTI-MEM, and siRNAs for p47 (RSS300253), Nox1 (RSS300165), Nox2 (RSS330363), Nox4 (RSS331680), p85 (RSS303756), Akt (RSS301983), and Nrf2 (RSS343557) were purchased from Invitrogen (Carlsbad, CA). Hybond-C membrane and enhanced chemiluminescence (ECL) western blotting detection systems were purchased from GE Healthcare Biosciences (Buckinghamshire, UK). Dimethyl sulfoxide (DMSO), tricarbonylchloro(glycinato)ruthenium (CORM-3), siRNAs for p110 (SASI_Rn02_00292737), FoxO1 (SASI_Rn02_00284211), and mTOR (SASI_Hs01_00203144), TRIzol, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2Htetrazolium-5-carbox-anilide (XTT) assay kit, and other chemicals were from Sigma (St. Louis, MO). Inactive form of CORM-3 (i-CORM-3) was prepared by dissolving CORM-3 in 0.01 mM phosphate-buffered saline (PBS) and CO was liberated at room temperature for 24 h. The bicinchoninic acid (BCA) protein assay reagents were from Thermo Scientific (Philadelphia, PA). Anti-phospho-Akt (Ser^{473}), anti-phospho-FoxO1 (Ser^{256}), anti-phospho-mTOR (Ser^{2448}), anti-mTOR, and anti-$p47^{phox}$ antibodies were from Cell Signaling (Danvers, MA). Anti-phospho-Nrf2 (Ser^{40}), anti-Nox1, anti-Nox2, and anti-Nox4 antibodies were from Abcam (Cambridge, UK). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Encor (Gainesville, FL). Anti-lamin A, anti-p110, anti-p85, anti-Akt, anti-FoxO1, and anti-Nrf2 antibodies were from Santa Cruz (Santa Cruz, CA). Anti-HO-1 antibody, N-acetyl-L-cysteine (NAC), diphenyleneiodonium chloride (DPI), apocynin (APO), rotenone, LY294002, SH-5, rapamycin, and AS1842856 were from Enzo Life Sciences (Farmingdale, NY).

2.2. Cell Culture and Treatment. RBA-1 cells originated from neonatal rat cerebrum astrocytes and naturally developed through successive cell passages [26]. The use of the cell lines had been approved by Chang Gung University Institutional Animal Care and Use Committee (IACUC Approval No.: CGU16-081). The cells were cultured in DMEM/F-12 containing 5% FBS at cell density of 2×10^5 cells/ml. Three days after the plating, 90% confluent cells were used for these experiments. and made quiescent at confluence by incubation in serum-free DMEM/F-12 for 24 h. Cells were incubated with CORM-3 or i-CORM-3 [27] at 37°C for the indicated time intervals, i-CORM-3 treatment had no effects on HO-1 induction, as compared with that of CORM-3 (Supplementary Figure 1). When inhibitors were used, cells were pretreated with the inhibitors for 1 or 2 h, as indicated before exposure to CORM-3, as previously described [28]. Treatment of RBA-1 cells with DMSO or the inhibitor alone had no significant effects on cell viability, as determined by an XTT assay kit (Supplementary Figure 2).

2.3. Protein Preparation and Western Blotting Analysis. RBA-1 cells were washed with ice-cold PBS and harvested in SDS-loading buffer (0.1 M Tris-HCl, pH 6.8; 1%SDS; 5% glycerol; 2.5% β-mercaptoethanol; and 0.02% bromophenol blue) to yield whole-cell extracts, as previously described [28]. Proteins were separated by SDS-PAGE and transferred by electrophoresis onto Hybond-C membranes. Membranes were incubated with antibodies at 1 : 1000 in Tween-Tris buffered saline (TTBS), and an anti-GAPDH antibody was used as an internal control. Membranes were washed with TTBS four times for 5 min and then incubated with 1 : 1500 secondary horseradish peroxidase-conjugated antibody for 1 h. Following washing, immunoreactive bands were detected by ECL

and captured using a UVP BioSpectrum 500 Imaging System (Upland, CA). Image densitometry analyses were quantified using UN-SCAN-IT gel software (Rem, UT).

2.4. Total RNA Extraction and Real-Time Polymerase Chain Reaction (RT-PCR) Analysis. Quiescent RBA-1 cells were incubated with 30 μM CORM-3 for 4 h in the presence or absence of the indicated inhibitors. Total RNA was extracted using TRIzol according to the manufacturer's protocol and was then reverse-transcribed to cDNA and analyzed by RT-PCR using a TaqMan gene expression assay system, as previously described [28], with sequences of primers and probes as follows:

HO-1: sense: 5′-TTTCACCTTCCCGAGCAT-3′, antisense: 5′-GCCTCTTCTGTCACCCTGT-3′, probe: 5′-CATGAACACTCTGGAGATGACC-3′; GAPDH: sense: 5′-AACTTTGGCATCGTGGAAGG-3′, anti-sense: 5′-GTGGATGCAGGGATGATGTTC-3′, probe: 5′-TGACCACAGTCCATGCCATCACTGC-3′.

RT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression was determined using the $^{\Delta\Delta Ct}$ method, with Ct indicating threshold cycles. All experiments were performed in triplicate.

2.5. Transient Transfection with Short Interfering RNA (siRNA). At 70%–80% confluence, cells were transiently transfected with siRNAs (100 nM) corresponding to p47, Nox1, Nox2, Nox4, p110, p85, Akt, mTOR, FoxO1, Nrf2, or scrambled siRNA. GenMute™ reagent was used, followed by mixing with 75 μl GenMute™ transfection buffer, as previously described [28]. After 10–15 min, 100 μl of the mixture was applied directly to the cells. The cells were washed with PBS and maintained in DMEM/F-12 with 5% FBS for 24 h. Next, cells were starved in a serum-free DMEM/F-12 medium for 24 h. The transfection efficiency (approximate 60%) was determined by transfection with an enhanced green fluorescent protein (EGFP).

2.6. Plasmid Construction, Transfection, and Luciferase Reporter Gene Assays. A rat HO-1 promoter (accession no. J02722.1; −766 to +20) was constructed (sense: GGTACC CAGGAAGTCACAGTGTGGCC; antisense: CCCGAG CTCGTCG AGCTGTGGGCG CTCCAT) and cloned into the pGL3-basic vector containing a luciferase reporter system, as previously described [28]. To obtain ARE-luciferase reporter construct, double-stranded oligonucleotides containing a single copy of the 41-bp pair murine GSTYa ARE (5′-TAGCTTGGAAATGACATTGCTAATGGTG ACAA AGCAACTTT-3′; the core sequence underlined) were cloned into the pGL3 promoter vector (Promega, Madison, WI). All sequences of pARE-Luci were confirmed and verified the presence of the correct sequence and the absence of any other nucleotide changes by DNA sequencing. ARE-Luci activity was determined using a luciferase assay system, as previously described [14].

RBA-1 cells were transfected with plasmid DNA using Lipofectamine 2000. Co-transfection with pCMV-gal encoding for β-galactosidase was used as a control for transfection efficiency. To assess promoter activity, cells were collected and disrupted by sonication in a lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM ethylenediaminetetraacetic acid, 1% Triton X-100 and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using a luciferase assay system (Promega, Madison, WI). Firefly luciferase activities were standardized to β-galactosidase activity.

ARE: 5′-TAGCTTGGAAATGACATTGCTAATGG TGACAAAGCAACTTT-3′(sense), 5′-AAAGTTGCTTT GTCACCATTAGCAATGTCATTTCCAAGCTA-3′(sense), 5′-CTAGCTTGGAAATGACATTGCTAATGGTGACAAA GCAACTTTC-3’′(Kpn sense), 5′-TCGAGAAAGTTGCT TTGTCACCATTAGCAATGTCATTTCCAAGCTAGGT AC-3′(Xho antisense).

2.7. Isolation of Subcellular Fractions. RBA-1 cells were seeded in 10-cm dishes and starved for 24 h in serum-free DMEM/F-12 medium. After incubation with CORM-3, the cells were washed once with ice-cold PBS. 200 μl of homogenization buffer A (20 mM Tris-HCl, pH 8.0, 10 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 10 μg/ml leupeptin) was added to each dish, and the cells were scraped into a 1.5 ml tube. The cytosolic and nuclear fractions were prepared by centrifugation, as previously described [14]. The protein concentration of each sample was determined by the BCA reagents. Samples from these supernatant fractions (30 μg protein) were denatured and then subjected to SDS-PAGE using a 12% (w/v) running gel and transferred to nitrocellulose membrane. The levels of Nrf2, phospho-Nrf2, and lamin A in the nuclear fraction were determined by western blotting using an anti-Nrf2, anti-phospho-Nrf2, or lamin A antibody.

2.8. Measurement of Intracellular ROS Accumulation. Cells were cultured in DMEM/F-12 for 24 h and then treated with CORM-3. When inhibitors were used, they were added 1 h before the application of CORM-3. After washing twice with warm PBS, the cells were incubated with H_2DCFDA (10 μM) or DHE (5 μM) for 30 min or 10 min, as previously described [29]. For ELISA assay, the fluorescence for DCF staining was detected at 495/529 nm, using a fluorescence microplate reader (Synergy^{H1} Hybrid Reader, BioTek). For immunofluorescence (IF) staining, washing thrice with cold-PBS, the images were observed under a fluorescence microscope (Axiovert 200 M, Zeiss).

2.9. Determination of NADPH Oxidase Activity Assay by Chemiluminescence Assay. After exposure to 30 μM CORM-3 for the indicated time intervals, cells were gently scraped and centrifuged at 400 x g for 10 min at 4°C, as previously described [29]. The cell pellet was resuspended with 35 μl of ice-cold PBS, and the cell suspension was kept on the ice. To a final 200 μl volume of prewarmed (37°C) PBS containing either NADPH (1 μM) or lucigenin (20 μM), 5 μl of cell suspension (2×10^4 cells) was added to initiate the reaction followed by immediate measurement of chemiluminescence in a luminometer (Synergy^{H1} Hybrid Reader, BioTek).

2.10. Chromatin Immunoprecipitation Assay. To detect the association of the nuclear protein with rat HO-1 promoter, chromatin immunoprecipitation analysis was conducted, as previously described [29] with some modifications. Briefly, RBA-1 cells were crosslinked with 1% (*v*/*v*) formaldehyde at 37°C for 30 min and stop this reaction with 0.125 M glycine, then washed thrice with ice-cold PBS containing 1 mM PMSF, 1% (*v*/*v*) aprotinin, and 1% (*v*/*v*) leupeptin. Soluble chromatin was prepared using a ChIP assay kit (Upstate) according to the instructions of the manufacturer and immunoprecipitated without (control) or with an anti-Nrf2 antibody and normal goat immunoglobulin G (IgG). Following washing and elution, immunoprecipitates were heated overnight at 65°C to reverse crosslinking of DNA and protein. To avoid the possibility of amplification artifacts, PCR products for all SYBR Green primer pairs were verified to produce single products by agarose electrophoresis and a high-resolution melt curve. The relative mRNA levels were calculated using the comparative Ct method ($^{\Delta\Delta Ct}$). The DNA was extracted and resuspended in H_2O and subjected to PCR amplification with the ARE primers:

(i) ARE1: forward, 5′-ACAGTG TGGCCCAGGTT CTA-3′, reverse, 5′-TTCTAGCTGT GAGATG CTGGT-3′

(ii) ARE2: forward, 5′- CTGGAGAATCTCAGGATTA AC-3′, reverse, 5′- ACCCTGTCTGGAAAAG ACAA-3′

DNA (2 μl) was extracted and resuspended in ddH_2O (5 μl), DMSO (1 μl), and 2x Screening Fire Taq Master Mix (10 μl) subjected to PCR amplification with the above primers ARE1 and ARE2. The amount of DNA-bound Nrf2 was expressed as a PCR product analyzed on 2% agarose 1× TAE gel containing ethidium bromide.

2.11. Immunofluorescent Staining. Growth-arrested cells were incubated with CORM-3 (30 μM) for the indicated time intervals with or without APO, DPI, NAC, or rotenone pretreatment for 1 h. These cells were fixed, permeabilized, stained using anti-p-Nrf2 antibodies (1 : 200 dilutions) and 4′,6-diamidino-2-phenylindole (DAPI) after washing with ice-cold PBS, and finally mounted, as previously described [14]. The images of p-Nrf2 and nucleus were detected with a fluorescence microscope (Zeiss, Axiovert 200 M).

2.12. Data and Statistical Analysis. GraphPad Prizm Program 6.0 software (GraphPad, San Diego, CA) was adopted to perform statistical analysis. We used one-way ANOVA followed by Dunnett's post hoc test or nonparametric Kruskal–Wallis test followed by Dunn's post hoc test when comparing multiple independent groups. *P* values less than 0.05 were statistically significant. Only if *F* achieved $P < 0.05$ and the assumption of homogeneity of variance was also achieved, post hoc tests were run. All experiments were performed at least three individual times (n = number of independent cell culture preparations). All the data were expressed as the mean ± SEM. Error bars were omitted when they fell within the dimensions of the symbols.

3. Results

3.1. ROS Generation Is Involved in CORM-3-Induced HO-1 Expression. ROS are important factors in many physiological and pathological processes. Intracellular ROS have been shown to induce HO-1 gene expression [30]. To investigate whether ROS are involved in the CORM-3-induced HO-1 expression in RBA-1 cells, NAC was used for this purpose. Pretreatment with NAC inhibited the CORM-3-induced HO-1 protein expression in a concentration-dependent manner (Figure 1(a)). NAC pretreatment also reduced the CORM-3-induced HO-1 mRNA expression and promoter activity, determined by real-time PCR and promoter-luciferase assay, respectively (Figure 1(b)). To ensure that the generation of ROS plays a role in the HO-1 expression induced by CORM-3, as shown in Figure 1(c), 30 μM CORM-3 time dependently induced ROS generation with an initial peak production within 10 min and followed by a second peak within 6 h. The production of ROS was reduced by NAC (Figure 1(d)). These results were further supported by using H_2DCF-DA or DHE staining observed under a fluorescent microscope (Figure 1(e)). These results suggested that CORM-3 induces HO-1 expression via ROS generation in RBA-1 cells.

3.2. CORM-3-Induced HO-1 Expression Is Mediated via NADPH Oxidase. ROS have been shown to participate in HO-1 expression induced by CORM-3. Thus, we explored whether Nox is involved in the CORM-3-induced HO-1 expression, RBA-1 cells were pretreated with either DPI or APO (Nox inhibitors) for 2 h before exposure to 30 μM CORM-3 for 6 h. As demonstrated in Figure 2(a), CORM-3-induced HO-1 protein expression was concentration dependently inhibited by pretreatment with either DPI or APO. CORM-3-induced HO-1 mRNA level and promoter activity were also attenuated by these inhibitors (Figure 2(b)). To uncover which isoform of Nox is involved in CORM-3-induced HO-1 expression, RBA-1 were transfected with p47, Nox1, Nox2, Nox4, or scrambled siRNA. As shown in Figure 2(c), downregulation of p47, Nox1, Nox2, or Nox4 protein levels attenuated the CORM-3-induced HO-1 expression. Besides, 30 μM CORM-3 stimulated NOX activity with an initial peak within 5 min, followed by a second peak within 1 h, and declined close to the basal level within 6 h (Figure 2(d)). We further ensured these results using H_2DCF-DA and DHE staining. As illustrated in Figure 2(e), CORM-3-stimulated ROS accumulation was attenuated by either DPI or APO. These findings suggested that the HO-1 expression induced by CORM-3 is mediated through p47, Nox1, Nox2, or Nox4 activation in RBA-1 cells.

3.3. CORM-3-Induced HO-1 Expression Is Mediated via Mitochondrial Respiratory Complex. The main sites of $\bullet O_2^-$ formation are the flavin mononucleotide (FMN) site of complex I and the Q cycle of complex III in the mitochondrial respiratory complex [31]. However, the role of

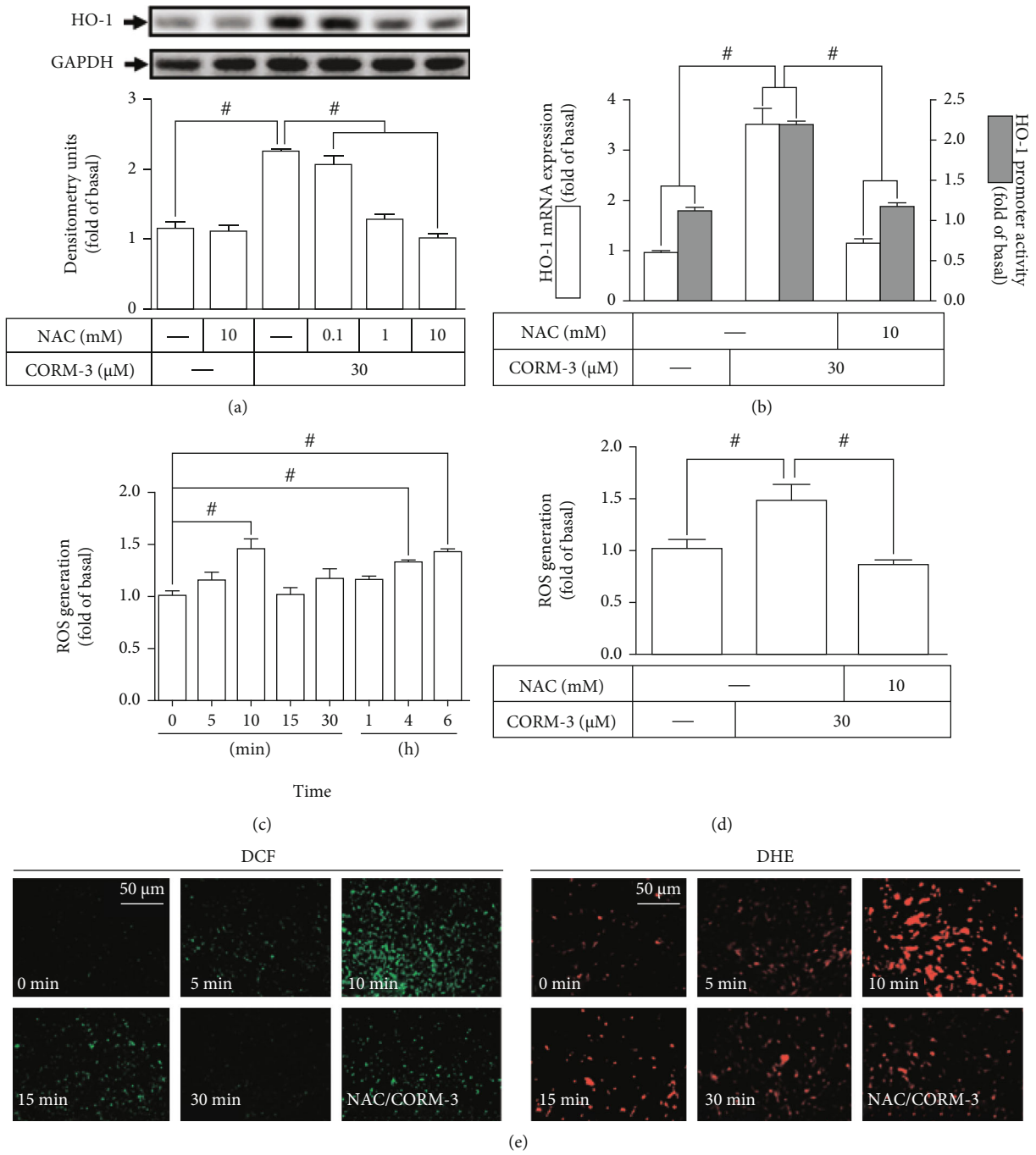


Figure 1: ROS generation involved in CORM-3-induced HO-1 expression. (a) RBA-1 cells were pretreated with various concentrations of NAC for 1 h and then incubated with 30 μM CORM-3 for 6 h. The levels of HO-1 and GAPDH (as an internal control) were determined by western blot. (b) The cells were pretreated with NAC (10 mM) for 1 h and then incubated with CORM-3 (30 μM) for 4 h. The levels of HO-1 and GAPDH mRNA were analyzed by real-time PCR (open bars). The cells were transiently transfected with HO-1 report gene together with a β-galactosidase plasmid, pretreated with NAC (10 mM) for 1 h, and then incubated with CORM-3 for 1 h. Promoter activity was determined in the cell lysates (solid bars). (c, d) The cells were pretreated with or without NAC (10 mM) for 1 h and then incubated with 30 μM CORM-3 for the indicated time intervals (c) or 10 min (d). ROS generation was determined by measuring fluorescence intensity of DCF-DA. (e) The cells were pretreated with NAC (10 mM) for 1 h, incubated with 30 μM CORM-3 for the indicated time intervals (CORM-3: 0, 5, 10, 15, and 30 min; NAC/CORM-3: 10 min), and then labeled with DCF-DA and DHE, respectively. The fluorescence intensity was observed under a fluorescence microscope. Data were expressed as mean ± S.E.M. of three independent experiments. Scale bar = 50 μm. $^{\#}P < 0.05$, as compared with the control or pretreatment with inhibitor indicated in the figure.

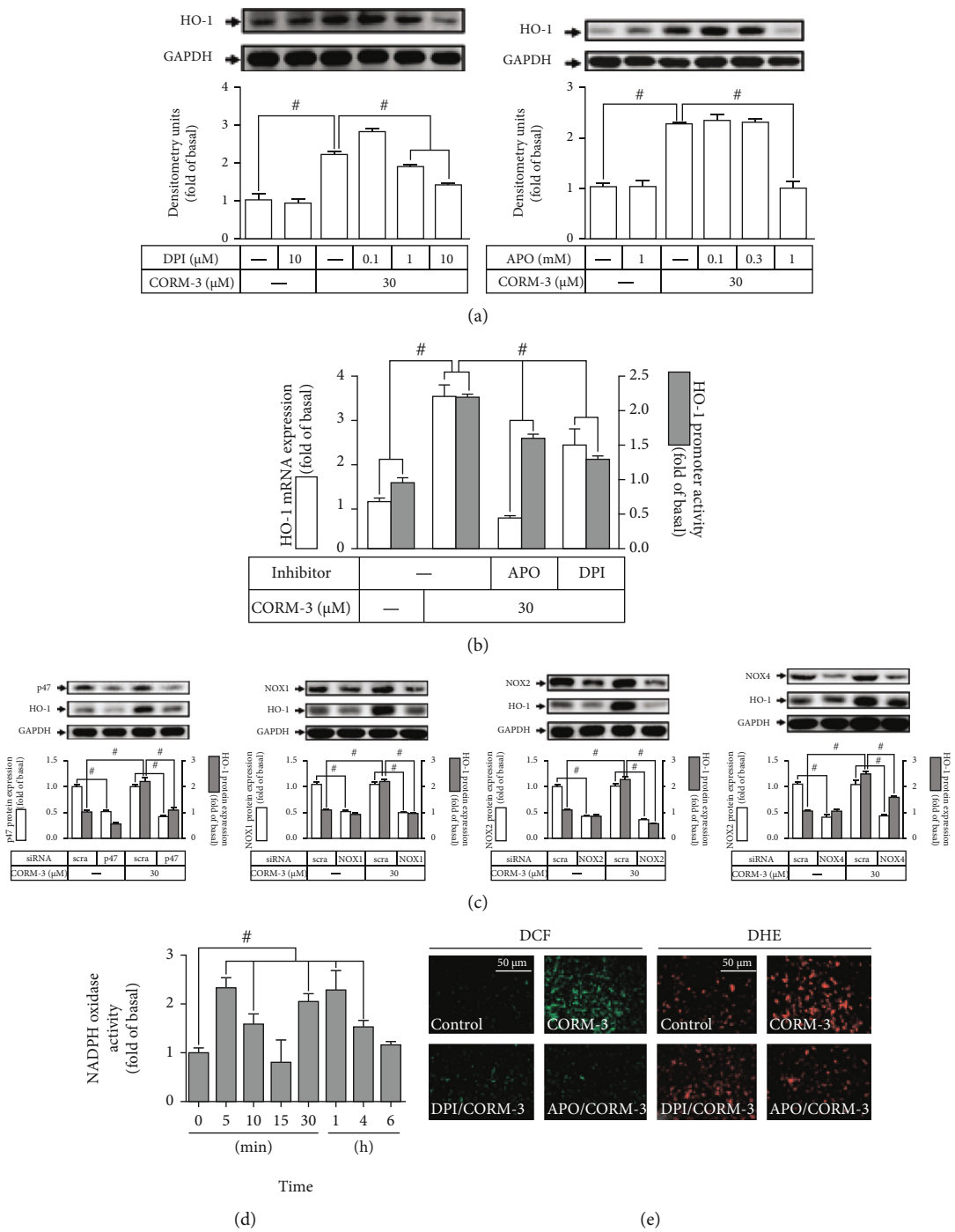


Figure 2: CORM-3-induced HO-1 expression is mediated via NADPH oxidase in RBA-1 cells. (a) The cells were pretreated with various concentrations of APO or DPI for 1 h and then incubated with 30 μM CORM-3 for 6 h. The levels of HO-1 and GAPDH (as an internal control) protein expression were determined by western blot. (b) The cells were pretreated with APO (1 mM) or DPI (10 μM) for 1 h and then incubated with 30 μM CORM-3 for 4 h. The levels of HO-1 and GAPDH mRNA were determined by real-time PCR (open bars). The cells were transiently transfected with HO-1 report gene together with a β-galactosidase plasmid, subsequently pretreated with APO (1 mM) or DPI (10 μM) for 1 h, and then incubated with 30 μM CORM-3 for 1 h. Promoter activity was determined in the cell lysates (solid bars). (c) The cells were, respectively, transfected with p47, Nox1, Nox2, or Nox4 siRNA and then incubated with 30 μM CORM-3 for 6 h. The levels of HO-1, p47, Nox1, Nox2, Nox4, and GAPDH (as an internal control) protein expressions were determined by western blot. (d) The cells were incubated with 30 μM CORM-3 for the indicated time intervals. NADPH oxidase activity was determined by an ELISA assay kit. (e) The cells were pretreated with either APO (1 mM) or DPI (10 μM) for 1 h, incubated with 30 μM CORM-3 for 10 min, and then labeled with H_2DCF-DA and DHE, respectively. The fluorescence intensity was observed under a fluorescence microscope. Scale bar = 50 μm. Data were expressed as mean ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, as compared with the control, pretreatment with inhibitor, or siRNA indicated in the figure.

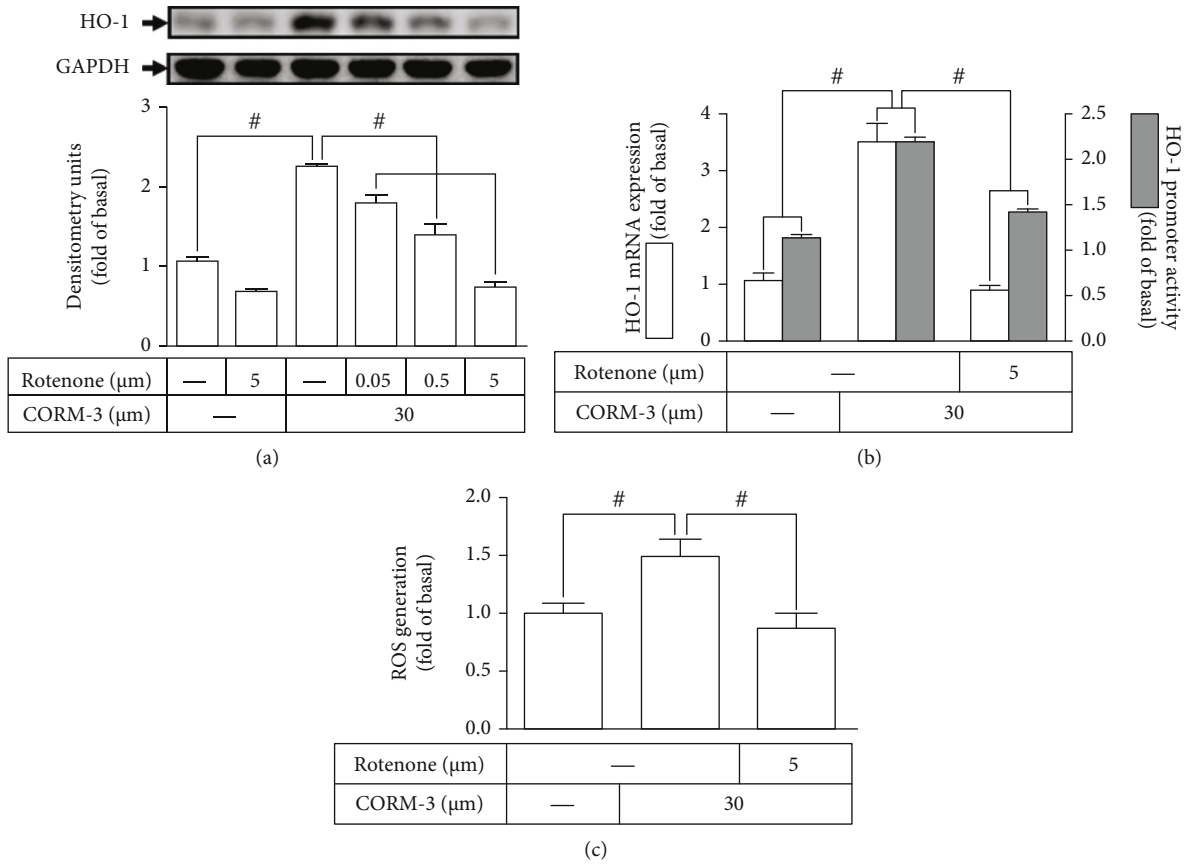
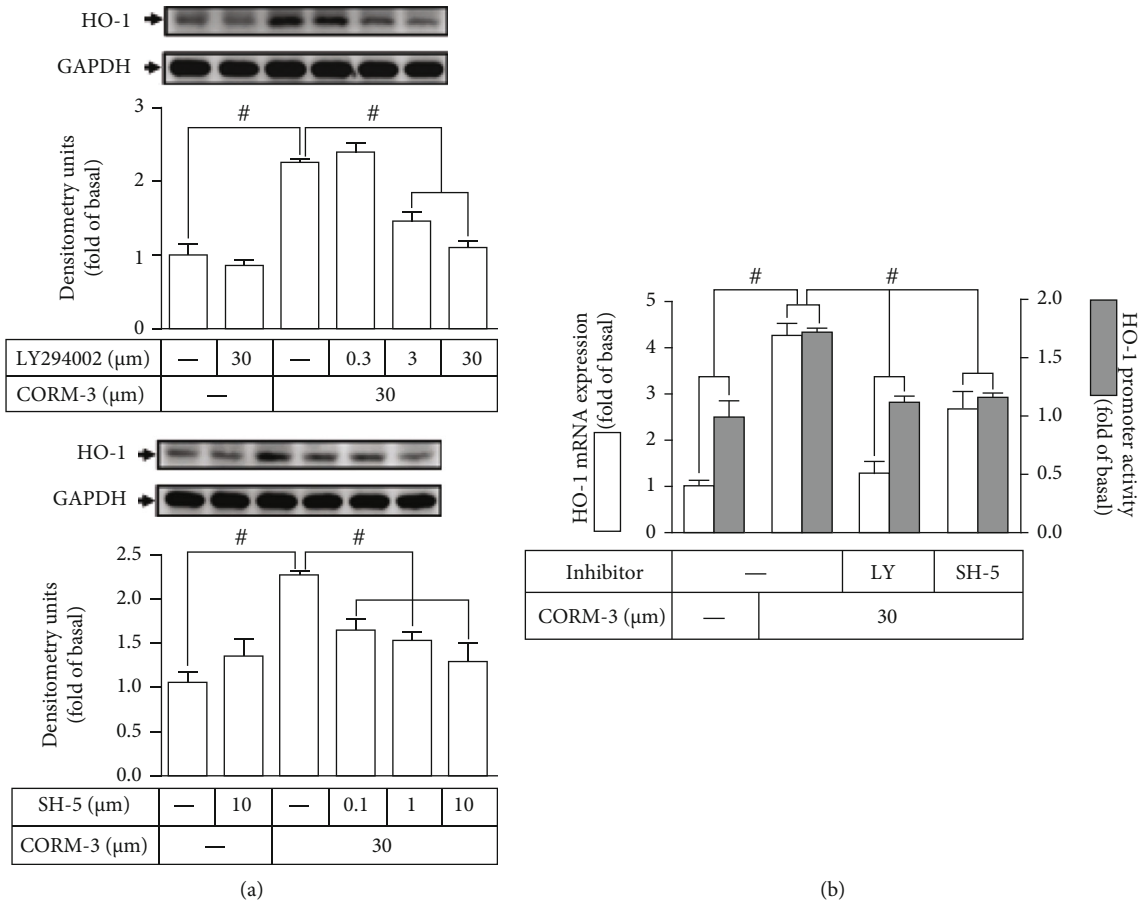


Figure 3: CORM-3-induced HO-1 expression is mediated via mitochondrial respiratory complex. (a) RBA-1 cells were pretreated with various concentrations of rotenone for 1 h and then incubated with 30 μM CORM-3 for 6 h. The levels of HO-1 and GAPDH (as an internal control) protein expressions were determined by western blot. (b) The cells were pretreated with 5 μM Rotenone for 1 h and then incubated with 30 μM CORM-3 for 4 h. The levels of HO-1 and GAPDH mRNA were determined by real-time PCR (open bars). The cells were transiently transfected with HO-1 report gene together with a β-galactosidase plasmid, subsequently pretreated with 5 μM Rotenone for 1 h, and then incubated with 30 μM CORM-3 for 1 h. Promoter activity was determined in the cell lysates (solid bars). (c) The cells were pretreated with rotenone (5 μM) for 1 h, incubated with 30 μM CORM-3 for 10 min, and then labeled with H2DCF-DA. The fluorescence of DCF staining was detected using an ELISA assay. Data were expressed as mean ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, as compared with the control or pretreatment with inhibitor indicated in the figure.

mitochondria-driven ROS in the CORM-3-induced HO-1 expression in RBA-1 cells was not understood. The cells were pretreated with rotenone (a mitochondrial complex inhibitor) for 2 h before exposure to 30 μM CORM-3 for 6 h. Rotenone concentration dependently inhibited the HO-1 protein expression induced by CORM-3 (Figure 3(a)). Moreover, the HO-1 mRNA expression and promoter activity induced by CORM-3 were also attenuated by pretreatment with rotenone (Figure 3(b)). To determine whether the production of ROS was mediated through activation of mitochondrial respiratory complex, CORM-3-stimulated mitochondrial ROS generation was blocked by pretreatment with rotenone (Figure 3(c)). These results implied that the HO-1 expression induced by CORM-3 is, at least partially, mediated through mitochondrial respiratory complex-driven ROS generation in RBA-1 cells.

3.4. CORM-3-Induced HO-1 Expression Is Mediated via PI3K/Akt Cascade. Several studies have revealed that the PI3K/Akt signaling pathway could trigger HO-1 upregulation in various types of cells [14, 32]. To investigate whether PI3K/Akt participated in HO-1 expression, the inhibitors of PI3K (LY294002) and Akt (SH-5) were used for these purposes. We found that pretreatment of RBA-1 cells with either LY294002 or SH-5 concentration dependently attenuated the HO-1 protein expression induced by CORM-3 (Figure 4(a)). Additionally, either LY294002 or SH-5 pretreatment also significantly reduced the HO-1 mRNA expression and promoter activity induced by CORM-3 (Figure 4(b)). To ensure the roles of PI3K/Akt in the CORM-3-induced HO-1 expression, cells were transfected with p110, p85, or Akt siRNA. Data in Figure 4(c) showed that knockdown of p110, p85, or Akt protein levels abolished the CORM-3-induced HO-1 expression. To prove whether CORM-3-stimulated PI3K/Akt activation was necessary for the HO-1 expression, the level of Akt phosphorylation was determined. Our data showed that CORM-3 time dependently stimulated Akt phosphorylation, which was attenuated by either SH-5 or LY294002 (Figure 4(d)). Moreover, pretreatment with NAC, APO, DPI, or rotenone also inhibited CORM-3-stimulated



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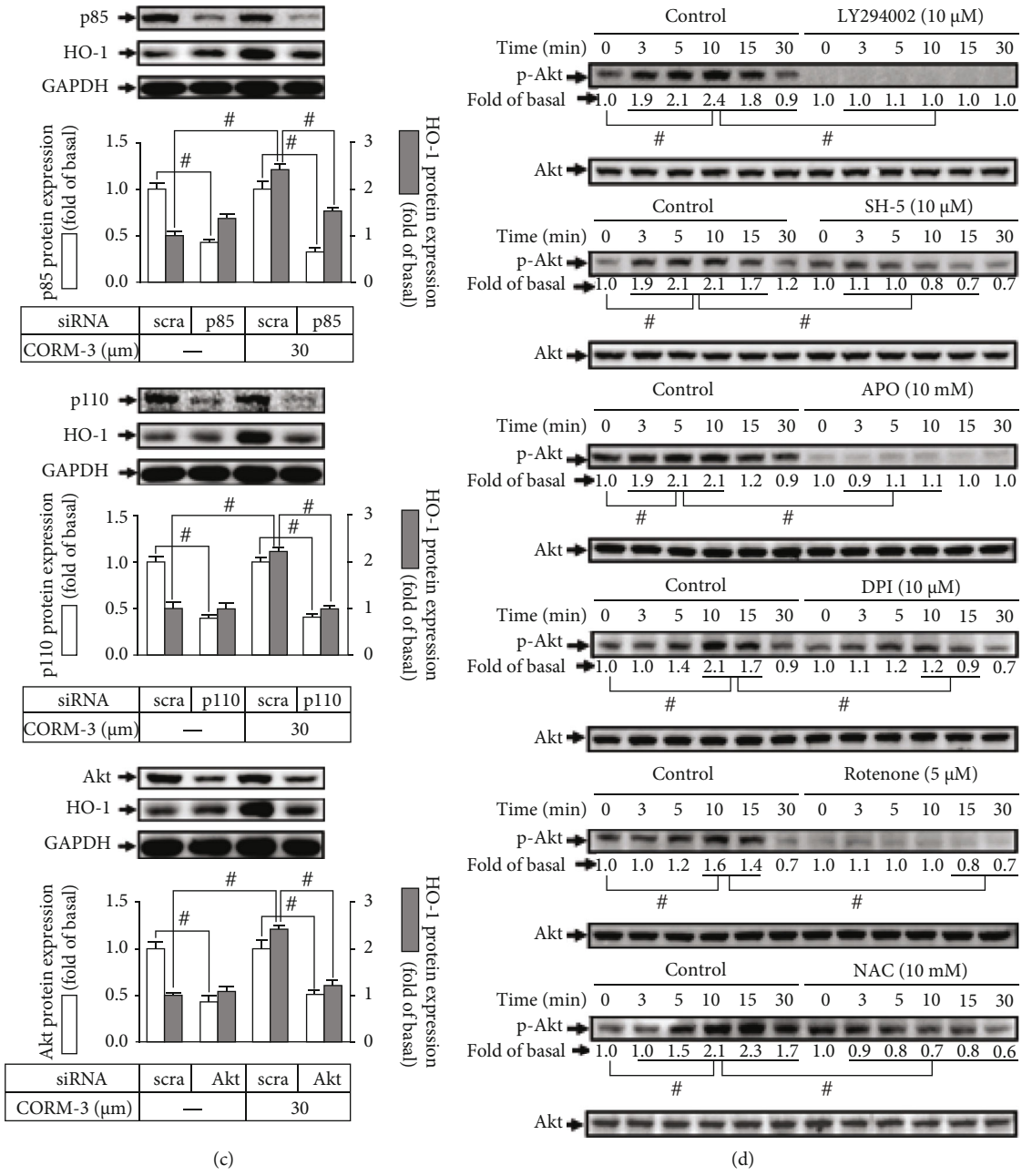
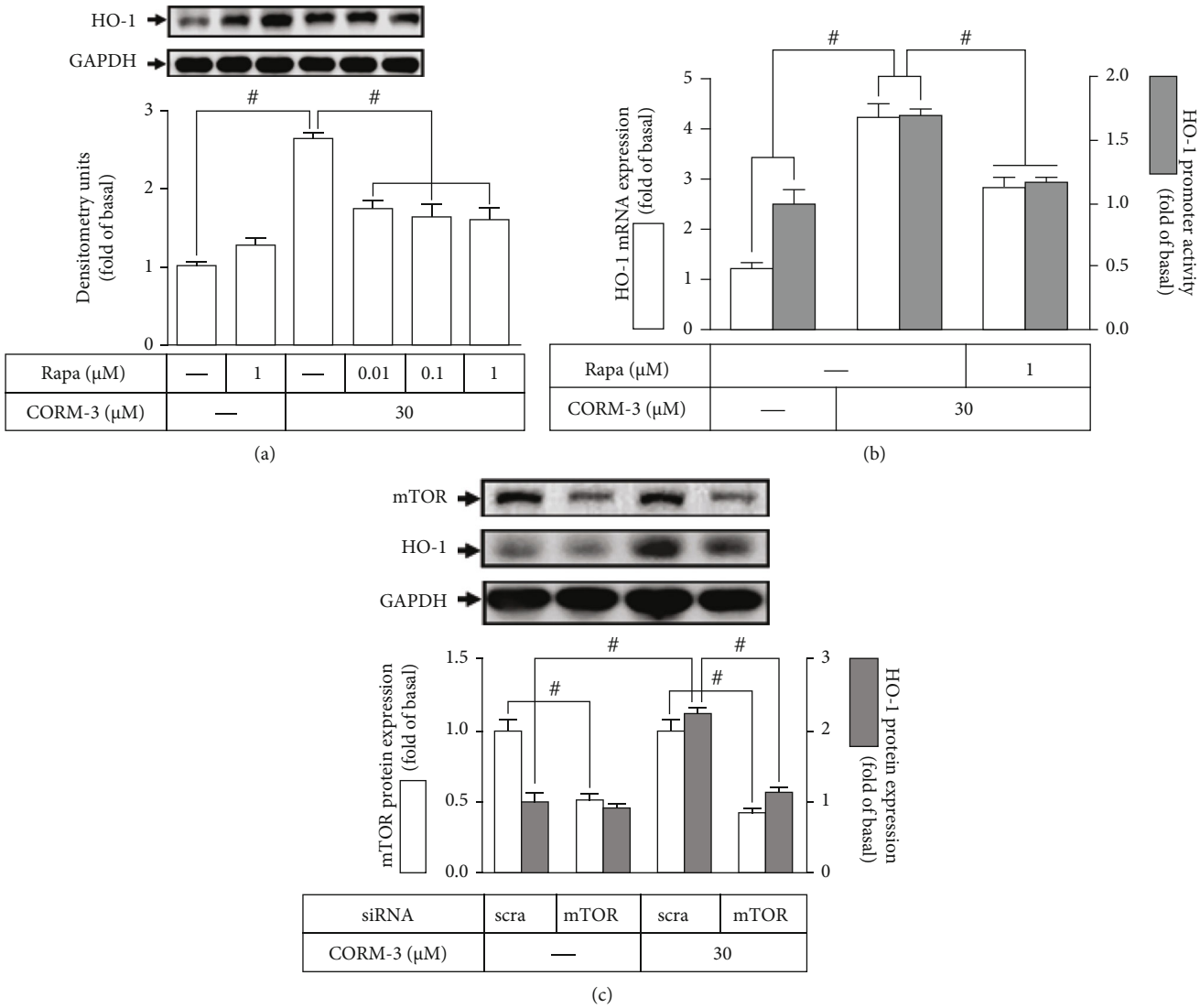


Figure 4: CORM-3-induced HO-1 expression is mediated via PI3K/Akt cascade. (a) RBA-1 cells were incubated with various concentrations of either LY294002 or SH-5 for 1 h and then incubated with 30 μM CORM-3 for 6 h. The levels of HO-1 and GAPDH (as an internal control) protein expressions were determined by western blot. (b) The cells were pretreated with SH-5 (10 μM) or LY294002 (10 μM) for 1 h and then incubated with 30 μM CORM-3 for 4 h. The levels of HO-1 and GAPDH mRNA were determined by real-time PCR (open bars). The cells were transiently transfected with HO-1 report gene together with a β-galactosidase plasmid, subsequently pretreated with SH-5 (10 μM) or LY294002 (10 μM) for 1 h, and then incubated with 30 μM CORM-3 for 1 h. Promoter activity was determined in the cell lysates (solid bars). (c) The cells were transfected with Akt, p110, or p85 siRNA and then challenged with 30 μM CORM-3 for 6 h. The protein levels of HO-1, Akt, p110, p85, and GAPDH (as an internal control) were determined by western blot. (d) The cells were pretreated without or with SH-5 (10 μM), LY294002 (10 μM), NAC (10 mM), APO (10 mM), DPI (10 μM), or rotenone (5 μM) for 1 h and then incubated with 30 μM CORM-3 for the indicated time intervals. The levels of phosphorylated Akt and total Akt protein were determined by western blot. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, as compared with the control, pretreatment with inhibitor, or siRNA indicated in the figure.

Akt phosphorylation (Figure 4(d)). These results suggested that in RBA-1, PI3K/Akt is involved in the HO-1 expression induced by CORM-3 and regulated by Nox- or mitochondrion-derived ROS signaling pathways.

3.5. CORM-3-Induced HO-1 Expression Is Mediated via mTOR. mTOR plays a critical role in diverse cellular functions including the expression of antioxidant enzymes such as HO-1 [33, 34]. To investigate whether mTOR is involved



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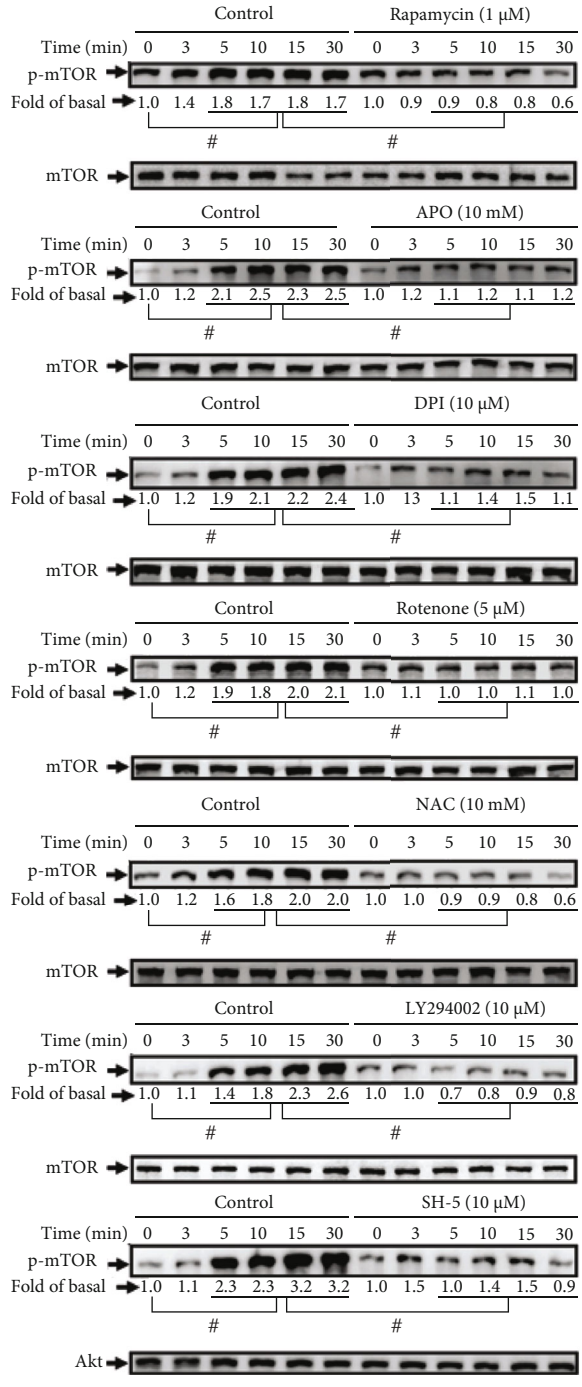
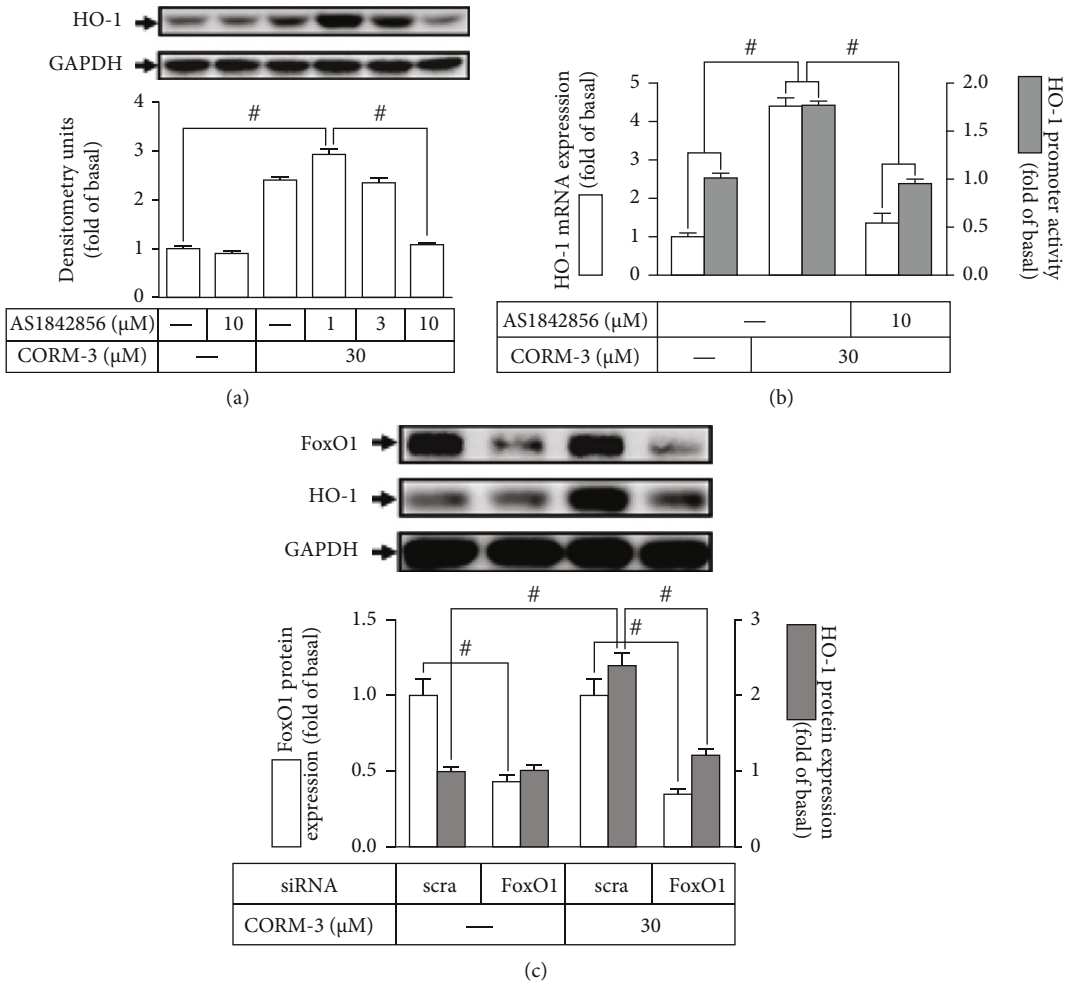


(d)

Figure 5: CORM-3-induced HO-1 expression is mediated via mTOR. (a) RBA-1 cells were incubated with various concentrations of rapamycin for 1 h and then incubated with 30 μM CORM-3 for 6 h. The levels of HO-1 and GAPDH (as an internal control) protein expressions were determined by western blot. (b) The cells were pretreated with 1 μM rapamycin for 1 h and then incubated with 30 μM CORM-3 for 4 h. The levels of HO-1 and GAPDH mRNA were determined by real-time PCR (open bars). The cells were transiently transfected with HO-1 report gene together with a β-galactosidase plasmid, subsequently pretreated with 1 μM rapamycin for 1 h, and then incubated with 30 μM CORM-3 for 1 h. Promoter activity was determined in the cell lysates (solid bars). (c) The cells were transfected with mTOR siRNA and then challenged with 30 μM CORM-3 for 6 h. The protein levels of HO-1, mTOR, and GAPDH (as an internal control) were determined by western blot. (d) The cells were pretreated with rapamycin (1 μM), NAC (10 mM), APO (10 mM), DPI (10 μM), rotenone (5 μM), LY294002 (10 μM), or SH-5 (10 μM) for 1 h and then stimulated by 30 μM CORM-3 for the indicated time intervals. The levels of phosphorylated mTOR and total mTOR proteins were determined by western blot. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, as compared with the control, pretreatment with inhibitor, or siRNA indicated in the figure.



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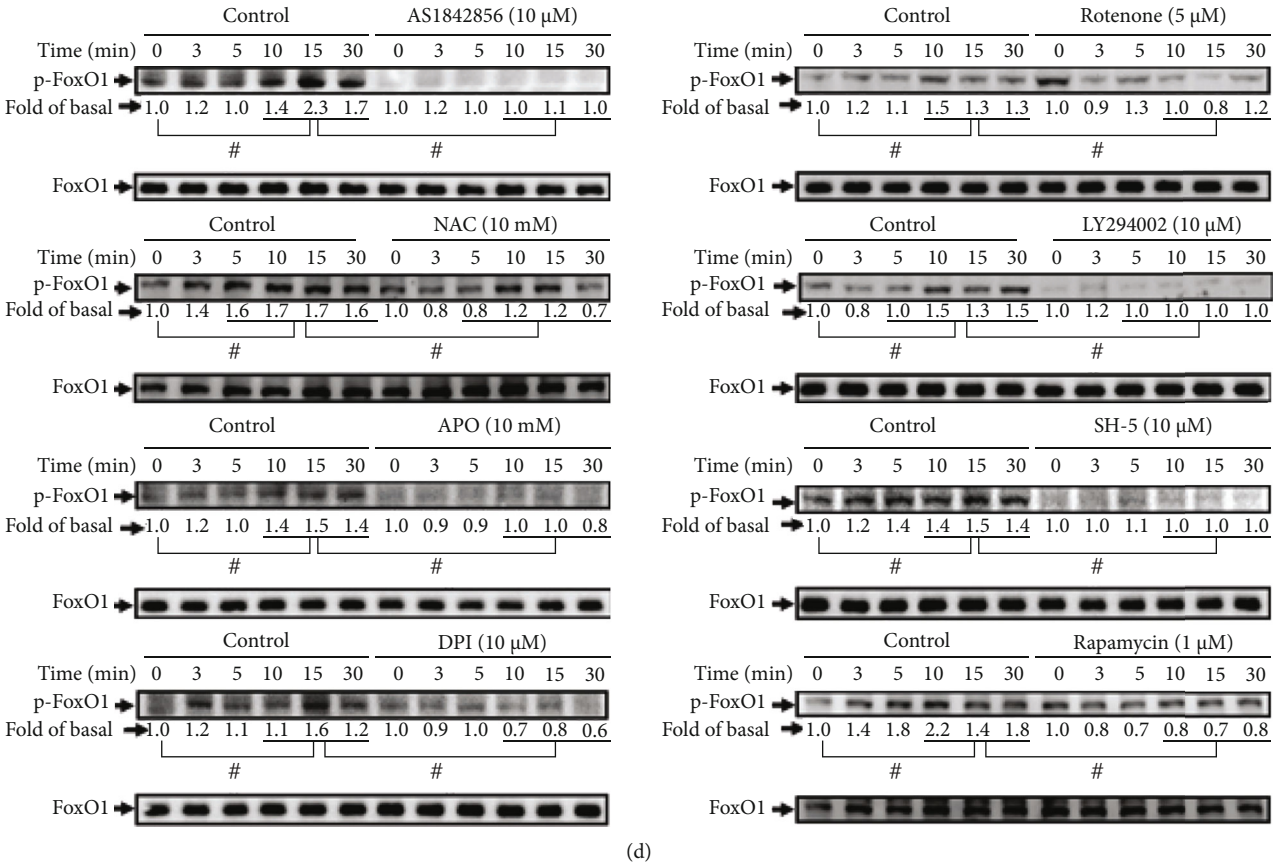
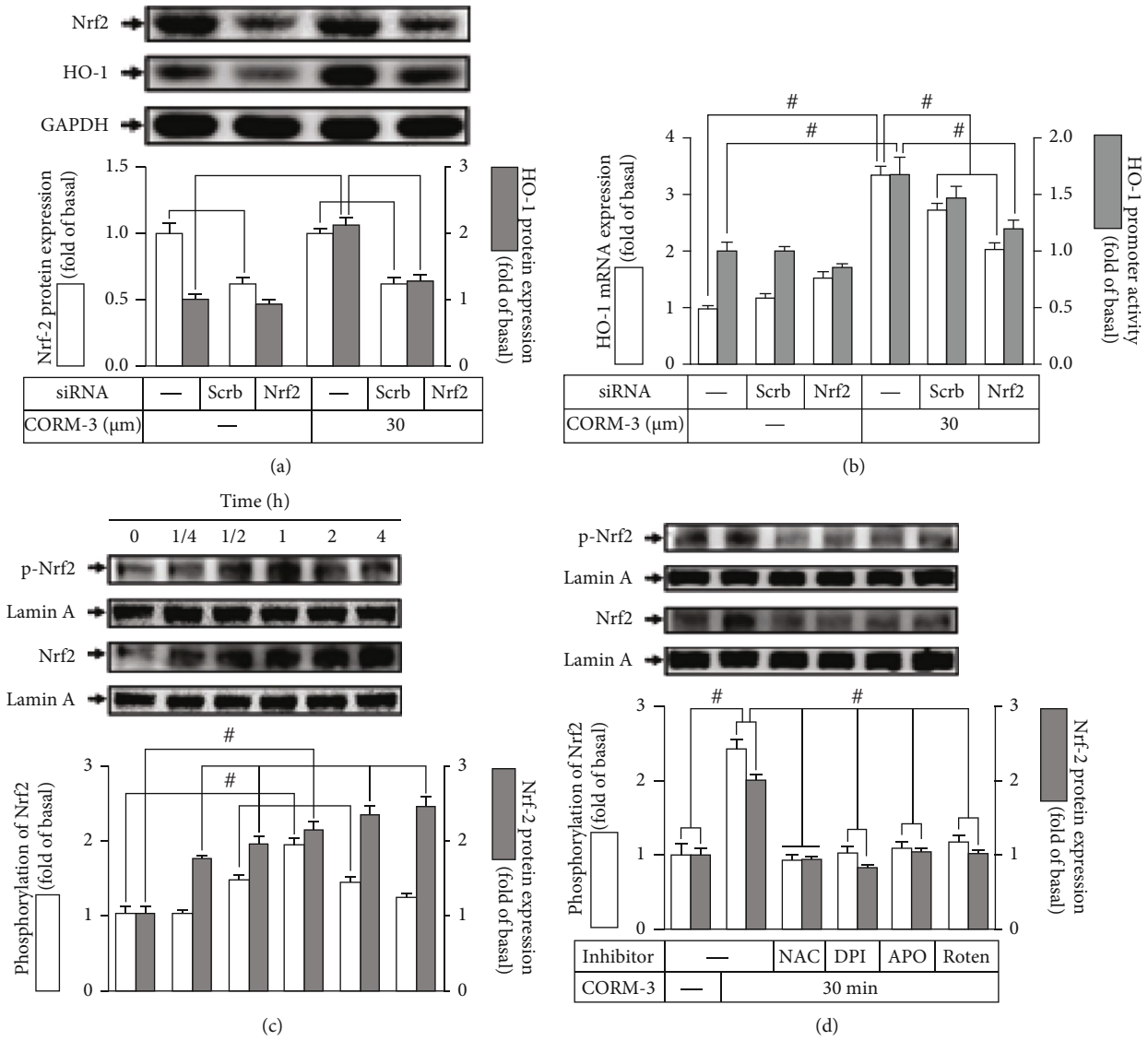


Figure 6: FoxO1 is involved in CORM-3-induced HO-1 expression. (a) RBA-1 cells were incubated with various concentrations of AS1842856 for 1 h and then incubated with 30 μM CORM-3 for 6 h. The levels of HO-1 and GAPDH (as an internal control) protein expressions were determined by western blot. (b) The cells were pretreated with 10 μM AS1842856 for 1 h and then incubated with 30 μM CORM-3 for 4 h. The levels of HO-1 and GAPDH mRNA were determined by real-time PCR (open bars). The cells were transiently transfected with HO-1 report gene together with a β-galactosidase plasmid, subsequently pretreated with 10 μM AS1842856 for 1 h, and then incubated with 30 μM CORM-3 for 1 h. Promoter activity was determined in the cell lysates (solid bars). (c) The cells were transfected with FoxO1 siRNA and then challenged with 30 μM CORM-3 for 6 h. The protein levels of HO-1, FoxO1, and GAPDH (as an internal control) were determined by western blot. (d) The cells were pretreated with AS1842856 (10 μM), NAC (10 mM), APO (10 mM), DPI (10 μM), rotenone (5 μM), LY294002 (10 μM), SH-5 (10 μM), or rapamycin (1 μM) for 1 h and then stimulated by 30 μM CORM-3 for the indicated time intervals. The levels of phosphorylated FoxO1 and total FoxO1 protein were determined by western blot. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, as compared with the control, pretreatment with inhibitor, or siRNA indicated in the figure.

in the CORM-3-induced HO-1 expression, pretreatment with rapamycin significantly inhibited the HO-1 protein expression induced by CORM-3 (Figure 5(a)). Rapamycin also attenuated the HO-1 mRNA expression and promoter activity induced by CORM-3 (Figure 5(b)). To ensure the role of mTOR in HO-1 expression, the cells were transfected with mTOR siRNA to downregulate the mTOR protein level which attenuated the CORM-3-induced HO-1 expression (Figure 5(c)). We investigated whether mTOR activation was necessary for the HO-1 expression induced by CORM-3, and the level of mTOR phosphorylation was determined. CORM-3-stimulated mTOR phosphorylation was attenuated by rapamycin, APO, DPI, rotenone, NAC, LY294002, or SH-5 (Figure 5(d)). These results suggested that CORM-3-induced HO-1 expression is dependent on mTOR activation mediated through a Nox/mitochondria complex/ROS/PI3-K/Ak cascade in RBA-1 cells.

3.6. FoxO1 Is Involved in CORM-3-Induced HO-1 Expression. Several studies have shown that the activation of FoxO1 leads to the expression of many genes in various types of cells. However, whether FoxO1 activation is involved in the expression of HO-1 is not fully understood [35]. The pharmacological inhibitor of FoxO1 (AS1842856) was used to assess the role of FoxO1 in HO-1 expression. Pretreatment with AS1842856 concentration dependently attenuated the expression of HO-1 in RBA-1 cells stimulated by CORM-3 (Figure 6(a)). The HO-1 mRNA expression and promoter activity were also attenuated by AS1842856 in RBA-1 cells stimulated with CORM-3 (Figure 6(b)). To ensure the role of FoxO1 in HO-1 expression, FoxO1 was knocked down by transfection with FoxO1 siRNA, which downregulated the CORM-3-induced HO-1 expression (Figure 6(c)). To verify whether phosphorylation of FoxO1 was required for the HO-1 expression, the level of FoxO1 phosphorylation



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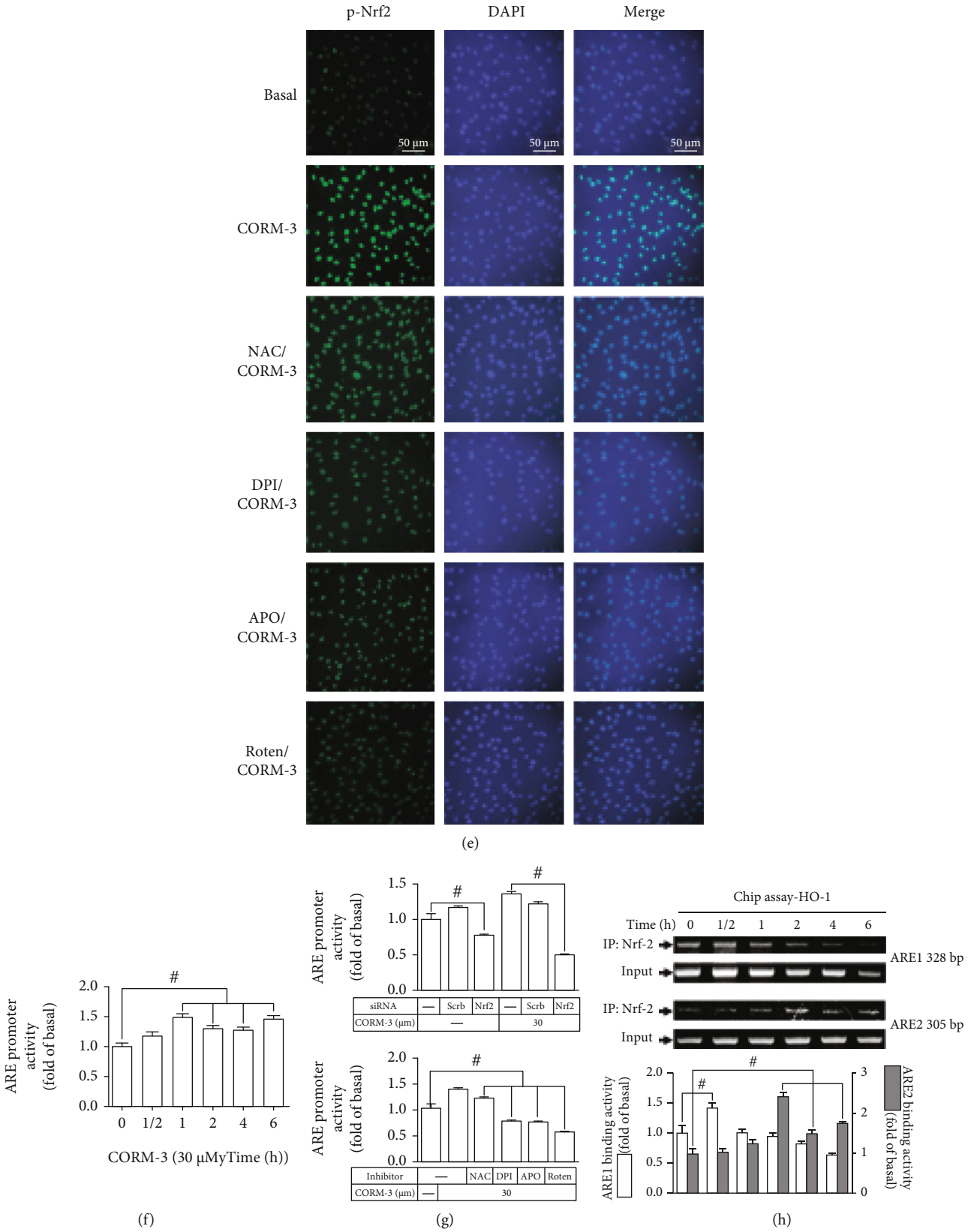

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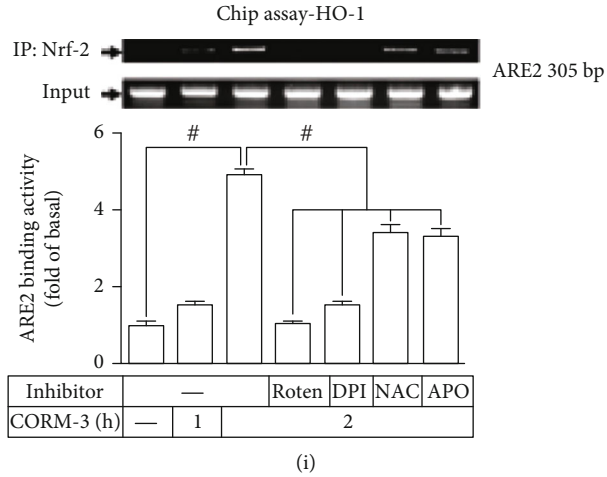

Figure 7: Nrf2 contributes to CORM-3-induced HO-1 expression. (a) RBA-1 cells were transfected with Nrf2 siRNA and then challenged with 30 μM CORM-3 for 6 h. The protein levels of HO-1, Nrf2, and GAPDH were determined by western blot. (b) The cells were transfected with Nrf2 siRNA and then incubated with 30 μM CORM-3 for 4 h. The levels of HO-1 and GAPDH mRNA were determined by real-time PCR (open bars). The cells were transiently transfected with HO-1 report gene together with a β-galactosidase plasmid, followed by transfected with Nrf2 siRNA, and then incubated with 30 μM CORM-3 for 1 h. Promoter activity was determined in the cell lysates (solid bars). (c) The cells were challenged with 30 μM CORM-3 for the indicated time intervals. The cell lysates were centrifuged to prepare nuclear fraction. The levels of Nrf2, phosphorylated-Nrf2, and lamin A were determined by western blot. (d) The cells were pretreated with NAC (10 mM), DPI (10 μM), APO (10 mM), or rotenone (5 μM) for 1 h and then incubated with 30 μM CORM-3 for 30 min. The nuclear fraction was prepared and analyzed by western blot. (e) Cells were pretreated with or without rotenone (5 μM), NAC (10 mM), APO (10 mM), or DPI (10 μM) for 1 h and then stimulated by CORM-3 for 30 min. These cells were stained using anti-p-Nrf2 antibodies and DAPI. The images of p-Nrf2 and nucleus were detected with a fluorescence microscope. Scale bar = 50 μm. (f) The cells were transiently transfected with ARE report gene together with a β-galactosidase plasmid and then incubated with 30 μM CORM-3 for the indicated time intervals. ARE promoter activity was determined by a luciferase reporter gene assay. (g) The cells were transfected with Nrf2 siRNA, followed by transiently transfected with ARE report gene together with a β-galactosidase plasmid, and then challenged with 30 μM CORM-3 for 1 h (upper panel). The cells transfected with ARE report gene together with a β-galactosidase plasmid were pretreated with NAC (10 mM), APO (10 mM), DPI (10 μM), or rotenone (5 μM) for 1 h and then stimulated by 30 μM CORM-3 for 1 h (lower panel). The levels of ARE promoter activity were determined in the cell lysates. (h, i) Cells were treated with 30 μM CORM-3 for the indicated time points (h) or pretreated with rotenone (5 μM), NAC (10 mM), APO (10 mM), or DPI (10 μM) for 1 h and then stimulated by CORM-3 for 2 h (i). The levels of Nrf2 binding to ARE region of the HO-1 promoter were detected by a ChIP assay. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{\#}P < 0.05$, as compared with the control, pretreatment with inhibitor, or siRNA indicated in the figure. DAPI: 4′,6-diamidino-2-phenylindole.

was examined. We found that CORM-3 time dependently stimulated FoxO1 phosphorylation which was attenuated by AS1842856 (Figure 6(d)). To differentiate whether CORM-3-stimulated FoxO1 phosphorylation was mediated via the Nox/mitochondria complex/ROS/PI3K/Akt/mTOR cascade, as shown in Figure 6(d), the level of FoxO1 phosphorylation stimulated by CORM-3 was inhibited by NAC, APO, DPI, rotenone, LY294002, SH-5, or rapamycin. These findings suggested that a Nox/mitochondria/ROS/PI3-K/Akt/mTOR-dependent FoxO1 activation is involved in HO-1 expression induced by CORM-3 in RBA-1 cells.

3.7. Nrf2 Contributes to CORM-3-Induced HO-1 Expression. It has been shown that activation of Nrf2 by external stimuli is a key player in the expression of HO-1 in various types of cells [36]. Some studies have revealed that CORMs activate the Nrf2/HO-1 axis to protect against the inflammatory responses under various pathological conditions [9, 37]. However, in RBA-1 cells, the involvement of Nrf2 in CORM-3-induced HO-1 expression was not completely defined. To evaluate the role of Nrf2 in HO-1 expression, Nrf2 protein expression was downregulated by transfection with Nrf2 siRNA, which abrogated the HO-1 expression induced by CORM-3 (Figure 7(a)). Transfection with Nrf2 siRNA also attenuated CORM-3-induced HO-1 mRNA expression and promoter activity (Figure 7(b)). To evaluate the function of Nrf2 in HO-1 expression, CORM-3 stimulated Nrf2 phosphorylation and translocation into the nuclear fraction (Figure 7(c)) which were attenuated by pretreatment with NAC, DPI, APO, or rotenone (Figure 7(d)). These results were further supported by the data of immunofluorescent staining to verify the role of Nrf2 in the CORM-3-mediated responses in RBA-1 cells (Figure 7(e)). These findings implied that activation of Nrf2 was mediated through a Nox/mitochondrial complex/ROS pathway.

Nrf2 can activate the antioxidant response element (ARE), leading to transcriptional activation of antioxidant genes. To determine whether ARE-driven transcriptional activity is involved in the HO-1 expression induced by CORM-3, data of ARE promoter-luciferase assay showed that CORM-3 time dependently enhanced ARE transcriptional activity which reached a maximal response within 1 h (Figure 7(f)). Moreover, transfection with Nrf2 siRNA and pretreatment with NAC, DPI, APO, or rotenone attenuated

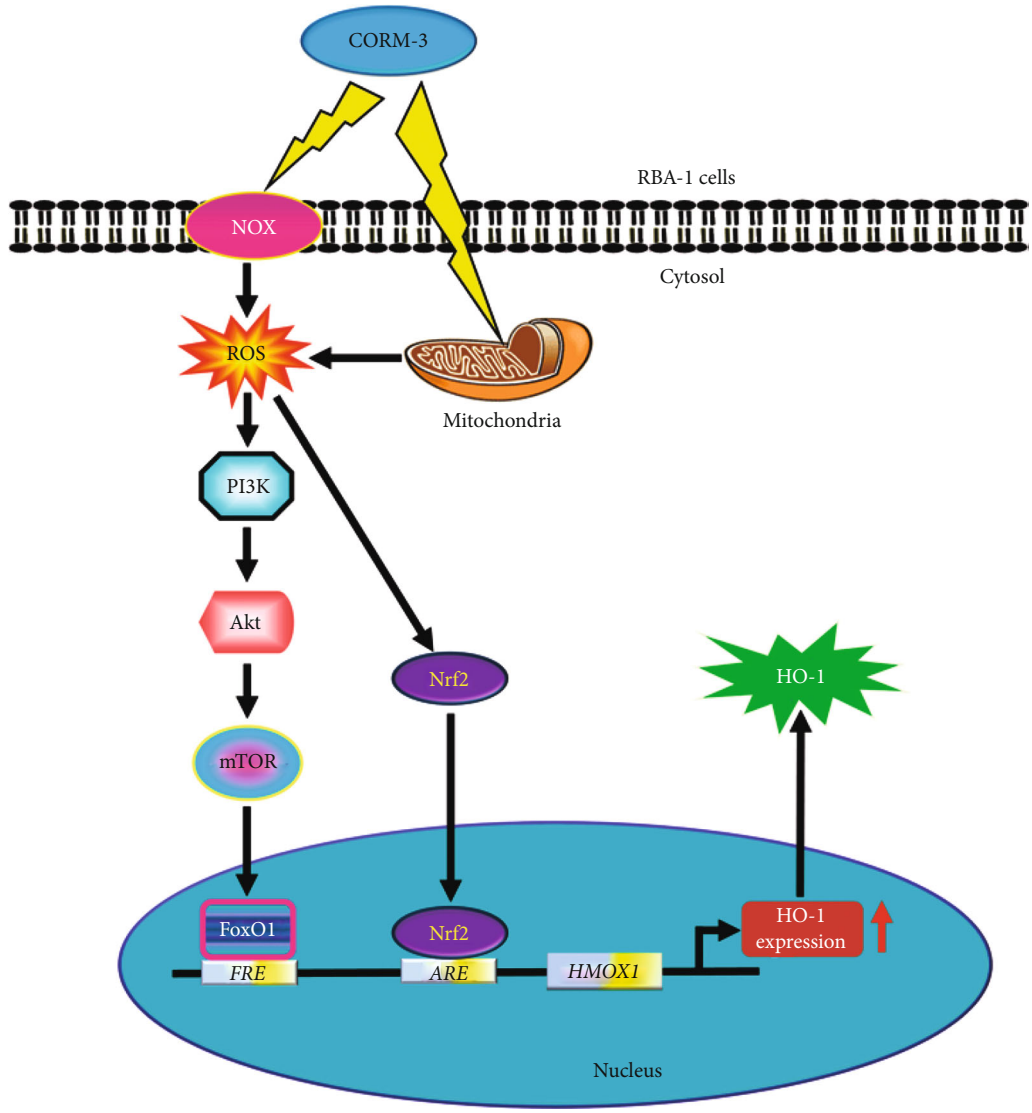

Figure 8: A schematic pathway for CORM-3-induced HO-1 expression in RBA-1 cells. CORM-3 enhanced NADPH oxidase and/or mitochondrial respiratory complex activity, which resulted in the accumulation of intracellular ROS. Oxidative stress promoted the phosphorylation of PI3K/Akt/mTOR and the activation of FoxO1 and Nrf2. After nuclear translocation, FoxO1 and Nrf2 bind to the ARE2 region of HO-1 promoter and increase the expression of the HO-1 gene in RBA-1 cells. Abbreviation: FRE: canonical forkhead response element; *HMOX1*: gene of HO-1.

CORM-3-stimulated ARE promoter activity (Figure 7(g)). Further, ChIP assay showed that CORM-3 enhanced the binding of Nrf2 to ARE2 on HO-1 promoter with a maximal response within 2 h, but not to ARE1 (Figure 7(h)). To further reveal whether ROS participate in Nrf2 recruitment to ARE2 promoter, pretreatment with rotenone, DPI, NAC, or APO attenuated the binding of Nrf2 to ARE2 on HO-1 promoters (Figure 7(i)). These results indicated that CORM-3 stimulated a Nox/mitochondrial complex/ROS-dependent Nrf2 activation, leading to transactivation of ARE2 and HO-1 transcription in RBA-1 cells.

4. Discussion

CO/HO-1 is recognized as a cytoprotective factor under various pathophysiological situations [5, 7, 8, 38, 39]. CO can activate the Nox activity and ROS generation to induce HO-1 expression and possess the effects of anti-inflammation and antioxidation [40]. Our previous study has shown that CORM-3 sequentially activated the c-Src/Pyk2/PKCα/p42/p44 MAPK/AP-1 pathway, thereby upregulating HO-1 in RBA-1 cells [28]. The results of the present study further indicated that CORM-3-induced HO-1 gene expression was attenuated by ROS scavenger, Nox inhibitors (DPI and APO), and rotenone. Further, the downstream components of Nox/ROS were differentiated by the application of selective genetic silencing and pharmacological inhibitors. Our results showed that, in RBA-1, CORM-3-enhanced HO-1 expression is mediated through activation of Nox-mitochondria/ROS-dependent either PI3K/Akt/mTOR/FoxO1 or Nrf2 pathways (Figure 8). To the best of our knowledge, this study is the first to address that the

ROS-mediated mTOR/FoxO1 pathway is involved in the CORM-3-induced HO-1 expression in RBA-1 cells.

CO can cause vasodilation through soluble guanylyl cyclase (sGC) and cyclic GMP (cGMP) [41]. Astrocytes have been supported to regulate the HO/CO pathway of cerebral vasodilation mediated through an increased intracellular Ca^{2+} in astrocytes themselves by activating the ionotropic glutamate receptors (iGluR) [42]. CO protects cerebral vascular endothelium from oxidative stress and apoptosis caused by proinflammatory mediators [43]. Thus, accumulating evidence has indicated that CO could act as a strategy for the management of ischemic and hemorrhagic stroke and multiple sclerosis, which is a major regulator of cerebrovascular hemodynamics and inflammatory reaction in the brain [44].

ROS function as second messengers that regulate intracellular signaling components associated with both inflammatory responses and normal physiological functions dependent on their levels [45, 46]. In astrocytes, Nox-dependent ROS generation is associated with the HO-1 expression induced by CORM-2 [14]. Thus, we investigated the causal relationship between Nox-derived ROS and HO-1 expression in RBA-1 challenged with CORM-3. NAC, a thiol-containing compound, possesses therapeutic effects on various psychiatric/neurological disorders, which are mediated through scavenging ROS and bind metal ions into complexes [47]. Both APO (a $p47^{phox}$ inhibitor) and DPI (a Nox inhibitor) belong to Nox-related inhibitors have been shown to inhibit Nox activation by preventing $p47^{phox}$ (a Nox subunit) translocation to the plasma membrane [48]. Our results showed that Nox/ROS plays an important role in HO-1 expression because CORM-3-induced ROS generation and HO-1 expression were inhibited by NAC, DPI, or APO. Nox is a multisubunit enzyme including membrane and cytosolic subunits. Nox family has seven members with various tissue distributions and activation mechanisms in many cells. According to our results, we revealed that Nox1, Nox2, and Nox4 are involved in ROS generation leading to the HO-1 expression induced by CORM-3. These results were supported by transfection with their respective siRNAs. The generation of mitochondrial ROS was also involved in the CORM-3-induced HO-1 expression, which was ensured by the downregulation of HO-1 expression by pretreatment with rotenone in RBA-1. CORM-3 can uncouple mitochondria and decrease the proton electromotive force, leading to reducing reverse electron flow and ROS production at the level of complex I [49]. In addition, previous studies have indicated that CORM-3/CO induces ROS generation by complex III due to inhibition of cytochrome *c* oxidase [49, 50]. These data are consistent with previous studies demonstrating that, in tracheal smooth muscle cells, Nox-derived ROS generation is involved in HO-1 induction [15, 16]. Our data suggested that Nox- and mitochondria-derived ROS are, at least partially, involved in the HO-1 expression induced by CORM-3 in RBA-1 cells.

The induction of HO-1 is regulated by Nox/ROS-dependent PI3K/Akt pathways [51]. Accumulating evidence has indicated that CO has cytoprotective effects on hepatic ischemia/reperfusion injury and enhancing neurotrophic factor expression via activating the PI3K/Akt pathway [22, 52]. In numerous cells, different inducers including CO can activate the PI3K/Akt pathway to mediate HO-1 gene expression [14, 32]. Our results demonstrated that PI3K/Akt phosphorylation is involved in the HO-1 expression stimulated by CORM-3. PI3K/Akt are also downstream targets of Nox and mitochondria-ROS generation since Akt phosphorylation stimulated by CORM-3 was attenuated by the inhibitor of either Nox or mitochondria. These results concluded that a Nox and mitochondria-ROS-dependent PI3K/Akt phosphorylation participates in the HO-1 expression induced by CORM-3.

PI3K/Akt or AMPK (AMP-activated protein kinase) which could regulate tuberous sclerosis complex (TSC) and Ras-homolog enriched in the brain (Rheb), which has emerged as a modulator of mTOR pathway to activate transcription and translation activities via phosphorylates 4E-BPs, which, in turn, stimulate eIF4E [53, 54]. Moreover, PI3K/Akt further activates transcription factors, such as FoxO [55]. mTOR integrates diverse signals leading to various cellular responses, including inflammation. PI3K/Akt inhibits the activity of TSCs by phosphorylating the TSC2 component [56], finally attenuating the inhibitory effect of the TSC complex toward mTOR complex (TORC)1 [57]. A recent study showed that salvianolic acid A-induced Nrf2/HO-1 activation and cytoprotection are mediated through Akt/mTORC1 activation [33]. Another study also revealed that melatonin activates an Akt/mTOR-dependent pathway to induce HO-1 expression, which prevents hemorrhagic shock-induced liver injury [34]. Our results indicated that CORM-3-induced HO-1 expression was attenuated by rapamycin or transfection with mTOR siRNA, compatible with these studies. We further demonstrated that mTOR is a downstream target of Nox and mitochondria-ROS generation/Akt cascade because CORM-3-stimulated mTOR phosphorylation was attenuated by NAC, rotenone, the inhibitors of Nox, LY294002, and SH-5. These results implied that a Nox and mitochondria-ROS/PI3K/Akt-dependent mTOR phosphorylation participates in the HO-1 expression induced by CORM-3 in RBA-1 cells.

FoxO members mainly comprise FoxO1, FoxO3, FoxO4, and FoxO6. These FoxO members specifically bind to conserved DNA sequence 5′-TTGTTTAC-3′ via their sharing conserved DNA-binding domains with a structure of helix-turn-helix [58]. FoxO regulates a series of downstream targets via transcriptional processes, thus involved in several pathophysiological activities [59]. Multiple signaling pathways converge on FoxOs, including PI3K/Akt [60]. Activated Akt can phosphorylate FoxO1 at three sites (Thr^{24}, Ser^{256}, and Ser^{319}) which results in increased binding of FoxO1 to the regulator 14-3-3, leading to export FoxO1 from nucleus to cytoplasm [23]. Previous studies indicated that FoxO1 is involved in the HO-1 expression which has cytoprotecting effects in various injury models [24, 25]. Our findings are the first to demonstrate that HO-1 expression induced by CORM-3 was attenuated by FoxO1 inhibitor AS1842856 or transfection with its siRNA. We observed that pretreatment with AS1842856, NAC, APO, DPI, rotenone, LY294002, or SH-5 attenuated FoxO1 phosphorylation stimulated by CORM-3. These findings concluded that FoxO1 is

downstream signaling of Nox and mitochondria-ROS/PI3-K/Akt-dependent mTOR cascade leading to HO-1 expression in RBA-1 cells.

Our recent research has reported that the MAPK-dependent AP-1 pathway is an essential mechanism for the CORM-3-induced HO-1 expression in RAB-1 cells [28]. Moreover, several redox-sensitive transcription factors including Nrf2 have been shown to bind with their binding sites in the regulatory elements of the HO-1 gene promoter [23]. Nrf2 is well known as a cellular defender against oxidative stresses and electrophilic insults through the ARE-mediated expression of HO-1. Under physiological conditions, Nrf2 is bound with Keap1 and degraded through Keap1-mediated ubiquitination. Upon cells exposed to oxidative or chemical stress, Nrf2 is liberated from its inhibitor Keap1, then leading to nuclear translocation and binding with the ARE located on the promoters of Nrf2 target genes. Interestingly, Nrf2/HO-1 can also be upregulated by lipopolysaccharides under glutamine depletion in microglial cells [61]. Some experiments have shown that CORMs/CO possesses anti-inflammatory and neuroprotective effects through the Nrf2-dependent HO-1 expression in various types of cells [9, 37]. Our previous study also demonstrated that CORM-2/CO induced HO-1 expression through ROS-dependent Nrf2 pathway [14]. In this study, our results uncovered that Nrf2 activation regulates the HO-1 expression induced by CORM-3 because the response is attenuated by its siRNA transfection. We further differentiated the role of Nrf2 in the signaling pathway of HO-1 expression and demonstrated that the phosphorylation and nuclear translocation of Nrf2 were attenuated by NAC, DPI, APO, and rotenone. Moreover, ChIP assay revealed the interaction between Nrf2 and ARE on HO-1 promoter, showing that the binding site of Nrf2 located on the ARE2 region but not ARE1 region on the HO-1 promoter and the binding ability of Nrf2 with ARE2 region was attenuated by NAC, DPI, APO, and rotenone.

5. Conclusions

In summary, we found that the HO-1 expression induced by CORM-3 is partially mediated through Nox-mitochondria/ROS-dependent PI3K/Akt/mTOR/FoxO1 cascade and Nrf2 activity in RBA-1 cells. Therefore, CORM-3 might be a potential strategy for upregulation of HO-1 and management of brain inflammatory and degenerative diseases.

Abbreviations

APO: Apocynin
ARE: Antioxidant response element
BSA: Bovine serum albumin
ChIP: Chromatin immunoprecipitation
CM-H_2DCFDA: 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester
CO: Carbon monoxide
CORM: Carbon monoxide-releasing molecules
CORM-3: Tricarbonylchloro(glycinato)ruthenium
CREB: Cyclic adenosine 3,5-monophosphate-responsive element-binding protein
DHE: Dihydroethidium
DMEM: Dulbecco's modified Eagle's medium
DNA: Deoxyribonucleic acid
DMSO: Dimethyl sulfoxide
DPI: Diphenyleneiodonium chloride
EAE: Experimental allergic encephalomyelitis
ECL: Enhanced chemiluminescence
EGFP: Enhanced green fluorescent protein
FMN: Flavin mononucleotide
FBS: Fetal bovine serum
FoxO1: Forkhead box O1
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
HIF1: Hypoxia-inducible factor 1
HO: Heme oxygenase
MAPK: Mitogen-activated protein kinase
Keap1: Kelch ECH associating protein 1
MCAO: Middle cerebral artery occlusion
mTOR: Mammalian target of rapamycin
NAC: N-acetyl-L-cysteine
Nox: NADPH oxidase
Nrf2: NF-E2-related factor 2
NO: Nitric oxide
PBS: Phosphate-buffered saline
PI3K: Phosphoinositide 3-kinase
PPAR: Peroxisome proliferator-activated receptor
RBA: Rat brain astrocytes
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RT-PCR: Reverse transcription-polymerase chain reaction
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEMs: Standard errors of the mean
siRNA: Small interfering RNA
STAT: Signal transducers and activators of transcription
TTBS: Tween-Tris-buffered saline
XTT: 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2Htetrazolium-5-carbox-anilide.

Data Availability

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

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

CCL, CCY, LDH, and CMY designed and conducted the study. CCL, CCY, and LDH performed and collected the data. CCL, CCY, LDH, and CMY analyzed and interpreted the data. CCY and CMY prepared the manuscript. CCL, CCY, LDH, and CMY reviewed the manuscript. All authors have read and agreed to the published version of the

manuscript. Chih-Chung Lin and Chien-Chung Yang contributed equally to this work.

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Supplementary Materials

Supplementary Figure 1: effects of either CORM-3 or inactive-CORM-3 on HO-1 expression on RBA-1 cells. Supplementary Figure 2: effects of CORM-3 on cell viability of RBA-1 cells. *(Supplementary Materials)*

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Review Article

Natural Phytochemicals as Novel Therapeutic Strategies to Prevent and Treat Parkinson's Disease: Current Knowledge and Future Perspectives

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Parkinson's disease (PD) is the second-most common neurodegenerative chronic disease affecting both cognitive performance and motor functions in aged people. Yet despite the prevalence of this disease, the current therapeutic options for the management of PD can only alleviate motor symptoms. Research has explored novel substances for naturally derived antioxidant phytochemicals with potential therapeutic benefits for PD patients through their neuroprotective mechanism, targeting oxidative stress, neuroinflammation, abnormal protein accumulation, mitochondrial dysfunction, endoplasmic reticulum stress, neurotrophic factor deficit, and apoptosis. The aim of the present study is to perform a comprehensive evaluation of naturally derived antioxidant phytochemicals with neuroprotective or therapeutic activities in PD, focusing on their neuropharmacological mechanisms, including modulation of antioxidant and anti-inflammatory activity, growth factor induction, neurotransmitter activity, direct regulation of mitochondrial apoptotic machinery, prevention of protein aggregation via modulation of protein folding, modification of cell signaling pathways, enhanced systemic immunity, autophagy, and proteasome activity. In addition, we provide data showing the relationship between nuclear factor E2-related factor 2 (Nrf2) and PD is supported by studies demonstrating that antiparkinsonian phytochemicals can activate the Nrf2/antioxidant response element (ARE) signaling pathway and Nrf2-dependent protein expression, preventing cellular oxidative damage and PD. Furthermore, we explore several experimental models that evaluated the potential neuroprotective efficacy of antioxidant phytochemical derivatives for their inhibitory effects on oxidative stress and neuroinflammation in the brain. Finally, we highlight recent developments in the nanodelivery of antioxidant phytochemicals and its neuroprotective application against pathological conditions associated with oxidative stress. In conclusion, naturally derived antioxidant phytochemicals can be considered as future pharmaceutical drug candidates to potentially alleviate symptoms or slow the progression of PD. However, further well-designed clinical studies are required to evaluate the protective and therapeutic benefits of phytochemicals as promising drugs in the management of PD.

1. Introduction

Parkinson's disease (PD) is a common progressive chronic neurodegenerative movement disorder that increases with age. PD prevalence is 315 per 100 000 persons of all ages in the Western world; this prevalence is expected to double by the year 2030, increasing mortality, morbidity, and socioeconomic burden worldwide [1]. The clinical symptoms commonly associated with PD disorder include bradykinesia, resting tremor, postural instability, rigidity, depression, and anxiety [2]. The important hallmarks of PD are progressive loss or damage of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and dopamine (DA) depletion in the striatum (ST), which is associated with the motor

impairments of PD [3]. In addition to the neuropathological process affecting dopaminergic and nondopaminergic systems, other pathological processes are also seen in Alzheimer's disease (AD) affecting cholinergic dysfunction and serotonergic, glutamatergic, and noradrenergic pathways associated with dopaminergic neuronal death and/or DA system dysfunction. People with PD also experience nonmotor symptoms such as sleep disturbance, cognitive changes, autonomic dysfunction, altered mood, depression, fatigue, and pain [4, 5]. PD is described as a synucleinopathy, as accumulation of misfolded α-synuclein becomes a central feature of Lewy bodies, which are an important pathological hallmark of PD. Moreover, α-synuclein appears to be linked to both sporadic and familial forms of the disease and carries unique importance in the etiology of PD [6]. Interestingly, α-synuclein accumulation has been broadly linked to several neurotoxin pathways, including posttranslational modifications, neuroinflammation, oxidative stress, mitochondrial dysfunction, altered mitochondrial morphology, synaptic dysfunction, phospholipids, induced endoplasmic reticulum (ER) stress, and metal ions [7]. The age-related failure of antioxidant defense system and overproduction of ROS exacerbate oxidative stress in the brain; these events may play a role of misfolded α-synuclein initiating aging process in PD [8, 9].

Currently, levodopa (L-dopa) is the most effective therapy for the early-stage motor symptoms of PD, but it is not considered a cure for PD [10]. Bradykinesia and rigidity respond best, whereas tremor may be only slightly reduced. Problems with balance and other symptoms may not be alleviated at all. However, L-dopa is not effective in relieving neuronal loss, nonmotor symptoms, or Lewy pathology. Over time, patients require higher doses of L-dopa, which are associated with increased side effects such as dyskinesia [11]. Anticholinergic drugs may help control rigidity and tremor in approximately 50% of cases, and the antiviral agent amantadine also seems to diminish motor symptoms [3]. Deep brain stimulation (DBS) and DA-based medications are also used to treat various neurologic motor symptoms with disease progression [12]. Hence, it is critical to develop new therapeutic approaches to prevent neuronal loss and nonmotor symptoms and to prevent the accumulation of α-synuclein aggregation or Lewy pathology in the brain. Moreover, only symptomatic treatment options are available for PD; none slow or prevent progressive neuronal loss in the dopaminergic system [13, 14]. Herbal preparation and phytochemicals isolated from plant food have been proposed as "herbal medicine" for the treatment of PD [15]. Myriad phytochemicals from nature have been documented as potential molecules, drug leads, and phytochemical formulations in treating several inflammatory disorders [16, 17]. Likewise, extensive pharmacological reports have demonstrated the effectiveness of phytochemicals in treating dementia, depression, and neurodegenerative disorders (NDDs) [18]. Biologically active phytochemicals produced in plants are of clinical importance as primary and secondary metabolites for the antioxidant defense mechanism against various stress-related disorders and other pathogenic conditions [19]. The therapeutic and beneficial effects of these phytochemicals provide nutrition for normal living cells, fight disease-causing agents, strengthen the immune system, and act as antioxidants [20]. Plant products and their bioactive phytochemicals can efficiently scavenge oxygen free radicals and boost the cellular antioxidant defense system and related molecules, thereby protecting cells from oxidative damage [20, 21]. Several findings indicate that these antioxidant phytochemicals have confirmed neuritogenic potential, reconstructing synaptic connectivity by restoring the loss of neuronal processes [22–25]. In fact, various preclinical reports have described a number of natural pharmacological candidates that can coactivate the antioxidant defense system and neurotrophic factor-mediated cell survival systems [26–31], suggesting that these phytochemicals have therapeutic potential for the treatment of oxidative stress-mediated NDDs, especially PD.

Thus, therapeutic approaches targeting oxidative stress, α-synuclein accumulation, neuroinflammation, and mitochondrial dysfunction may hold great promise as a cure for PD. Numerous antioxidant phytochemicals have displayed potentially neuroprotective properties by targeting several mechanisms beyond those mentioned above. Phytochemicals are biologically active compounds that usually correspond to the secondary metabolites present in plants like alkaloids, flavonoids, and terpenoids [32]. Many epidemiological studies have suggested a proportional relationship between consumption of a diet rich in antioxidant phytochemicals and improved health outcomes, including reduced risk for AD, PD, and other NDDs [33–35]. Other epidemiological studies have associated the consumption of various food groups and beverages such as fruits, vegetables, tea, and coffee with reduced risk of development of PD [36, 37]. Recent research on the dietary intake of phenolic phytochemicals has been presented in several European countries, with results showing that the average intake is 820 mg/day in Spain, 1193 mg/day in France, and 1756.5 mg/day in Poland. The main dietary sources of the total polyphenols in Spain and France are fruits and nonalcoholic beverages (principally coffee and tea). In Spain, fruits accounted for 44% and nonalcoholic beverages for 23% of total polyphenol intake, whereas in France fruit accounted for only 17% and nonalcoholic beverages for 55%. Considered individually, the main source of total dietary polyphenols is food with 18% and 44% of contribution in Spain and France, respectively. In Spain, olives and olive oils are also important sources of polyphenols, accounting for 11% of the total polyphenol intake. Nonalcoholic beverages were the main food contributors to polyphenol intake in Poland and accounted for fully 67% of the total polyphenol intake due to high consumption of coffee and tea. The third main contributor to total polyphenol intake is chocolate, whereas fruits accounted for a lower percentage of intake [38–40].

In the present study, we describe the phytochemicals present in dietary sources, using chrysin, vanillin, ferulic acid (FA), thymoquinone (TQ), ellagic acid (EA), caffeic acid (CA), epigallocatechin-3-gallate (EGCG), theaflavin (TF), and other plant-derived antioxidant phytochemicals (asiatic acid (AA) and α- and β-asarone) as examples and discuss their beneficial neuroprotective effects and relevance to potential treatment strategies of PD. Importantly, phytochemicals

have thus far been investigated primarily in both cellular and rodent experimental studies for their potential benefits in brain metabolism; these studies have provided some encouraging results indicating antioxidant, anti-inflammatory, and cognitive enhancing effects of these phytochemicals coupled with a wide range of tolerability [41, 42]. Additionally, phytochemicals also have been confirmed to reduce mitochondrial dysfunction and inhibit formation of α-synuclein accumulation-induced oxidative stress and inflammatory responses [43, 44]. Several studies have also provided evidence that the antioxidant activity of some phytochemicals can activate nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathways. Furthermore, phytochemicals contribute to the activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and extracellular signal-regulated kinase (ERK) pathways and inhibit nuclear factor kappa B (NF-κB) pathways [22, 45]. Similarly, a number of studies have suggested that phytochemicals confer neuroprotection in experimental parkinsonism by reducing oxidative stress and mitochondrial dysfunction and fostering degradation of α-synuclein toxic species through activation of autophagy [46–48]. This study provides information about the neuroprotective properties and mechanisms of action of recently discovered naturally derived phytochemicals that target oxidative stress and neurodegeneration through cellular- and molecular-level changes in the progression of PD. In addition, we explore Nrf2/ARE and autophagy signaling-related pharmacological mechanisms. Moreover, we highlight some potentially neuroprotective active derivatives of antioxidant phytochemicals and phytochemical-based nanodelivery systems that fight pathological conditions associated with aging-related oxidative stress. The sources and chemical structures of phytochemicals are presented in Figure 1.

2. Role of Oxidative Stress in PD

Oxygen is a crucial molecule that may produce free radicals during metabolic reactions. These free radicals constitute an important fundamental molecule in any biochemical and/or biological reduction reaction [49]. However, these free radicals are highly unstable and easily cross biomolecules like nucleic acid, lipids, cellular membranes, and proteins [50]. In addition, recent investigations have observed novel developments in the biology of free radicals and their impact on brain health and the development of various pathological conditions, since the brain uses a disproportionately large volume of oxygen compared to other organs, making it more vulnerable to free radical attacks [51, 52]. Free radicals like reactive nitrogen species (RNS) and reactive oxygen species (ROS) are prime stimulators of oxidative stress and imbalance in the antioxidant capability of cells [53]. ROS, such as hydrogen peroxide (H_2O_2), are key precursors of superoxide radicals ($O^{\cdot}-2$) produced in the brain mitochondria that restrict mitochondrial movement and damage DNA, leading to impaired brain function and NDDs [54]. Considerable evidence from recent *in vivo* experiments has confirmed that excessive production of ROS contributes significantly to neuronal cell death and altered brain function resulting from DA metabolism, low levels of glutathione (GSH), and high levels of calcium and iron in the substantia nigra (SN) [55, 56]. Moreover, the brain contains a high level of membrane polyunsaturated fatty acids, which, under oxidative stress conditions, leads to higher lipid peroxidation and the generation of neurotoxic products. These deficient enzymatic antioxidant systems and increased oxidative stress markers are common pathological hallmarks of PD [57]. ROS contributions to PD pathogenesis have been supported by extensive evidence (Figure 2).

Several studies have previously reported that PD tissues show an oxidant status that is broadly associated with both DA autooxidation and mitochondrial complex I dysfunction [58]. In addition, preclinical and clinical findings have revealed that brain and cerebrospinal fluid samples from PD patients contain significantly higher levels of oxidized coenzyme Q10 and the nuclear DNA oxidation biomarker 8-OHdG, establishing the existence of oxidative damage to DNA and mitochondria in PD patients [59]. Moreover, reduced mitochondrial complex I catalytic activity, downregulated mitochondrial biogenesis, and reduced mitochondrial DNA levels in the prefrontal cortex were observed contributing to excessive ROS production and oxidative stress in PD brains [60]. Recent findings have identified excess production of ROS and diminished mitochondrial complex I activity in SH-SY5Y neuroblastoma cells [61]. Similarly, one model using 6-hydroxydopamine- (6-OHDA-) induced rats found increased levels of 8-OHdG in the urine, serum, and SN of rats when compared with control groups; this finding was consistent with research involving human tissue [62]. In addition, earlier studies have demonstrated that increased ROS production causes significant and simultaneous dysregulation of several signaling pathways, such as RAS-MEK-ERK1/2 [63], PI3K/AKT/GSK3β [64], Keap1-Nrf2-ARE [65], NF-κB [66–68], and JAK/STAT [69].

3. Role of Autophagy in PD

Autophagy is the key physiological cellular catabolic process in response to cellular starvation and the degradation of damaged organelles [70]. Autophagy describes the process by which diverse cellular mechanisms encompassing nucleic acids, whole organelles, lipids, proteins, sugars, and cytoplasmic compartments are sequestered into a double-membrane budding vacuole called a phagophore, which later matures to seal in a vesicle called an autophagosome [71, 72]. Damaged or dysregulated autophagy has been linked with several pathological processes, including inflammation, cancer, lipid metabolism, and NDDs [73–76]. In fact, autophagy helps clear damaged organelles, protein aggregates, and lipid droplets, which constitute unwanted and often toxic cargo that may lead to cellular dysfunction. Importantly, damaged or dysregulated autophagy has been recognized as a critical pathogenic process, particularly in PD and other NDDs [73, 77].

In PD, the role of autophagy is significant in neuronal quality control and brain maintenance. Recent confirmations have demonstrated that mice lacking key autophagy genes like AuTophaGy ATG5 and ATG7 show spontaneous neurodegeneration, accumulation of protein aggregates, and motor

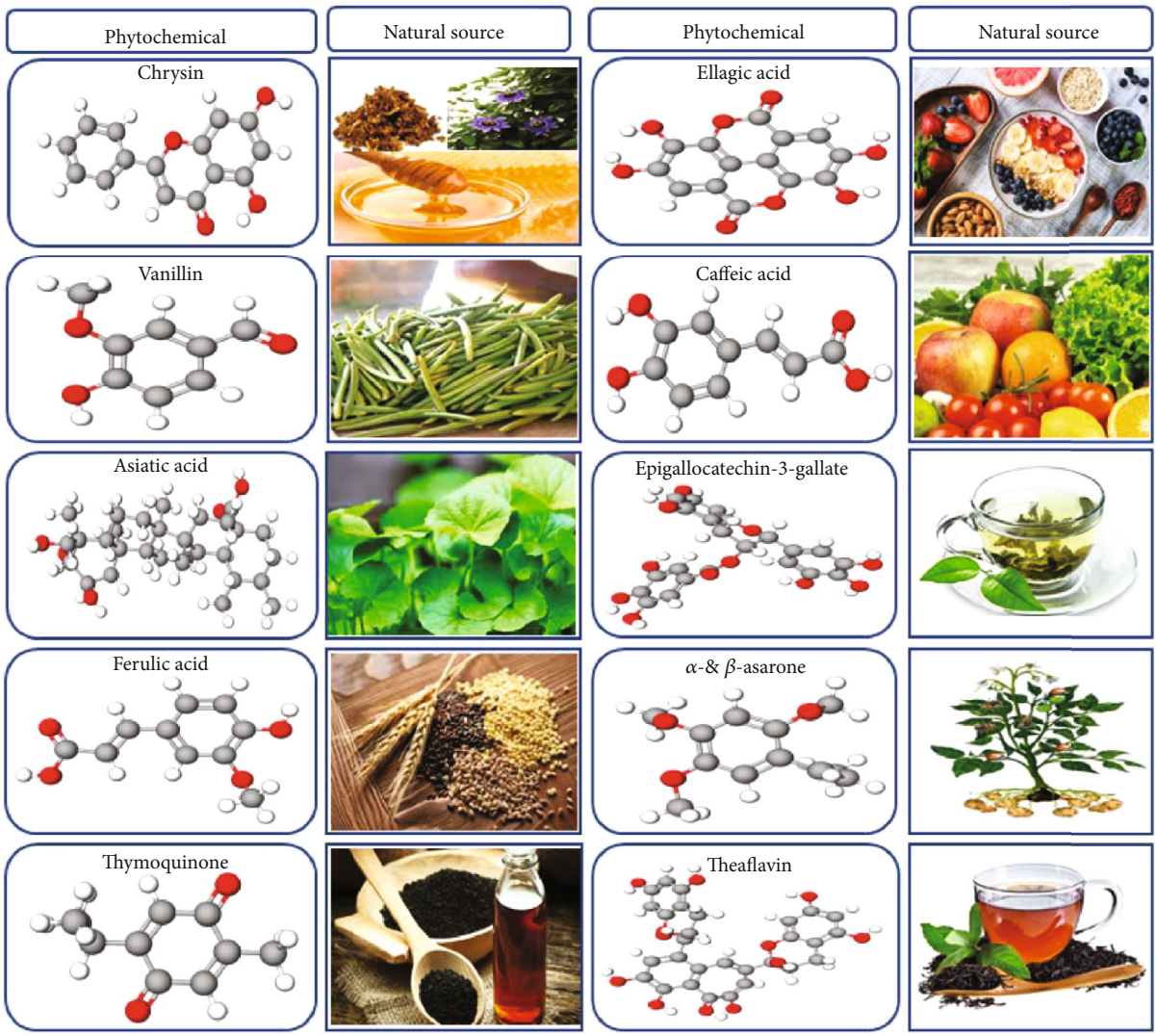

Figure 1: Structures of phytochemicals present in dietary sources (chrysin, vanillin, ferulic acid (FA), thymoquinone (TQ), ellagic acid (EA), caffeic acid (CA), epigallocatechin-3-gallate (EGCG), and theaflavin (TF)) and other plant-derived phytochemicals (asiatic acid (AA) and α- and β-asarone), belonging to different classes of phenolics and nonphenolics. These phytochemicals have demonstrated several mechanisms of action by which they protect the brain from neurodegeneration. The structures were regenerated from http://molview.org/. Here, the different colors of different atoms in the structures represent specific molecules, where grey = carbon, white = hydrogen, and red = oxygen.

neuron dysfunction [78, 79]. 6-OHDA, 1-methyl-4-phenylpyridinium (MPP^+), rotenone, and 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP)—a neurotoxin associated with mitochondrial dysfunction, ATP depletion, and accumulation of ROS such as H_2O_2, hydroxyl radicals, and superoxide—are commonly used in experimental models of PD in both *in vitro* and *in vivo* research [80, 81]. Changes in autophagy status have been reported in 6-OHDA-induced PD models [82, 83]. Recently, it was reported that a mild increase in ROS levels could activate mucolipin 1, a key calcium-conducting channel located on the lysosome membrane, to initiate calcineurin-dependent activation of transcription factor EB, which is identified as a master regulator of the autophagy–lysosome pathway (ALP) [84]. In turn, the transcription factor EB-mediated induction of autophagy promotes clearance of damaged mitochondria and removal of excess ROS [73]. Excessive ROS levels may cause lysosomal dysfunction and autophagic failure and lead to oxidative stress or cell death; however, oxidative stress generally mediates autophagic pathway activation [85]. Moreover, the activation levels and induction time of autophagy also play critical roles in the survival or death of cells [86].

As mentioned previously, DJ-1 has been confirmed to regulate basal autophagy and mitophagy in a manner similar to Parkin and PINK1 [87, 88]. Recently, it has been demonstrated that DJ-1 loss-of-function mutations were first identified as generating oxidative stress in mice and *Drosophila* exposed to the toxicity of rotenone, paraquat (PQ), and MPTP [89, 90]. Interestingly, DJ-1 overexpression has been shown to protect against oxidative insults. In dopaminergic cell lines, the overexpression of DJ-1 was able to protect dopaminergic cells through reduced levels of protein oxidation,

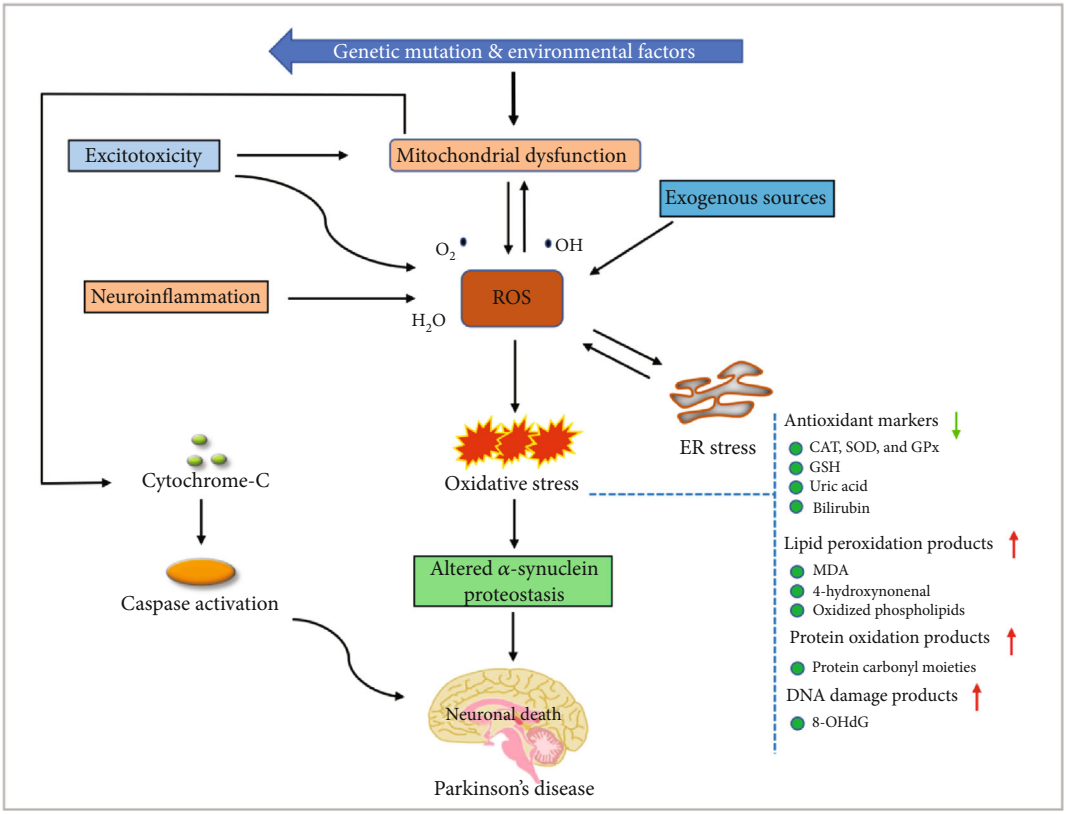

Figure 2: Oxidative stress and its implications in the pathogenesis of neurodegeneration in PD. When the production of ROS generation overwhelms intracellular antioxidant defenses, brain cells are exposed to oxidative stress, which may lead to mitochondrial dysfunction and further ROS production. Oxidative stress impairs the protein degradation system and hinders the clearance and results in the subsequent deposition of misfolded protein, which in turn results in lipid peroxidation, protein oxidation, and DNA damage, leading to neuronal death. These events constitute the pathological basis of PD. CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; GSH: glutathione; MDA: malondialdehyde; 8-OHdG: 8-hydroxydeoxyguanosine.

ROS, and cell death in H_2O_2- and 6-OHDA-exposed wild-type, but not mutant cells [91, 92]. In animal models, DJ-1 overexpression has been shown to protect against dopaminergic neuronal degeneration in mice exposed to MPTP and rats exposed to 6-OHDA in wild-type, but not mutant murine models [91, 93]. Similarly, novel drugs activating DJ-1 protected against rotenone- and 6-OHDA-induced degeneration of SN dopaminergic neurons in murine PD models [94]. The findings of the previous studies suggested that these neuroprotective effects of DJ-1 may diminish ROS production in response to local L-calcium channels' pacemaking activity in dopaminergic neurons through regulation of mitochondrial functions [95]. Overall, the excess ROS and RNS are generated when autophagy and mitophagy are impaired. Consequently, in the present study, we explore evidence of the autophagy-based neuroprotective effects of some natural phytochemicals in experimental PD.

4. Oxidative Stress and Antioxidant Defense Mechanisms

Nrf2 is a ubiquitous transcription factor that binds to the ARE in the maintenance of intracellular homeostasis and protects cells from harmful chemical agents by inducing the detoxifying agent and responding to oxidative stress. Under normal conditions, three known ubiquitin ligase systems, E3 ubiquitin ligase Hrd1, glycogen synthase kinase (GSK3β), and Kelch-like ECH-associated protein 1 (Keap1), contribute extensively to the degradation of Nrf2 [96]. The electrophilic nature of these three activator genes enables the modification of thiol group (-SH) groups in Keap1 via oxidation, alkylation, and reduction processes [97]. Under oxidative stress condition, the Nrf2-ubiquitin ligase system's interaction is disturbed, and newly synthesized Nrf2 is transported into the nucleus, where it binds to the ARE region of specific target genes [98]. These targets are typically considered to be antioxidant genes; however, in recent decades, several scientific findings have demonstrated the extensive involvement of Nrf2 in the regulation of various physiological functions through its antioxidant, anti-inflammatory, autophagic, detoxifying, and proteasomal actions [99, 100]. Some of the physiological functions are directly or indirectly linked with PD pathogenesis. Previous research has examined the Nrf2 signaling-mediated antioxidant protection of neurons in numerous PD models, finding that Nrf2 directly regulates the activities of catalase (CAT), sulfiredoxin-1 (SRXN1), NAD(P) H:quinone oxidoreductase-1 (NQO1), superoxide dismutase 2 (SOD2), heme oxygenase-1 (HO-1), peroxiredoxins 3

and 5 (PRDX3 and PRDX5), and enzymes involved in GSH metabolism [101–103]. Genetic variation in the Nrf2 gene has been associated with PD progression [98]. Recently, microchip analysis revealed that Nrf2-dependent signaling cascades play a vital role during PD; this role was well illustrated in various types of tissues of patients with PD. The expression of 31 genes that contain ARE sequences in the promoter decreased significantly, while the expression of Nrf2 increased in all of the tissue samples of PD patients [104].

Recently, several *in vitro* and *in vivo* findings provided evidence for a possible protective role of the Nrf2-ARE pathway in PD. For instance, apomorphine, which acts a dopamine D(1)/D(2) receptor agonist, activates the Nrf2-ARE signaling pathway to protect against ROS-mediated damage, thereby bolstering the cellular defense system. Pretreatment of SH-SY5Y cells with apomorphine stimulated the translocation of Nrf2 into the nucleus and the transactivation of the ARE. The expression of HO-1 was induced by apomorphine in a dose-dependent manner. Moreover, the authors found that the activation of the ARE and the induction of HO-1 mRNA caused by apomorphine were suppressed in the presence of intracellular ROS in 6-OHDA-induced SH-SY5Y cells [105]. Transplantation of astroglia cells overexpressing Nrf2 into mouse brain showed a significant decline in vulnerability to 6-OHDA-mediated neurotoxicity [106]. Similarly, astrocytic Nrf2 overexpression diminished MPTP-mediated toxicity in mice, demonstrating that astrocytic modulation of the Nrf2-ARE pathway indicates decreased or attenuated neuronal death in PD [107]. Deprenyl, a drug used in the treatment of PD, was shown to stimulate Nrf2 activity as part of its cytoprotective mechanism of action [108]. Another study reported that flavonoid luteolin effectively protects against the mitochondrial toxin MPP^+ by activation of the Nrf2 pathway in cultured rat neuronal PC12 and glial C6 cells [109]. In a more recent study, the knockdown of Nrf2 nullified this protective effect, showing that luteolin-triggered protection is mediated solely through Nrf2. Similarly, the Nrf2 transcriptional activator tBHQ protected against neurotoxin deltamethrin-induced PC12 in a cellular PD model [110]. In a very recent study using an MPTP-induced PD model, uric acid increased Nrf2-responsive genes, including g-glutamate-cysteine ligase catalytic subunit (g-GCLC), HO-1, NQO1, and mRNA, and protein expressions of Nrf2 [111]. Sulforaphane, a natural phytochemical, significantly attenuated cell damage and reversed the reduction of Nrf2, HO-1, and NQO1 expression induced by MPP^+ neurotoxicity; these protective effects are due in part to the activation of the Nrf2-ARE signaling pathway [112]. A similar protective effect against the decreased levels Nrf2 and Keap1 caused by 6-OHDA has been reported in rats treated with puerarin; the puerarin decreased lipid peroxidation and oxidative stress in the SN, significantly increased brain-derived neurotrophic factor (BDNF) expression, and activated the Nrf2/ARE signaling pathway [113]. Another study reported that dietary bioflavonoid pinostrobin significantly enhanced Nrf2 expression and nuclear accumulation, improved ARE promoter activity, and upregulated expression of HO-1. Interestingly, authors then found that pinostrobin promoted phosphorylation of PI3K/AKT and ERK, and pharmacological inhibition of PI3K/AKT or ERK signaling diminished pinostrobin-induced Nrf2/ARE activation and neuroprotective actions against MPTP/MPP^+-induced neurotoxicity in PD models [114]. Together, these findings strongly indicate that treatment of PD via activation of the Nrf2-ARE signaling pathway may be possible. Moreover, these studies demonstrate the protective effects of naturally derived antioxidant phytochemicals on Nrf2-ARE-induced antioxidant protection in experimental models of PD.

5. Structure-Activity Relationship between Phytochemicals and Neuroprotection in PD

Recent reports have shown that polyphenols, terpenoids, flavonoids, ascorbic acid, alpha-tocopherol, catechins, and beta-carotene may offer a potential neuroprotective effect and play a role in alleviating the progression of PD [39, 115]. Polyphenolic compounds have been found in a variety of edible and nonedible plants; to date, approximately 8000 different flavonoids have been identified in different organs of plant tissues [116, 117]. ROS generation and RNS in mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase can trigger oxidative damage harmful to cells and decrease antioxidant defense, resulting in age-associated NDDs such as PD [118, 119]. Moreover, free radical-mediated oxidation of nucleic acids, lipid oxidation, and oxidation of proteins can damage cell membranes and trigger protein crosslinking [120]. The health benefits of flavonoids and terpenoids are attributed to their antioxidant and chelating abilities. Through their capacity for oxidation, terpenoids and flavonoids have confirmed unique neuroprotective effects [39, 121]. Flavonoids give a proton to form a phenoxyl radical and a singlet oxygen and scavenge superoxide as well as hydroxyl and peroxyl radicals by the release of an additional proton. Indeed, a complex structure was formed by the diol group with copper, various transition metal ions, and ferric iron, which plays a crucial role in preventing the production of ROS [122]. Furthermore, recent research revealed that flavonoids, terpenoids, and nonflavonoids chelate copper and iron ions and reduce the generation of free radicals [123].

The structure required for neuroprotective activity is not always linked with antioxidant properties [124]. Previous studies have demonstrated that EGCG, TF, and EA and its derivatives have good free-radical-scavenging effects and relatively high hydrophobic properties; thus, these compounds showed much higher antioxidant activity [125, 126]. These results suggest the relationship of independent antioxidant mechanisms in their neuroprotective activity. Furthermore, glutathione reductase (GR), GPx, CAT, SOD, and glutathione S-transferases (GSTs) are polyphenols that upregulate ROS-scavenging enzymes [127]. Polyphenols may enhance the levels of these antioxidants by activation of cellular signaling pathways [128]. Phenolic acids of FA, EA, and CA have 4-hydroxy and electron-donating 3-methoxy groups on the benzene ring and can scavenge hydroxyl, peroxynitrite, and superoxide and terminate radical chain reactions.

These phenolic acids effectively bind to the lipid bilayer and prevent lipid peroxidation with an adjacent unsaturated carbon–carbon double bond [129, 130]. There is evidence that phenolic acids and their ethyl ester derivatives can upregulate protective genes, such as heat shock protein-70 (HSP-70), HO-1, and ERK1/2, which are beneficial therapeutic and preventive agents in NDDs, particularly in PD [129, 131].

The chemical properties of chrysin, due to a lack of B- and C-ring oxygenation, are linked with several pharmacological properties that range from antioxidant to anti-inflammatory effects [132]. Differences in the chemical structure of flavones have been shown to enhance the activity of antioxidant enzymes and to provide an inhibitory effect on the proinflammatory mediator of COX-2 expression [133]. In addition, xanthine oxidase inhibitory assay has shown that chrysin significantly suppresses xanthine oxidase production of ROS [134, 135], and amentoflavone and blueberry potentially inhibit iNOS expression [136]. Interestingly, in rodent models, 10–100 μM concentrations are required to display the potential antioxidant and anti-inflammatory activities of polyphenols [134, 135].

6. Neuroprotective Roles of Phytochemicals: Antioxidant, Anti-Inflammatory, and Other Signaling Pathways

6.1. Neuroprotective Mechanisms of Chrysin in PD. Chrysin is a natural polyphenolic compound known as a flavonoid; flavonoids are ubiquitous in vegetables, fruits, mushrooms, blue passion flowers, plants, and especially in honey [137]. Numerous epidemiological, cellular, and animal studies have substantiated the potential health benefits of flavonoids. The main sources of flavonoids in Westernized countries—specifically Australia, the United States, and many European nations—are tea, fruit or vegetable juices, blue passion flowers, and wine, with the estimated total intake of flavonoids ranging from 200 mg/day in Australia [138] to 500 mg/day in parts of Europe where tea consumption is high [139]. In countries such as Spain, Poland, Mexico, and Greece, where diets are rich in citrus fruits and wine, dietary flavanones can range from 30 mg/day (Greece) to 170 mg/day (Poland) [139–142]. Similarly, in Asian countries (China, Japan, and Korea), dietary flavanone intake ranged from 36 mg/day (Korea) to 5 mg/day (China) [143–146]. Chrysin has been explored for its neuroprotective effects, which are attributable to its antioxidant, anti-inflammatory, and other pharmacological properties. The various therapeutic activities of chrysin depend on its bioavailability and attainable concentrations in cells and target tissues in rodents. Following administration of 400 mg of chrysin to human subjects, bioavailability was found to be extremely low due to rapid metabolism, poor intestinal absorption, and rapid excretion [147]. Scientific evidence has established that chrysin is found conjugated approximately 99% in the plasma protein binding [147]. However, it was reported that chrysin is considered to have very low oral bioavailability and a distribution volume of approximately 0.003%–0.02% [147]. Furthermore, a recent investigation demonstrated that chrysin can cross biological membranes easily; however, extensive sulfation and glucuronidation in the intestinal cells limit its absorption [148]. Although chrysin is considered safe for human consumption at a daily dose of 500 mg to 3 g without any toxic effects, higher doses may induce undesirable side effects [149]. Chrysin is especially important for polyphenols targeting the brain; penetration of the blood-brain barrier (BBB) is crucial for their therapeutic benefits [150].

The neuroprotective activity of chrysin shows that increases in DA levels in both *in vitro* and *in vivo* experiments are inversely associated with dopaminergic neuronal loss (Table 1) [151, 152]. It was found that chrysin enhanced DA levels by acting as an Monoamine oxidase B (MAO-B) inhibitor in MPP^+- and MPTP-treated CGN cells and mouse models of PD [152]. In this way, chrysin treatment can increase DA levels and subsequently suppress DA metabolism in the ST through the inhibition of MAO-B [152]. Several neurotoxin-induced *in vivo* experiments also demonstrated that chrysin increased DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) levels [153, 154]. Moreover, chrysin administration very significantly attenuated the cognitive dysfunction and motor impairment in these animals as evaluated by passive avoidance, rotational behavior, and Barnes maze tests [154], as well as beam walk and horizontal and vertical grid tests [153]. Therefore, chrysin presents as a potential disease-modifying agent that may also prove beneficial for symptom relief in PD.

Several preclinical studies have revealed that chrysin provides antioxidant effects by reducing oxidative stress and modulating or boosting cellular antioxidant enzyme levels, thus decreasing the lipid peroxidation triggered by numerous oxidative insults [155–157]. In aged mice, chrysin administration significantly blocked the elevation of ROS levels and boosted antioxidant enzyme activities [158]. In the MPTP-induced PD mouse model, chrysin pretreatment diminished oxidative stress as evaluated by lipid peroxidation levels; the pretreatment also inhibited reduction of SOD activity and GSH content in the SN region [153]. In agreement with these results, chrysin administration inhibited the increase of NO and NADPH oxidase activity and prevented the reduction of GSH in the ST of 6-OHDA-treated mice [154]. Additionally, chrysin treatment attenuated 6-OHDA-induced decreases in the levels of Na^+ and K^+-ATPase in the ST of mice. Na^+ and K^+-ATPase play a central role in maintaining ionic gradients and neuronal excitability and are more susceptible to oxidative damage [159].

RNS generated by iNOS may be significantly associated with oxidative stress in PD, and excessive production of NO can lead to loss of dopaminergic neurons and impaired motor functions [160]. NADPH oxidase activation can stimulate ROS and plays a vital role in dopaminergic neurodegeneration [161]. In this regard, pretreatment of PC12 cells with chrysin may reduce the intracellular NO level, decrease iNOS expression, and subsequently inhibit NF-κB phosphorylation and decrease its transcriptional activity induced by 6-OHDA neurotoxicity [162]. In particular, chrysin administration significantly enhanced the anti-inflammatory markers interleukin-4 (IL-4) and interleukin-10 (IL-10), diminished

Table 1: Promising studies of antioxidant phytochemicals for the management of PD.

Phytochemicals	Studied materials	Dose	Neurotoxins	Physiological effects	References
Chrysin	Male C57BL/6J mice	10 mg/kg	6-OHDA	↑Behavioral functions ↑TH-positive cells in the SN and ST ↑DA, DOPAC, and HVA levels	[151]
	Male C57BL/6 mice	50 and 100 mg/kg	MPTP	↑DA and its metabolites ↑AKT/GSK3β/MEF2D pathway ↓MAO-B activity	[152]
	Male C57BL/6J mice	50, 100, and 200 mg/kg	MPTP	↑BDNF and GDNF protein expression ↓IL-10, IL-6, TNF-α, and NF-κB protein expression	[153]
Vanillin	Male Wistar albino rats	5, 10, and 20 mg/kg	LPS	↓iNOS, COX-2, IL-1β, and IL-6 protein expression ↓ERK1/2, p38, and NF-κB signaling ↓Microglia activation	[167]
	Male Wistar albino rats	5, 10, and 20 mg/kg	Rotenone	↑Striatal DA and its metabolite levels ↑Behavioral function ↓Cyto-C, Bax, and caspase protein expression ↑Bcl-2 protein expressions	[168]
Asiatic acid	Male C57BL/6 mice	20, 40, and 80 mg/kg	MPTP	↑Striatal DA levels ↑Striatal TH, TLR4, BDNF, and GFAP protein expression ↓α-Synuclein and lowered AIF protein expression	[169]
	Male Wistar albino rats	100 mg/kg (*in vivo*) and 0.1–10 nM (*in vitro*)	MPTP/p and MPP^+	↑Motor functions ↑PI3K, Akt, GSK-3β, and mTOR phosphorylation ↑TrKB protein expression ↓NLRP3 inflammasome expression in microglia cells	[170]
Ferulic acid	Male Wistar albino rats	100 mg/kg	6-OHDA	↓Mitochondrial Drp1 expression ↑PGC1α gene and protein expression ↑Mfn2 and mitochondrial dynamics	[171]
	Male C57BL/6 mice	100 mg/kg	Rotenone	↑Motor function ↑HSP-70 protein expression ↑TH-positive fibers in corpus striatum	[172]
	Male C57BL/6 mice	20 mg/kg and muscle exercise	MPTP	↑Motor behavior ↑CAT, SOD, GPx, and GSH activity ↓TBARS activity ↑Activation of the Nrf2 signaling	[173]
	Male C57BL/6 mice	40 mg/kg	MPTP	↑CAT, SOD, GPx, and GSH activity ↓TBARS activity ↓iNOS, COX-2, IL-1β, and IL-6 protein expression	[174]
Thymoquinone	Male Wistar albino rats	7.5 and 15 mg/kg	Rotenone	↑Behavioral functions ↑Parkin, Drp1, TH-positive cells in the SN and ST ↑ DA, DOPAC, and HVA levels	[175]
	Male Wistar albino rats	5 and 10 mg/kg	6-OHDA	↑Behavioral functions ↑DA level in the SN ↓MDA level	[176]
Ellagic acid	Male Wistar albino rats	50 mg/kg	6-OHDA	↑Motor function and electrophysiological performance ↑CAT, SOD, GPx, and GSH cerebral activity	[177]
	Male Wistar albino rats	50 mg/kg	6-OHDA	↑CAT, SOD, GPx, and GSH cerebral activity ↓TBARS activity ↓MAO-B activity ↑ERβ/Nrf2/HO-1 signaling cascade	[178]

Table 1: Continued.

Phytochemicals	Studied materials	Dose	Neurotoxins	Physiological effects	References
Caffeic acid	Drosophila melanogaster	0.5, 1, and 2 mg/g	Paraquat	↑CAT, SOD, GPx, and GSH cerebral activity ↓TBARS activity ↑Nrf2-Keap1 signaling	[179]
	A53T transgenic mice	5 mg/kg	A53T Tg mice	↓A53T α-synuclein ↑Bcl-2-mediated autophagy pathway ↑Behavioral functions	[180]
	Male C57BL/6 mice	0.5, 1, and 2 g/kg	MPTP	↑DA synthesis ↑TH-positive cells ↑BDNF and GDNF protein expression, maintained loss ↓IL-1β, IL-6, TNF-α, iNOS, and COX-2 expression ↓GFAP protein expression	[181]
Epigallocatechin-3-gallate	Male C57BL/6 mice	50 mg/kg	MPTP	↑Iron-export protein ferroportin in SN ↑CAT, SOD, GPx, and GSH cerebral activity ↓TBARS activity ↑DA synthesis	[182]
	Male C57BL/6J mice	25 mg/kg	MPTP	↑Movement behavior ↑TH-positive cells in the SN region ↑$CD3^+CD4^+$ to $CD3^+CD8^+$ T-cell lymphocyte ratio in the peripheral blood ↓TNF-α and IL-6 cytokine expression in serum	[183]
	Postmortem PD tissue	100 nM	—	↓α-Synuclein aggregates	[184]
	Male C57BL/6 mice	10 mg/kg	MPTP	↑Movement behavior ↓Microglial activation	[185]
α- and β-Asarone	Male C57BL/6 mice	10 mg/kg	6-OHDA	↑HVA, DOPAC, 5-HIAA levels ↑TH-positive cells in the ST region ↓JNK and p-JNK expression ↑Bcl-2 protein expression	[186]
	Sprague Dawley rats	15 mg/kg	6-OHDA	↑CAT, SOD, GPx, and GSH cerebral activity ↓TBARS activity ↑PERK/CHOP/Bcl-2/Beclin-1 pathway ↓GRP78 levels	[187]
Theaflavin	Male C57BL/6 mice	10 mg/kg	MPTP/p	↑DAT and VMAT-2 expression ↑Behavioral functions ↑CAT, SOD, GPx, and GSH cerebral activity ↓TBARS activity	[188]
	Male C57BL/6 mice	10 mg/kg	MPTP/p	↑Behavioral characterization ↑TH-positive cells in the ST region ↓Caspase-3, caspase-8, and caspase-9 activity ↓Bax expression ↑Bcl-2 protein expressions	[189]
	Male C57BL/6 mice	10 mg/kg	MPTP/p	↑Behavioral characterization ↓IL-4 and IL-10 protein expressions	[190]

expression of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), and inhibited myeloperoxidase (MPO), COX-2, and iNOS expression, which are highly implicated in anti-inflammatory responses [163]. In a cerebral ischemic mouse model, chrysin inhibited neuroinflammation by reducing the level of IL-1β and TNF-α expression, prevented cognitive deficits, and downregulated the activation of NF-κB [164]. In addition to inhibiting NF-κB activation and downregulating gene-related inflammation, chrysin also interacts with iNOS enzymes at a molecular level and COX-2 and decreases levels of arachidonic acid-induced prostaglandins, thromboxanes, and leukotrienes in the SN and ST of 6-OHDA- and MPTP-treated PD mice [151, 153]. Hence, one may conclude that chrysin's protective effects may carry promising anti-inflammatory properties in PD that are at least partially mediated by NF-κB inhibition.

In an experimental model using MPTP, chrysin administered to mice was demonstrated to attenuate dopaminergic neuronal loss in the SN region [152]. Consequently,

immunohistochemical analysis revealed that oral administration of chrysin significantly restored the loss of TH-positive cells in the ST and protected nigrostriatal morphology against 6-OHDA-induced mouse models of PD [151]. A very recent study showed that oral administration of chrysin improved locomotor activity and protected against dopaminergic neurodegeneration in MPTP-injected PD mouse models [153]. Chrysin played a neuroprotective role at a molecular level, at least in part through the suppression of proapoptotic proteins such as caspase-3, caspase-9, and Bax, and enhanced antiapoptotic protein Bcl-2 expression against MPP^+ neurotoxicity [152]. Moreover, chrysin treatment provided neuroprotectivity by regulating or restoring BDNF and glial cell-derived neurotrophic factor (GDNF) levels in the ST region in 6-OHDA- and MPTP-induced PD mouse models [151, 153]. Importantly, PD animal models showed that neurotrophic factors such as BDNF and GDNF could partially inhibit neurodegeneration as reported by previous studies [165, 166]. Hence, the multiple protective roles of chrysin in PD, both neuroprotective and symptom-relieving, are of great value and may open new horizons for novel therapeutic management of PD; further clinical studies are needed.

6.2. Neuroprotective Mechanisms of Vanillin in PD. Vanillin is a phenolic aldehyde compound used as an important flavoring agent worldwide. It is found in abundance in plant species and is often used in the food production, beverage, pharmaceutical, perfume, and cosmetic industries [191]. Currently, approximately 50% of worldwide synthetic vanillin production is used as an intermediate in both food and nonfood applications and in pharmaceutical industries for the production of herbicides, antifoaming agents, or drugs such as papaverine, L-dopa, L-methyldopa, and antimicrobial agents [191]. Acceptable daily intake of vanillin is consumed in the form of food and beverage worldwide, and its ubiquity suggests that nearly every human consumes vanillin-containing products. A daily intake of vanillin 10 mg/kg has been approved by the Food and Agriculture Organization of the United Nations (FAO)/WHO and European Union. For a person weighing 70 kg, the daily recommended intake is 700 mg of vanillin, which amounts to a minimum of 700 g of chocolate or 700 g of ice cream [192].

Vanillin has been studied extensively for its pharmacological properties, which are attributable to its structure and main bioactive metabolites, including vanillyl alcohol and vanillic acid; vanillin's bioactive properties include antioxidant, anti-inflammatory, and neuroprotective abilities [193]. Vanillin can easily penetrate the BBB and demonstrates significant brain-neuroprotective potential by enhancing the activities of antioxidant enzymes and reducing the levels of lipid peroxidation and NO production [194, 195]. This polyphenolic flavoring agent can scavenge the $O^{\cdot}-2$ and $^{\cdot}OH$ intermediates implicated in biological membrane damage [196]. It alleviates renal oxidative stress by decreasing lipid peroxidation levels, increasing levels of enzymatic (SOD, CAT, GPx, and GSH) and nonenzymatic antioxidants (vitamin C and nonprotein thiol), and protecting against DNA damage and histopathological changes in maneb-induced mice [197]. Another study demonstrated the neuroprotective properties of vanillin in a rotenone-induced PD model. Administration of vanillin in SH-SY5Y cells inhibited rotenone-induced ROS generation, mitochondrial dysfunction, and caspase activation and reduced the expression of signaling molecules [198].

A previous study elucidated the relationship between striatal DA depletion and motor impairments [199]. Furthermore, intraperitoneal injection of rotenone displayed a significant diminishment in locomotion activity, akinetic movement, and cataleptic ability [200]. Oxidative stress-mediated degeneration of neurons was further exacerbated by high levels of DA metabolism, greater prevalence of glial cells, and low levels of antioxidant enzyme activity in the SN and ST regions [201]. The successful antioxidant actions of vanillin are due to its structure by reducing the levels of lipid peroxidation and enhancing the antioxidant enzyme activities [168, 202]. A recent study determined that vanillin treatment increases the striatal DA and its metabolites, subsequently improving behavioral function. It has also been reported to prevent Cyto-C release, diminish Bax expression, inhibit caspase activation, and enhance Bcl-2 expression in rotenone-induced rat models of PD [168]. Vanillin has also exhibited improvement in chronic stress-induced rat models by reducing the depressive-like motor symptoms via elevation of serotonin and DA levels in brain tissue [203].

Other study findings recently revealed that mice that inhaled vanillin had a decreased pain response to the hot plate test; no significant differences were observed between the inhaled vanillin group and the control group in the tail suspension, Y-maze, forced swimming, open field, and aggression tests. These results suggested that inhaled vanillin has potential antinociceptive properties similar to other routes of administration [204]. In LPS-lesioned PD models, oral administration of vanillin reduced IL-1β, IL-6, and TNF-α expression through inhibition of the p38-MAPK signaling pathway, suppressing activation of NF-κB and inflammatory genes like iNOS that produce NO and COX-2. Consequently, LPS-lesioned rats experienced increased degeneration of dopaminergic neurons and decreased motor function, and microglial activation triggered in the SN and ST was restored or improved significantly following vanillin treatment [167]. Similar findings also confirmed that vanillin reduced the inflammatory mediators of iNOS and COX-2, as well as the mRNA expression levels of proinflammatory cytokines. In addition, vanillin effectively inhibited the phosphorylation of MAPK signaling and NF-κB activation in LPS-lesioned microglia cells [205]. Together, these data suggest the neuroprotective and anti-inflammatory role of vanillin in protecting dopaminergic neurons and improving behavioral function by inhibiting oxidative stress, inflammation, and apoptosis; thus, it is possible that vanillin may act as a natural therapeutic drug for PD.

6.3. Neuroprotective Mechanisms of Asiatic Acid in PD. Asiatic acid (AA), a naturally pentacyclic triterpenoid, shows promise as neuroprotective drug candidate due to its several pharmacological properties. Interestingly, a number of bioactive components of AA were found to have therapeutic

potential in curing various diseases [206, 207]. AA exhibited the ability to modulate several enzymes, growth factors, receptors, transcription factors, apoptotic proteins, and cell signaling molecules [208, 209]. In experimental studies, AA has displayed a wide range of biological activities, including antioxidant, hepatoprotective, antidiabetic, anticancer, anti-inflammatory, and neuroprotective properties [210–213]. Recently, AA tromethamine-loaded solid lipid nanoparticles have been shown to prevent proteolytic degradation and to facilitate sustained release of the drug, thereby improving its bioavailability [214]. Another study injected formulations containing glutathione-conjugated BSA nanoparticles of AA intravenously into rats at a dose of 75 mg/kg; after 5 hours, the nanoformulation displayed ten times greater bioavailability in the brain than AA alone [215].

AA provides neuroprotection by maintaining the stability of the BBB and by protecting mitochondrial functions. A recent *in vitro* study showed that AA administration reduced intracellular mitochondrial ROS production and altered MMPs to regulate mitochondrial function, subsequently decreasing NLRP3 inflammasome expression in microglia cells [216]. In addition, AA treatment directly improved SH-SY5Y cell viability and maintained mitochondrial function in an MPP^+-induced PD model [216]. Furthermore, administration of AA attenuated ROS overproduction, mitochondrial dysfunction, and apoptotic expressions of proapoptotic and antiapoptotic indices in a rotenone-induced SH-SY5Y PD model [217]. A similar study reported that AA pretreatment protected cell viability, inhibited the upregulation of voltage-dependent anion channel mRNA and protein expression levels, and prevented MMP damage following exposure to H_2O_2 [218]. Further recent *in vitro* and *in vivo* experiments also observed that the administration of AA significantly decreased apoptotic cell death, lessened mitochondrial ROS production, stabilized MMPs, and promoted the expression of PGC1α and Sirt1 to mitigate toxicity induced by glutamate in a dose-dependent manner. In the *in vivo* model, oral administration of AA significantly attenuated cognitive deficits, decreased lipid peroxidation level, and restored GSH content and SOD activity in the hippocampus and cortex; subsequently, AA effectively attenuated glutamate-induced neuronal damage of the pyramidal layer in the CA1 and CA3 regions [219].

Moreover, the anti-inflammatory activity of AA significantly decreases the level of MDA, NO, and inflammatory mediators iNOS and COX-2 and has also been shown to inhibit NF-κB activation in paw edema by increasing the antioxidant enzyme levels of CAT, GPx, and SOD in λ-carrageenan-induced mice [220]. In the MPTP/p-induced PD mouse model, AA administered was shown to improve behavioral function, enhance DA levels, and increase expressions of neurotrophic factors and tyrosine kinase receptors (TrKB). Moreover, AA significantly inhibited phosphorylation of P38, JNK, and ERK protein expression and significantly increased phosphorylated PI3K, Akt, GSK-3β, and mTOR activation [170]. Indeed, experimental studies showed that AA treatments increased striatal DA levels and upregulated striatal TH, TLR4, BDNF, and GFAP expression, subsequently decreasing striatal upregulation of α-synuclein, apoptotic markers, and Bcl-2 expression in MPTP-induced PD-like neurotoxicity in mice. In addition, posttreatment of AA significantly suppressed NF-κB activation [169]. Interestingly, recent studies have also found that AA prevented decreases in Nrf2 expression, counteracted the downregulation of neurogenesis within the hippocampus, and mitigated memory deficits produced by 5-fluorouracil through inhibiting oxidative stress and boosting the antioxidant defense system [221]. These novel findings suggest that AA may be developed as an agent for PD prevention or therapy.

6.4. Neuroprotective Mechanisms of Ferulic Acid in PD. Ferulic acid (FA) is a natural phenolic phytochemical commonly found in apples, oranges, peanuts, wheat, rice, barley, coffee, and many other dietary sources [222]. Investigations have confirmed the beneficial effect of FA-rich foods and drinks. Recent reports revealed that consumption of vegetables, fruits, and cereals is beneficial in the prevention of diabetes, cancer, obesity, cardiovascular disease, AD, and PD [129, 223, 224]. In Japan, FA has been approved as a food additive and is used as a natural antioxidant in foods, beverages, and cosmetics. In the United States and most European countries, numerous medical essences and natural extracts of herbs, coffee, vanilla beans, spices, and other botanicals are selected for their high content of FA and are added to foods as an FDA-approved antioxidant concoction [225]. Accurate nutritional surveys about FA intake are lacking, but consumption of FA from food sources can be estimated at a daily intake of approximately 200–1000 mg per person [222, 226, 227].

In recent years, scientific findings have reported the health benefits of FA, which exhibits low toxicity and possesses several pharmacological properties including immunomodulatory, antioxidant, anti-inflammatory, antiapoptotic, anticancer, antidiabetic, and neuroprotective functions. FA achieves these functions through inhibition of lipid peroxidation and ROS generation by its phenolic hydroxyl group. Moreover, FA has been reported to downregulate the expression of enzymes that promote inflammation [129, 222]. The physiological benefits of FA depend on its bioavailability for absorption and consequent interaction with target tissues. Recent preclinical investigations estimated that for subjects who consumed phenolic acid-containing drinks, vegetables, and fruits in the daily diet, total daily intake of polyphenols equaled about 1 g [227]. In animal models, FA has shown greater bioavailability compared to other various phenolic ingredients [222, 228].

Based on its structural similarity to another phenolic compound, salicylic acid, that can enter the brain, it is speculated that FA can easily penetrate the BBB. It has also been reported to be a successful neuroprotective agent [229]. Recent *in vitro* and *in vivo* findings have demonstrated that FA exhibited increased levels of protective HO-1 activity in SH-SY5Y cells, upregulated the levels of CAT, SOD, GPx, and GSH, and reduced the lipid peroxidation level in MPTP-injected PD mouse models, which confirms FA's potential antioxidant effects in the prevention of PD [230]. In another study, FA led to upregulation of antioxidant enzyme status through induced HO-1 gene expression,

enhanced ARE promoter activity, promoted ERK1/2 phosphorylation, and Nrf2 translocation in PC12 cells exposed to lead acetate [231]. Moreover, oxidative stress stimulated by 6-OHDA and rotenone was alleviated effectively by FA treatment via its ability to scavenge radicals generated by increased lipid peroxidation, decreased antioxidant GSH content, and mitochondrial oxygen radical production, thus substantiating FA's preventive effect against oxidative damage in PD [171, 232].

Anti-inflammation has been proposed as one of the main mechanisms underlying FA-induced neuroprotection in PD, and several studies have shown that FA can potentially inhibit neuroinflammation and neuronal apoptosis in NDDs. Furthermore, histological findings revealed that FA administration suppressed microglial cell activation and the Bax/Bcl-2 ratio, reflecting a reduction in inflammation and apoptosis, respectively. It was also discovered that FA effectively prevented MPTP-induced neuronal loss-triggered declines in behavioral function and motor coordination [233]. Additionally, FA reduced proinflammatory cytokines and inflammatory mediators such as iNOS and COX-2. Further, the results confirmed that FA mitigates activation of microglial and astrocytes by a remarkable reduction in GFAP and Iba-1 hyperactivity [232]. In addition to their role in mitochondrial propagation and function, dynamin-related protein 1 (Drp1) and mitofusin 2 (Mfn2) have also been linked with excitotoxicity and are thought to play a vital role in programmed cell death [234]. In fact, enhanced expression of Drp1 has been linked to neuronal damage in animal models of PD [235]. Moreover, the study's author reported that FA administration attenuated 6-OHDA-induced morphological changes and DNA damage and blocked caspase activity. FA also reduced mitochondrial expression of Drp1 and increased expression of the PGC1α gene and protein, thereby regulating expression of its downstream target Mfn2 and restoring mitochondrial dynamics in 6-OHDA-lesioned PD animal models [171]. Hence, the combination of FA and muscle exercise effectively improved motor function and increased HSP-70 expression and TH-positive fibers in the corpus ST in rotenone-induced PD mice [172].

Sirtuin 2 (SIRT2) is a potential culprit in PD pathology and modulates the α-synuclein accumulation that is critical to several pathological processes in PD. Recent *in vitro* and *in vivo* studies using PD models have revealed that pharmacological inhibition of SIRT2 activity potentially ameliorates the α-synuclein-mediated toxicity reported previously [236]. Deacetylation of Nrf2 by SIRT2 leads to a reduction in both nuclear and total cellular levels of Nrf2 through its degradation [237]. Interestingly, FA treatment prevents MPTP-induced oxidative stress through activation of ERK1/2 signaling and inhibition of SIRT2, processes that are facilitated by independent mechanisms. Additionally, FA attenuated motor impairments in MPTP-injected α-synuclein knockout mice and wild-type mice, but not in Nrf2 knockout mice [173]. Therefore, these antioxidant and anti-inflammatory properties add to the value of FA as a therapy for PD.

6.5. Neuroprotective Mechanisms of Thymoquinone in PD. Thymoquinone (TQ) is a pharmacologically active compound found in black cumin seeds and plants from the Lamiaceae family [238]. Black cumin has been used in medicine since ancient times; more recently, interest in this compound has increased significantly [239]. Several investigations have demonstrated that black cumin seeds and their active constituent, TQ, may be suitable for clinical trials because most of the major effects of TQ have been shown to be beneficial. Intake of any black cumin seed has a recommended daily intake range of approximately 250–1000 mg [240]. Previous experimental studies have confirmed that TQ and its derivatives evince several pharmaceutical activities, including antioxidant, anti-inflammatory, antihypertensive, antiasthmatic, antidiabetic, and antitumor properties [241–243]. Interestingly, the effects of TQ were studied for the α-synuclein-induced synaptic toxicity in the cultures of rat hippocampal neurons and neurons differentiated from human induced pluripotent stem cells (IPSCs). In both types of cultures, TQ protected the neurons against α-synuclein-induced synaptic damage, increased the level of synaptophysin (a synaptic density marker), and prevented inhibition of synaptic vesicle recycling induced by the mutant β-synuclein (P123H). Moreover, using cells cultured on the multielectrode arrays, the authors demonstrated that TQ maintained normal bioelectrical activity in the neuronal network that was damaged by the actions of α-synuclein [244]. As previously described, autophagy is a natural cellular mechanism for eliminating unnecessary or damaged organelles and molecules and can be induced by oxidative or toxic stress. The disturbance of the autophagy mechanism can lead to the development of neurodegenerative diseases. TQ at a concentration of 0.0110 μM prevented the MPP^+-induced death of mesencephalic dopaminergic neurons *in vitro* by reducing the release of lactate dehydrogenase and maintaining MMP. The effect of TQ was accompanied by the activation of autophagy, which contributed to the reduction of the apoptotic neuron death [245].

The neuroprotective effect of TQ was demonstrated in a rotenone-induced PD model, in which TQ treatment prevented the death of primary dopaminergic neurons [246]. Furthermore, TQ significantly upregulated the expression of neuroprotective proteins, significantly downregulated the expression of proinflammatory cytokines, and inhibited activation of NF-κB against LPS/IFNγ-activated BV-2 microglial cells [247]. Importantly, TQ manifested the ability to improve the course of PD in *in vivo* experiments. In mice with MPTP-induced PD accompanied by the development of oxidative stress and neuroinflammation in the brain, TQ restored the activities of anti-inflammatory enzymes, prevented GSH exhaustion, inhibited lipid peroxidation, and decreased levels of proinflammatory cytokines [174]. In a rotenone-induced PD model, TQ prevented the development of motor impairments and changes in the content of Parkin and Drp1 proteins and increased DA levels in the SN and ST areas of rat brain [175]. The neuroprotective action of TQ was observed in the animals after injection of 6-OHDA into the ST which led to the loss of neurons and behavioral functions [176].

6.6. Neuroprotective Mechanisms of Ellagic Acid in PD. Ellagic acid (EA) is a natural antioxidant phenol found in several

vegetables and fruits, in particularly large quantities in persimmon, pomegranates, nuts, black raspberry, raspberry, peach, strawberry, and plumes [248]. There is a relatively high content of EA in raspberries (1500 mg/g dry weight), strawberries (630 mg/g dry weight), cranberries (120 mg/g dry weight), walnuts (590 mg/g dry weight), pecans (330 mg/g dry weight), and a number of other plant foods [249]. The daily intake of total polyphenols is the highest in Denmark at approximately 1786 mg/day, followed by Japan at 1500 mg/day. European countries and the Americas average about 900 mg/day and 800 mg/day, respectively. In Europe, Poland and France have the highest intake—after Denmark—at above 1000 mg/day; Italy averages approximately 650 mg/day, Greece about 584 mg/day, and Spain has the lowest intake at 300 mg/day [250]. In Asian countries, such as China and Korea, apples and vegetables seem to serve as the main sources of polyphenols, while green tea provides the highest intake for the Japanese population [251]. The appropriate dose of an EA supplement depends on several factors, including the age and health of the consumer. Currently, there are no standard dosing recommendations for EA.

Moreover, EA has been shown to have potentially antioxidant, anti-inflammatory, antiviral, antisteatotic, antibacterial, antihepatotoxic, anticholestatic, anticancer, antidiabetic, immunomodulatory, and antiproliferative properties, representing an extensive range of beneficial effects that may be applied to improve human health [248, 252–254]. The bioavailability of EA is low compared with that of other phenols; however, punicalagin, which is a source of EA, was detected in human plasma after consumption of pomegranate juice [255, 256]. Evidence indicates that pomegranate juice containing 318 mg of punicalagin and 25 mg of EA consumption led to a plasma concentration of 31–33 ng/mL (maximum concentration) EA 1 h after absorption [255, 256]. After oral administration to rats, both punicalagin and EA were detected in the plasma after consumption of pomegranate leaf extract [257–259].

Several preclinical trials have suggested that EA effectively attenuates renal oxidative damage in LPS/D-galactosamine-induced PD rats by reducing the levels of lipid peroxidation and boosting the antioxidative defense system via increased levels of enzymic and nonenzymic antioxidant activities [260]. Moreover, treatment with EA enhanced the GSH content and mRNA expression of renal and hepatic SOD and GPx activity. In addition, EA treatment also prevented elevated renal and hepatic levels of MDA and NO production [261]. Of note, EA administration prevented 6-OHDA-induced increased levels of MDA and decreased GPx and SOD activities in both ST and hippocampus tissues. EA treatment also improved motor function and electrophysiological performance in medial forebrain bundle-lesioned rats by raising cerebral antioxidant content [177]. Other studies discovered that combined treatment with three compounds such as EA, α-lipoic acid, and myrtenal attenuated decreases in DA levels, substantially decreased lipid peroxidation levels, and restored CAT activity in 6-OHDA-induced PD rats [262].

Interestingly, a study involving the kinetics of enzyme inhibition demonstrated that EA combined with curcumin inhibited MAO-B activity via both competitive and noncompetitive inhibition [263]. Importantly, previous research has shown that dimethyl fumarate, a pharmacological activator of Nrf2 that is presently used in multiple sclerosis, may also exert beneficial effects in PD [264]. EA was also utilized to confer neuroprotection against rotenone-induced neurotoxicity through activation of Nrf2 signaling, which was involved in EA-mediated DA neuroprotection [265]. Furthermore, similar research also revealed that EA reduced striatal MDA levels, ROS, and DNA fragmentation and improved Nrf2, HO-1, and behavioral functions. Meanwhile, EA prevented the loss of TH-positive neurons within the SN in 6-OHDA-induced rat models of PD [178]. These results suggest EA is a potential Nrf2 activator, and that by restoring antioxidant mechanisms, EA may serve as a promising therapeutic candidate for PD in the future.

6.7. Neuroprotective Mechanisms of Caffeic Acid in PD. Caffeic acid (CA) is a phenolic acid extracted from numerous plant species and is present in beverages such as coffee, wine, and tea. CA carries medicinal properties such as those found in propolis, which has been studied extensively for its strong antioxidant potential [266–269]. Emerging epidemiological evidence suggests that greater coffee consumption may reduce the risk of NDDs. The daily intake of CA has been estimated to be about 500–1000 mg in humans consuming fruits, vegetables, beer, and coffee [226]. Drinking a single cup of coffee provides as much as 70-350 mg of chlorogenic acid or CA [270]. Recent evidence revealed the highest category of coffee consumption (Netherlands, 6 cups/day; Sweden, 2-5 cups/day; Spain, 1-2 cups/day; Japan, 3-5 cups/day; Italy, 1-3 cups/day; Finland, 1-4 cups/day; and China, 1-3 cups/day) [33, 271].

CA has been reported to have several pharmacological activities, including anticancer, anti-inflammatory, and neuroprotective properties [180, 272]. It has also been reported that CA may react with peroxyl radicals involved in lipid peroxidation to efficiently mitigate several disease conditions of PD [180, 273]. CA is believed to remove excess ROS/RNS generation, and CA is known to stimulate antioxidative enzyme activities including SOD, GPx, GSH, and CAT [273]. In PQ-induced fruit fly, using a *Drosophila melanogaster* model, a molecular docking study demonstrated the strong interaction of CA with *Drosophila melanogaster* transcriptional activation of Nrf2. In observations of the binding of CA to the Keap1 domain of Nrf2, results show that the protective effect of CA reduction of lipid peroxidation level and ROS in *Drosophila melanogaster* was possible through its coordination, which delays Nrf2-Keap1 binding and leads to an enhanced antioxidant defense system [179]. In the 6-OHDA-induced SH-SY5Y cellular model, CA-phenethyl ester significantly improved cell viability, diminished apoptotic cell death, and prevented changes in damaged nuclear morphology. Furthermore, treatment with CA-phenethyl ester could maintain mitochondrial function, inhibit ROS production, upregulate Bcl-2 and Akt levels, and downregulate Bax expression [274]. Similarly, in MPP^+-induced neurotoxicity *in vitro* PD models, CA-phenethyl ester directly inhibited release of Cyto-C and apoptosis-inducing factor (AIF) from mitochondria [275].

Furthermore, one highlight resulting from CA administration was a significant increase in the efficiency of striatal DA content and, consequently, a decrease in microglia expressions and inflammatory mediators. In addition, histopathological assessment of SN neurons demonstrated high immunostaining for TH-positive cells and improved motor performance in rotenone-treated mice [276]. In seven-month-old A53T α-synuclein transgenic mice, CA alleviated cell damage and reduced A53T α-synuclein expression by activating the JNK/Bcl-2-mediated autophagy signaling pathway. It was also observed that the CA's protective efficacy on A53T α-synuclein degradation was prevented by the JNK inhibitor SP600125 and the autophagy inhibitor bafilomycin A1. In A53T Tg mice, CA attenuated loss of dopaminergic neurons, improved behavioral function, enhanced autophagy activation, and reduced α-synuclein accumulation in the SN [180]. In MPTP-induced PD mice, CA lowered the production of proinflammatory cytokines, diminished the expression of inflammatory mediators and GFAP, and decreased the production of NO and PGE_2. In addition, CA restored the expression of BDNF and GDNF and maintained TH-positive cells and DA synthesis [181]. Previous research discovered that metallothionein- (MT-) 1 and MT-2 were upregulated, particularly in ST astrocytes, by activation of Nrf2 signaling in response to oxidative stress and acted to protect SN neurons [277]. Treatment with CA protected both SN and intestinal enteric neurons and upregulated MT-1 and MT-2 antioxidative molecules in the ST astrocytes of rotenone-induced C57BL/6J mice [278]. Interestingly, the inhibitory effect of CA against escitalopram-induced α-synuclein accumulation and other neurotoxins may prove to be promising novel therapeutic drugs for PD [279].

6.8. Neuroprotective Mechanisms of Epigallocatechin-3-Gallate in PD. Epigallocatechin-3-gallate (EGCG), a polyphenol isolated from green tea, is known for its myriad physiological beneficial effects against inflammatory disorders, cancer, and NDDs in humans [280–282]. Several pharmacological activities including antioxidant, anti-inflammatory, metal-chelating, radical-scavenging, antiapoptotic, and anticarcinogenic properties by modulation of several transcription factors, proteins, and other important growth factors are attributed to EGCG [282, 283]. EGCG's broad potential for improving healthy aging derives from its promotion of morphologic and functional alterations that occur naturally in an aging brain; these alterations increase memory and learning ability, suppress cognitive dysfunction, and reduce oxidative damage in the brain [284–286]. The typical daily intake of EGCG resulting from the consumption of green tea infusions ranges from 90 to 300 mg/day, while exposure in high-level consumers is estimated to be as much as 866 mg EGCG/day in the adult population in Europe. Food supplements containing green tea catechins provide a daily dose of EGCG in the range of 5–1000 mg/day for the adult population. Based on available data on the potential adverse effects of green tea catechins on the liver, the evidence from interventional clinical trials of doses equal to or above 800 mg/day in the form of a food supplement has shown increased bioavailability of EGCG in treated subjects compared to control [287–290]. A few studies have previously demonstrated that EGCG can cross the BBB easily, which is crucial for the development of therapeutic agents for PD [291, 292].

Several studies of human subjects have described an inverse dose-response relationship between green tea consumption and cognitive impairments in PD disorders [293, 294]. Recent advancements in case-controlled joint studies revealed that daily consumption of two or more cups of tea reduced cognitive dysfunction and decreased the prevalence of PD [295, 296]. A Finnish longitudinal study conducted over 13 years with 30 000 adults aged 25–74 years old showed that daily consumption of three or more cups of tea reduced the risk of PD [297]. Similarly, a large-scale-cohort, 20-year follow-up study involving nearly 50 000 male and 80 000 female volunteers demonstrated that EGCG intake was associated with a 40% lower risk of PD [298]. Moreover, several cohort- and case-controlled studies across Asian, European, and North American countries have reported a link between tea consumption and lower risk of PD [299]. In Asian populations, particularly among the Chinese, 28% of populations experienced a decreased risk of developing PD with daily consumption of three cups of tea for ten years [299].

Currently, several findings have demonstrated the antioxidative capacity of EGCG under experimental conditions in both cellular and animal models [300, 301]. In rotenone-induced rat PD models, EGCG treatment reduced lipid peroxidation production and NO levels, increased succinate dehydrogenase (SDH) activity, improved mitochondrial function, and raised ATPase and ST catecholamine levels. In addition, EGCG treatment decreased the level of neuroinflammatory cytokines and apoptotic markers and improved motor performance [302]. Administration of EGCG in MPTP-induced mice prevented neurotoxin-induced reduction in ST antioxidant enzymes SOD and CAT and increased the activities of both enzymes in the total brain [303]. Furthermore, EGCG treatment wholly prevented STAT3 activity and stimulated neuronal cell proliferation induced by 6-OHDA in SH-SY5Y cells [304]. Most recent findings have confirmed that the neurorescue effect of EGCG regulated the iron-export protein ferroportin in the SN, reduced oxidative stress, and attenuated functional and neurochemical shortages against MPTP-induced PD mice [182]. In the MPTP-injected mouse model, EGCG restored movement behavior and protected TH-positive cells in the SN region. Flow cytometric analysis showed that the ratio of $CD3_+CD4_+$ to $CD3_+CD8_+$ T-cell lymphocytes in the peripheral blood increased with EGCG treatment and reduced expression of inflammatory factors such as TNF-α and IL-6 in the serum [183]. Furthermore, EGCG was shown to display antiapoptotic effects in PQ-induced PC12 cell models: EGCG maintained MMP and inhibited the upregulation of caspase-3 activity and the downregulation of the proapoptotic SMAC protein in cytosol expression [305]. It was also observed that EGCG treatment reduced TNF-α and NO inflammatory mediators and attenuated loss of midbrain DA levels triggered by LPS-induced neurotoxicity [306]. Furthermore, *in vitro* and *in vivo* studies demonstrated that cotreatment with EGCG lowered glutamate-induced oxidative cytotoxicity in HT22 cells through inhibition of NF-κB activation. In

addition, EGCG treatment moderated the effect of decreased accumulation of 3-O-methyldopa in the plasma and ST of rats that were administered carbidopa+L-Dopa; EGCG also exerted a strong therapeutic effect against kainic acid-induced oxidative neuronal death in the hippocampus of PD rats [307].

The process of α-synuclein and other protein deposition has been strongly linked with numerous NDDs, including PD. Recently, several studies have attracted attention by showing that α-synuclein accumulation formation might be mediated by small molecules, such as the polyphenol EGCG, which may offer a potential therapeutic option for management of α-synucleinopathies [308, 309]. Other research found that EGCG potentially interacted with α-synuclein amino acid sites found on peptide membranes. It was implicated that EGCG binds to α-synuclein via unstable hydrophobic interactions; these findings support the assertion that EGCG could be a potent remodeling agent of α-synuclein accumulation and a potential disease-modifying agent for the treatment of PD [184].

6.9. Neuroprotective Mechanisms of α- and β-Asarone in PD. Alpha- (α-) asarone and beta- (β-) asarone compose an important antioxidant aromatic chemical constituent that is extracted from the rhizomes of *Acorus calamus*. Consequently, both α- and β-asarone have been reported to have one or more similar pharmacological properties that may offer beneficial effects in the therapeutic management of several diseases [310–313]. Importantly, the delivery of α- and β-asarone in the brain is extensive, demonstrating its ability to cross the BBB, a desirable characteristic of compounds used for the treatment of numerous NDDs [310]. A recent Swiss ADME predictor study revealed that absorption, distribution, metabolism, and excretion results showed that α- and β-asarone possess good oral bioavailability; the study also showed a good binding affinity towards dopaminergic receptors. Further, α- and β-asarone were found to interact with different amino acid residues of disease-modifying D2 and D3 receptors through hydrogen bonding [314].

In one PD model, α-asarone treatment reduced neural inflammation and suppressed IL-β, IL-6, and TNF-α production in LPS-stimulated BV-2 cells. In addition, α-asarone treatment effectively inhibited the LPS-stimulated activation via regulation of NF-κB by blocking degradation of inhibitor NF-κB signaling in BV-2 microglial cells. *In vivo* studies also demonstrated that prophylactic administration with α-asarone inhibited microglial activation and attenuated PD-like behavioral deficits in MPTP-injected PD mice [185]. In the 6-OHDA-induced PD model, β-asarone improved the behavioral function of rats in the initiation time, open field, stepping time, and rotarod tests. Research has also found that β-asarone increases the levels of HVA, DOPAC, and 5-HIAA in the ST region. In addition, administration with β-asarone elevated the level of TH-positive neurons and inhibited the expression of LC3-II in SN4741 cells. Moreover, *in vivo* experimental results showed that β-asarone affected the expression of Bcl-2, Beclin-1, JNK, and p-JNK in 6-OHDA-injected PD rats. The neuroprotective effect of β-asarone occurs primarily by downregulating JNK and p-JNK expressions and then indirectly increasing Bcl-2 expression. Additionally, β-asarone may inhibit the function of Beclin-1, thereby inhibiting autophagy activation [186]. Activated autophagy is an important process that may play a defensive role through clearance of toxic aggregated α-synuclein in neurons [315]. On the other hand, dysfunction of the autophagy-lysosomal pathway has been associated with the development of PD [316]. Recent studies also proposed that endoplasmic reticulum (ER) stress may induce autophagy [317]. In 6-OHDA-induced PD rat models, β-asarone administration may decrease the levels of Beclin-1, CHOP, GRP78, and p-PERK while significantly increasing the level of Bcl-2. β-Asarone may increase Bcl-2 by inhibiting the p-ERK pathway, and Bcl-2 may inhibit the expression of Beclin-1. The results of that study suggested that β-asarone may regulate autophagy and ER stress via the PERK/-CHOP/Bcl-2/Beclin-1 pathway [187]. Very recent findings have shown that β-asarone can effectively inhibit neuronal apoptosis through the CaMKII/CREB/Bcl-2 signaling pathway and regulate Bcl-2 family proteins [318]. Moreover, β-asarone significantly lowered the expression levels of MALAT1 and α-synuclein in the midbrain of MPTP-injected PD mice. In addition, immunoprecipitation and RNA pull-down assays confirmed that MALAT1 was associated with α-synuclein, leading to the increased stability of α-synuclein and its expression in SH-SY5Y cells. β-Asarone treatment could increase the viability of cells exposed to MPP^+[319]. Similarly, another study demonstrated that β-asarone exerted antioxidative effects on H_2O_2-stimulated PC12 cells by reducing oxidative stress via activation of the protective Nrf2/HO-1 pathway [320].

Based on the pharmacological effect of β-asarone, previous scientific studies demonstrated that combined treatment with β-asarone and L-dopa carried potentially therapeutic value. Interestingly, combined treatment with β-asarone and L-dopa reduced the level of creatinine and increased the level of HVA, DA, DOPAC, and 5-HT, while β-asarone also enhanced TH and DAT protein expression in madopar-induced PD rats [321]. Similar findings also show that LC3B and Beclin-1 expression decreased, while p62 expression increased after coadministration with β-asarone and L-dopa. In addition, the group that received coadministered β-asarone and L-dopa exhibited a significant decrease in autophagosome activity when compared with the 6-OHDA-injected PD control group [322]. However, further experimental validation using *in vitro* and *in vivo* studies is needed before clinical trials may commence.

6.10. Neuroprotective Mechanisms of Theaflavin in PD. Theaflavin (TF) is representative of a group of polyphenols that are found in black tea, comprising theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate, which contribute to the quality and color of black tea [323]. TF is known for its several therapeutic effects owing to its antioxidant properties: removal of excess free-radical formation and metal chelation ability [324–327]. In the past few years, several scientific reports have shown that TF has potential neuroprotective effects against NDDs. TF has been found equal in efficiency to EGCG at inhibiting β-amyloid- and

α-synuclein-induced neurotoxicity due to its potential antioxidant properties [328]. Recently, a randomized, double-blind, placebo-controlled study showed that the beneficial TF doses of 50 mg/day and 100 mg/day provide health benefits. These TF doses also appeared to be more effective than similar doses of tea catechin [329]. Recently, a clinical survey from the European Union demonstrated that the daily intake of TF ranged from 181 mg/day (Czech Republic) to 793 mg/day (Ireland). The highest intakes of TF were observed in Ireland (191–505 mg/day) and the lowest intakes in Spain (9–24 mg/day) [330]. Little data exists on the pharmacokinetic profile of TF in humans: after the consumption of 700 mg of TF once a day, corresponding to about 30 cups of black tea, achieved maximum concentration observed in blood plasma and betterment of oral bioavailability [331].

Cell lines were used to investigate the mechanism of action of TF, 6-OHDA-induced SH-SY5Y, and the findings revealed attenuated loss of cell viability, reduced nuclear morphology, decreased apoptosis, increased MMPs, and diminished intracellular NO levels. These results suggested that TF had a protective effect against 6-OHDA-induced apoptosis through inhibition of NO and ROS production [332]. In the PC12 cell line treated with H_2O_2, oxidative stress was eliminated by administration of TF, which decreased Bax and caspase-3 protein expression and increased Bcl-2 expression. This finding indicates that TF possesses antiapoptotic properties, providing both cytoprotection and neuroprotection [333]. Another study demonstrated that TF acted as a potent inhibitor of β-amyloid and α-synuclein fibrillogenesis and stimulated the β-amyloid and α-synuclein assembly into a nontoxic form. These results suggest that TF could be used to remove toxic amyloid deposits [328].

In the PD mouse model, TF increased the expression of DAT and VMAT-2 in addition to downregulating the effects of oxidative stress in MPTP-induced neurotoxicity in mice. TF has also been shown to ameliorate dopaminergic neuronal loss and behavioral deficits [188]. In mice treated with MPTP/p, TF administration increased the expression of nigral TH and DAT and lowered caspase-3, caspase-8, and caspase-9; these results were accompanied by increases in regulated behavioral function [189]. Moreover, dysfunction of the cholinergic system was also observed to trigger the production of proinflammatory cytokines and activation of microglia: the levels of IL-4 and IL-10 anti-inflammatory markers were raised in MPTP-injected mice as a compensatory mechanism against neuronal inflammation [334]. In a recent *in vivo* study, treatment with TF ameliorated the chronic MPTP-induced neurotoxicity in the SN and ST of mice, as evidenced by significantly decreased neuroinflammation and apoptosis. In addition, treatment with TF attenuated the MPTP-injected behavioral impairments such as catalepsy and akinesia and significantly reduced the excess formation of IL-4 and IL-10 anti-inflammatory cytokines [190].

7. Neuroprotective Roles of Antioxidant Phytochemicals and Their Analogs

As the present study has explained, some naturally derived phytochemical constituents are potential neuroprotective agents that may have applications for treating PD. The chrysin derivatives 6,8-bis(*o*-tolylselanyl)-chrysin, 6,8-bis(*p*-anisoylselanyl)-chrysin, and 6,8-bis(*p*-fluorophenylselanyl)-chrysin were semisynthesized and studied for their antioxidant and neuroprotective activity [335]. The author of that study suggested that the structure-activity relationship, with all three compounds containing a fluorine atom in the *para* position to selenium, showed the greatest antioxidant activity through its ability to inhibit lipid peroxidation and ROS generation in mouse cortex and hippocampus. Scipioni et al. [336] demonstrated that synthesized novel vanillin derivatives of 4,4′-(((3-hydroxypropyl)azanediyl)bis(methylene))bis(2-methoxyphenol) and 4,4′,4″,4‴-((1,4-phenylenebis(azanetriyl))tetrakis(methylene))tetrakis(2-methoxyphenol) having a tertiary amino group, accompanied by the number of vanillin moieties, have confirmed ability to protect from oxidative damage and potential antioxidant activity in H_2O_2-induced neuroblastoma SH-SY5Y cells. Another study reported that semisynthesized AA derivatives including AS-2, AS-2-9-006, and AS-9-006 exhibited the greatest activity in the active avoidance, passive avoidance, and the Morris water maze tests and cognitive-enhancing activity [337]. 1-Feruloyl glycerol (FA-G1) and 1-feruloyl diglycerol (FA-DG1), two water-soluble derivatives of FA, exhibited neuroprotective effects against β-amyloid-induced neurodegeneration in both *in vitro* and *in vivo* experiments. This neuroprotection was evidenced by inhibition of NO production and reduction in iNOS expression in a dose-dependent manner mediated by suppression of NF-κB nuclear translocation in primary astrocytes, by beneficial effects against abnormal activation of astrocytes, and by a reduction in neurodegeneration [338].

A very recent study demonstrated that lipophilic butyl ferulate, a derivative of FA, binds to amide NH in Gln15 and Lys16 via a hydrogen bond. This binding significantly attenuated intracellular ROS formation and could potentially upregulate antioxidant enzyme activity by modulating the Keap1-Nrf2-ARE signaling pathway [339]. Another study reported the neuroprotective effect of two CA derivatives known as caffeic acid phenethyl ester and danshensu ((R)-4 2-hydroxy-3-(3,4-dihydroxyphenyl)propionic acid), which contain numbers of hydroxyl groups in an aromatic ring (A) that are conjugated with double bond. These study results demonstrated that these two compounds significantly boost the endogenous antioxidant defense system and modulation of the PKA/CREB signaling pathway. Furthermore, these compounds significantly improved behavioral performance in both the step-down avoidance test and Morris water maze test [340]. A similar study also revealed that CA-phenethyl ester 4-O-glucoside, which is synthesized from CA, suppressed H_2O_2-induced oxidative stress by inhibiting ROS generation, protein carbonylation, and MDA content, in addition to significantly enhancing GSH and SOD activities in both SH-SY5Y and PC12 cells. Mechanistically, it prevented impairments in learning and cognition *in vivo* by reducing neuronal cell death and protecting against hippocampus and cortex dysfunction [341]. The most promising polyphenolic TF derivatives, namely, TF-3-gallate, TF-3′-gallate, and TF-3,3′-gallate, exhibited significant rescue from

the metabolic inhibition induced by α-synuclein aggregates in PC12 cells [328].

8. Neuroprotective Role of Phytochemical Nanoformulation

Several nanodelivery systems loaded with naturally derived antioxidant phytochemicals have been demonstrated to be effective in modulating oxidative stress, α-synuclein aggregation, chronic inflammation, and various signaling pathways that mediate most aging-associated NDDs, particularly in PD. For instance, in a study by Giacomeli et al. [342], chrysin-loaded lipid-core nanocapsules showed higher antioxidant ability and reduced neurotoxicity through reduction of oxidative stress and neuroinflammation and through modulation of neurochemical and behavioral changes in an aged animal, compared with free chrysin. A very recent study observed that chrysin-loaded poly (lactic-co-glycolic acid) nanoparticles less than 150 nm in size in pentylenetetrazol-induced epilepsy mice showed chrysin nanoparticle treatment counteracted oxidative stress, reduced neuronal apoptosis, and upregulated Nrf2, HO-1, and NAD(P)H quinone oxidoreductase [343]. Trans-FA-loaded solid lipid nanoparticles could enable the uptake of FA by cells due to of their lipophilic nature, thereby increasing FA bioavailability and concentration-dependent reduction of lipid peroxidation and enhancement of antioxidant enzyme activities in rat brain [344]. Anti-inflammatory effects of FA-loaded nanoparticles such as modified glycol chitosan nanoparticles have also been reported previously [345]. Another study demonstrated that rats that received intravenous injections of an FA-loaded nanostructured lipid carrier exhibited significantly attenuated neurobehavioral deficits, oxidative stress, and cellular damage; the study showed that activating the PI3K pathway may be of beneficial effect in cerebral stroke [346].

There is evidence that nanoencapsulated TQ carry greater antioxidant and neuroprotective properties. In a rat model, TQ-loaded mesoporous silica nanoparticles 90 nm in size and spherical in shape were able to cross the BBB. Results showed that the encapsulated TQ-loaded mesoporous silica nanoparticles enhanced drug target delivery to all brain areas (ST, cortex, thalamus, midbrain, and hypothalamus) and significantly reduced oxidative stress biomarkers [347]. Numerous studies have reported that different nanoparticle-loaded phytochemicals (e.g., vitamin E, resveratrol, curcumin, and hyaluronic acid) with an average particle size of 100 nm resulted in higher ROS scavenging efficiency and lower lipid peroxidation in patients with PD [348–350]. Similarly, TQ-loaded PLGA-chitosan nanoparticles (particle size from 183 nm) delivered via the nose-to-brain route in rodents improved their pharmacokinetic profile in the brain and enhanced grip strength and locomotor activity. In addition, these effects were supported by a significant reduction in levels of lipid peroxidation and increase in antioxidant enzyme activity in the brain of middle cerebral artery-occluded rats [351]. The anti-inflammatory and neuroprotective potential of stress-induced TQ-loaded solid lipid nanoparticles has been demonstrated by significantly enhanced antidepressive-like behavioral function, hippocampus BDNF levels, and reduced levels of hippocampal IL-6 and TNF-α expression compared with free TQ [352]. TQ-loaded solid lipid nanoparticle treatment also attenuated the overexpression of GFAP, proinflammatory cytokines, and p-p65 NF-κB nuclear translocation; improved the number of TH-positive neurons; and ameliorated motor deficits in neurotoxin-intoxicated animals when compared with the free TQ-treated group [353].

Recently, El-Missiry et al. [354] studied the neuroprotective and antiepileptic efficacy of EA-loaded calcium-alginate nanoparticles (sized approximately 150 nm) in pentylenetetrazol-induced seizures in male mice. These EA-loaded calcium-alginate nanoparticles were also able to ameliorate oxidative stress, as evidenced by enhanced antioxidant efficiency and decreased 4HNE levels in the brain. Furthermore, the nanoformulation outperformed free EA in several activities: amelioration of apoptosis, inhibition of Cyto-C release, activation of caspases, regulation of P53, Bax, and Bcl-2 protein expression, and protection against DNA damage. Moreover, the EA and chitosan-coated combination mitigated rotenone-induced ROS overproduction and reduced cytotoxicity [355]. The antioxidant and antiaging properties of CA-loaded nanotransfersomes were found to enhance cell viability, reduce intracellular ROS generation, attenuate lipid peroxidation, and modulate MMP expression [350]. In addition, anticonvulsive and neuroprotective effects of EGCG-loaded PEGylated-PLGA nanoparticles (particle size from 169 nm) safe for brain cells significantly reduced neuroinflammatory marker expression and were capable of increasing drug integrity and bioavailability [356]. The α-asarone-loaded lactoferrin-modified mPEG-PLA nanoparticles delivered intranasally to the brain showed increased nasal permeability, brain targeting, and brain systemic exposure and reduced toxicity without affecting bioavailability [357].

9. Conclusion

In recent years, research into naturally derived phytochemicals targeting several pathogenic conditions of age-associated NDDs has increased, and clinically, no serious adverse reactions of currently available phytochemicals have been documented. Moreover, PD has been therapeutically cured by natural products, in particular by naturally food-derived phytochemicals with antioxidant potential that may present a reliable source of medicine. The clinical evidence of the health benefits of phytochemicals is not yet fully accepted. However, naturally derived lipophilic phytochemicals can enter the brain and cross the BBB easily, offering increased bioavailability, faster metabolism, and higher affinity to receptors. The regular administration of these naturally derived phytochemicals is an imperative approach to enhance the reversal of neuron function decline and disease resistance competency. Much of the *in vivo* experimental evidence presented in the current study of the potential neuroprotective effects of antioxidant phytochemicals was supported by the results of histopathological and immunohistochemical investigation, which indicated the protection of dopaminergic neurons and attenuation of the loss of

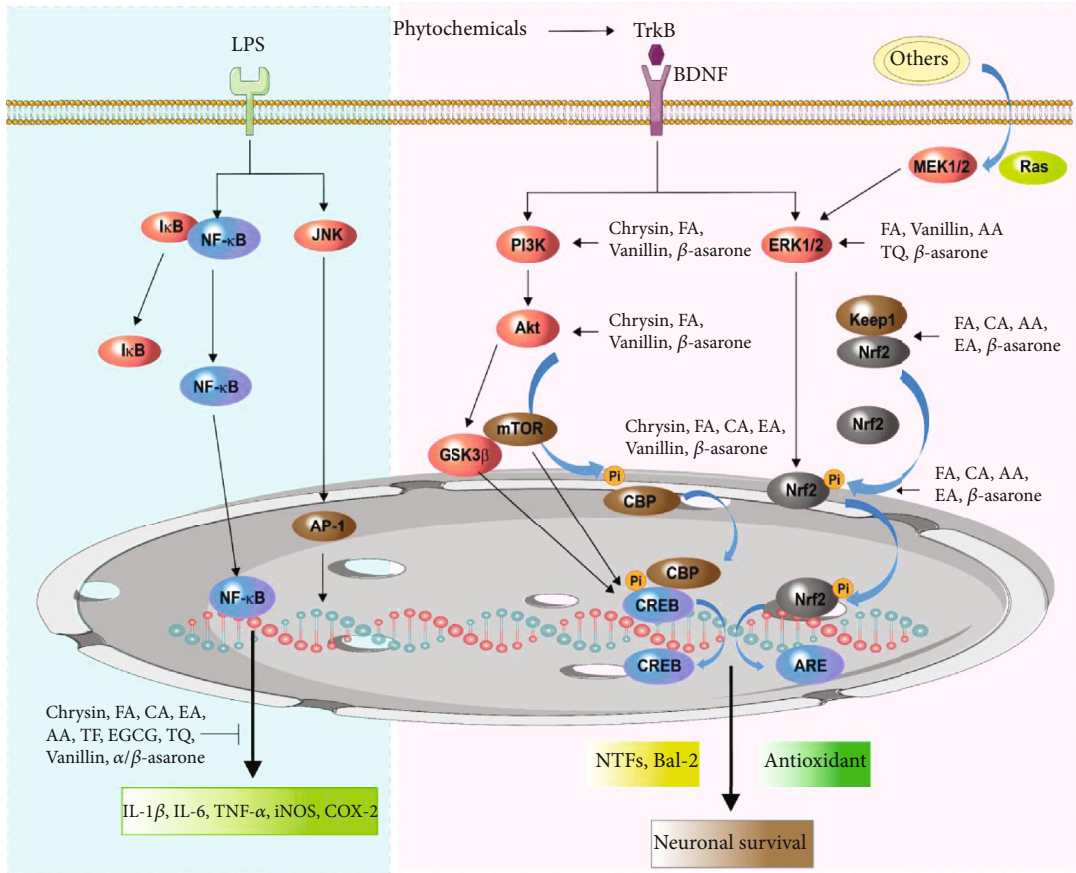

Figure 3: Intracellular targets of neuroprotective antioxidant phytochemicals by activation of Keap1/Nrf2/ARE signaling pathways to increase the expression of antioxidant enzymes. The modulation of these pathways by natural antioxidant phytochemicals such as chrysin, vanillin, asiatic acid (AA), ferulic acid (FA), thymoquinone (TQ), ellagic acid (EA), caffeic acid (CA), epigallocatechin-3-gallate (EGCG), α- and β-asarone, and theaflavin (TF).

TH-positive cells. Furthermore, these changes were accompanied by improvement of several neurotoxin-induced motor balance and cognitive deficits of study animals, as evaluated by rotational behavior, open field test, beam walking, and horizontal and vertical grid tests, as well as by passive avoidance, Barnes maze, and other nonmotor behavioral patterns. Table 1 provides a summary of well-recognized and reported antioxidant phytochemicals and their molecular mechanisms of action in PD.

Collectively, the antiparkinsonian effects of antioxidant phytochemicals have been demonstrated in the reports cited in the present study. It is known that oxidative stress and neuroinflammation are important factors responsible for the progression of PD. Hence, our study provides evidence that the anti-inflammatory activities of antioxidant phytochemicals offer a safe approach to protect against the neuronal damage by reducing oxidative stress, inhibiting lipid peroxidation activity, enhancing the content of GSH, modulating the secretion of proinflammatory cytokines, regulating inflammatory mediators such as COX-2, iNOS, and NO, and regulating anti-inflammatory molecules and pathways (Figure 3). Abundant evidence suggests that oxidative stress and α-synuclein accumulation trigger activation of microglia and astrocytes; this activation is associated with the complex neuroinflammatory pathways leading to neurodegeneration in PD. A number of antioxidant phytochemicals have been shown to clear α-synuclein accumulation and to inhibit microglial activation accompanied by suppressed IBA-1 and GFAP expression. Simultaneously, several antioxidant phytochemicals discussed enhanced the expression of GDNF and BDNF levels involved in the survival of DA neurons. Recently, several experimental findings demonstrated that the JNK signaling pathway involved in apoptotic actions belonging to the superfamily of MAPKs responds to induced ROS and plays a significant role in triggering apoptosis. Several antioxidant phytochemicals are being directed towards some molecular pathways underlying the neuroprotective properties; thus, phytochemicals have been observed to restore the reduced level of antiapoptotic Bcl-2, lowering the expression of proapoptotic Bax and inhibiting caspase activity. Furthermore, phytochemicals have been confirmed to reduce JNK activation and transcription factor c-Jun, resulting in the reduction of dopaminergic neuron apoptosis.

Together, oxidative stress, neuroinflammation, and Nrf2/ARE deregulation are common major situations in the pathogenesis of NDDs, particularly in PD, resulting in impaired motor function and neuronal cell death. Moreover, some important modulators of Nrf2/ARE pathways and autophagy

are altered in PD. Again, neuroprotection by antioxidant phytochemicals is associated with the activation of Nrf2/ARE pathways and autophagy signaling, which appear to be the most well-studied mechanisms for PD treatments. Moreover, inhibition of NF-κB activation should be investigated further as a useful therapeutic approach to the treatment of PD. Accumulated data strongly suggest that antioxidant phytochemicals' potential for activating Nrf2/ARE pathways and autophagy signaling, shown to enhance the expression of Nrf2/ARE and autophagy-related genes, proves protective in several experimental models of PD (Figure 3).

In summary, naturally derived phytochemicals and their derivatives play a potential neuroprotective role in their multidimensional ability to regulate and modulate chronic inflammation, oxidative stress, and downstream signaling, the hallmarks of PD. In addition, to prevent the occurrence of NDDs and their threat to the population, it is essential to explore novel interventional procedures in the clinic for direct employment of dietary phytochemicals as supplements in everyday use. Moreover, the evident lack of toxicity and easy availability from natural resources highlight their advantages in adopting them in the diet. Future research should be aimed at increased clinical acceptance of claims from *in vitro* and *in vivo* preclinical studies and further clinical trial studies of several more potential compounds and the combinations thereof, to observe and prevent any undesirable side effects. The success of phytochemicals in clinical research will thereby be decisive in the evaluation of their pharmacological relevance in humans, and nutritional intervention programs will thereby decrease oxidative neuroinflammatory damage and reduce/slow down the progression of PD.

Abbreviations

AA: Asiatic acid
AD: Alzheimer's disease
AKT: Serine/threonine protein kinase
ALP: Autophagy-lysosome pathway
ARE: Antioxidant response element
Apo: Apomorphine
BBB: Blood-brain barrier
BDNF: Brain-derived neurotrophic factor
BSA: Bovine serum albumin
CAT: Catalase
COX-2: Cyclooxygenase-2
CREB: cAMP-response element binding protein
DA: Dopamine
DAT: Dopamine transporter
DBS: Deep brain stimulation
DOPAC: 3,4-Dihydroxyphenylacetic acid
Drp1: Dynamin-related protein 1
EGCG: Epigallocatechin-3-gallate
EA: Ellagic acid
Epo: Erythropoietin
ERK: Extracellular signal-regulated kinase
ER: Endoplasmic reticulum
FA: Ferulic acid
GFAP: Glial fibrillary acidic protein
GR: Glutathione reductase
GSH: Glutathione
GSK3β: Glycogen synthase kinase-3 beta
GST: Glutathione S transferases
HO-1: Heme oxygenase-1
HSP-70: Heat shock protein-70
HVA: Homovanillic acid
iNOS: Inducible nitric oxide synthase
IFNγ: Interferon-gamma
IL-1β: Interleukin-1β
IL-4: Interleukin-4
IL-6: Interleukin-6
IL-10: Interleukin-10
Keap1: Kelch-like ECH-associated protein
LRRK2:: Leucine-rich repeat kinase 2
mtDNA: Mitochondrial DNA
MAPK: Mitogen-activated protein kinase
MDA: Malondialdehyde
MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+: 1-Methyl-4-phenylpyridinium
NADPH: Nicotinamide adenine dinucleotide phosphate
NDDs: Neurodegenerative disorders
NF-κB: Nuclear factor-κB
NADPH: Nicotinamide adenine dinucleotide phosphate
NO: Nitric oxide
Nrf2: Nuclear factor erythroid 2-related factor 2
PD: Parkinson's disease
PGC: Peroxisome proliferator-activated receptor gamma coactivator
PI3K: Phosphoinositide 3-kinase
PINK1: PTEN-induced kinase 1
PKC: Protein kinase C
PPAR-γ: Peroxisome proliferator-activated receptor gamma
PQ: Paraquat
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SN: Substantia nigra
SOD: Superoxide dismutase
SOD2: Superoxide dismutase 2
ST: Striatum
TF: Theaflavin
TH: Tyrosine hydroxylase
TNF-α: Tumor necrosis factor-alpha
TQ: Thymoquinone
TrkB: Tropomyosin receptor kinase B
UPS: Ubiquitin proteasome system
VMAT-2: Vesicular monoamine transporter 2
6-OHDA: 6-Hydroxydopamine.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

RB contributed to the design, conceptualization, organization, and writing—original draft preparation, and visualization; SA and DYC helped design and review this paper; ISK interpreted the figures; DKC was responsible for the supervision of the review and approval of the final draft. All authors have read and agreed to the published version of the manuscript.

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Research Article

Antioxidant and Anti-Inflammatory Profiles of Spent Coffee Ground Extracts for the Treatment of Neurodegeneration

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Spent coffee grounds (SCGs), waste products of coffee beverage production, are rich in organic compounds such as phenols. Different studies have demonstrated phenol beneficial effects in counteracting neurodegenerative diseases. These diseases are associated with oxidative stress and neuroinflammation, which initiates the degeneration of neurons by overactivating microglia. Unfortunately, to date, there are no pharmacological therapies to treat these pathologies. The aim of this study was to evaluate the phenolic content of 4 different SCG extracts and their ability to counteract oxidative stress and neuroinflammation. Caffeine and 5-*O*-caffeoylquinic acid were the most abundant compounds in all extracts, followed by 3-*O*-caffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid. The four extracts demonstrated a different ability to counteract oxidative stress and neuroinflammation in vitro. In particular, the methanol extract was the most effective in protecting neuron-like SH-SY5Y cells against H_2O_2-induced oxidative stress by upregulating endogenous antioxidant enzymes such as thioredoxin reductase, heme oxygenase 1, NADPH quinone oxidoreductase, and glutathione reductase. The water extract was the most effective in counteracting lipopolysaccharide-induced neuroinflammation in microglial BV-2 cells by strongly reducing the expression of proinflammatory mediators through the modulation of the TLR4/NF-κB pathway. On these bases, SCG extracts could represent valuable nutraceutical sources for the treatment of neurodegeneration.

1. Introduction

The food industry generates considerable amounts of waste products that require to be appropriately managed to reduce their negative sustainability impacts. An appropriate waste management helps to reduce not only the negative effects on the environment but also has got an important economic impact, since there is less production of nonrenewable resources and less energy is used in the production of new goods. Among food industry wastes, coffee by-products have been extensively taken into consideration for recycle [1–5]. Coffee is made by roasting and grinding coffee beans to produce a powder that is extracted with hot water or brewed. During the preparation of coffee beverages, a solid residue known as spent coffee grounds (SCG) is produced and this is the most abundant coffee waste (55–67%) [6].

About 650 kg of SCG are produced from 1000 kg of green coffee beans, and nearly 2 kg of wet SCG are obtained by the preparation of 1 kg of soluble coffee [7]. SCG is a nonedible resource, which is not entering into the food chain, and its disposal in the environment is dangerous since SCG contains caffeine, tannins, and polyphenols that make it a toxic residue

[6, 7]. On these bases, numerous authors have suggested different ways to recycle SCG, to manage and reduce its disposal [8–10]. SCG can be used as a source of oil for biodiesel production [11–13] or as a source of recoverable sugars which can be employed as food addictive or for bioethanol production [13–16]. Moreover, different papers focused on SCG constituents and their application in the food and nutraceutical industry [1, 17–19]. The main constituents of SCG are polysaccharides, proteins, and lipids, as well as minerals, caffeine, melanoidins, and phenols [20]. Phenols of SCG are mainly represented by different highly bioavailable and bioactive phenolic acids such as chlorogenic, caffeic, ellagic, *trans*-ferulic, gallic, p-hydroxybenzoic, p-coumaric, protocatechuic and tannic acids, and flavonoids such as catechin, epicatechin, rutin, and quercetin [1, 21, 22]. Phenolic compounds are well known for their beneficial effects on human health, e.g., in the prevention of different chronic degenerative diseases such as cancer, cardiovascular, and neurodegenerative diseases [23–25]. Neurodegenerative diseases, mainly including Parkinson's and Alzheimer's diseases, are a health problem primarily affecting the elderly. These disorders share common cellular and molecular events such as oxidative stress, abnormal protein deposition, damaged mitochondrial function, induction of apoptosis, impairment of proteostasis, and neuroinflammation [26]. Neuron cells are particularly vulnerable to oxidative damage due to their high polyunsaturated fatty acid content in membranes, high oxygen consumption, and weak antioxidant defenses [27]. Oxidative damage results in an increase in reactive oxygen species (ROS), which leads to further oxidative damage and feeds this self-propagating cycle. ROS may also trigger protein misfolding, potentially leading to protein aggregation, which is a classical hallmark of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [28].

In addition to oxidative damage, in recent years, the immune system is emerging as a key determinant in the onset and progression of neurodegeneration [29, 30] as it triggers modification of cytokine signaling, immune cell proliferation and migration, impaired phagocytosis, and reactive gliosis [31]. Neuroinflammation, caused by the activation into proinflammatory states of the brain immune cells, namely, microglia and astrocytes, represents a fundamental defense system that protects neurons from toxic substance and microorganisms. In normal physiological conditions, this is commonly a positive mechanism aimed at preserving the brain integrity by removing threats and reestablishing homeostasis [32]. However, chronic neuroinflammation can stimulate a series of events that induce progressive neuronal damage that characterizes many neurodegenerative disorders [33]. Unfortunately, currently, no drugs capable of slowing down or blocking the progression of these debilitating pathologies have been identified. This is why the research is turning its attention to the identification of natural compounds with a preventive/protective activity against neurodegenerative disorders. As we previously demonstrated that extracts obtained by coffee silverskin, another coffee by-product, are rich in bioactive compounds with antioxidant and antibacterial activities, we assumed that also SCG could be rich in bioactive phytochemicals with potential neuroprotective activity [5, 34].

The present study was undertaken to evaluate the phenolic content of 4 different SCG extracts and their ability to counteract oxidative stress and neuroinflammation in neuron-like SH-SY5Y and microglial BV-2 cells.

2. Materials and Methods

2.1. Chemicals and Reagents. Cyanidin 3-glucoside chloride, delphinidin 3,5-diglucoside chloride, and kaempferol 3-glucoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). The other 27 analytical standards of the 30 bioactive compounds and high-glucose Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, glutamine, LPS from Escherichia coli serotype O127: B8, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), H_2O_2, dimethyl sulfoxide (DMSO), were purchased from Sigma Aldrich–Merck (Milan, Italy). The 30 analytical standards were dissolved in pure standard compounds in HPLC-grade methanol at a concentration of 1000 mg L^{-1} and stored in glass stoppered bottles at 4°C. The standard working solutions were obtained by appropriate dilution of the stock solution with methanol. HPLC-grade formic acid 99–100% was purchased from Merck (Darmstadt, Germany) while HPLC-grade methanol (MeOH) and ethanol (EtOH) were supplied by Carlo Erba (Milano, Italy). Deionized water was obtained from a Milli-Q Reagent Water System (Bedford, MA, USA). All other solvents and chemicals were of analytical grade. Before HPLC analysis, all samples were filtered with Phenex RC 4 mm 0.2 μm syringeless filter, Phenomenex (Castel Maggiore, Italy). Low-endotoxin FBS was purchased from Euroclone (Milan, Italy).

2.2. Spent Coffee Ground Sample and Extract Preparation. Roasted beans of 100% Coffea arabica L., Ethiopian origin, were supplied by Simonelli Group S.p.A. (Belforte del Chienti, Italy). Roasted beans were grinded by Mythos 1 grinder (Simonelli Group S.p.A.), and spent coffee ground (SCG) was obtained after a series of replicates of espresso coffee preparations using a VA833 Black Eagle espresso coffee machine (Victoria Arduino, Simonelli Group S.p.A., Belforte del Chienti, Italy). The extraction of espresso coffee was carried out as follows: 7 ± 0.05 g of roasted and ground (R&G) coffee per cup, 25 ± 1 s of extraction, water pressure and temperature 9 bar and 92.0°C, respectively, and 25 ± 2 g in cup. SCG samples were collected and oven-dried at 50°C until constant weight (about 48 h). Dried SCG sample was stored at 4°C up to use. The extract preparation was carried out following a previous work [5] with some adjustments. For the current research, four extracts were selected on the base of their high performance in terms of bioactive compound recovery and extraction yield [5, 21]. Briefly, 10 g of SCG were extracted with 50 mL of solvent assisted by a FALC ultrasonic bath (FALC, Treviglio, Italy) at a frequency of 40 kHz for 120 min at 20°C. Four different solvents were tested, i.e., H_2O, MeOH, a mixture of MeOH : H_2O (50 : 50, *v*/*v*), and a mixture of EtOH : H_2O (30 : 70, *v*/*v*). After extraction, the sample was filtered with a filter paper and

lyophilized with a LyovaporTM L-200 (Buchi, Cornaredo, Italy). The lyophilized SCG extracts were kept in darkness at -20°C until use. Before high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) analysis, 5 mL of MeOH (1 mg mL^{-1}) was added to the lyophilized extract (5 mg), and the mixture was sonicated for 10 min and filtered with a 0.2 μm pore size filter.

2.3. HPLC-MS/MS Triple Quadrupole. HPLC-MS/MS studies were performed following a previous procedure [35]. Briefly, the system was composed of an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an electrospray ionization (ESI) source operating in the negative and positive ionization modes. The separation of 30 analytes was achieved on a Kinetex PFP analytical column (100 × 2.1 mm, particle size 2.6 μm) from Phenomenex (Torrance, CA, USA). The mobile phase was obtained mixing (W) water and (M) methanol, both with 0.1% of formic acid. The elution was carried out in gradient mode (flow rate of 0.2 mL min^{-1}). The composition of the mobile phase varied as follows: 0–2 min, isocratic condition, 20% M; 2–15 min, 80% M; 15–18 min, isocratic condition, 80% M; 18–23 min, 100% M; and 23–35 min, 20% M. The injection volume was 2 μL, and the column was set at 30°C. The drying gas in the ionization source was at 350°C. The flow rate of the gas was 10 L min^{-1}, the nebulizer pressure was 25 psi, and the capillary voltage was 4000 V. The dynamic "multiple reaction monitoring" (dynamic MRM) mode was used for detection, and the quantification was realized by integrating the dynamic MRM peak areas. The most abundant product ion was used for quantitation, and the other to confirm the analyte. In Table 1, the selected ion transitions and the mass spectrometer parameters comprising the definite time window for each compound (Δ retention time) are listed.

2.4. Total Phenolic and Flavonoid Contents and DPPH Radical Scavenging Activity. The total phenolic content (TPC) was measured spectrophotometrically according to the method developed by Siatka and Kašparová [36] with some modifications. In particular, 0.5 mL of extract solution (1 mg mL^{-1} in methanol), 2.5 mL of Folin–Denis reagent solution, and 7 mL of Na_2CO_3 (7.5% *w*/*w* in water) solution were added to the test tubes. The reaction mixture was maintained at 25°C for 2 h in the dark, and the absorption was measured at 765 nm. Gallic acid was used as a reference compound, and the TPC in the extracts was calculated using gallic acid calibration curve and expressed as mg of gallic acid equivalents (GAE) per g of dry weight of SCG extract.

The total flavonoid content (TFC) of each extract was evaluated as reported in [37] with some modification. 0.5 mL of extract solution (1 mg mL^{-1}), 0.15 mL of $NaNO_2$ (0.5 M), 3.2 mL of methanol (30% *v*/*v*), and 0.15 mL of $AlCl_3{\cdot}6H_2O$ (0.3 M) were added in a 15 mL test tube. 5 min later, 1 mL of NaOH (1 M) was added and the solution was mixed well before measuring the absorbance at 506 nm. Rutin (0 to 100 mg L^{-1}) was used to make the standard calibration curve for TFC following the procedure described above. TFC was reported as mg of rutin equivalents (RE) per g of dried extract.

The in vitro antioxidant activity of the extracts was measured as ability to scavenge the radical 2,2-diphenyl-1-picrydrazyl (DPPH) as reported in [38] with some modifications. Briefly, 0.5 mL of extract solution (1 mg mL^{-1} in methanol) was added to 4.5 mL of ethanolic solution of DPPH (0.1 mM) in a 15 mL test tube and allowed to stand for 30 min in the dark at 25°C. The DPPH reduction was evaluated spectrophotometrically at 517 nm. The % of DPPH scavenging was obtained following the formula: $\%I = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$. A_{control} and A_{sample} indicate the absorbance obtained in the absence and presence of antioxidants, respectively. The scavenging activity of the extracts was reported as the IC_{50} value (μg mL^{-1}), the extract concentration which causes a 50% DPPH inhibition. The IC_{50} value was calculated by interpolation from the linear regression analysis. Trolox® (1–50 μg mL^{-1}) was considered as a reference antioxidant.

2.5. Cell Cultures and Treatments. The SH-SY5Y cell line was purchased from Sigma-Aldrich (ECACC 94030304) (St. Louis, MO, USA) and was grown in high-glucose DMEM supplemented with 10% (*v*/*v*) of FBS, 2 mM L-glutamine, 50 U/mL of penicillin, and 50 μg/mL of streptomycin, as previously reported [39]. Cells were used for experiments after inducing their differentiation with all-trans retinoic acid (10 μM) for 7 days.

Differentiated SH-SY5Y were treated with different concentrations of the SCG extracts for 24 h and then exposed to 700 μM H_2O_2 for 1.0 h in 1% FBS DMEM.

BV-2 murine microglial cells were a kind gift of Prof. Elisabetta Blasi (University of Modena and Reggio Emilia, Modena, Italy). Cells were cultured in high-glucose DMEM supplemented with 10% (*v*/*v*) of low-endotoxin FBS (Euroclone, Milano), 2 mM L-glutamine, 50 U/mL of penicillin, and 50 μg/mL of streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2 and subcultured using Trysin-EDTA.

BV-2 cells were pretreated with the SCG extracts at different concentrations for 24 h before the addition of 100 ng mL^{-1} LPS for 24 h.

2.6. Cell Viability Assay. Cell viability was evaluated by measuring MTT reduction as previously reported [40]. Briefly, at the end of each experiment, the cell medium was removed from 96-well tissue culture plates, and the cells were incubated with 0.5 mg mL^{-1} of MTT solution. The incubation time was 30 min for BV-2 cells and 90 min for SH-SY5Y cells. After removing the MTT solution, DMSO was added to lyse the cells. The presence of formazan was evaluated spectrophotometrically at 570nm using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; PerkinElmer, Wellesley, MA, USA). Data are reported as percentage with respect to controls. Control cells are considered as 100% cell viability.

2.7. Trypan Blue Assay. SH-SY5Y were differentiated and treated with the extracts (50 μg mL^{-1}) and after 24 h cells were stained with 0.4% trypan blue. The viability was evaluated in

Table 1: HPLC-MS/MS acquisition parameters, working as a dynamic "multiple reaction monitoring" mode, including retention time (Rt) and delta retention time (Δ Rt) for each transition.

No.	Compounds	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)	Polarity	Retention time (Rt) (min)	Delta retention time (Δ Rt)
1	Shikimic acid	173	173 —	87 —	0 —	Negative —	1.40	3
2	Gallic acid	169	125[a] 51	92	12 36	Negative	2.37	3
3	Loganic acid	375	213[a] 113	126	8 16	Negative	3.13	3
4	3-Caffeoylquinic acid	353	191[a] 179	102	12 12	Negative	3.58	3
5	Swertiamarin	419	179[a] 89	100	4 16	Negative	4.89	3
6	Gentiopicroside	357	177[a] 73	50	10 28	Positive	5.33	3
7	(+)-Catechin	289	245[a] 109	121	8 24	Negative	5.48	3
8	Delphinidin-3,5-diglucoside	463	300[a] 271	165	24 48	Negative	5.64	3
9	Sweroside	403	125[a] 179	102	12 4	Negative	5.95	3
10	5-Caffeoylquinic acid	353	191[a] 85	92	12 48	Negative	6.22	3
11	Caffeine	195	138[a] 110	107	20 24	Positive	6.50	3
12	Cyanidin-3-glucoside	449	287[a] 403	121	20 16	Positive	6.50	3
13	Vanillic acid	167	108[a] 152	78	16 8	Negative	6.70	3
14	Caffeic acid	179	135[a] 134	87	12 24	Negative	6.87	3
15	(-)-Epicatechin	289	245[a] 109	126	8 20	Negative	7.03	3
16	Syringic acid	197	182[a] 123	92	8 20	Negative	7.48	3
17	p-Coumaric acid	163	119[a] 93	83	12 32	Negative	8.47	3
18	Ferulic acid	193	134[a] 178	88	12 8	Negative	9.16	3
19	3,5-Dicaffeoylquinic acid	515	353[a] 191	117	8 28	Negative	9.82	3
20	Quinine	325	79[a] 81	135	44 32	Positive	10.1	5
21	Naringin	579	271[a] 151	210	32 48	Negative	10.17	3
22	Rutin	609	300[a] 271	195	40 50	Negative	10.34	3
23	Hyperoside	463	300[a] 271	160	24 44	Negative	10.43	3

Table 1: Continued.

No.	Compounds	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)	Polarity	Retention time (Rt) (min)	Delta retention time (Δ Rt)
24	*trans*-Cinnamic acid	149	131[a]	44	8	Positive	10.79	3
			77		36			
25	Resveratrol	227	185[a]	131	12	Negative	10.92	3
			143		20			
26	Amarogentin	585	227[a]	145	16	Negative	11.05	3
			245		16			
27	Kaempferol-3-glucoside	447	284[a]	163	24	Negative	11.24	3
			227		50			
28	Quercitrin	447	300[a]	155	24	Negative	11.24	3
			301		16			
29	Quercetin	301	151[a]	126	16	Negative	13.03	3
			179		12			
30	Isogentisin	257	242[a]	116	16	Negative	16.31	3
			214		24			

[a]These product ions were used for quantification; the others to confirm the analytes.

Countess™ Cell Counting Chamber Slides (Invitrogen, Carlsbad, CA, USA) using the Countess® Automated Cell Counter (Invitrogen). The number of the cells was determined for each sample, and dead cells were discriminated by the incorporation of trypan blue. Percent of viability was calculated as follows:

2.8. DCFH-DA Assay. Intracellular ROS levels were evaluated using the fluorescent DCFH-DA probe as previously reported [41]. Briefly, at the end of each experiment, 10 μM DCFH-DA solution in DMEM 1% FBS without phenol red was added to the cells allowed to stand for 30 min. PBS was added after removing DCFH-DA solution. Cell fluorescence was measured using 485 nm excitation and 535 nm emission on a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, PerkinElmer).

2.9. RNA Extraction. RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to extract total RNA. The quality and quantity of RNA were evaluated by a NanoVue Spectrophotometer (GE Healthcare, Milano, Italy).

2.10. Real-Time Polymerase Chain Reaction (PCR). Reverse-transcription of 1 μg of the extracted RNA to cDNA was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to the supplier's instructions. To perform PCR, 2.5 μL (12.5 ng) of cDNA, 5 μL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), and 0.5 μL (500 nM) of each primer were added to a PCR tube. In Tables 2 and 3, the primers used (Sigma-Aldrich, Milan, Italy) are listed. Two different reference genes were used: GAPDH rRNA for microglial cells and RPS18 for neuronal cells. cDNA amplification was started at 95°C for 30 s to activate the polymerase, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Normalized expression levels were calculated relative to control cells according to the $2^{-\Delta\Delta CT}$ method.

2.11. Western Immunoblotting. Cells were washed with ice-cold PBS and lysed on ice using 50 mM Tris, 0.1% Triton X-100, 150 mM NaCl, and 2 mM EGTA/EDTA containing mammalian protease inhibitor mixture (1 : 100 dilution), 1 mM sodium pyrophosphate, 10 mg/mL phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 50 mM sodium fluoride. Samples were boiled for 5 min before separation on 4-20% SDS-polyacrylamide gels (20 μg/lane). A nitrocellulose membrane was used to transfer proteins (Hybond-C; GE Healthcare, Buckinghamshire, UK) in Tris-glycine buffer at 110 V for 90 min. The membranes were incubated in blocking buffer prepared with 5% (w/v) bovine serum albumin (BSA) and then incubated with anti-HO1 (Cell Signaling Technology, Beverly, MA) (1 : 1000 dilution) and anti-β-actin (Sigma Aldrich–Merck) (1 : 5000 dilution) as internal loading control, overnight at 4°C on a three-dimensional rocking table. Targeted proteins were visualized using ClarityTM Western ECL Substrate (Bio-Rad). Densitometric analysis of specific immunolabeled bands was performed using ImageJ software.

2.12. Flow Cytometry. To evaluate the surface expression of TLR4 receptor on BV-2 cells, 1×10^5 cells were seeded in 12-well tissue culture plates. At the end of each experiment, cells were washed with PBS and detached with accutase solution. The cells were centrifuged at 300 g for 5 min. The cell pellet was washed twice by centrifugation and resuspension in washing buffer (0.2% BSA-PBS), in 1.5 mL tubes. After removing the supernatant, the cells were resuspended with FITC-conjugated rabbit anti-TLR4 antibody (Stressmarq, cat. no. SPC-200), 1 : 100 dilution in 0.2% BSA-PBS, then incubated for 30 min in the dark at 37°C according to the manufacturer's instructions. After antibody incubation, the cells were washed twice as above. After supernatant aspiration, the samples were appropriately diluted to 5×10^5 cells mL^{-1} and finally resuspended in BSA 0.1% PBS for flow cytometry reading. Guava® easyCyte™ 5 HT instrument

Table 2: List of primers for real-time PCR in SH-SY5Y cells.

Gene	Primer
RPS18 forward	5′CAGAAGGATGTAAAGGATGG3′
RPS18 reverse	5′TATTTCTTCTTGGACACACC3′
GR forward	5′GACCTATTCAACGAGCTTTAC3′
GR reverse	5′CAACCACCTTTTCTTCCTTG3′
NQO1 forward	5′AGTATCCACAATAGCTGACG3′
NQO1 reverse	5′TTTGTGGGTCTGTAGAAATG3′
HO1 forward	5′CAACAAAGTGCAAGATTCTG3′
HO1 reverse	5′TGCATTCACATGGCATAAAG3′
TRX forward	5′AGACAGTTAAGCATGATTGG3′
TRX reverse	5′AATTGCCCATAAGCATTCTC3′

Table 3: List of primers for real-time PCR in BV-2 cells.

Gene	Primer
GAPDH forward	5′ACCACAGTCCATGCCATCAC3′
GAPDH reverse	5′TCCACCACCCTGTTGCTGTA3′
IL-1β forward	5′GTTCCCATTAGACAACTGCACTACAG3′
IL-1β reverse	5′GTCGTTGCTTGGTTCTCCTTGTA3′
TNF-α forward	5′CCCCAAAGGGATGAGAAGTTC3′
TNF-α reverse	5′CCTCCACTTGGTGGTTTGCT3′
iNOS forward	5′CCTCCTCCACCCTACCAAGT3′
iNOS reverse	5′CACCCAAAGTGCTTCAGTCA3′
COX2 forward	5′TGGGGTGATGAGCAACTATT3′
COX2 reverse	5′AAGGAGCTCTGGGTCAAACT3′

was used to collect all raw data. FlowJo software was used to analyze the mean fluorescence intensity (MFI). Unstained samples were used as negative controls.

2.13. Immunofluorescence Confocal Microscopy. BV-2 cells were cultured directly on glass coverslips in 6-well plates. Cells were then fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and permeabilized with Triton 0.1% for 10 min, after which they were treated with a polyclonal antibody (1 : 500) against NF-κB p65 overnight. Following extensive washing with PBS, cells were incubated with a secondary Alexa Fluor 488-conjugated anti-rabbit IgG antibody diluted 1 : 1000 in PBS for 1 h at room temperature. Nuclei were stained with 1 μg mL^{-1} of 4′-6-diamidino-2-phenylindole (DAPI). Slides were analyzed with a C2 Plus confocal laser scanning microscope (Nikon Instruments, Firenze, Italy). Images were processed using NIS Element Imaging Software (Nikon Instruments, Firenze, Italy).

2.14. Statistical Analysis. The experiments were carried out at least in triplicate, and values were reported as mean ± standard error. The differences among groups were evaluated by one-way ANOVA followed by Dunnett's or Bonferroni's test (Prism 5; GraphPad Software, San Diego, CA) (for cell culture data). Differences at the level $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Bioactive Compounds in Different SCG Extracts. Before extract analysis, the analytical method has been validated testing linearity, limit of detection (LOD), limit of quantification (LOQ), and repeatability. The calibration curves were plotted on seven points by injecting seven different concentrations of mixtures of 30 analytes, and the respective determination coefficients (R^2) were calculated. The R^2 for each monitored molecule was ≥0.9937, which implied good linearity. LOD and LOQ were evaluated by injecting gradually lower concentration of standard mixtures, and the concentration with signal-to-noise ratio (SNR) of 3 was assigned to LOD that with SNR of 10 was assigned to LOQ. The LODs obtained ranged from 0.3 to 50 μg L^{-1}, while the LOQs were between 1 and 200 μg L^{-1}. The repeatability has been tested by injecting five replicates of three different concentrations of the standard solutions on the same day (run-to-run precision) and on three consecutive days (day-to-day precision). Relative standard deviation (RSD) % was utilized to define the intraday repeatability or run-to-run precision and interday repeatability or day-to-day precision. Run-to-run precision was between 1.7% and 3.9%, whereas day-to-day precision was between 4.3% and 7.4%.

Four SCG extracts were prepared, i.e., MeOH (E1), H_2O (E2), MeOH : H_2O (50 : 50, *v*/*v*) (E3), and EtOH : H_2O (70 : 30, *v*/*v*) (E4). The content of bioactive compounds (μg g^{-1} of dry weight extract) measured in each SCG extract is listed in Table 4. All extracts were prepared using ultrasound-assisted extraction (UAE), and the analytes were quantified using an HPLC-MS/MS system. The higher content of bioactive compounds was found in EtOH : H_2O extract (71629.19 ± 3025.85 μg g^{-1}) followed by MeOH : H_2O (69891.35 ± 3102.12 μg g^{-1}), MeOH (58796.31 ± 2756.32 μg g^{-1}), and H_2O (56792.60 ± 2521.98 μg g^{-1}). Therefore, the solvent type significantly influenced the analyte extraction, and the EtOH : H_2O and MeOH : H_2O were shown to be the most efficient. Similar outcomes were reported in another recent work [21] which dealt with the chemical composition and some biological properties of different SCG and coffee silverskin (CS) extracts. Caffeine (41047.71-52346.41 ± 1896.25-2536.98 μg g^{-1}) and 5-*O*-caffeoylquinic acid (5-CQA) (7569.25-13256.35 ± 305.21 -499.74 μg g^{-1}) were the most abundant in all extracts followed by chlorogenic acids, i.e., 3-*O*-caffeoylquinic acid (3-CQA) (2324.33-4317.31 ± 100.89-185.42 μg g^{-1}) and 3,5-*O*-dicaffeoylquinic acid (3,5-diCQA) (902.34-1325.98 ± 58.12 -88.23 μg g^{-1}). Andrade et al. [42] have reported similar levels of caffeine in SCG, using UAE with different solvents, finding the best results with dichloromethane (38200.00 μg g^{-1}) and ethanol (25700.00 μg g^{-1}). Considering that the use of dichloromethane should be discouraged since it is associated with both acute and chronic toxicity in humans, including respiratory, central nervous system, and cardiovascular toxicity, carcinogenicity, and genotoxicity [43], the use of ethanol was

Table 4: Bioactive compounds content ($\mu g\ g^{-1}$ of dry weight extract) in spent coffee ground extracts.

No.	Analytes[a]	E1 (MeOH)	E2 (H_2O)	E3 (MeOH : H_2O)	E4 (EtOH : H_2O)
1	Shikimic acid	38.52 ± 1.84	23.11 ± 1.23	86.70 ± 3.26	71.15 ± 3.12
2	Gallic acid	87.65 ± 3.33	57.62 ± 2.65	112.29 ± 4.26	75.91 ± 2.81
3	Loganic acid	n.d.[c]	n.d.	n.d.	n.d.
4	3-CQA[b]	3637.65 ± 157.21	2324.33 ± 100.89	3587.15 ± 163.24	4317.31 ± 185.42
5	Swertiamarin	n.d.	n.d.	n.d.	n.d.
6	Gentiopicroside	n.d.	n.d.	n.d.	n.d.
7	(+)-Catechin	0.95 ± 0.04	n.d.	1.25 ± 0.05	1.02 ± 0.04
8	Del 3,5-diglu[b]	n.d.	n.d.	n.d.	n.d.
9	Sweroside	n.d.	n.d.	n.d.	n.d.
10	5-CQA[b]	12699.32 ± 483.26	7569.25 ± 305.21	13256.35 ± 499.74	12868.75 ± 401.68
11	Caffeine	41047.71 ± 1896.25	45568.32 ± 2121.56	51236.74 ± 2036.15	52346.41 ± 2536.98
12	Cya 3-glu[b]	1.56 ± 0.07	1.02 ± 0.05	1.85 ± 0.08	2.03 ± 0.09
13	Vanillic acid	65.23 ± 2.36	82.65 ± 3.33	122.36 ± 5.14	105.41 ± 4.21
14	Caffeic acid	81.58 ± 1.65	103.28 ± 4.78	170.83 ± 5.98	220.71 ± 10.36
15	(-)-Epicatechin	87.23 ± 2.98	n.d.	85.11 ± 2.22	n.d.
16	Syringic acid	23.56 ± 1.01	44.15 ± 1.87	43.65 ± 2.10	78.63 ± 3.88
17	*p*-Coumaric acid	8.36 ± 0.32	9.45 ± 0.29	15.23 ± 1.12	28.12 ± 1.15
18	Ferulic acid	82.47 ± 3.45	87.54 ± 2.65	118.96 ± 4.13	155.32 ± 5.89
19	3,5-diCQA[b]	915.43 ± 55.32	902.34 ± 58.12	1025.84 ± 64.32	1325.98 ± 88.23
20	Quinine	1.44 ± 0.07	1.69 ± 0.06	2.75 ± 0.10	3.23 ± 0.12
21	Naringin	n.d.	0.62 ± 0.03	0.40 ± 0.02	0.47 ± 0.02
22	Rutin	3.33 ± 0.15	5.36 ± 0.33	8.75 ± 0.52	10.11 ± 0.61
23	Hyperoside	0.98 ± 0.04	0.86 ± 0.03	0.75 ± 0.03	1.23 ± 0.06
24	*trans*-Cin acid[b]	6.27 ± 0.24	5.44 ± 0.30	6.49 ± 0.32	8.11 ± 0.35
25	Resveratrol	n.d.	n.d.	n.d.	n.d.
26	Amarogentin	n.d.	n.d.	n.d.	n.d.
27	Kae 3-glu[b]	1.54 ± 0.06	1.03 ± 0.05	1.97 ± 0.08	2.84 ± 0.11
28	Quercitrin	0.47 ± 0.02	0.28 ± 0.01	0.74 ± 0.03	1.12 ± 0.05
29	Quercetin	3.42 ± 0.12	3.15 ± 0.13	3.96 ± 0.15	3.87 ± 0.11
30	Isogentisin	1.65 ± 0.06	1.12 ± 0.04	1.23 ± 0.05	1.45 ± 0.05
Total compounds		58796.31 ± 2756.32	56792.60 ± 2521.98	69891.35 ± 3102.12	71629.19 ± 3025.85

[a]Each sample was analyzed in triplicate ($n = 3$); [b]3-CQA: 3-caffeoylquinic acid; 3,5-diCQA: 3,5-dicaffeoylquinic acid; 5-CQA: 5-caffeoylquinic acid; Del 3,5-diglu: delphinidin 3,5-diglucosiede; Cya 3-glu: cyanidin 3-glucoside; *trans*-Cin acid: *trans*-cinnamic acid; Kae 3-glu: kaempferol 3-glucoside; [c]n.d.: not detectable.

shown to be a good choice according to the extraction efficiency and at the same time the environmental impact. As reported in Table 1, all seven unconjugated phenolic acids were recovered in all extracts and the most abundant were caffeic (81.58-220.71 ± 1.65-10.36 $\mu g\ g^{-1}$), ferulic (82.47-155.32 ± 3.45-5.89 $\mu g\ g^{-1}$), and vanillic acid (65.23-122.36 ± 2.36 -5.14 $\mu g\ g^{-1}$). Such molecules were also the most abundant in different SCG extracts prepared using a filter coffeemaker preceded by a defatting process with petroleum ether, as reported by Monente et al. [44]. At slightly lower concentrations, we identified gallic acid (57.62-112.29 ± 2.65-4.26 $\mu g\ g^{-1}$) and shikimic acid (23.11-86.70 ± 1.23-3.26 $\mu g\ g^{-1}$), an important metabolite involved in the biosynthesis of aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan in microorganisms and plants (shikimate pathway) [45, 46]. Among the eleven flavonoids monitored in the current study (kampferol 3-glucoside, quercetin, quercitrin, hyperoside, rutin, (+)-catechin, (-)-epicatechin, cyanidin 3-glucoside, delphinidin 3,5-diglucoside, naringin, and resveratrol), nine of them have been found in the SCG extracts. The most abundant was (-)-epicatechin, a flavonoid of flavan-3-ol subclass, which occurred only in MeOH (87.23 ± 2.98 $\mu g\ g^{-1}$) and MeOH : H_2O (85.11 ± 2.22 $\mu g\ g^{-1}$) extracts and two molecules of flavonol subclass, i.e., quercetin (3.15-3.96 ± 0.15-0.13 $\mu g\ g^{-1}$) and its glycoside rutin (3.33-10.11 ± 0.15-0.61 $\mu g\ g^{-1}$). Interestingly, cyanidin 3-glucoside, an anthocyanin that occurs in

coffee skin and pulp [47], has been found in all extracts ranging from 1.02 to 2.03 ± 0.05-$0.09\,\mu g\,g^{-1}$ but not delphinidin 3,5-diglucoside. As already reported by Angeloni et al. (2020), iridoids and secoiridoids did not occur in spent coffee and probably in coffee beans. On the other hand, an alkaloid first isolated from the *Cinchona* tree known as a potent antimalarial agent, namely, quinine (1.44-3.23 ± 0.07 $-0.12\,\mu g\,g^{-1}$) [48], and a xanthone of Gentian plant [49], namely, isogentisin (1.12-1.65 ± 0.04-$0.06\,\mu g\,g^{-1}$), were detected in all SCG extracts.

3.2. Total Phenolic and Flavonoid Contents and DPPH Radical Scavenging Activity of SCG Extracts. Table 5 reports the content of the phenolic and flavonoid compounds and the radical scavenging activity of different SCG extracts. The TPC has been spectrophotometrically measured, and data are reported as mg of gallic acid equivalents (GAE) per g of dry weight of SCG extract. The highest levels of phenolic compounds were found in E4 (112.65 ± 4.53 mg GAE/g) followed by E3 (95.12 ± 3.56 mg GAE/g), E1 (88.75 ± 2.13 mg GAE/g), and E2 (69.32 ± 2.11 mg GAE/g) extract. These levels were higher than those reported by other works when a simply solid-liquid extraction was employed [50–52]. For instance, Bravo et al. found SCG extracts with TPC of 17.44 ± 0.26 mg GAE/g using water for analyte extraction [52]. On the other hand, Al-Dhabi et al., who performed UAE at different conditions, obtained higher levels of TPC (32.81-36.23 mg GAE/g) [53]. The use of ultrasound during the extraction process increases the mass transfer due to acoustic cavitation effect generated by ultrasonic waves [54], and this can be the reason together with the coffee variability of higher TPC obtained in the current research. The total content of chlorogenic acids, one of the most important class of phenolic compounds in coffee, measured by the HPLC system, was characterized by the same abovementioned ranking, i.e., EtOH : H_2O ($18512.04 \pm 895.32\,\mu g\,g^{-1}$) followed by MeOH : H_2O ($17869.34 \pm 925.26\,\mu g\,g^{-1}$), MeOH ($17252.40 \pm 823.12\,\mu g\,g^{-1}$), and H_2O ($10795.92 \pm 772.65\,\mu g\,g^{-1}$). In contrast, the highest level of TFC, expressed as mg of rutin equivalents (RE) per g of dried extract, was obtained in MeOH : H_2O (6.29 ± 0.23 mg RE/g) followed by MeOH (6.17 ± 0.16 mg RE/g), EtOH : H_2O (5.56 ± 0.12 mg RE/g) and H_2O (3.15 ± 0.14 mg RE/g) extract. These data are consistent with HPLC-MS/MS studies on the total content of monitored flavonoids since they can be ranked in the following order MeOH : H_2O ($107.53 \pm 7.25\,\mu g\,g^{-1}$) > MeOH ($100.92 \pm 5.98\,\mu g\,g^{-1}$) > EtOH : H_2O ($25.92 \pm 1.08\,\mu g\,g^{-1}$) > H_2O ($14.01 \pm 0.65\,\mu g\,g^{-1}$). The radical scavenging activity of SCG extracts has been evaluated by DPPH assay, and it was expressed as the IC_{50} value ($\mu g\,mL^{-1}$) which is the concentration of the extract necessary to cause 50% of DPPH inhibition. The solvent type influenced the antioxidant capacity of the extracts, and the highest radical scavenging activities were obtained with EtOH : H_2O (196.25 ± 6.87 $\mu g\,mL^{-1}$) and MeOH ($215.35 \pm 7.42\,\mu g\,mL^{-1}$). Notably, the H_2O extract ($585.32 \pm 25.32\,\mu g\,mL^{-1}$) was the worst in terms of antioxidant capacity and it was characterized by lower content of bioactive compounds as well. The latter together with an inefficient extraction of low-polar compounds could be the reason of lower antioxidant activity. In fact, some lipophilic compounds which usually occur in coffee, e.g., diterpenes and tocopherols, are known as powerful antioxidants [55, 56].

Table 5: Total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity of the different spent coffee ground extracts.

Extracts	Total phenolic content (mg GAE/g)	Total flavonoid content (mg RE/g)	DPPH IC50 (μg/mL)
E1 (MeOH)	88.75 ± 2.13	6.17 ± 0.16	215.35 ± 7.42
E2 (H_2O)	69.32 ± 2.11	3.15 ± 0.14	585.32 ± 25.32
E3 (MeOH : H_2O)	95.12 ± 3.56	6.29 ± 0.23	298.44 ± 13.12
E4 (EtOH : H_2O)	112.65 ± 4.53	5.56 ± 0.12	196.25 ± 6.87

3.3. Neuroprotective Activity of SCG Extracts

3.3.1. Antioxidant Activity. The in vitro antioxidant activity of E1, E2, E3, and E4 has been investigated in neuron-like SH-SY5Y cells differentiated with retinoic acid. To study the potential cytotoxicity of E1, E2, E3, and E4, cells were treated with 1–$200\,\mu g\,mL^{-1}$ of the four extracts for 24 h and MTT assay was used to measure cell viability (Figures 1(a)–1(d)). The extracts were not cytotoxic up to $200\,\mu g\,mL^{-1}$ except the E1 extract that induced a significant reduction of cell viability at $200\,\mu g\,mL^{-1}$. Interestingly, the treatment with the extracts led to a significant increase of cell viability. As MTT evaluates cell viability as the enzymatic conversion of the tetrazolium compound to water-insoluble formazan crystals by dehydrogenases occurring in the mitochondria of living cells [57], we can suppose that this cell viability increase could be caused by an intensification of mitochondrial respiration. All extracts are rich in caffeine that has been associated to an increased mitochondrial content due to the upregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) that modulates the nuclear respiratory factors 1 and 2 (NRF1/2) and mitochondrial transcription factor A (TFAM) [58–61]. Moreover, the treatment with caffeine of isolated human muscle fibers showed a direct effect on the mitochondrial activity by increasing the respiration rate and concomitantly decreasing the mitochondrial membrane potential [62]. In a previous study, we observed a significant increase of cell viability of differentiated SH-SY5Y cells treated with extracts containing caffeine, and these new data reinforce the hypothesis that caffeine could be the compound responsible for this effect [5]. Of course, further investigations are needed to determine a direct involvement of caffeine in the enhancement of mitochondrial respiration in SH-SY5Y cells. To clarify if the observed increase in cell viability is only linked to an increase in mitochondrial activity, we measured cell viability

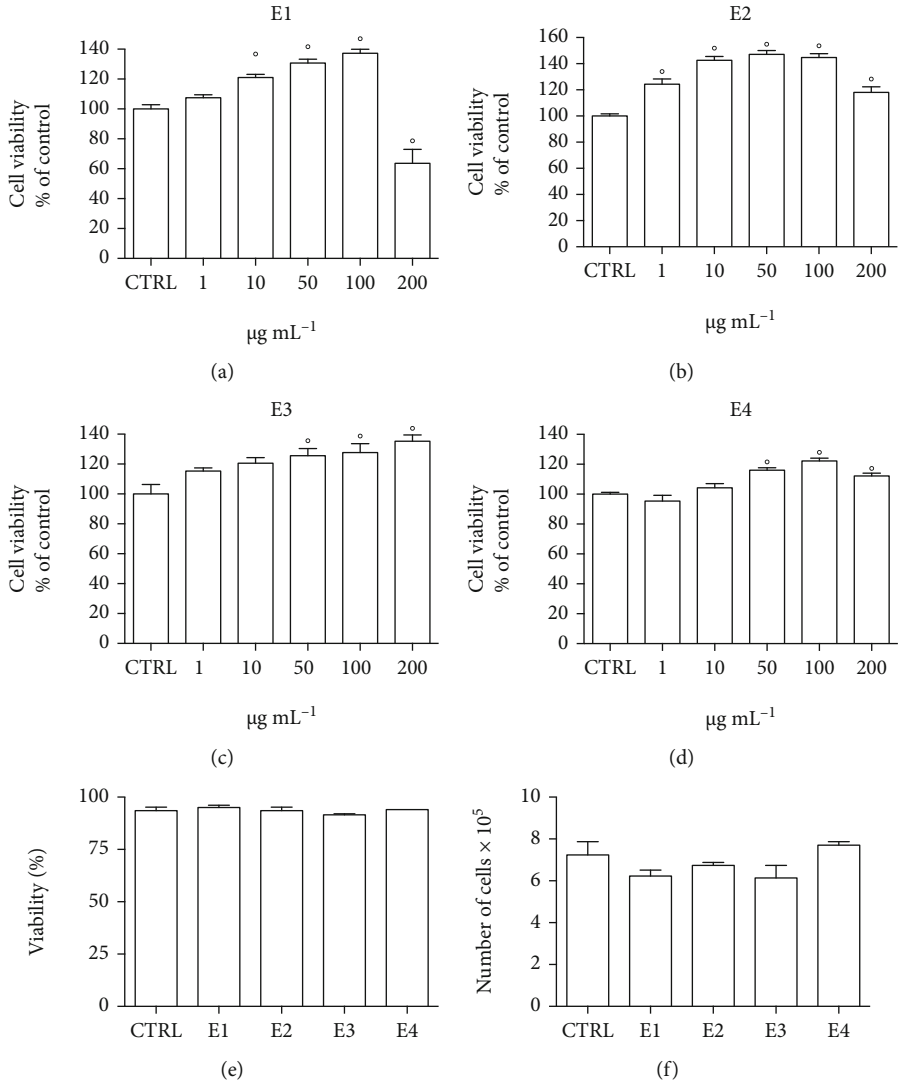

Figure 1: Effect of the different extracts on viability of SH-SY5Y cells. (a–d) Cells were treated with 1–200 μg mL^{-}1 of each extract for 24 h, and cell viability was evaluated by MTT assay. (e, f) Cells were treated with 50 μg mL^{-1} of each extract for 24 h, and the trypan blue assay was used to measure cell number and cell viability. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. $^{\circ}p < 0.05$ with respect to CTRL.

with a different viability assay. Differentiated SH-SY5Y cells were treated with 50 μg mL^{-1} of each extract for 24 h, and cell viability was measured by the trypan blue assay (Figure 1(e)) that is based on the principle that living cell membranes are intact and exclude trypan blue, whereas the dead cells are permeable to the dye. Of note, all treatments did not increase cell viability suggesting that the increase in cell viability measured by MTT assay is related to an increase in mitochondrial activity. Another hypothesis to explain the observed increase in cell viability could be a corresponding increase in cell proliferation. To investigate this aspect, differentiated SH-SY5Y cells were treated with 50 μg mL^{-1} of each extract for 24 and the total cell number was counted (Figure 1(f)). Interestingly, the treatments did not modify the cell number, confirming that the observed increase in cell viability measured by MTT assay is related to a higher rate of mitochondrial respiration and not to an increased proliferation.

The antioxidant activity of the extracts has been evaluated pretreating SH-SY5Y cells with 1-100 μg mL^{-1} of the extracts for 24 h before exposing the cells to 700 μM H_2O_2 to induce oxidative stress (Figure 2). At the lowest concentrations, only the E4 extract significantly increased cell viability with respect to H_2O_2-treated cells, meanwhile, at 10 μg mL^{-1}, E1 was also able to significantly increase cell viability. E3 significantly counteracted oxidative stress at 50 μg mL^{-1} and E2 only at the highest tested concentrations. Of note, at 50 μg mL^{-1}, E1 increased cell viability with respect to peroxide-treated cells by about 22%, meanwhile E3 and E4 by about 16%, evidencing a higher ability of E1 in counteracting oxidative stress-induced damage in SH-SY5Y cells.

To further investigate the antioxidant activity of the extracts, SH-SY5Y cells were treated with 1-100 μg mL^{-1} of each extract and the DCFH-DA assay was used to evaluate the effect on intracellular ROS production (Figure 3). The

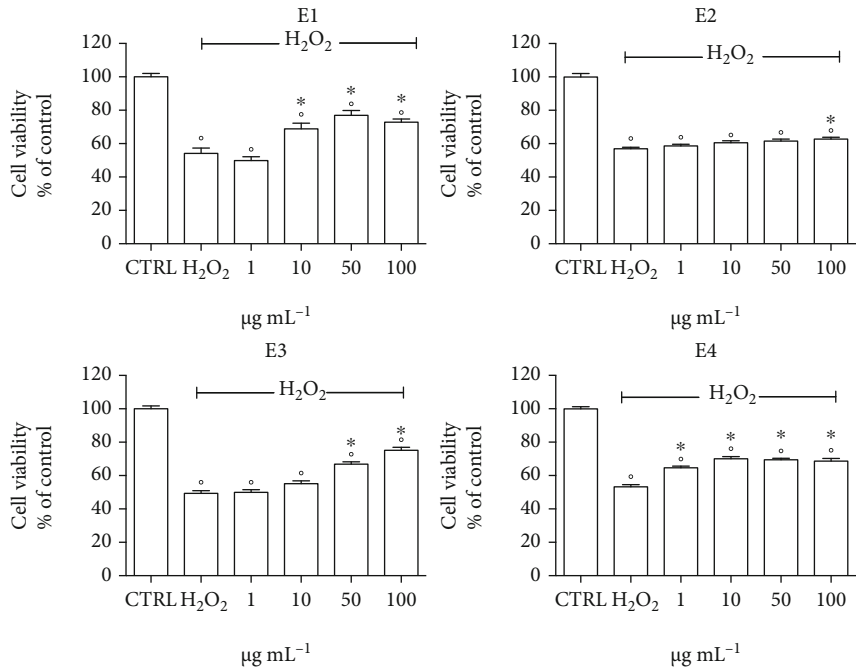

Figure 2: Cytoprotective activity of the extracts in SH-SY5Y cells exposed to H_2O_2. Cells were pretreated with 1–100 μg mL^{-1} of each extract for 24 h, exposed to 700 μM H_2O_2 for 1 h before measuring cell viability by MTT assay. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^{\circ}p < 0.05$ with respect to CTRL; $^{*}p < 0.05$ with respect to H_2O_2.

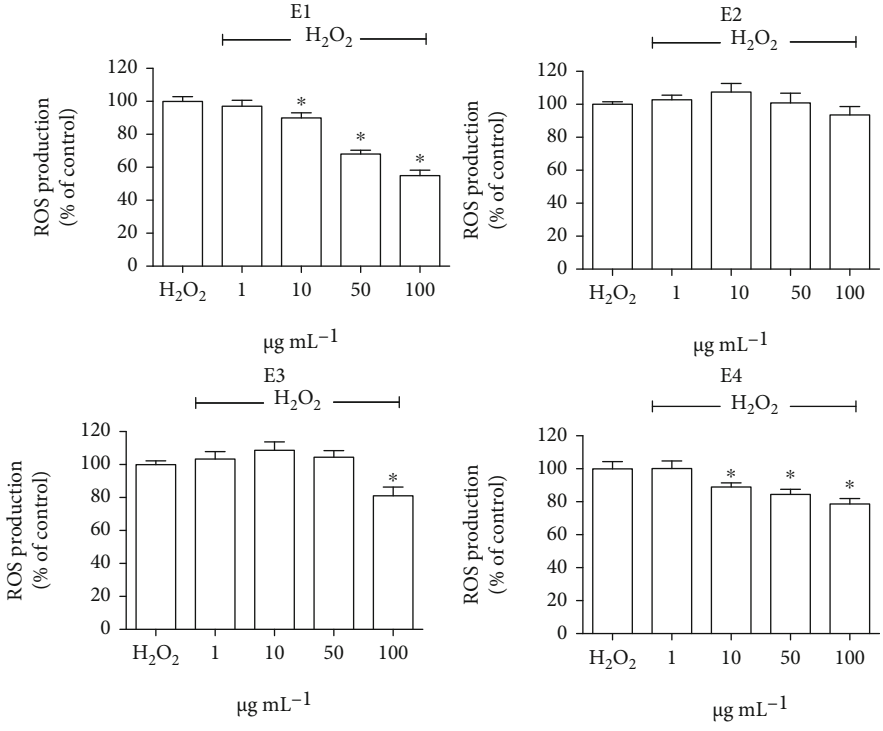

Figure 3: ROS levels of SH-SY5Y cells treated with the extracts and exposed to H_2O_2. Cells were pretreated with 1–100 μg mL^{-1} of the different extracts for 24 h and then treated with H_2O_2. The peroxide-sensitive probe DCFH-DA was used to measure ROS production. Data are expressed as percentage with respect to H_2O_2-treated cells. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. $^{*}p < 0.05$ with respect to H_2O_2.

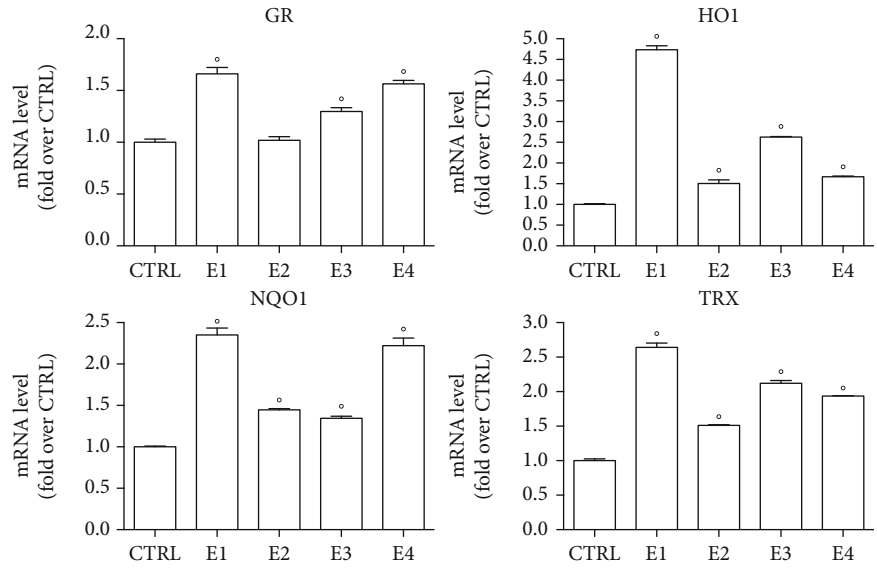

Figure 4: Effect of the extracts on the mRNA level of antioxidant enzymes in SH-SY5Y cells. Cells were treated with 50 μg mL^{-1} of each extract for 5 h. GR, HO1, NQO1, and TRX mRNA levels were determined by RT-PCR. Data are reported as relative abundance in respect to control cells (CTRL). Each bar represents mean ± SEM of three independent experiments. Data were analyzed with a one-way ANOVA followed by the Dunnett's test. $^{\circ}p < 0.05$ vs. CTRL.

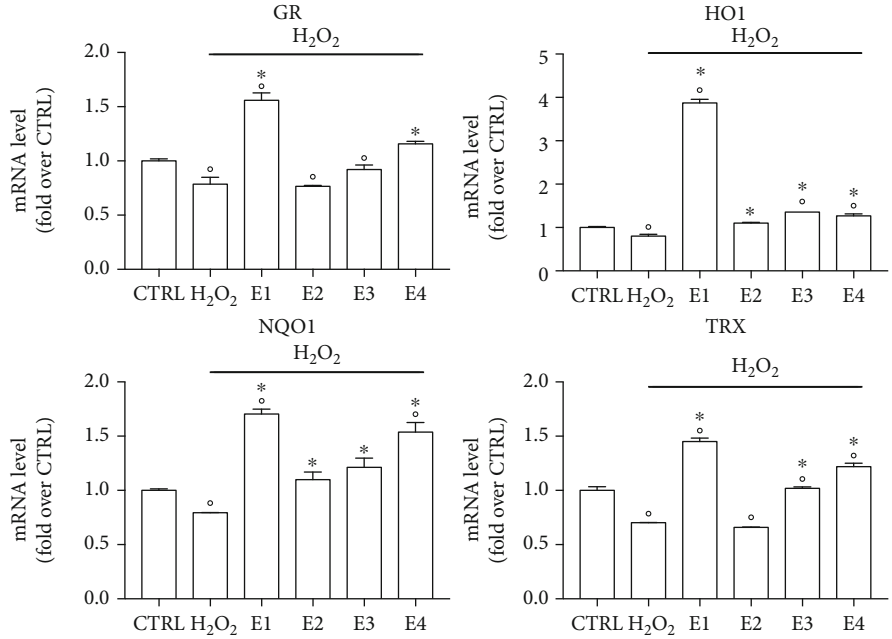

Figure 5: Effect of the extracts on the mRNA level of SH-SY5Y in the presence of H_2O_2. Cells were pretreated with 50 μg mL^{-1} of each extract and after 5 h exposed to H_2O_2 700 μM for 1 h. GR, HO1, NQO1, and TRX mRNA levels were determined by RT-PCR. Data are reported as relative abundance in respect to control cells (CTRL). Each bar represents mean ± SEM of three independent experiments. Data were analyzed with a one-way ANOVA followed by the Bonferroni's test. $^{\circ}p < 0.05$ vs. CTRL; $^{*}p < 0.05$ vs. H_2O_2.

results showed that E1 and E4 were the most effective ones in reducing ROS levels, meanwhile E3 reduced ROS levels only at the 100 μg mL^{-1} and E2 did not influence this parameter. These results confirm that E1 and E4 are the extracts with the strongest antioxidant activity.

These biological results on the antioxidant activity of the extracts are in agreement with the results obtained by DPPH assay (Table 2). In particular, in SH-SY5Y cells, E1 and E4 extracts were the most effective ones in terms of antioxidant activity, meanwhile E2 showed the lowest activity. As previously underlined, the low antioxidant capacity of E2 could be caused by an inefficient extraction of low-polar compounds, which usually occur in coffee and are known for their elevated antioxidant activity [55, 56].

Evidence is cumulating which shows that many phytochemicals exert antioxidant activity through an indirect antioxidant mechanism, i.e., enhancing the expression of antioxidant enzymes and cytoprotective proteins [63–66]. To verify if the extracts modulate the endogenous antioxidant system, we treated SH-SY5Y cells with 50 μg mL^{-1} of each extract before analyzing the expression of the antioxidant enzymes glutathione peroxidase (GR), heme oxygenase 1 (HO1), NADP(H) oxidoreductase 1 (NQO1) and thioredoxin reductase by RT-PCR (Figure 4). All the extracts significantly upregulated HO1, NQO1, and TRX, meanwhile GR expression was significantly increased only by E1, E3, and E4.

We also evaluated the expression of these antioxidant enzymes in the presence of H_2O_2. In particular, SH-SY5Y cells were pretreated with 50 μg mL^{-1} of each extract and then exposed to H_2O_2 before analyzing mRNA levels of GR, HO1, NQO1, and TRX (Figure 5). H_2O_2 exposure significantly reduced the expression of all the tested genes in respect to control cells. Considering the short H_2O_2 exposure, the observed downregulation of these genes could be probably ascribed to the H_2O_2-induced oxidation of the corresponding mRNA. E1 was able to significantly upregulate all the four genes with respect to both H_2O_2 and controls. E2 treatment did not influence GR and TRX expressions with respect to H_2O_2-treated cells, meanwhile slightly but significantly upregulated HO1 and NQO1 expressions. E3 significantly increased mRNA levels of HO1, NQO1, and TRX with respect to H_2O_2-treated cells and upregulated HO1 and TRX with respect to control cells. E4 significantly increased the expression of HO1, NQO1, and TRX with respect to both H_2O_2 and controls, meanwhile significantly upregulated GR only with respect to H_2O_2.

Considering the strong upregulation of HO1 with respect to the other tested genes, we performed an immunoblotting analysis to confirm HO1 induction also at a protein level. SH-SY5Y cells were pretreated with 50 μg mL^{-1} of each extract and then exposed to H_2O_2 before western blot analysis (Figure 6). H_2O_2 exposure reduced HO1 protein levels with respect to control cells, even if not significantly. On the contrary, E1 strongly and significantly increased the expression of HO1, confirming the expression data.

Interestingly, E1, with respect to the other extracts, showed a marked ability to upregulate the four antioxidant enzymes both in the absence and in the presence of H_2O_2 suggesting that the higher ability of E1 to protect SH-SY5Y cells against oxidative stress could be ascribed to its ability to strongly upregulate the endogenous antioxidant system.

Considering the characterization of the extracts in terms of bioactive compound content (Table 1), E1 showed the highest content of (-)-epicatechin and isogentisin with respect to the other extracts. Of note, no correlation was evidenced between (-)-epicatechin content and the different parameters tested to analyze the antioxidant activity of the extracts. On the other hand, isogentisin content was positively correlated with the protection against H_2O_2 ($r = 0.9745$, $p < 0.05$) and GR expression ($r = 0.9575$, $p < 0.05$) and inversely correlated with ROS levels ($r = -0.9604$, $p < 0.05$). The xanthone isogentisin is a characteristic constituent found in plants such as Gentianaceae [67]. Very few studies investigated its bio-

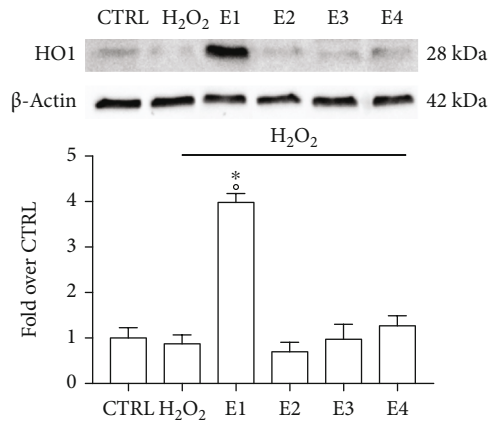

Figure 6: Protein level of HO1 in SH-SY5Y cells treated with the extracts and exposed to H_2O_2. Cells were treated with E1, E2, E3, and E4 (50 μg mL^{-1}) and after 24 h exposed to H_2O_2 700 μM for 1 h. Immunoblotting was performed using anti-HO1. Data are expressed as fold over CTRL and normalized by β-actin. Each bar represents mean ± SEM of three independent experiments. Data were analyzed with a one-way ANOVA followed by the Bonferroni's test. $^{\circ}p < 0.05$ vs. CTRL; $^{*}p < 0.05$ vs. H_2O_2.

logical activity. In particular, isogentisin has been shown to counteract smoking-caused injury in human umbilical vein endothelial cells (HUVECs) [68] and to inhibit monoamine oxidase types A and B in rat brains [69, 70]. Monoamine oxidase inhibitors are considered important agents for the treatment of depression, anxiety, and neurodegenerative disorders, including Alzheimer's and Parkinson's diseases [71, 72]. From this point of view, further studies will be necessary both to investigate the antioxidant activity of pure isogentisin in neuron cells and to verify if the E1 extract also can act as a monoamine oxidase inhibitor.

3.3.2. Anti-Inflammatory Activity. The in vitro anti-inflammatory activity of the extracts has been investigated in microglial BV-2 cells. Microglia are equivalent to macrophages in the brain and represent the first and most important line of defense in the central nervous system. Under physiological conditions, microglia have a key role in neuronal survival through the production of neurotrophic factors and the phagocytosis of dead cells, cellular debris, protein aggregates, and invading pathogens [73]. However, excessively activated microglia can lead to neurotoxicity through the production of proinflammatory mediators such as tumor necrosis factor alpha (TNF-α), nitric oxide, interleukin-1β (IL-1β), IL-6, and ROS [74]. Different studies have shown that microglia play an important role in the onset and progression of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [75–77].

Prior to investigating the effect of the extracts on BV-2 microglia-mediated neuroinflammation, we assessed the potential cytotoxicity of E1, E2, E3, and E4 on BV-2 microglial cells using MTT assay (Figure 7). The extracts were not cytotoxic up to 100 μg mL^{-1}, meanwhile all extracts were cytotoxic at 200 μg mL^{-1} as demonstrated by a significant

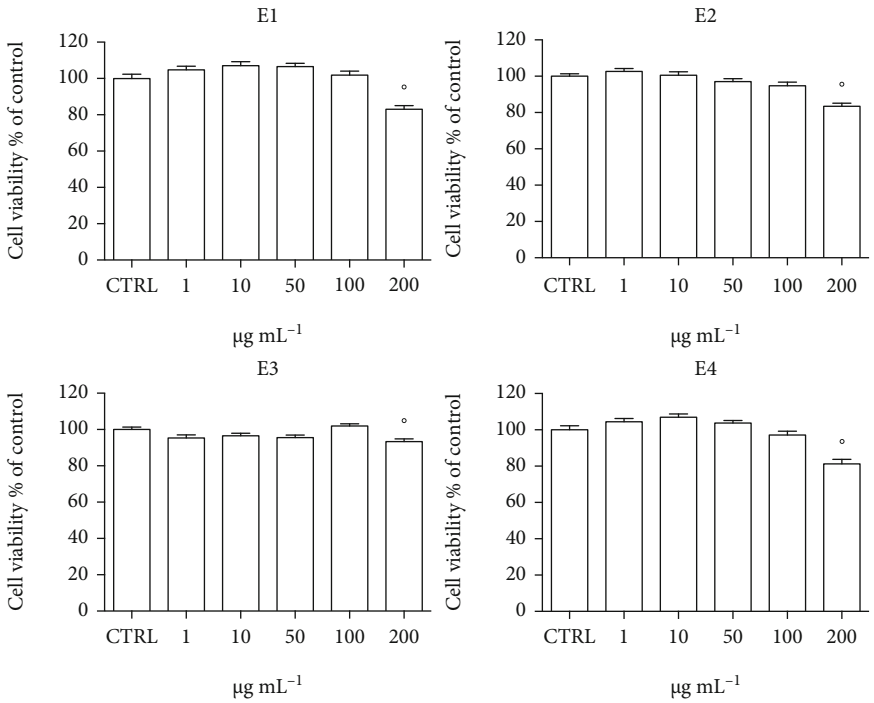

Figure 7: Effect of the different extracts on cell viability of BV-2 cells. Cells were treated with 1–200 μg mL^{-1} of each extract for 24 h, and MTT assay was used to obtain cell viability. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. $^{\circ}p < 0.05$ with respect to CTRL.

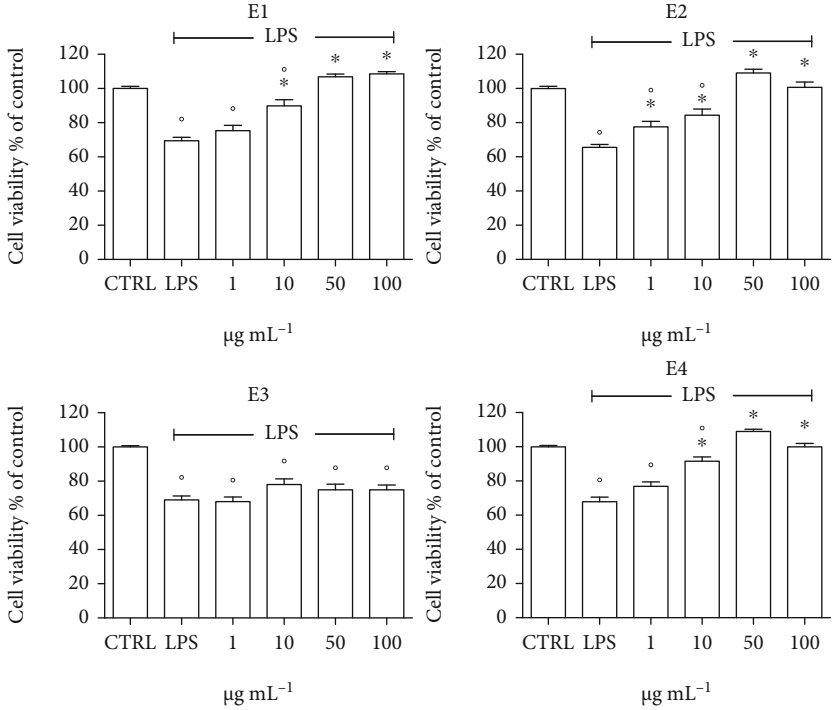

Figure 8: Cytoprotective activity of the extracts in BV-2 cells activated by LPS. Cells were pretreated with 1–100 μg mL^{-1} of each extract for 24 h, activated with 100 ng mL^{-1} LPS for 24 h, and MTT assay was used to measure cell viability. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^{\circ}p < 0.05$ with respect to CTRL; $^{*}p < 0.05$ with respect to LPS.

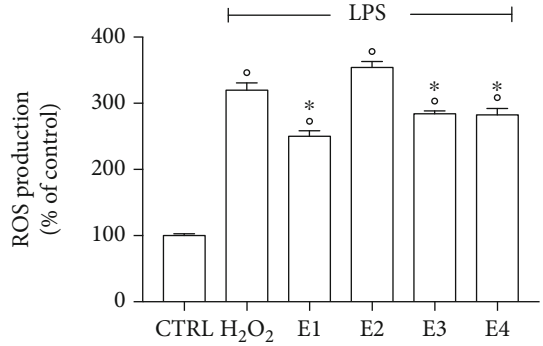

Figure 9: ROS levels of BV-2 cells treated with the extracts and activated by LPS. Cells were pretreated with 50 μg mL^{-1} of the different extracts for 24 h and then activated by LPS. The peroxide-sensitive probe DCFH-DA was used to measure ROS production. Data are expressed as % compared to control cells (CTRL). Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. $^{\circ}p < 0.05$ with respect to CTRL; $^{*}p < 0.05$ with respect to H_2O_2.

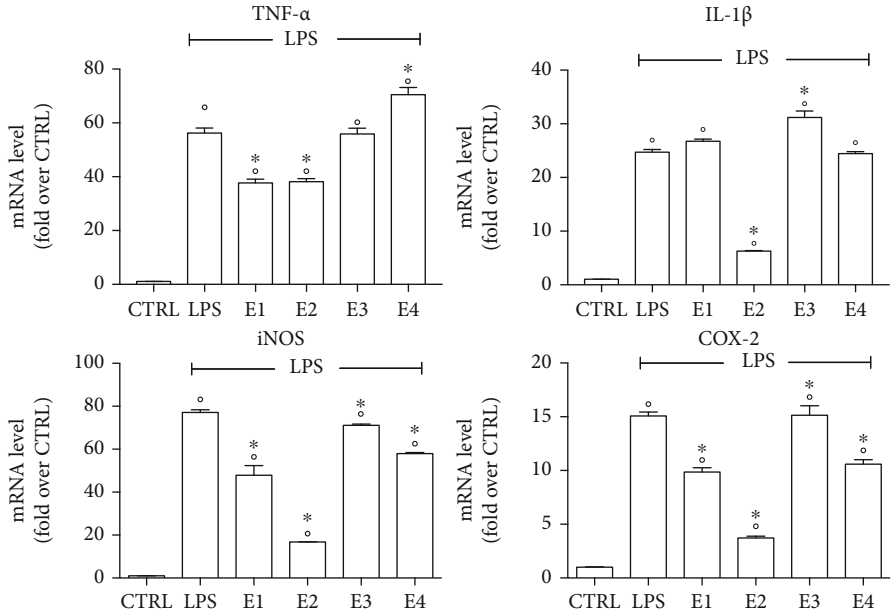

Figure 10: Expression of proinflammatory cytokines and enzymes in BV-2 cells treated with the extracts. Cells were treated with E1, E2, E3, and E4 (50 μg mL^{-1}) for 24 h, exposed to 100 ng mL-1 LPS for 24 h and TNF-α, IL1-β, iNOS, and COX-2 mRNA levels were measured by RT-PCR. Data are expressed as relative abundance compared to untreated cells. Each bar represents the mean ± SEM of three independent experiments. Data were analyzed with a one-way ANOVA followed by Bonferroni's test. $^{\circ}p < 0.05$ vs. CTRL; $^{*}p < 0.05$ vs. LPS.

reduction of cell viability at this concentration with respect to control cells.

The anti-inflammatory activity of the extracts was evaluated pretreating BV-2 cells with different concentrations (1-100 μg mL^{-1}) of the extracts for 24 h before exposing the cells to lipopolysaccharide (LPS) to induce inflammation (Figure 8). LPS is the most widely used inflammatory mediator to activate microglial cells in vitro and triggers the proinflammatory signaling cascade [78, 79]. LPS treatment significantly reduced cell viability with respect to control cells by ~40%. Interestingly, E1, E2, and E4 were able to significantly increase cell viability with respect to LPS-treated cells, and at 50 μg mL^{-1}, all of them were able to maintain cell viability to a value comparable to control cells. On the other hand, E3 did not show any protective effect against LPS-induced damage.

As it has been shown that LPS induces oxidative stress [80, 81], we measured intracellular ROS levels in BV-2 cells pretreated with 50 μg mL^{-1} of the extracts for 24 h and then activated by LPS (Figure 9). As expected, LPS significantly increased intracellular ROS levels with respect to controls. E1, E3, and E4 significantly reduced ROS levels compared to LPS-treated cells, meanwhile E2 did not modify ROS levels with respect to LPS-treated cells, in agreement with the results obtained in SH-SY5Y cells.

Since proinflammatory cytokines and enzymes including tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS) are crucial mediators of neuroinflammation, we next measured the effects of the extracts on these inflammatory mediators in LPS-stimulated BV-2 microglial cells

(Figure 10). BV-2 microglial cells were pretreated with 50 μg mL^{-1} of E1, E2, E3, and E4, followed by LPS for 24 h. Total RNA was isolated, and proinflammatory cytokine and enzyme expressions were measured using RT-PCR. As expected, LPS significantly increased the expression of TNF-α, IL-1β, COX-2, and iNOS with respect to control cells. In agreement with the MTT data, E3 did not show any ability to inhibit LPS-induced expression of TNF-α and COX-2 and significantly increased the expression of IL-1β with respect to LPS. Moreover, E3 was able to significantly reduce iNOS expression with respect to LPS, but the extent of this reduction was very small, maintaining iNOS expression to levels strongly higher than those measured in control cells. E1 and E4 had no effect on LPS-induced IL-1β expression, meanwhile they were able to significantly reduce iNOS and COX-2 expression with respect to LPS-treated cells. These two extracts showed opposite behaviors with respect to TNF-α expression: E1 significantly inhibited LPS-induced expression of this cytokine, on the contrary E4 significantly increased its expression with respect to LPS-treated cells. Of note, E2 was the most effective extract and significantly and strongly inhibited the expression of all proinflammatory mediators analyzed. These results are partially in agreement with the data on cell viability that showed that E1, E2, and E4 had a similar effect in counteracting LPS-induced damage, and all of them were able to completely protect cells against neuroinflammation at 50 μg mL^{-1}. This discrepancy could be explained by the different mechanisms by which these extracts counteract LPS-induced damage. The mechanism behind E2 protection is easy to understand as this extract exerts a strong anti-inflammatory activity that significantly and strongly reduces the expression of all proinflammatory mediators investigated. On the contrary, the protective activity of E1 and E4 cannot be explained only in terms of their ability in reducing proinflammatory cytokine and enzyme expression levels. Taking into consideration the results also obtained in SH-SY5Y, we can suggest that E1 and E4 were able to protect BV-2 cells against LPS-induced damaged thanks to their antioxidant activity. In fact, it is widely accepted that LPS generates ROS that trigger oxidative stress and cell damage [82–84]. Of note, E1, E3 and E4, but not E2 at 50 μg mL^{-1}, significantly reduced ROS levels in BV-2 cells.

The NF-κB pathway is a key mediator of inflammation and is activated via Toll-like receptors (TLRs) resulting in increased cytokine and chemokine production [85]. It has been observed that the activation of NF-κB and release of its subunits play a crucial role in the onset and progression of neurodegenerative disorders [86, 87]. Moreover, transcription of TNF-α, IL-1β, iNOS, and COX-2 is regulated by the transcription factor NF-κB. To further elucidate the mechanisms of the extracts on the inhibition of the expression of these proinflammatory mediators in BV-2 cells, the effect of E1, E2, E3, and E4 on NF-κB activation was investigated by confocal microscopy (Figure 11). BV-2 cells were pretreated with 50 μg mL^{-1} of the extracts, exposed to LPS for 24 h and immunostained with a primary antibody against NF-κB p65, followed by Alexa Fluor 488-conjugated secondary antibody. LPS induced a strong increase in NF-κB protein levels and triggered its translocation to the nucleus with respect to control cells. In agreement with the previous data, E1, E2, and E4 reduce NF-κB protein levels with respect to LPS-treated cells, confirming their anti-inflammatory ability. Of note, E1 and E2 maintained NF-κB protein levels to values comparable to control cells. On the other hand, E3 maintained NF-κB protein levels to a value comparable to LPS-treated cells.

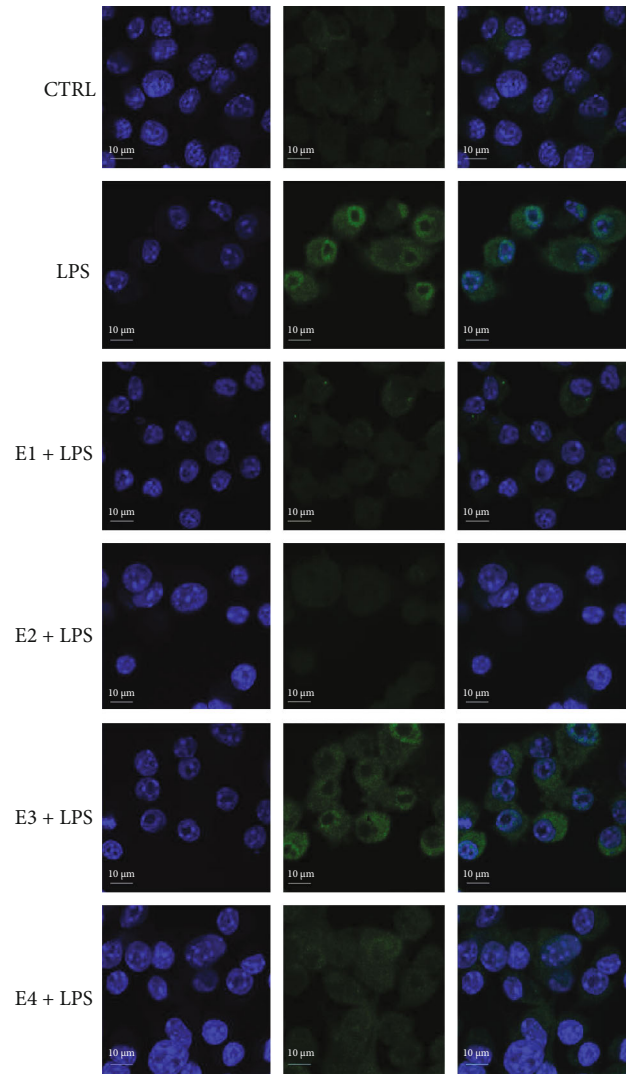

Figure 11: NF-κB nuclear translocation in BV-2 cells treated with the extracts. Cells were treated with 50 μg mL^{-1} of each extracts for 24 h, activated by LPS for 24 h. Cells were immunostained with a primary antibody against NF-κB p65, followed by secondary Alexa Fluor 488-conjugated anti-rabbit IgG antibody (green), and cell nuclei (blue) were visualized with DAPI. Scale bar: 10 μm. Tests were performed in triplicate.

One of the main receptors mediating the activation of microglia and release of proinflammatory mediators is Toll-like receptor 4 (TLR4). LPS is a well-characterized ligand of TLR4 [88]. Dimerization of TLR4 induces the downstream activation of NF-κB signaling, triggering the activation of immune cells such as microglia [89]. On these bases, we further investigated the effects of the extracts on TLR4 cell surface expression by cytofluorimetric analysis (Figure 12).

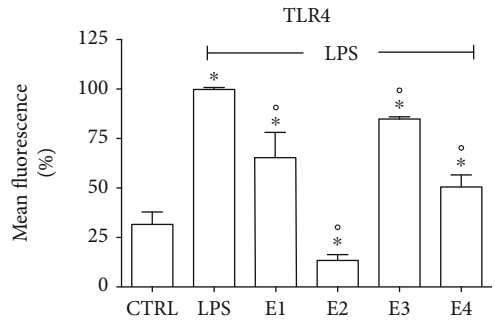

Figure 12: Effect of the extracts on cell surface expression of TLR4 in BV-2 cells. Cells were treated with E1, 50 μg mL^{-1} of each extract for 24 h, exposed to 100 ng mL^{-1} LPS for 24 h, and TLR4 surface expression was evaluated by flow cytometry. Data are expressed as percentage compared to LPS-activated cells. Each bar represents the mean ± SEM of three independent experiments. Data were analyzed with one-way ANOVA followed by Bonferroni's test. $^{\circ}p < 0.05$ vs. CTRL, $^{*}p < 0.05$ vs. LPS.

LPS induced a strong and significant increase of TLR4 surface expression with respect to control cells. According to the previous results, E2 showed the strongest effect in significantly reducing TLR4 surface expression both with respect to LPS-treated cells and control cells. E1, E3, and E4 significantly reduced TLR4 surface expression with respect to LPS-treated cells and, in agreement with the previous results, E3 was the least effective.

Considering the characterization of the extracts in terms of bioactive compound content (Table 2), it is not possible to find any correlation among the anti-inflammatory activity and the presence of specific compounds in the extracts. E2, the most effective extract in counteracting neuroinflammation, does not contain compounds that are not present in the other extracts; moreover, all characterized compounds are present at a lower concentration with respect to the other extracts. Therefore, we can hypothesize that E2 could contain some bioactive compounds that we have not identified. Further researches are needed to better characterize the composition of this extract.

4. Conclusions

The extract analysis evidenced that the different solvents had a profound impact on the composition of the extracts. In particular, the higher content of potential bioactive compounds was found in EtOH : H_2O and MeOH : H_2O extracts. Interestingly, the biological data revealed that the richest extracts in terms of compounds were not the most effective in counteracting oxidative stress and inflammation. The MeOH extract showed the strongest antioxidant activity in neuron-like SH-SY5Y cells, reducing intracellular ROS levels and upregulating endogenous antioxidant enzymes such as NQO1, GR, TRX, and HO1. This effect seems to be related to its higher content of isogentisin with respect to the other extracts. The H_2O extract elicited the highest anti-inflammatory activity, markedly reducing the expression of proinflammatory mediators by the TLR4/NF-κB pathway. Of note, none of the identified compounds in the H_2O extract can explain its higher anti-inflammatory activity with respect to the other extracts. For this reason, further studies should be carried out to better characterize this extract and identify potential bioactive compounds responsible for its anti-inflammatory activity. In conclusion, the antioxidant and anti-inflammatory properties of the extracts suggest that SCGs are a valuable source of nutraceuticals that could be used to prevent/counteract neurodegeneration.

Data Availability

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

Conflicts of Interest

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

Authors' Contributions

Simone Angeloni and Michela Freschi contributed equally to this work.

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Review Article

Targeting the Mitochondrial Permeability Transition Pore to Prevent Age-Associated Cell Damage and Neurodegeneration

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The aging process is associated with significant alterations in mitochondrial function. These changes in mitochondrial function are thought to involve increased production of reactive oxygen species (ROS), which over time contribute to cell death, senescence, tissue degeneration, and impaired tissue repair. The mitochondrial permeability transition pore (mPTP) is likely to play a critical role in these processes, as increased ROS activates mPTP opening, which further increases ROS production. Injury and inflammation are also thought to increase mPTP opening, and chronic, low-grade inflammation is a hallmark of aging. Nicotinamide adenine dinucleotide (NAD+) can suppress the frequency and duration of mPTP opening; however, NAD+ levels are known to decline with age, further stimulating mPTP opening and increasing ROS release. Research on neurodegenerative diseases, particularly on Parkinson's disease (PD) and Alzheimer's disease (AD), has uncovered significant findings regarding mPTP openings and aging. Parkinson's disease is associated with a reduction in mitochondrial complex I activity and increased oxidative damage of DNA, both of which are linked to mPTP opening and subsequent ROS release. Similarly, AD is associated with increased mPTP openings, as evidenced by amyloid-beta (Aβ) interaction with the pore regulator cyclophilin D (CypD). Targeted therapies that can reduce the frequency and duration of mPTP opening may therefore have the potential to prevent age-related declines in cell and tissue function in various systems including the central nervous system.

1. Introduction

The number of older adults is growing worldwide. As a result, the incidence of age-associated diseases including AD, osteoporosis, sarcopenia, and osteoarthritis is also increasing. This increase in age-related disorders has a significant, negative impact on the quality of life for patients and their families and also places a substantial burden on healthcare systems. A better understanding of the cellular and molecular mechanisms underlying aging is central to the successful development and clinical translation of novel therapies and prevention strategies. Recent work has demonstrated that changes in mPTP function may contribute directly to cellular dysfunction with aging [1–3]. These changes include increases in ROS production, induction of cellular senescence (particularly in aging stem cells), and activation of the inflammasome, the latter contributing directly to the chronic state of inflammation often referred to as "inflammaging" [1–3]. mPTP dysfunction has been cited as a key factor in neurodegenerative pathologies through its role in collapsing mitochondrial membrane potential, repressing mitochondrial respiratory function, releasing mitochondrial Ca^{2+} and cytochrome c, and enhancing ROS generation [4–7]. Thus, the mPTP has received increased attention as a potential therapeutic target.

The relationship between the mPTP and the generation of mitochondrial reactive oxygen species (mROS) has attracted significant interest within the context of aging and age-related tissue degeneration [8]. Recently, it was found that mROS can stimulate the opening of the mPTP, which can lead to further mROS production and release [9]. This positive feedback mechanism ultimately leads to an excessive amount of ROS accumulation. ROS accumulation in turn damages nuclear DNA, activates proapoptotic signaling

pathways, and drives cellular aging [10–12]. On the other hand, ROS can in some cases activate protective pathways, decrease stress on the mitochondria, and increase lifespan [1, 11]. It is currently thought that the mPTP plays an important role in integrating the effects of mROS and hence may play a vital role in the aging process [8]. In this review, we discuss the various mechanisms inducing activation of the mPTP and the age-associated cell damage seen as a byproduct of mPTP activation. Furthermore, we discuss potential therapies that target the mPTP and may therefore inhibit the effects of aging and injury.

1.1. Structure and Formation of the mPTP. Various structural components of the mPTP are implicated in permeability transition (PT); however, the overall structure of the mPTP is still not completely understood. It was previously thought that the pore consisted of several components including a voltage-dependent ion channel (VDAC), an adenine nucleotide transporter (ANT), and a peripheral benzodiazepine receptor [13, 14]. These elements are described to perform specific roles: VDAC is associated with the benzodiazepine receptor and regulates the extramitochondrial transfer of cholesterol to the intermembrane space whereas ANT permits the inflow of phosphorylated and nonphosphorylated derivatives of adenine nucleotides [15]. Except for ANT, which is thought to act as a potential regulatory molecule, recent genetic experiments have ruled out the aforementioned elements as components of the mPTP [16]. Thus, we present here the most recent models regarding mPTP composition with the understanding that these may be revised in the near future.

Recent models of pore composition posit that the F_1F_0 (F)-ATP synthase is the main component of the pore and that the regulatory molecule CypD is a protein modulator of the mPTP [17]. In this model, the mPTP originates from a conformational change occurring on the F_1F_0 (F)-ATP synthase after Ca^{2+} binding, possibly by replacing Mg^{2+} at the catalytic site [18]. Whether the dimeric form or the monomeric form of F_1F_0 (F)-ATP synthase is necessary to increase PT is still of great debate [19, 20]. Nevertheless, F_1F_0 (F)-ATP synthase's status as a pore component is supported by genetic manipulation of F_1F_0 (F)-ATP synthase [20, 21], by electrophysiological measurements [20, 22–24], and by mutagenesis of specific residues of F_1F_0 (F)-ATP synthase [18, 25–27]. On the other hand, Walker and colleagues have proposed that the F_1F_0 (F)-ATP synthase is not an essential component of the pore [28, 29]. Their hypothesis is based on the observation that, even after ablating subunits b and OSCP of F_1F_0 (F)-ATP synthase, mitochondrial PT still occurred [29]. Matrix swelling was used to determine PT because long-lasting mPTP opening in vitro is followed by solute diffusion with matrix swelling [30].

Questions have, however, been raised regarding these findings. Bernardi [17] in particular noted the absence of replicates and calibration with pore-forming agents like alamethicin may complicate interpretation of the data. The effects on respiration following F_1F_0 (F)-ATP synthase knockout raise additional questions. Respiratory activity was dramatically decreased to between 10 and 20% of the rate observed in wild-type cells after F_1F_0 (F)-ATP synthase knockout [29]. The driving force in respiring mitochondria for Ca^{2+} accumulation is the inside-negative membrane potential generated by respiration [31, 32]. Furthermore, Ca^{2+} uptake is charge-compensated by increased H+ pumping by the respiratory chain [17]. Thus, it is important to note that the maximal rate of Ca^{2+} uptake is limited by the maximal rate of H+ pumping by the respiratory chain [33]. When extramitochondrial Ca^{2+} levels exceed $2\,\mu$M, the latter becomes rate-limiting [34]. He et al. [28] used $10\,\mu$M pulses of Ca^{2+} to induce PT; therefore, Ca^{2+} uptake by mitochondria lacking subunits c, b, and OSCP should have been significantly lower and not identical to wild-type mitochondria [17]. This raises questions about the Ca^{2+} retention capacity, a measurement used by He et al. [28] to determine mPTP opening. It is possible that respiratory inhibition due to absence of certain subunits may not be constant over time. Potential mechanisms may exist that restore the expression of F_1F_0 (F)-ATP synthase and by consequence the respiratory chain. When considering the above findings, F_1F_0 (F)-ATP synthase cannot necessarily be ruled out as a pore component.

The most compelling experiments supporting F_1F_0 (F)-ATP synthase as a pore component focus on the mutagenesis of specific residues of F-ATP synthase. Specifically, it was found that matrix H+ leads to inhibition of mPTP and complete channel block at pH 6.5 [25, 35]. It was found that the mPTP block is mediated by reversible protonation of matrix-accessible His residues [35]. Recently, H112 of the OSCP subunit has been implicated as the unique His responsible for the PTP block by H+ [25]. Although these findings are intriguing with respect to mPTP activity, they serve a dual purpose in also supporting OSCP and by consequence F_1F_0 (F)-ATP synthase as potential components of the mPTP. Further controversial components include ANT, which may serve a regulatory role by binding CypD and reconstituting into proteoliposomes, producing Ca^{2+}-activated pores similar to the mPTP [36, 37] and the mitochondrial phosphate carrier PiC [38, 39]. Thus, potential constituents of the mPTP include ANT, PiC, and F_1F_0 (F)-ATP synthase (Figure 1). Although its role is controversial, we emphasize the potential role of F_1F_0 (F)-ATP synthase in mitochondrial permeability.

F_1F_0 (F)-ATP synthase's various interactions with molecules such as CypD result in increased mitochondrial permeability. The specific subunits of F_1F_0 (F)-ATP synthase have been studied in relation to their interaction with regulatory molecules such as CypD. It is thought that mammalian F_1F_0 (F)-ATP synthase is a protein complex composed of the following: an F_1 region composed of $(\alpha\beta)3$, γ, δ, and ε subunits, which protrudes in the matrix and synthesizes/hydrolyzes ATP; an F_0 sector, formed from *a* subunit, the c8-ring, two membrane-inserted α-helices of b subunit, and supernumeraries subunits e, f, g, k, A6L, diabetes-associated protein in insulin-sensitive tissue (DAPIT) and 6.8 kDa proteolipid, which allows H+ flow across the IMM; the central stalk complex; and the peripheral stalk subcomplex composed of the following: oligomycin sensitivity conferral protein (OSCP), d, F6, and the extrinsic α-helices of A6L and b subunits (Figure 1) [40].

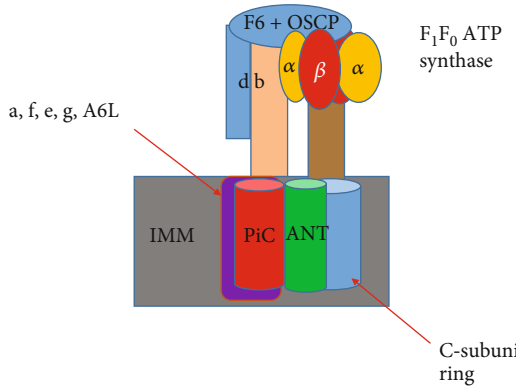

FIGURE 1: Prevailing model concerning the makeup of the mPTP as formed by the following potential components: mammalian F_1F_0 (F)-ATP synthase, Adenine nucleotide translocator (ANT), and mitochondrial phosphate carrier (PiC). The figure is redrawn and adapted based on reference [138]. Although ANT and PiC remain controversial potential components of the mPTP, they are shown as both red and green components overlaying the inner mitochondrial membrane (IMM). Similarly, although F_1F_0 (F)-ATP synthase is a controversial component, it is labeled as follows. Subunits of the F_0 component labeled in purple include a, e, f, g, and A6L. F_1 components include α and β subunits labeled in yellow and red, respectively. The C ring subunit is labeled in blue represented by a cylinder. The F_1 peripheral stalk is composed of the subunits b, d, F6, and oligomycin sensitivity conferring protein (OSCP) labeled represented by a peach rectangle, a blue rectangle, and a blue circle, respectively. The mPTP is the point at which ROS, Ca^{2+}, and other molecules can escape from the matrix of the mitochondria.

OSCP and CypD interact to promote the opening of the mPTP, and further, mPTP opening is increased with aging and oxidative stress [41–43]. Oxidative stress induces the translocation of the tumor suppressor p53 to the mitochondrial matrix where it interacts with CypD to aid in the formation of the mPTP [44]. Like the oxidative stress-induced formation of the mPTP, Ca^{2+} can also induce formation of the mPTP. It has been found that soluble matrix peptidylprolyl isomerase F cyclophilin D (PPIF) is involved in the Ca^{2+}-induced opening of the mPTP [15]. The interaction between the aforementioned molecules, oxidative stress, and Ca^{2+} overloading can change significantly across the lifespan.

1.2. Role of the mPTP in Cellular Aging. A range of studies indicates that mPTP activation is altered with age in a variety of cell and tissue types. These include permeabilized myofibrils in humans [45], myocytes in rats [46], and osteocytes in mice [47]. It should be noted, before discussing the various effects of aging on mPTP activation, that Ca^{2+} is a well-established activator of the mPTP [48]. Specifically, concerning the permeabilized myofibrils in humans, Gouspillou et al. [28] found that Ca^{2+} retention and time to mPTP opening were significantly decreased in skeletal muscle of older active men [45]. Decreased Ca^{2+} retention is indicative of mPTP openings [45]. It was also found that the mPTP of older, active men maintains an increased sensitivity to Ca^{2+}, further supporting the idea that increased mPTP activation is a byproduct of aging. These results are further reinforced by work showing that oxidative damage to Ca^{2+} transporters leads to Ca^{2+} leakage into the cytosol and subsequent mitochondrial matrix Ca^{2+} overloading, which then leads to activation of the mPTP [49, 50].

Activation of the mPTP can also be seen as a product of increased ROS production. Notably, ROS production increases with age [51], and it is thought that ROS production is increased in complexes I and III with the inhibition of electron transport [52]. Oxidative damage to mtDNA and/or electron transport complexes is suggested to result in defective ROS-producing complexes. A cycle is established in which ROS produced by damaged mtDNA and/or electron transport complexes further damages electron transport complexes with age [51] (Figure 2). The increase in ROS production with age is noteworthy because increased mPTP activation is associated with elevated levels of ROS. This is based on the study conducted by Zorov and colleagues, who found that ROS accumulation within the mitochondria of cardiac myocytes leads to increased mitochondrial permeability transition and release of ROS from the mitochondria (ROS-induced ROS release) [53]. Thus, a clear relationship between age, elevated ROS levels, and increased mPTP openings is established. As will be discussed later, ROS released from the mitochondria can damage nuclear DNA and lead to proapoptotic signals which increase mPTP openings [54–56]. Due to the scope of this article, changes in the respiratory chain with aging will not be discussed further.

ROS-induced ROS release is observed during aging and after injury. Inflammation, a process typically associated with injury, induces extracellular acidification [57]. This acidification can in turn lead to increased ROS production within the cell [58]. Increased ROS production in the cell instigates mROS release from the matrix of the mitochondria [9], specifically by means of the mPTP [59]. Thus, inflammation can effectively alter the function of the pore by increasing PT. These effects are not, however, limited to inflammation. Ischemia is also known to decrease extracellular pH [60], in turn launching the same ROS stimulating pathway described above in which the release of mROS further stimulates the production of ROS leading to a positive feedback mechanism in which normal pore function is disrupted [8].

Intracellular pH, like extracellular pH, plays a role in the interaction between inflammation, ischemia, and mPTP activation. Kerr et al. [61] used 2-deoxy-d-[3H]glucose (2-DG) mitochondrial entrapment to show that recovery of Langendorff-perfused rat hearts from ischemia is accompanied by a reversal of the mitochondrial PT [61]. This connection hinges on pyruvate, which is suggested to inhibit the mPTP by decreasing intracellular pH. The beneficial effects of mPTP inhibition included recovery of left ventricular pressure [61]. When considering the results of their study, it is clear that mPTP function is altered in ischemic injury, specifically by means of increased permeability. Yet, it is this same alteration of increased permeability that further stimulates injury, as evidenced by the beneficial effects observed upon mPTP inhibition. The protective effects of mPTP inhibition are further evidenced by Na(+)-H(+) exchanger-1 (NHE-1) inhibition. NHE-1 inhibition in hearts subjected to

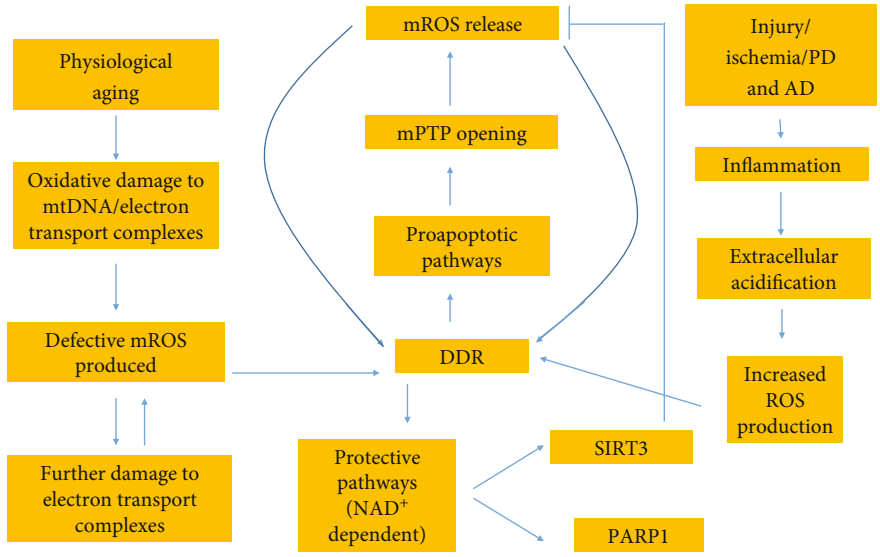

Figure 2: All of the following contribute to ROS production: physiological aging, injury, ischemia, PD, and AD. Injury, ischemia, PD, and AD do so by means of inducing inflammation. Extracellular acidification is a pathological effect of inflammation. A decrease in extracellular pH leads to increased ROS production within the cell which in turn instigates DDR. Aging results in oxidative damage to either mtDNA or electron transport complexes. This instigates defective mROS production. Upon ROS-induced ROS release, ROS can damage nuclear DNA, again inducing DDR. DDR results in proapoptotic pathways that induce mPTP opening and further mROS release. A positive feedback mechanism is initiated in which mPTP openings allow for mROS release which instigates DDR. Simultaneous to the proapoptotic mechanism is the NAD^+-dependent protective pathways. SIRT3 in particular acts as an inhibitor to mROS release. It is important to note that these mechanisms are opposing and upon depletion of NAD^+, the proapoptotic pathways dictate mROS release as the protective pathways are unable to perform their function.

ischemia/reperfusion using the same 2-DG mitochondrial entrapment method described above is associated with attenuation of mPTP opening [62]. The beneficial effects of mPTP attenuation also included recovery of left ventricular pressure [62]. A careful analysis of these studies shows that increased PT is observed with injury, inhibition of the mPTP can lead to a decrease in PT, and decreased PT can improve cardiac function.

ROS production within the cell leads to mPTP opening and subsequent mROS release. It is thought that the outer-membrane anion channel, VDAC, plays a role in allowing the release of ROS from the intramembranous space of the mitochondria [63]. The ROS that are released by VDAC include superoxide and H_2O_2, as they are both small enough (less than 1500 kDa) to pass through the channel [64]. Once released into the cytosol, ROS damages nuclear DNA [11] and triggers the DNA damage response (DDR). DDR induces both proapoptotic signaling in postmitotic pathways [12] and protective pathways [11] (Figure 2). Proapoptotic signals include p53, which targets the mitochondrial matrix, and p66Shc, which targets the intermembrane space. p66Shc induces apoptosis specifically by means of generating H_2O_2. H_2O_2 reacts with cytochrome c and induces oxidation of the mPTP leading to mitochondrial swelling and ultimately mPTP activation [54–56]. Thus, the increase in ROS production seen as a byproduct of aging initiates mPTP opening, but mPTP opening leads to further ROS production (H_2O_2) via proapoptotic signals. This positive feedback mechanism is a means by which continued opening of the mPTP leads to a destruction of the membrane potential, swelling, and rupture of the outer mitochondrial membrane. The mPTP exacerbates the effects of aging as the rupture of the outer mitochondrial membrane leads to the release of ROS, Ca^{2+}, and other metabolites which can, in turn, induce oxidative damage to proteins, transporters, and nuclear DNA ultimately disrupting cellular homeostasis [9, 50].

The frequency of mPTP opening is further increased by Ca^{2+} overloading in the matrix [49, 50, 65] Ca^{2+} concentration within the mitochondria is driven by cytosolic Ca^{2+} levels and mediated by the Ca^{2+} uniporter MCU [66, 67]. It is known that aging disrupts Ca^{2+} homeostasis [68, 69] and interferes with the interaction between ER and mitochondria [70]. The disruption in Ca^{2+} homeostasis is thought to be a byproduct of oxidative damage to Ca^{2+} transporters which increases the leak of Ca^{2+} into the cytosol and subsequently increases Ca^{2+} overload of the mitochondria [71, 72] (Figure 3). Since oxidative damage to Ca^{2+} transporters is a byproduct of increased ROS levels, the continued opening of the mPTP would lead to further damage first initiated by cellular aging. In addition to damaged Ca^{2+} transporters, the direct transfer of calcium from the ER to the mitochondria increases Ca^{2+} overloading within the matrix [73]. To counter calcium overloading resulting from mPTP openings, MICU1, a subunit of MCU, limits calcium accumulation in the matrix as it maintains a threshold for calcium uptake [66, 74]. In aged cells, however, cytosolic free calcium often exceeds the MICU1 threshold for calcium uptake while the calcium threshold controlling mPTP activation is lower than the normal threshold [75]. This would indicate that more Ca^{2+}-induced mPTP openings are to be observed in aged

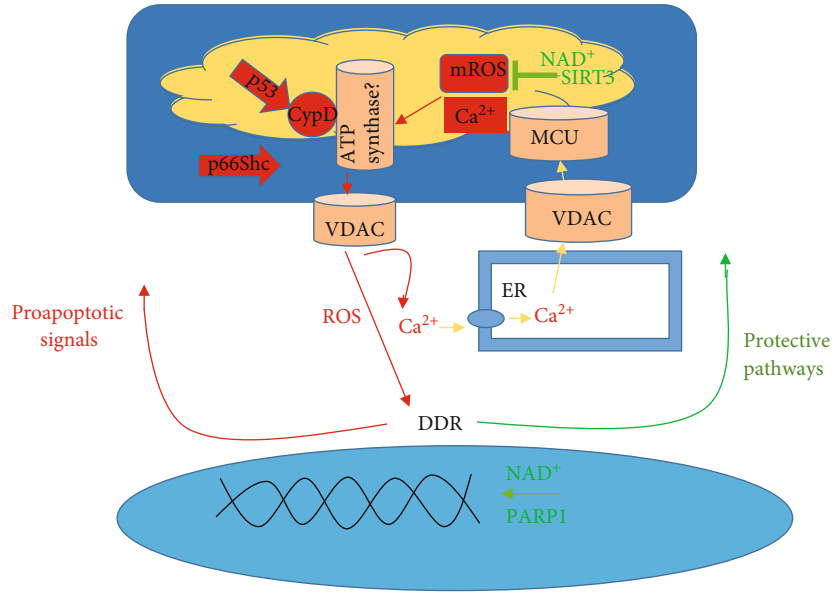

Figure 3: The components of the mPTP are of great controversy. However, despite this, CyPD and the controversial F_1F_0 (F)-ATP synthase are shown as pore constituents. VDAC, while not considered to be part of the mPTP, is thought to be how mROS, Ca^{2+}, etc. are shuttled from the intermembrane space to the cytosol. mROS release through the mPTP leads to DNA and Ca^{2+} transporter damage. DNA damage induces DDR or DNA damage response. DDR subsequently induces both proapoptotic signals and protective pathways. Proapoptotic signals recruit p53 and p66Shc which act upon the mPTP (p53 specifically interacts with CypD, and p66Shc targets the intermembrane space generating ROS) to further induce mPTP openings. Oxidative damage to Ca^{2+} transporters can lead to calcium overloading and subsequent increased mPTP openings. MCU in particular can be affected by oxidative damage, leading to a disruption in mitochondrial Ca^{2+} levels. Protective pathways such as PARP1 aid in DNA repair, and SIRT3 inhibit mROS production. As further oxidative damage to DNA takes place, both protective pathways continue to utilize NAD^+. NAD^+ depletion can result, leading to an inactivation of protective pathways. In turn, the proapoptotic signals are left unchallenged and mPTP openings become more frequent.

cells. Since ROS release can lead to oxidative damage of Ca^{2+} transporters and consequently Ca^{2+} overloading, increased mPTP sensitivity with age can be seen as a byproduct of both Ca^{2+} overloading and ROS release. Furthermore, mPTP opening can be seen as a key driver of the processes (oxidative damage to Ca^{2+} transporters, etc.) first initiated by aging.

1.3. Protective Pathways Involving PARP1 and SIRT3 Can Inhibit mPTP Opening. Although aging can increase ROS production, ROS do not always invoke damaging effects. This is because protective pathways exist to counter oxidative damage by ROS. Thus, due to the dual nature of ROS, which can have both protective and damaging effects, it is necessary to address the interplay between both to examine the overall effects of mPTP opening. Before the interplay can be discussed in regard to aging, it is necessary to examine the protective pathways stimulated by mROS that exist to maintain cellular homeostasis. One of the most important pathways begins with the intranuclear protein PARP1. When DNA is damaged through oxidative stress, PARP1 repairs DNA in a manner that is dependent on NAD^+ [76]. Working in conjunction with PARP1 are the sirtuins, which are similarly classified as NAD-dependent deacetylases [77]. It is thought that SIRT3 plays a particularly important role in inhibiting ROS production and mPTP activation in stem cells [78]. Through metabolic reprogramming, SIRT3 is suggested to effectively increase efficient electron transport away from carbohydrate catabolism, resulting in reduced ROS production [79, 80]. Thus, because ROS production is decreased and mPTP activation is inhibited, the protective pathways above antagonize the mPTP from exacerbating the processes initiated by aging. With this in mind, it is clear that any downregulation of the protective pathways described above through depletion of NAD^+ would lead to the domination of mPTP openings and subsequent positive feedback regarding the release and production of mROS.

As aging proceeds, NAD+ depletion, mPTP openings, and mROS production and release lead to DNA damage. The protective pathways noted above, as well as activation of nuclear factor erythroid 2-related factor 2 (Nrf2), are initiated to counter oxidative damage; however, the continued activation of these pathways leads to a depletion of NAD^+. Since both the PARP1 and sirtuin protective pathways are dependent upon NAD^+, they are no longer able to perform their function as an inhibitor of mPTP opening and subsequent mROS release and production [81, 82]. Ultimately, as aging progresses, oxidative damage to nuclear DNA results in the activation of protective pathways which in turn leads to depletion of NAD^+. Without NAD^+, the protective pathways involving PARP1 and SIRT3 are unable to perform their function as an mPTP opening inhibitor. As a consequence, the mPTP can effectively exacerbate processes initiated by aging.

SIRT3 serves a protective role to inhibit ROS production but it can also act to deacetylate CypD and inhibit mPTP opening [83]. Since SIRT3 is dependent upon NAD^+ levels,

CypD openings are therefore also dependent at least in part on NAD^+ levels. Consequently, as NAD^+ concentration declines as a byproduct of protection pathway activation, SIRT3 is unable to perform inhibition of CypD-induced mPTP opening [81, 83]. SIRT3's simultaneous effects on ROS production and CypD lead to an interplay between the two, which enables further mPTP openings. As discussed earlier, a decrease in SIRT3 activity leads to proapoptotic pathway activation through the ROS-induced DDR [12, 77]. In particular, p53 binds to CypD to form a complex triggering mPTP openings [44]. Thus, ROS production and CypD activation are connected via SIRT3 inhibition.

Pore openings are not limited to CypD's interaction with p53. Recently, a relationship was established between metformin, AMP kinase (AMPK), the peroxisome proliferator-activated receptor-α (PPARα)/mitochondria pathway, and CypD in cardiomyocytes [84]. It is known that activation of AMPK protects the heart from myocardial infarction and heart failure [84]. Thus, because it was found that metformin activates AMPK, metformin can be a potential impetus in driving myocardial protection. Metformin abolished oxidative stress-induced physical interactions between PPARα and cyclophilin D (CypD), and the abolishment of these interactions was associated with inhibition of mPTP formation [84]. Thus, the myocardial protective effects of metformin were found to converge at the mPTP.

1.4. Aging, Decreased Lifespan, and Neurodegenerative Diseases as Byproducts of mPTP Opening. mPTP openings become more frequent and longer in duration as a byproduct of increased ROS production with age and subsequent ROS-induced ROS release [51, 53]. mPTP openings lead to the release of ROS which in turn stimulates proapoptotic pathways leading to further openings [12, 54–56]. Due to the protective pathway dependence on NAD^+, depletion of NAD^+ leads to the inhibition of protective pathways leaving the counter effects of proapoptotic signals to proceed unchallenged [81, 83]. Thus, it is necessary to address the effects of mPTP opening in regard to the overall phenomenon of aging. It has long been held that ROS accumulation leads to oxidative stress and the subsequent observable phenomenon of aging [85]. Recently, ROS involvement in cellular senescence has received significant attention with regard to organismal aging. Cellular senescence is thought to be initiated by genomic damage which activates DDR and subsequent pathways leading to growth arrest [86]. The accumulation of senescent cells in organismal tissue is commensurate with advancing age, and senescent cells reduce stem and progenitor cell numbers leading to impaired capacity for tissue regeneration [87–89]. Considering that ROS play a vital role in cellular senescence and the mPTP plays a vital role in the release of ROS, it is therefore likely that the mPTP contributes to the progression of senescence.

Despite knowledge on the interaction between ROS and the mPTP, little work has been done with regard to the relation between the mPTP and cellular senescence. Hofer and colleagues investigated rat ventricular subsarcolemmal (SSM) and interfibrillar (IFM) mitochondrial susceptibility to Ca^{2+}-induced mPTP openings with aging and calorie restriction [90]. They found that IFM exhibited an increased susceptibility towards mPTP openings during senescence. A decline in Ca^{2+} retention was observed with aging, particularly during senescence [90]. It is important to note that SSM did not exhibit these same results, and mPTP's association with senescence may be dependent on the tissue type. SSM aside, these results would suggest that the mPTP plays a role in the induction of cellular senescence and thus tissue aging, as evidenced by the decline in Ca^{2+} retention. As described above, genomic damage initiates the cellular pathway inducing senescence. Since it is known that ROS release through the mPTP is capable of inducing DDR, it is clear that a relation between mPTP opening, induction of cellular senescence, and cell and tissue aging exists.

Another proposed mechanism by which mPTP opening leads to cellular aging is through increased levels of autophagy. While autophagy is commonly thought to increase longevity due to its ability to clear damaged proteins and dysfunctional organelles, it can be detrimental at very high levels [91]. Elevated autophagy shortened lifespan in *C. elegans* lacking serum/glucocorticoid-regulated kinase-1 (sgk-1) because of increased mitochondrial permeability [91]. Furthermore, mice maintaining sgk-1 displayed lower levels of mitochondrial permeability, normal levels of autophagy, and normal lifespan. Based on these results, sgk-1 is suggested to modulate mPTP opening, which in turn mediates mitochondrial permeability, autophagy, and lifespan [91]. Since mitochondrial permeability is enhanced in the absence of sgk-1, it can be concluded that lifespan reduction as a byproduct of elevated autophagy is likely due to increased mPTP activity.

Research on neurodegenerative diseases, particularly on PD, has uncovered significant findings regarding mPTP openings and aging. PD is characterized by two phenomena including loss of dopaminergic neurons in the *substantia nigra* [92] and accumulation of highly insoluble fibrillar aggregates of the protein alpha-synuclein [93]. Recently, Ludtmann and colleagues [64] investigated the relationship between monomeric and oligomeric α-synuclein encoded by the gene SNCA and their subsequent effects on mPTP openings and cellular death. While α-synuclein in its monomeric form improves ATP synthase efficiency, upon protein aggregation and subsequent formation of the oligomeric form, a toxic gain of function is observed. Specifically, as it relates to the mPTP, the oligomers induce selective oxidation of the ATP synthase beta subunit resulting in an increased probability of mPTP opening. This finding is significant as induced pluripotent stem cell- (iPSC-) derived neurons bearing SNCA triplication generate α-synuclein aggregates that interact with ATP synthase and induce mPTP opening, leading to neuronal death [94].

PD is, however, not fully characterized by neuronal death alone. Loss of the antioxidant protein (protein-disulfide reductase) glutathione (GSH), a reduction in mitochondrial complex I activity, increased oxidative damage of DNA, and elevated free iron levels in the *substania nigra* have all been documented in patients suffering from PD [95, 96]. As mentioned earlier, ROS production increases with age, specifically in complexes I and III with the inhibition of electron

transport [51, 52]. Furthermore, ROS accumulation within the mitochondria can lead to ROS-induced ROS release via the mPTP [53]. ROS released from the mitochondria can damage nuclear DNA and lead to proapoptotic signals which stimulate further mPTP openings [54–56]. Thus, the mPTP links two key processes associated with PD: a reduction in mitochondrial complex I activity leading to increased mitochondrial ROS, which in turn prompts mPTP openings and subsequent ROS release inducing increased DNA damage [51–56]. It is also important to note that neuroinflammation is observed in PD [64], and inflammation leads to extracellular acidification [33] which in turn leads to increased ROS production in the cell driving further mROS release from the matrix of the mitochondria via the mPTP [5, 34, 35]. The etiology of PD is complex and multifactorial involving environmental factors, genetic susceptibility, and aging that together promote disease progression [97]. The findings reviewed above suggest that the mPTP is also likely to have a potential role in the pathogenesis of PD.

Age-related dysfunctions of the mPTP extend to and are prevalent in age-related pathologies mediated by various factors such as inflammation. Inflammation is an early step in the pathogenesis of AD [98], and neuroinflammation is a process that occurs in PD [99]. As was noted earlier, extracellular acidification can increase ROS production, which leads to increased PT via the opening of the mPTP [59]. mPTP dysfunction may also be involved in the progression of AD. In its later stages, AD is characterized by massive amyloid-beta (Aβ) deposition in the parenchyma and the cerebrovascular walls [100, 101]. Recent findings show that mitochondrial damage in AD is linked to Aβ toxicity [102–105]. Some examples include decreased mitochondrial respiratory chain function [105, 106], increased mitochondrial ROS generation [105, 107], and changes in mitochondrial structure [108]. The interaction of Aβ species with certain regulators of the mPTP is likely responsible for the aforementioned damage. Specifically, the interaction of Aβ species with CypD and the upregulation of CypD expression were found to decrease the threshold of mPTP activation [109]. An AD mouse model overexpressing a mutant human form of amyloid precursor protein (mAPP) has also been shown to demonstrate increased CypD levels [109]. Thus, Aβ appears to be an important mediator connecting AD to the mPTP.

CypD is considered a crucial component for mitochondrial permeability transition pore (mPTP) formation [4, 110]. Du et al. found that mitochondrial function and learning/memory were significantly improved in CypD-deficient mice [109, 111]. These results suggest that pore formation is a necessary step in the pathogenesis of AD and that the ablation of CypD in mice gives lifelong protection against Aβ-induced mitochondrial and behavioral dysfunction [111]. Other studies have shown that Aβ oligomers induce a massive entry of Ca(2+) in neurons and promote mitochondrial Ca(2+) overload and mitochondrial PT [112]. This is significant because, as mentioned earlier, Ca^{2+} overload can lead to mPTP activation [49, 50]. Nonsteroidal anti-inflammatory drugs (NSAIDs), including salicylate and sulindac sulfide, were able to inhibit mitochondrial Ca^{2+} overload through mitochondrial depolarization. These studies highlight the role of mPTP dysfunction in neurodegenerative disease.

1.5. Potential Therapies to Mitigate mPTP Opening. Previous work suggests that mPTP opening plays a role in both injury and aging, thus targeted therapies to inhibit continued and frequent opening of the mPTP may serve to promote longevity and healthspan (Table 1). As discussed earlier, PD, AD, and other age-related disorders are thought to be byproducts of mPTP openings. Research targeting the mPTP whether directly or indirectly is divided into two areas. The first area involves therapeutics that require some form of interaction with CypD, and the second area involves therapeutics that require no interaction with CypD [113].

Of the therapies that inhibit CypD, cyclosporin A (CsA) has evoked great interest as it has shown cytoprotective properties in cellular models due to its ability to interfere with the interaction of CypD with the mPTP [114]. Specifically, CsA has been shown to block mitochondrial Ca^{2+} efflux and allow mitochondria to accumulate large amounts of Ca^{2+} [115]. The mechanism which is responsible for increased Ca^{2+} retention is indicative of mPTP closure [45]. This point is supported by Crompton and colleagues, who found that ability of mitochondria to retain Ca^{2+} in the presence of CsA was due to CsA inhibition of the mPTP [116]. CypD in particular was shown to be the target of CsA [117]. mPTP openings were studied in ischemic reperfusion injury in rat hearts to determine the efficacy of CsA with regard to cardioprotection. Cardioprotection was observed in a narrow range, between 0.2 and 0.4 μM, as benefits were lost at concentrations above 0.4 μM [118]. Despite these promising results, a recent clinical trial showed that CsA failed to improve clinical outcomes and prevent adverse left ventricular remodeling in patients with an acute anterior ST-segment elevation myocardial infarction (STEMI) [117]. This raises questions regarding the viability of targeting CypD to promote cardioprotection. It is possible that these seemingly conflicting studies on the cardioprotection offered by CsA could be explained by the means of drug administration. Since cardioprotection was observed in a narrow range in rat hearts, it is possible that the dosage administered in the clinical trial, 2.5 mg/kg body weight, was too low/high of a concentration [117].

Further research supporting CypD as a viable cardioprotective target was conducted by Parodi-Rullman et al. on induced myocardial infarction in rats [119]. They found that CypD inhibition exerts cardioprotective effects in reperfused but not in nonreperfused infarcted hearts of female rats, and the effects are observed only during acute postinfarction injury. CypD remains a viable target for age-related pathologies, although the timing and dosage of drug administration should be refined and optimized to demonstrate clear benefits for the patient. The CypD inhibitor, CsA derivative N-methyl-isoleucine-4-cyclosporin (NIM811), has been investigated as a therapeutic alternative to CsA alone. In a study conducted to determine the efficacy of NIM811 with regard to inhibiting the mPTP, it was found that both mitochondrial permeability transition onset and apoptosis were prevented when NIM811 was added to rat hepatocytes [114]. The

Table 1: Small molecules targeting the mPTP, directly or indirectly, and their potential impact on age-associated diseases.

Classification	Compound	Experimental model	Effect
CypD inhibition independent	Melatonin	Rat stroke model	Decreased neuron loss and reduced infarct volume [124]
CypD inhibition dependent	Cyclosporin A (CsA)	Ischemic reperfusion injury rat heart [118]; anoxia-induced injury in rat heart myocytes [139]	Cardioprotection [118]; reduced proportion of necrosis in rat heart myocytes [139]
	N-Methyl-isoleucine-4-cyclo-sporin (NIM811)	Isolated mitochondria in rat hepatocytes (TNFα-induced permeable transition onset)	Mitochondrial permeability transition onset and apoptosis prevented [114]
CypD inhibition independent	(E)-3-(4-Fluoro-3-hydroxy-phenyl)-N-naphthalen-1-yl-acrylamide (compound 22)	Rabbit model of acute myocardial infarction	Cardioprotective; reduced infarct size; inhibitor of mPTP openings [127]
	Edaravone (Radicut™)	Ischemia/reperfusion injury in rat brain	Neuroprotective; inhibited Ca^{2+}- and H_2O_2-induced swelling in mitochondria; inhibited Ca^{2+} generation of ROS [140]
N-Phenylbenzamides (CypD inhibition independent)	Compound 4, (3-(benzyloxy)-5-chloro-N-(4-(piperidin-1-ylmethyl)phenyl)benzamide)	Ca^{2+} retention capacity (CRC) assay in HeLa cells	Induced a concentration-dependent increase in the CRC of permeabilized HeLa cells (indicative of mPTP inhibition) [129]
Isoxazoles (CypD inhibition independent)	Compound 1, 5-(3-hydroxyphenyl)-N-(3,4,5-trimethoxyphenyl)isoxazole-3-carboxamide	mPTP openings measured by CRC in isolated mouse liver mitochondria	Inhibitory activity against mitochondrial swelling; no interference on the inner mitochondrial membrane potential [130]
Cinnamic anilides (CypD inhibition independent)	GNX-4728	Mouse model of amyotrophic lateral sclerosis (ALS)	Slowed disease progression and significantly improved motor function; displayed a nearly 2-fold extension of lifespan; established mitochondrial calcium retention [128]
Electron scavengers/antioxidants (CypD inhibition independent)	SS-31	15-month-old male mice exposed to isoflurane [141]; antioxidant properties of SS peptides in neuronal N_2A cells treated with t-butylhydroperoxide (tBHP) [131]	Enhances synaptic plasticity and provides protective effects on cognitive function [141]; reduced intracellular ROS and increased cell survival [131]
	XJB-5-131	Cardiolipin oxidation as a byproduct of experimental traumatic brain injury in rats [133]; cardiac function in aged rats [132]; muscle contractility of aged adult rats [142]	Inhibition of cardiolipin oxidation and subsequent improvement in motor skills and cognitive operations [133]; improved postischemic recovery of aged hearts, reduced Ca(2+)-induced swelling in the mitochondria, attenuated the H_2O_2-induced depolarization of the mitochondrial inner membrane as well as the total and mitochondrial ROS levels in cultured cardiomyocytes [132]; higher muscle contractility [142]
	MitoQ	Cardiac ischemia-reperfusion injury in rats	Decreased heart dysfunction, cell death, and mitochondrial damage after ischemia-reperfusion [134]
	EUK-8	Oxidative stress-sensitized harlequin (Hq) mutant mice and their wild-type (WT) counterparts [143]; presymptomatic heart/muscle-specific manganese-superoxide dismutase-(Mn-SOD-) deficient mice [135]	Improved left ventricular end-systolic dimensions and fractional shortening, prevented myocardial oxidant stress, attenuated necrotic and apoptotic cell death, and attenuated cardiac hypertrophy and fibrosis in both Hq and WT [143]; suppressed the progression of cardiac dysfunction and diminished ROS production and oxidative damage [135]

Table 1: Continued.

Classification	Compound	Experimental model	Effect
	MitoTEMPO	Amyloid-beta toxicity in primary cultured mouse neurons	Neuronal lipid oxidation was significantly suppressed; demonstrated protective effects on mitochondrial bioenergetics evidenced by preserved mitochondrial membrane potential and cytochrome c oxidase activity as well as ATP production [136]

potential of NIM811 for reducing mitochondrial permeability and improving cell survival has also been shown in animal models of spinal cord injury [120], traumatic brain injury [121], and hindlimb ischemia-reperfusion injury [122].

CypD-independent therapeutics have received attention (Table 1). Melatonin in particular has been studied as a potential inhibitor to the mPTP that does not require CypD interaction. Melatonin has been shown to inhibit mPTP activation as evidenced by reduced mitochondrial swelling and increased Ca^{2+} capacity [123]. This is further supported by Andrabi et. al who studied the effects of melatonin on mPTP openings in rat brain models [124]. The release of cytochrome c was used to assess pore opening, and rats treated with melatonin displayed a marked decrease in cytochrome c release [124]. These results would support the assertion that melatonin does indeed inhibit mPTP activation. Postmortem analyses of cerebrospinal fluid shows a marked decrease in melatonin concentration with age [125], which could in theory contribute to increased mPTP opening with aging. mPTP openings lead to swelling of the mitochondria, rupture of the outer mitochondrial membrane, and subsequent release of intermembranous proteins [126]. Melatonin supplementation may therefore represent one option to suppress mPTP opening in older adults who are likely to have relatively low endogenous levels of melatonin.

In addition to melatonin, other CypD-independent therapeutics include mitotargeted compounds (Table 1). Mitotargeted therapeutics acting in the absence of CypD interaction include electron scavengers, cinnamic anilides, N-phenylbenzamides, and isoxazoles. One small molecule that directly targets the mPTP is (E)-3-(4-fluoro-3-hydroxy-phenyl)-N-naphthalen-1-yl-acrylamide (compound 22), a cinnamic anilide that inhibits oxidative stress and chemical crosslinker-induced mPTP opening [127]. Other novel CypD-independent therapies exist, classified in the same cinnamic anilide series and performing similar functions as compound 22. One example is GNX-4728 which was administered in a mouse model of amyotrophic lateral sclerosis (ALS). GNX-4728 was found to slow disease progression, improve motor function, and extend lifespan by nearly twofold. Furthermore, Ca^{2+} retention was established, which is again indicative of mPTP closure [128]. Regarding N-phenylbenzamides, the most prominent therapeutic candidate is compound 4, (3-(benzyloxy)-5-chloro-N-(4-(piperidin-1-ylmethyl)phenyl)benzamide). Compound 4 induced a concentration-dependent increase in the calcium retention capacity (CRC) of permeabilized HeLa cells suggesting mPTP inhibition [129]. The isoxazole, compound 1, 5-(3-hydroxyphenyl)-N-(3,4,5-trimethoxyphenyl)isoxazole-3-carboxamide, produced similar results in an isolated mouse liver mitochondria model. Compound 1 was shown to inhibit mitochondrial swelling without interfering with the inner mitochondrial membrane potential [130].

Electron scavengers are mitotargeted therapeutics acting in the absence of CypD interaction. Some of the most studied drug therapies in this category include SS-31, XJB-5-131, MitoQ, EUK-8, and MitoTEMPO. SS-31 can increase cell survival and reduce intracellular ROS in neuronal N2A cells treated with t-butylhydroperoxide (tBHP) [131]. XJB-5-131 can improve postischemic recovery of aged hearts, reduce Ca^{2+}-induced swelling in mitochondria, and reduce total mROS levels in cardiomyocytes [132]. It was also observed that XJB-5-131 improved motor skills and cognitive functions in rats with traumatic brain injury. These results are seen as a byproduct of decreased levels of mROS and subsequent prevention of cardiolipin oxidation [133]. Both MitoQ and EUK-8 employ the same electron-scavenging mechanisms as the therapies above. MitoQ's therapeutic effects were examined in rat cardiac ischemia-reperfusion injury and MitoQ decreased cell death and decreased mitochondrial damage [134]. EUK-8's effects were examined in presymptomatic heart/muscle-specific manganese-superoxide dismutase- (Mn-SOD-) deficient mice. It was observed that EUK-8 suppressed the progression of cardiac dysfunction and diminished ROS production and oxidative damage [135]. Again, while the above therapies do not interact directly with the mPTP, they do reduce ROS levels or production within the mitochondria, which leads to inhibition of mPTP opening.

MitoTEMPO has been investigated as a potential therapeutic in the treatment of AD. A recent study was performed in which Aβ toxicity, a hallmark of AD, was measured in primary cultured mouse neurons. Upon treatment with MitoTEMPO, it was found that Aβ-induced mitochondrial superoxide production and neuronal lipid oxidation were significantly decreased. Furthermore, a protective effect on mitochondrial bioenergetics was observed as evidenced by preserved mitochondrial membrane potential [136]. These results would indicate that MitoTEMPO has the potential to protect neuronal function in AD. While the previous therapies are in the developing stages, one therapy had been approved for treating acute ischemic stroke in Japan. Edaravone is a free radical scavenger that produces neuroprotective effects. The mechanism in which this is accomplished is similar to the other scavengers in that edaravone captures and reduces excessive ROS [137]. Similarly, as with the other

scavengers, the therapy acts on the relationship between ROS and mPTP activation. Thus, as a byproduct of edaravone administration, a decrease in ROS is observed and a decrease in mPTP activation occurs.

2. Summary and Conclusions

Mitochondrial dysfunction is now thought to play a significant role in the tissue degeneration and loss of function that occurs in multiple organ systems with advanced age. A key factor in this process is the generation of reactive oxygen species in mitochondria of aged cells, which is in turn associated with cell death, senescence, and tissue damage. The mitochondrial permeability transition pore appears to play a significant role in ROS production with aging. For example, the continued opening of mPTP and release of mROS lead to DNA damage. Cytoprotective pathways are activated to counter oxidative damage; however, the continued activation of these pathways leads to a depletion of NAD+. Since both the PARP1 and sirtuin protective pathways are dependent on NAD+, they lose their ability to suppress mPTP opening and inhibit mROS release and production. These findings point to mPTP inhibition as a potential therapeutic target for age-related disorders. Mitotargeted compounds and small molecules such as NIM811 have, at least in animal models, demonstrated success in promoting cell survival in settings associated with significant cellular damage such as spinal cord injury, traumatic brain injury, and ischemic stroke. Despite this, the application of mPTP targeted drugs in a medical setting remains elusive. This is evidenced by CsA, which failed to improve clinical outcomes and prevent adverse left ventricular remodeling in patients with an acute myocardial infarction. The electron scavenger edaravone remains one of the only mPTP-targeted drugs approved for clinical use as a neuroprotective agent. Future studies should be directed at exploring more long-term use of these small molecules in older animals to determine their effects on the development and progression of chronic age-associated disorders of the brain, musculoskeletal, and cardiovascular systems.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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