Oxidative Medicine and Cellular Longevity

Interplay of Inflammatory Cytokines and Oxidative Stress in Neurodegenerative Diseases

Lead Guest Editor: Roman Fischer
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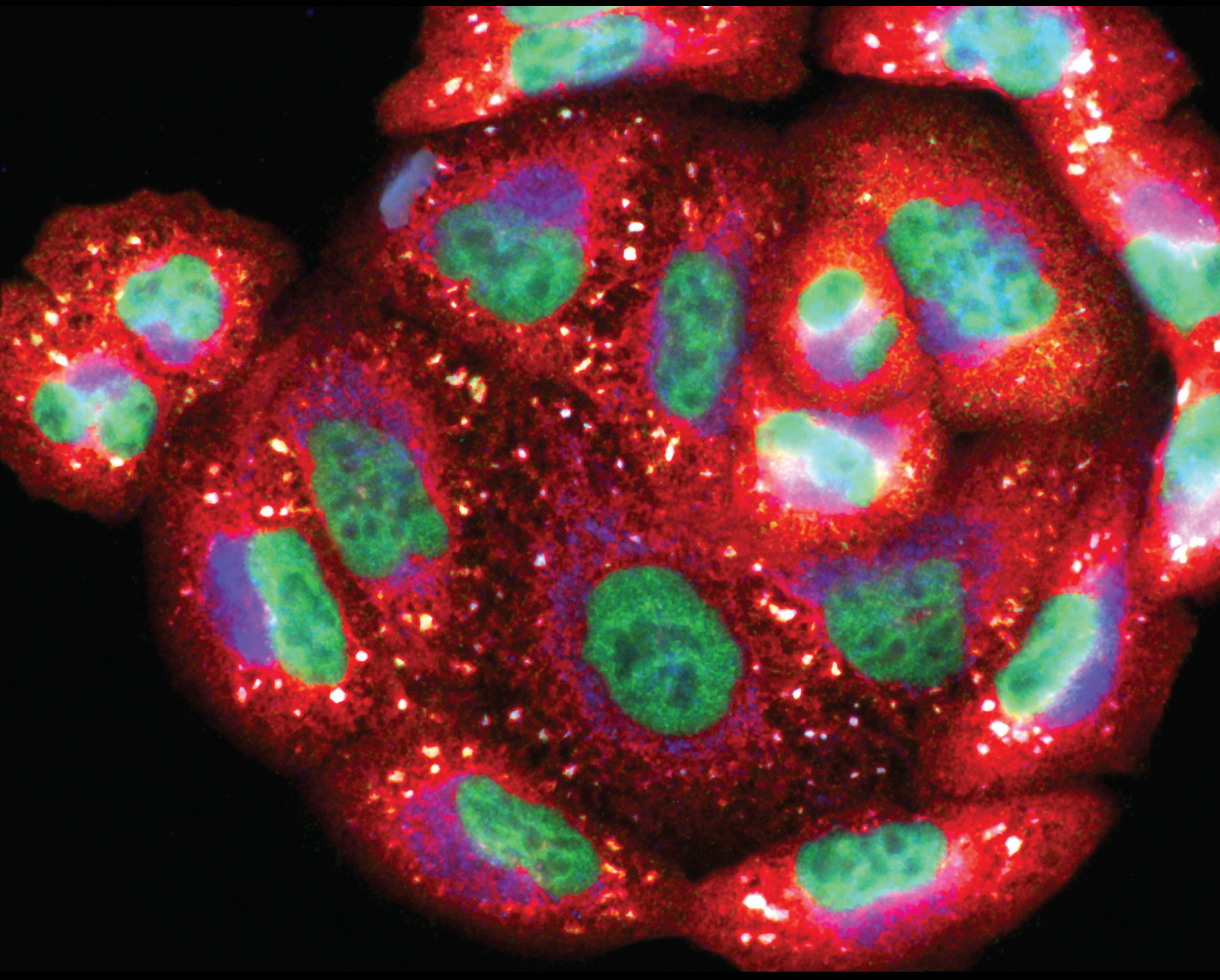

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Review Article

Inflammation and Oxidative Stress in Multiple Sclerosis: Consequences for Therapy Development

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CNS inflammation is a major driver of MS pathology. Differential immune responses, including the adaptive and the innate immune system, are observed at various stages of MS and drive disease development and progression. Next to these immune-mediated mechanisms, other mediators contribute to MS pathology. These include immune-independent cell death of oligodendrocytes and neurons as well as oxidative stress-induced tissue damage. In particular, the complex influence of oxidative stress on inflammation and vice versa makes therapeutic interference complex. All approved MS therapeutics work by modulating the autoimmune response. However, despite substantial developments in the treatment of the relapsing-remitting form of MS, approved therapies for the progressive forms of MS as well as for MS-associated concomitants are limited and much needed. Here, we summarize the contribution of inflammation and oxidative stress to MS pathology and discuss consequences for MS therapy development.

1. Introduction

Multiple sclerosis (MS) is a multifactorial autoimmune disease of the central nervous system (CNS) that is characterized by chronic inflammation, demyelination, and axon and neuronal loss. Depending on the location of the demyelinating lesions, MS patients can develop almost any neurological sign or symptom, including motor, sensory, and cognitive impairment [1]. The most common symptoms are numbness, muscle spasms, ataxia, walking difficulties, bladder or visual problems, fatigue, pain, depression, and MS-related dementia [1]. One of the most frequent nonmotor MS-associated symptoms is chronic neuropathic pain (CNP), a long-lasting chronic pain that affects approx. 60% of MS patients and dramatically reduces their quality of life [2, 3]. As a multifactorial disease, the etiology of MS is complex. However, inflammation is a major driver of the pathology. In addition, oxidative stress contributes to tissue injury and promotes existing inflammatory response. Due to the inflammatory nature of MS, targeting of the immune response is the most widely used therapeutic approach. Acute attacks are treated with corticosteroids; however, due to dose-limiting severe side effects, steroids cannot be used for chronic treatment. Currently, 12 immunomodulatory agents are approved as disease-modifying therapies for MS. Adjuvant drugs, such as antidepressants, are typically used to treat MS-associated CNP [4]. However, all of these therapeutics show either a limited efficiency or severe side effects. Further, they do not target all MS symptoms, and treatment options for sensory impairments are limited and often not very effective [2, 4]. Therefore, novel therapeutics that target both motor and sensory MS disease are an urgent medical need. In this review,

we will summarize the contribution of inflammation and oxidative stress to MS pathology and discuss current therapeutic developments that may improve MS therapy.

2. Multiple Sclerosis

2.1. Etiology and Epidemiology. Worldwide over 2.5 million people are living with MS, a number that is constantly growing [5]. Even though MS can develop at any time in life, most people get diagnosed with MS around age 20 to 40 years. Women are more often affected with MS than men, with a two- to threefold higher prevalence and incidence [1]. Similar sex differences were found for MS-related CNP as well as CNP in general [2]. The incidence of MS is impacted by ethnicity, geographical location, and environmental factors, resulting in a variable epidemiology around the world. The general population has a lifetime risk of 0.2% to develop MS. However, siblings of MS patients have a 10- to 20-fold higher risk of developing the disease [6], indicating that genetic factors play an important role for MS development. The first identified mutations that impact MS susceptibility were specific human leukocyte antigen (HLA) variants within the major histocompatibility complex (MHC) gene complex, outlining the important role of the immune system for MS development. However, like other autoimmune diseases, MS is a complex genetic disorder following a polygenic etiology and a multitude of MS-associated genes outside the MHC locus were identified during large genome-wide association studies [7].

2.2. Clinical Manifestation. The most common form of MS is the relapsing-remitting course (RRMS), which is dominated by peripheral and central inflammation leading to axonal injury and neuronal loss. Due to the accumulation of neurological signs and symptoms, the RRMS form may evolve years later into the secondary progressive MS (SPMS). Up to 15% of MS patients do not experience relapses and develop directly a primary progressive (PPMS) disease after clinical onset [1]. The mean age of onset is approx. 40 years and is similar in SPMS and PPMS patients [8]. Around 60% of MS patients suffer from CNP which is typically associated with significant disability and depression [2, 3]. MS pain syndromes are divided into primary pain caused by inflammation, demyelination, or neurodegeneration and secondary pain due to indirect consequences of the CNS lesion [9]. MS patients can experience a wide range of CNP symptoms. The most common MS-associated CNP conditions include ongoing dysaesthetic pain in the lower extremities, paroxysmal pain, which is divided into L'hermitte's phenomenon and trigeminal neuralgia, as well as thermal and mechanical sensory abnormalities [2, 4, 9].

2.3. Pathology. The central hallmarks of MS pathology are demyelinating plaques within the white and grey matter of the CNS [1]. The location of these lesions within the CNS is quantitatively and qualitatively variable over time and a crucial determinant of the clinical outcome. An inflammatory reaction of autoimmune nature is believed to be the driving force of the demyelinating lesions. Classically, MS is regarded as a T cell-mediated autoimmune disorder [10], and for a long time, it was widely accepted that MS is initiated by an adaptive immune response directed against CNS antigens. Indeed, activated autoreactive T cells infiltrate the CNS, where they upregulate proinflammatory mediators and activate microglia/macrophages, leading to inflammation and demyelination. However, there is now increasing evidence that also B lymphocytes and the innate immune response contribute to the pathogenesis of MS [11, 12]. Other data suggest that oxidative injury and subsequent mitochondrial damage play a pathogenic role for neurodegeneration [13]. Next to the hypothesis that MS is a primary inflammatory disease, in which demyelination and tissue injury are driven by immune-mediated mechanisms throughout all different stages and in all different courses [14], other data indicate that MS is a primary neurodegenerative disease, which is modified and amplified by the inflammatory process [15]. Indeed, oligodendrocyte apoptosis in MS lesions and tissue damage can occur independently of lymphocytes or peripheral macrophages [16], indicating that nonimmune-mediated mechanisms contribute to MS pathology.

Similarly, central inflammation, demyelination, and neurodegeneration lead to the development of MS-associated CNP [2, 17]. Further, data from the rodent experimental autoimmune encephalomyelitis (EAE) model of MS indicate that next to neurodegeneration in the CNS, peripheral nerves undergo major pathologic changes with disease progression [18–20]. Lymphocyte infiltration into peripheral nerves and macrophage activity in the dorsal root ganglion represent a hallmark of peripheral CNP pathology [2], indicating that peripheral inflammation and demyelination may contribute to MS-associated CNP. Indeed, peripheral nerve lesions were observed in MS patients [21]. However, there are no clinical data on association of peripheral neuropathy with occurrence of MS-associated CNP.

3. Inflammation in Multiple Sclerosis

3.1. Role of Adaptive Immune Cells. The inflammatory lesions within the CNS have been reported to contain $CD4^+$ and $CD8^+$ T cells, and the meninges in progressive MS contain ectopic germinal centers that include B cells and other immune populations [22], indicating that the adaptive immune system plays a major role in pathogenesis (Tables 1 and 2). The work of Lassmann's group suggests that two types of inflammation occur in MS patients. In acute and relapsing MS, the blood-brain barrier (BBB) becomes leaky and focal bulk invasion of T and B cells into the white matter leads to the classical active demyelinated plaques [10]. The lymphocyte invasion correlates with cytokine activity in the CNS, with disease activity linked to higher expression of inflammatory cytokines. In contrast, the expression of anti-inflammatory cytokines varies more and clinical studies suggest that the phase of RRMS may determine their expression levels. In early stages of MS, a slow but gradually increasing accumulation of T cells and B cells, in the absence of major BBB damage, is observed in the connective tissue spaces of the brain. This second type of inflammation is associated with the formation of subpial demyelinated lesions in the

Table 1: Overview of the cellular immune contribution to MS pathology.

Cell	Effect	Role
B cells	Proinflammatory	Antibody production, antigen presentation to T cells, cytokine production Participate in the adaptive immune response [25, 43, 45, 46]
$CD4^+$ T cells	Proinflammatory	Recognize and proliferate in response to autoantigens, cytokine production, drive the inflammatory process [22, 26]
$CD8^+$ T cells	Proinflammatory	Recognize and proliferate in response to foreign/self-antigens, target cell cytotoxicity, main T cell type present in MS lesions [24, 26, 32]
CNS dendritic cells	Proinflammatory	Involved in (re)presentation of MS autoantigens to active T cells [61–63]
Macrophage (M1)	Proinflammatory	Activated in response to T cell infiltration, phagocytosis, antigen presentation to T cells, production of proinflammatory cytokines, chemokines, and nitric oxide, increase neuropathy, represent the majority of macrophages in active MS [48, 66]
Macrophage (M2)	Anti-inflammatory	Phagocytosis, antigen presentation to T cells, production of anti-inflammatory cytokines, involved in repair mechanisms, low numbers found deep inside MS lesions [66]
Microglia	Both	CNS surveillance and host defense, activated in MS lesions, production of cytokines, roles in tissue damage and repair, but differential roles to infiltrating macrophages not well understood [48, 52, 56, 58, 67, 71]
T helper (Th17) cells	Proinflammatory	Significant initiator of inflammation in CNS [64]
Tregs	Anti-inflammatory	Suppress autoimmunity, low expression in MS brain tissue [37, 38, 41, 42]

Table 2: Overview of cytokines and other immune proteins that contribute to MS pathology.

Protein	Type	Effect	Role
Activin A	Cytokine	Anti-inflammatory	APC costimulation of T cell responses [71]
B7-1	APC membrane protein	Proinflammatory	APC costimulation of T cell responses [69]
B7-2	APC membrane protein	Proinflammatory	APC costimulation (inhibitory) of T cell responses [69]
CTLA4	Receptor	Anti-inflammatory	T cell produced cytokine, associated with increased pathology [22, 27, 36]
IFNγ	Cytokine	Proinflammatory/inflammation associated	Anti-inflammatory cytokine, produced by macrophages, Th2 cells, and regulatory T cells, promotes expression of immune-modulating Tregs [70]
IL-10	Cytokine	Anti-inflammatory	Produced by T cells, neutrophils, and other immune cells, associated with pathogenesis [22, 27, 36]
IL-17	Cytokine	Proinflammatory	Proinflammatory cytokine produced by activated macrophages/microglia [52, 64]
IL-1β	Cytokine	Proinflammatory	Produced during inflammation, proinflammatory and tissue protective functions, barrier maintenance [29]
IL-22	Cytokine	Proinflammatory	Produced by immune cells including T cells and type M2 microglia and macrophages Pivotal role in shaping immune responses [70]
IL-4	Cytokine	Anti-inflammatory	Produced by proinflammatory macrophages, high levels in CSF associated with greater severity of MS [52]
iNOS	ROS-related enzyme	Proinflammatory	Multifunctional cytokine involved in immune regulation, inflammation, and repair Produced by T cells and type M2 microglia and macrophages, some role in modulation of Th17 cell differentiation [70]
TGF-β	Cytokine	Anti-inflammatory	Multifunctional cytokine with proinflammatory and cytotoxic (soluble TNF) and beneficial (tmTNF) effects in the CNS [52]
TNF	Cytokine	Proinflammatory	APC costimulation of T cell responses [69]

cortex, which are associated with diffuse neurodegeneration in the white or grey matter [10].

Experimental evidence from the EAE rodent model suggests that $CD4^+$ T cells are the major drivers of the inflammatory process [22]. Even though a pathogenic role of $CD4^+$ T cells in MS would be supported by the genetic association of MS with MHC class II haplotypes and associated molecules [23], the inflammatory cells from the adaptive immune system within MS lesions mainly consist of MHC class I restricted $CD8^+$ T cells [24] while MHC class II restricted $CD4^+$ T cells are rare and restricted to locations deep within CNS lesions and the cerebrospinal fluid (CSF) [25, 26]. In particular, $CD4^+$ T cells are described to contribute to the initiation of the immune response in MS patients, but not to play a major role for the effector stage of CNS inflammation and immune-mediated demyelination and neurodegeneration [22]. Indeed, interferon gamma- (IFNγ-) and interleukin-17- (IL-17-) secreting $CD4^+$ T cells are believed to be the pathogenic initiators of MS [22], and in MS patients, the increased production of either IFNγ or IL-17 is associated with pathology [27]. MS patients also show elevated IL-22 concentration in the CNS, and higher concentrations of this cytokine were observed during the remitting stage [28]. Indeed, secretion of IL-22 promotes CNS infiltration of additional lymphocytes, thus amplifying the inflammatory cascade [29]. The pathogenic role of IFNγ in MS is further supported by a clinical MS trial, where IFNγ administration exacerbated disease [30]. Similarly, neutralization of IL-17 in MS patients resulted in reduced lesion formation [31]. These clinical data indicate that T helper cells play a role in the induction of CNS autoimmunity in MS. However, it is not completely understood how IFNγ and IL-17 initiate or augment disease.

In contrast to $CD4^+$ T cells, $CD8^+$ T cells are the major lymphocytes found in active MS lesions and $CD8^+$ T cells have been identified as potential major contributors to MS pathology. MHC class I expression and presentation are necessary for $CD8^+$ T cells to carry out their cytotoxic function. While all cells constitutively express MHC class I, expression was gradually upregulated on astrocytes, oligodendrocytes, neurons, and axons in active MS lesions, making these cells potential targets for $CD8^+$ T cells in the context of the disease [32]. Consistent with this, $CD8^+$ T cells are able to mediate axonal transection after neuronal MHC class I expression *in vitro* [33]. Further mechanisms of $CD8^+$ T cell-mediated neuronal injury may include cytotoxicity by secretion of granzymes, as elevated levels of granzymes were detected in the CSF of relapsing MS patients compared to those in remission [34]. Indeed, histopathological analysis from MS patients revealed that axonal injury correlated with the infiltration of $CD8^+$ T cells into lesions [35]. In addition to direct oligodendrocyte death and neuronal injury, $CD8^+$ T cells can secrete the cytokines IFNγ and IL-17 [36] and may potentiate T helper cell-mediated pathology.

Next to $CD4^+$ and $CD8^+$ T effector cells, regulatory T cells (Tregs) impact MS pathology. Tregs are master regulators of the immune system that can suppress autoimmunity and contribute to tissue regeneration. In contrast to the effector arm of the immune system, Tregs express TCRs that recognize self-antigens and thereby are activated by self-antigens. In mouse models of MS, Tregs suppress CNS autoimmunity [37, 38], and MBP-reactive, disease-ameliorating Tregs have been identified in mice [39]. In MS patients, Tregs showed functional deficits. Whereas no changes in the frequency of Tregs were observed in the peripheral blood of MS patients, the immunomodulatory function of Tregs is impaired in MS patients [38]. Indeed, whereas Tregs exhibit enhanced migratory characteristics compared to non-Treg cells, this feature is impaired in MS patients [40]. This is in line with data that Treg levels are rather low in the brain tissue of MS patients [41]. In contrast, a highly apoptosis-sensitive Treg subpopulation was observed in the CSF of MS patients [41, 42], indicating that immunomodulatory Tregs might be eliminated by cell death within MS lesions.

Next to T cells, cells from the B cell lineage contribute to adaptive immune inflammation in the CNS of MS patients [25]. Clonally expanded B cells are found in the CSF, the meninges, and the brain parenchyma of MS patients [43]. In early disease stages, $CD20^+$ B cells are major components of the lesions, while plasma cells dominate in later stages during lesion maturation and in the progressive disease stage [25]. This is in line with the long-standing observation that immunoglobulin synthesis occurs in the CNS of MS patients [44]. B cells may impact MS through a variety of mechanisms, including the establishment of ectopic lymphoid follicles within the CNS, presentation of antigens to T cells, cytokine/chemokine secretion, and autoantibody production in the CNS [22]. The direct pathogenic role of B cells for MS is supported by data that show that B cells from the CNS of MS patients produce factors that can trigger demyelination and neurodegeneration *in vitro* [45, 46]. In recent years, the essential role of B cells for MS has been validated by successful clinical trials that use anti-CD20 therapy to deplete B cells.

3.2. Role of Macrophages/Microglia. Experimental and clinical investigations have demonstrated that microglia- and monocyte-derived macrophages play important roles in MS and EAE [47]. In particular, their interaction and activation by encephalitogenic T cells are critical for inflammatory demyelination in EAE and possibly MS. When fully activated, they can exacerbate neuroinflammation and neuropathology through the production of cytokines, chemokines, and other inflammatory mediators [48]. However, while monocyte-derived macrophages and CNS-resident microglia are heavily implicated in promoting neuroinflammation and degeneration in diseases such as MS, they also hold so-far understudied immunoregulatory, tissue repair, and neuroprotective properties that represent important therapeutic targets for drugs to treat chronic neurodegeneration.

Microglia are CNS resident immune cells. Unlike bone marrow-derived macrophages, they originate from the embryonic yolk sac and represent a self-perpetuating CNS-specific glial cell population [49]. Under physiological conditions, they are important for clearance of apoptotic cells, synaptic pruning, and the formation of mature neuronal circuits during development and are involved in diverse brain processes such as synaptic plasticity, cognition, learning, and memory

in the adult [50, 51]. As CNS immune cells, they represent a first line of CNS host defense and are essential for brain protection and homeostasis. They rapidly sense damage- or pathogen-associated signals and become activated to release a host of proinflammatory mediators, such as IL-1β, TNF, iNOS, and chemokines, eventually activating and recruiting peripheral immune system cells to infiltrate the CNS. However, microglia immune activity is tightly regulated by inhibitory mechanisms that resolve inflammation to prevent unnecessary tissue damage [52].

In the context of chronic neurodegenerative disease, diverse microglia phenotypes have been detected and their functions are multiplex [53]. On one extreme, they are believed to perpetuate neuroinflammation and disease pathogenesis. Studies in a toxin-induced demyelination model show that microglia are sufficient to drive chronic neuroinflammation in the absence of BBB breakdown and in the absence of significant infiltrating immune cells, a situation similar to that seen in progressive forms of MS [54, 55]. The homeostatic role of microglia in maintenance of neuronal synaptic plasticity is also lost, resulting in synaptic loss in MS and eventually cognitive decline [56, 57]. On the other extreme, there is now compelling evidence that microglia are critical for resistance to EAE onset, a function that involves microglia-specific TNF receptor 2 (TNFR2) [58] as well as tissue repair and recovery, in part through phagocytic clearance of dead cells and debris and in part by production of immunoregulatory mediators [59, 60].

The healthy CNS is also populated by several types of nonmicroglia myeloid cells, including barrier-associated macrophages (BAMs), and CNS dendritic cells (DC) [61]. Both BAMs and CNS DC are mainly located in boundary regions, including perivascular spaces, the meninges, and the choroid plexus [47, 61]. Like microglia, BAMs are long-lived, while CNS DC are bone marrow-derived and short-lived. The precise functions of these nonmicroglia myeloid cells in autoimmune disease are not clear although CNS DC are required for representation of CNS autoantigens to activated T cells, a function critical for the initiation of CNS-directed T cell autoimmune disease [62, 63].

After CNS injury, CNS-resident microglia and macrophages are activated and if additional blood-born monocytes are recruited into the CNS, the BBB is disrupted and neurological symptoms become apparent. In particular, during the effector stage of EAE, monocytes infiltrate the CNS, differentiate into monocyte-derived macrophages, and produce proinflammatory mediators and directly contribute to demyelination. IL-1β, an inflammatory cytokine primarily expressed in activated macrophages, monocytes, and microglia, significantly contributes to MS development. IL-1β promotes differentiation of T cells into Th17 cells via the STAT3 pathway and thereby promotes and aggravates the inflammatory environment in the CNS [64]. Similarly, monocyte-derived macrophages are mainly found in demyelinated lesions of MS patients [65]. In general, activated monocyte-derived macrophages are thought to be harmful in MS. Indeed, the majority of lesional macrophages belong to the proinflammatory M1 phenotype with only a small percentage of M2 polarized cells [66].

Microglia are the first cells that can take up myelin antigens [67] and become APCs that can activate and intensify adaptive immunity. As antigen-presenting cells (APCs), microglia in turn activate T cells during the course of demyelination and remyelination in MS [56]. Indeed, microglia play a key role for the recruitment of adaptive immune cells to the CNS [68]. After activation, microglia express class I and II MHCs and can activate adaptive immune cells through antigen presentation. In addition, they express costimulatory molecules, such as B7-1 and B7-2, which can interact with CD28 on T cells to stimulate proliferation, differentiation, and cytokine secretion and CTLA4 to promote T cell anergy or apoptosis [69]. Next to their interaction with adaptive immune cells, activated microglia can secrete cytotoxic cytokines and oxidative products, such as ROS and NO radicals in MS lesions thereby promoting oxidative stress and contributing to myelin destruction [56].

Until recently, studies concerning the deleterious disease-inducing effects of chronically activated microglia/macrophages in the CNS have overshadowed the understanding of their powerful endogenous repair potential. Macrophages and microglia show a high plasticity and have been arbitrarily classified into "M1" (proinflammatory) and "M2" (prorepair, anti-inflammatory) phenotypes depending on their activation state, although it is now widely accepted that this classification is hugely oversimplified, particularly for microglia, and only partially reflects the real situation. According to the M1/M2 model, M1 polarized cells are characterized by the release of proinflammatory mediators, such as TNF, IL-1β, and IFNγ. In addition, they are potent APCs and can activate adaptive immunity. In contrast, M2 polarized cells express a variety of anti-inflammatory mediators, such as IL-4, IL-10, and transforming growth factor-β (TGF-β), and contribute to immunoregulation [70]. Other data have shown that M2 microglia promote oligodendrocyte differentiation and that microglia depletion impairs remyelination [71]. Multiple intermediate and different microglia/macrophage phenotypes exist that await functional classification.

Indeed, studies aimed at differentiating the effects of microglia and macrophages in the pathogenesis of EAE in mice revealed a significant neuroprotective effect of microglia, via TNFR2, at the onset of disease [58]. Also, administration of a CNS-penetrating inhibitor of soluble TNF in a toxin-induced mouse demyelination model promoted macrophage/microglia phagocytosis of myelin debris and remyelination [60, 72]. Myelin debris is known to be a potent inhibitor of oligodendrocyte precursor cell (OPC) differentiation into myelin-forming oligodendrocytes [73]. In addition, anti-inflammatory mediators secreted from M2 polarized microglia promote remyelination in EAE, for example IL-4, which enhances oligodendrogenesis [74] and suppresses Th1 macrophage reaction, including release of macrophage inflammatory protein (MIP) and activin A, which promotes oligodendrocyte differentiation [71]. Recently, in a mouse model of Alzheimer's disease, a novel disease-associated phagocytic microglial cell phenotype, termed disease-associated microglia (DAM), was associated with restricting neurodegeneration [75].

It is clear that better understanding of the cellular and molecular mechanisms that control the polarization of microglia between proinflammatory to prorepair phenotypes will be critical for the design of drugs that will promote the beneficial functions of these cells and hopefully reverse the inflammatory demyelinating process in MS and provide neuroprotection and CNS repair in other neurodegenerative diseases.

4. Oxidative Stress and Mitochondrial Dysfunction in Multiple Sclerosis

4.1. Redox Homeostasis and Oxidative Damage. Under physiological conditions, mitochondrial oxidative metabolism produces energy as the end-product of the mitochondrial electron transport chain. Moreover, mitochondria incorporate components of the respiratory transport chain and a set of enzymes, which are the major producers of free radicals within the cell. Free radicals are chemical species with an unpaired electron in their outer orbital which is able to induce reactivity. When oxygen receives an electron, superoxide anion radical ($^{\cdot}$O2-) is formed and the further addition of other molecules generates secondary reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}$OH). Cellular ROS are also generated in response to endogenous and exogenous stimuli such as cytokines, pathogens, radiations, and xenobiotics [76]. Similarly, to ROS, nitric oxide ($^{\cdot}$NO) is a free radical with an unpaired electron belonging to the reactive nitrogen species (RNS) family.

At moderate concentrations, nitric oxide, superoxide anion, and other ROS play an important role as regulatory mediators in signaling processes. For instance, free radicals and their derivatives are able to regulate vascular tone, sense oxygen tension, enhance the signal transduction from various membrane receptors including the antigen receptor of lymphocytes, and modulate oxidative stress responses in order to maintain redox homeostasis [77]. When cells are challenged by metabolic and temporary environmental stressors, they prevent oxidative damage and maintain redox homeostasis through endogenous feedback mechanisms aimed at continuously balancing electrophiles and nucleophiles [78]. An example of redox signaling is the self-inhibition of neuronal NO synthases which converts to a catalytically inactive ferrous-nitrosyl complex upon NO stimulation [79]. When the feedback loop is disturbed either by a permanent harmful challenge or an inappropriate defense response or inefficient nucleophilic feedback, physiological redox steady state is breached and oxidative damage occurs. In order to avoid this, a complex system of antioxidants is able to effectively support the maintenance of the redox homeostasis. Antioxidants are substances that are able to delay or inhibit oxidation of a substrate at low concentrations. These include both enzymatic and nonenzymatic compounds. Together with cofactors such as copper, zinc, manganese, and iron, enzymatic antioxidants convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water. Increased levels of enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSHPx) increase the number of endogenous antioxidants. The nonenzymatic antioxidants such as vitamins C and E, plant polyphenol, and carotenoids interrupt free radical chain reactions [80]. An important regulator of the antioxidant defense is the nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a transcription factor that binds to a DNA sequence called antioxidant response element (ARE). When drug-metabolizing enzymes (e.g., cytochrome P450) are activated, Nrf2 detoxifies and eliminates dangerous metabolites by regulating the response against high electrophiles and oxidants [81]. Another important function of Nrf2 is the inhibition of inflammation through inhibition of the NF-κB pathway thereby decreasing cytokine production and oxidative responses [82].

When the antioxidant system is overwhelmed, high levels of free radicals can damage essentially all macromolecules in the cells. ROS may oxidize polyunsaturated fatty acids in lipids by sequestering an electron to increase their stability: an event called lipid peroxidation. A chain reaction is triggered in which a lipid takes an electron from its neighbouring lipid thus leading to the loss of membrane fluidity and elasticity, impaired cellular functioning, and even cell rupture [83]. Moreover, ROS may have dramatic genotoxic actions, which causes the alteration of DNA bases directly contributing to carcinogenesis [84]. It has been estimated that metabolism-generated ROS can induce approximately 10,000 lesions per day in the genome of a human nonneuronal cell [85]. Further, ROS can damage proteins. Even though all amino acids can be targeted by ROS, tryptophan, tyrosine, histidine, and cysteine are particularly sensitive to denaturation [86]. Protein oxidation generates fragmentation at amino acid residues, formation of protein-protein cross-linkages, and oxidation of the protein backbone, which ultimately leads to loss of function. Intracellular pathways are affected by damaged proteins which then contribute to the etiology of different diseases. If protein degradation does not function properly due to altered proteolytic mechanisms, affected proteins accumulate in the cell, developing pathological conditions [87].

4.2. Mitochondrial Oxidative Damage and Cell Death. ROS can promote tissue damage by directly activating the apoptosis via the intrinsic mitochondrial pathway, which promotes outer membrane permeabilization and translocation of cytochrome c, apoptosis-inducing factor (AIF), or second mitochondria-derived activator of caspases (Smac/-Diablo) from mitochondria to the cytosol. These factors trigger cytosolic apoptotic signaling events or induction of nuclear chromatin condensation and DNA fragmentation by translocation of AIF from the cytosol to the nucleus [88, 89]. To favour this mitochondrial permeabilization and the release of apoptotic signals, the permeability transition pore (PTP) is essential. This is a huge pore spanning the inner and outer mitochondrial membrane, and it is composed mainly of three proteins: the voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophilin D (CypD) [90, 91]. The mitochondrial permeability transition pore (mtPTP) is a voltage- and calcium-dependent channel that allows the entry of solutes up to

<1.5 kD through the generally impermeable IMM. Alteration in membrane permeability causes depolarization of the transmembrane potential, release of small solutes and proteins, mitochondrial swelling, and loss of oxidative phosphorylation [92]. Evidence shows that there might be both direct and indirect effects of ROS on mtPTP formation. Changes in the membrane conformation can occur due to oxidation of the thiol groups of the IMM that induces disulphide bond and protein aggregation [93]. Moreover, VDAC was shown to regulate the mtPTP and mediate ROS-induced apoptosis. As a matter of fact, VDAC exposes amino acids to the intermembrane space or to the cytosol which are therefore easily accessible for oxidation [94]. Likewise, ANT might also be directly targeted by ROS and this has an effect mainly on its binding to CypD [95]. Nonetheless, recent findings suggested that ROS might affect indirectly the mtPTP. Isolated mitochondria from $CypD^{-/-}$ mice were protected from permeabilization in the presence of H_2O_2 or mitochondrial Ca^{2+} overload. In these knockout mice, the mitochondrial membrane potential (MMP) is still generated by TNF, suggesting that CypD involvement is specific to the apoptotic inducer [96].

Although the mechanism and targets of action are still unknown, another important inducer of PTP opening is mitochondrial Ca^{2+} overload. When large quantities of Ca^{2+} accumulate in the mitochondrial matrix, Ca^{2+} interacts with CypD [97]. This interaction could induce the opening of the mtPTP which in turn causes ROS and free fatty acid formation thereby exacerbating the mtPTP opening. Loss of membrane permeabilization causes dissipation of MMP, and if the Ca^{2+} overload persists, the mtPTP will stay open allowing accumulation of solutes in the mitochondrial matrix. Eventually, the outer mitochondrial membrane will rupture releasing the intermembrane space content and proapoptotic signals will leak into the cytoplasm causing death of the cell [98].

It seems that both ROS and Ca^{2+} have key roles in determining oxidative stress-induced mitochondrial dysfunction and cell death. In addition to apoptosis, increased ROS levels lead to other cellular fates including senescence [99], necroptosis [100], and autophagy [101].

4.3. Oxidative Damage in Multiple Sclerosis. As previously mentioned in this review, oxidative stress is heavily involved in several MS pathological hallmarks such as myelin destruction, axonal degeneration, and inflammation [102]. In an EAE model, CNS regions characterized by perivascular inflammatory infiltrates show higher mitochondrial dysfunction, fragmentation, and impaired trafficking than other CNS regions [103]. Likewise, active MS lesions show profound mitochondrial protein alterations and DNA deletions in neurons [104]. In these lesions, oligodendrocytes show high levels of oxidized DNA while oxidized phospholipids are preferentially accumulating in axons with disturbed transport. Moreover, the severity of oxidative damage seems to correlate with the extent of inflammation [105]. Furthermore, *in vivo* imaging of EAE-induced axonal damage showed that macrophage-derived ROS can trigger mitochondrial dysfunction and focal axonal degeneration also in axons with intact myelin [106]. This holds true for human multiple sclerosis CNS autopsies where mitochondrial damage is restricted to the lesion area even in the absence of demyelination [106]. In this line, another study shows that accumulation of amyloid precursor protein (APP), a marker for acute axonal damage, occurs not only in active demyelinating but also in remyelinating and inactive demyelinated lesions with a large interindividual variability. APP expression in damaged axons correlates with the numbers of infiltrating leukocytes at the lesion site [107].

Conversely, other studies show extensive oxidative damage to proteins, lipids, and nucleotides in active demyelinated MS regions, specifically in reactive astrocytes and myelin-loaded macrophages [108]. In the same lesions, scavenging activity is also enhanced due to the increased activity of antioxidant enzymes such as SOD1, SOD2, CAT, and heme oxygenase 1 [108] and upregulation of the transcription factor Nrf2 in infiltrating macrophages [109]. In addition, fluorescence life imaging to detect functional NADPH oxidase in an EAE model showed that inflammatory monocytes, activated microglia, and astrocytes are the major sources of oxidative damage within the CNS [110]. Hence, there are discrepancies in literature regarding the cellular localization of oxidative damage within MS and EAE lesions. The reasons for such differences are not clear but they may generate from the high cellular heterogeneity at the lesions' site [105].

Under physiological conditions, neurons, astrocytes, and oligodendrocytes display molecules that bind to microglial receptors, inhibiting their activation state [111]. Decreased expression of these molecules (e.g., myelin CD47) leads to increased microglial activation, which may trigger myelin debris phagocytosis and delivery of neurotrophic factors [111, 112]. Sustained injury, systemic inflammation, proinflammatory cytokine release, and ROS signaling turn microglial physiological functions into toxic inflammatory insults [113]. Taken together, these findings suggest that activated microglia and macrophages are orchestrating tissue injury through their oxidative burst during the development and progression of EAE and MS lesions. Even though a complex antioxidant response is simultaneously triggered, this is insufficient to revert degeneration and apoptotic processes.

The CNS is highly vulnerable to oxidative stress due to several factors such as great energy demand and mitochondrial activity, restricted cell renewal, and large quantity of iron and poly unsaturated fatty acids. Hence, these features increase CNS susceptibility for typical neurodegenerative hallmarks linked with oxidative stress such as impaired mitochondrial function, increased oxidative damage, defect in ubiquitin-proteasome system, changes in iron metabolism, presence of abnormal, aggregated proteins, inflammation, and excitotoxicity [114]. Nevertheless, oxidative damage is not only regulating MS disease within the CNS but it also shapes the immune response developing in the periphery. Firstly, high ROS levels damage the brain endothelium by decreasing its electrical resistance thereby affecting its permeability [115]. In MS patients, nitric oxide metabolites are found upregulated in CSF samples and correlated with relapses suggesting a deleterious role of nitric oxide in inflammatory BBB dysfunction [116]. Furthermore, it has

been suggested that interaction of monocytes with the brain endothelium produces ROS facilitating the following intrusion of leukocytes within the CNS [117]. Infiltrating leukocytes are also producing massive amounts of ROS, which induces myelin phagocytosis by activated microglia and macrophages [118], as mentioned above.

The immune system has developed resistance mechanisms and is less sensitive to high ROS levels. Generating H_2O_2 and hypochlorous acid enables neutrophils and phagocytes to kill bacteria [119]. ROS signaling is also essential in target cell killing by neutrophils and cytotoxic T cells [120]. Further, T cell receptor activation induces intracellular ROS production [121]. Undoubtedly, ROS signaling is a major contributor in the organism's defense system, but if homeostasis is breached, a vicious circle that comprises inflammation and degeneration will initiate. Similar to MS [122], excessive or sustained ROS levels are involved in the pathogenesis of other neurodegenerative disease [123, 124]. Moreover, the long-standing free radical theory of ageing proposes that ROS are also heavily involved in this natural process and in age-associated diseases [125]. Therefore, therapeutic treatments for MS and other diseases should be aimed at restoring general homeostasis, including redox balance, in order to prevent physiological ROS signaling from being revert.

5. Targeting Inflammation and Oxidative Stress to Treat Multiple Sclerosis

5.1. Approved MS Therapies. The clinical management of MS addresses three major challenges: (1) prevention of relapses and progressive worsening of disease, (2) handling acute relapses and MS-related symptoms efficiently, and (3) treatment of drug's adverse side effects. Corticosteroids have been used in clinical practice for more than 70 years as immune suppressants. A high-dose intravenous injection of methylprednisolone is the current treatment for acute MS exacerbations. Methylprednisolone immediately decreases $CD4^+$ lymphocytes and results in a short-term decrease of IFNγ production and chemokine expression levels [126]. This rapid effect has also been linked to transient tightening of the BBB during and shortly after corticosteroid treatment [127]. Even though the resolution of the acute relapse is fast, long-lasting effects of steroid treatment have not been detected.

However, most of the MS preclinical and clinical studies are mainly focused on the prevention of exacerbations and disease progression. Currently, 12 disease-modifying therapies are approved by the US Food and Drug Administration (FDA) to treat MS (Table 3). Three are injectable medications: interferon beta-1a, interferon beta-1b, and glatiramer acetate; 5 are oral small molecule medications: teriflunomide, fingolimod, dimethyl fumarate, cladribine, and siponimod; 4 are administered via infusion: alemtuzumab, mitoxantrone, ocrelizumab, and natalizumab. In 1993, interferon beta-1b was the first drug to ever be approved for MS, soon to be followed by interferon beta-1a and glatiramer acetate [128]. Since then, interferon beta and glatiramer acetate are typically used as first-line treatment after MS diagnosis (Figure 1). Interferon beta-1a and interferon beta-1b are cytokine derivatives that reduce T cell infiltration into the CNS resulting in alleviated central inflammation [129]. Glatiramer acetate is a random-sized peptide mixture consisting of glutamic acid, lysine, alanine, and tyrosine, 4 amino acids that are enriched in myelin basic protein, a central component of the myelin sheaths [128]. Treatment with glatiramer acetate results in a shift from proinflammatory Th1 cells to anti-inflammatory Th2 cells [130] and an expansion of regulatory T cells [131]. Two disease-modifying drugs are used as second-line treatment in relapsing-remitting MS, natalizumab and fingolimod. Natalizumab is a humanized monoclonal antibody (mAb) against the cell adhesion molecule α4-integrin that blocks trafficking of immune cells over the blood-brain barrier into the CNS parenchyma (Figure 1). Fingolimod is a sphingosine-1-phosphate receptor modulator, which sequesters lymphocytes in lymph nodes, preventing them from contributing to an autoimmune reaction, and shifts macrophages into an anti-inflammatory phenotype [129]. Mitoxantrone, teriflunomide, and cladribine are small molecules that inhibit rapidly dividing cells and therefore suppress the replication of T cells and B cells in MS patients [129]. Dimethyl fumarate (DMF) is a small molecule that shifts various immune cell subsets towards an anti-inflammatory state and promotes neuronal survival [132]. Alemtuzumab is a humanized monoclonal antibody (mAb) directed against CD52, a glycoprotein present on the surface of mature lymphocytes, which leads to a rapid, but long-lasting depletion of mature T and B cells [133]. Recently, ocrelizumab, a humanized anti-CD20 mAb, was the first FDA-approved drug for the primary progressive form of MS. Ocrelizumab targets B lymphocytes and kills the cells via antibody-dependent cell-mediated cytotoxicity (ADCC) and, to a lesser extent, complement-dependent cytotoxicity (CDC) [134]. In 2019, the FDA approved siponimod, a sphingosine-1-phosphate receptor modulator and follow-up product of fingolimod, for use in RRMS and SPMS [135]. Indeed, the past 25 years have witnessed substantial developments in the treatment of RRMS. However, approved therapies for the progressive forms of MS, especially PPMS, are limited and much needed.

In general, medications for CNP are limited and often not very effective. Although conventional pain medications can lead to some pain relief, no current therapy provides more than 50% pain relief in the clinic and large randomized and controlled clinical trials for MS-associated chronic neuropathic pain are lacking [4]. Temporary pain relief can be achieved through antidepressants and anticonvulsants. However, these therapies have long-term complications and only a short-term efficacy that leaves patients with untreated and constant pain [2]. As described earlier, TCAs and SSRIs are typically used as first-line drug therapy for MS-associated CNP whereas second-line treatments include opioid analgesics and tramadol [2, 4]. Summarizing, the number of medications to treat MS-associated CNP is limited and their use is often associated with severe adverse events.

5.2. Current Developments

5.2.1. Failed Clinical Trials. Despite encouraging results in preclinical disease models, several compounds that modulate

Table 3: List of FDA-approved disease-modifying therapies to treat multiple sclerosis, adapted from [128, 129, 188, 189].

Drug	Route of administration	Drug class	Mechanism of action	Treatment strategy	Main possible side effects when compared to placebo	Approved indication
Interferon beta-1a	Injection	Protein biologic	Immunomodulatory	First line	Injection site reaction, influenza-like symptoms, lymphopenia, depression	RRMS
Interferon beta-1b	Injection	Protein biologic	Immunomodulatory	First line	Injection site reaction, influenza-like symptoms, lymphopenia, depression	RRMS
Glatiramer acetate	Injection	Peptide polymer	Immunomodulatory	First line	Injection site reactions, vasodilatation, rash, dyspnea, chest pain	RRMS
Teriflunomide	Oral	Small molecule	Immune suppressive	First line	Hepatotoxicity, alopecia, diarrhea, influenza, nausea, and paresthesia	RRMS
Fingolimod	Oral	Small molecule	Immunomodulatory	First line	Headache, liver transaminase elevation, diarrhea, cough, influenza, sinusitis, pain	RRMS
Dimethyl fumarate	Oral	Small molecule	Immunomodulatory	First line	Flushing, abdominal pain, diarrhea, nausea	RRMS
Cladribine	Oral	Small molecule	Immune suppressive	First or second line	Upper respiratory tract infection, headache, lymphopenia	RRMS, SPMS
Siponimod	Oral	Small molecule	Immunomodulatory	First line	Headache, hypertension, transaminase increases	RRMS, SPMS
Alemtuzumab	Infusion	Humanized mAb	Immune suppressive	Second or third line	Infusion reactions, infections, rash, headache, pyrexia	RRMS
Mitoxantrone	Infusion	Small molecule	Immune suppressive	Second or third line	Nausea, alopecia, urinary tract infection, cardiotoxicity, menstrual disorders	RRMS, SPMS
Ocrelizumab	Infusion	Humanized mAb	Immune suppressive	First or second line	Infusion reactions, skin and respiratory tract infections	RRMM, PPMS
Natalizumab	Infusion	Humanized mAb	Inhibits immune cell trafficking into CNS	Second line	Delayed infusion reactions, progressive multifocal leukoencephalopathy (PML), hypersensitivity, immunosuppression/ infections, headache, fatigue	RRMS

the immune system failed in clinical MS trials. A prominent example is targeting of the master proinflammatory cytokine tumor necrosis factor receptor (TNF) with nonselective inhibitors that inhibit both proinflammatory and beneficial functions of this cytokine. Such anti-TNF drugs are blockbuster drugs for use in several autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis [136, 137]. However, the approved anti-TNF therapeutic infliximab and the TNF inhibitor Lenercept failed in clinical trials with MS patients [138, 139], demonstrating that nonselective targeting of TNF is contraindicative in MS.

Despite the clinical success of ocrelizumab, atacicept, a recombinant fusion protein that neutralizes the B-lymphocyte stimulator (BLysB) and A-proliferation-inducing-ligand (April) and inhibits maturation, function, and survival of B cells [140], failed in a randomized, placebo-controlled, double-blind, phase 2 trial. This study had to be terminated early, since atacicept increased relapse rates in MS patients [141], suggesting that the role of B cells and humoral immunity in multiple sclerosis is more complex than currently appreciated.

5.2.2. Antioxidant Therapy. The development of neurodegeneration in MS is a complex process with a multitude of contributing mechanisms, including but not limited to inflammation, primary apoptosis, synaptopathy, mitochondriopathy, and oxidative stress. As described earlier, inflammation and oxidative stress are tightly linked and impact each other. Therefore, next to anti-inflammatory and immunomodulatory treatments, neutralizing free radicals might be a promising therapeutic approach (Table 4). Indeed, DMF was shown to activate antioxidative pathways and to increase expression of the transcription factor Nrf2 [142]. In human oligodendrocytes, DMF stabilizes the cell metabolism resulting in protection from oxidant challenge, providing a mechanism by which DMF may preserve myelin integrity [143] (Figure 1). Even though the mechanism of action of DMF in MS treatment is not well understood, it has been confirmed as a safe antioxidant treatment for MS. In this line, many antioxidant dietary compounds can exert similar functions and boost the beneficial effects of DMFs if used as complementary therapies [144].

In general, antioxidants protect the body against free radicals and are divided into enzymatic and nonenzymatic substances. Enzymes include catalase GPx, GR, and SOD. Nonenzymatic antioxidants may be classified into low molecular weight (e.g., melatonin, vitamins, glutathione, and coenzyme Q) and antioxidant elements (ions) [122]. Melatonin is a neurohormone and important antioxidant that also activates antioxidant enzymes such as SOD,

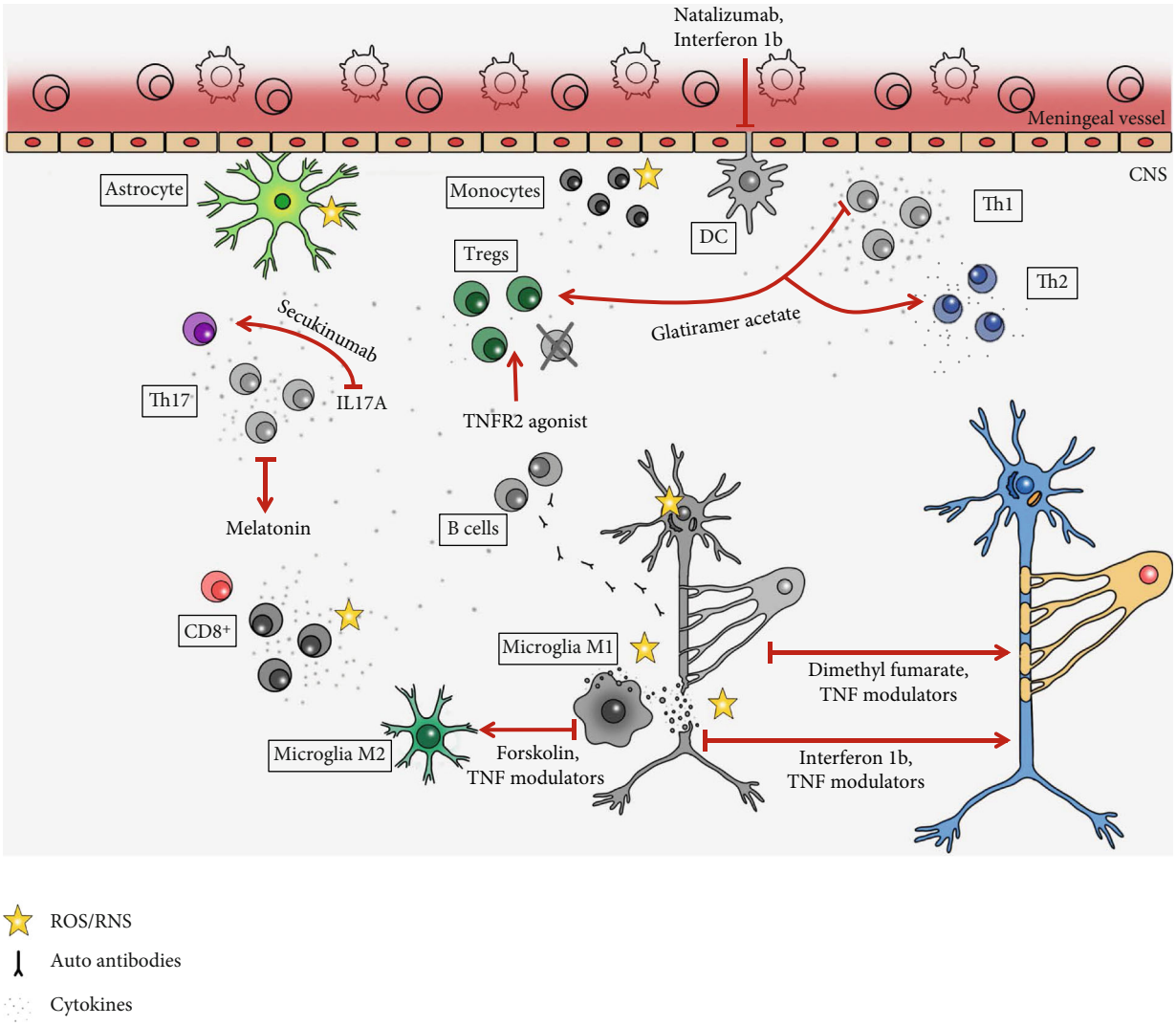

Figure 1: Approved and exploratory immunomodulatory and antioxidant therapeutic strategies to treat MS. MS pathological hallmarks are shown in grey and effect of the therapeutics in color.

catalase, and GPx [145]. Indeed, melatonin supplementation improved antioxidant defense in MS through upregulation of catalase, manganese superoxide dismutase (MnSOD), and sirtuin 1 (SIRT1), an inhibitor of oxidative stress [146]. Similarly, in a small clinical trial, melatonin supplementation caused a statistically significant increase in SOD and GPx in erythrocytes of SPMS patients. A correlation analysis revealed a positive correlation between SOD levels and the Expanded Disability Status Scale (EDSS) score, both before and after melatonin treatment [145], indicating the importance of antioxidant defense to control MS disability. Another study indicated that levels of melatonin, whose production is modulated by seasonal variations in night length, negatively correlated with MS activity in humans [147]. Further, melatonin treatment ameliorated EAE and directly interfered with the differentiation of human and mouse T cells (Figure 1). In particular, it blocked the differentiation of Th17 cells and promoted expansion of type 1 regulatory T cells (Tr1) [147]. Altogether, these and other studies indicate that melatonin has both immunomodulatory and antioxidant activities. However, the impact of melatonin supplementation on MS disability was modest and larger clinical trials are lacking.

Coenzyme Q10 supplementation for 12 weeks resulted in increased SOD and decreased malondialdehyde A activity in a randomized small clinical trial with RRMS patients, indicating that coenzyme Q10 supplement increases antioxidant enzyme activity and decreases oxidative stress [148]. However, a preclinical study using the EAE model of MS demonstrated that the antioxidant idebenone, a synthetic analog of coenzyme Q10, failed to prevent or attenuate motor disease even when administered preventively [149], suggesting that coenzyme Q10 supplementation may not have an impact on MS disease.

Altogether, this shows that interfering with oxidative stress is a promising therapeutic strategy to treat MS, but might not be sufficient as a single treatment. The combination of antioxidant therapy with other immunosuppressive or immunomodulatory therapies might be superior to current approved therapies (Figure 1).

Table 4: Antioxidant complementary therapies and their relevance for MS. Complementary antioxidant therapies for MS were reviewed in detail in [144].

Compound	Specification	Antioxidant characteristics	Relevance for MS
Coenzyme Q10	Coenzyme	Energy transfer molecule, cofactor in mitochondrial electron transport chain	Increases SOD and decreases malondialdehyde A in RRMS patients; synthetic analog has no effect on EAE
Curcumin	Natural pigment	ROS, RNS, and peroxyl radical scavenger; it also modulated GSH, catalase, and SOD activities [190]	Decreases EAE clinical severity, demyelination, and inflammation in the spinal cord and IL-12 production by macrophages/microglia through Janus kinase-STAT pathway [191]
Melatonin	Neurohormone	Activates SOD, catalase, and GPx	It increases SOD and GPx levels in erythrocytes of SPMS patients. Its levels negatively correlate with lesion activity. It ameliorates EAE symptoms, blocks Th17 differentiation and promotes Tr1 expansion.
Vitamin A	Essential nutrient (retinoic acid)	Hydrophobic polyene chain quenches singlet oxygen and neutralizes thiyl radicals stabilizing peroxyl radicals [192].	Serum levels are low in MS patients during relapses [193]. It increases TGFbeta and FoxP3 expression in PBMCs in Avonex-treated RRMS patients [194]. Retinoic acid inhibits cytokine production by Th17 cells in EAE [195].
Vitamin C	Essential nutrient (ascorbic acid)	Scavenges ROS and RNS [196]	Serum levels are low in MS patients during relapses [193]. It promotes OLGs generation and remyelination [197].
Vitamin D	Essential nutrient	Inhibits iron-dependent lipid peroxidation [198]	Serum levels are low in MS patients with elevated relapse frequency [199]. It diminishes risk of MS although the therapeutic value is still debated [200].
Vitamin E	Essential nutrient (alpha-tocopherol)	Peroxyl radical scavenger [201]	Serum levels are low in MS patients during relapses [193]. During IFNβ treatment, decreased MRI activity in RRMS patients is associated with higher levels of alpha-tocopherol [202]. It decreases IFNγ production, inflammation, and demyelination in the spinal cord of EAE mice [203].

5.2.3. Selective Modulation of the Immune System. As mentioned before, treatment using nonselective TNF inhibitors failed in clinical trials with MS patients [138, 139]. The failure of these studies might be explained with the pleiotropic actions of TNF. TNF exists in two forms, soluble (sTNF) and transmembrane bound (tmTNF), and activates two receptors, TNF receptor 1 (TNFR1) and TNFR2. Whereas sTNF/TNFR1 signaling promotes inflammation and tissue degeneration, tmTNF/TNFR2 contributes to immune suppression as well as tissue homeostasis and neuroprotection [136, 150]. Blocking all effects of TNF therefore can be counterproductive and exacerbate MS. Given the opposing effects induced by TNFR1 and TNFR2, a more effective therapeutic approach to treat MS therefore is the selective blocking of sTNF/TNFR1 signaling, which leaves TNFR2 signaling functional. Indeed, several studies have shown that neutralization of sTNF/TNFR1 signaling is therapeutic in rodent models of spinal cord injury [151], Parkinson's disease [152], and neuropathic pain [153]. Further, various studies demonstrated the therapeutic potential of sTNF/TNFR1 blocking in the EAE model of MS [154–156]. Therapeutic administration of a selective inhibitor of sTNF in a chronic EAE model rapidly reduced the neurological symptoms of disease, inhibiting spinal cord inflammation and promoting remyelination and neuroprotection [155, 156]. The mechanisms by which sTNF inhibition promote CNS repair were further studied in a cuprizone demyelination/remyelination model where it was found that sTNF inhibits the capacity of microglia to phagocytose and clear myelin debris [60]. Clearance of myelin debris is essential for OPC to be recruited and form new myelin in demyelinated lesions, a function that is critically mediated by tmTNF/TNFR2 [157, 158].

Next to inhibition of sTNF/TNFR1 signaling, specific activation of TNFR2 may hold promise as a new MS therapy. Indeed, TNF promotes proliferation of oligodendrocyte progenitors and remyelination via TNFR2 [157–159]. Further, data from our laboratories indicate that selective agonism of TNFR2 rescues neurons from oxidative stress-induced cell death [160] and excitotoxic cell death [161, 162]. Similarly, TNFR2 activation induces expression of antiapoptotic and detoxifying proteins and protects OPCs against oxidative stress [163]. *In vivo*, TNFR2 agonist administration promoted immunomodulation via expansion [164] and alleviated autoimmune disease [165]. Studies in the EAE model demonstrated that exogenous activation of TNFR2 was therapeutic for motor and sensory disease [166]. Indeed, a recent study in a model of peripheral nerve injury confirmed that TNFR2 is therapeutic for neuropathic pain via an immunomodulatory mechanism [167]. Altogether, these data suggest that selective modulation of TNF-TNFR signaling may hold great promise as a new therapeutic intervention to treat MS [168] (Figure 1).

An important downstream mediator of TNF pathology is the cytokine interleukin 6 (IL-6), which like TNF is found in elevated concentrations in plasma samples [169] and acute and chronic active plaques of MS patients [170]. The pathogenic role of IL-6 was highlighted by data demonstrating that IL-6-deficient animals are fully resistant to EAE [171] and

that blocking of IL-6 signaling using an IL-6R-blocking antibody or inhibition of trans-signaling in the periphery led to diminished motor symptoms in EAE [172]. Further, data indicate that IL-6R antagonism is therapeutic for CNP in EAE mice [173], indicating the general suitability of targeting IL-6 to treat MS. Indeed, an exploratory open-label study using the humanized anti-IL-6R monoclonal antibody tocilizumab indicates that RRMS patients receiving tocilizumab had reduced number of relapses, but tocilizumab increased disability in SPMS [174]. Indeed, another study described a patient with rheumatoid arthritis who developed MS during anti-IL-6 therapy [175]. This neuroprotective role of IL-6 is supported by findings that indicate that IL-6 together with TGFβ restrains Th17 cell-mediated pathology. In particular, stimulation of myelin-reactive T cells with TGFβ and IL-6 completely abrogated their pathogenic function and Th17 cells failed to upregulate the proinflammatory chemokines crucial for central nervous system inflammation after IL-6/TGFβ stimulation [176]. This is supported by data indicating that IL-6 contributes to controlling the balance between Th17 cells and Tregs [177]. The clinical importance of IL-17 is further outlined by the first promising clinical results with secukinumab, a fully human monoclonal antibody that neutralizes IL-17A (Figure 1). A randomized proof-of-concept study indicated that secukinumab reduced lesion activity in MS patients and showed a trend toward reduced relapse rates [31]. Further clinical evaluation will reveal whether targeting of IL-17A can be used to treat MS.

Next to direct interference with specific inflammatory cytokines, several preclinical products are developed for MS therapy that promote Treg function. However, laquinimod, an orally available carboxamide derivative that induces Tregs and secretion of anti-inflammatory cytokines as well as direct neuroprotection, failed in a clinical trial. Even though the compound was well tolerated and impacted brain atrophy in a phase III trial, it failed to meet its primary clinical trial goal of slowing progression of RRMS [178]. Clinical evaluation of other therapies that promote Treg function, such as low-dose IL-2 [179], will be necessary to evaluate if correcting Treg function in MS patients is therapeutic.

5.2.4. Microglia Repolarization as a Therapeutic Target. Ablation of microglia impaired development of EAE, indicating the important role of microglia for disease [180]. However, microglia also promote remyelination through the expression of anti-inflammatory molecules, phagocytosis of debris, and repair of tissues [181]. Indeed, microglia were shown to differentiate into different phenotypes during demyelination and remyelination [182]. Whereas M1 microglia contribute to inflammation and oxidative stress-induced oligodendrocyte damage, M2 microglia regulate immune functions and drive oligodendrocyte differentiation during CNS remyelination. In particular, in EAE a switch from a M1- to a M2-dominant response occurred in microglia and peripherally derived macrophages as remyelination started [71]. The important role of M2 microglia/macrophages is supported by experiments demonstrating that *in vitro* OPC differentiation was enhanced in the presence of M2 cell conditioned media. Similarly, blocking M2 activity impaired oligodendrocyte differentiation during remyelination in cerebellar slice cultures and *in vivo* [71]. Indeed, genetic depletion of microglia resulted in inefficient clearance of myelin debris thereby impairing remyelinating processes [183]. Therefore, inhibiting microglia to prevent their proinflammatory and tissue destructive activity might be counterproductive. In contrast, modulation of the inflammatory environment of the lesion, e.g., by repolarization of M1 into M2 microglia, might provide a more promising therapeutic approach. Indeed, the neuroprotective effects of the approved MS drug glatiramer acetate are suggested to be mediated by activated M2 microglia [184]. The sTNF inhibitor XPro1595 [185] also promotes remyelination and neuroprotection in demyelinated lesions by increasing the repair potential of microglia [60]. Several other compounds that modulate microglia/macrophage polarization are currently in preclinical development. The adenylyl cyclase activator Forskolin for example alleviates EAE motor disease by suppressing the expression of CD86 while enhancing M2 macrophage polarization at the site of inflammation [186] (Figure 1). Another example is the clinically approved immunomodulatory agent lenalidomide, which promotes M2 macrophage polarization to regulate CNS autoimmunity resulting in abolished progression of EAE [187].

6. Conclusion

MS is a multifactorial disease with a complex etiology. Even though MS is considered an immune-driven disease, several other mechanisms contribute to its pathology, including oxidative stress, immune-independent demyelination, and neuronal cell death. All approved MS therapeutics modulate the immune system thereby suppressing adaptive autoimmunity. However, they are often not effective for all aspects of MS, i.e., sensory deficits, and lead to severe side effects due to unspecific modulation of the immune system. Research of the last decade has shown that selective modulation of the immune system, such as targeting microglia polarization or specific cytokines, might be superior to the currently approved therapies. Two examples are selective targeting of cytokines or microglia. In particular, the cytokines TNF and IL-6, historically considered to be proinflammatory mediators that contribute to MS pathology, contribute to neuroprotection, and neutralization of these cytokines was detrimental in clinical MS trials. Similarly, microglia are cells with a high plasticity and contribute to neurodegeneration, but are also necessary for tissue regeneration. Therefore, selectively targeting the inflammatory activity of these mediators might result in superior therapeutic strategies. Several of the approved MS therapeutics lead to reduction of oxidative stress, and it is hypothesized that this effect contributes to their therapeutic activity. However, different strategies that interfere with oxidative stress failed in clinical evaluation. Nevertheless, these antioxidants may prove to be beneficial as cotreatments with anti-inflammatory reagents resulting in superior clinical outcome. Altogether, several promising novel therapeutic strategies that specifically target components of the neuroinflammatory process are currently under preclinical and clinical evaluation and may lead to the

development of novel MS therapeutics with better activity and safety profiles.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Therapeutic Potential of Diosgenin and Its Major Derivatives against Neurological Diseases: Recent Advances

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Diosgenin (DG), a well-known steroidal sapogenin, is present abundantly in medicinal herbs such as *Dioscorea rhizome*, *Dioscorea villosa*, *Trigonella foenum-graecum*, *Smilax China*, and *Rhizoma polgonati*. DG is utilized as a major starting material for the production of steroidal drugs in the pharmaceutical industry. Due to its wide range of pharmacological activities and medicinal properties, it has been used in the treatment of cancers, hyperlipidemia, inflammation, and infections. Numerous studies have reported that DG is useful in the prevention and treatment of neurological diseases. Its therapeutic mechanisms are based on the mediation of different signaling pathways, and targeting these pathways might lead to the development of effective therapeutic agents for neurological diseases. The present review mainly summarizes recent progress using DG and its derivatives as therapeutic agents for multiple neurological disorders along with their various mechanisms in the central nervous system. In particular, those related to therapeutic efficacy for Parkinson's disease, Alzheimer's disease, brain injury, neuroinflammation, and ischemia are discussed. This review article also critically evaluates existing limitations associated with the solubility and bioavailability of DG and discusses imperatives for translational clinical research. It briefly recapitulates recent advances in structural modification and novel formulations to increase the therapeutic efficacy and brain levels of DG. In the present review, databases of PubMed, Web of Science, and Scopus were used for studies of DG and its derivatives in the treatment of central nervous system diseases published in English until December 10, 2019. Three independent researchers examined articles for eligibility. A total of 150 articles were screened from the above scientific literature databases. Finally, a total of 46 articles were extracted and included in this review. Keywords related to glioma, ischemia, memory, aging, cognitive impairment, Alzheimer, Parkinson, and neurodegenerative disorders were searched in the databases based on DG and its derivatives.

1. Introduction

With an increasingly aging population, human neurological disorders have become a great burden in terms of impact on quality of life and living costs [1]. In developed countries, a dramatic improvement in average life expectancy has led to substantial increases in the prevalence of diseases that mainly afflict the elderly [2, 3]. Human neurological disorders,

including stroke, Alzheimer's disease, Parkinson's disease, depression, ischemic brain injury, and spinal cord injury, resulting from gradual and progressive loss of neural cells in CNS can lead to nervous system dysfunction [4]. Currently, no clinically effective treatments are available for these diseases. The only options to treat CNS disorders are by using drugs and performing surgery [5, 6]. However, most patients with neurological diseases need lifelong medication and long-term use of drugs is associated with serious side effects. Surgical treatment often increases the chance of infection and leads to other dysfunctions. In recent years, natural medicine has shown a great potential to treat nervous system disorders in many western countries [7, 8].

Natural products (NPs) derived from medicinal herbs, plants, vegetables, and fruits play an important role in the prevention and treatment of various human diseases, including cancer, cardiovascular disorders, diabetes, obesity, metabolic syndromes, and neurological disorders. NPs isolated from Chinese herbs have been widely used in traditional medicine over centuries. Many natural products derived from herbs exhibit a wide range of pharmacological properties, including the antimalarial drug artemisinin from *Artemisia apiacea* [9], the anticancer drug paclitaxel from *Taxus brevifolia* [10], and quercetin found in various vegetables and fruits [11]. Diosgenin (DG) is a naturally occurring steroidal sapogenin isolated from *Agavaceae*, *Dioscoreaceae*, *Liliaceae*, *Solanaceae*, *Scrophulariaceae*, *Amaryllidaceae*, *Leguminosae*, and *Rhamnaceae* [12–17]. It has been extensively studied for the management and treatment of different types of cancer [18], osteoporosis [19], cardiovascular diseases [20], atherosclerosis [21], diabetes mellitus [22], and skin diseases [23]. DG is being increasingly investigated in the treatment of neurological diseases [24]. Numerous studies have demonstrated that DG and its derivatives have preventive and therapeutic effects against various neurological disorders. Animal experiments have shown that DG is active in the treatment of nervous system diseases such as Parkinson's disease and Alzheimer's disease [25–27].

Despite its pharmacological activities in the treatment of various diseases, the clinical application of DG is severely hindered by its low aqueous solubility, poor bioavailability and pharmacokinetics, and rapid biotransformation under physiological conditions [28]. Several recent reviews have provided a comprehensive account of its pharmacological effects in cancer [18], diabetes mellitus, metabolic syndrome [29], and others [24, 30]. In this review, we will discuss recent progress of DG as a therapeutic agent against various neurological diseases along with its mechanisms of action in CNS. This review also critically evaluates existing limitations of DG solubility and bioavailability. It briefly recapitulates recent advances involving structural modification and formulations to increase its therapeutic efficacy.

2. Chemistry of Diosgenin

Diosgenin (Figure 1, DG, 25*R*-spirost-en-3β-ol) is a C27 spiroketal steroid sapogenin belonging to a family of spirostanol steroidal compounds. Its molecular formula is $C_{27}H_{42}O_3$ with a relative molecular mass of 414.62. DG is a white needle crystal or light amorphous powder with a proven thermal and chemical stability under various physical conditions [31]. DG is relatively stable against temperature and light exposure. However, DG is destabilized when it is exposed to hydrochloric acid [31]. DG is strongly hydrophobic (with Log $P = 5.7$), and it is insoluble in water [32, 33]. The solubility of DG is around 0.7 ng/mL in aqueous medium [34]. However, it is highly soluble in most nonpolar organic solvents (such as chloroform, dichloroethane, propanol, ethyl acetate, and propylacetate) and in partially polar solvents (such as acetone, methanol, and anhydrous ethanol).

3. Sources of Diosgenin

Primary sources of diosgenin (DG) include the *Dioscorea* species, *Heterosmilax* species, and *Trigonella foenum-graecum*, although DG and related steroidal sapogenins can be commercially obtained from tubers of various *Dioscorea* species [17]. DG is present in high levels in tubers of various wild yams (*D. villosa* Linn). A total of 137 types of *Dioscorea species* contain DG. Of them, 41 contain DG at more than 1%. The seeds of fenugreek (*T. foenum graecum* Linn) [35] and the rhizomes of *D. zingiberensis* are also important sources of DG. In addition, *Trillium govanianum* and *Costus speciosus* contain around 2.5% and more than 2.12% of DG, respectively [36–38]. DG is mainly generated by the hydrolysis of steroidal saponins in the presence of a strong acid, base, or enzyme catalyst [39]. Currently, microbial transformation is a promising method for the production of DG because of its environmentally friendly, highly specific, and mild reaction conditions at a low cost [36, 40].

4. Biosynthesis of Diosgenin

DG is biosynthesized from cholesterol via the isoprenoid pathway in several plant species [41, 42]. The biosynthesis of DG starts with acetyl CoA. It involves several steps to generate squalene that cyclizes to yield lanosterol. Lanosterol is further catalyzed to cholesterol by various enzymes. Cholesterol is sequentially converted to glucoside furostanols and spirostanols. These glycosides are eventually converted to spirostanols after the elimination of the glucose molecules at C26, resulting in ring closure during the catalysis of glucosidases. DG aglycone may convert to glycoside forms with mono-, di-, or trisaccharides known as saponins (Figure 1, Compounds **2** and **3**; Compound **3** is also called dioscin). The attachment of a carbohydrate moiety improves both the solubility and potency of DG.

5. Toxicity and Safety of Diosgenin

Diosgenin (DG) shows high biocompatibility and low toxicity. For instance, in an acute study, the oral administration of a single dose of 112.5–9000 mg/kg ethanol extracts of *Dioscorea* sp. containing 28.34% DG (31.7-2550.6 mg/kg) did not result in any signs of acute toxicity in rats. In a subchronic toxicity study, Sprague-Dawley rats orally administrated with DG at doses of 127.5, 255, and 510 mg/kg/day for 30 days did not show any significant changes in biochemical or

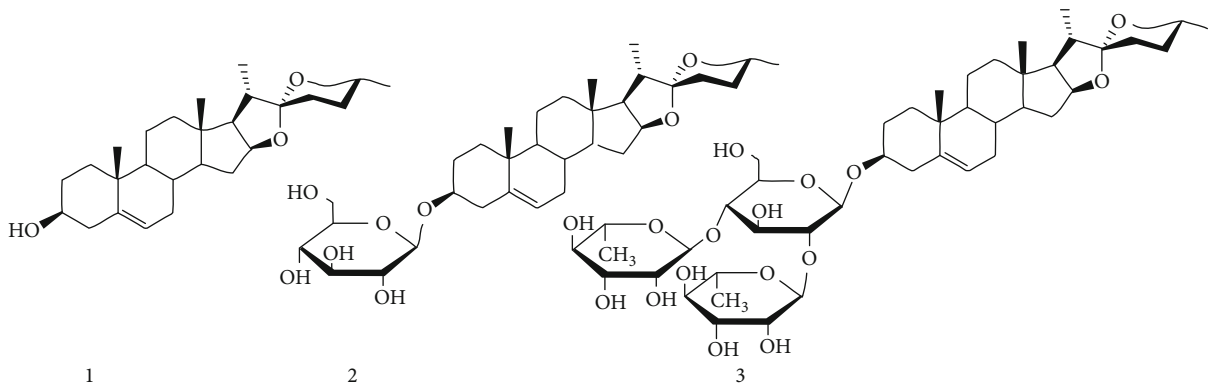

Figure 1: Structures of diosgenin (DG) and its glycosides.

hematological parameters. DG was the main metabolite in the serum [43, 44]. Additionally, a toxicological assay using *D. villosa* (DV) root extract showed that both acute (5 g/kg, single dose) and subchronic (1 g/kg/day, 30 days) treatments of rats resulted in only unremarkable changes in hematological, biochemical, and histopathological parameters [45]. Wojcikowski et al. reported that no acute renal or hepatic toxicity was observed with a crude extract of DG obtained from *D. villosa* at a dose of 0.79 g/kg/day administered orally. However, an increase in kidney fibrosis and liver inflammation was found when mice received a continuous treatment for 28 days in the same study. In a 90-day subchronic study, no toxic sign was found in mice fed fenugreek seeds at doses of DG ranging from 1% to 10% [46]. However, an *in vitro* study showed deleterious effects of DG mediated via genetic instability. At concentrations greater than 30 μM, DG reduced cell viability and increased micronucleus frequency. It also has a significant cytostatic effect with DNA damage in HepG2 cells [47].

6. Bioavailability and Pharmacokinetic Studies of Diosgenin

Using a rat model, Okawara et al. have elucidated the pharmacological effect of cyclodextrin-bound diosgenin (DG). The peak level of DG in the skin was observed at 6 h after oral administration. The plasma concentration of an orally administered DG reached C_{max} (132.5 ± 48.2 ng/mL) at 5.01 ± 0.55 h with an AUC of 4121.9 ± 1354.7 ng · h/mL and an absolute oral bioavailability of 4.45 ± 1.46% [32, 33]. Similarly, Liu et al. have demonstrated that treatment with DG resulted in a C_{max} of 42.1 ± 30.4 ng/mL at 11.3 ± 3.9 h along with an $AUC_{0-60\,h}$ of 1309.3 ± 849.8 ng h/mL and $t_{1/2}$ of 10.4 ± 4.2 h [48]. It was suggested that a single dosage of DG administered to rats, monkeys, and dogs was mostly excreted into feces, while the amount absorbed was rapidly eliminated via bile. Tissue distribution of DG in rats most notably occurred in the liver, adrenals, and gastrointestinal walls. Unchanged DG at concentrations up to 15 μg/mL was found when multiple doses (100 mg/kg/day for 4 weeks) were administered to dogs. Several metabolites of DG were found in the bile of rats and dogs with a pattern of metabolites different in the two tested species. One of its major biliary metabolites was monohydroxylated diosgenin in the F ring. In humans, oral administration of DG at 3 g/day for 4 weeks did not alter the levels of DG in human serum (less than 1 mg/mL) [43].

7. Semisynthetic Derivatives of Diosgenin against Neurological Diseases

Although DG possesses numerous pharmacological activities against various diseases, it has weak biological activity, low aqueous solubility, poor pharmacokinetic profile, and instability under physiological conditions which greatly hinder its clinical application. Covalent modification of therapeutic agents is a clinically proven strategy that can enhance treatment efficacies. Semisynthetic modification of DG at C3 can address these issues by altering its physicochemical characteristics, thus improving its metabolic profile in terms of adsorption, distribution, metabolism, elimination, and biological activities.

To date, a variety of DG derivatives have been designed and synthesized, and most of them have shown improved physicochemical properties and enhanced pharmacological activities compared to parent drug DG. Semisynthetic DG derivatives can be roughly divided into four major categories on the basis of covalent linkage and attached functional entities. First, an amino acid prodrug strategy has been successfully used in the oral delivery of drugs that have low solubility and permeability [49, 50]. The introduction of an amino acid, either natural or its analog, to a parent drug generally can increase the aqueous solubility by orders of magnitude through an ionized carboxylate anion or the formation of amine salts. Moreover, various amino acid transporters are expressed in brush-border membranes of intestinal epithelial cells known to play a significant role in the absorption of several amino acid prodrugs [51]. In the past decade, a series of DG amino acid derivatives have been synthesized for the treatment of cancer, inflammation, diabetes, thrombosis, and neurodegenerative disorders [52]. Representative structures of diosgenin-arginine derivatives (Compound 4, Arg-DG) are presented in Figure 2 [50]. Second, the carbohydrate moiety plays a critical role in biological functions of

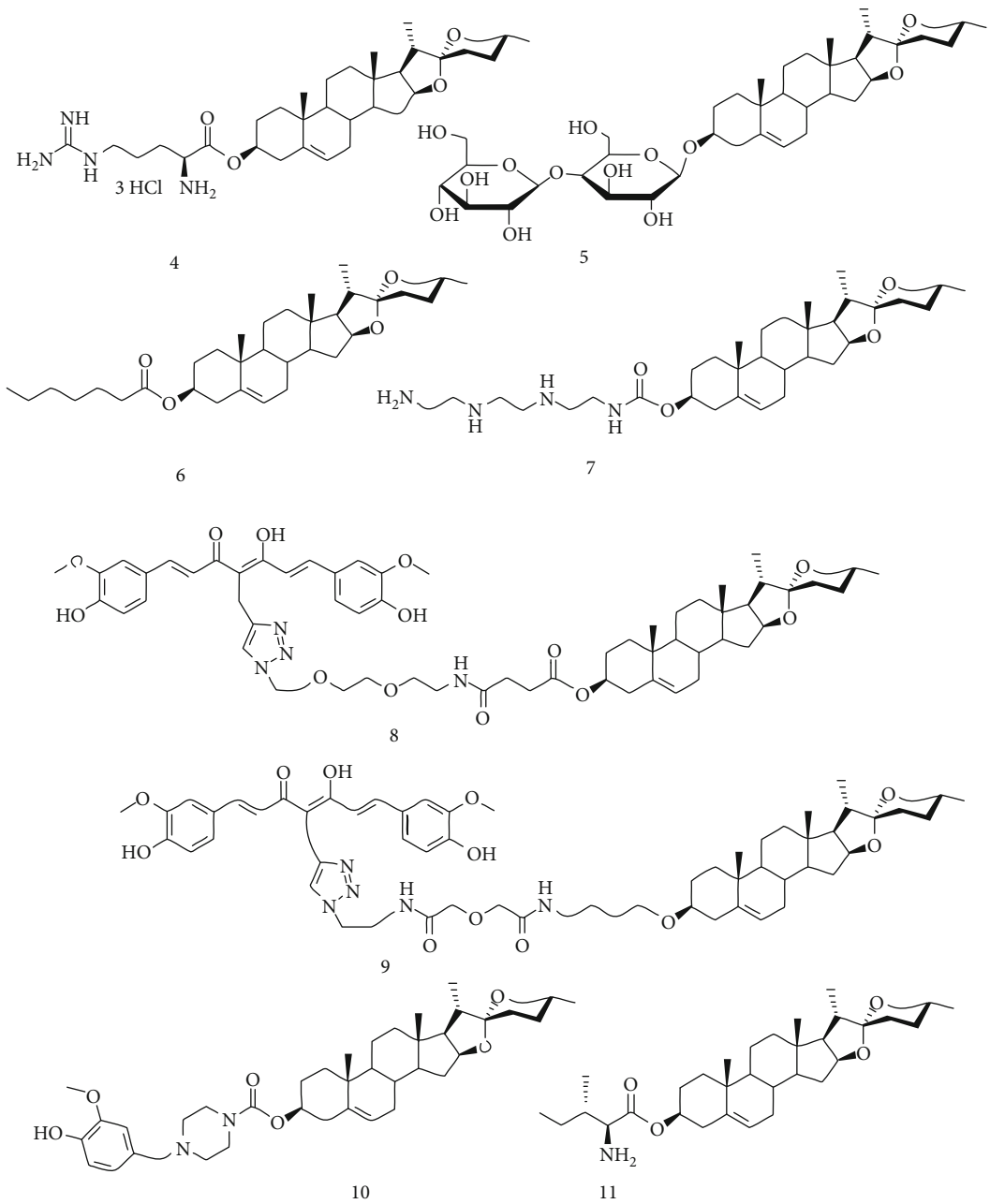

Figure 2: Chemical structures of semisynthetic diosgenin analogs.

steroidal saponins [53, 54]. An increasing number of synthetic steroidal saponins (DG-carbohydrates) have been created and tested for their biological activities mainly against inflammation and cancer (Compound 5) [55]. Third, DG-fatty acid derivatives with a hydrophobic moiety have been designed and prepared based on DG 3-caproate, also known as caprospinol, a naturally occurring compound in *Gynura japonica* that can protect neuronal cells from Aβ1–42 neurotoxicity [56]. Additionally, a series of DG derivatives with hydrophilic moieties such as PEG oligomers [28] and polyamines [57] have been synthesized to improve its solubility and biological activity (Compound 7). Fourth, DG-drug conjugates including DG moieties have been covalently linked to other therapeutic agents directly or via a linkage, forming codrugs with enhanced physicochemical, biopharmaceutical, and drug delivery properties (Compounds 8 and 9) [58, 59]. Several representatives of DG-drug conjugates reported in previous studies are presented in Figure 2.

8. Novel Formulations and Increased Bioavailability of Diosgenin

In addition to structural modification, drug delivery system, particularly nanotechnology, can be used to develop novel formulations with improved solubility, enhanced pharmacokinetics, and/or target delivery. Okawara et al. have prepared

DG-cyclodextrin (CD) complexes to improve the skin concentration of DG and its pharmacokinetic profile, resulting in about 4- to 11-fold higher oral bioavailability of DG in the inclusion complex of DG/β-CD compared to a DG suspension [32, 33]. In addition, the bioavailability of DG has been further improved by combining β-CD and liquid crystal DG to enhance the bioavailability of poorly water-soluble drugs [34]. Recently, DG nanocrystals have been prepared via a media-milling method using a combination of pluronic F127 and sodium dodecyl sulfate as surface stabilizers, resulting in a significant improvement in the dissolution rate and pharmacokinetic profile compared to DG alone as well as increased bioavailability of DG [60]. An eight-arm-PEG-DG conjugate has been prepared for hydrophobic drug delivery via self-assembly to nanoparticles [61]. It has been reported that hyaluronate-DG conjugation via esterification can promote self-assembly into stable, negatively charged nanoparticles measuring 159-441 nm in water, which significantly enhances its solubility [62]. The DG-PEG conjugate can self-assemble into micelles in water, thus significantly enhancing the therapeutic efficacy for the prevention of arterial thrombus and venous thrombus [63].

9. Pharmacological Activity and Mechanism of Diosgenin and Its Derivatives in Central Nervous System (CNS) Diseases

The experimental design, pharmacological evidence, and underlying mechanism for diosgenin, dioscin, and diosgenin derivatives against various diseases in the central nervous system are summarized in Table 1.

9.1. Alzheimer's Disease and Parkinson's Diseases. Alzheimer's disease (AD) is one of the most common neurodegenerative disorders characterized by learning disabilities and declining cognitive function. It is a multifactorial disease caused by multiple etiological and pathogenic mechanisms. However, the exact mechanism underlying AD remains unclear. Several hypotheses such as amyloid-β (Aβ) accumulation, hyperphosphorylation of Tau, altered energy metabolism, oxidative stress, and neuroinflammation have been proposed [64].

Extracellular aggregation of Aβ leading to the formation of plaques via stepwise formation of oligomers and fibrils is a neuropathological hallmark of AD brains. Reduction in Aβ has been considered as a major therapeutic strategy against AD [65]. Tohda et al. have reported that DG can significantly improve memory loss and spike firing in the medial prefrontal cortex and hippocampal CA1 in 5XFAD mice. The accumulation of Aβ plaques and neurofibrillary tangles in the cerebral cortex and hippocampus was significantly decreased after DG treatment. Additionally, DG treatment decreased the number of degenerated axons and presynaptic terminals in regions surrounding amyloid plaques. These events were mediated by 1,25D_3-membrane-associated, rapid response steroid-binding protein (1,25D_3-MARRS) [66, 67], or heat shock cognate 70 by normalization of α-tubulin expression, which is a potentially critical event in axonal formation [25]. Koh et al. have reported that DG can ameliorate multiple types of brain injury in transgenic 2576 (TG) mouse models, in which the accumulation of Aβ plaques is induced by Aβ-42 peptides and neurotoxicant trimethyltin (TMT). Their results demonstrated that the numbers of Aβ plaques and dead cells in the granule cell layer of the dentate gyrus were significantly decreased by pretreatment with DG for 21 days. Additionally, a significant increase in the expression of nerve growth factor (NGF) and variation in corresponding components of NGF signaling pathways were found, suggesting that DG could stimulate NGF biosynthesis in multiple types of brain damage [68].

Although extracellular accumulation of neurotoxic Aβ species in the brain has been proposed as one of the main events in early stages of AD, continued failure of clinical trials involving Aβ-targeting drugs have prompted scientists to explore alternative mechanisms and therapeutic strategies [69]. Neurofibrillary tangles (NFTs) mainly composed of hyperphosphorylated Tau are another histopathological hallmark of AD and associated tauopathies. Hyperphosphorylation of Tau protein can lead to neurodegenerative disorders. Tohda et al. found that DG treatment can also decrease the hyperphosphorylation of Tau protein in the cortex and hippocampus in an AD mouse model [66, 67].

Teper et al. have identified the diosgenin analog (22*R*,25*R*)-20α-spirost-5-en-3β-yl hexanoate (Figure 2, diosgenin 3-caproate; caprospinol, Compound 6) using the 22*R*-hydroxycholesterol chemical structure as a probe. Compound 6 is a naturally occurring compound in *Gynura japonica*, a plant belonging to the Asteraceae family. It can protect neuronal PC12 cells against Aβ1-42-induced neurotoxicity [70]. In later studies, Lecanu et al. and Tillement et al. have screened potential candidates among the prepared DG derivatives in human NT2N neuronal cells and PC12 cells against β-amyloid (1-42)- (Aβ-) induced neurotoxicity. Their results showed that DG and its derivatives exert their neuroprotective effect via inhibiting the formation of neurotoxic amyloid-derived diffusible ligands and preferentially binding to two binding sites of Aβ identified by computational docking simulations in contrast to 22*R*-hydroxycholesterol that could only bind a single site. A subsequent study has revealed that Compound 6 has a direct effect on mitochondrial function. It blocked Aβ uptake by mitochondria in neuronal cells and protected SK-N-AS cells from Aβ-induced mitochondrial impairment by targeting Complexes 4 and 5 of the respiratory chain, indicating that DG derivatives might have potential in AD therapy [71, 72]. Compound 6 can prevent the formation of amyloid-derived diffusible ligands (ADDLs) by binding to Aβ42, decreasing amyloid accumulation in mitochondria, and directly targeting the mitochondrial respiratory chain [73]. Lecanu et al. have found that the neuroprotective effect of Compound 6 against AD is mediated by decreasing the level of tau phosphorylation [74]. Compound 6 can significantly reduce neurodegeneration and attenuate memory loss and cognitive disorders in a rat model of AD. The recovery from cognitive impairment is accompanied by a reduction in amyloid accumulation in the hippocampus [74]. A series of DG derivatives with different lengths of lateral carbon chains at C3 have been investigated. The structure-activity relationship has revealed that a six-carbon

Table 1: Key pharmacological effects and mechanisms of action of DG and its major derivatives in neurological diseases.

Entry	Active ingredient	Experimental model	Pharmacological effect	Mechanisms of action	Ref
1	Diosgenin	5XFAD transgenic mouse model of AD; Rat cortical neurons and mouse cortical neuron primary culture	Increased memory and decreased axonal degeneration; Reduced amyloid plaques and neurofibrillary tangles in the cerebral cortex and hippocampus	1,25D_3-membrane-associated, rapid response steroid-binding protein (1,25D_3-MARRS)	[65]
2	Diosgenin	Normal mouse	Improved memory and axonal density; Increased c-Fos expression in the medial prefrontal and perirhinal cortices	1,25D_3-MARRS-triggered axonal growth	[66]
3	Diosgenin	Trimethyltin- (TMT-) injected transgenic 2576 (TG) mice	Decreased the number of Aβ-stained plaques and dead cells in the granule cell layer of the dentate gyrus; Reduced acetylcholinesterase (AChE) activity and Bax/Bcl-2 expression; increased expression of nerve growth factor (NGF) and superoxide dismutase (SOD) activity	Increased phosphorylation of downstream members in TrkA signaling; Evaluated p75(NTR) expression and JNK phosphorylation in the NGF signaling pathway	[67]
4	Compound 6	Memory-impaired Long-Evans rats induced by infusion of Fe^{2+}, Aβ42, and buthionine-sulfoximine (FAB) into the left cerebral ventricle for 4 weeks	Enhanced cognitive function; Decreased amyloid deposits, astrogliosis, and Tau protein phosphorylation in hippocampus		[68]
5	Compound 6	Aβ-induced neurotoxicity in rat PC12 and human NT2N neuronal cells	Protected against 0.1 μM Aβ in PC12 cells; Reversed 0.1-10 μM Aβ-induced decrease in ATP levels	Physicochemical interaction with Aβ inhibited the formation of neurotoxic amyloid-derived diffusible ligands	[70, 71]
6	Compound 6	Aβ1-42-induced SK-N-AS cells	Protected MPT and inhibited accumulation of the Aβ1-42 in the mitochondrial matrix	Directly targeting complexes IV and the mitochondrial respiratory chain	[72]
7	Compound 8	A cellular AD model using MC65 neuroblastoma cells from TC withdrawal-induced cytotoxicity	Antioxidative ability and inhibitory effects on amyloid-β oligomer (AβO) formation	Bind directly to Aβ	[56]
8	Compound 9	A cellular AD model using MC65 neuroblastoma cells from TC withdrawal-induced cytotoxicity; Neuronal N2a cells and rat primary cortical neurons	Significant stimulating activity on neurotic outgrowth and the state 3 oxidative rate of glutamate while preserving the coupling capacity of the mitochondria	Interfere with glutamate uptake or its redox reaction	[27]
9	Diosgenin-rich yam extracts	Senescent mice induced by D-galactose	Improve their learning and memory abilities; Increase the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) and decrease malondialdehyde (MDA) level	Enhancing endogenous antioxidant enzymatic activities	[56, 58]
10	Diosgenin	PD model using Sprague-Dawley rats using intrastriatal injection of lipopolysaccharide (LPS)	Attenuate the inflammatory and oxidative stress response; Restore LPS-induced motor deficits; Decrease the expression levels of TLR2, TLR4, and NF-κB	Inhibiting the TLR/NF-κB pathway	[26]
11	Diosgenin	*In vitro* model of HIV-induced dementia using human neuronal cultures with E4 allele of ApoE	Protected against the neurotoxicity of Tat+morphine; Tat-induced oxidative stress impaired morphine metabolism		[76]

Table 1: Continued.

Entry	Active ingredient	Experimental model	Pharmacological effect	Mechanisms of action	Ref
12	Diosgenin	A rat model with brain aging through subcutaneous injection of D-galactose	Improve learning and memory; Upregulating Rheb and downregulating mTOR	Rescuing dysfunctional autophagy mediated by Rheb-mTOR signal pathway	
13	Compound 3	Neuroinflammation induced by intraperitoneal injection of LPS	Enhanced the serotonergic system and produced the antidepressant effect	Protects the hippocampus from LPS-induced neuroinflammation by the neurotransmitter 5-HT and the HMGB-1/TLR4 signaling pathway	[80]
14	Compound 2	Neuroinflammation model using rat microglia and BV2 cells induced by LPS	Suppressed the expression levels of proinflammatory M1 markers, such as NO, IL-6, and TNF-α; Repressed IκB-α, ERK, MAPK, and p38 MAPK phosphorylation	Inhibiting NF-κB, ERK/MAP, and p38/MAPK signaling	[81]
15	Compound 7	Neuroinflammation model using BV2 cells induced by LPS	Inhibition of the inflammatory mediators such as NO, iNOS, COX-2, IL-6/1b, and TNF-α in protein and mRNA levels; Suppressed the NF-κB activity and phosphorylation level of JNK	Inactivation of NF-κB and JNK MAPK signaling	[82]
16	Compound 4	Neuroinflammation model using BV2 cells or mice by I.C.V. injection of LPS	Improved the cognitive function impaired by LPS and attenuated LPS-impaired neurogenesis; Suppressed the production of proinflammatory cytokines in hippocampal DG	Blocking microglial activation; Underlying NF-κB and JNK MAPK; Signaling in LPS-induced adult mice	[57]
17	Diosgenin	C57BL/6J mice model of experimental autoimmune encephalomyelitis	Inhibit the activation of microglia and macrophages, suppress $CD4^+$ T cell proliferation, and hinder Th1/Th17 cell differentiation		[84]
18	Diosgenin	Rat primary oligodendrocyte progenitor cell (OPC) culture model, a cuprizone-induced demyelination C57BL/6J mice model	Significantly and specifically promotes OPC differentiation; Enhances remyelination; Increases the number of mature oligodendrocytes in the corpus callosum	Differentiation of OPC into mature oligodendrocytes through an ER-mediated ERK1/2 activation pathway to accelerate remyelination	[85]
19	Compound 2	Sprague-Dawley rats with traumatic spinal cord injury	Significantly less tissue injury and edema; Functional recovery	Significantly attenuated p62 expression and upregulated the Rheb/mTOR signaling pathway due to the downregulation of miR-155-3p	[87]
20	Compound 3	Ischemic stroke rat model	Improved infarct volume and neurological scores; Reduced inflammatory responses, and suppressed the expression of TLR4, MyD88, NF-κB, TGF-β1, HMGB-1, IRAK1, and TRAF6	Inhibition of TLR4/MyD88/NF-κB induced inflammation	[89]
21	Compound 5	Thrombosis model using male balb/C mice	Prolonging the bleeding time; Inhibited platelet aggregation, prolonged partial thromboplastin time (APTT), and inhibited factor VIII activities		[90]
22	Diosgenin	Transient focal cerebral ischemia-reperfusion (I/R) injury model by middle cerebral artery occlusion (MCAO) using the intraluminal thread for 90 min	Inhibited the death rate and improved the impaired neurological functions, neurological deficit scores, and cerebral infarct size;	Antiapoptosis, anti-inflammation, and intervening NF-κB signaling pathway	[92]

Table 1: Continued.

Entry	Active ingredient	Experimental model	Pharmacological effect	Mechanisms of action	Ref
23	Compound 3	*In vitro* oxygen-glucose deprivation and reoxygenation (OGD/R) model and an *in vivo* middle cerebral artery occlusion (MCAO) model	Reduced cell apoptosis in the hippocampus CA1 and cortex; Suppressed the production of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in blood serum Prevented OGD/R insult and cerebral I/R injury; Inhibition in the expression and the nuclear-to-cytosolic translocation of HMGB-1; Blockade of the TLR4/MyD88/TRAF6 signaling pathway; Inhibited NF-κB and AP-1 transcriptional activities, inhibited MAPK and STAT3 phosphorylation, inhibited proinflammatory cytokine responses, and upregulated the levels of anti-inflammatory factors	HMGB-1/TLR4 signaling	[93]
24	Compound 3	Cerebral ischemia-reperfusion model by middle cerebral artery occlusion (MCAO) ischemic mice	Enhanced spatial learning memory in ischemic mice; An improvement in deficient ability and reduction in infarct volume		[94]
25	Diosgenin	Ovariectomized (OVX) female Wistar rats	Dose-dependently influences IL-2 levels in the brain of OVX rats and affects depressive behavior in OVX with high-anxiety rats		[95]
26	Diosgenin	Neuropathic pain model induced by chronic constriction injury (CCI) in rats	Reversed the mechanical withdrawal threshold and thermal withdrawal latency; Inhibited the expression levels of proinflammatory cytokines TNF-α, IL-1β, and IL-2; Suppressed oxidative stress	Inhibiting activation of p38 MAPK and NF-κB signaling pathways	[96]
27	Diosgenin	Diabetic neuropathy mice model	Increased NGF levels in the sciatic nerve, enhanced neurite outgrowth in PC12 cells, and improved nerve conduction velocities; Reduced disarrangement of the myelin sheath, increased area of myelinated axons, and an improvement in the damaged axons	Increased the nerve conduction velocity by induction of NGF	[97]
28	Diosgenin	Peripheral nerve injury model using male Sprague-Dawley rat to crush the right sciatic nerve for 30 sec	Increased sciatic function index (SFI) value; Suppressed nerve injury-induced c-Fos expression in the ventrolateral periaqueductal gray (vlPAG) and paraventricular nucleus (PVN); Increased expression levels of BDNF, TrkB, COX-2, and iNOS		[99]

Table 1: Continued.

Entry	Active ingredient	Experimental model	Pharmacological effect	Mechanisms of action	Ref
29	Diosgenin	C6 rat glioma cells	Reduced the dosage regimen of TMZ and overcome temozolomide resistance in TMZ-resistant GBM cells; Underwent apoptosis and early cell cycle arrest with significant reduction in MMP-2 levels	Upregulation of MMP-2 level and apoptosis signaling pathway	[103]
30	Compound 3	*In vitro* study using GBM, U87MG, A172, LN18, NBRC, T98G, and LN229 cell lines	Inhibited proliferation of C6 glioma cells, ROS generation caused mitochondrial damage and cell apoptosis; Inhibited tumor size and extended the life cycle of rats	Increase in ROS accumulation, DNA damage, and mitochondrial-mediated apoptosis signaling	[104]

Compound 6 is the most effective one among all tested derivatives at different concentrations. The closest analogs with chain lengths of 4 to 5 carbons failed to exhibit any neuroprotective activity at the lowest concentration of 10 μM, while the analogs with chains longer than C6 were less effective than Compound 6 [73]. Papadopoulos and Lecanu have reviewed the pharmacological activity of Compound 6 [56, 73].

Apart from the naturally occurring diosgenin derivatives such as Compound 6, a series of multifunctional, bivalent diosgenin-curcumin conjugates have been developed as neuroprotectants to combat AD [27, 58]. The results showed a clear structural preference for the introduction of the methylene carbon between diosgenin and curcumin. The conjugate with a spacer length of 17 atoms showed the highest protective activity in MC65 neuroblastoma cells and a decrease in neuroprotective activity was observed when the spacer length was extended to 28 atoms. The most potent Compounds 8 and 9 are presented in Figure 2. Their mechanism of action involves antioxidant activity and inhibitory effects on amyloid-β oligomer formation by directly binding to Aβ [58]. Additionally, DG can act as a membrane-anchoring moiety to improve the access to the cell membrane for the conjugates, suggesting that DG is a novel anchor that can facilitate the multifunctional role of bivalent conjugates for further development as potential therapeutic agents for AD treatment [27]. Recently, Yang et al. synthesized a series of multifunctional DG derivatives and evaluated their effect from Aβ-induced damage in PC12 cells and improved learning and memory impairments in Aβ-injected mice. Among them, Compound 10 (AA36) significantly prevented Aβ-induced PC12 cell damage and restored the cognitive impairment in Aβ-injected mice, suggesting that DG is a promising skeleton structure for anti-AD drug development [59].

Parkinson's disease (PD) is another neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra. The protective effect of DG against LPS-induced PD has been evaluated using a rat model. DG can attenuate LPS-induced motor deficits in rats by suppressing the TLR4/NF-κB signaling pathway [26]. Recently, Cai et al. has synthesized and evaluated the neuroprotective potential of the diosgenin-amino acid derivative and the diosgenin derivative conjugated with L-isoleucine (Compound 11) displayed a neuroprotective role on damaged SH-SY5Ycells by reducing apoptosis as well as promoting angiogenesis at 4 mg/mL on the chorioallantoic membrane model [75].

9.2. Cognitive Effects. Oxidative stress is intimately associated with cognitive function in neurodegenerative disorders such as Alzheimer's disease. Chiu et al. have acknowledged the neuroprotective effects of DG-rich yam in senescent mice induced by D-galactose (D-gal). Compared with D-gal treatment alone, DG treatment for 4 weeks significantly restored learning and memory impairment in mice starting from week six. The mechanism is partly mediated through an increase in endogenous antioxidant enzyme activities [76]. They found similar results after treatment with DG at the concentration range of 5 to 125 mg/kg [77]. Turchan-Cholewo et al. have reported that DG may improve the cognitive impairment associated with human immunodeficiency virus (HIV) infection. Increased levels of oxidative stress and the E4 allele of apolipoprotein E (ApoE) have been found in the CNS of an HIV-infected population or in individuals with a history of intravenous drug abuse, and were considered as the risk factors contributing to the development of dementia. The results revealed that HIV proteins such as gp120, Tat, and Tat+morphine treatment increased the neurotoxicity in cultured human neuronal cells with ApoE4. DG can protect against neurotoxicity induced by Tat+morphine treatment, and the Tat-induced oxidative stress-impaired morphine metabolism can be prevented by DG treatment [78]. Moreover, the learning and memory capacity of Compound 2 derived from *Trillium tschonoskii* Maxim has been investigated in aging rats induced by D-gal with impaired cognitive function using the Morris water maze test. Treatment with Compound 2 improved the learning and memory capacity in aging rats induced by D-gal and the mechanism might be associated with rescuing dysfunctional autophagy via the upregulation of Rheb and the downregulation of mTOR signaling, suggesting that Compound 2 has potential in health promotion and aging-related disease therapy [79].

9.3. Neuroinflammation. Overactivated microglia are present in large numbers in several neurodegenerative disorders. Overproduction of various proinflammatory cytokines may result in neurotoxicity in neurodegenerative disorders. Cumulative evidence has suggested that microglial activation is an early and ongoing stage in neurodegenerative disorders. Anti-inflammatory drugs might be beneficial during the early stages of diseases in several animal models. They can inhibit microglial activation and result in the suppression of proinflammatory cytokines in the hippocampus, finally reversing the decline in memory and learning. It is a practical strategy to develop therapies by preventing the progression of neurodegenerative disorders via the modulation of the neuroinflammation markers in the hippocampus [80].

Binesh et al. have investigated the efficacy of DG in the amelioration of atherosclerotic progression in the heart and inhibition of inflammatory mediators in the liver and brain of Wistar rats treated with an atherogenic diet. DG can inhibit the inflammatory mediators triggered by atherogenic diet in the heart, liver, and brain of rats via the downregulation of COX-2, TNF-α, and NF-κBp65, thereby preventing atherosclerotic disease progression [81]. Yang et al. have reported that Compound 3 can rescue endotoxemia-induced neuroinflammation and has a neuroprotective effect on hippocampal neurogenesis impaired by neuroinflammation, which is consistent with the results obtained in the behavior test showing that Compound 3 reversed cognitive impairment. The endotoxemia-triggered neuroinflammation cascade involves the neurotransmitter 5-HT and the HMGB-1/TLR4 signaling pathway [82]. Wang et al. has reported that Compound 2 extracted from *Tritulus terrestris* L. can selectively inhibit inflammatory M1 markers (NO, IL-6, and TNF-α) in activated rat microglia and BV2 cells induced by LPS, without affecting the production of inflammatory M2 markers (IL-10, IL-1Rα, and CD206) in LPS- and IL-4-treated microglia. Its mechanism involves the inactivation of NF-κB, ERK1/2/MAPK, and p38/MAPK signaling pathways indicating that DG glycoside might be a potential candidate for the treatment of various neurodegenerative disorders mediated by neuroinflammation [83].

Recently, we reported that DG derivatives carrying primary amine (Compound 7, DGP) or the amino acid L-arginine (Compound 4, Arg-DG) at the C3 show a significant increase in aqueous solubility and anti-inflammatory activity in LPS-induced BV2 cells compared to DG. The possible mechanisms of both derivatives involve the inhibition of NF-κB activation and JNK/MAPK signaling [57, 84]. Arg-DG can also rescue hippocampal neurogenesis and cognitive function impaired by LPS via the inhibition of microglial overactivation, the expression of the TLR4 receptor and downstream signaling of NF-κB and JNK/MAPK, and the ultimate suppression of proinflammatory cytokines. Our results suggested that the chemical modification of DG might be an effective approach to improve its physicochemical properties and pharmacological activities in neurodegenerative disorders resulting from microglia-mediated neuroinflammation [84]. Interestingly, although the DG glycoside and DG derivatives including DGP and Arg-DG exhibited strong anti-inflammatory activity in LPS-induced microglial BV2 cells, they differed in inhibitory activity against MAPK signaling. Structural differences involving the substituted groups of DG at C3 might have led to the selective inhibition of MAPK subfamily members (ERK1/2, JNK, and p38).

9.4. Multiple Sclerosis. Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease involving the central nervous system. Currently, curative drugs are unavailable for MS in clinics. Phagocytosis by microglia or macrophages is considered a hallmark of MS lesions. Activated microglia with different phenotypes exhibit either neuroprotective or neurotoxic effects in MS depending on the disease stage and severity of disease. They might lead to a relapsing-remitting MS [80, 85].

Recently, Liu et al. have reported the therapeutic potential of DG in an experimental autoimmune encephalomyelitis (EAE) model of mice using myelin oligodendrocyte glycoprotein. Their results showed that DG significantly alleviated the progression of EAE in mice and obviously reduced the inflammation and demyelination in the CNS. A mechanistic study has shown that DG can inhibit the microglial/-macrophage activation, reduce $CD4^+$ T cell proliferation, and suppress Th1/Th17 cell differentiation [86]. Additionally, in their earlier study involving a purified rat OPC culture model, DG significantly and specifically promoted the differentiation of oligodendrocyte progenitor cells (OPCs) into mature oligodendrocytes, which is considered as a prerequisite for remyelination after demyelination. Moreover, DG administration can enhance remyelination in a demyelination model induced by cuprizone. DG can also significantly increase the number of mature oligodendrocytes in the corpus callosum without affecting the number of OPCs. The underlying mechanism for the accelerated remyelination is attributed to the ER-mediated ERK1/2 activation. DG not only attenuates the progression of EAE, and reduces demyelination and inflammation of CNS as an immunomodulator, but also promotes the differentiation of OPCs, and enhance remyelination and the number of OPCs in demyelinating lesions of the CNS, suggesting that DG is a promising therapeutic candidate in the treatment of MS [87].

9.5. Spinal Cord Injury. Spinal cord injury (SCI) is a severe neurological disorder of CNS that usually causes permanent disability or motor deficit and sensory loss in patients, and it also leads to numerous complications associated with complex pathological mechanisms. Very few restorative therapeutic options are available clinically to improve the neurologic deficits in the SCI. Chen et al. has reported that the DG-rich extract of *Trillium tschonoskii* Max has a neuroprotective role against the spinal cord in rats by upregulating the expression of ciliary neurotrophic factor (CNTF) and its receptor (CNTFRα) at mRNA and protein levels [88]. Chen et al. has further evaluated the neuroprotective effect of the bioactive component of Compound 2 in *T. tschonoskii* Max on motor function recovery and the underlying mechanism after SCI in rats. Compound 2 could significantly reduce tissue injury and edema. The underlying mechanism might be associated with autophagy via the suppression of p62 expression and upregulation of Rheb/mTOR signaling due to the

downregulation of miR-155-3p, leading to the prevention of neuronal cell damage and apoptosis [89].

9.6. Stroke and Thrombosis. Stroke is a major public health concern with high morbidity and mortality. The main stroke pathological types include ischemic stroke, primary intracerebral hemorrhage, and subarachnoid hemorrhage. Stroke is the third leading cause of death worldwide with a progressively increasing incidence and involving younger individuals. Thus, developing therapies to treat stroke is highly desirable [90]. Zhu et al. have reported that Compound 3 has therapeutic potential against ischemic stroke in rats. Compound 3 can significantly reduce the infarct volume and neurological scores in rat models of ischemic stroke. Compound 3 can inhibit the expression levels of TLR4, myeloid differentiation factor 88 (MyD88), and activation of NF-κB, leading to the inhibition of inflammatory responses in a rat model of ischemic stroke, suggesting that Compound 3 acts as an anti-inflammatory agent against ischemic stroke via the inhibition of the TLR4/MyD88/NF-κB signaling pathway [91].

Thrombosis of cerebral arteries is the major cause of morbidity and mortality worldwide. Zhang et al. have designed several monomers of diosgenyl saponin using a simple and convenient method. The antithrombotic effects of a synthetic disaccharide saponin of DG attached to two glucose units (Compound 5) along with the naturally occurring DG saponin have been examined. Their results showed that Compound 5 exhibited a strong efficiency in prolonging bleeding time and altering platelet aggregation both *in vitro* and *in vivo*. Moreover, their results demonstrated that Compound 5 could inhibit platelet aggregation, prolong the activated partial thromboplastin time, reduce factor VIII activities in rats, and significantly enhance the protection in mice. However, its mechanism of action was not determined in their study [55]. It needs to be examined in further studies.

9.7. Cerebral Brain Ischemia-Reperfusion Injury. Cerebral I/R injury refers to cerebral ischemia-induced brain damage that occurs if blood supply is restored [92]. It often causes a series of consequences including neurotoxicity induced by excitatory amino acids (EAA), mitochondrial dysfunction, overproduction of reactive oxygen species (ROS), inflammatory reaction, and neuronal cell death, which ultimately leads to irreversible brain injury. Great strides have been made in treatment modalities for ischemic stroke. However, an effective therapeutic strategy is currently unavailable for ischemic stroke clinically. Ischemic stroke is still a major cause of deaths in developed countries [93]. A recent study has uncovered that DG is effective in treating transient cerebral I/R injury via different mechanisms. Intragastric administration of DG once daily for 7 days prior to surgery can significantly inhibit the death rate in rats, restore motor impairment, and reduce neurological deficit scores along with cerebral infarct size. DG decreased the cellular apoptosis in the hippocampus CA1 and cortex via suppression of caspase-3 activity and Bax/Bcl-2 ratio. Moreover, DG can suppress the overproduction of proinflammatory cytokines including TNF-α, IL-1β, and IL-6 in the blood serum of I/R insulted rats. Its action is mediated by blocking the NF-κB signaling pathway via the downregulation of IκBα, suggesting that DG has a great potential to combat similar diseases in the clinic [94].

Compound 3 (Figure 1), a saponin of diosgenin, also possesses neuroprotective activities against ischemia-reperfusion injury. It can protect PC12 cells and primary cortical neurons in oxygen-glucose deprivation and reoxygenation (OGD/R) insults *in vitro*. It can also significantly attenuate cerebral I/R injury in the middle cerebral artery occlusion (MCAO) model. Further studies have indicated that the neuroprotective mechanism of Compound 3 is related to a blockade of the HMGB-1/TLR4/MyD88/TRAF6 signaling pathway via the inhibition of transcriptional activities of NF-κB and AP-1, the phosphorylation of MAPK and STAT3, and the proinflammatory cytokine responses and augmentation of anti-inflammatory mediator levels. These findings indicate that Compound 3 is a potential therapeutic agent for the prevention of cerebral I/R injury [95]. Furthermore, a combination treatment of Compound 3 and baicalein has resulted in a significant improvement in spatial memory and reduction in the infarct volume in a mice model of cerebral I/R injury. Hippocampal gene expression profiles of MCAO ischemic mice using cDNA microarray analysis of 1176 known genes have shown that numerous genes including those involved in cell cycle regulation, DNA binding, signal transduction pathways, and neuronal transcription factors are associated with neuroprotective effects [96].

9.8. Antidepressant Effects. Depression is becoming a common neuropsychiatric disorder. However, there is no effective antidepression therapy available clinically. Ho et al. reported that chronic diosgenin administration at a dosage of 10 mg/kg/day can improve avoidance behavior in a learned helplessness test involving ovariectomized rats with high anxiety levels but not in low-anxiety rats. Chronic administration of DG can reduce the expression of IL-2, an indicator of neuroimmune function, in the brains of ovariectomized rats, suggesting that DG might relieve depressive behavior via the modulation of the neuroimmune system [97]. Moreover, Yang et al. have reported that Compound 3 exhibits the antidepressant effect by enhancing 5-HT levels in endotoxemia-induced acute neuroinflammation in mice [82].

9.9. Neuropathic Pain. Neuropathic pain is a prevalent and complicated condition arising from diseases such as diabetes mellitus (DM), postherpetic neuralgia, and brain injury affecting the peripheral or central nervous system. It is often resistant to treatment. It is associated with poor treatment satisfaction in patients [98]. Zhao et al. has shown the effect of DG on allodynia and the underlying mechanism in a neuropathic pain model of rats induced by chronic constriction injury (CCI). Their results showed that DG could significantly reverse mechanical allodynia and thermal hyperalgesia induced by CCI. DG can alleviate CCI-induced neuropathic pain in rats by inhibiting the

activation of the p38MAPK and NF-κB pathways, ultimately leading to the suppression of CCI-induced overexpression of proinflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin- (IL-) 1β, and IL-2, and reduction of oxidative stress induced by CCI [99]. In addition, Zhao et al. has shown the efficacy of DG in the alleviation of neuropathic pain in streptozotocin- (STZ-) induced diabetic rats. Their study revealed that daily administration of DG at a dose of 40 mg/kg over 5 weeks obviously increased the mechanical and thermal nociceptive thresholds and lowered the pain score at delayed stages of the formalin test, but not in the early stage. An antinociceptive mechanism of DG is that it can lower oxidative stress and inflammation in diabetic rats via the restoration of the malondialdehyde (MDA) level, the activity of superoxide dismutase (SOD) and catalase, and the expression of TNF-α and IL-1β via NF-κB signaling [100]. Moreover, Kang et al. has reported that DG from *Dioscorea nipponica* can ameliorate diabetic neuropathy in diabetic rats. DG can increase levels of the nerve growth factor (NGF) that are reduced in diabetic rats and enhanced nerve conduction velocities in a mouse model of diabetic neuropathy. Additionally, DG can increase neurite outgrowth in PC12 cells, improve damaged axons, reduce disorganization of the myelin sheath and increase the area of myelinated axons. Beneficial effects of DG in a diabetic neuropathy model in restoring ultrastructural changes and neural regeneration might be associated with increased expression of NGF [101].

Lee et al. have examined the effect of DG on chronic pain and functional deficit resulting from sciatic crushed nerve injury in rats. DG treatment increased the sciatic function index and suppressed the nerve injury-induced overexpression of BDNF, TrkB, COX-2, iNOS, and c-Fos in the ventrolateral periaqueductal gray and paraventricular nucleus, suggesting that DG treatment could prolong pain control and extended functional recovery after peripheral nerve injury [102].

9.10. Glioblastoma. Glioblastoma is the most aggressive and malignant primary central nervous system cancer, characterized by rapid proliferation and high invasion. Surgical resection is still the mainstay of treatment for glioblastoma. Currently, no effective treatments are available to cure patients with glioblastoma due to its exceptionally heterogeneous nature and unique microenvironment. Temozolomide (TMZ) and bevacizumab are the only FDA-approved therapeutic agents for the treatment of primary and recurrent glioblastoma, respectively [105]. Acquired TMZ resistance seriously restricts the therapeutic index and fails to prolong the overall survival. DG can significantly reduce the dosage regimen of TMZ in the combinatorial therapy of DG and TMZ. It can also overcome TMZ resistance in glioblastoma cells as well. The underlying mechanism involves the downregulation of matrix metalloproteinase-2 (MMP-2) and the promotion of apoptosis [103]. The antitumor activity and the underlying mechanism of dioscin has been examined both *in vitro* and *in vivo*. Dioscin exhibited a growth inhibitory effect on C6 glioma cancer cells. It significantly inhibited tumor size and prolonged the life cycle of rats. The mechanism of action of dioscin involves the promotion of ROS accumulation, DNA damage, and mitochondrial-mediated apoptosis signaling [104].

9.11. Clinical Studies. Tohda et al. have investigated the effects of a DG-rich yam extract on cognitive enhancement in 28 healthy volunteers aged between 20 and 81 years recruited from the Toyama Prefecture, Japan. The administration of DG-rich yam extract for 12 weeks significantly improved the semantic fluency without any adverse effects, indicating that DG could enhance the cognitive function in healthy adults [106].

10. Conclusion and Future Perspectives

Diosgenin and its derivatives have attracted considerable attention from researchers worldwide. Several studies have described the pharmacological effects of DG and its derivatives against a variety of diseases such as cancer, diabetes, osteoporosis, AD, and stroke. Several reviews have emphasized the pharmacological advances of DG in the treatment of cancer and described the analytical methodology. In recent years, increasing experimental evidence has shown that DG and its derivatives exhibit promising therapeutic potential in several neurodegenerative and neurological disorders. Therefore, the present review mainly addressed recent progresses of DG and its potent derivatives against multiple diseases of CNS including AD, PD, stroke, neuroinflammation, multiple sclerosis, spinal cord injury, ischemia-induced brain damage, depressive disorders, neuropathic pain, glioblastoma, and cognitive impairment, along with their underlying mechanisms of action at the molecular and cellular levels. This review will facilitate the exploration of new horizons for further research of DG or its derivatives at the preclinical and clinical levels for potential treatment of neurodegenerative disorders.

Although DG is abundant in nature, with high biocompatibility and with thousands of reports elaborating the remarkable pharmacological properties of DG and its derivatives documented in the literature, most of the current results are derived from *in vitro* or animal studies which prevents definitive conclusions about its clinical efficacy. Clinical testing and validation of preclinical data are still insufficient, especially in the treatment of specific neurological disorders. Additional data from clinical trials are highly desired, including test period, dosage, formulation, ethical approvals, adverse effects, drug interactions, and food interactions. Additionally, most patients suffering from neurodegenerative disorders require lifelong medication due to the slow progression of such diseases. Therefore, a systematic experimental design to assess the long-term outcomes of DG and/or its derivatives for the treatment of neurodegenerative disorders and the management of related symptoms is highly recommended in future studies. Furthermore, the risk assessment and safety evaluation of the pharmacological use of DG or its derivatives in the treatment of neurodegenerative disorders need to be investigated in depth.

The successful development of a therapeutic candidate against neurological disorders is a challenge. First, DG suffers

from several drawbacks including low solubility and poor pharmacokinetic profiles which severely restrict its clinical application. Structural modifications or drug delivery systems are reliable techniques to solve these limitations. For structural modification, a balanced analysis of biological activity, solubility, cytotoxicity, and the permeability of blood-brain barrier after modification should be conducted to screen for potential lead compounds for further study. Second, the pathogenesis of neurodegenerative diseases such as AD and PD is complex and multifactorial. The prevention and treatment of those diseases using DG or its derivatives alone might be unsatisfactory. Combination therapies of DG with compounds possessing multiple mechanisms of action are expected to be more effective than individual drugs to treat varied pathological aspects of these diseases. Further, a multitarget drug strategy against multiple risk factors in the development of therapies for neurodegenerative disorders is an essential paradigm and an innovative approach to treat neurological diseases with complex pathogenesis. Numerous studies have indicated that the multifunctional compounds can enhance therapeutic effectiveness and minimize side effects, subsequently leading to better patient compliance via simultaneous modulation of multiple targets in a selective manner.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

CIRBP Ameliorates Neuronal Amyloid Toxicity via Antioxidative and Antiapoptotic Pathways in Primary Cortical Neurons

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It is generally accepted that the amyloid β (Aβ) peptide toxicity contributes to neuronal loss and is involved in the initiation and progression of Alzheimer's disease (AD). Cold-inducible RNA-binding protein (CIRBP) is reported to be a general stress-response protein, which is induced by different stress conditions. Previous reports have shown the neuroprotective effects of CIRBP through the suppression of apoptosis *via* the Akt and ERK pathways. The objective of this study is to examine the effect of CIRBP against Aβ-induced toxicity in cultured rat primary cortical neurons and attempt to uncover its underlying mechanism. Here, MTT, LDH release, and TUNEL assays showed that CIRBP overexpression protected against both intracellular amyloid β- (iAβ-) induced and A$\beta_{25\text{-}35}$-induced cytotoxicity in rat primary cortical neurons. Electrophysiological changes responsible for iAβ-induced neuronal toxicity, including an increase in neuronal resting membrane potentials and a decrease in K^+ currents, were reversed by CIRBP overexpression. Western blot results further showed that A$\beta_{25\text{-}35}$ treatment significantly increased the level of proapoptotic protein Bax, cleaved caspase-3, and cleaved caspase-9 and decreased the level of antiapoptotic factor Bcl-2, but were rescued by CIRBP overexpression. Furthermore, CIRBP overexpression prevented the elevation of ROS induced by A$\beta_{25\text{-}35}$ treatment by decreasing the activities of oxidative biomarker and increasing the activities of key enzymes in antioxidant system. Taken together, our findings suggested that CIRBP exerted protective effects against neuronal amyloid toxicity via antioxidative and antiapoptotic pathways, which may provide a promising candidate for amyloid-based AD prevention or therapy.

1. Introduction

Alzheimer's disease (AD) is an irreversible age-related neurodegenerative disorder, mainly characterized by progressive memory loss and cognitive decline [1]. One of the typical histological hallmarks associated with AD brains is the extracellular plaque deposits of the amyloid β (Aβ) peptides, which is produced by the cleavage of the transmembrane amyloid precursor protein (APP) [2–4]. Studies showed that Aβ peptides rapidly self-aggregates into Aβ dimmers, fibrils, and amyloid plaques and induced neuronal apoptosis in the cultured neuron [5, 6]. On the other hand, recent reports also demonstrated the accumulation of intracellular Aβ (iAβ), especially Aβ_{1-42}, at the early stage of AD development, which occurs earlier than the appearance of Aβ plaques [7]. Direct evidence for iAβ cytotoxicity is that microinjection of A$\beta_{1\text{-}42}$ induces neuronal cell death in cultured human primary neurons [8]. This cytotoxicity effect of causing neuronal cell death is found to be more potent than extracellular Aβ [9]. Currently, the toxicity of Aβ peptides is thought to contribute to the neuronal loss in the cerebral cortex and hippocampus and is involved in the initiation and progression of AD [10]. Although the underlying mechanisms by which Aβ production leads to the cytotoxicity and neuronal loss remains elusive, searching for strategies that can ameliorate Aβ toxicity may be potentially beneficial to AD treatment.

Cold-inducible RNA-binding protein (CIRBP) is a stress-responsive gene, which belongs to a family of cold-shock proteins [11]. In addition to upregulation of CIRBP induced by hypothermia, the expression of CIRBP can also be regulated by other stress conditions, such as hypoxia, UV radiation, glucose deprivation, and osmotic pressure [11]. In response

to stress, CIRBP generally modulates mRNA stability at the posttranscriptional level through its binding site on the 3′-untranslational region (UTR) of its targeted mRNAs [12, 13]. Recent studies have demonstrated that CIRBP exerts neuroprotective effects against H_2O_2-induced cell death through the Akt and ERK pathways in primary rat cortical neurons and neuro2a (N2a) cells [14–16]. The aim of the present study is to investigate how CIRBP reacts to the intracellular and extracellular Aβ treatment, and whether CIRBP can protect against Aβ-induced toxicity in cultured rat primary cortical neurons.

In the present study, we found that CIRBP protected against both intracellular amyloid β- (iAβ-) induced and A$\beta_{25\text{-}35}$-induced cytotoxicity in rat primary cortical neurons. These neuroprotective effects of CIRBP were mediated through antioxidative and antiapoptotic pathways.

2. Material and Methods

2.1. Culture of Primary Cortical Neurons. Newborn Sprague-Dawley (SD) rats were used in these experiments. After cervical dislocation and sterilization by immersion in 75% ethanol, the whole brains were taken out from the head. Cortical tissues then were dissected from the brains in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA). The tissues were mechanically dissociated by gently chopping for about 15 times, and then were digested with 0.25% trypsin (Invitrogen) for 25 minutes at 37°C. After terminating the digestion with DMEM containing 10% fetal bovine serum, the mixture was gently triturated through the pipette to obtain single-cell suspension. The suspension then was filtered through nylon meshes and centrifuged at 500 g for 5 minutes. Single cells were resuspended in DMEM with 10% fetal bovine serum (FBS), 2 g/l HEPES, penicillin G (100 U/ml), and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and plated at a density of 1×10^6/ml on poly-L-lysine-coated plates or coverslips. To inhibit glia cell growth and increase the purity of neurons, 10 μM cytosine arabinoside (Sigma) was added to the medium 24 h after plating. Cells were used for experiments 6 days after culture. All animal experiments were approved by the Animal Care and Use Committee of Harbin Medical University.

2.2. Adenovirus Infection. The rat CIRBP gene was PCR amplified according to the following primers and then was subcloned into the pAdTrack-CMV plasmid (a gift from Bert Vogelstein) [17] through KpnI and XhoI restriction sites. The forward primer is ATGGCATCAGATGAAGGCAA and the reverse primer is TTACTCGTTGTGTGTAGCA TA. The human intracellular Aβ_{1-42} cDNA was synthesized directly, and then was also subcloned into pAdTrack through BglII and XhoI restriction sites. Adenovirus packaging and quality testing were performed in HEK293 cells. The neurons after 6 days in culture were infected by directly adding the adenovirus into the culture medium with an optimized multiplicity of infection (MOI) for 12~24 h. Cellular and biochemical experiments were performed 48 h after infection.

2.3. A$\beta_{25\text{-}35}$ Treatments. A$\beta_{25\text{-}35}$ and a control peptide A$\beta_{35\text{-}25}$ are from Sigma. Before use, 2 mM A$\beta_{25\text{-}35}$ stock solution was prepared by water and aged in a humidified chamber at 37°C for 5 days to obtain aggregates of Aβ peptides. The control peptide A$\beta_{35\text{-}25}$ followed the same procedure. After 12 h infection of adenovirus, the cells were treated with 20 μM A$\beta_{25\text{-}35}$ or A$\beta_{35\text{-}25}$ by directly adding to the medium.

2.4. ELISA Assay. ELISA assay was used to determine the concentrations of A$\beta_{1\text{-}42}$ in the cultured rat cortical neurons after the infection of recombinant adenoviruses using the ELISA kit (R & D Systems) according to the manufacturer's instruction. The microplate reader (Bio-Rad) was used to evaluate the intensity of each well at 480 nm.

2.5. Cell Cytotoxicity Analysis. In this study, the cytotoxicity of the cells after Aβ treatment was assessed by MTT assay and lactate dehydrogenase (LDH) release assay. For MTT assay, cells were seeded in 96-well plates. After treatment, media of the culture neurons were carefully removed by aspiration. After gently washing with PBS, 100 μl cell culture medium containing 0.5 mg/ml MTT was added to each well and incubated at 37°C for 4 h. Then, the medium in the wells was discarded and 150 μl dimethyl sulfoxide was added to each well. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad). LDH release assay was measure using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). This experiment was performed according to the manufacturer's instructions.

2.6. Electrophysiology. The cortical neurons were bathed in an extracellular solution containing (in mM) 140 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 10 HEPES, and 10 glucose, pH 7.35 adjusted with NaOH. 1 μM TTX and 100 μM $CdCl_2$ were also included in the bath solution to block voltage-gated Na^+ and Ca^{2+} channels. The patch pipette solution contained (in mM) 115 K-gluconate, 5 KCl, 5 Na_2-ATP, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, and 10 HEPES, pH 7.2 adjusted with KOH. Pipettes with a resistance of 3–5 M were used in this experiment. The neurons were held at -70 mV, and then depolarized in a whole-cell patch clamp configuration by 1000 ms from -60 to 80 mV with 10 mV steps using an Axon 200B amplifier at room temperature.

2.7. Western Blot Analysis. After washing with PBS for 2 times, the cultured cells were treated with cell lysis buffer to extract the total proteins. A total of 20 μg of protein samples was separated on a 10% SDS-PAGE, and then transferred to a polyvinylidene fluoride (PVDF, Millipore) membrane (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk in Tris-buffered saline Tween-20 (TBST) for 1 h at room temperature, the PVDF membranes were incubated with the primary antibodies at 4°C overnight. After washing, the membranes were further incubated with horseradish peroxidase-(HRP-) conjugated secondary antibodies for 1 h at room temperature. β-Actin was used as a loading control. The intensities of the lanes were quantified using ImageJ software.

2.8. Cell Apoptosis Analysis. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (Roche) was used to evaluate the rate of cell apoptosis according to the manufacturer's instructions. Briefly, cells

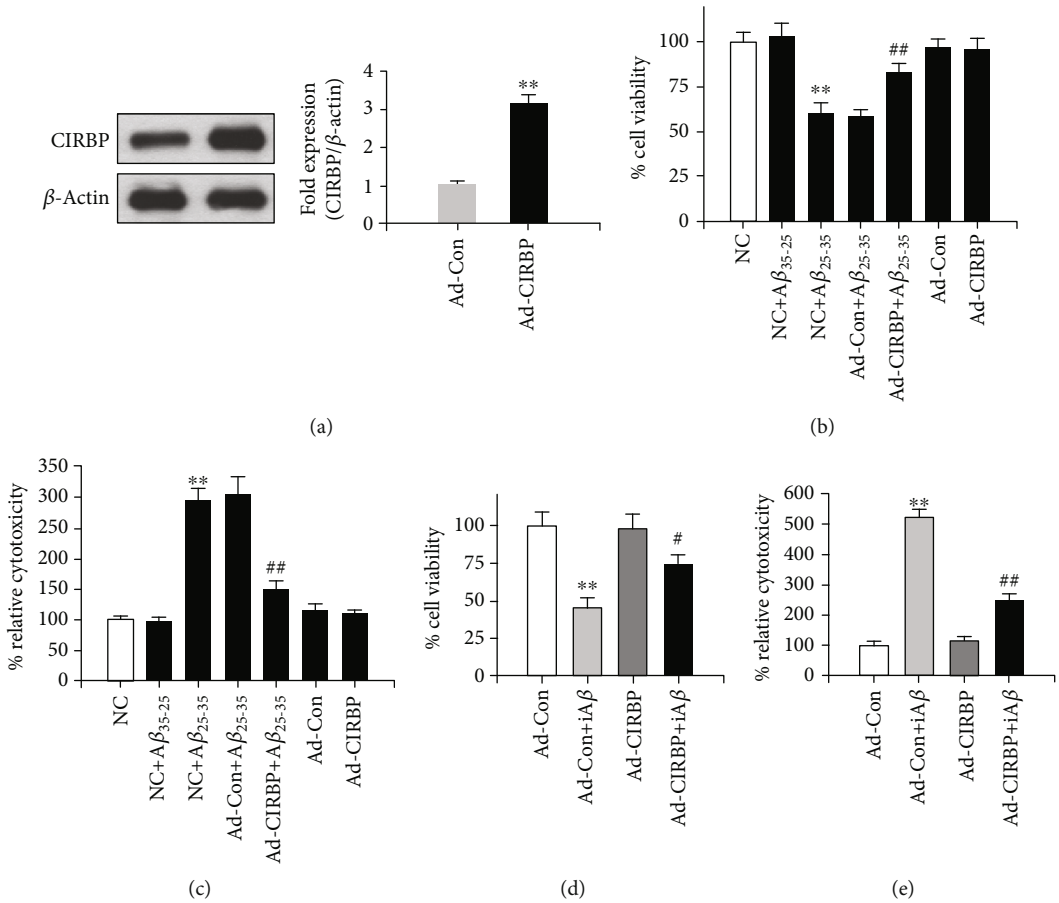

Figure 1: CIRBP protected against Aβ-induced neurotoxicity in rat primary cortical neurons. (a) Western blot result shows CIRBP overexpression in cultured cortical neurons. $^{**}p < 0.01$ *vs.* Ad-Con. (b) Cell viability was measured by MTT assay after treatment with 20 μM $A\beta_{25\text{-}35}$. (c) Cell cytotoxicity was tested by LDH release assay after treatment with 20 μM $A\beta_{25\text{-}35}$. $^{**}p < 0.01$ *vs.* NC, $^{\#\#}p < 0.01$ *vs.* Ad-Con+$A\beta_{25\text{-}35}$. (d) Cell viability was measured by MTT assay after $iA\beta_{1\text{-}42}$ adenovirus infection. (e) Cell cytotoxicity was tested by LDH release assay after $iA\beta_{1\text{-}42}$ adenovirus infection. $^{**}p < 0.01$ *vs.* Ad-Con, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* Ad-Con+iAβ.

were fixed in 4% parafomaldehyde solution for 30 minutes at room temperature and permeabilized in 0.1% Triton X-100 for 5 min. TUNEL reagents then were added and incubated for 1 h at 37°C. After washing with PBS, the cells were mounted with DAPI in a mounting solution.

2.9. Caspase Activity Measurement. The activities of caspase-9 and caspase-3 were measured by using the fluorometric assay kit according to the manufacturer's instructions (Cell Signaling Technology). The protein samples were incubated with the reaction buffer and initiated by the DEVD-AMC substrate in a 96-well plate. The fluorescence intensity was measured using a fluorescence reader with excitation at 380 nm and emission at 460 nm.

2.10. Intracellular ROS Measurement. The measurement of intracellular ROS level was performed using a 2,7-dichlorofluorescein diacetate (DCF-DA) detection kit (Abcam) according to the manufacturer's instruction. Briefly, after washing twice with PBS buffer, adherent neurons were digested with 0.25% trypsin. Then, the cells were resuspended and incubated with 10 μM DCF-DA at 37°C for 30 min. After staining, the DCF fluorescence was detected using a fluorescence spectroscopy with excitation/emission at 495 nm/529 nm (BD Biosciences).

2.11. ELISA. After different treatments, cortical neurons were homogenized in lysis buffer to obtain the protein extracts. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), 4-hydroxy-2-nonenal (4-HNE), and malondialdehyde (MDA) were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions.

2.12. Statistical Analysis. Results were expressed as mean ± SE. Statistical analysis was performed using Student's *t*-test or the Mann–Whitney rank sum test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. CIRBP Overexpression Reduced Aβ-Induced Neurotoxicity in Rat Primary Cortical Neurons. To examine whether CIRBP could reduce Aβ-induced neurotoxicity, rat primary cortical neurons cultured for 6 days were infected with an

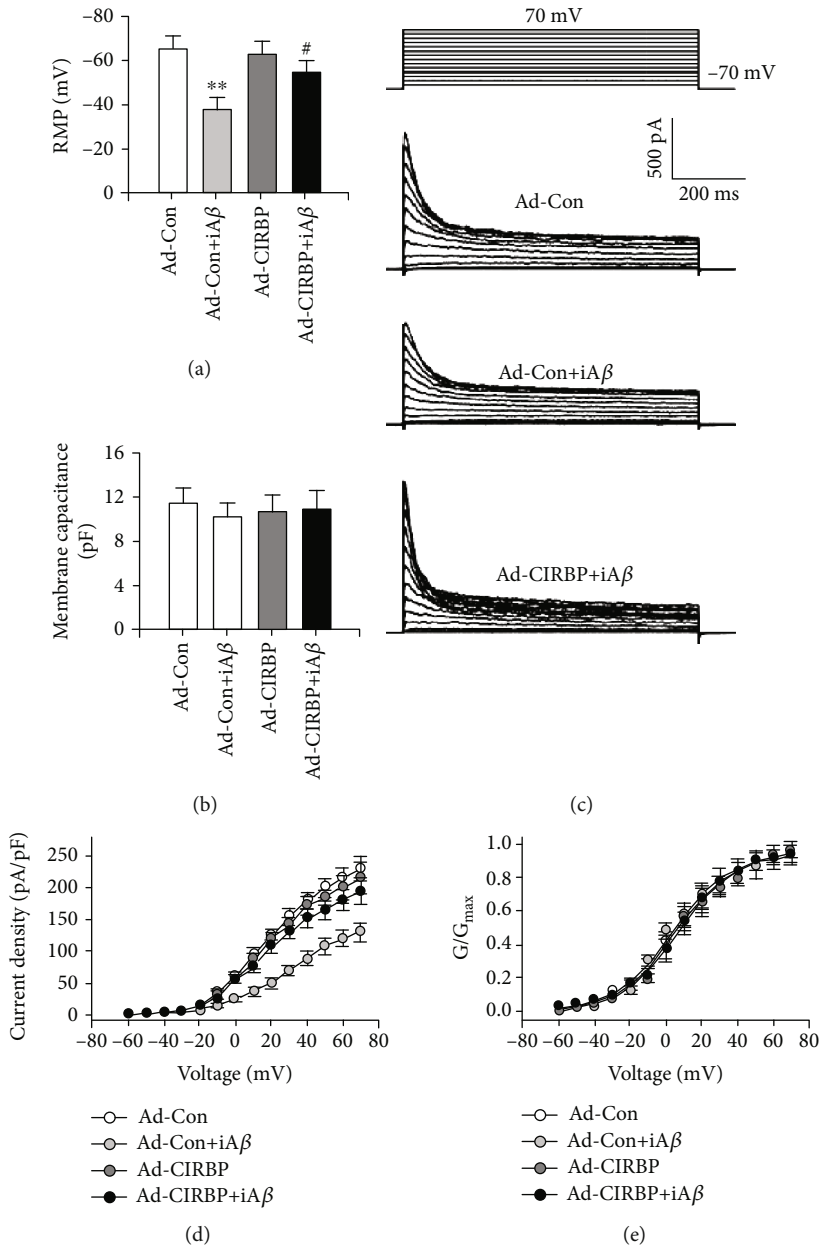

Figure 2: CIRBP reversed iAβ-induced electrophysiological changes that are responsible for neuronal toxicity. (a, b) Resting membrane potentials (RMP) (a) and membrane capacitance (b) were recorded among different groups. $^{**}p < 0.01$ *vs.* Ad-Con, $^{\#}p < 0.05$ *vs.* Ad-Con +iAβ. (c) Typical recordings of whole-cell K^+ current in the Ad-Con, Ad-Con+iAβ, and Ad-CRIPB+iAβ groups by depolarizing the membrane from -70 mV to +70 mV from a holding potential at -70 mV with 10 mV increasing steps. (d) Current-voltage relationship of the peak K^+ current density. $^{**}p < 0.01$ *vs.* Ad-Con. (e) Voltage-dependent activation curve of K^+ current showing there was no difference among different groups.

adenovirus-carrying CIRBP gene (Ad-CIRBP) at a MOI of 2 for 24 h, and then exposed to 20 μM A$\beta_{25\text{-}35}$ or a control peptide A$\beta_{35\text{-}25}$ for another 24 h. Cell viability was analyzed by MTT assay, and cell cytotoxicity was analyzed by LDH release assay. First, CIRBP expression was analyzed by western blot. In the Ad-CIRBP group, CIRBP expression was 3-fold higher than in the control group (Ad-Con) (Figure 1(a)). Although the biochemical organization of A$\beta_{25\text{-}35}$ (monomeric or oligomeric) was not evaluated, MTT assay results showed that compared to the control peptide A$\beta_{35\text{-}25}$, exposure to 20 μM A$\beta_{25\text{-}35}$ dramatically reduced cell viability to about 60%, which was prevented by CIRBP expression in the Ad-CIRBP group, but not in the Ad-Con group (Figure 1(b)). Notably, CIRBP overexpression itself has no effect on cell viability (Figure 1(b)). LDH release assay showed similar results (Figure 1(c)), suggesting that there is a neuroprotective effect of CIRBP against Aβ-induced toxicity.

Here, we also checked the effects of CIRBP on iAβ-induced neurotoxicity. After infection with the adenovirus-

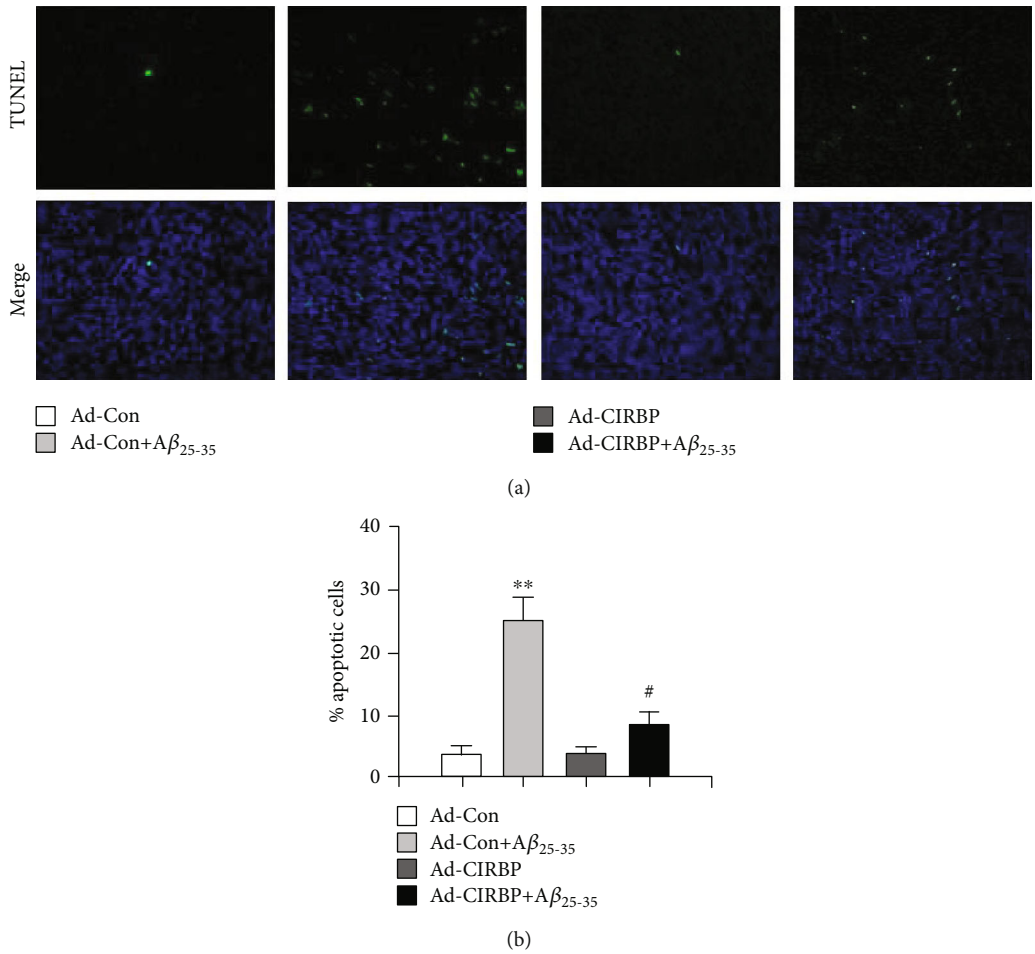

Figure 3: CIRBP prevented $A\beta_{25\text{-}35}$-induced neuronal apoptosis. (a) Apoptotic cells were evaluated by TUNEL staining. Representative TUNEL staining images showing blue nuclear staining by DAPI and green TUNEL staining. (b) The percentage of apoptotic cells was calculated following the formula: Apoptosis index = apoptotic cells/(apoptotic cells + normal cells). $^{**}p < 0.01$ *vs.* Ad-Con, $^{\#\#}p < 0.01$ *vs.* Ad-Con+$A\beta_{25\text{-}35}$.

carrying CIRBP gene for 24 h, rat primary cortical neurons were infected with another $iA\beta_{1\text{-}42}$ adenovirus containing the human $A\beta_{1\text{-}42}$ sequence without any signal peptide for another 24 h. As shown in a previous study [18], this construct mainly expresses $A\beta_{1\text{-}42}$ in the cytosol and has higher toxicity than the construct coding human $A\beta_{1\text{-}42}$ sequence with an addition signal peptide in human neurons. Our result in ELISA assay showed that the production of $A\beta_{1\text{-}42}$ in the cultured rat cortical neurons after the infection of recombinant adenoviruses at the MOI of 0.1 was about 11.2 ng/ml (Supplemental Figure 1). Compared with the results above, $iA\beta_{1\text{-}42}$ dramatically reduced cell viability to about 40% tested by MTT and increased cytotoxicity tested by LDH release assay in the Ad-Con+iAβ group, showing more toxicity of $iA\beta_{1\text{-}42}$ than $A\beta_{25\text{-}35}$ (Supplemental Figure 2). However, this effects of $iA\beta_{1\text{-}42}$ were significantly rescued in the Ad-CIRBP+iAβ group (Figures 1(d) and 1(e)), although the form of intraneuronal Aβ (monomeric or oligomeric) was still unclear. These results indicated that CIRBP overexpression prevented against the $iA\beta_{1\text{-}42}$-induced cytotoxicity.

3.2. CIRBP Overexpression Reversed iAβ-Induced Electrophysiological Changes That Are Responsible for Neuronal Toxicity. The change of electrophysiological properties induced by Aβ treatment, such as an increase in neuronal resting membrane potentials and a decrease in K^+ currents, is thought to be responsible for neuronal toxicity [18–22]. Here, to test whether CIRBP could reverse the iAβ-induced electrophysiological changes, we recorded the resting membrane potentials and K currents in rat primary cortical neurons that were infected with $iA\beta_{1\text{-}42}$ adenovirus with or without CIRBP overexpression. Our results showed that the resting membrane potentials dramatically increased after $iA\beta_{1\text{-}42}$ treatment (-38.2 ± 4.5 mV) compared with the Ad-Con group (-64.8 ± 6.2 mV) (Figure 2(a)). Moreover, $iA\beta_{1\text{-}42}$ also reduced the whole-cell K^+ current density in the Ad-Con+iAβ group (Figures 2(c) and 2(d)) but did not alter the voltage-dependent activation of K^+ currents (Figure 2(e)). These results were consistent with previous studies [18, 20–22]. In this study, we found that CIRBP overexpression rescued the loss of resting membrane potentials (-55.4 ± 4.2 mV) and the decrease of K^+ currents induced

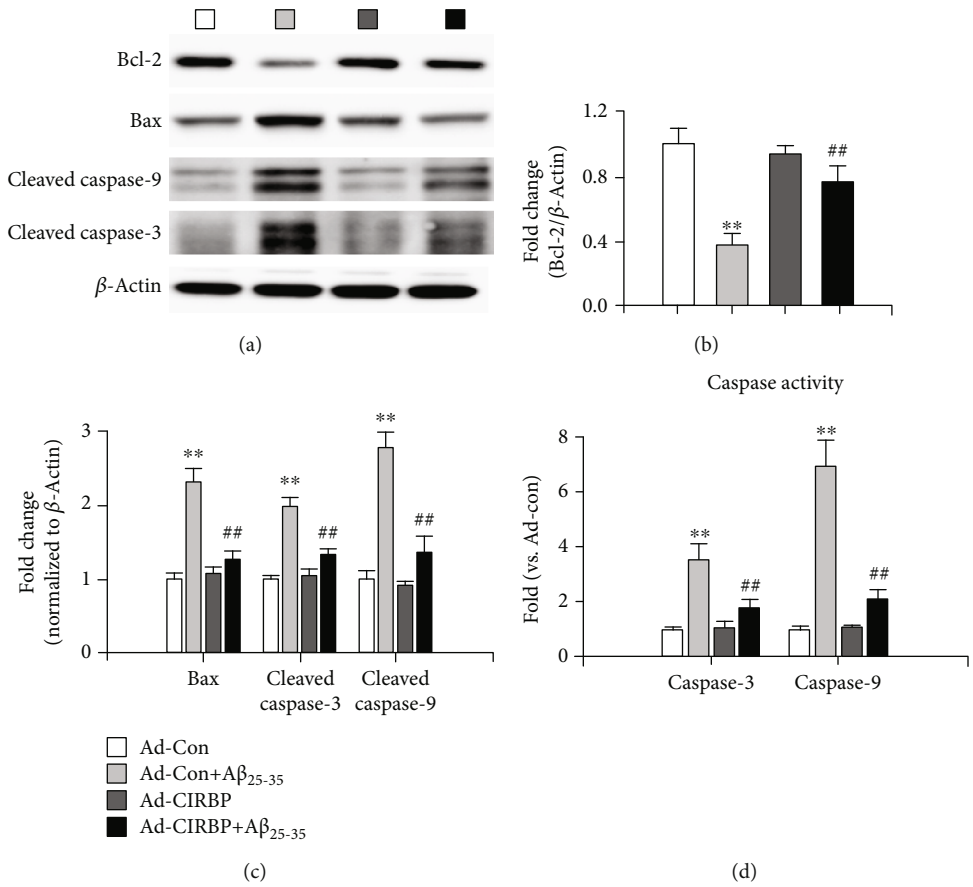

Figure 4: CIRBP inhibited A$\beta_{25\text{-}35}$-induced neurotoxicity via the antiapoptotic pathway. (a) Representative imaging of western blot assay. (b) The expression level of Bcl-2 normalized to the expression level of β-actin. (c) The expression level of Bax, cleaved caspase-3, and cleaved caspase-9. (d) The caspase-3 and caspase-9 activities measured by a commercial fluorescent quantitative detection kit. $^{**}p < 0.01$ *vs*. Ad-Con, $^{\#\#}p < 0.01$ *vs*. Ad-Con+A$\beta_{25\text{-}35}$.

by iA$\beta_{1\text{-}42}$ (Figures 2(a) and 2(d)). However, CIRBP overexpression itself had no effect on the resting membrane potentials (-63.1 ± 5.2 mV) and K^+ current density (Figures 2(a) and 2(d)). The membrane capacitance did not change either in iAβ treatment or CIRBP overexpression (Figure 2(b)). These results indicated that CIRBP ameliorated Aβ-induced neurotoxicity by rescuing the electrophysiological properties of the neurons.

3.3. CIRBP Overexpression Prevented Aβ-Induced Activation of Cell Apoptosis Pathway in Cortical Neurons. To elucidate how CIRBP protected against Aβ-induced neurotoxicity in rat primary cortical neurons, we used TUNEL assay to measure the cell apoptosis in each group. Compared with the Ad-Con group, 20 μM A$\beta_{25\text{-}35}$ treatment significantly increased the percentage of cell apoptosis in the Ad-Con+A$\beta_{25\text{-}35}$ group, while overexpression of CIRBP markedly attenuated cell apoptosis induced by A$\beta_{25\text{-}35}$ (Figure 3). However, no significant difference was observed between the Ad-Con and Ad-CIRBP groups (Figure 3).

Next, western blot was performed to evaluate the expression of some key apoptosis-related genes, such as antiapoptotic factor, B cell leukemia/lymphoma-2 (Bcl-2) and proapoptosis factors, Bcl-2 associated X protein (Bax), cleaved caspase-9, and cleaved caspase-3. As shown in Figures 4(a) and 4(b), the protein level of antiapoptotic factor, Bcl-2, was significantly decreased after A$\beta_{25\text{-}35}$ treatment but was reversed by CIRBP overexpression. In contrast, the proapoptosis factors, Bax, cleaved caspase-9, and cleaved caspase-3, were all upregulated in the Ad-Con+A$\beta_{25\text{-}35}$ group, which were inhibited by CIRBP overexpression (Figures 4(a) and 4(c)). We further measured the activities of caspase-9 and caspase-3 by using commercial fluorescent quantitative detection kit. Consistent with the western bolt result, 20 μM A$\beta_{25\text{-}35}$ treatment significantly increased the activities of caspase-9 and caspase-3, while CIRBP overexpression ameliorate this effect (Figure 4(d)). Thus, these results indicated that the underlying mechanism of CIRBP protective effect against neuronal amyloid toxicity may be mediated by the antiapoptotic pathway.

3.4. CIRBP Overexpression Inhibited Aβ-Induced Oxidative Stress in Cortical Neurons. Oxidative stress, such as reactive oxygen species (ROS) generation, induced by Aβ is one of the major causes that lead to neuronal apoptosis [23]. Therefore, we further examined whether CIRBP could suppress Aβ-induced oxidative stress in cortical neurons. Intracellular ROS level in cortical neurons was assessed by using a ROS-

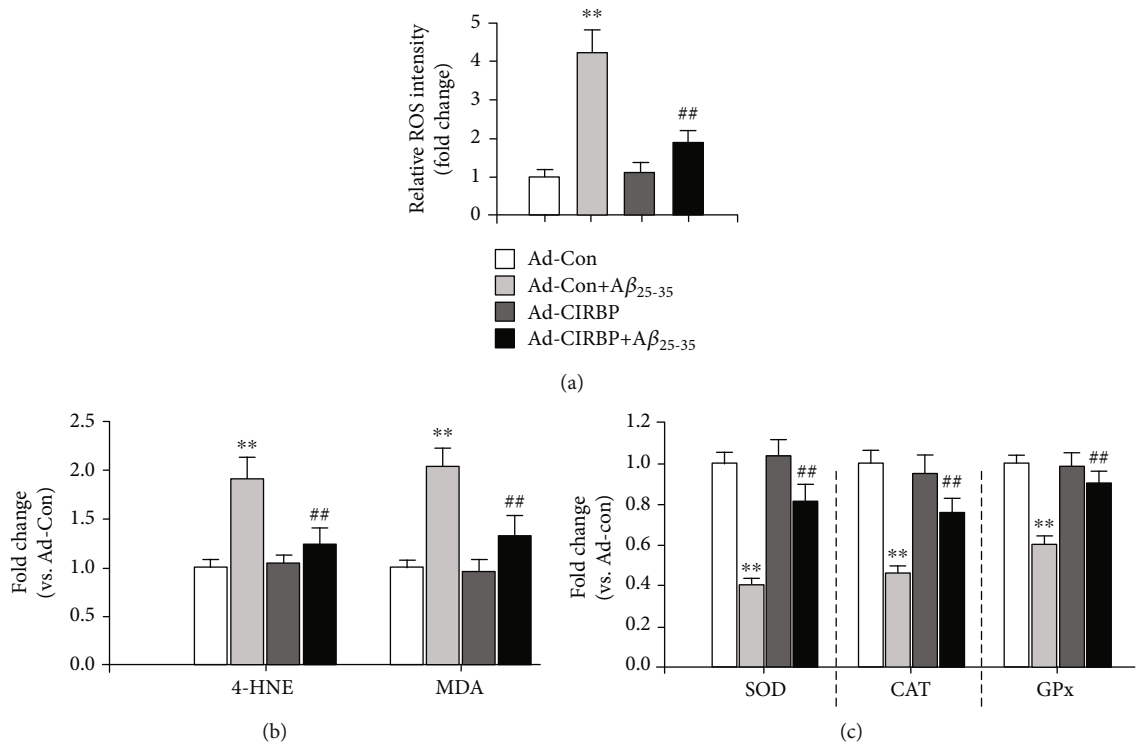

Figure 5: CIRBP inhibited $A\beta_{25\text{-}35}$-induced neurotoxicity via the antioxidative pathway. (a) Intracellular ROS level was measured using a ROS-detecting fluorescence dye DCF-DA kit. (b) The activities of oxidative stress biomarkers, such as 4-HNE and MDA, measured by ELISA assay. (c) The activities of key enzymes in an antioxidant system, such as SOD, CAT, and GPx, were measured by ELISA. $^{**}p < 0.01$ *vs.* Ad-Con, $^{\#\#}p < 0.01$ *vs.* Ad-Con+$A\beta_{25\text{-}35}$.

detecting fluorescence dye DCF-DA kit. As expected, the level of ROS in cortical neurons treated with 20 μM $A\beta_{25\text{-}35}$ was significantly elevated to 4.3-fold compared with the Ad-Con group. However, CIRBP overexpression remarkably inhibited the ROS generation induced by $A\beta_{25\text{-}35}$ (Figure 5(a)). Next, the activities of oxidative marker, such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) and key enzymes in the antioxidant system, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), were measured by ELISA. As shown in Figures 5(b) and 5(c), CIRBP overexpression significantly rescued $A\beta_{25\text{-}35}$-induced upregulation of oxidative marker and $A\beta_{25\text{-}35}$-induced downregulation of antioxidase activities of these three antioxidant enzymes, indicating that the antioxidative pathway mediated the protective effect of CIRBP against $A\beta$-induced neuronal apoptosis.

4. Discussion

AD is one of the neurodegenerative disorders in the elderly with extremely deficient clinical therapies and is associated with high morbidity and mortality [1]. AD is clinically characterized by progressive memory loss and cognitive decline [1]. One of the major pathological hallmarks in AD is the appearance of amyloid plaques that is enriched in $A\beta$ [2–4]. Extensive studies suggest that the neurotoxicity of $A\beta$ contributes to the neuronal loss and involves in the pathogenesis of neuronal dysfunction in AD [5, 24]. Although the underlying mechanisms are still largely unknown, accumulated evidences have showed that natural products or genetical manipulation that protect against $A\beta$-induced neuronal loss are beneficial to the treatment of AD [18, 25]. In the present study, we found that CIRBP, a stress-response protein, attenuated $A\beta$-induced cytotoxicity via antioxidative and antiapoptotic pathways in cultured rat primary cortical neurons, which may provide a promising candidate for amyloid-based AD prevention or therapy.

$A\beta$-mediated electrophysiological changes have been extensively studied in human primary neurons, rodent neurons, and different cell lines [18–22]. Dysfunction of neuronal excitability is responsible for the neuronal toxicity induced by $A\beta$ [26]. Here, we found that i$A\beta$ induced significant increase in resting membrane potential and decrease in K^+ current density in the Ad-Con+i$A\beta$ group, which is consistent with previous reports [18, 20–22]. Suppression of K^+ current by $A\beta$ was reported to trigger a large increase of Ca^{2+} influx by activating voltage-gated Ca^{2+} channels in the distal dendrites of rat hippocampal neurons [20]. The Ca^{2+} imbalance is suggested to initiate neuronal dysfunction and lead to cell death in rat hippocampus cells. In the present study, CIRBP overexpression rescued the resting membrane potential, probably by increasing the K^+ current density, to maintain normal neuronal excitability. A previous report in the heart showed that CIRBP regulated cardiac repolarization by reducing the expression and function of transient outward K^+ current [27]. Our present study identified a role of CIRBP in regulating another K^+ channel, although the underlying mechanism still needs further investigation.

CIRBP, a cold-shock protein found in mammals, expressed in various cell types and is involved in multiple biological and cellular processes, such as cell survival, apoptosis, cell proliferation, circadian rhythm, immune response, reproduction, and cancer [11]. Upregulation of CIRBP at mild hypothermia is reported to suppress cell death and contribute to cell survival [16, 28, 29]. Li et al. first demonstrated that CIRBP upregulation in rat cortical neurons at low temperature inhibited H_2O_2-induced neuronal apoptosis [16]. Liu et al. also found that CIRBP protected against oxidative stress via the Akt and ERK signal transduction pathways in N2a cells [15]. Sakurai et al. observed that mild hypothermia-induced elevated CIRP levels inhibited tumor necrosis factor-alpha-induced apoptosis by caspase-8 activation and ERK phosphorylation [30]. Zhang et al. revealed a neuroprotective effect of CIRBP during mild hypothermia by inhibiting neuron apoptosis via the suppression of the mitochondria apoptosis pathway [14]. Consistent with these previous reports, we found that CIRBP overexpression significantly attenuated Aβ-induced neuronal apoptosis and exerted a neuroprotective effect in cultured rat cortical neurons. Mitochondrial signaling pathway plays an important role in promoting apoptotic cell death [31]. In our study, CIRBP overexpression rescued the decreased protein expression of antiapoptotic factor-Bcl-2 and also inhibited the increased protein expression of proapoptosis factor Bax, cleaved caspase-9, and cleaved caspase-3. All these results indicated that CIRBP suppressed Aβ-induced neuronal apoptosis, probably via mitochondrial signaling pathway.

Aβ-induced neuronal apoptosis is associated with the generation of ROS and plays an important role in the pathogenesis of AD [23]. Various compounds with antioxidant ability have been proposed to attenuate Aβ-induced oxidative stress in studies done *in vitro* and *in vivo* [32]. In this study, we showed that A$\beta_{25\text{-}35}$ treatment dramatically elevated intracellular ROS levels in culture primary cortical neurons, which is consistent with previous studies [23, 32]. However, CIRBP overexpression largely prevented A$\beta_{25\text{-}35}$-induced excessive ROS release, confirming the antioxidative activity of CIRBP. In addition, we evaluated the activities of primary antioxidant enzyme, such as SOD, CAT, and GPx. SOD can scavenge superoxide anions by catalyzing them to hydrogen peroxide and molecular oxygen. CAT can detoxify hydrogen peroxide by converting them to oxygen and water. GPx can remove hydrogen peroxide by catalyzing them to water. Our results suggested that CIRBP overexpression significantly suppressed the downregulation of SOD, CAT, and GPx activities induced by Aβ. Thus, CIRBP inhibits oxidative damage-induced mitochondrial dysfunction and cell apoptosis.

5. Conclusions

In conclusion, we demonstrate a neuroprotective effect of CIRBP against Aβ induced-cytotoxicity through antioxidative and antiapoptotic pathways in rat primary cortical neurons, which may provide a novel therapeutic strategy for amyloid-based AD prevention or therapy. To the best of our knowledge, this is the first study to evaluate the function of CIRBP against Aβ-induced neurotoxicity.

Data Availability

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

Conflicts of Interest

All authors declare no conflict of interest.

Authors' Contributions

Fang Su and Shanshan Yang contributed equally to the work.

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Supplementary Materials

Supplemental Figure 1: The production of Aβ1-42 in the cultured rat cortical neurons after the infection of recombinant adenoviruses at the MOI of 0.1. ND: no detection. $^{**}p < 0.01$ vs. Control. Supplemental Figure 2: MTT results showed no toxicity in the control peptide Aβ35-25, and more toxicity is in the intraneuronal Aβ1-42 than Aβ25-35. $^{**}p < 0.01$ vs. NC, $^{\#\#}p < 0.01$ vs. NC+Aβ25-35. *(Supplementary Materials)*

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Review Article

Application of Acupuncture to Attenuate Immune Responses and Oxidative Stress in Postoperative Cognitive Dysfunction: What Do We Know So Far?

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Postoperative cognitive dysfunction (POCD) is a common sequela following surgery and hospitalization. The prevention and management of POCD are important during clinical practice. POCD more commonly affects elderly patients who have undergone major surgery and can result in major decline in quality of life for both patients and their families. Acupuncture has been suggested as an effective intervention for many neurological disorders. In recent years, there are increasing interest in the use of acupuncture to prevent and treat POCD. In this review, we summarized the clinical and preclinical evidence of acupuncture on POCD using a narrative approach and discussed the potential mechanisms involved. The experimental details and findings of studies were summarized in tables and analyzed. Most of the clinical studies suggested that acupuncture before surgery could reduce the incidence of POCD and reduce the levels of systematic inflammatory markers. However, their reliability is limited by methodological flaws. Animal studies showed that acupuncture reduced cognitive impairment and the associated pathology after various types of surgery. It is possible that acupuncture modulates inflammation, oxidative stress, synaptic changes, and other cellular events to mitigate POCD. In conclusion, acupuncture is a potential intervention for POCD. More clinical studies with good research design are required to confirm its effectiveness. At the same time, findings from animal studies will help reveal the protective mechanisms, in which systematic inflammation is likely to play a major role.

1. Introduction

Postoperative cognitive dysfunction (POCD) is a common complication following surgery and hospitalization [1]. In 1955, Dr. Bedford first reported symptoms of cognitive changes and behavioral abnormalities in elderly patients who underwent general anesthesia, resulting in increasing research regarding cognitive impairment that occurs following various types of surgery, particularly cardiac surgery [2]. POCD can have profound impact on patients and their families. The most immediate effect can be observed for in-hospital education, where POCD can reduce the ability of the patient to understand and recall detailed instructions, such as wound care and drug treatment, putting the patient at higher risks of postsurgical complications. Over the long term, patients with POCD require more assistance in their

daily lives than before surgery, due to the partial loss of abilities to perform normal daily activities [3]. POCD also decreases the quality of life for patients [4], and it is associated with higher mortality after surgery [5, 6]. Recently, researchers have also begun to suspect that POCD can be a harbinger for dementia because POCD and dementia share common mechanisms and there is considerable overlap in the risk factors for both diseases [7]. Alzheimer's disease, in particular, may be accelerated by POCD [8]. Therefore, prevention and management of POCD are important in clinical practice. Pharmacological interventions and advances in perioperative management are continually devloped, and their implications for POCD have been reviewed elsewhere [9, 10]. This article focuses on the use of acupuncture for POCD management. In the last decade, more than 30 clinical studies have been conducted to investigate whether acupuncture can prevent or treat POCD. A number of animal studies have also revealed the potential mechanisms underlying the effects of acupuncture during POCD prevention and treatment. In this article, we will summarize and analyze the findings from clinical trials and animal studies examining acupuncture. Thereafter, we will discuss the effects of acupuncture on inflammation, oxidative stress, synaptic changes, and other cellular events, which may potentially explain its effects during the treatment and prevention of POCD.

2. Effects of Acupuncture on POCD

Acupuncture has frequently been used to treat neurological and mental disorders. According to the Traditional Chinese Medicine theory, acupuncture balances the body and restores its physiological functions by stimulating specific acupoints through the insertion and manipulation of thin needles. The two most commonly used types of acupuncture techniques are manual acupuncture (MA) and electroacupuncture (EA). During MA, clinical efficacy can be achieved by lifting, thrusting, or rotating the needles by hand until "De-qi" (an irradiating feeling considered to be indicative of effective needling) is attained. During EA, electrical currents are passed through the needles, resulting in the combined therapeutic effect of MA and continuous electric pulses. Data from clinical trials and basic sciences studies suggest that MA and EA are different in terms of clinical outcomes and underlying physiology, but it is difficult to conclude which one is superior [11]. To stimulate acupoints, some researchers also use transcutaneous electrical nerve stimulation (TENS) in which low-voltage electric currents are applied to the skin surface by conducting gel pads. TENS does not involve the use of thin metal needles for stimulation. It is generally not regarded as acupuncture and therefore will not be discussed in this review. Since acupuncture is nonpharmacologically based, there are no concerns regarding dependence, addiction, tolerance, and neurological toxicity, nor will acupuncture increase the metabolic burdens of the liver and kidney, making acupuncture a potentially attractive therapy for treating POCD.

3. Evidence from Clinical Trials

Many clinical trials have been conducted to investigate whether acupuncture is beneficial for POCD. All of the studies were conducted in China, and most of them were published in Chinese within the last 10 years in line with the increased awareness and concern regarding POCD. As this is not a systematic review, a narrative summary on the findings followed by comments on the methodology of these studies will be provided. Table 1 summarizes the main findings from these clinical studies. We have classified the studies into two categories, according to the time when acupuncture treatment was given and when POCD was diagnosed.

In the first category, researchers focused on the preconditioning effects of acupuncture and asked whether acupuncture pretreatment could reduce the incidence of POCD. The majority of the studies listed in Table 1 belong to this category. Acupuncture was given to the patients either before surgery or was started before and then continued throughout the entire operation. We also included a subgroup under this category, in which the acupuncture session started after the surgery (usually on the next day) and was continued daily for a few days. We considered this to be a preconditioning protocol because acupuncture was applied before any diagnosis of POCD. The timing of POCD development in these patients was also unclear; however, acupuncture was given to all patients in the study, regardless of a POCD diagnosis. The acupuncture treatment protocol varied greatly among the studies, and it is difficult to conclude which protocol provided the best results. However, almost all of the studies reported a positive effect for the treatment, except those conducted by Yang et al. [12] and Zhou et al. [13]. The incidence of POCD was evaluated for one week in most of the studies and was reported to be significantly lower in the acupuncture groups than the control groups. The long-term effects of acupuncture remain unclear, as only a few studies included follow-up periods longer than 2 weeks. Overall, DU20 (Baihui), PC6 (Neiguan), and ST36 (Zusanli) were the most commonly used acupoints for POCD pretreatments.

In the second category, there was only one study identified that focused on whether acupuncture can treat POCD [14]. Acupuncture was given to patients who had been diagnosed with POCD and were receiving conventional rehabilitation treatments. The authors reported that patients who received acupuncture in addition to usual rehabilitation treatments had higher Mini-Mental State Examination (MMSE) scores than those who only received the usual rehabilitation treatments. However, this study focused on patients who received surgery to treat traumatic brain injury, which is well-known to cause cognitive dysfunction. The results of this study must therefore be carefully interpreted, as it includes more confounding variables, such as the types and severity of the underlying traumatic brain injury. Since no detailed descriptions of the underlying injuries were provided in the paper, the reliability of the results remains questionable [14].

Although the data from clinical studies appears to be promising, it is too early to draw any conclusions. We found that many reports have missing components in their

Table 1: Summary of clinical trials examining the effects of acupuncture on POCD.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
Yang et al./2009 [12]	Coronary artery bypass grafting or cardiac valve replacement surgery	(i) Control: usual care/$n = 37$ (ii) Intervention: usual care+EA/$n = 38$	EA, 5 days before surgery, 30 min/day	LU2, LU7, PC6	MMSE, digit span subtest, digit symbol subtest, trail making test, short story memory test	(i) No differences in POCD incidence rates at postoperative days 7 and 14 between the two groups
Zhou et al./2011 [13]	Off-pump coronary artery bypass grafting	(i) Control: usual care +sham EA/$n = 18$ (ii) Intervention: usual care+EA/$n = 18$	EA, 30 min before surgery to the end of surgery	PC6	MMSE	(i) No differences in POCD incidence rates at the postoperative time points of 1 week and 1 month between the two groups
Zhang et al./2012 [100]	Hip or knee replacement surgery	(i) Control: usual care/$n = 47$ (ii) Intervention: usual care+EA/$n = 41$	EA, 30 min before surgery	DU20, DU24	MMSE, serum S100β	(i) Lower POCD incidence rate at postoperative day 1 in the intervention group (ii) No differences in postoperative serum S100β levels between the groups
Gao et al./2012 [92]	Noncardiac surgery	(i) Control: usual care/$n = 60$ (ii) Intervention: usual care+EA/$n = 60$	EA, 30 min before surgery to the end of surgery	DU20, PC6, ST36, LI4	MMSE, PONV incidence	(i) Incidence rates of POCD were lower at postoperative days 2 and 4 in the intervention group (ii) Lower PONV incidence in the intervention group
Lin et al./2013a [69]	Intestinal cancer resection	(i) Control: usual care/$n = 24$ (ii) EA1: usual care +EA, continuous wave, 2 Hz/$n = 26$ (iii) EA2: usual care +EA, sparse-dense wave, 2-100 Hz/$n = 25$ (iv) EA3: usual care +EA, continuous wave, 100 Hz/$n = 24$ (v) TENS: usual care +TENS, sparse-dense wave, 2-100 Hz/$n = 25$	EA/TENS, 30 min before surgery to the end of surgery	DU20, EX-HN3, PC6	MMSE, serum S100β	(i) Lower POCD incidence rate at postoperative day 3 in the EA and TENS group (ii) Lower postoperative serum S100β levels in the EA and TENS group

Table 1: Continued.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
Lin et al./2013b [71]	Intestinal cancer resection	(i) Control: usual care/$n = 37$ (ii) Intervention: usual care+EA/$n = 38$	EA, 20 min before surgery to the end of surgery	DU20, PC6, SP6, ST36	MMSE, serum S100β	(i) Lower POCD incidence rate at postoperative day 3 in the intervention group (ii) Lower postoperative serum S100β levels in the intervention group
Lin et al./2014 [86]	Gastrointestinal cancer resection	(i) Control: usual care/$n = 41$ (ii) Intervention: usual care+EA/$n = 42$	EA, 30 min before surgery to the end of surgery	DU20, PC6, ST36	MMSE, serum IL-1β, IL-6, TNF-α	(i) Lower POCD incidence rate at postoperative day 3 in the intervention group (ii) Lower postoperative serum IL-6, IL-1β, and TNF-α levels in the intervention group (iii) Reduced amounts of anesthetic agents were used in the intervention group
Wang et al./2014 [101]	Hip replacement surgery	(i) Control: usual care/$n = 40$ (ii) Intervention: usual care+EA/$n = 40$	EA, 1 day before and 1 day after surgery, once daily, 30 min EA during surgery	Scalp acupuncture lines MS1, MS5, 2/5 middle of MS7, MS10	Neuropsychological test (did not mention the details)	(i) Lower POCD incidence rates at the postoperative time points of 6 days, 1 week, 3 months, and 6 months in the intervention group (ii) Incidence rates of POCD at the postoperative time points of 3 days, 1 week, 3 months, and 6 months were lower in the intervention group
Zhang et al./2014 [89]	Abdominal surgery	(i) Control: usual care/$n = 60$ (ii) Intervention: usual care+EA/$n = 60$	EA, 30 min before surgery to the end of surgery	DU20, DU24, PC6	MMSE, PONV incidence	(i) Lower POCD and PONV incidence rates at the postoperative time point of 48 h in the intervention group (ii) Reduced amounts of anesthetic agents were used in the intervention group (iii) Lower PONV incidence in the intervention group

Table 1: Continued.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
Zhang et al./2014 [102]	Knee replacement surgery	(i) Control: usual care/$n = 45$ (ii) Intervention: usual care+EA/$n = 34$	EA, 30 min before surgery	DU20, DU24	MMSE, serum IL-1β, IL-6, TNF-α, S100β	(i) Lower POCD incidence rate at postoperative day 1 in the intervention group (ii) No differences in postoperative serum IL-1β, IL-6, TNF-α, and S100β levels between the groups
Zhou et al./2014 [38]	Lumbar spinal stenosis surgery	(i) Control: usual care/$n = 30$ (ii) Intervention: usual care+EA/$n = 30$	EA, 30 min before surgery to the end of surgery	LI4, PC6, SP6, ST36	MMSE, serum IL-6, IL-10, S100β	(i) Lower POCD incidence rate at the postoperative time point of 72 h in the intervention group (ii) Lower postoperative serum IL-6, IL-10, and S100β levels in the intervention group (iii) Reduced amounts of anesthetic agents were used in the intervention group
Chen/2015 [90]	Gynecological laparoscopic surgery	(i) Control: usual care/$n = 30$ (ii) Intervention: usual care+EA/$n = 30$	EA, 30 min before and 30 min after surgery	LI4, PC6	MMSE, QoR-40, serum IL-6, β-EP, 5-HT, PONV incidence	(i) Higher MMSE scores and lower PONV incidence at postoperative day 2 in the intervention group (ii) Higher QoR-40 scores at postoperative days 1 and 2 in the intervention group (iii) Lower postoperative serum IL-6 and 5-HT levels, higher β-EP levels in the intervention group (iv) Lower PONV incidence in the intervention group
Chen/2015 [65]	Laparoscopic cholecystectomy	(i) Control: usual care/$n = 62$ (ii) Intervention: usual care+EA/$n = 62$	EA, 15-30 min before surgery to the end of surgery	GB34, LI4, PC6, ST36	MMSE, serum S100β, NSE	(i) Lower POCD incidence rate at postoperative day 3 in the intervention group (ii) Lower postoperative serum S100β and NSE levels in the intervention group (iii) Reduced amounts of anesthetic agents were used in the intervention group

Table 1: Continued.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
Jiang et al./2015 [103]	Hip or knee replacement surgery	(i) Control: usual care/$n = 45$ (ii) Intervention: usual care+EA/$n = 43$	EA, 5 days before surgery, 30 min/day	DU14, DU20	MMSE	(i) Lower POCD incidence rate at postoperative day 1 in the intervention group
Jiang/2015 [67]	Coronary artery bypass grafting	(i) Control: usual care +sham EA/$n = 40$ (ii) Intervention: usual care+EA/$n = 40$	EA, 30 min before surgery to the end of surgery	PC6	MMSE, serum S100β, NSE	(i) Lower POCD incidence rate at postoperative day 3 in the intervention group (ii) Lower postoperative serum S100β and NSE levels in the intervention group
Qing and Jiang/2015 [14]	Surgery for brain trauma	(i) Control: usual care/$n = 80$ (ii) Intervention: usual care+MA/$n = 80$	MA (scalp acupuncture) after surgery, 30 min/day for 24 days	DU17, DU24, GB13, GB19, and other scalp acupoints	MMSE	(i) Higher MMSE scores at postoperative day 24 in the treatment group
Yang et al./2015 [104]	Gastrointestinal cancer resection	(i) Normal: patients without diabetes/$n = 45$ (ii) Control: usual care/$n = 45$ (iii) Intervention: usual care+EA/$n = 45$	EA, 20 min before surgery to the end of surgery	DU20, LI10, LI11, PC6	MMSE, serum IL-1β, IL-6	(i) Lower POCD incidence rate at postoperative day 3 in the intervention group (ii) Serum IL-1β and IL-6 levels at the postoperative time points of 1 and 24 were lower in the intervention group compared with those in the control group but higher compared with those in the normal group
Zhang et al./2015 [105]	Laparoscopic cholecystectomy	(i) Control: usual care +sham EA/$n = 35$ (ii) Intervention: usual care+EA/$n = 35$	EA after surgery, 20 min/day for 7 days	DU20, PC6	MMSE	(i) Lower POCD incidence rate at postoperative days 1 and 3 in the intervention group
Dong et al./2016 [91]	Intestinal cancer resection	(i) Control: usual care/$n = 30$ (ii) Intervention: usual care+EA/$n = 30$	EA, 30 min before surgery to the end of surgery	DU20, PC6	MMSE, PONV incidence	(i) Lower POCD incidence rates at postoperative days 1 and 3 in the intervention group (ii) Lower PONV incidence rate at postoperative day 7 in the intervention group

Table 1: Continued.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
Xie et al./2016 [106]	Hip replacement surgery	(i) Control: usual care/$n = 60$ (ii) Intervention: usual care+EA/$n = 60$	EA, 30 min before the end of surgery+after surgery, daily for 2 days, 30 min	DU24, GB13	PQRS	(i) Lower POCD incidence rates at postoperative days 1, 2, 3, and 5 in the intervention group
Yu et al./2016 [107]	Intestinal cancer resection	(i) Control: usual care/$n = 59$ (ii) Intervention: usual care+EA/$n = 59$	EA during surgery	DU20, PC6, SP6, ST36	MMSE	(i) Lower POCD incidence rate at the postoperative time points of 6 and 12 h in the intervention group
Yuan et al./2016 [72]	Extracerebral intervention	(i) Control: usual care/$n = 61$ (ii) Intervention: usual care+EA/$n = 61$	EA, 30 min before surgery	DU20, PC6, EX-HN3	MMSE, serum IL-1β, IL-6, TNF-α, S100β, NSE	(i) Lower POCD incidence rate at postoperative day 1 in the intervention group (ii) Lower postoperative serum IL-1β, IL-6, TNF-α, S100β, and NSE levels in the intervention group
Lin et al./2016 [70]	Carotid endarterectomy	(i) Control: usual care/$n = 25$ (ii) Intervention: usual care+EA/$n = 25$	EA, 30 min before surgery to the end of surgery	DU20, PC6, ST36	MoCA, plasma TNF-α, S100β, BDNF	(i) Higher MoCA scores at postoperative days 1, 3, and 7 in the intervention group (ii) Lower postoperative serum TNF-α and S100β levels in the intervention group (iii) Plasma BDNF levels in the intervention group plasma
Li/2017 [108]	Various types	(i) Control: usual care/$n = 40$ (ii) Intervention: usual care+EA/$n = 40$	EA, 30 min before surgery to the end of surgery	DU20, PC6, ST36	Serum TNF-α, IL-1β, IL-6	(i) Lower postoperative serum IL-1β and TNF-α levels in the intervention group
Li et al./2016 [68]	Hip replacement surgery	(i) Control: usual care/$n = 42$ (ii) Intervention: usual care+EA/$n = 42$	EA during surgery	MS1, MS5	Neuropsychological test, serum S100β, NSE	(i) Lower POCD incidence rates at the postoperative time points of 3 days, 1 week, 3 months, and 6 months in the intervention group (ii) Lower postoperative serum NES and S100β levels in the intervention group

Table 1: Continued.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
Liu et al./2017 [73]	Hip replacement surgery	(i) Control: usual care/$n = 40$ (ii) Intervention: usual care+EA/$n = 40$	EA, 3 days before and 3 days after surgery, once daily, 30 min+30 min before surgery to the end of surgery	LI4, LR3	MMSE, serum IL-1β, TNF-α, S100β, NSE	(i) Lower POCD incidence rate at postoperative day 4 in the intervention group (ii) Lower postoperative serum IL-1β, TNF-α, NSE, and S100β levels in the intervention group
Liu and Teng/2017 [109]	Tumor resection	(i) Control: usual care/$n = 49$ (ii) Intervention: usual care+EA/$n = 49$	EA, 30 min before surgery to the end of surgery	DU20, PC6, ST36	MMSE, serum IL-1β, IL-6, TNF-α	(i) Lower POCD incidence rates at postoperative days 1 and 3 in the intervention group
Tao et al./2017 [110]	Knee replacement surgery	(i) Control: usual care/$n = 30$ (ii) Intervention: usual care+EA/$n = 30$	EA during surgery	DU20, DU24, PC6	MMSE	(i) Lower POCD incidence rate at postoperative day 1 in the intervention group
Wang/2017 [111]	Nonspecific type	(i) Control: usual care/$n = 28$ (ii) Intervention: usual care+EA/$n = 29$	EA, after surgery, once daily for 7 days, 30 min	DU20, PC6	MMSE, FAQ	(i) Higher MMSE and lower FAQ scores at postoperative days 1 and 3 in the intervention group
Xiao et al./2017 [88]	Cardiac valve replacement with cardiopulmonary bypass	(i) Control: usual care/$n = 22$ (ii) Intervention: usual care+EA/$n = 22$	EA, 20 min before surgery to the end of surgery	DU20, HT7, PC4, PC6	MMSE, PONV incidence	(i) Lower POCD incidence rate at postoperative day 3 in the intervention group (ii) Reduced amounts of anesthetic agents were used in the intervention group (iii) Lower PONV incidence in the intervention group
Zhang et al./2017 [93]	Cardiac valve replacement with cardiopulmonary bypass	(i) Control: usual care +sham EA/$n = 20$ (ii) Intervention: usual care+EA/$n = 20$	(i) EA, 20 min before surgery to the end of surgery	DU20, DU24, PC4, PC6	MMSE, QOR-9, PONV incidence	(i) Higher MMSE scores at postoperative days 1 and 3 in the intervention group (ii) Improved QOR-9 scores in the intervention group (iii) Lower PONV incidence in the intervention group

Table 1: Continued.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
Zhang et al./2017 [39]	Spine surgery	(i) Control: usual care/$n = 45$ (ii) Intervention: usual care+EA/$n = 45$	EA, 30 min before surgery to the end of surgery	DU14, DU20, ST36	MMSE, serum IL-6, IL-10, S100β	(i) Higher MMSE scores at postoperative day 7 in the intervention group (ii) Lower postoperative serum IL-6, IL-10, and S100β levels in the intervention group (iii) Reduced amounts of anesthetic agents were used in the intervention group
Zhao and Li/2017 [112]	Laparoscopic cholecystectomy	(i) Control: usual care/$n = 28$ (ii) MA: usual care +MA/$n = 29$ (iii) EA: usual care +EA/$n = 29$	MA or EA after surgery, 30 min/day for 7 days	DU20, PC6	MMSE, FAQ	(i) Higher MMSE scores at postoperative days 1 and 3 in the EA and MA groups (ii) Lower FAQ scores at postoperative day 3 in the EA and MA groups
Zheng/2017 [113]	Intestinal cancer resection	(i) Control: usual care/$n = 56$ (ii) Intervention: usual care+EA/$n = 56$	EA during surgery	DU20, PC6, SP6, ST36	MMSE	(i) Lower POCD incidence rates at the postoperative time points of 6 and 12 h in the intervention group
Dong et al./2018 [114]	Hip replacement surgery	(i) Control: usual care/$n = 20$ (ii) Drug: usual care +dexmedetomidine/ $n = 20$ (iii) MA: usual care +dexmedetomidine +MA/$n = 20$	MA, 20 min before surgery	DU20, LI4, PC6, ST36	MMSE	(i) Lower POCD incidence rate at postoperative day 1 in both intervention groups (ii) Lower POCD incidence rate in the MA+drug group compared with the drug-only group (iii) Incidence rates of POCD at postoperative day 1 were the lowest in the MA group and the highest in the control group
Han/2018 [66]	Intestinal cancer resection	(i) Control: usual care/$n = 45$ (ii) Intervention: usual care+EA/$n = 45$	EA SW 20 min before surgery to the end of surgery	DU20, PC6, SP6, ST36	MMSE, serum S100β	(i) Higher MMSE scores at the postoperative time points of 12, 24, and 36 h in the intervention group (ii) Lower postoperative serum S100β levels in the intervention group

Table 1: Continued.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
						(iii) Reduced amounts of anesthetic agents were used in the intervention group
Liu et al./2018 [115]	Hip replacement surgery	(i) Control: usual care/$n = 60$ (ii) Intervention: usual care+EA/$n = 60$	EA, 3 days before surgery and 3 days after surgery, once daily, 30 min+30 min before surgery to the end of surgery	LI4, LR3	MMSE, serum IL-1β, TNF-α, cortisol, epinephrine, norepinephrine, $CD3^+$, $CD4^+$, $CD8^+$, CD4/CD8 ratio, $CD16^+$, $CD56^+$	(i) Lower POCD incidence rate at postoperative day 4 in the intervention group (ii) Lower postoperative serum levels of IL-1β, TNF-α, cortisol, epinephrine, and norepinephrine (iii) Higher postoperative serum levels of $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+/CD8^+$, $CD16^+$, and $CD56^+$ in the intervention group
Sun et al./2018 [116]	Gastrectomy for gastric carcinoma	(i) Control: usual care/$n = 20$ (ii) EA: usual care +EA/$n = 20$ (iii) Sham: usual care +sham EA/$n = 20$	EA, 20 min before surgery to the end of surgery	LI4, PC6, ST36, ST37	MMSE, serum IL-6, TNF-α	(i) Lower POCD incidence rate at postoperative day 3 in the EA group (ii) Lower postoperative serum IL-6 and TNF-α levels in the intervention group
Wang et al./2018 [117]	Subtotal gastrectomy	(i) Control: usual care/$n = 48$ (ii) Intervention: usual care+EA/$n = 48$	EA, 15-20 min before surgery	LI4, PC6, ST36, ST37	MMSE, MoCA, $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+/CD8^+$ ratio	(i) A trend of higher MMSE and MoCA scores at postoperative day 1 in the intervention group (ii) Higher $CD3^+$, $CD4^+$, and $CD4^+/CD8^+$ levels at certain postoperative time points
Wang/2018 [87]	Nonspecific type	(i) Control: usual care/$n = 29$ (ii) Intervention: usual care+EA/$n = 29$	EA, 30 min before surgery	DU20, PC6, ST36	MMSE, serum IL-1β, IL-6, TNF-α	(i) Higher MMSE scores at postoperative day 3 in the intervention group (ii) Lower postoperative serum IL-1β, IL-6, and TNF-α levels in the intervention group

Table 1: Continued.

Author/year	Types of surgery	Study groups/no. of participants	Acupuncture intervention	Acupoints	Outcome measures	Results (compared with the control group)
						(iii) Reduced amounts of anesthetic agents were used in the intervention group
Zeng and Wang/2018 [118]	Lower abdomen surgery and lower limb surgery	(i) Control: usual care/$n = 50$ (ii) Intervention: usual care+EA/$n = 50$	EA, after surgery, daily for 30 days	BL23, DU20, GB20	MMSE, acetylcholine and cholinesterase activity in cerebrospinal fluid	(i) Higher MMSE scores at postoperative days 1, 3, and 7 in the intervention group (ii) Lower POCD incidence rates at postoperative days 3 and 7 in the intervention group (iii) Higher acetylcholine levels and lower cholinesterase activities after surgery in the intervention group

Abbreviations: MA: manual acupuncture; EA: electroacupuncture; TENS: transcutaneous electrical nerve stimulation; MMSE: Mini-Mental State Examination ((preoperative scores-postoperative scores) ≥ 2 indicates occurrence of POCD); FAQ: Functional Activities Questionnaire; MoCA: Montreal Cognitive Assessment; PONV: postoperative nausea and vomiting; QoR-40: 40-item quality of recovery score; QOR-9: quality of recovery-9; PQRS: postoperative quality recovery scale; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-10: interleukin-10; TNF-α: tumor necrosis factor-α; S100β: S100 calcium-binding protein β; NSE: neuron-specific enolase; BDNF: brain-derived neurotrophic factor; 5-HT: 5-hydroxy tryptophan; β-EP: β-endorphin; BL23: Shenshu; DU14: Dazhui; DU17: Naohu; DU20: Baihui; DU21: Qianding; DU24: Shenting; EX-HN3: Yintang; GB4: Hanyan; GB6: Xuanli; GB7: Qubin; GB13: Benshen; GB19: Naokong; GB20: Fengchi; GB34: Yanglingquan; HT7: Shenmen; LI4: Hegu; LI10: Shousanli; LI11: Quchi; LR3: Taichong; LU2: Yunmen; LU7: Lieque; PC4: Ximen; PC6: Neiguan; SP6: Sanyinjiao; ST36: Zusanli; ST37: Shangjuxu.

methodology. Many of them did not clearly describe how randomization was performed, nor did they describe their blinding methods. These issues could lead to potential bias and placebo effects. Some important variables for the patients, such as the number of years of education, social activities, and the presence of chronic illnesses, were not documented. All of these variables should be taken into consideration when the data are analyzed. Mood changes, such as surgery-associated anxiety, in particular, can also affect preoperative performance [15]. Therefore, mood and anxiety scales, together with the neuropsychological tests, should be administered prior to surgery to allow the statistical adjustment of cognitive test scores according to the mood state of subjects[16]. Many studies did not examine this important covariant.

Another common problem was the use of the MMSE as the only outcome measure for cognitive performance. The diagnosis of POCD should be verified by psychometric tests, comparing pre- and postoperative cognitive performances. Although there is no gold standard test for the assessment of POCD, a number of neuropsychological tests have been recommended [4], including the Montreal Cognitive Assessment (MoCA) for global cognitive change, the Digit Span Forward and Backward test, the Stroop Color Word test, and the Trail Making A & B test for executive function [4, 17, 18]. Although the MMSE test is sometimes used to quantify POCD, it is not recommended for POCD as it has a marked learning effect [19]. In fact, the MMSE test has two versions, and the use of these parallel versions in conjunction can reduce potential learning effects, resulting in greater sensitivity for the detection of functional changes associated with surgery. However, none of the studies included in Table 1 described the use of the parallel version. Compared with the MoCA, the sensitivity of the MMSE for mild cognitive changes is lower. Therefore, a more comprehensive neuropsychological battery should be used in the future for this type of study. An important issue for the detection of POCD is the standardization cognitive test administration across occasions and subjects. These tests should be administered to all subjects by the same suitably qualified and trained staff to minimize subjectivity and improve reliability [19]. Unfortunately, this standardization was likely not addressed in most of the studies described here.

The timing of postoperative cognitive tests is also a crucial element. After surgery, postoperative pain, opioids, sleep disturbance, nausea, limited mobility, and fatigue are common during the immediate postoperative period and can affect cognitive performance. Therefore, some researchers have argued that patients should not be evaluated for POCD until at least one week after the operation. However, no consensus on this issue has been reached, as findings suggest that limiting the screening period for POCD to seven days after surgery could result in missed POCD diagnoses in many surgical patients [20]. While most of the studies only detected POCD within 1 week after surgery, there is limited data regarding intermediate POCD, which may occur within 3 months, and long-term POCD, for changes 1-2 years following surgery. More studies are necessary to provide evidence on the long-term effects of acupuncture on POCD.

4. Potential Mechanisms of Acupuncture against POCD

Studying the mechanisms underlying the effects of acupuncture on the development of POCD in randomized, controlled trials may be challenging. To improve our understanding, animal studies may be a good option. Together with clinical findings, animal studies can provide insights for the future direction of research. We have summarized the findings of animal studies in Table 2, and we will discuss the potential mechanisms underlying the effects of acupuncture on POCD.

4.1. Attenuation of Systemic Inflammation and Neuroinflammation. Inflammation appears to play a major role in the development of POCD. Both systemic inflammation and neuroinflammation, particularly in the hippocampus, triggered by peripheral surgery trauma or anesthesia, have been proposed to be involved in the observed cognitive deficits [21–24]. Elevated levels of proinflammatory cytokines, such as interleukin- (IL-) 6, IL-1β, and tumor necrosis factor (TNF)-α, have been reported after surgery and may be related to POCD [25]. For instance, Xu et al. observed elevated levels of IL-6 following abdominal surgery in the elderly, which positively correlated with decline in cognitive function [26]. Geng et al. reported increased levels of IL-6, IL-1β, and TNF-α among patients undergoing laparoscopic cholecystectomy, and these increases appeared to be correlated with the choice of anesthetic agent and the incidence of POCD [27]. Consistent findings have also been reported for other types of surgery, where the levels of TNF-α and IL-6 in the perioperative period have been positively correlated with the development of POCD in aged patients [28, 29]. IL-1β and its upstream marker, TNF-α, are released from phagocytes and endothelial cells following tissue trauma [30]. Elevated levels of IL-1β can interfere and inhibit hippocampal long-term potentiation (LTP), a primary cellular mechanism that underlies memory and learning [31, 32]. IL-1β can also enhance glutamate neurotoxicity, which is related to cognitive dysfunctions [32].

The effects of acupuncture on inflammation have been studied extensively for various diseases and the reader if referred to the review by Park and Namgung [33]. It is therefore reasonable to speculate that acupuncture may also protect against POCD by modulating inflammatory responses, and this idea is supported by both clinical and preclinical findings.

Clinical trials have revealed that acupuncture can attenuate surgery-induced elevation of serum proinflammatory factors, such as IL-1β, IL-6, and TNF-α (please refer to Table 1). Since these cytokines are known to be associated with cognitive decline [28, 29], the clinical data appears to support a role for acupuncture in the suppression of systemic inflammation during POCD [34]. Similar findings have been observed in animals [35–37]. Rather than simply suppressing the inflammatory response, acupuncture may play a dual role in the modulation of the immune system. Data from two studies demonstrated that acupuncture reduced the levels of IL-10 (classically viewed as anti-inflammatory) and IL-6(classically viewed as proinflammatory) during the 3- to 7-day

Table 2: Summary of animal studies examining the effects of acupuncture on POCD.

Author/year	Type of surgery	Animals and study groups/group size	Acupuncture interventions	Acupoints	Results
Ye et al./2014 [119]	Partial hepatectomy (PH)	(i) Male SD rats, 12 months old (ii) Control: no treatment/$n = 30$ (iii) PH only/$n = 30$ (iv) PH+EA/$n = 30$ (v) PH+minocycline/$n = 30$	EA, 30 min/day after surgery, for 1, 3, or 7 days	DU20, DU14	(i) EA improved performance in MWM (ii) EA reduced serum levels of CHR, ACTH, and corticosterone
Yuan et al./2014 [34]	Acute myocardial ischemia-reperfusion (AMIR)	(i) Male, SD rats, 18–24 months old (ii) Sham/$n = 30$ (iii) AMIR/$n = 30$ (iv) AMIR+EA/$n = 30$	EA, immediately after reperfusion started, for 30 min	DU20, ST36	(i) EA group showed a trend of improvement in working and reference memory in the 8-arm maze task (ii) EA improved survival rates, reduced the excitability of the sympathetic nerve, and attenuated neural apoptosis and microglial activation (Iba-1 ↓) (iii) EA suppressed hippocampal oxidative stress (MDA and SOD ↓), reduced peripheral inflammation (IL-6 and TNF-α ↓)
Yin et al./2015 [50]	Splenectomy (ST)	(i) Male SD rats, 20 months old (ii) Control: no procedure/$n = 5$ (iii) Sham: anesthesia only/$n = 15$ (iv) Anesthesia+ST/$n = 15$ (v) Anesthesia+ST+EA/$n = 15$	EA, 20 min before surgery	DU20, PC6, EX-HN3	(i) EA improved performance in Y-maze (ii) EA reduced serum concentration levels of IL -1β, IL-6, and TNF-α, reduced hippocampal Bcl-2/Bax ratio
Wang et al./2016 [35]	Splenectomy (ST)	(i) Male SD rats, 18–20 months old (ii) Control: no treatment/$n = 30$ (iii) ST only/$n = 30$ (iv) ST+EA sham/$n = 30$ (v) ST+EA/$n = 30$ (vi) ST+EA+AMPK inhibitor	EA, 30 min/day, 5 days before surgery	DU20	(i) EA improved performance in MWM (ii) EA reduced hippocampal expression of NF-$\kappa\beta$, IL-1β, and TNFα
Xie et al./2016 [61]	No surgery	(i) Male and female SD rats, 20 months old (ii) Control: no treatment/$n = 16$ (iii) Isoflurane only/$n = 16$ (iv) Isoflurane+EA/$n = 16$	EA, during isoflurane anesthesia for 4 h	DU20	(i) EA improved performance in MWM (ii) EA reduced hippocampal LTP lesion

Table 2: Continued.

Author/year	Type of surgery	Animals and study groups/group size	Acupuncture interventions	Acupoints	Results
Chen et al./2017 [120]	Trigeminal neuralgia (TN) model	(i) Male SD rats, 200–260 g (ii) Control: no treatment/$n = 10$ (iii) TN only/$n = 10$ (iv) TN+EA/$n = 10$ (v) TN+pregabalin/$n = 10$	EA, 30 min each time, once every 2 days for 11 consecutive days	LI10, L111	(i) EA improved performance in MWM (ii) EA reduced demyelination in the Gasserian ganglion and medulla oblongata (iii) EA reduced vacuolar degeneration and swelling of mitochondria in hippocampal neurons (iv) EA increased fEPSP slope in electrophysiological study
Chen et al./2017 [75]	Hepatic ischemia reperfusion (HIR)	(i) Male SD rats, 18–20 months old (ii) Control: no treatment/$n = 20$ (iii) HIR only/$n = 20$ (iv) HIR+EA/$n = 20$	EA, 30 min/day, 7 days before surgery	DU20, ST-36, PC6, LI11	(i) EA improved performance in MWM (ii) EA reduced the levels of Aβ and p-tau (181) in cerebrospinal fluid (iii) EA increased the levels of β-catenin, Wnt, and GSK-3β in the hippocampus
Feng et al./2017 [121]	Hepatolobectomy (HT)	(i) Male SD rats, 1 month old (ii) Young control: no treatment/$n = 10$ (iii) D-Galactose-induced aged (DA) group/$n = 10$ (iv) DA+HT/$n = 30$ (v) DA+HT+EA for 1/3/7 days (3 groups, $n = 10$ in each group)	EA, 30 min/day after surgery, for 1, 3, or 7 days	DU20, DU14	(i) EA improved performance in Y-maze (ii) EA reduced levels of Ang II and AT1R in the hippocampus
Feng et al./2017 [36]	Partial hepatectomy (PH)	(i) Male SD rats, 21–23 months old (ii) Control: saline i.p. injection/$n = 20$ (iii) PH only/$n = 20$ (iv) PH+EA/$n = 20$ (v) PH+minocycline/$n = 20$	EA, 30 min/day after surgery, once every 2 days for 3 or 7 days	DU20, GV14	(i) EA improved performance in MWM (ii) EA reduced the levels of the proinflammatory cytokines IL-1β, IL-6, TNF-α, and HGMB1 in the hippocampus (iii) EA decreased the expression levels of TLR 4/2 in the hippocampus
Liu et al./2017 [37]	Partial hepatectomy (PH)	(i) Male SD rats, 18–20 months old (ii) Sham/$n = 30$ (iii) PH only/$n = 30$ (iv) PH+EA/$n = 30$	EA, 30 min preoperative to the end of surgery+30 min/day for 7 days after surgery	DU20, PC6, LI4	(i) EA improved performance in MWM (ii) EA increased hippocampal expression of α7-nAChR and downregulated TNFα and IL-1β

Abbreviations: PH: partial hepatectomy; AMIR: acute myocardial ischemia reperfusion; ST: splenectomy; TN: trigeminal neuralgia; HIR: hepatic ischemia reperfusion; HT: hepatic lobectomy; SD: Sprague Dawley; i.p.: intraperitoneal; EA: electroacupuncture; MWM: Morris water maze; IL-1β: interleukin-1β; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; MDA: malondialdehyde; SOD: superoxide dismutase; Iba-1: ionized calcium-binding adaptor molecule-1; α7-nAChR: α7-nicotinic acetylcholine receptors; p-GSK-3β: phosphorylated glycogen synthase kinase-3β; Aβ-24: amyloid β-protein-42; p-AMPK: phosphorylated adenosine 5′ monophosphate-activated protein kinase; NF-κB: nuclear factor κB; Ang II: angiotensin II; AT1R: angiotensin II type 1 receptor; Bcl-2: B-cell lymphoma/leukmia-2; Bax: Bcl-associated x protein; fEPSP: field excitatory postsynaptic potential; CHR: corticotropin-releasing hormone; ACTH: adrenocorticotropic hormone; LTP: long-term potentiation; HGMB1: high mobility group protein B1; TLR: toll-like receptor.

postoperative period [38, 39]. This finding is of special interest because the two cytokines are repressed simultaneously, indicating the suppression of opposing mechanisms. The plasma levels of IL-6 and IL-10 are commonly elevated after tissue damage. Some studies support the hypothesis that IL-6 and IL-10 can have both pro- and anti-inflammatory properties [40, 41]. Overall, it is not fully understood how these two cytokines, as well as other anti- and proinflammatory mechanisms, interact with each other during the postoperative period to affect cognition.

Recent studies on macrophages may further support the idea that acupuncture can result in the bidirectional modulation of the immune system. M1 and M2, two subtypes of macrophages, process proinflammatory and anti-inflammatory functions, respectively. The activation level of macrophages can affect the types and amounts of cytokines to be released. In rats with spinal cord injuries, EA treatment altered the ratio of M1/M2 macrophages. EA treatment suppressed the M1 subtypeby downregulating the marker protein CD68 and reducing TNF-α, IL-1β, and IL-6 levels, while simultaneously enhancing the proportion of M2, upregulating the marker proteins CD206 and NT-3 and increasing expression levels of IL-10 [42]. Similar findings were revealed in a study conducted by da Silva and colleagues [43], where they demonstrated that MA resulted in analgesic and anti-inflammatory effects in a rat model of muscle pain, accompanied by an M1 to M2 shift in the macrophage population and increased IL-10 expression levels. These findings are consistent with those in four other studies, where acupuncture improved cognition in rats that received various types of surgery and suppressed systemic inflammation and neuroinflammation [34].

4.2. Reduction of Oxidative Stress Levels. Surgical procedures inevitably produce reactive oxygen species (ROS) [44] in the body. Perioperative BBB dysfunction promotes the entry of inflammatory factors and oxidative species into the brain enabling activation of microglia and vascular endothelial cells by cytokines via surface receptors [9]. These activated microglia in turn release ROS with proinflammatory factors, creating a toxic environment in which neuronal damages are induced [9, 45]. A number of animal studies support this hypothesis. For example, elevated levels of expression of Nox2 (a key source of ROS production in the CNS) and the inflammatory cytokine IL-1β were found in the hippocampi of mice undergoing exploratory laparotomy. These elevated levels contributed to the production of ROS and microglial activation, eventually leading to memory dysfunction [46]. Lipid peroxidation and oxidative DNA damage can also promote neuronal dysfunction and apoptosis. Increased 8-isoprostane : creatinine ratios, an indicator of lipid peroxidation, were found in the urine of elderly patients with POCD after orthopedic surgery when compared to the non-POCD patients [47]. Moreover, researchers showed that pretreatment with the free radical scavenger edaravone resulted in a reduction in occurrence of cognitive impairment following carotid endarterectomy and spinal surgery [48, 49] suggesting that the amelioration of cerebral oxygen metabolism may be a potential management strategy for POCD.

Acupuncture has been suggested to affect oxidation levels during different medical conditions. However, most of the evidence has come from animal studies. In aged rats, EA attenuated POCD induced by acute myocardial ischemia-reperfusion, splenectomy, or partial hepatectomy. This attenuation was accompanied by reduced levels of malondialdehyde (MDA) and increased superoxide dismutase (SOD) activity in the hippocampus [34–37, 50]. A few clinical trials have also provided supportive data. A two-armed, randomized controlled trial provided direct evidence of the effect of acupuncture on oxidative stress, reporting that scalp acupuncture given before the introduction of general anesthesia in intestinal cancer patients resulted in reduced levels of MDA and SOD activity during the operation stage [51]. Indirect evidence was provided by a randomized controlled trial targeting overweight and obese subjects. Participants who received a 6-week acupuncture intervention had significantly reduced serum prooxidant/antioxidant ratio values compared with the sham acupuncture controls [52]. Similarly, in patients with rheumatoid arthritis, laser acupuncture significantly reduced plasma MDA, serum nitrate and nitrite, serum C-reactive protein, and IL-6 levels, as well as glutathione peroxidase (GPx) activity [53].

The mechanism through which acupuncture modulates the redox pathways and reduces oxidative stress remains unclear. Acupuncture likely affects a cluster of oxidative stress-related enzymes, inducing a nonspecific response [54, 55]. By using a proteomics approach, researchers found that acupuncture can induce a cluster of proteins related to oxidative stress and reduced ROS production in a rat vascular dementia model. The same study also reported decreased neuronal apoptosis and improved LTP and cell survival in the acupuncture group, suggesting the multitargeted effect of the intervention [55]. Similarly, in a study conducted by Han and colleagues, EA was found to effectively improve cognition in mice injected with lipopolysaccharide, which is widely used to induce inflammation. This group reported that EA significantly decreased MDA and hydrogen peroxide levels and increased the catalase and glutathione levels. They also reported the suppression of proinflammatory cytokine levels in the hippocampi of the treated animals [56]. Since oxidative stress and inflammation are closely related, these results are consistent with the proposed inflammation-suppressing effects of acupuncture.

4.3. Improvement of Synaptic Plasticity. Reduced synaptic proteins and changes in synaptic plasticity have been reported in animals after surgery. Synaptic proteins assist during the normal release of neurotransmitters and during synaptic transmission, which is critical for cognitive functions [57]. In Alzheimer's disease model transgenic mice, the levels of postsynaptic density protein 95 (PSD-95) and synaptophysin were decreased after laparotomy [58]. Exposure to high concentrations of sevoflurane was also shown to result in markedly decreased expression levels for synaptotagmin-1 in the rat hippocampus, which hindered the release of presynaptic neurotransmitters and decreased the efficiency of synaptic transmission [59]. In aged mice, laparotomy reduced the expression levels of plasticity-

related proteins, such as brain-derived neurotrophic factor (BDNF), cAMP response element-binding protein (CREB), and Arc. These changes in protein levels, as well as cognitive impairments, could be attenuated by the pharmacological blockade or the genetic suppression of the inflammatory prostaglandin E2 pathway. Therefore, neuroinflammation is thought to be an upstream event of the synaptic changes observed during POCD [60].

There is little direct evidence regarding the effects of acupuncture on synaptic function during POCD. A study conducted by Xie and colleagues reported that EA can reduce impairment in spatial memory and learning and recover hippocampal LTP in aged rats that received repeated exposures to the anesthetic agent isoflurane [61]. Reports focusing on cognition-related diseases have provided indirect evidence of the effects of acupuncture. For example, when rats were injected with amyloid beta protein into their ventricles, those receiving EA showed reduced cognitive impairment and markedly different synapse morphology than those receiving no acupuncture treatment. Increased synaptic curvatures, decreased widths of synaptic clefts, and thickened postsynaptic densities were observed in the brains of rats treated with acupuncture [62]. These changes in synapse morphology may explain, at least partly, the observed cognition improving effects conveyed by EA treatment.

4.4. Reduction of Neuronal Injury. Neuronal damage has been suspected in humans with POCD. The S100 calcium-binding protein β (S100β) protein is a frequently used peripheral neuronal marker of neuronal damage and blood-brain barrier disruption. The protein is concentrated in glial cells and can also be detected in other nonneural cell types. During brain injury, S100β can leak from or be secreted by damaged cells [63]. A meta-analysis of 13 clinical studies reported that the level of serum S100β is correlated with the incidence of POCD [64]. Ten of the included studies reported reduced levels of serum S100β in the acupuncture groups [38, 39, 65–72]. Neuron-specific enolase (NSE) is another common maker used for POCD studies. NSE is an enzyme involved in the glycolytic pathway and can be found in neurons and neuroendocrine cells. Four of the included studies reported reduced levels of serum NSE in the acupuncture groups [65, 67, 68, 73]. Both S100β and NSE have been proposed to act as predictors of cognitive dysfunction after surgery [74].

Acupuncture provides neuroprotection through various mechanisms. The antioxidative and anti-inflammatory effects discussed above are likely to be partially responsible for its protective effects. However, acupuncture may also modulate apoptotic pathways. In aged rats that underwent splenectomy, acupuncture reduced the hippocampal Bcl-2/Bax ratio [50]. Acupuncture also increased the levels of β-catenin, Wnt, and glycogen synthase kinase- (GSK-) 3β in the hippocampi of aged rats that underwent hepatic ischemia reperfusion procedures [75], and these proteins are involved in the neuronal survival signaling pathways [76].

4.5. Reducing the Use of Anesthetic Agents, Promoting Patient Recovery, and Reducing Postoperative Nausea and Vomiting (PONV). Exposure to anesthetic agents has been proposed to induce undesirable effects. Several animal studies have reported the deleterious effects of general anesthesia in the absence of surgical insults. For instance, aged rats exposed to isoflurane were found to have increased escape latencies and impaired spatial memory during the Morris water maze test. This result may be related to the metabolism of the beta-amyloid (Aβ) peptide, a key protein involved in the development of Alzheimer's disease. Isoflurane has been reported to increase the production of Aβ and promote Aβ oligomerization and accumulation in the hippocampus, which resembles the neuropathology of Alzheimer's disease [77]. Others have reported that exposure to desflurane resulted in transient spatial reference memory impairment in aged rats [78]. However, conflicting data has also been reported, showing that an anesthetic agent alone (isoflurane, sevoflurane, and propofol) was unable to impair cognition or to reduce hippocampal neurogenesis and cell survival [79–81]. Data from clinical studies have also been inconclusive, and there have been conflicting reports of the effects of anesthesia exposure on the development of POCD in elderly patients [82, 83]. There are many confounding variables, such as age, hypotension, body temperature, and hypoxia during surgery, which can make the interpretation of findings and the identification of a causal linkage between general anesthetics and cognitive and behavioral deficiencies quite challenging.

Regardless of these limitations, several studies have suggested that acupuncture could potentially reduce the required dosages of volatile anesthetic used during surgery. A meta-analysis performed by Asmussen and colleagues reported that the complementary use of acupuncture could reduce the amounts of volatile anesthetics used during craniotomy and cardiac surgery, leading to faster extubation and postoperative recovery times. They also reported a lower incidence of postoperative nausea and vomiting (PONV) in patients receiving acupuncture [84, 85]. Many of the POCD studies included in Table 1 also reported these beneficial effects of acupuncture. Reduced dosages of anesthetic agents during surgery were reported in eight studies [38, 39, 65, 66, 86–89], while a lower incidence of PONV was reported in six studies [88–93], accompanied by a lower incidence of POCD. By definition, PONV occurs during the first 24-48 h after surgery in inpatients [94]. However, a number of the studies included in Table 1 conducted their cognitive assessments during this period, and any discomfort or pain is likely to impact cognitive test performance.

Acupuncture can lead to sensations of relaxation and it may have anxiolytic properties. In addition, acupuncture may modulate the neural circuits involved in nausea. In a group of anesthetized healthy human subjects, acupuncture was reported to decrease the blood flow to the right medial frontal gyrus and to the left putamen, which are responsible for pain processing and nausea [95].

It is worth to note that pain reduction by acupuncture may also partly explain why it helps to lower the incidence of POCD. Although pain level was not assessed in most of the papers cited in this review, acupuncture is known to reduce postoperative pain [96], which is a risk factor of POCD [97]. As indicated in the systematic view conducted by Wu and colleagues in 2016 [98], acupuncture was

associated with less postoperative pain one day after surgery than control treatment, although it could not reduce the use of opioid analgesic in patients. Pain research on animals showed that acupuncture could suppress inflammation and reduce the levels of reactive oxidative species. For example, in a mouse model of chronic inflammatory pain, electroacupuncture modulated the inflammatory mediators such as glial fibrillary acidic protein (GFAP) and the receptor of advanced glycation end products (RAGE) [99]. Collectively, acupuncture seems to be able to promote postoperative recovery through an array of mechanisms.

5. Conclusion

Based on the studies included in this review, we believe that acupuncture has the potential to be used as a complementary therapy to reduce the incidence of POCD. However, current clinical data may not be reliable as there are methodological problems for almost all of the studies, which can lead to potential bias. More clinical studies with better study designs are necessary. Regardless of the results of cognitive assessment tests, which are more susceptible to bias, the observed reductions in inflammatory and oxidative stress markers, as well as neuronal injury markers, provide solid evidence that acupuncture may protect the brain during surgery. The multiple effects of acupuncture on surgery-related conditions and mechanisms make it an interesting direction for future research. Further investigation on the types of acupuncture (MA or EA) and treatment protocol (e.g., duration, start and end time) for POCD will be clinically meaningful.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yuen-Shan Ho and Fei-Yi Zhao contributed equally to the manuscript.

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Research Article

Chronic Systemic Inflammation Exacerbates Neurotoxicity in a Parkinson's Disease Model

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Systemic inflammation is a crucial factor for microglial activation and neuroinflammation in neurodegeneration. This work is aimed at assessing whether previous exposure to systemic inflammation potentiates neurotoxic damage by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and how chronic systemic inflammation participates in the physiopathological mechanisms of Parkinson's disease. Two different models of systemic inflammation were employed to explore this hypothesis: a single administration of lipopolysaccharide (sLPS; 5 mg/kg) and chronic exposure to low doses (mLPS; 100 μg/kg twice a week for three months). After three months, both groups were challenged with MPTP. With the sLPS administration, Iba1 staining increased in the striatum and substantia nigra, and the cell viability lowered in the striatum of these mice. mLPS alone had more impact on the proinflammatory profile of the brain, steadily increasing TNFα levels, activating microglia, reducing BDNF, cell viability, and dopamine levels, leading to a damage profile similar to the MPTP model *per se*. Interestingly, mLPS increased MAO-B activity possibly conferring susceptibility to MPTP damage. mLPS, along with MPTP administration, exacerbated the neurotoxic effect. This effect seemed to be coordinated by microglia since minocycline administration prevented brain TNFα increase. Coadministration of sLPS with MPTP only facilitated damage induced by MPTP without significant change in the inflammatory profile. These results indicate that chronic systemic inflammation increased susceptibility to MPTP toxic effect and is an adequate model for studying the impact of systemic inflammation in Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and is characterized by a chronic progressive neuronal loss mainly in the substantia nigra, which causes a decrease in the production and availability of dopamine and manifests as a loss of movement control [1]. Despite the amount of research on this neurodegenerative disease, its origin remains unclear. Only 5-10% of cases have a genetic background [2–5], while the rest are of idiopathic origin [6], although some risk factors have been identified, such as age, environmental toxins, and infections [7, 8].

The inflammatory process, oxidative stress, and microglia activation are essential components in the pathogenesis of many neurodegenerative disorders such as PD [9]. Microglia are vital in the maintenance of immune homeostasis in the central nervous system (CNS). Nevertheless, during aging, microglia are activated, secrete inflammatory cytokines, and also promote the release of secondary inflammatory mediators such as prostaglandins and nitric oxide

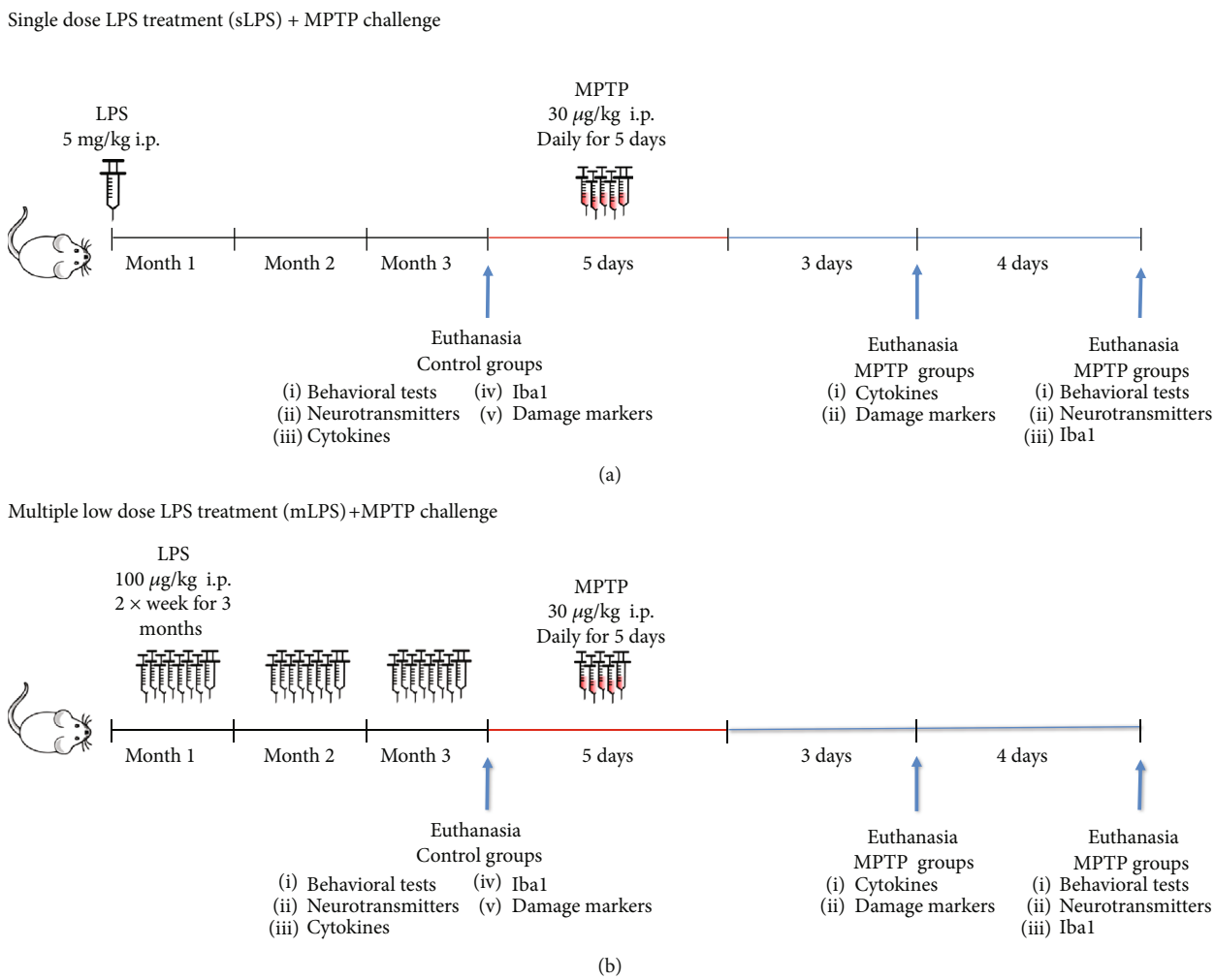

Figure 1: Schematic representations of systemic LPS administration regimens. For the single LPS (sLPS) treatment (a), mice were injected intraperitoneally once with 5 mg/kg. After three months, they were challenged with MPTP (30 mg/kg for five consecutive days) and were then analyzed. For the repeated LPS (mLPS) treatment (b), mice were injected intraperitoneally 100 μg/kg twice a week for three months. They were then challenged with MPTP (30 mg/kg for five consecutive days) and analyzed. Control groups (saline, sLPS, and mLPS) were euthanized after three months of the challenges. MPTP groups were euthanized either three days after MPTP exposure for damage markers (MTT assay, cleaved caspase-3, and TBARS) and cytokine levels or seven days after MPTP challenge for behavioral tests, neurotransmitter levels, and Iba1 staining.

(NO) [10, 11]. Additionally, they facilitate the production of reactive oxygen species (ROS) through the induction or activation of NADPH oxidase and the release of NO [12, 13].

Microglia also respond and propagate inflammatory signals initiated in the periphery by producing the proinflammatory cytokines IL1β, IL6, and TNFα [14–16]. High levels of systemic TNFα can cross the blood-brain barrier (BBB), stimulating the microglia to secrete more TNFα as well as other proinflammatory factors and thus creating persistent and self-generated neuroinflammation [15]. Metabolic diseases such as obesity, hypertension, dyslipidemia, diabetes, and insulin resistance are associated with chronic systemic inflammation and a higher risk of developing neurodegenerative diseases such as Alzheimer's disease and PD [17–23]. Due to the importance of peripheral inflammatory processes in PD development [24–26], it is relevant to investigate more thoroughly the mechanisms involved.

In this work, we evaluated whether systemic inflammation increases susceptibility and further damage after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure. For this purpose, we employed two systemic lipopolysaccharide (LPS) administration models that induce neuroinflammation, one consisting of a single high dose of LPS (5 mg/kg; Figure 1(a)) and the other of multiple low doses for three months (100 μg/kg twice a week; Figure 1(b)). To test this hypothesis, we evaluated systemic inflammation through the measurement of cytokines, its impact on neuroinflammation by assessing brain cytokines, microglial response, brain NFκB expression, and inflammation-induced BBB permeability. To ensure that neuroinflammation induced by peripheral LPS stimulation causes damage, we determined cell viability, cleaved caspase-3, lipid peroxidation, and neurotransmitter levels (dopamine and serotonin). Finally, we evaluated whether

these phenomena are reflected in the behavior of the animals exposed to the different treatment schemes.

Both LPS models were challenged with the neurotoxin MPTP or vehicle at the end of three months of LPS treatment (Figure 1). Our results show that chronic systemic inflammation induced sustained neuroinflammation with microglia activation, TNFα production, BBB compromise, and cell death, inducing a parkinsonism model and conferring additional susceptibility to MPTP damage.

2. Methods

2.1. Chemicals. All reagents were of analytical grade. 3,3′,5,5′-Tetramethylbenzidine (TMB; T4444), protease inhibitor cocktail (11836153001), LPS (Lipopolysaccharides from *Escherichia coli* O111:B4; L4391), and MPTP (M0896) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Alexa Fluor 488-coupled donkey anti-rabbit (ab150073) antibody was purchased from Abcam (Cambridge, UK). Rabbit anti-Iba1 antibody (CP 290) was acquired from Biocare Medical (Pacheco, CA, USA). Human/Mouse Cleaved Caspase-3 (Asp175), DuoSet IC ELISA (DYC835), and Human/Mouse BDNF DuoSet ELISA (DY248) were purchased from R&D Systems (Minneapolis, MN, USA). 4,6-Diamidino-2-phenylindole (DAPI) antifade solution was obtained from Millipore (MA, USA). ELISA kits were bought from eBioscience (TNFα Mouse 88-7324; IL6 Mouse 88-7064; IL10 Mouse 88-7104; IFNγ Mouse 88-7314; TGFβ-1 Human/Mouse 88-8350; and IL1β Mouse 88-7013).

All required solutions were prepared with deionized water from a Milli-RQ system (Millipore, MA).

2.2. Experimental Animals. All experiments were carried out with male CD1 (ICR) mice of 8 weeks of age, maintained under standard conditions with a 12:00 h light-dark cycle and free access to water and food. CD1 mice (ICR), as previously reported, develop a stronger proinflammatory response than C57BL/6J mice; these differences do not originate from alterations in the expression levels of TLR4 or CD14, the LPS receptors [27]. Also, CD1 mice showed depletion of the neurotransmitter dopamine and serotonin, as well as dopaminergic neuron loss in the substantia nigra, when treated with the proneurotoxin MPTP [28].

Animal handling and experimentation strictly followed the Guidelines for Care and Use of Laboratory Animals published by the National Institutes of Health and the Guidelines of the Mexican Law of Animal Protection for the use and care of laboratory animals (Norma Oficial Mexicana NOM-062-ZOO-1999). All experimental procedures were approved by the research and ethics committees of the Facultad de Medicina, Universidad Nacional Autónoma de México (Approval 043/2015). We minimized the number of mice used and their suffering or pain as much as possible.

2.3. LPS and MPTP Administration. Systemic inflammation was induced either with a single dose of 5 mg/kg of LPS (sLPS) administered intraperitoneally (i.p.) [15] or with multiple low doses (mLPS; 100 μg/kg i.p. twice a week) for three months [29, 30] (Figure 1). Both models induce a persistent chronic neuroinflammatory state with increased brain TNFα levels and microglial activation in a period from three, six, up to ten months [15, 29, 30]. The control groups received saline solution twice a week for three months. After three months, sLPS and mLPS groups were challenged with MPTP (30 mg/kg i.p.) or saline solution administered daily for five consecutive days [31, 32] (Figure 1).

Animals were euthanized at two different times, depending on the type of the test to which they were assigned. The biological material used for cytokine levels and damage markers (mitochondrial function, TBARS, and cleaved caspase-3) was obtained three days post-MPTP administration; for neurotransmitter detection, Iba1, and NFκB expression, this biological material was obtained seven days post-MPTP administration.

2.4. Motor Coordination Test with Equilibrium Bar. Motor coordination with a horizontal bar allows measuring forelimb strength and coordination [33]. For this we used an equilibrium bar of 2 mm diameter, 38 cm long, and 49 cm away from the floor. Each mouse was taken from the tail and placed quickly with the front paws at the center of the bar. The time on the bar was registered. For analysis, scores were established as follows: score 1 (1-5 seconds on the bar), 2 (6-10 s), 3 (11-20 s), 4 (21-30 s), and 5 (more than 30 s). In the case that the mouse fell from the bar, the mouse was placed again and the longest time on the bar was recorded. Lower scores show a less efficient test result. If the mouse reached the end of the column with its front paw within the 30 s, it received the score of 5. Every mouse was tested three times, and the highest score obtained from the three attempts was used for the analysis [33]. Eight to fifteen animals per group were used for statistical analysis.

2.5. Sucrose Consumption Preference Test. Two mice from the same experimental group were separated from the cage littermates and then housed together for the sucrose consumption preference test. Mice were evaluated for three consecutive days. Each cage contained a drinking bottle with water and one with 2% sucrose. The bottle position was changed daily to avoid preference for a place. Every day at the same time new bottles were placed, and the volume consumed in each of the previous bottles was measured. The percentage of water taken per day was calculated based on the total water consumed per cage. A percentage lower than 65% of consumption was considered anhedonia [34]. Nine to eleven animals per group were analyzed.

2.6. Evaluation of Dopamine and Serotonin Levels. Neurotransmitter levels were analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection [32]. Briefly, animals were sacrificed by cervical dislocation, the striatum dissected and placed in microtubes with 300 μL of 0.4 N perchloric acid and 0.1% of sodium metabisulfite to homogenize the tissue. Subsequently, the samples were centrifuged at 4000 × g at 4°C for 10 minutes. The supernatant was taken from each tube and stored at -80°C for further analysis on HPLC. The levels of neurotransmitters were detected in a Perkin Elmer liquid chromatograph with a

BAS CC-5 electrochemical detector. An Alltech Adsorbosphere column for catecholamines (100 Å~4.6 mm) was employed. The mobile phase was composed of phosphate buffer (0.1 M, pH 3.1), 0.9 mM sodium octyl sulfate, 0.1 mM EDTA, and 15% methanol. Twenty microlitres of perchloric acid metabisulfite solution was used as a vehicle in samples and standard. All samples were analyzed by duplicate. At least five animals per group were analyzed.

2.7. Monoamine Oxidase B Activity Assay. The MAO-B activity was measured spectrophotometrically and is based on the formation of H_2O_2 from the conversion of benzylamine to benzaldehyde. The formation of H_2O_2 is detected by the oxidation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in its presence. The substantia nigra of the mice was homogenized in a 1 M monosodium phosphate buffer at pH 7.2 and centrifuged at 10,000 × g. To perform the kinetics, each well of a 96-well plate contained 20 μL of 1 M sodium phosphate buffer, 20 μL of 50 mM benzylamine, 20 μL of sample, and 40 μL of water. A control well with the sample without benzylamine and a control well without sample were also included. The samples were allowed to incubate for 3 min and were placed in a BioTek plate reader to take readings every 3 minutes for 30 minutes at a wavelength of 560 nm. At least seven animals per group were used for statistical analysis.

2.8. BDNF and Cytokine Titration. The levels of BDNF, TNFα, IL6, IL10, IFNγ, TGFβ, and IL1β were measured in serum and brain tissue by sandwich ELISA following the instructions of the provider. Mice were euthanized with sodium pentobarbital (50 mg/kg). Blood sampling was performed with a heart puncture, and after clotting, blood was centrifuged for the obtention of the serum (2500 × g, 15 min at 4°C). The whole brain was recovered and homogenized in 500 μL of lysis buffer (20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100) containing a protease inhibitor [32]. Samples, serum and brain tissue homogenates, were incubated for 18 h at 4°C with PBS-Tween 20 (0.05%)/0.5% BSA, washed three times, and incubated with the corresponding detection antibody for 2 h at room temperature. Bound detection antibodies were detected using TMB as the substrate. Optical density readings were made at 450 nm. All assays were performed by duplicate and sensitivities were 23.4 pg/mL for BDNF, 8 pg/mL for IL1β, 4 pg/mL for IL6, 30 pg/mL for IL10, 8 pg/mL for TNFα, 8 pg/mL for TGFβ, and 15 pg/mL for IFNγ. At least four animals per group were used for statistical analysis.

2.9. Iba1 Immunofluorescence. The staining of Iba1 evaluated the microglial response in the caudal striatum and substantia nigra; both brain areas are primarily affected in Parkinson's disease [35]. For this, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) and transcardially perfused with 100 mL of phosphate-buffered saline (PBS) followed by 100 mL of a 4% paraformaldehyde solution. The brains were dissected and cryopreserved in 30% sucrose. Coronal brain sections (20 μm) were obtained in a cryostat and adhered to silanized slides. Slices were permeabilized in 0.1% Triton X-100 and incubated in a 4% blocking buffer with bovine serum albumin (BSA) for 20 min at room temperature. Slices were incubated overnight with the primary antibody for Iba1 (1 : 300) at 4°C. The following day, brain slices were washed three times with PBS and incubated with the secondary antibody (1 : 500) diluted in PBS containing 2% BSA for 2 hours at room temperature, followed by three times with PBS. The slides were mounted with a DAPI antifade solution. Images were taken using a Nikon fluorescent microscope and analyzed using ImageJ software (ImageJ v1.38x; NIH, US).

The mean of fluorescence intensity was calculated using three consecutive slices from each region per animal. At least three animals per group were used for statistical analysis. The coordinates for the identification of the caudal striatum (ML = 1.5 mm, AP = 1.25 mm, and DV = 3.0 mm from Bregma) and the substantia nigra (ML = 1.5 mm, AP = −3.16 mm, and DV = 4.5 mm from Bregma) were based on the Paxinos Mouse Brain Atlas.

2.10. MTT Assay. 3-[4,5-Dimethylthiazol-2yl]-diphenyltetrazolium bromide (MTT) reduction to formazan was measured in brain tissue homogenate as an index of the functional status of the mitochondrial respiratory chain [32, 36]. Briefly, 100 μL of tissue homogenate in PBS was incubated with 10 μL of MTT (5 mg/mL) for 30 minutes at 37°C protected from light. The samples were centrifuged at 15,300 × g for 3 minutes. The supernatant was removed, and the pellets were dissolved in 500 μL of isopropanol. The optical density was detected at 560 nm in a plate reader BioTek (Winooski, VT, USA). Six to eleven animals per group were analyzed.

2.11. Determination of Cleaved Caspase-3. Cleaved caspase-3 was measured in brain homogenates by sandwich ELISA following the instructions of the provider. Samples were incubated for 18 h in a sensitized with capture antibody plate at 4°C, washed, and incubated with the detection antibody for 2 h at room temperature. Bound detection antibodies were detected using TMB as the substrate. Optical density was measured at 450 nm. All assays were made by duplicate. Four to five animals per group were used for statistical analysis.

2.12. Determination of Lipid Peroxidation. Lipid peroxidation was evaluated by the production of substances reactive to thiobarbituric acid (TBARS). The homogenate of the substantia nigra or the striatum was placed in a microtube with PBS and TBA reagent (0.375 g of TBA+15 g of trichloroacetic acid+2.54 mL of concentrated HCl). The samples were put in a boiling bath (94°C) for 20 min. Subsequently, the samples were centrifuged at 3000 × g for 15 min, and the optical density of the supernatant was determined with a BioTek plate reader at a wavelength of 532 nm. Six to ten animals per group were used for statistical analysis.

2.13. Statistical Analysis. The results are expressed as mean values ± SEM. Statistical analyses were performed using Prism (GraphPad Software 8). We used two- and one-way ANOVA to compare more than two groups of normally distributed datasets and Kruskal-Wallis test to compare more than two groups for nonnormally distributed datasets.

Post hoc multiple comparison tests included Tukey's and Dunn's. The motor behavioral test was analyzed with the chi-square test and logistic regression. Differences were considered significant with $P \leq 0.05$.

3. Results

3.1. Systemic LPS and MPTP Exposure Affects Behavior and Dopamine Levels. The presence of motor symptoms characterizes Parkinson's disease due to dopamine depletion after the neuronal loss of dopaminergic neurons, mainly in the substantia nigra [1]. To explore the possible effect of systemic LPS stimulation on motor impairment, we evaluated motor coordination and forelimb strength. Motor coordination was only affected in mice groups exposed to MPTP (Figure 2(a)). Mice showed less motor coordination and forelimb strength since they presented lower scores than the control groups (Figure 2(a)). Interestingly, all groups exposed to MPTP also displayed less success in reaching the end of the support bar ($P = 0.0457$; Figure 2(b)).

Depressive disorders are frequent neuropsychiatric complications present in Parkinson's disease [37]. Also, systemic inflammation secondary to LPS exposure has been documented to induce depressive symptoms in mice [38, 39]. To explore the impact of systemic LPS exposure on depressive behavior in our model, we employed the sucrose preference test as an indicator of anhedonia. mLPS and MPTP groups showed significantly less sucrose consumption than the saline group ($P = 0.0021$ and $P = 0.0002$, respectively; Figure 2(c)). The sLPS and mLPS groups exposed to MPTP showed less preference for sucrose consumption when compared to the control group (sLPS ($P < 0.0001$), mLPS ($P < 0.0001$)). mLPS displayed less sucrose preference than the sLPS group ($P = 0.001$). The sLPS group presented less sucrose preference than the MPTP group ($P < 0.0001$). MPTP exposure in the mLPS group also showed diminished sucrose preference when compared to the mLPS group ($P = 0.0148$; Figure 2(c)). Two-way ANOVA showed that the interaction of LPS and MPTP exposure was significant in sucrose preference (interaction: $P < 0.0001$, $F = 13.19$; LPS: $P < 0.0001$, $F = 12.18$; MPTP: $P < 0.0001$, $F = 113.3$). In conclusion, LPS exposure and MPTP coadministration led to an increased preference for water consumption over the 2% sugar solution, suggesting anhedonia (Figure 2(c)).

Since motor impairment and depressive disorders are secondary to neuronal loss in the MPTP model, we determined the levels of dopamine and serotonin in the striatum of the different experimental groups. The two-way ANOVA showed that administration of LPS did not modify the dopamine nor serotonin, whereas MPTP only modified the dopamine ($F\,1, 28 = 17.49$; $P = 0.0003$). However, LPS and MPTP challenges presented a significant interaction for both neurotransmitters (serotonin ($F\,2, 28 = 9.02$; $P = 0.0009$); dopamine ($F\,2, 28 = 5.997$; $P = 0.0068$)). The administration of mLPS caused a significant reduction in dopamine when compared to the saline group ($P = 0.014$; Figure 2(d)). Also, MPTP exposure significantly decreased the levels of both neurotransmitters compared to the saline-treated group (dopamine ($P = 0.0008$), serotonin ($P = 0.0441$); Figures 2(d) and 2(e)). LPS and MPTP coadministration also led to dopamine reduction (sLPS ($P = 0.0058$); mLPS ($P = 0.0132$), although this decrease was not significantly different from the MPTP group (Figure 2(d)). Curiously, previous LPS exposure to MPTP challenge restored serotonin levels (Figure 2(e)), and mLPS presented significantly higher levels of serotonin than the MPTP-exposed group ($P = 0.0406$; Figure 2(e)).

To ensure that the mice can correctly metabolize the MPTP after LPS challenge (sLPS and mLPS), we measured the activity of the enzyme monoamine oxidase B (MAO-B), which is the primary enzyme in transforming the MPTP into 1-methyl-4-phenylpyridinium (MPP+), the neurotoxic metabolite that enters neurons via the dopamine transporter. The activity of this enzyme was similar in all groups, and only the mLPS group presented significantly increased MAO-B activity ($P = 0.023$; Figure 2(d)).

Our data show that indeed MPTP challenge induces a motor deficit and anhedonic behavior in all experimental groups exposed to the neurotoxin. Anhedonic symptoms worsen when exposed to LPS beforehand. These phenomena are likely to be secondary to the decrease in dopamine [37], which is involved in the reward system [40], since the experimental sLPS and mLPS groups with MPTP presented an increase in serotonin compared to the MPTP group.

3.2. Multiple Low Doses of LPS Induce Persistent Systemic Inflammation. NFκB activation via the toll-like receptor 4 (TLR4) pathway by LPS administration is a well-known event and widely documented [41]. NFκB nuclear translocation leads to the production of inflammatory cytokines such as TNFα, IL1β, and IL6. However, prolonged exposure to LPS can sometimes induce tolerance [42, 43]. To rule out the possible LPS-induced tolerance effect in our multiple low doses of LPS administration (mLPS) experimental group, we measured both pro- and anti-inflammatory cytokines in serum samples.

The two-way ANOVA showed that administration of LPS modified the serum levels of pro- and anti-inflammatory cytokines IL1β ($F\,2, 25 = 16.50$; $P = 0.0002$), IL6 ($F\,2, 32 = 37.09$; $P < 0.0001$), TNFα ($F\,2, 28 = 66.47$; $P < 0.0001$), and TGFβ ($F\,2, 37 = 45.28$; $P < 0.0001$), while MPTP modified IL1β ($F\,1, 25 = 8.779$; $P = 0.0066$), IL6 ($F\,1, 32 = 27.48$; $P < 0.0001$), IFNγ ($F\,1, 22 = 9.409$; $P = 0.0056$), TNFα ($F\,1, 28 = 91.2$; $P < 0.0001$), IL10 ($F\,1, 41 = 102.9$; $P < 0.0001$), and TGFβ ($F\,1, 37 = 61.64$; $P < 0.0001$). LPS challenge acted synergistically with MPTP administration in IL1β ($F\,2, 25 = 7.815$; $P = 0.0023$), IL6 ($F\,2, 32 = 8.458$; $P = 0.0011$), TNFα ($F\,2, 28 = 5.665$; $P = 0.0086$), and TGFβ ($F\,2, 37 = 84.95$; $P < 0.0001$).

Multiple low doses of LPS (mLPS) increased the serum levels of IL1β ($P < 0.0001$), IL6 ($P = 0.0094$), TNFα ($P < 0.0001$), and TGFβ ($P < 0.0001$) compared to saline, while the single LPS dose (sLPS) did not induce any changes in the cytokine levels (Figure 3). mLPS administration also raised IL1β ($P = 0.0095$), IL6 ($P = 0.0258$), TNFα ($P < 0.0001$), and TGFβ ($P < 0.0001$) compared to the sLPS group (Figures 2(a), 2(b), 2(d), and 2(f)). The administration of MPTP also elevated the levels of IL6

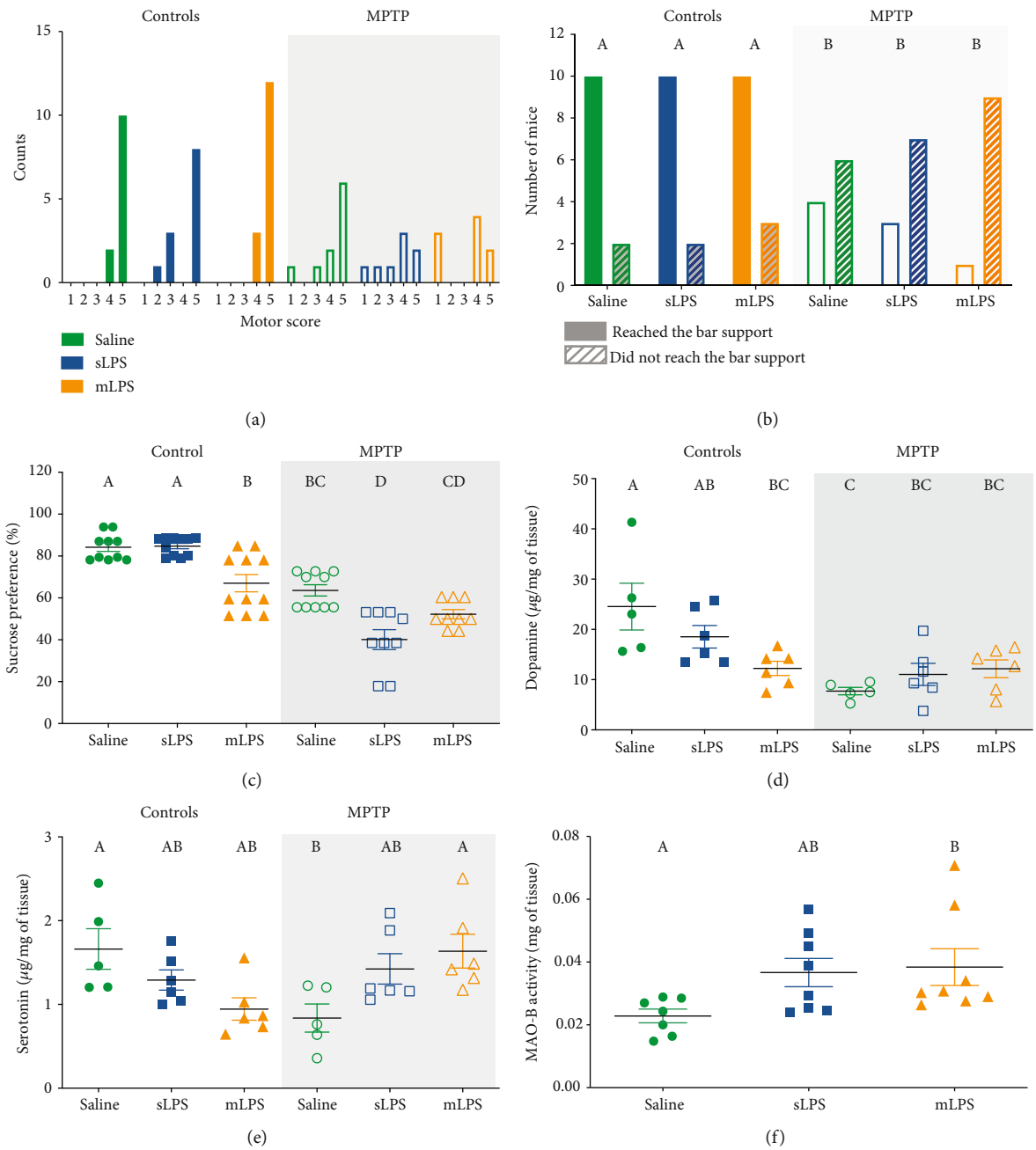

Figure 2: Reduction of dopamine levels relates to a decreased sucrose consumption preference, forelimb strength, and motor coordination. Mice were administered with saline solution, mLPS (100 μg/kg twice a week for three months), sLPS (5 mg/kg), and MPTP (30 mg/kg for five consecutive days). Motor coordination score in mice of the different experimental groups (a). The number of mice that successfully reached the bar support (b) is indicated with colored bars. Mice that did not reach the bar support are indicated with gray stripes. Data represent mean ± SEM (n = 10-12) and were analyzed by chi-square and logistic regression. Percentage of sucrose consumption preference in mice of the different experimental groups (c). Data represent mean ± SEM (n = 9-11) and were analyzed by two-way ANOVA, followed by a Tukey's *post hoc* test. Different letters indicate significant differences among the experimental groups ($P < 0.05$). Striatal dopamine (d) and serotonin (e) levels were determined in mice administered with saline solution, mLPS, sLPS, and MPTP by HPLC. Data represent mean ± SEM (n = 5-6) and were analyzed by two-way ANOVA, followed by a Tukey's *post hoc* test. Different letters indicate significant differences among the experimental groups ($P < 0.05$). Determination of MAO-B activity in the substantia nigra of mice administered with saline solution, treated with mLPS and sLPS (f). Data represent mean ± SEM (n = 7-8) and were analyzed by Kruskal-Wallis, followed by Dunn's test. Different letters indicate significant differences among the experimental groups ($P < 0.05$).

($P = 0.0007$), TNFα ($P < 0.0001$), and IL10 ($P < 0.0001$) compared to vehicle (Figures 2(b), 2(d), and 2(e)). However, when the MPTP stimulus followed a previous challenge with mLPS, it induced a significantly higher response in TNFα ($P < 0.0001$) and IL6 ($P = 0.0015$), while TGFβ was decreased ($P = 0.0002$) when compared

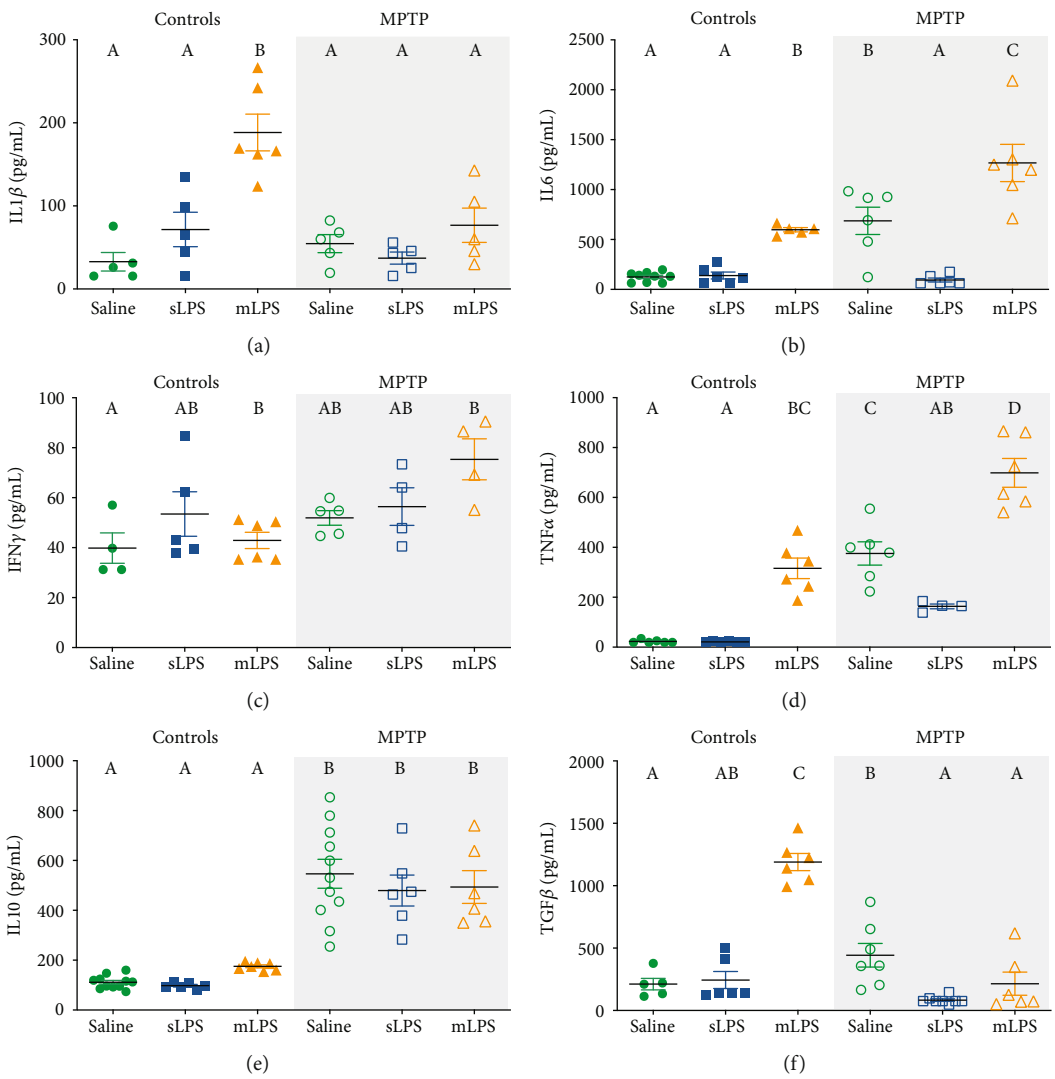

Figure 3: Serum inflammatory cytokines increase after LPS administration and MPTP challenge. Mice were administered with saline solution, mLPS (100 μg/kg twice a week for three months), or sLPS (5 mg/kg), followed by MPTP (30 mg/kg for five consecutive days). The levels of IL1β (a), IL6 (b), IFNγ (c), TNFα (d), IL10 (e), and TGFβ (f) were analyzed by ELISA. Data represent mean ± SEM (n = 5-8) and were analyzed by two-way ANOVA, followed by a Tukey's *post hoc* test. Different letters indicate significant differences among the experimental groups ($P < 0.05$).

with the group administered only with MPTP (Figures 2(b), 2(d), and 2(f)). The sLPS and MPTP coadministration behaved similarly to the MPTP group. Interestingly, sLPS in conjunction with MPTP significantly decreased IL6 ($P = 0.0011$), TNFα ($P = 0.0103$), and TGFβ ($P < 0.0001$) when compared with the group administered with MPTP only (Figures 2(b), 2(d), and 2(f)). The MPTP challenge in the mice previously exposed to mLPS induced a more pronounced response in IL6 ($P < 0.0001$) and TNFα ($P < 0.0001$) than the MPTP coadministration with sLPS (Figures 3(b) and 3(d)).

These results show that indeed chronic exposure to LPS maintains increased serum levels of the inflammatory IL6 and TNFα cytokines.

3.3. Systemic Inflammation Exacerbates Neuroinflammation after MPTP Challenge. After chronic systemic LPS challenge, an increase in proinflammatory cytokine production was observed (Figure 3). Peripheral proinflammatory cytokines can signal the brain by active transport through the BBB, the choroid plexus, or by afferent nerves such as the vagus nerve [44]. These peripheral inflammatory signals stimulate innate immune brain cells like microglia and astrocytes to respond with the same proinflammatory cytokines [15, 44]. To confirm this, we also assessed the presence of brain proinflammatory cytokines.

Two-way ANOVA showed that LPS modified the brain levels of IL1β ($F\,2, 25 = 15.65$; $P < 0.0001$), IL6 ($F\,2, 34 = 28.25$; $P < 0.0001$), TNFα ($F\,2, 36 = 56.42$; $P < 0.0001$), IFNγ ($F\,2, 26 = 8.474$; $P = 0.0015$), and TGFβ ($F2, 30 = 9.515$; $P = 0.0006$), while MPTP modified IL1β ($F\,1, 25 = 44.4$; $P < 0.0001$), IL6 ($F\,1, 34 = 92.88$; $P < 0.0001$), TNFα ($F\,1, 36 = 61.89$; $P < 0.0001$), IL10 ($F\,1, 34 = 101.5$; $P < 0.0001$), and TGFβ ($F\,1, 30 = 69.44$; $P < 0.005$). The

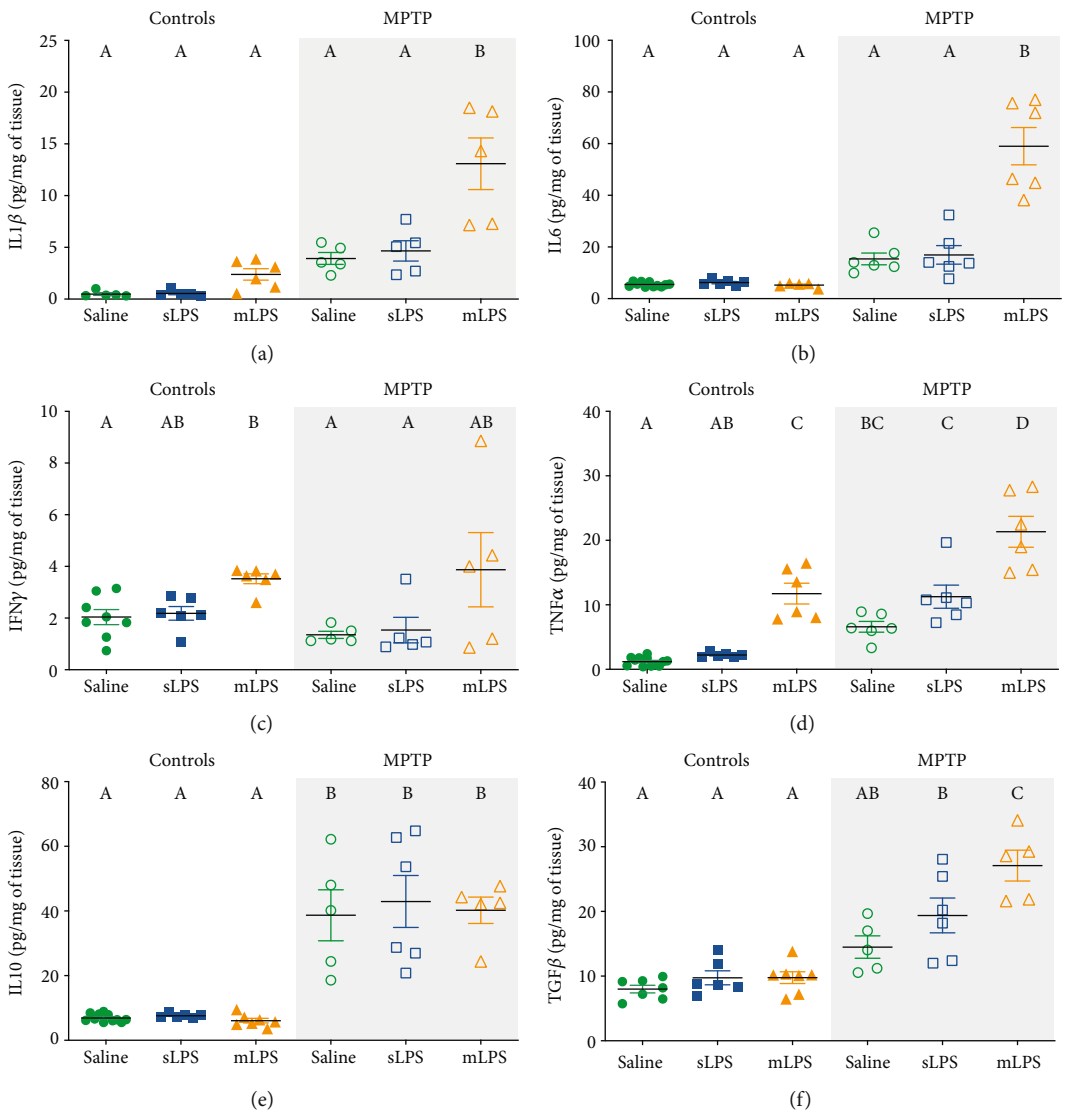

FIGURE 4: Brain inflammatory cytokines rise after LPS administration and MPTP challenge. Mice were administered with saline solution, mLPS (100 μg/kg twice a week for three months), or sLPS (5 mg/kg), followed by MPTP (30 mg/kg for five consecutive days). The levels of IL1β (a), IL6 (b), IFNγ (c), TNFα (d), IL10 (e), and TGFβ (f) were analyzed by ELISA. Data represent mean ± SEM (n = 5-8) and were analyzed by two-way ANOVA, followed by a Tukey's *post hoc* test. Different letters indicate significant differences among the experimental groups ($P < 0.05$).

interaction of LPS and MPTP exposure was significant for IL1β ($F\,2, 25 = 6.609$; $P = 0.0001$), IL6 ($F\,2, 34 = 29.64$; $P < 0.0001$), and TGFβ ($F\,2, 30 = 5.729$; $P = 0.0078$).

Mice administered with mLPS showed a significant rise in the brain levels of TNFα ($P < 0.0001$; Figure 3(d)) and IFNγ ($P = 0.0158$; Figure 4(c)), whereas MPTP administration induced a significant increase of TNFα ($P = 0.0175$; Figure 4(d)) and IL10 ($P < 0.0001$; Figure 4(e)). sLPS showed no significant changes in the brain cytokines measured compared to the saline group (Figure 4). The administration of mLPS in conjunction with the MPTP challenge led to higher levels of IL1β ($P < 0.0001$), IL6 ($P < 0.0001$), TNFα ($P < 0.0001$), and TGFβ ($P = 0.0003$) compared to the MPTP group (Figures 4(a), 4(b), 4(d), and 4(f)). This group also presented similar levels of IL10 to the MPTP group (Figure 4(e)). The mice exposed to sLPS and MPTP stimuli behaved similarly to the MPTP group (Figure 4). MPTP challenge in the sLPS group significantly increased TNFα ($P < 0.0001$), IL10 ($P < 0.0001$), and TGFβ ($P = 0.0002$) compared to the saline group (Figures 4(d)–4(f)). The mLPS group treated with the MPTP proneurotoxin significantly raised the inflammatory cytokines IL1β ($P < 0.0001$), IL6 ($P < 0.0001$), and TNFα ($P < 0.0001$) as well as IL10 ($P < 0.0001$) and TGFβ ($P < 0.0001$) compared to the saline group (Figures 4(a), 4(b), and 4(d)–(f)). The main differences between the sLPS and mLPS groups was the presence of higher levels of TNFα in the mLPS group ($P = 0.0001$; Figure 4(d)) and that the mLPS group rises significantly the levels of IL1β

($P = 0.0003$), IL6 ($P < 0.0001$), TNFα ($P < 0.0001$), and TGFβ ($P < 0.0001$) after the MPTP challenge (Figures 4(a), 4(b), 4(d), and 4(f)).

High TNFα levels may lead to the activation and nuclear translocation of NFκB [45]. NFκB, in turn, favors the transcription of inflammatory cytokines such as TNFα, IL1β, and IL6, perpetuating the inflammatory process [45]. We determined brain NFκB p105 levels to show if proinflammatory cytokine production is related to NFκB expression. The two-way ANOVA showed that administration of LPS ($F\,2, 16 = 4.743$; $P = 0.0241$) and MPTP ($F\,1, 16 = 16.82$; $P = 0.0008$) modified the NFκB expression. No significant changes were observed in mice administered only with sLPS, mLPS, or MPTP (Supplementary Figure 1S) compared to the saline group, though the levels of NFκB p105 were significantly increased in the groups previously administered with sLPS or mLPS and then challenged with MPTP ($P = 0.0317$ and $P = 0.0029$, respectively; Supplementary Figure 1S). Interestingly, all MPTP-treated groups behaved similarly (Supplementary Figure 1S), and only the MPTP and mLPS-cotreated group was significantly different from mLPS ($P = 0.0371$; Supplementary Figure 1S). It would have been more appropriate to evaluate the expression of phosphorylated NFκB or its nuclear translocation since the increase in NFκB p105 only indicates that there is more precursor for NFκB p50 [46], which could be then translocated to the nucleus. However, a dual role for p105 has been established [47], and it could be possible that its increase could also indicate an attempt to regulate/inhibit the NFκB pathway. This last hypothesis can be ruled out, since indirectly we observed a rise in IL1β, IL6, and TNFα, suggesting an active translocation of NFκB to the nucleus, since the latter is responsible for the transcription of these molecules.

Single peripheral LPS challenge leads to BBB leakage [48], MPTP administration favors BBB dysfunction, probably secondary to the effect of TNFα [49, 50]. To evaluate if the systemic inflammation had any effect on the BBB, albumin coupled to FITC was administered intravenously 24 h before sacrifice. Striatal sections from control and sLPS mice did not show any presence of albumin (Supplementary Figure 2S). Nevertheless, the mLPS group showed significantly higher levels of albumin compared to the saline ($P = 0.0064$) and sLPS groups ($P = 0.0066$; Supplementary Figure 2S). These results provide evidence that chronic systemic inflammation by itself affects BBB integrity.

Since chronic systemic inflammation compromises BBB integrity, we next evaluated whether the changes observed in brain cytokines were the consequence of the systemic inflammation and not secondary to the entrance of LPS to the brain. To this end, LPS coupled to FITC was injected in the same mLPS scheme to explore this possibility (Supplementary Figure 3S). The brain showed no FITC-LPS fluorescence (Supplementary Figure 3S), reinforcing our observation that the neuroinflammatory effect is secondary to the systemic inflammation.

Our results show that chronic exposure to LPS induces persistent systemic and brain TNFα, which comprises the BBB integrity. Despite BBB leakage, circulating LPS does not enter the brain, suggesting its stimulation is in the periphery, and the cytokine synthesis afterward is secondary to circulating TNFα.

3.4. MPTP Intoxication after Systemic Inflammation Increases Iba1 Staining. Our results show that chronic stimulation with LPS does increase the expression of TNFα (Figure 4); in fact, the MPTP challenge after chronic exposure to LPS significantly raised the synthesis of IL1β, IL6, and TNFα. Since microglial cells respond to peripheral inflammatory signals by producing more inflammatory molecules such as TNFα [15, 44], we evaluated the morphology of these in substantia nigra and striatum, the two main areas that show dopamine depletion in Parkinson's disease [35].

Iba1, a microglial marker, was modified by LPS ($F\,2, 21 = 34.84$; $P < 0.0001$) and MPTP ($F\,1, 21 = 134$; $P < 0.0001$) in the substantia nigra as shown by the two-way ANOVA. LPS and MPTP showed a positive interaction in increasing Iba1 staining ($F\,2, 21 = 5.785$; $P = 0.01$). This marker was significantly higher in the substantia nigra in all experimental groups when compared with the saline group ($P \leq 0.0002$; Figures 5(a) and 5(b)). Interestingly, the MPTP group previously exposed to mLPS had significantly higher levels of Iba1 compared to the group treated with MPTP alone ($P = 0.0002$; Figure 5(b)). MPTP challenge in both LPS groups increased significantly Iba1 staining (mLPS ($P < 0.0001$), sLPS ($P = 0.0044$)) when compared to their respective control groups (Figures 5(a) and 5(b)).

In the striatum, Iba1 staining showed a similar profile to that in the substantia nigra (Figures 6(a) and 6(b)). The two-way ANOVA showed that administration of LPS modified Iba1 staining in the striatum ($F\,2, 27 = 47$; $P < 0.0001$) as well as MPTP ($F\,1, 27 = 408.7$; $P < 0.0001$). Also, the two-way ANOVA showed a significant LPS and MPTP interaction ($F\,2, 27 = 9.422$; $P = 0.0008$). The mLPS and sLPS groups have a similar Iba1 staining (Figures 6(a) and 6(b)), and MPTP exposure increased Iba1 when compared to saline, sLPS, and mLPS (all with a $P < 0.0001$; Figures 6(a) and 6(b)). MPTP treatment in the previously exposed LPS groups significantly increased Iba1 compared to the MPTP group (mLPS ($P = 0.0404$), sLPS ($P = 0.0107$); Figures 6(a) and 6(b)).

To further discern the role of microglia, minocycline was administered during the same three months as mLPS. Inhibition of microglia M1 polarization, the classically activated microglia with proinflammatory functions, reduced significantly IL1β and TNFα brain levels when challenged with MPTP and MPTP coadministered with mLPS almost to physiological levels (Supplementary Figure 4S).

Additionally, we explored brain brain-derived neurotrophic factor (BDNF) levels. In the aging brain and under neuroinflammatory conditions, BDNF levels are reduced secondary to the M1 polarization of microglia and proinflammatory astrocytes [51–54]. The two-way ANOVA showed that administration of LPS modified the brain BDNF levels ($F\,2, 21 = 6.85$; $P = 0.0051$) as well as MPTP ($F\,1, 21 = 69.57$; $P < 0.0001$). Interaction between MPTP and LPS administration was significant ($F\,2, 21 = 14.17$; $P = 0.0001$). Chronic

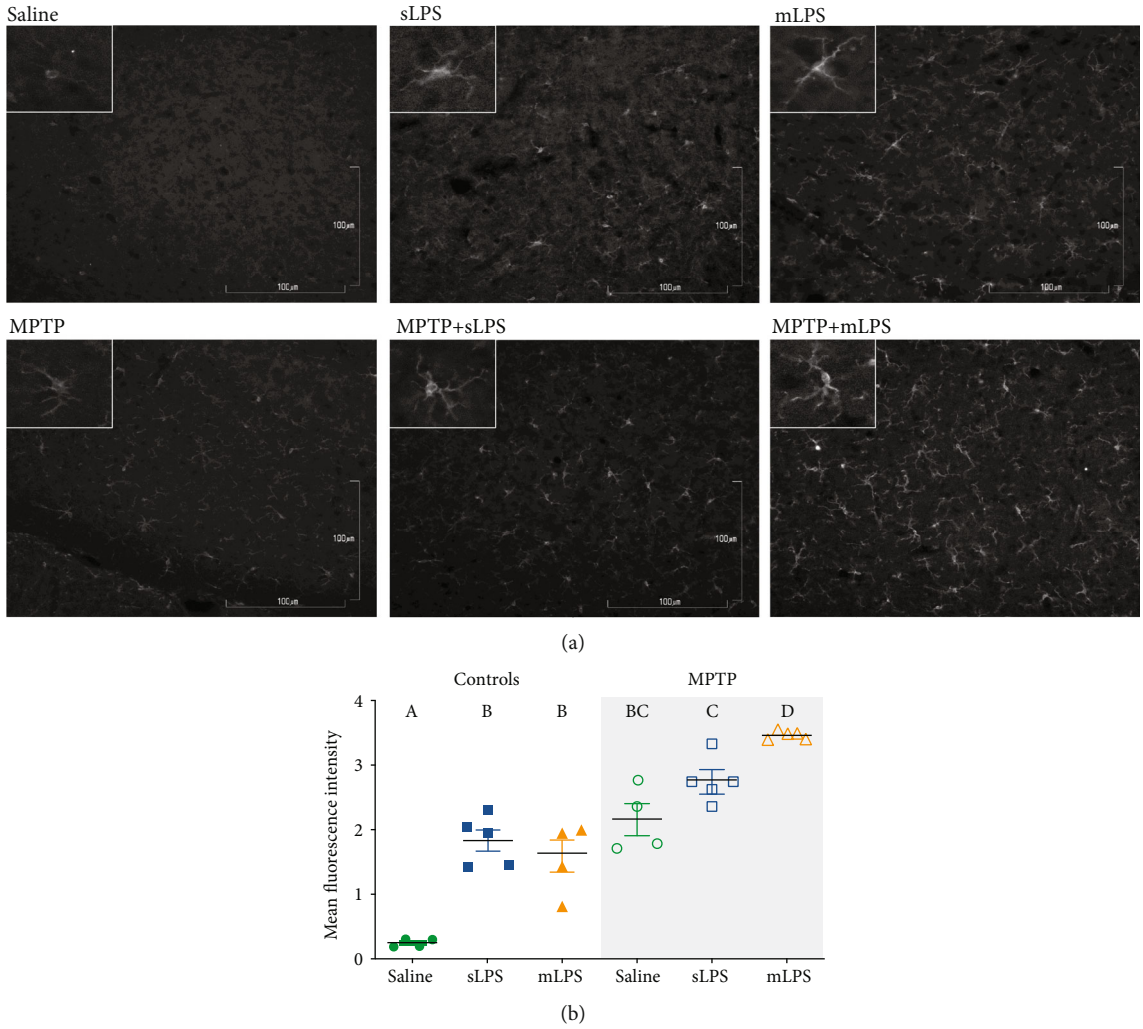

Figure 5: Iba1 staining increases in the substantia nigra after LPS administration and MPTP challenge. Representative images showing Iba1 immunodetection in grayscale in the substantia nigra of mice administered with saline solution, mLPS (100 μg/kg twice a week for three months), sLPS (5 mg/kg), and MPTP (30 mg/kg for five consecutive days) (a). Quantification of the fluorescence intensity of Iba1 staining in the substantia nigra from the different experimental groups (b). Data represent mean ± SEM (n = 4-6) and were analyzed by two-way ANOVA, followed by a Tukey's *post hoc* test. Different letters indicate significant differences among the experimental groups ($P < 0.05$).

exposure to systemic inflammation reduced BDNF significantly when compared to saline ($P = 0.001$; Figure 6) and sLPS groups ($P < 0.0001$; Figure 7), while MPTP exposure in all experimental models decreased further BDNF ($P < 0.001$; Figure 7).

Our data support that systemic inflammation leads to microglia activation possibly favoring an inflammatory M1 profile with increased Iba1 staining accompanied by increased brain IL1β, IL6, and TNFα production with a BDNF reduction, probably secondary to NFκB activation.

3.5. Systemic Inflammation Exacerbates Damage after MPTP Challenge. Cytokine and BDNF levels were evaluated in whole brain lysates. Nevertheless, after the measurement of these molecules, we decided it would be more precise to evaluate in the primary two affected brain structures (striatum and substantia nigra), since the administration of peripheral LPS can induce neurodegeneration in the substantia nigra secondary to the microglial synthesis of TNFα and ROS [15, 55]. To test whether this happens with chronic exposure to systemic LPS, we evaluated cell viability with the MTT assay, cleaved caspase-3, and lipid peroxidation as damage markers.

A decrease in cell viability determined by MTT reduction was observed in both substantia nigra and striatum of mice. In the substantia nigra, the two-way ANOVA showed that administration of LPS modified the cell viability ($F\,2, 42 = 5.339$; $P = 0.0086$), as well as MPTP ($F\,1, 42 = 28.46$; $P < 0.0001$). In the striatum, the two-way ANOVA also showed that administration of LPS modified the cell viability ($F\,2, 40 = 18.98$; $P < 0.0001$), as well as MPTP ($F\,1, 40 = 92.32$; $P < 0.0001$). Additionally, LPS and MPTP coadministration had a significant interaction in the striatum ($F\,2, 40 = 19.75$; $P < 0.0001$). A reduction in cell viability in both substantia nigra and striatum was observed in mice treated with mLPS ($P = 0.0099$

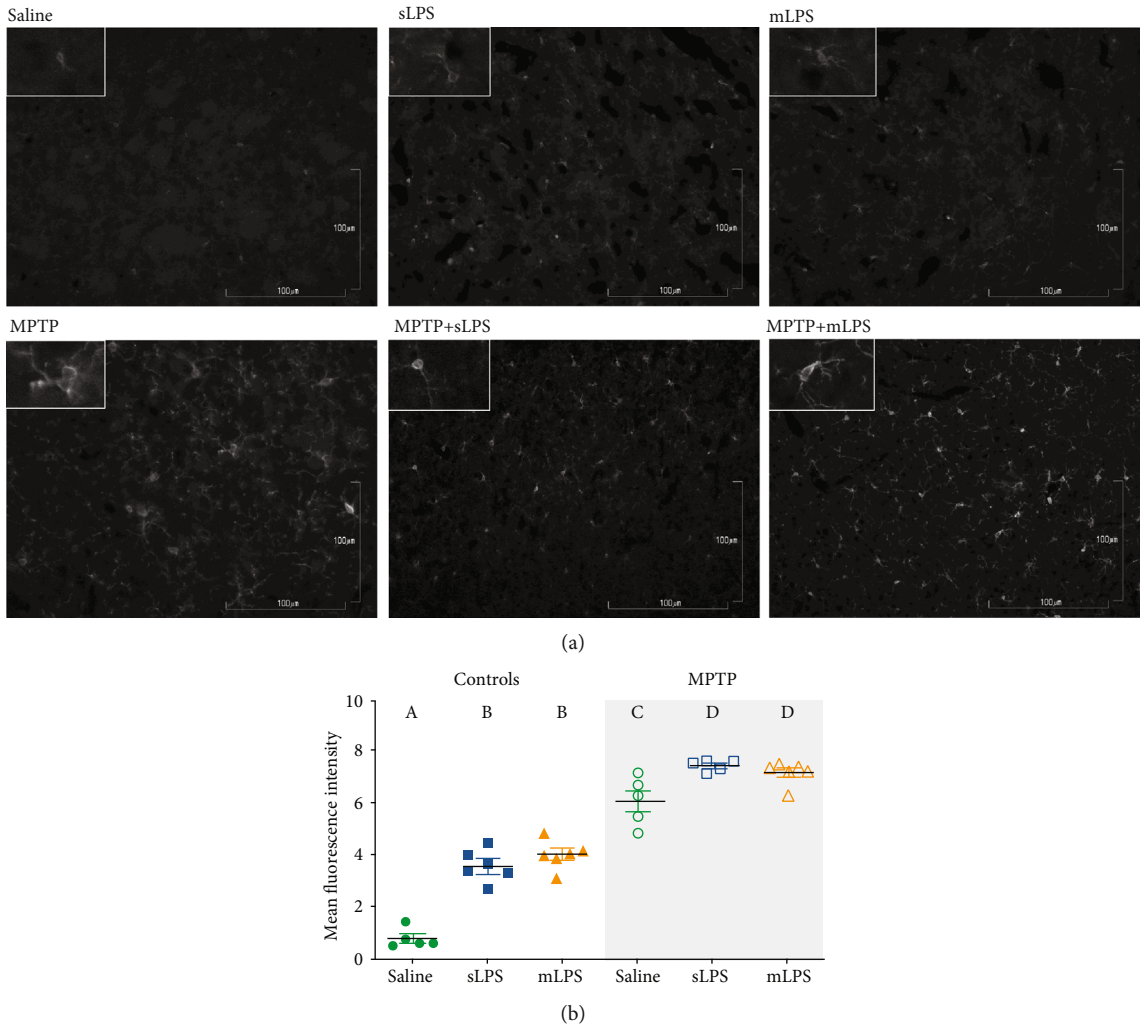

Figure 6: Iba1 staining increases in the striatum after LPS administration and MPTP challenge. Representative images showing Iba1 immunodetection in grayscale in the striatum of mice administered with saline solution, mLPS (100 μg/kg twice a week for three months), sLPS (5 mg/kg), and MPTP (30 mg/kg for five consecutive days) (a). Quantification of the fluorescence intensity of Iba1 staining in the striatum from the different experimental groups (b). Data represent mean ± SEM (n = 4-6) and were analyzed by two-way ANOVA, followed by a Tukey's *post hoc* test. Different letters indicate significant differences among the experimental groups ($P < 0.05$).

and $P < 0.0001$, respectively; Figures 8(a) and 8(b)), whereas the sLPS dose only decreased cell viability in the striatum ($P < 0.0001$; Figure 8(b)). Similarly, MPTP reduced cell viability significantly when compared to the control (substantia nigra ($P = 0.0002$), striatum ($P < 0.0001$); Figures 8(a) and 8(b)). However, the previous exposure to LPS did not further diminish the reduction observed in the MPTP group (Figures 8(a) and 8(b)). Also, the sLPS-coadministered MPTP group presented significantly lower levels of MTT reduction in the striatum when compared to sLPS ($P = 0.0182$; Figure 8(b)).

The two-way ANOVA showed that administration of LPS modified the cleaved caspase-3 in the substantia nigra ($F\,2, 24 = 5.852$; $P = 0.0085$), whereas MPTP modified the cleaved caspase-3 in the substantia nigra ($F\,1, 24 = 66.39$; $P < 0.0001$) and the striatum ($F\,1, 22 = 34.03$; $P < 0.0001$). MPTP exposure increased cleaved caspase-3 in the substantia nigra ($P = 0.0142$) and in the striatum ($P = 0.0337$) when compared to the saline control group (Figures 8(c) and 8(d)). The group previously administered with mLPS and subsequently with MPTP showed a significant increase in caspase-3 levels compared to the group administered only with MPTP ($P = 0.0268$); this effect was only observed in the substantia nigra of these mice (Figure 8(c)).

As an additional damage marker, we determined lipid peroxidation assessed by thiobarbituric acid reactive substances (TBARS) in the substantia nigra and the striatum (Figures 8(e) and 8(f)). The two-way ANOVA showed that administration of LPS did not modify the lipid peroxidation in the substantia nigra nor striatum, whereas MPTP modified the TBARS ($F\,1, 38 = 45.92$; $P < 0.0001$) in the substantia nigra and the striatum ($F\,1, 40 = 96.66$; $P < 0.0001$). We observed an increase of lipid peroxidation in the substantia nigra in mice exposed to MPTP alone, sLPS, and mLPS with MPTP challenge ($P = 0.0003$, $P = 0.0004$, and $P = 0.0036$, respectively; Figures 8(e) and 8(f)) compared to the saline

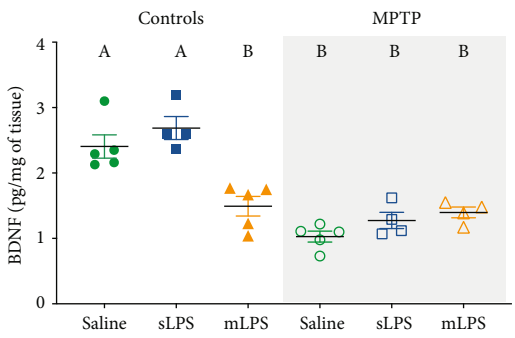

Figure 7: After LPS and MPTP challenges, brain BDNF levels are reduced. Mice were administered with saline solution, mLPS (100 μg/kg twice a week for three months), sLPS (5 mg/kg), and MPTP (30 mg/kg for five consecutive days). The levels of BDNF were analyzed by ELISA. Data represent mean ± SEM (n = 4-6) and were analyzed by two-way ANOVA, followed by a Tukey's *post hoc* test. Different letters indicate significant differences among the experimental groups ($P < 0.05$).

group. In the striatum, lipid peroxidation showed a similar pattern (all with a $P \leq 0.0001$) when compared to the control group (Figures 8(e) and 8(f)). However, the sLPS group challenged with MPTP presented with significantly raised lipid peroxidation in the substantia nigra ($P = 0.0045$) and the striatum ($P < 0.0001$) when compared to its sLPS control (Figures 8(e) and 8(f)). The mLPS-coadministered with MPTP group presented the same levels of lipid peroxidation in the striatum as the MPTP group and was significantly different from the mLPS group ($P < 0.0001$; Figures 8(e) and 8(f)).

Our results show that indeed with chronic LPS exposure, cell viability decreased in the substantia nigra and the striatum and was further reduced after MPTP challenge; these observations are in accordance with the higher levels of the active form of caspase-3. On the other hand, lipid peroxidation only was observed after MPTP administration, indicating that probably oxidative stress is not involved in the synergistic action of LPS and MPTP.

4. Discussion

Neurodegenerative diseases are chronic inflammatory and oxidative processes associated with aging that lead to neuronal death. The role of systemic inflammation has been established in neuroinflammation, but the contribution of chronicity of these inflammatory processes in the development of PD requires additional studies for their understanding. To explore this possibility, we used two different models of systemic LPS administration that induce neuroinflammation with a subsequent proneurotoxin MPTP challenge.

The sLPS administration scheme induces a well-known neuroinflammatory process [15]; however, we found that mLPS is a more complete neuroinflammatory model that *per se* resembles the MPTP model with higher brain TNFα levels, increased Iba1 staining in the substantia nigra and striatum, and diminished brain BDNF and dopamine levels, as well reduced cell viability and striatal dopamine. Our results are in accordance with other LPS models [56] that also induce nigrostriatal neurodegeneration secondary to neuroinflammation and microglial activation after LPS administration in the striatum [26] and substantia nigra [57] and of a single or repeated (four doses) systemic exposure [15, 25].

Chronic stimulation with LPS increased serum proinflammatory cytokines (IL1β, IL6, and TNFα), TGFβ, but not IL10 (Figure 2). Interestingly, when the animal is subsequently challenged with MPTP, IL10 raised, and IL1β and TGFβ decreased (Figure 2). Prolonged stimulation of TLRs can lead to reduced synthesis of IL1β through the action of IL10 in macrophages [58]. This event is likely to be secondary to the IL10 negative regulation of NLRP3 of the inflammasome pathway, which, in conjunction with caspase-1, are responsible for converting pro-IL1β to mature and functional IL1β [59]. TGFβ lowering in the mLPS group after MPTP administration might be the result of the action of elevated IFNγ and TNFα. TGFβ reduction in the mLPS group after MPTP administration might be the result of the action of elevated IFNγ and TNFα. IFNγ via STAT1 and TNFα via NFκB induce inhibitory Smad7 expression, an antagonist of TGFβ signaling pathway, thus lowering TGFβ secretion [60, 61].

Although it has been shown that the repeated administration of LPS causes innate immune tolerance that could induce tolerant microglia [62], our data show the opposite. The mLPS administration induces a significant increase of the inflammatory cytokines (IL1β, IL6, and TNFα) secondary to the constant stimulation with LPS of the peripheral innate immune system. However, when peripheral immune tolerance to LPS has been demonstrated, it has been in models that employ higher doses of LPS (e.g., 300 μg/kg daily for four days versus 100 μg/kg twice a week for three months) and do or do not receive a second stimulus in the brain or *ex vivo* [63–65]. This peripheral immune tolerance is mainly by the downregulation of TLR4 expression and upregulation of CD200-CD200R and anti-inflammatory cytokines after epigenetic reprogramming [63–65]. On the contrary, our data support that the systemic TNFα induced by mLPS leads to microglial priming, exacerbating the inflammatory response after a stimulus such as MPTP and potentiating the damage induced by the neurotoxin. Systemic TNFα is transported through the BBB by TNFα receptors, promoting the activation of microglial cells, releasing additional TNFα and other cytokines establishing chronic neuroinflammation [15].

Unsurprisingly, we found compromised BBB permeability in mice administered with mLPS (Supplementary Figure 2S), since previous reports have shown the disruptive effects of LPS on the BBB [66, 67]. The evidence shows that LPS stimulates the cerebrovascular endothelium and surrounding cells via prostanoids and NO [68–70]. Nevertheless, we did not detect LPS presence in the CNS after its intravenous administration (Supplementary Figure 3S). Despite this, endogenous LPS can cross the BBB and reach the CNS under physiological conditions [71]. On the other hand, Banks et al. [70, 72] showed that disruption of the BBB with repeated injections of LPS did not enhance LPS entry into the CNS, and the effects of peripherally

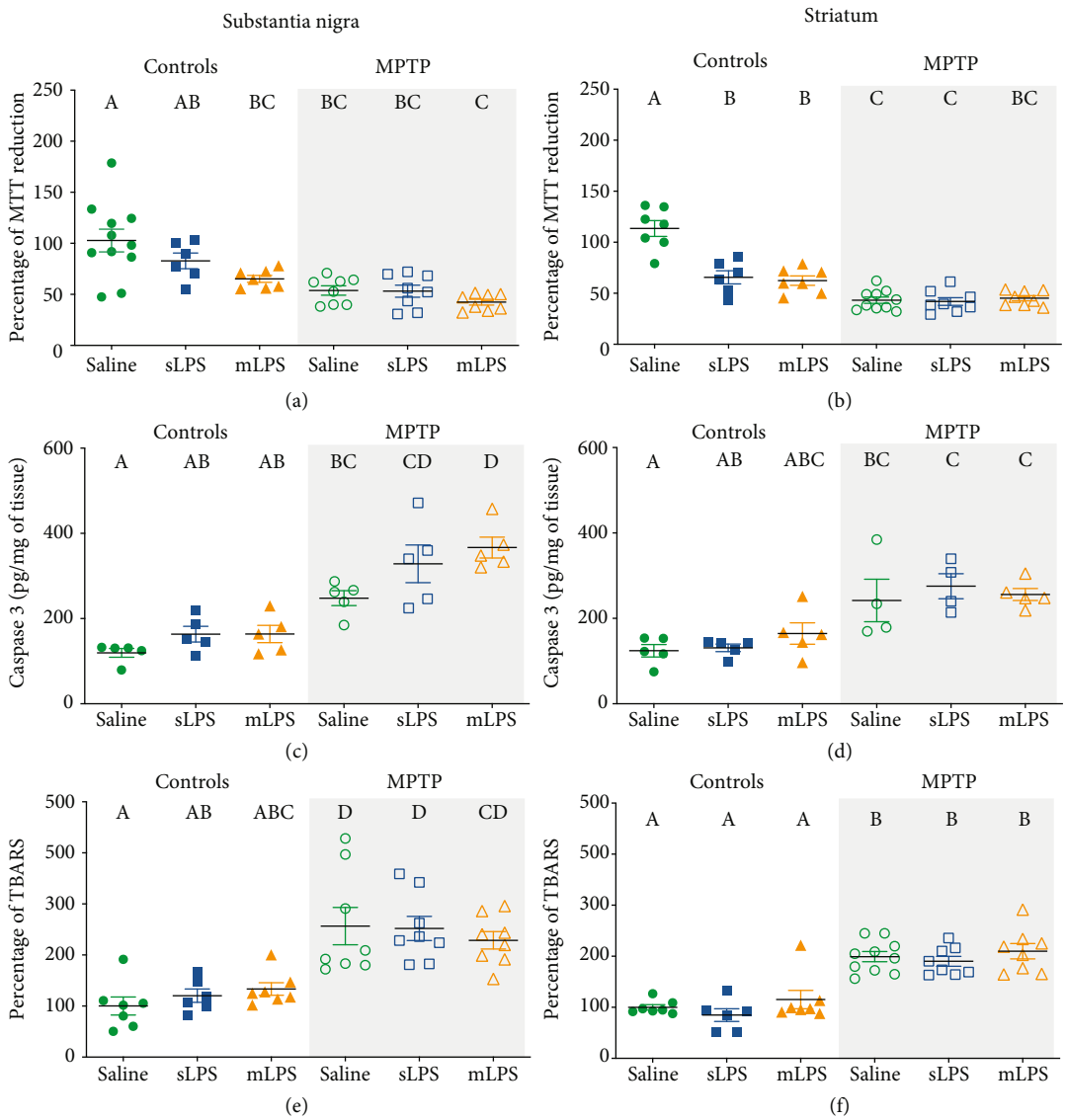

Figure 8: Cell viability decreases and lipid peroxidation and cleaved caspase-3 rise after LPS and MPTP challenges. Mice were administered with saline solution, mLPS (100 μg/kg twice a week for three months), sLPS (5 mg/kg), and MPTP (30 mg/kg for five consecutive days). Cell viability assessed by MTT reduction (a, b), cleaved caspase-3 (c, d), and lipid peroxidation determined by TBARS method (e, f) were determined in the substantia nigra (a, c, e) and striatum (b, d, f). Data represent mean ± SEM (n = 4-10) and were analyzed by two-way ANOVA, followed by a Tukey's *post hoc* test. Different letters indicate significant differences among the experimental groups ($P < 0.05$).

administered LPS are likely mediated through LPS receptors located outside the BBB. Thus, the effect observed in the brain results from the chronic systemic inflammation induced by the LPS treatment and not by direct LPS microglial stimulation.

mLPS generated an increase of brain TNFα but not of IL10 and TGFβ, which are both anti-inflammatory cytokines, suggesting an imbalance of the anti-inflammatory mechanisms which causes a chronic proinflammatory state. It would be interesting to evaluate in the mLPS mice the levels of Parkin, since microglia exposed to LPS or TNFα downregulate Parkin, an anti-inflammatory regulator, increasing the vulnerability of the nigrostriatal pathway to degeneration [29, 73, 74].

Microglia can respond to damage by expressing specific growth factors. After a lesion in the striatum where dopaminergic neurons are mainly affected, microglia respond by releasing BDNF and glial cell-derived neurotrophic factor, both neuroprotective factors [75]. However, IL1β suppresses the release of BDNF [76]. Furthermore, the presence of M1 polarized microglia, the proinflammatory microglial cell, is associated with low BDNF brain levels in an LPS-induced depression-like model [77]. These observations could explain the reduction of BDNF in mLPS mice, simulating the changes related to aging and probably conferring susceptibility to damage in the experimental MPTP-treated groups [78].

MAO-B is the primary enzyme responsible for transforming MPTP to MPP+ in glial cells, and MPP+ enters into

the dopaminergic neurons via dopamine transporter [79, 80]. In our models, MAO-B was not affected by the administration of LPS since the activity of MAO-B was not altered (Figure 2(f)), suggesting that astrocytes correctly metabolized MPTP and ensuring that the results observed are not due to a failure in the metabolism of the MPTP toxin. Moreover, our data show that mLPS increases MAO-B activity. MAO-B activity can be upregulated by p38 mitogen-activated protein kinases (p38 MAPK) in activated glial cells, microglia, and astrocytes [81, 82], and its overexpression in astrocytes causes dopaminergic neurodegeneration [83]. It is likely that in the mLPS model, the increase of MAO-B activity secondary to the p38 MAPK pathway, which plays a role in PD pathogenesis [84], could increase the amount of MPP+ generated, effectively increasing the neurotoxin dose.

The sLPS-coadministered MPTP model behaved very similar to the MPTP model presenting a similar cytokine and damage profile even though the sLPS increased the expression of Iba1. It is plausible that prior exposure to an acute inflammatory stimulus generates some protection or tolerance to the second proinflammatory stimulus, in this case, MPTP exposure, so the damage observed is mainly secondary to the neurotoxin. Nevertheless, MPTP challenge after mLPS administration led to significantly higher levels of IL1β, IL6, and TNFα than the other MPTP-treated groups, probably as a consequence of microglial priming since Iba1 staining was elevated in the substantia nigra when compared to MPTP- and sLPS-coadministered MPTP groups (Figures 4 and 5). Monocyte chemoattractant protein-1 (MCP-1/CCL2) secreted by astrocytes plays a role in microglia recruitment in the MPTP model [85], but it also increases after systemic LPS stimulation [42, 44]. It is probable that secreted MCP-1 favored microglial recruitment to the substantia nigra and striatum, thus increasing Iba1 staining in these structures (Figures 4 and 5). During aging, microglia generate higher levels of IL6, TNFα, and IL1β [86], possibly remaining in a chronic inflammatory state, similar to our model of mLPS.

Interestingly, mLPS also significantly raised IFNγ in the brain, which enhances the phagocytic activity of microglia [87] and induces the expression of MHC class II and costimulatory molecules, probably polarizing towards an M1 profile, therefore promoting persistent neuroinflammation with increased brain TNFα levels. With the administration of minocycline, an inhibitor of microglial M1 polarization, brain TNFα and IL1β levels were equal to those of the control group (Supplementary Figure 4S). Even the increase of TNFα observed after mLPS and MPTP administration lowered to levels similar to control mice without minocycline treatment, indicating that microglial source of TNFα may play a crucial role in the development of neurodegenerative diseases. Deficiency of TNFα receptors in mice suppressed microglial activation and modified the brain susceptibility to MPTP damage, further supporting the role of microglia in TNFα production and participation in neuronal degeneration [88].

LPS exposure generates an inflammatory state very similar to that observed in depressive patients [89], and many studies claim that this inflammation, given by high levels of TNFα, IL6, and IL1β, can impact on serotonin metabolism [90]. Since proinflammatory cytokines can lead to the overactivation of the enzyme indoleamine-2,3-dioxygenase, which uses tryptophan as a substrate for the formation of kynurenine, tryptophan lowers, causing a decrease in serotonin synthesis [90]. The serotonin transporter is responsible for the recapture of serotonin; its overregulation caused by inflammation can also lead to a decrease in serotonin levels [91]. These observations are congruent with our results, where mLPS decreases serotonin inducing less sucrose preference similar to the MPTP model. Like in serotonin, cytokines have been shown to influence both the synthesis and the recapture of dopamine [92]. Mice from the different groups exposed to MPTP showed a decrease in motor coordination and forelimb strength when they were subjected to the balance bar test (Figures 2(a) and 2(b)), probably secondary to dopamine depletion comparable to other studies [29, 36, 93].

During PD, bradykinesia, rigidity, and resting tremor are the most common symptoms secondary to the depletion of dopamine levels [94, 95]. Notwithstanding, PD also presents nonmotor symptoms, including cognitive and psychiatric abnormalities, like dementia, depression, apathy, anxiety, and hallucinations [94]. Depression presents symptoms like anhedonia, the inability to perceive pleasure. Both our models previously exposed to LPS (mLPS and sLPS) and subsequently challenged with MPTP presented diminished preference in sucrose consumption, suggesting anhedonia (Figure 2(c)). This behavior arises from a deterioration of noradrenergic/serotonergic function that occurs secondarily to systemic inflammation. Evidence shows that brain and cerebrospinal fluid levels of norepinephrine [96] and serotonin in PD patients are decreased compared to healthy persons [97]. Systemic inflammation evoked by LPS could be generating a neuronal loss in multiple brain areas such as serotonergic neurons in the raphe nuclei, noradrenergic neurons in the locus coeruleus, dopaminergic neurons in the substantia nigra, and cortical neurons in regions interconnected with limbic structures, causing a depressive behavior in these mice similar to PD development [37]. We show that MPTP, in combination with LPS exposure, generated reduced cell viability, inflammation, and progressive neural degeneration in an *in vivo* model, suggesting MPTP and LPS cointeraction. MPTP and LPS interaction favors the NADPH oxidase-mediated release of superoxide free radicals in neuron-glia cultures; the pharmacological inhibition and genetic inactivation of NADPH oxidase prevented superoxide production and synergistic neurotoxicity [98]. MPTP and LPS have been proven to act synergistically [50, 98], stimulating microglia activation with inflammatory cytokine production and inducing neurotoxic A1 astrocytes [50]. Neurotoxic A1 astrocytes are present in the striatum of elderly [99], as well as in the substantia nigra and striatum of Parkinson's disease patient [100], and are considered to be coresponsible for neuronal loss since its blocking protects dopaminergic neurons in a Parkinson's disease murine model [101]. Further experiments on A1 astrocyte participation in these LPS models could be useful to elucidate

more *in vivo* mechanisms that lead to dopaminergic neuron degeneration.

In conclusion, we demonstrated that chronicity in systemic inflammation generated by the repeated intraperitoneal administration of LPS induced elevated serum TNFα levels, leading to enhanced microglial neurotoxic response, subsequently increasing brain TNFα levels and raising MAO-B activity, consequently exacerbating MPTP damage. This chronic neuroinflammatory state results from the persistence of microglia activation and the subsequent production of proinflammatory cytokines, mainly TNFα. This model resembles more the impact of systemic inflammation in developing Parkinson's disease in humans than the model of the single LPS dose.

Data Availability

All 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 conflicts of interest.

Authors' Contributions

All authors meet the criteria for authorship and have approved the final article, and all those entitled to authorship are listed as authors. The roles for each and every author are described as follows: (1) the conception and design of the study were carried out by P.U.M. and A.C.; (2) the acquisition of data and analysis and interpretation of data were carried out by P.U.M., I.F.P., E.G, L.N. and A.C.; (3) the drafting of the article or revising of it critically for important intellectual content was carried out by I.F.P., L.N., and A.C.

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Supplementary Materials

Figure 1S: brain expression of NFκB. (a) Representative Western blot bands for NFκB p50 and p105 in the whole brain of mice challenged with saline solution, mLPS, sLPS, and MPTP. (b) Densitometric analysis of the expression levels of NFκB p105. Figure 2S: increased permeability of the blood-brain barrier after chronic LPS administration. (a) Representative images showing the presence of labeled FITC-albumin in the striatum of mice treated with saline, sLPS, and mLPS. (b) Quantification of FITC-albumin present in the whole brain lysates obtained from the mice treated with saline, sLPS, and mLPS. Figure 3S: absence of LPS entry to the CNS. (a) Representative images showing the absence of labeled FITC-LPS in the striatum of mice treated with mLPS. (b) Quantification of FITC-LPS present in the total brain. Figure 4S: effect of minocycline administration on the levels of brain TNFα (a) and IL1β (b) in mice administered with saline, mLPS, and challenged with MPTP. *(Supplementary Materials)*

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Research Article

Oxidative Stress and Dementia in Alzheimer's Patients: Effects of Synbiotic Supplementation

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Background. Alzheimer's disease (AD) is the most common cause of dementia in elderly patients. Recently, several studies have shown that inflammation and oxidative stress precede the cardinal neuropathological manifestations of AD. In view of the proven antioxidant effects of probiotics, we proposed that continuous dietary supplementation with milk fermented with kefir grains might improve cognitive and metabolic and/or cellular disorders in the AD patients. *Methods*. This study was designed as an uncontrolled clinical investigation to test the effects of probiotic-fermented milk supplementation (2 mL/kg/daily) for 90 days in AD patients exhibiting cognitive deficit. Cognitive assessment, cytokine expression, systemic oxidative stress levels, and blood cell damage biomarkers were evaluated before (T0) and after (T90) kefir synbiotic supplementation. *Results*. When the patients were challenged to solve 8 classical tests, the majority exhibit a marked improvement in memory, visual-spatial/abstraction abilities, and executive/language functions. At the end of the treatment, the cytometric analysis showed an absolute/relative decrease in several cytokine markers of inflammation and oxidative stress markers ($^{\cdot}O_2^-$, H_2O_2, and $ONOO^-$, ~30%) accompanied by an increase in NO bioavailability (100%). In agreement with the above findings by using the same technique, we observed in a similar magnitude an improvement of serum protein oxidation, mitochondrial dysfunction, DNA damage/repair, and apoptosis. *Conclusion*. In conclusion, we demonstrated that kefir improves cognitive deficits, which seems to be linked with three important factors of the AD—systemic inflammation, oxidative stress, and blood cell damage—and may be a promising adjuvant therapy against the AD progression.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in elderly patients and is clinically defined as a progressive, global, and strong cognitive decline leading to an emotional distress and codependence [1–4]. Unfortunately, the number of AD patients has been rapidly growing worldwide (increasing by 117% in the last 26 years), with the highest age standardized prevalence in Turkey and Brazil [5].

The pathophysiology of AD is multifactorial, involving microglial activation, excessive proinflammatory cytokines, vascular disorder, disrupted mitochondrial function accompanied

by overproduction of reactive oxygen species (ROS), and oxidized molecules [6–13]. In this scenario, the cardinal neuropathological manifestations of AD culminate with amyloid-β (Aβ) and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein leading to synapse degeneration [9, 14, 15].

As the actual pharmacotherapy for dementia (using cholinesterase and/or glutamate inhibitors) is insufficient in slowing down completely the AD progression, new treatment strategies are still necessary to improve AD patient care [5, 16, 17]. In parallel, there is growing evidence that disturbances along the "brain-gut-microbiota axis" are involved in the pathogenesis of neurodegenerative diseases enhancing inflammation at the gut, systemic, and central nervous system (CNS) levels [15, 18]. In this context, recent data have shown the beneficial effects of probiotic supplementation on intestinal epithelial integrity, immunomodulation, oxidative stress, and even procognition [15, 19, 20]. However, the role of safe and inexpensive nutraceutical synbiotic kefir (a synergistic mixture of prebiotics and probiotics) in AD is still not documented [15, 20, 21].

The kefir-fermented milk is a functional food originally from the Northern Caucasus and currently distributed worldwide either commercially (e.g., Russia, Spain, Germany, United States, Canada, and Brazil) or "in-house" [22–26]. Milk fermentation with kefir grains is made up by bioactive compounds (peptides, vitamins, and polysaccharides) originally generated by acid lactic bacteria and yeast species present in these grains [23, 24, 26–28]. Recently, we and others have shown that administration of kefir and/or their bioproducts was able to prevent the cardiac and vascular dysfunctions in experimental models of hypertension [23, 26, 29], atherosclerosis [30], and gastric ulcers [27], which was justified, at least in part, by its antioxidative and immunomodulatory properties.

The present study addressed the hypothesis that kefir supplementation would provide cognitive benefit by attenuating systemic inflammation and oxidative stress in AD patients. The novel data revealed relevant new insights into the effects of this synbiotic on cognitive function through biochemical, molecular, and cellular parameters related to neurodegenerative diseases.

2. Methods

2.1. Patients. This uncontrolled clinical trial evaluated AD patients selected by convenience sampling. The diagnosis of AD was according to the clinical diagnostic criteria of dementia due to probable Alzheimer's disease with increased level of certainty defined by the Alzheimer's Association and the National Institute on Aging (NIA) published in 2011 [31]. The criteria evaluated in our study were based on the presence of insidious cognitive progressive decline or behavior symptoms involving a minimum of two cognitive domains (e.g., memory, language, attention, and constructive abilities) besides impairment of usual activities. It is important to emphasize that unexplained symptoms by delirium or major psychiatric disorders are detected through a combination of history taking and objective cognitive assessment. The sample included individuals of both sexes, without age restriction, who were assisted at a reference center in Vila Velha, Espírito Santo, Brazil, specialized in AD. The inclusion criteria were the following: (1) patients without previous neurological and/or psychiatric comorbidities associated with cognitive impairment, (2) patients without clinical depression and/or with a depression index < 17 using the Hamilton scale, and (3) patients without several clinical comorbidities (decompensated diabetes) and patients with autoimmune diseases taking or not immunosuppressive medication, neoplasms, and/or inflammatory bowel diseases. The exclusion criteria were the following: (1) patients unable to make use of probiotic supplementation because of organic or environmental causes, (2) patients using substances that might affect neurocognitive assessment, and (3) patients not using the maximum dose of acetylcholinesterase inhibitor (donepezil, 10 mg/day). The research was approved by the ethics committee of Vila Velha University (#1.804.392), and a signed free informed consent form was obtained from each of the subjects and/or their tutors after complete information about the nature and possible risks and benefits of the study, for him/herself, the community, and medical science was given.

In the beginning of the study, thirty-four subjects fulfilled the inclusion criteria. However, eighteen subjects were excluded from the study due to the elevated depression indexes (three subjects), due to clinical and laboratory signs of decompensated diabetes (six subjects), and due to the intervention with antibiotic therapy and/or hospitalization during the follow-up (nine subjects). During the study, 3 out of 16 patients in the final group died and a total of 13 subjects completed the experiments receiving the probiotic supplementation for 90 days and being evaluated, as summarized in Figure 1.

2.2. Production of Fermented Milk by Kefir Grains. The fermented material was prepared by inoculating pasteurized milk with 4% kefir grains containing the species *Acetobacter aceti*, *Acetobacter* sp., *Lactobacillus delbrueckii delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus fructivorans*, *Enterococcus faecium*, *Leuconostoc* spp., *Lactobacillus kefiranofaciens*, *Candida famata*, and *Candida krusei* and incubating the culture at 25°C–28°C for 24 h. After the incubation period, the fermented product was filtered and refrigerated at 2°C–6°C for 24 h, as previously described by us [23]. In order to improve organoleptic characteristics, the product was blended with organic strawberries in the proportion of 500 g of fruit for every 2 L of fermented milk without added sugars or preservatives.

2.3. Experiment Protocol. At first (T0), the participants were submitted to a battery of tests for the screening of the identification of cognitive deficits and their venous blood was collected for analysis of inflammation, oxidative stress, and molecular and cellular integrity. The blood samples were collected in EDTA-containing Vacutainer glass tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and centrifuged at 2000 g for 10 min, and the serum was then stored at −20°C. In addition, erythrocytes were lysed and

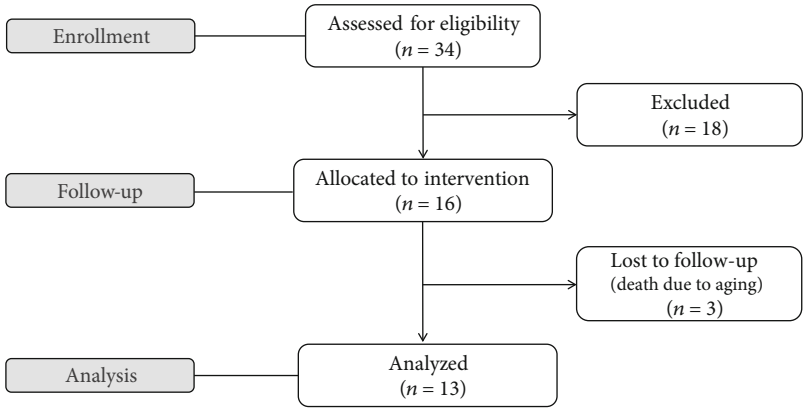

Figure 1: Summary of exclusion criteria used in the present study and patient flow.

white blood cells were stored at -80°C. All the measurements were obtained via an automatic biochemical analyzer (AU 400 or 680, Olympus/Beckman Coulter, Munich, Germany) or a flow cytometer (FACSCanto II, BD, CA, USA). These data were considered the control values in this paired study. After, the patients were subjected to fermented milk supplementation at the daily dose of 2 mL per kilogram of body weight. At the end of the study (T90), the cognitive, biochemical, molecular, and cellular parameters were once again evaluated.

2.4. Cognitive Assessment. Cognitive assessment was made before (T0) and after 90 days (T90) of the probiotic supplementation, using the tests recommended by the Department of Cognitive Neurology and Aging of the Brazilian Society of Neurology for screening dementia syndromes [32], as well as by the American Society of Neurology [33] and the National Institute on Aging and Alzheimer's Association [34]. The following functions were analyzed: "global cognitive functions," using the (1) Mini-Mental State Examination (MMSE); "memory," using a recall board with 10 concrete objects to promote the (2) immediate memory test and (3) delayed memory test, according to Nitrini et al. [32], which is recommended for evaluating populations with different levels of education; "visual-spatial and abstraction abilities," using the (4) Cookie Theft Picture Test, according to the consensus recommendations published by Nitrini et al. [32] suggesting the use of description of thematic figures justified by the absence of studies in the area with the Brazilian population, and the (5) Similarity Test, using the recommendations published by Nitrini et al. [32] to apply NEUROPSI subsection where the respondent is asked to say the similarity between three pairs of nouns (orange and pear, dog and horse, and eye and nose); "executive and language functions," using the (6) Boston Naming Test and (7) verbal fluency test; "attentive function," using (8) Trail Making Test A; and "visuoconstructive abilities," using the (9) clock-drawing test. To avoid the *learning effect bias*, the cognitive assessment trials were spaced by 90 days and the various domain tests were applied on a different order.

2.5. Determination of Cytokines Using the Cytometric Bead Array. Concentrations of proinflammatory (IL-6, IL-8, IL-1b, IL-12p70, and TNF-α) and anti-inflammatory (IL-10) cytokines were analyzed in the serum of patients using a Cytometric Bead Array Human Inflammation kit (CBA, BD Biosciences, USA) according to the manufacturer's instructions. Samples were analyzed with a FACSCanto II flow cytometer (BD, San Jose, CA, USA). Data acquisition was performed with FACSDiva software (BD), and the analysis of the events acquired was performed with the help of FCAP Array software (BD). Samples were measured by comparing them with the standard curves of recombinant cytokines using FCAP Array software (BD). All results are expressed as pg/mL.

2.6. ROS Analysis. Quantification of ROS components was also performed by flow cytometry, using a FACSCanto II (Becton Dickinson, BD, CA, USA) instrument to analyze the intracytoplasmic ROS content, as previously described by us [27, 35, 36]. Peripheral blood was drawn from the Alzheimer's patients, and the red blood cell lysis was induced by the addition of ammonium chloride. Superoxide anion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), peroxynitrite/hydroxyl radical ($ONOO^-/^{\cdot}OH^-$), and nitric oxide ($^{\cdot}NO$) were monitored separately by measuring changes in median fluorescence intensity (MFI) emitted by dihydroethidine (DHE), dichlorofluorescein (DCF), hydroxyphenyl fluorescein (HPF), and diaminofluorescein (DAF), respectively. Briefly, 10^6 cells were incubated with 160 mmol/L of DHE, 20 mmol/L of DCF, 10 μmol/L of HPF, or 2 μmol/L of DAF at 37°C for 30 min (DHE, DCF, and HPF) or 180 min (DAF) in the dark. The samples were then washed, resuspended in PBS, and kept on ice until the acquisition of 10,000 events by flow cytometry, which were subsequently analyzed using FCS Express software (De Novo).

2.7. Advanced Oxidation Protein Products. To determine advanced oxidation protein products (AOPP), 40 μL of plasma diluted in PBS (1.5) was added to 10 μL of KI (1.16 mol/L) and 20 μL glacial acetic acid and the absorbance was read at 340 nm in a microplate reader (Spectra-MAX-190, Molecular Devices, Sunnyvale, CA, USA). The formation of triiodide ion through the oxidation of KI with chloramine-T was used to quantify AOPP levels. Data are

expressed as μmol/mg of chloramine-T per mg of proteins, according to Witko-Sarsat et al. [37].

2.8. Mitochondrial Membrane Potential (MMP). Estimation of mitochondrial membrane potential (MMP) was performed by flow cytometry using JC-10, a fluorogenic probe (Sigma-Aldrich, USA), following the manufacturer's instructions. Briefly, 2×10^6 cells were loaded with 500 μL of JC-10 solution at 37°C for 60 min, protected from light. For the positive control, cells were previously incubated with carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP, 5 μM), while unmarked cells were set as the negative control. JC-10 forms red J-aggregates in healthy cells but stays as a green monomer in cells that have lost mitochondrial integrity. The fluorescence intensities of JC-10 aggregates (red, FL2 channel) and monomers (green, FL1 channel) were measured with flow cytometer detectors and analyzed after compensation for spectral overlap. Data are expressed as the relative aggregate/monomer (FL2/FL1) ratio, which was assumed to be proportional to MMP intensity [38].

2.9. p53 and Cleaved PARP Expression. To determine the expression of p53 and cleaved PARP, 2×10^6 cells were resuspended in Cytofix/Cytoperm (BD) solution and washed twice with Perm/Wash buffer (BD). Blood cells were separately incubated with 5 μL of anti-p53-FITC (BD) or anticleaved PARP-PE, during 30 min in the dark. As the positive control, an aliquot of cells was treated with doxorubicin (25 μg/mL, Sigma-Aldrich, USA) before antibody incubation. For the staining control, we used specific immunoglobulins (IgG) conjugated with FITC or PE. After antibody incubation, the samples were stained with 10 μL of 7-amino-actinomycin D (7-AAD, BD). The determination of protein expression was acquired with the FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using FCS Express software (De Novo). Data are expressed as the percentage of positive cells.

2.10. Cell Cycle Analysis. For the determination of cell cycle distribution, 2×10^6 cells were resuspended in Cytofix/Cytoperm (BD) solution, washed with Perm/Wash buffer (BD), and incubated with 10 μL of 7-amino-actinomycin D (7-AAD, BD) for 30 min, at 4°C, in the dark. The cell cycle profile was determined by the acquisition of 10,000 events per sample using the FACSCanto II flow cytometer for acquisition and the FCS Express software for analysis. The sample flow rate during acquisition did not exceed 200-300 cells per second. Data are expressed as the percentage of cells in each cell cycle phase, which are sub-G_0-representing cells with fragmented DNA (DNA content < 2n), G_0/G_1-representing cells with 2n DNA, and $S/G_2/M$-representing cells with DNA content > 2n [35, 36].

2.11. Cell Viability and Apoptosis. Apoptotic cells were identified and quantified by flow cytometry using the Annexin V-FITC/PI Apoptosis Detection kit (BD Bioscience) according to the manufacturer's instruction. Briefly, 10^6 blood cells were resuspended in binding buffer and incubated for 15 min at room temperature, in the dark, with 5 μL annexin V-FITC and 5 μL propidium iodide (PI). Data were acquired using the FACSCanto II flow cytometer (BD) and analyzed by the FCS Express software (De Novo). Double-negative cells were considered viable, while annexin V-FITC-positive cells were considered apoptotic [27, 35, 36]. Data are expressed as percentage of cells.

Table 1: Patient characteristics.

Parameters	Women ($n = 11$)	Men ($n = 2$)	p value
Age (years)	78.7 ± 3	78 ± 7	0.93
BMI (kg/m^2)	25.8 ± 0.6	27 ± 1.7	0.61
Education level (years)	5.9 ± 0.6	5 ± 1.0	0.51
Treatment duration (years)	1.85 ± 0.7	0.6 ± 0.1	0.08

*The values are presented as mean ± SD.

2.12. Statistical Analysis. All data are expressed as the mean ± SEM (except for the characteristics of patients, expressed as means ± SD). The Kolmogorov-Smirnov test was applied to assess the normal distribution of data. Considering that all the samples had Gaussian distribution, Student's t-test for paired samples was used for the statistical analysis of cytokine concentration, ROS, apoptosis indexes, and cell viability before and after the administration of probiotic. p values < 0.05 were considered statistically significant. Statistical analysis was performed using the GraphPad Prism software, version 7.0.

3. Results

3.1. Characteristics of Patients (Demographic, Anthropometric, and Social Characteristics). Table 1 shows the clinical characteristics of elderly patients included in this study. No significant differences between gender groups were observed in relation to age, body mass index (BMI), treatment duration, and education level.

3.2. Cognitive Assessment. Figure 2 summarizes the results of the cognitive tests divided into T0 and T90 time points. We observed an improvement of performance in the MMSE in 28% (T0: 17.4 ± 1.03 hits and T90: 22.3 ± 0.82 hits, $p < 0.0001$), indicating the benefits of kefir-fermented milk on "global cognitive status" (Figure 2(a)). A similar impact was observed on "memory analysis" (Figure 2(b), left panel) through the immediate memory test (~66%, $p < 0.05$) and late memory test (~62%, $p < 0.05$) between T0 and T90. In the center of Figure 2(b), we also demonstrate the improvement of "visual-spatial and abstraction abilities" using the Similarity Test (~2-fold increase, $p < 0.05$) and Cookie Theft Picture Test (~2-fold increase, $p < 0.05$). Concerning the "executive and language functions" (Figure 2(b), right panel), we also note a significant increment through the Boston Test (~30%, $p < 0.05$) and verbal fluency test (~25%, $p < 0.05$). Finally, the "cognitive battery assay" showed a significant amelioration on the constructive abilities, evidenced by the improvement on the clock-drawing test (T0: 9.0 ± 2.4 hits and T90: 13.2 ± 2.3 hits,

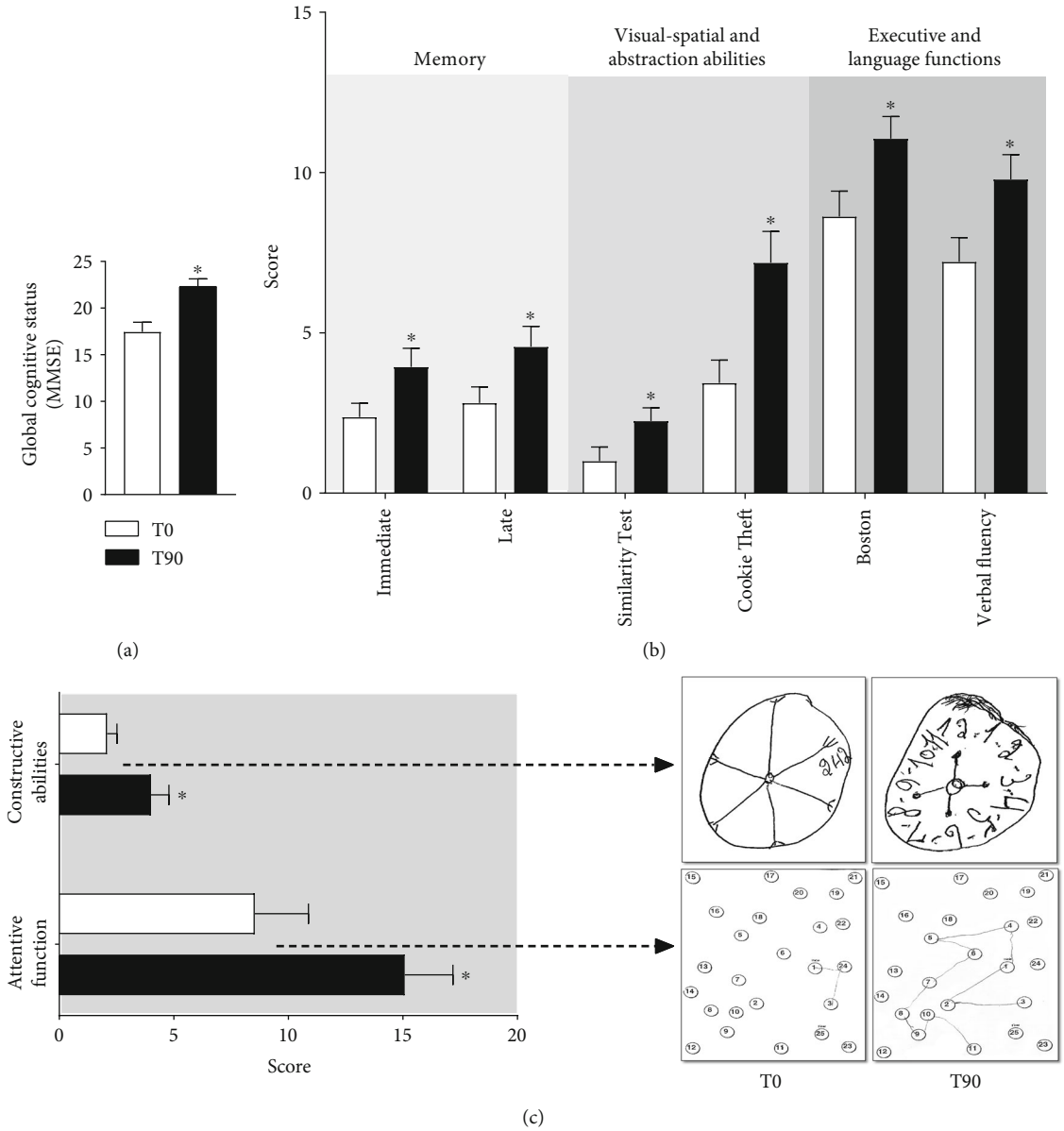

Figure 2: Series of panels showing the results of the evaluation of cognitive tests before and after treatment using kefir (a fermented milk with synbiotics). (a) Global cognitive status (by Mini-Mental State Examination (MMSE)) comparing the effect of the treatment with the previous observed values. (b) Memory analysis (by immediate and late tests, left panel), visual-spatial and abstraction abilities (by Similarity Test and Cookie Theft Picture Test, center panel), and executive and language functions (by Boston Naming Test and verbal fluency test, right panel). (c) Cognitive battery assay, evaluated through constructive abilities and attentive function, with typical hand drawings of the patients during the applied test. The results are expressed as mean ± SEM ($n = 13$). $^*p < 0.05$ compared to T0.

$p < 0.05$), and on attentive function testified by the Trail Making Test (40%, $p < 0.05$).

3.3. Cytokines. Figure 3 shows the quantification of some cytokines involved in pathogenesis of neurodegenerative diseases. The levels of proinflammatory cytokines TNF-α, IL-8, and IL12p70 were lower at T90 than at T0 (~1.5-fold decrease, respectively). However, other proinflammatory (such as IL-1b and IL-6) or anti-inflammatory (IL-10) cytokines did not show difference between T90 and T0. Interestingly, analyzing the balance between proinflammatory and anti-inflammatory cytokines (Figure 3(g)), we verified that the probiotic supplementation was able to reduce the IL-8/IL-10 and IL-12/IL-10 ratios (T0: 2.3 ± 0.2 vs. T90: 1.7 ± 0.1 pg/mL and T0 : 0.95 ± 0.05 vs. T90: 0.72 ± 0.08 pg/mL, respectively, $p < 0.05$).

3.4. Direct and Indirect Oxidative Stress Biomarkers. Oxidative stress was evaluated by flow cytometry trough DHE, DCF, HPF, and DAF fluorescence. Figure 4 shows systemic ROS production measured before and after kefir treatment. We observed a significant decrease in serum levels of $^{\cdot}O_2^-$, H_2O_2, and $ONOO^-/OH^-$ (Figure 4(a)) with a simultaneous increase in NO levels (Figure 4(b)). The bar graph in

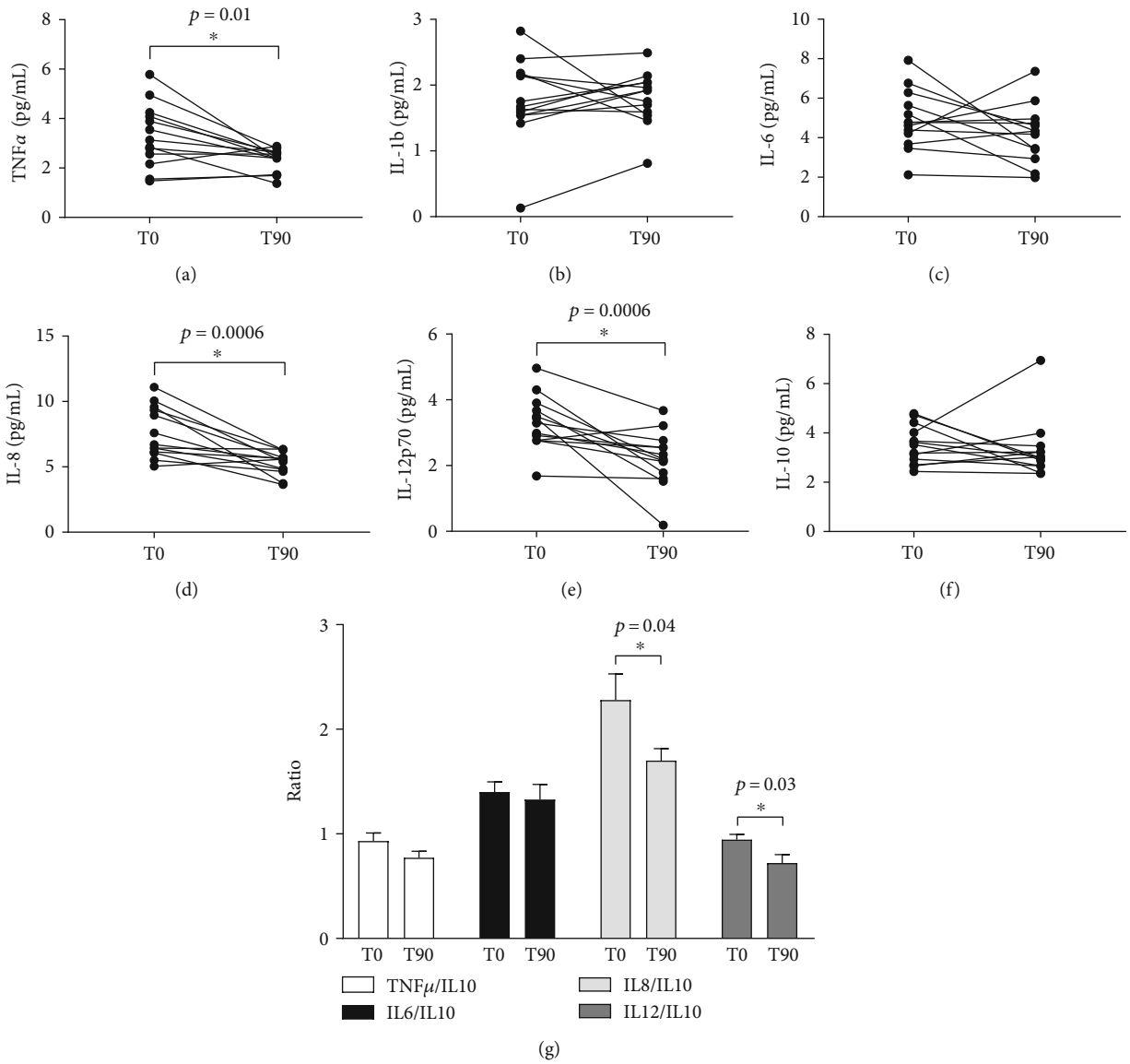

Figure 3: Cytokine levels measured by the Cytometric Bead Array (CBA) using protocols performed through flow cytometry analysis. TNF-α (a), IL-1b (b), IL-6 (c), IL-8 (d), IL-12p70 (e), IL-10 (f), and the ratio of proinflammatory/anti-inflammatory markers (g) were measured before and after probiotic supplementation. The results are expressed as mean ± SEM ($n = 13$). $^*p < 0.05$ compared to T0.

Figure 4(c) represents the mean values of systemic $^{\cdot}O_2^-$ (T0: 5953 ± 999 vs. T90: 3622 ± 707, a.u.), H_2O_2 (T0: 4580 ± 611 vs. T90: 3202 ± 286, a.u.), $ONOO^-/^{\cdot}OH^-$ (T0: 1161 ± 70 vs. T90: 874 ± 34, a.u.), and NO (T0: 3493 ± 304 vs. T90: 1799 ± 158) between T0 and T90.

Figure 5 shows the assessment of systemic protein oxidation by AOPP (an important indirect biomarker of oxidative stress). The results revealed that kefir administration leads to a remarkable decrease in protein oxidation (T0: 8.4 ± 0.6 vs. T90: 2.9 ± 0.3 μmol/mg).

3.5. Mitochondrial Membrane Potential (MMP). We further evaluated whether the decrease in ROS production was accompanied by a recovery of mitochondrial membrane potential (MMP) due to kefir administration. The level of membrane polarization after kefir treatment is shown in Figure 6(a) (left panel). JC-10 green fluorescence (reflecting mitochondrial dysfunction) significantly decreased, and JC-10 red fluorescence (reflecting mitochondrial integrity) increased after kefir consumption. Most of the cells shifted towards the red fluorescence after kefir consumption indicating a significant preservation of mitochondrial function (T0: 0.11 ± 0.03 vs. T90: 2.0 ± 0.14, FL2/FL1, a.u.).

3.6. p53 Expression. The protein p53 is a transcription factor that plays an important role in maintaining the genome integrity by controlling cell apoptosis and cell cycle arrest through signaling of genotoxic stress, like oxidative stress. Figure 6(a) (right panel) shows the values of p53 expression levels measured in the blood samples before and after kefir

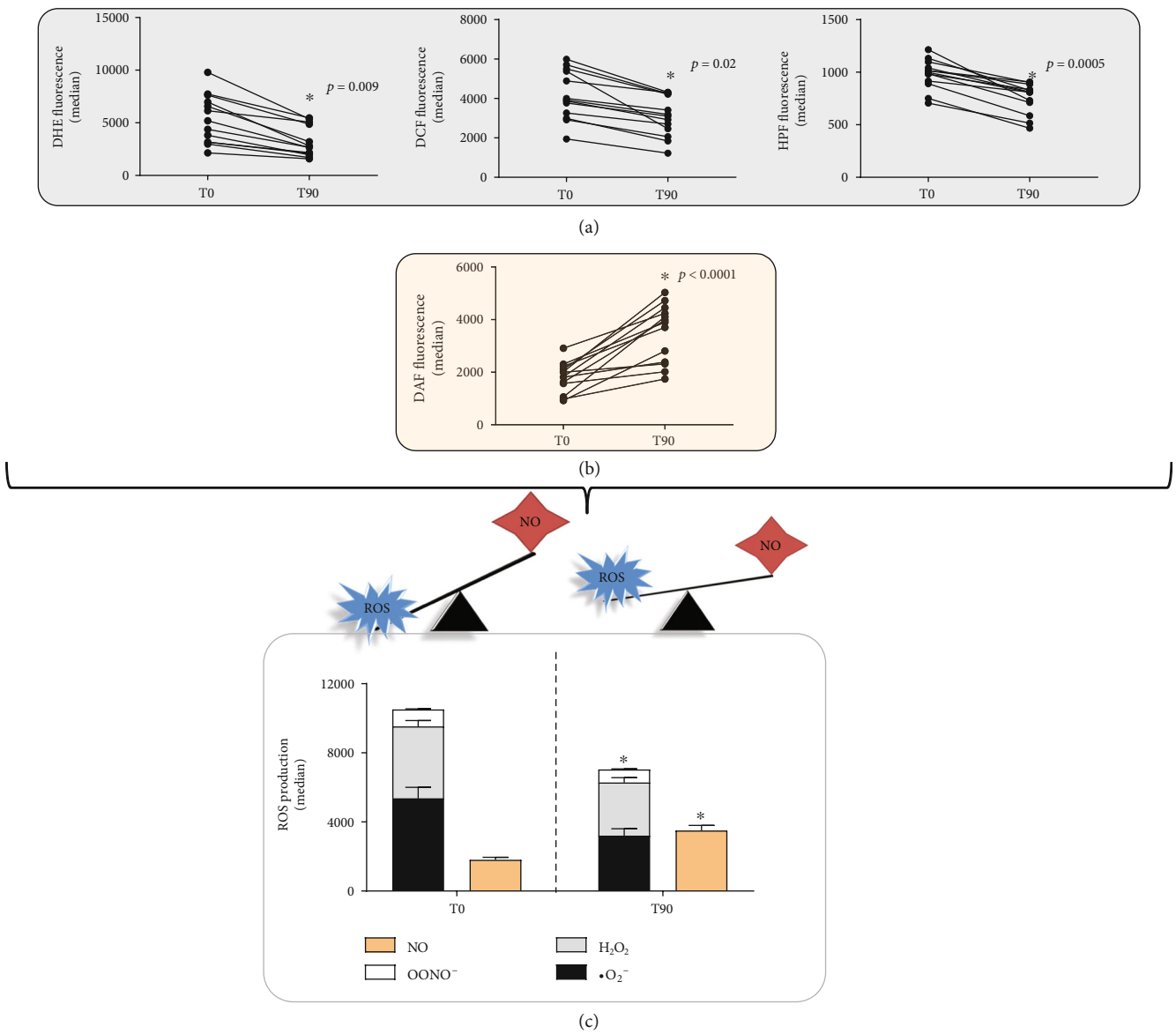

Figure 4: Superoxide anion, hydrogen peroxide, peroxynitrite/hydroxyl radical, and nitric oxide levels measured by specific biomarkers commonly used to evaluate ROS (DHE, DCF, HPF, and DAF staining, respectively). Records of ROS production were made before and after 90 days of the probiotic supplementation. Note a marked recovery of the ROS imbalance after the treatment. The results were expressed as mean ± SEM ($n = 13$). $^*p < 0.05$ compared to T0.

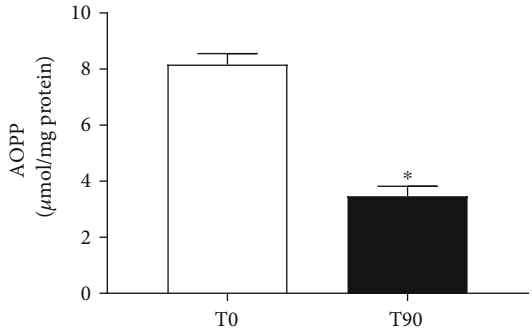

Figure 5: Levels of advanced oxidative protein products before and after probiotic supplementation. The results are expressed as mean ± SEM ($n = 13$). $^*p < 0.05$ compared to T0.

treatment. As shown, p53 expression increased from T0 (10.1 ± 1.8%) to T90 (29.6 ± 2.9%).

3.7. Cell Cycle Arrest. Cell cycle distribution was determined using flow cytometric analysis of blood cells. As shown in Figure 6(b) (left panel), kefir consumption induced an increase in the G_0/G_1 phase, indicating a cell cycle arrest in T90 in comparison to T0 (T0: 61 ± 3.7 vs. T90: 92 ± 1.0%). Simultaneously, we observed a decrease in the percentage of cells in $S/G_2/M$ phases of the cell cycle after kefir consumption (data not shown).

3.8. DNA Fragmentation. Results showed a significant decrease in cells with sub-G_0 DNA content after kefir treatment. A DNA fragmentation assay was performed to

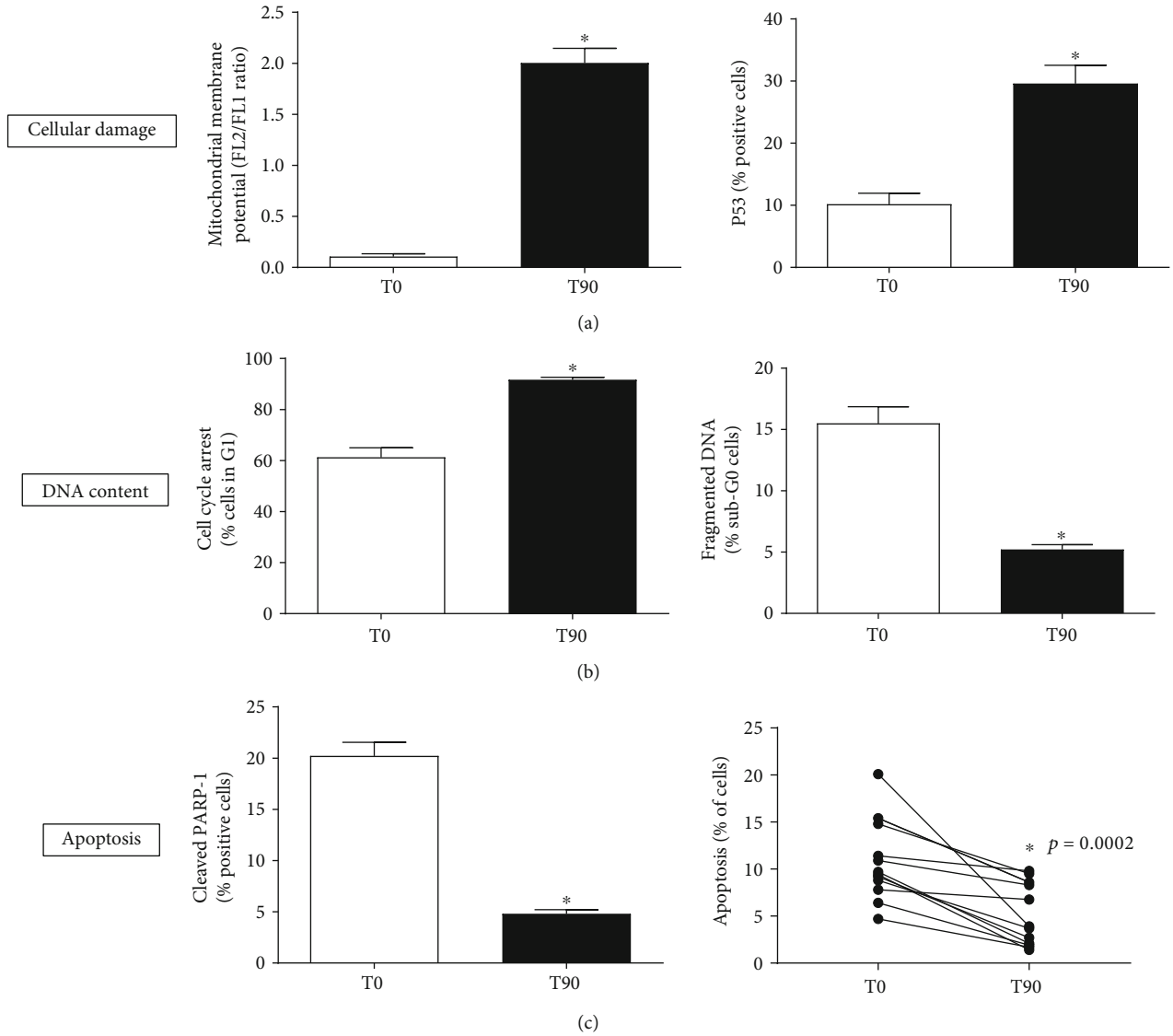

Figure 6: Different evidences that kefir supplementation protects against cellular damage. The mitochondrial membrane potential (MMP) and p53 expression (a), followed by induced cell cycle arrest and DNA repair (b), accompanied by a marked decrease in apoptosis (c). The results are expressed as mean ± SEM ($n = 13$). $^*p < 0.05$ compared to T0.

determine whether kefir was capable of protecting DNA from damage in cells undergoing increased ROS production. As shown in Figure 6(b) (right panel), kefir administration was able to reduce DNA fragmentation from 15.5 ± 1.3% in T0 to 5.2 ± 0.4% in T90. This finding suggests significant changes in cell cycle progression and induction of apoptosis.

3.9. Cleavage of PARP-1. Poly (ADP-ribose) polymerase 1 (PARP-1) is involved in several biological processes, such as cell cycle progression, DNA repair and regulation of transcription, and programmed cell death. Proteolytic cleavage of PARP is considered a hallmark of apoptosis, since PARP-1 is cleaved by activated apoptotic caspases. Our flow cytometry analysis showed a significant reduction in the percentage of cleaved PARP-1 after kefir consumption (T0: 20.2 ± 1.3 vs. T90: 4.8 ± 0.4%) (Figure 6(c), left panel).

3.10. Apoptosis Assay. Flow cytometry was used to determine the antiapoptotic effect of kefir by quantification of annexin V-positive cells. Phosphatidylserine (PS) is externalized and available for detection by annexin V when cells undergo apoptosis. After kefir treatment, the percentage of annexin V-positive cells decreased in T90 (6.86 ± 1.91%) compared to T0 (12.88 ± 1.91%) (Figure 6(c), right panel); consequently, majority of cells were negative for annexin V indicating their healthy status. The viable-to-apoptotic cell ratio (V/A ratio) was 2.05, and the mean V/A ratio increased from 6.53 at T0 to 13.39 at T90.

4. Discussion

In 1908, a Russian zoologist named Metchnikoff (the Nobel laureate who discovered phagocytosis) popularized for the

first time the consumption of probiotics in the form of yogurt as a "healthy food" [39–41]. However, only 100 years later, it has been recognized that probiotics may influence CNS function via the microbiota-gut-brain axis [19, 42, 43]. Even so, clinical investigations using probiotics in elderly patients with dementia are still scarce in medical literature [15]. In this context, our study is the first to evaluate the beneficial effects of kefir supplementation (at the minimum dose of 2 mL/kg for 90 days) on cognitive function, biomarkers of systemic oxidative stress, inflammation, and cell damage in elderly patients with AD.

Although it is known that AD patients are susceptible to multiple complications related to cognitive performance, the innovative therapeutic strategies to reverse this progression are still scant. Interestingly, our results with kefir supplementation for 3 months improved all cognitive tests applied in our experiment (memory, visual-spatial function and abstraction abilities, executive and language functions, constructive abilities, and attentive function). Our results corroborate the findings of Akbari et al. [19] that also demonstrated that probiotic milk (containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum*) for 12 weeks are able to improve the cognitive function. More recently, Kobayashi et al. [44] reported that oral 24-week supplementation with *Bifidobacterium breve* A1 improved cognitive function in AD patients. As suggested by others, we speculated that kefir supplementation also could alter the gut microbiota contributing to neuromodulation through neuroactive and neuroendocrine synthesis (e.g., acetylcholine, dopamine, serotonin, norepinephrine, adrenaline, glutamate, gamma-aminobutyric acid, and brain-derived neurotrophic factor (BDNF)) besides their related receptor expression [15, 19, 45–49]. This hypothesis is partially based on early findings in AD patients using multispecies probiotic intake (for 1 month) improving gut bacteria composition and serum tryptophan levels, an amino acid precursor in serotonin and melatonin biosynthesis [50]. Moreover, experimental evidences support this idea demonstrating decreased level of serum serotonin, BDNF, and NMDA receptors in germ-free mice compared to conventional mice [19, 51, 52] and that *Lactobacilli* supplementation can increase GABA availability from glutamate [53]. More recently, another data demonstrated in mice revealed that the gut microbiota is a potent influencer of BDNF in cortical and hippocampal areas, besides increasing striatal monoamine turnover and modulating the expression of serotonin receptor 1A [48, 54]. Despite all these neuromodulatory benefits previously described, it is believed that the influence on inflammation and oxidative stress by probiotics may also contribute to the neuroprotective effect, thus justifying the next step of this study.

Neuroinflammation has been observed as another relevant player in AD pathogenesis in both experimental and clinical studies [11, 12, 55–59]. Numerous data demonstrate positive associations between proinflammatory cytokines (e.g., IL-1, IL-6, TNF-α, IL-8, and IL-12) and the progression of AD [58, 60]. Moreover, recent investigations reported that these neuroinflammatory cytokines can compromise the clearance of Aβ, accumulating this toxic protein in the brain [57, 58, 61–63]. Thus, cytokine balance has been an important research target for understanding the pathophysiology of AD and identifying new potential therapeutic targets. At the same time, emerging data have shown that probiotics can secrete metabolites and factors with immunomodulatory properties [24, 48, 64]. For example, relevant studies demonstrated a decrease in proinflammatory cytokines using multistrain probiotic supplementation [21, 65, 66]. The novelty in our study was that we used an inexpensive food (and easily produced at home) to reduce serum proinflammatory cytokines and possibly contribute to neuroprotective effect in AD patients. This immunosuppressive profile is reinforced by other recent study from our lab using only nonbacterial fraction of kefir in dyslipidemic mice [30], which allows us to speculate that this immunomodulation could be the result of a synergistic effect between microorganisms and soluble products present in kefir.

Increased levels of serum oxidative stress biomarkers reported in neurodegenerative diseases [11, 19, 67–71] seem to be an interesting approach to evaluate the impact of new therapeutic strategies in AD patients. At the same time, there is a strong correlation of antioxidant-rich diets as an "easy" strategy for neuroprotection [11, 17, 72, 73], including probiotics [19, 21]. In this study, the assessment of serum oxidative stress by direct and indirect methods (ROS and AOPP) demonstrates that kefir has significant antioxidant effects, helping to explain the favorable result concerning cognition in AD patients. It is known that excessive ROS are involved as a cause and consequence of proinflammation contributing directly and indirectly to the pathogenesis of AD [8, 17, 74–76]. More specifically, due to their electronegativity and reactivity, $^{\cdot}O_2^{-}$, H_2O_2, and $ONOO^{-}/^{\cdot}OH^{-}$ can oxidize lipids, nucleic acids, and proteins leading to mitochondrial destruction, neuroexcitotoxicity (e.g., excessive Ca^{2+} influx and formation of aggregates of toxic proteins), and stimulation of microglia and astrocytes to develop inflammatory response [17, 24, 36, 77]. On the other hand, NO (by eNOS or nNOS isoforms) seems to be a "ROS-gasotransmitter" with important neuroprotective properties such as antioxidant (acting as a scavenger of $^{\cdot}O_2^{-}$), vasodilator (increasing cerebral blood supply to neurons), inhibitor of NMDA receptors at glutamatergic synapses (thereby preventing neuroexcitotoxicity), and preventing the deposition of Aβ [10, 78–81]. Interestingly, our findings demonstrate for the first time that kefir reduces serum ROS bioavailability accompanied by an NO increase reflecting the reduction of plasma protein oxidation in AD patients. These data may be supported by experiments of Musa et al. [21] that revealed an increase in the activity of antioxidant enzymes (SOD, GSH, and GPx) in the brain tissue of mice treated with *Lactobacillus*. Therefore, all these results motivated us to investigate the impact of mitochondrial and cell damage obtained by the same blood samples from the same subjects.

It is known that AD brain mitochondria develop diminished membrane potential, disrupting the electron transfer chain, favoring excess ROS production, alteration in cytosolic calcium homeostasis, and Aβ accumulation leading to neurodegeneration [11, 72, 82–85]. In parallel,

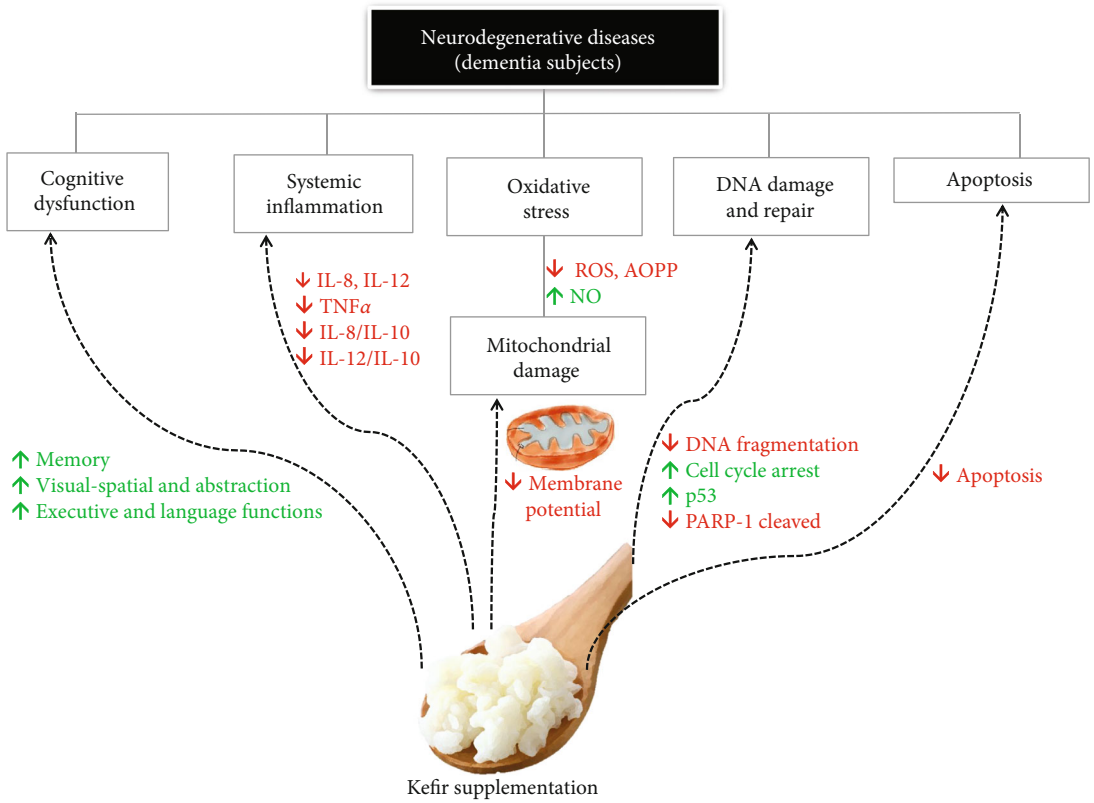

Figure 7: Beneficial effects of kefir on dementia in AD patients. Simplified scheme of main effects after 90 days with kefir supplementation on Alzheimer's subjects.

Delbarba et al. [86] demonstrated that mitochondrial DNA can decrease in peripheral blood in the early stages of neurodegenerative disease progression, being a potential "blood-based signature" in AD patients. Therefore, since several studies of mitochondrial damage in Alzheimer's patients are performed postmortem, the possibility of exploring blood samples in clinical investigations (as in the present study) may be a promising strategy for establishing mechanisms related to cognitive improvement with immunomodulatory, antioxidant, and/or mitochondrial function. Interestingly, we observed that kefir was able to reverse the compromised mitochondrial membrane potential in blood cells of AD patients, helping to explain (at least in part) the reduction of ROS bioavailability in the same patients. Furthermore, in this same context, as it is known that p53 under lower ROS levels can contribute to cellular survival [87–89], we confirmed this protective effect by kefir in AD patients through 2 evidences: (1) by induction of DNA repair and (2) by reduction of apoptosis. Firstly, we may affirm this beneficial effect through a decrease in DNA fragmentation accompanied by induction of cell cycle arrest after 90 days of supplementation. Secondly, the reduction of apoptosis by kefir was detected through a reduction of cleaved PARP-1 (considered to be a classical hallmark of apoptosis) and by a decrease in annexin V-positive cells [36, 90]. In summary, our data indicate that kefir supplementation has also a mitochondria-protective effect in addition to cytoprotective and antiapoptotic action, whose effects try to mitigate the progression of neurodegeneration (Figure 7).

This study had some potential methodological limitations. First, due to difficulties in obtaining fresh fecal samples, we had problems with analysis of the fecal microbiota before and after the kefir supplementation in our AD patients. Second, our study was conducted without control participants using other type of fermented milk. Third, the sample size was small, but justified by age of the patients, and it was accompanied by severe exclusion criteria. Additionally, the impact of learning effect bias was minimized through randomized procedures applied in the cognitive tests in the present study. Lastly, but not least, we could have enriched the data exploring imaging biomarkers (e.g., magnetic resonance imaging and PET) or specific biomarkers (e.g., brain-derived neurotrophic factor, neuronal butyrylcholinesterase, and apolipoprotein A1) which would extend the possible beneficial effects induced by chronic kefir administration in these AD patients. Despite these limitations, the novelty of our study is to demonstrate the beneficial effects of chronic kefir supplementation on the cognitive function in the elderly with AD.

5. Conclusion

The current study demonstrated that synbiotic supplementation for 90 days to older patients with AD had reparatory favorable effects on cognitive dysfunction (improving memory, language, executive functions, visual-spatial function, conceptualization, and abstraction abilities), systemic inflammation (by reduction of proinflammatory

cytokines), systemic oxidative stress (verified by a decrease in ROS and AOPP), and blood cell damage (analyzed by DNA damage/repairment and apoptosis). Therefore, the data of the present study is opening a great opportunity for the evaluation of the clinical benefits of probiotics/synbiotics at larger randomized controlled clinical trials, strengthening the present valuable findings.

Abbreviations

DA: Alzheimer's disease
CNS: Central nervous system
ROS: Reactive oxygen species
MMSE: Mini-Mental State Examination
CBA: Cytometric Bead Array
MFI: Median fluorescence intensity
DHE: Dihydroethidine
DCF: Dichlorofluorescein
HPF: Hydroxyphenyl fluorescein
DAF: Diaminofluorescein
AOPP: Advanced oxidation protein products
MMP: Mitochondrial membrane potential
CCCP: Carbonyl cyanide 3-chlorophenyl-hydrazone
PI: Propidium iodide
BMI: Body mass index
PARP-1: Poly (ADP-ribose) polymerase 1
PS: Phosphatidylserine
V/A ratio: Viable-to-apoptotic cell ratio
BDNF: Brain-derived neurotrophic factor
SEM: Standard error of the mean
UVV: Vila Velha University.

Data Availability

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

Ethical Approval

All subjects and their tutors were fully educated on the nature of the project. The research was approved by the ethics committee of Vila Velha University (#1.804.392).

Consent

All subjects and their tutors signed the free informed consent form.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

ECV, AMMT, BPC, CMA, and SSM conceived and designed the study. AMMT, BPC, GAA, RA, LZC, and CMA conducted the clinical research and the laboratory tests. AMMT, BPC, MCT, ECV, and TMCP analyzed the data and drafted the manuscript. All authors approved the final version of the manuscript.

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Research Article

Generation of Cellular Reactive Oxygen Species by Activation of the EP2 Receptor Contributes to Prostaglandin E2-Induced Cytotoxicity in Motor Neuron-Like NSC-34 Cells

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Amyotrophic lateral sclerosis (ALS) is a devastating motor neuron disease characterized by progressive degeneration of motor neurons in the central nervous system. Prostaglandin E2 (PGE2) plays a pivotal role in the degeneration of motor neurons in human and transgenic models of ALS. We have shown previously that PGE2 directly induces neuronal death through activation of the E-prostanoid (EP) 2 receptor in differentiated NSC-34 cells, a motor neuron-like cell line. In the present study, to clarify the mechanisms underlying PGE2-induced neurotoxicity, we focused on generation of intracellular reactive oxygen species (ROS) and examined the effects of N-acetylcysteine (NAC), a cell-permeable antioxidant, on PGE2-induced cell death in differentiated NSC-34 cells. Dichlorofluorescein (DCF) fluorescence analysis of PGE2-treated cells showed that intracellular ROS levels increased markedly with time, and that this effect was antagonized by a selective EP2 antagonist (PF-04418948) but not a selective EP3 antagonist (L-798,106). Although an EP2-selective agonist, butaprost, mimicked the effect of PGE2, an EP1/EP3 agonist, sulprostone, transiently but significantly decreased the level of intracellular ROS in these cells. MTT reduction assay and lactate dehydrogenase release assay revealed that PGE2- and butaprost-induced cell death were each suppressed by pretreatment with NAC in a concentration-dependent manner. Western blot analysis revealed that the active form of caspase-3 was markedly increased in the PGE2- and butaprost-treated cells. These increases in caspase-3 protein expression were suppressed by pretreatment with NAC. Moreover, dibutyryl-cAMP treatment of differentiated NSC-34 cells caused intracellular ROS generation and cell death. Our data reveal the existence of a PGE2-EP2 signaling-dependent intracellular ROS generation pathway, with subsequent activation of the caspase-3 cascade, in differentiated NSC-34 cells, suggesting that PGE2 is likely a key molecule linking inflammation to oxidative stress in motor neuron-like NSC-34 cells.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a type of motor neuron disease characterized by progressive atrophy of skeletal muscle resulting from selective degeneration of motor neurons. The molecular mechanisms underlying this selective vulnerability are still unknown, but inflammation is considered to be an important factor contributing to the pathogenesis of both patients and animal models of ALS [1–3]. Prostaglandins are small lipid inflammatory mediators derived from arachidonic acid via multienzymatic reactions. Five primary prostaglandins are synthesized *in vivo*—prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin F2, prostaglandin I2, and thromboxane A2—and their biological functions occur through seven distinct transmembrane-spanning G-protein-coupled receptors (GPCRs), termed D-prostanoid receptor (DP) 1 and 2, E-prostanoid receptor (EP) 1 to 4, F-prostanoid receptor (FP), I-prostanoid receptor (IP), and thromboxane-prostanoid receptor (TP) [4, 5]. Among them, PGE2 plays a pivotal role in the degeneration of motor

neurons in humans and transgenic models of ALS. PGE2 is increased in cerebrospinal fluid from patients with sporadic ALS [6] and in the spinal cord of ALS model mice [7]. We have revealed that the level of microsomal PGE synthase-1 (mPGES-1), the enzyme catalyzing the final step of PGE2 biosynthesis, is significantly increased in motor neurons in the mouse model of ALS [8]. Importantly, inhibition of mPGES-1 by AAD-2004, a dual-function drug derived from aspirin and sulfasalazine, has been reported to exhibit significant neuroprotective effects and to prolong survival in ALS model mice [9]. More recently, we reported that positive-feedback regulation of EP2 in spinal motor neurons may exacerbate PGE2-induced damage to neurons during the progression of ALS in the murine model [10]. These results suggest that PGE2-EP2 signaling is a critical mediator of motor neuron death in the pathogenesis of ALS.

A growing amount of evidence now suggests that oxidative stress contributes to motor neuronal death in ALS [11]. In fact, edaravone, a free radical scavenger, was approved as a therapeutic drug for ALS in 2015 in Japan and South Korea and in 2017 in the United States [12]. More importantly, oxidative stress and chronic inflammation are linked and perpetuate each other, resulting in progression of a number of neurodegenerative diseases including ALS [13, 14]. Oxidative stress leads to exacerbated inflammatory responses, and, conversely, uncontrolled inflammation is known to cause cellular accumulation of reactive oxygen species (ROS), which are associated with oxidative stress [15, 16]. Several previous observations have also indicated that PGD2 and its J2 series metabolites (such as PGJ2, Δ12-PGJ2, and 15d-PGJ2) act as potential inducers of intracellular oxidative stress [17–19]. Especially, J2-series prostaglandin-induced ROS production is well correlated with cytotoxicity in a human neuroblastoma cell line, SH-SY5Y [17]. Despite numerous studies suggesting important roles for PGE2 in neuron cell death, very little is known about the ability of PGE2 to act as a ROS inducer/source in motor neurons. Earlier studies at our laboratory revealed that PGE2 directly induces cell death in differentiated NSC-34 cells, which possess the unique morphological and physiological characteristics of motor neurons [20], and that the neurotoxic effect of PGE2 is mediated by activation of EP2 in motor neurons [10, 21]. In the present study, to identify endogenous inducers of intracellular oxidative stress and clarify the molecular mechanism underlying the interaction of oxidative stress with the inflammatory response in ALS neurodegeneration, we examined the ability of PGE2 to induce intracellular ROS production in the mouse motor neuron-like cell line NSC-34, and found that EP2 receptor-dependent ROS production contributes to PGE2-induced cytotoxicity.

2. Materials and Methods

2.1. Cell Culture and Reagents. NSC-34 cells were seeded at a density of 25,000 cells/cm^2. To enhance cell differentiation, the medium was exchanged for Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 1:1Mixture (Life Technologies Corporation, Carlsbad, CA, USA) containing 0.5% fetal bovine serum (FBS; Life Technologies Corporation), 1% (*v*/*v*) MEM nonessential amino acid solution (100X) (Life Technologies Corporation), and 1% (*v*/*v*) penicillin-streptomycin solution (100X) (Life Technologies Corporation) in a humidified atmosphere containing 5% CO_2 at 37°C. PGE2 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), butaprost (Cayman Chemical, Ann Arbor, MI, USA), sulprostone (Cayman Chemical), PF-04418948 (Cayman Chemical), and L-798,106 (Cayman Chemical) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, USA). N-acetyl-L-cysteine (NAC; Sigma-Aldrich) and dibutyryl-cAMP (Daiichi Sankyo, Tokyo, Japan) were dissolved in water. Untreated NSC-34 cells were used as controls for all our comparative analyses.

2.2. MTT Reduction Assay. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) reduction assay in 96-well plates as described previously [21]. Briefly, the cells were incubated with MTT (0.25 mg/mL) for 3 h at 37°C. The MTT formazan product was solubilized by adding a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (SDS) (pH 4.7), and its amount was determined by measuring the absorbance with a microplate reader SH-1000Lab (Corona Electric, Ibaraki, Japan) at a test wavelength of 570 nm and a reference wavelength 655 nm. The relative cell viability was calculated as the percentage of untreated cells.

2.3. LDH Assay. The extent of cytotoxicity was quantified by measurement of lactate dehydrogenase (LDH) released into the medium during exposure to drugs. After NSC-34 cells had been incubated with PGE2 for 48 h, the supernatants were collected in new plates and LDH release was determined using a LDH-Cytotoxic Test kit (Wako Pure Chemical Industries) as described previously [22]. The absorbance of samples was measured at 570 nm using a microplate reader SH-1000Lab (Corona Electric). The background absorbance obtained from the culture medium was subtracted from the absorbance of each sample. The LDH release in each treatment group was calculated as a percentage of the LDH release from the cells treated with 0.2% Tween-20. In all cases, cell death was confirmed by microscopy.

2.4. Live/Dead Assay. Amount of viable and dead cells was evaluated with LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, 4 μM calcein-AM and 2 μM ethidium homodimer-1 (EthD-1) were added to culture medium for 40 min. The images were collected with inversed fluorescence microscope (IX70, Olympus, Tokyo, Japan). Cell mortality was determined by calculating the percentage of EthD-1-positive cells to the total number of cells (the sum of calcein-positive live cells and EthD-1-positive dead cells).

2.5. Measurement of Intracellular ROS. Generation of ROS was evaluated by measurement of dichlorofluorescein (DCF) fluorescence as described previously [23]. The cells were preincubated with a standard artificial cerebrospinal fluid (aCSF; 136 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MaCl_2$, 10 mM HEPES, 10 mM glucose, and 12 mM

$NaHCO_3$, pH 7.4 buffered by Tris) containing 1 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Life Technologies Corporation) and 25 μg/mL Hoechst 33258 (Sigma-Aldrich) at 37°C for 60 min. The cells were washed with the medium and then exposed to drugs. The cells were then washed with aCSF, and the DCF fluorescence intensities were measured at excitation and emission wavelengths of 485 and 520 nm, respectively, using a fluorescence plate reader (FLUOstar OPTIMA, BMG Lab Technologies, Germany). Hoechst 33258 fluorescence was measured at excitation and emission wavelengths of 355 and 460 nm. The intracellular ROS level was calculated as the DCF fluorescence intensity normalized to the number of cells, as determined from the Hoechst 33258 fluorescence intensity as described previously [24]. The results are expressed as fold change relative to vehicle-treated cells which were assigned a value of 1.

2.6. Western Blotting. NSC-34 cells were plated at a density of 2.0×10^5 cells per 35 mm dish. Western blot analysis was performed as described previously [21, 25]. Briefly, differentiated NSC-34 cells were harvested in a lysate buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0, 1% Triton, 5 mM EDTA, and protease inhibitor cocktail (Roche, Switzerland). Protein extracts were separated on SDS-polyacrylamide gel with Tris/glycine running buffer. After electrophoretic separation, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked in blocking buffer (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.05% Tween-20, 5% skim milk) for 1 h at room temperature and then treated with a polyclonal antibody against pro-caspase-3 (1 : 1000; Cell Signaling Technology, Danvers, MA, USA) and cleaved caspase-3 (1 : 1000; Cell Signaling Technology), and a monoclonal antibody against β-actin antibody (Sigma-Aldrich) overnight at 4°C. The membranes were washed repeatedly in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 137 mM NaCl) containing 0.05% Tween-20, and then a HRP-conjugated secondary antibody (Santa Cruz Biotechnology, USA) was added for 1 h. Immunoreactive bands were visualized with enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The blots are scanned, and the optical density of the blots was measured using Scion imaging analysis software (Scion, Frederick, MD, USA). Quantitative results were expressed as the ratio of the band intensity of the protein of interest relative to the band intensity of β-actin.

2.7. Extraction of Total RNA and Real-Time Semiquantitative PCR Analysis. Gene expression of the EP3 isoforms was determined by semiquantitative real-time RT-PCR as described previously with modifications [26]. Briefly, total RNA was extracted using a High Pure RNA Isolation kit (Roche Diagnostics, GmbH, Penzberg, Germany) in accordance with the manufacturer's instructions. After treatment with DNase I, cDNA was synthesized using Oligo (dT)18 primers and a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Aliquots of cDNA were amplified on a MX3000P real-time PCR system (Stratagene, La Jolla, CA, USA) using SYBR® Premix Ex Taq™ II (Takara Biotechnology, Shiga, Japan). All samples were assayed in duplicate. The levels of mRNA expression were normalized to an internal standard (β-actin; $\Delta\Delta$CT method). The mouse primer sequences were 5′-CGG AAG TTC TGC CAG ATC AGA-3′ (forward) and 5′-TCC AGC TGG TCA CTC CAC ATC-3′ (reverse) for EP3α; 5′-CGG AAG TTC TGC CAG ATG ATG-3′ (forward) and 5′-CAG GGA AAC AGG TAC TGC AAT G-3′ (reverse) for EP3β; 5′-AGT TCT GCC AGG TAG CAA ACG-3′ (forward) and 5′-GCC TGC CCT TTC TGT CCA T-3′ (reverse) for EP3γ; 5′-CAT CCG TAA AGA CCT CTA TGC CAA C-3′ (forward) and 5′-ATG GAG CCA CCG ATC CAC A-3′ (reverse) for β-actin. The RT-PCR products were electrophoresed on 2.5% agarose gels and visualized with GelRed™ Nucleic Acid Gel Stain (Biotium, VWR, Leuven, Belgium).

2.8. Statistical Analysis. Data analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± S.E.M. or mean ± S.D. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple tests. Differences at $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. PGE2- and EP2 Agonist-Induced Intracellular ROS Production in Differentiated NSC-34 Cells. We first examined the effect of PGE2 on DCF fluorescence intensity as a marker of intracellular ROS production in differentiated NSC-34 cells preloaded with DCFH-DA. Intracellular ROS levels were markedly and time-dependently increased in cells treated with 80 μM PGE2 (Figure 1(a)). The levels of intracellular ROS in these cells were significantly higher than those detected under vehicle treatment conditions after incubation for 120 to 300 min. We have reported previously that EP2 and EP3 are highly expressed in differentiated NSC-34 cells as well as motor neurons in the mouse spinal cord [21]. In order to clarify the type of EP receptor contributing to PGE2-induced ROS induction in differentiated NSC-34 cells, we investigated the effects of two well-characterized and selective EP agonists in the DCF fluorescence quantification assay. Exposure to an effective concentration range of butaprost (40 μM), an EP2-selective agonist, resulted in a time-dependent increase of intracellular ROS production, and a statistically significant increase of DCF fluorescence was observed after incubation for 60 to 300 min (Figure 1(a)). In contrast, the level of intracellular ROS after treatment with 40 μM sulprostone, an EP1/EP3 agonist, was transiently but significantly decreased after incubation for 120 to 180 min (Figure 1(a)). Moreover, DCF analysis showed that the generation of ROS caused by PGE2 at 80 μM was attenuated significantly in the presence of PF-04418948, an EP2-selective antagonist, at 30 μM (Figure 1(b)). In contrast, L-798,106, an EP3-selective antagonist, at 10 μM did not suppress PGE2-induced ROS production (Figure 1(b)). PF-04418948

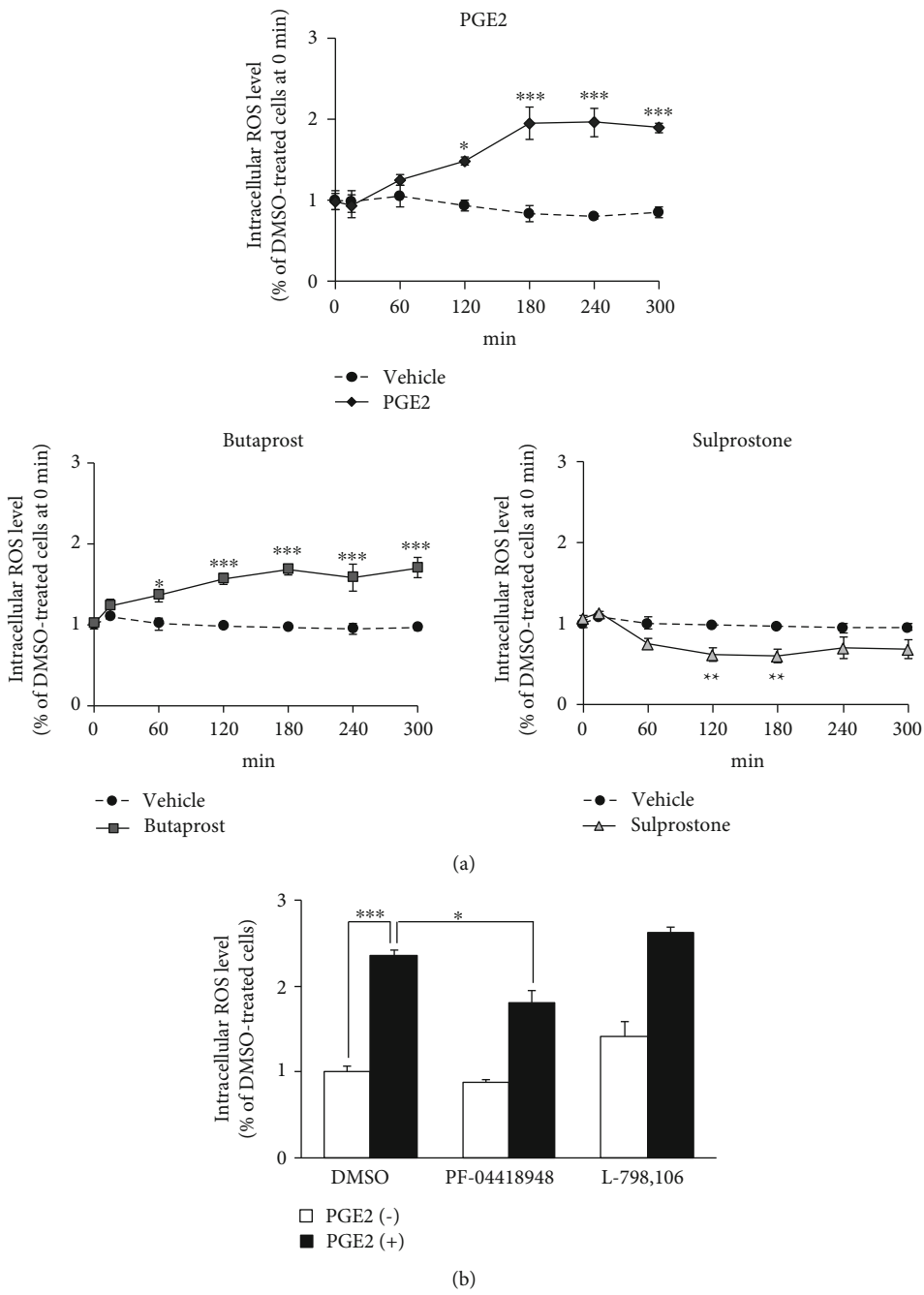

Figure 1: The intracellular ROS level in differentiated NSC-34 cells treated with PGE2 and EP agonist. (a) The intracellular levels of ROS were measured over time by monitoring DCF fluorescence intensity in differentiated NSC-34 cells incubated in vehicle (0.15% DMSO) or in the presence of PGE2 (80 μM), butaprost (40 μM), or sulprostone (40 μM). Graphs show time courses of DCF fluorescence intensity in the four groups, respectively. Values represent means ± S.E.M. for four separate experiments. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to vehicle-treated cells at each time point. (b) Differentiated NSC-34 cells were treated with 30 μM PF-04418948 (an EP2 selective antagonist) or 10 μM L798,106 (an EP3 selective antagonist) with or without 80 μM PGE2 for 180 min. The graph illustrates the endpoint fluorescence values after these incubations. Each value represents the mean ± S.E.M. for four separate experiments. $^{***}p < 0.001$, $^{*}p < 0.05$ compared to vehicle-treated cells.

and L-798,106 did not change ROS formation in differentiated NSC-34 cells.

Mouse EP3 has three different isoforms (EP3α, EP3β, and EP3γ), and we have previously shown that expression of EP3γ mRNA is predominant in mouse motor neurons, whereas EP3α and EP3β are not detectable [26]. Therefore, we sought to identify the distribution of EP3 receptor isoforms in differentiated NSC-34 cells. As shown in Figure 2,

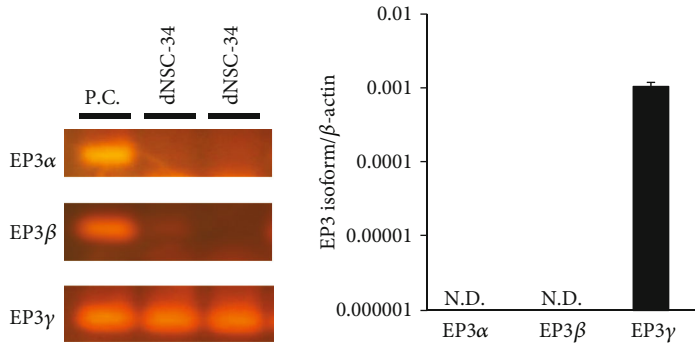

Figure 2: Characterization of mRNA expression for the EP3 isoform in motor neuron-like NSC-34 cells. Photographs show RT-PCR products of EP3α (101 bp), EP3β (63 bp), and EP3γ (61 bp). RT-PCR product amplified from differentiated NSC-34 cells (dNSC-34) and mouse hippocampus (positive control: P.C.) were electrophoresed on 2.5% agarose gels and visualized with GelRed™ Nucleic Acid Gel Stain. Representative data from at least four independent experiments are shown. Graphs show the expression profile of mRNAs for EP3 isoforms in differentiated NSC-34 cells. Expression of the mRNA for each EP3 isoform was normalized to the level of β-actin mRNA. Values represent the mean ± S.E.M. for four separate experiments. N.D.: not detected.

semiquantitative real-time PCR demonstrated predominant expression of EP3γ in the cells, whereas EP3α and EP3β were undetectable.

3.2. N-Acetyl-L-Cysteine (NAC) Protects against PGE2- and EP2 Agonist-Induced Cell Death in Differentiated NSC-34 Cells. Next, we evaluated the effect of the antioxidant N-acetyl-L-cysteine (NAC) on PGE2- and butaprost-induced cell death using the MTT reduction assay (Figure 3) and LDH-based cytotoxicity assays (Figure 4). Consistent with previous results [21], exposure of the differentiated NSC-34 cells to 80 μM PGE2 and 40 μM butaprost for 48 h resulted in a significant decrease (35% and 33%, respectively) of cell survival in the MTT reduction assay (Figure 3). Unlike PGE2 and butaprost, exposure to 40 μM sulprostone for 48 h had no significant effect within the concentration range for selective interaction with EP3. Pretreatment with NAC at 0.1–3 mM protected differentiated NSC-34 cells against PGE2-induced cell death in a concentration-dependent manner, although a high concentration of NAC (6 mM) yielded false-positive results in the MTT reduction assay due to their possible reducing activity on the MTT compound (Figure 3). Likewise, butaprost-induced cell death was rescued by pretreatment with NAC in a concentration-dependent manner, whereas NAC had no effect on the MTT levels in sulprostone-treated cells (Figure 3).

As shown in Figure 4, exposure to PGE2 and butaprost resulted in decreased cell viability (55% and 59%, respectively) when determined by LDH release assay, whereas sulprostone had no effect on the viability of these cells. Pretreatment with NAC at 0.1–3 mM protected these cells against PGE2- and butaprost-induced decreases in cell viability in a concentration-dependent manner (Figure 4), although the LDH assay also produced a false-positive result in cells treated with 6 mM NAC. In contrast, NAC had no effect on the level of LDH release in sulprostone-treated cells, as was the case in the MTT reduction assay (Figure 4).

Phase-contrast images showed no difference in morphology between the vehicle (DMSO)-treated cells and 6 mM NAC-treated cells within 48 h after the treatment (Figure 5). Exposure to PGE2 and butaprost, but not sulprostone, caused extensive alterations in cell morphology: the cells appeared clearly shrunken and rounded and were detached from the bottom of the culture plate (Figure 5). NAC pretreatment of these cells preserved their neuron-like cell morphology in a concentration-dependent manner upon treatment with PGE2 and butaprost (Figure 5).

In order to investigate the change of cell mortality after treatment with NAC, Live/Dead assay was performed after various treatments. As shown in Figure 6, exposure of differentiated NSC-34 cells to 80 μM PGE2 and 40 μM butaprost, but not 40 μM sulprostone, for 48 h led to an increase in the percentage of dead cells stained by EthD-1. Pretreatment with 3 mM and 6 mM NAC markedly reduced dead cells. Quantitative analysis showed that NAC significantly protected NSC-34 cells against PGE2- and butaprost-induced cell death.

3.3. NAC Suppresses the PGE2- and EP2 Agonist-Induced Increase in Cleaved Caspase-3 Protein in Differentiated NSC-34 Cells. PGE2 (5–25 μM) has been reported to induce caspase-3 activation and apoptosis in cultured rat hippocampal neurons [27]. Therefore, we examined whether exogenously applied PGE2 and selective EP2 agonists are able to induce activation of caspase-3 in differentiated NSC-34 cells. Caspase-3 activation was detected by determining the level of caspase-3 cleavage fragments (17 kDa) using Western blotting. As shown in Figure 7, the levels of cleaved caspase-3 (17 kDa) were markedly increased after treatment of differentiated NSC-34 cells with PGE2 and butaprost, whereas sulprostone had no such effect. We also assessed the effects of NAC on the increased levels of cleaved caspase-3 after exposure to PGE2 and butaprost. Although NAC (3 mM) alone had no effect, the increase in the generation of cleaved caspase-3 induced by PGE2 and butaprost was significantly suppressed when the cells were pretreated with NAC (Figure 7). Unlike the results of cleaved caspase-3, the difference in the level of pro-caspase-3 was not detected following

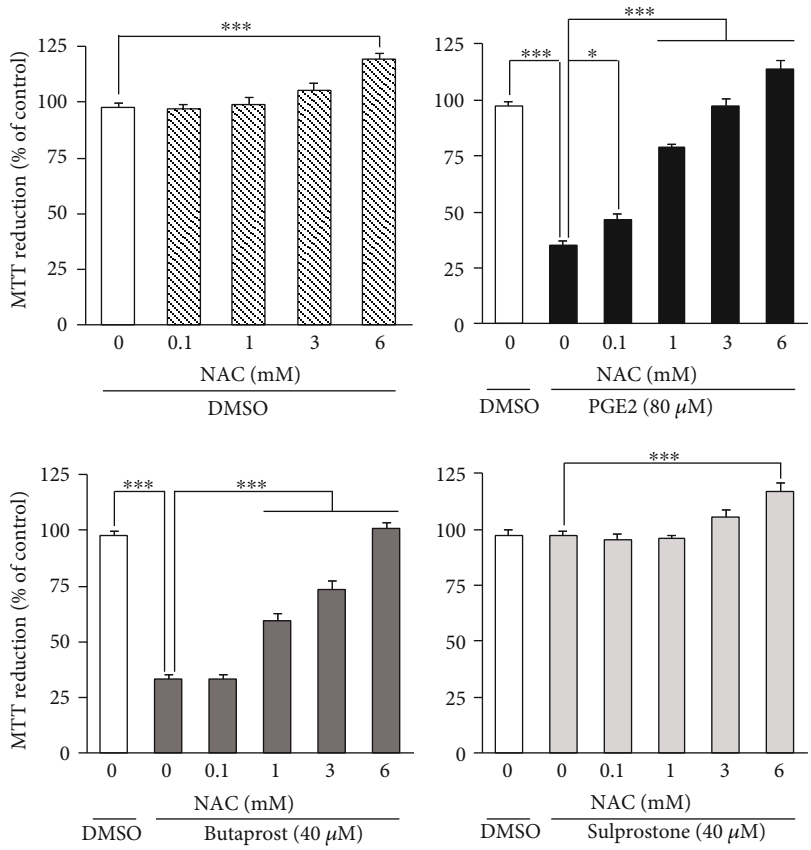

Figure 3: Effects of NAC pretreatment on PGE2- and EP agonist-induced cell death detected by MTT reduction assay. Differentiated NSC-34 cells were exposed to various concentrations of NAC for 4 h and then treated with vehicle (0.15% DMSO), 80 μM PGE2, 40 μM butaprost (an EP2 agonist), or 40 μM sulprostone (an EP1/3 agonist) for 48 h. The viability of the cells was assessed by the MTT reduction assay. Results are expressed as percentages relative to the control (nontreated) cells. Values represent means ± S.E.M. for four separate experiments. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

in any kind of treatment in differentiated NSC-34 cells shown in Figure 7.

3.4. Exogenously Applied Cyclic Adenosine Monophosphate (cAMP) Analog Induces Cell Death and Facilitates Intracellular ROS Production in Differentiated NSC-34 Cells. It is generally accepted that EP2 is a Gs-coupled GPCR and increases the level of cyclic adenosine monophosphate (cAMP) [28]. In order to assess the functional significance of cAMP in the cell death induced by oxidative stress, we investigated the effect of dibutyryl-cAMP (dbcAMP), a cell-permeable cAMP analog, on cell survival and intracellular ROS production in differentiated NSC-34 cells. When differentiated NSC-34 cells were exposed to dbcAMP (1–100 mM) for 48 h, cell viability decreased in a concentration-dependent manner (Figure 8(a)). Statistically significant attenuation of cell viability was observed at doses of 3 mM and higher. Moreover, treatment with submaximal concentrations of dbcAMP (at 30 mM) transiently and significantly enhanced the production of intracellular ROS at 60 min (Figure 8(b)). Pretreatment with NAC at 3 mM abrogated the dbcAMP (30 mM)-induced decrease of MTT reduction activity and increase of LDH release (Figure 8(c)).

4. Discussion

PGE2 is known to be a potent proinflammatory mediator that is increased in postmortem brain tissue, cerebrospinal fluid, and serum from patients with sporadic ALS [6, 29] and in both the cerebral cortex and spinal cord in the G93A mutant SOD1 transgenic mouse model of ALS [7, 30]. A previous study from our laboratory showed that the PGE2-induced cytotoxicity is mediated by activation of EP2 in differentiated NSC-34 cells [21]. However, details of the mechanisms of cell injury triggered by PGE2 are not yet fully clear. The present results showed that PGE2 induced a dramatic increase of intracellular ROS generation in differentiated NSC-34 cells, strongly suggesting that PGE2-induced cell death in differentiated NSC-34 cells is attributable to intracellular ROS generation. We also showed that pretreatment of these cells with NAC reversed the decrease in MTT reduction activity, increases in the release of LDH, and the level of cleaved caspase-3 protein and cell death induced by

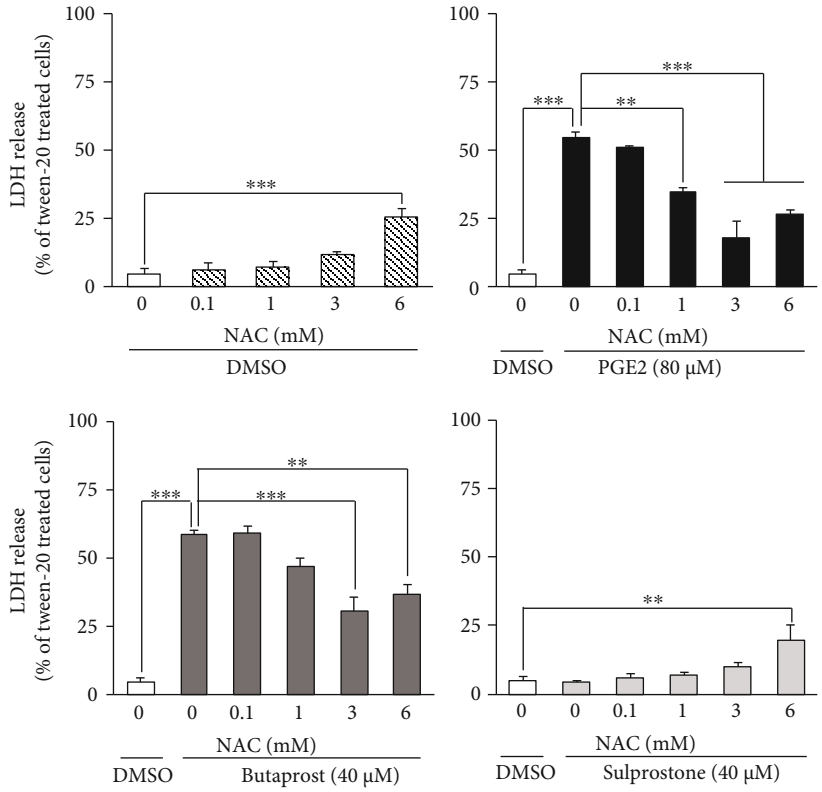

Figure 4: Effect of NAC pretreatment on PGE2- and EP agonist-induced LDH leakage from differentiated NSC-34 cells. Differentiated NSC-34 cells were exposed to various concentrations of NAC for 4 h, and then the cells were treated with vehicle (0.15% DMSO), 80 μM PGE2, 40 μM butaprost (an EP2 agonist), or 40 μM sulprostone (an EP1/3 agonist) for 48 h. After exposure, the amount of LDH released into the medium was assayed as described in Experimental Procedures. Graphs show the relative levels of LDH in these cells. Values are calculated as percentages of LDH released relative to that of cells treated with 0.2% Tween-20. Values represent means ± S.E.M. for four separate experiments. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

PGE2 and butaprost. Although the level of cleaved caspase-3 increased in differentiated NSC-34 cells treated with PGE2, the decrease in pro-caspase-3 was not detected in these cells. Similar to our present results, several studies have reported increased level of cleaved caspase-3 without a decreased level in pro-caspase-3 [31, 32]. One possible explanation of this conflict is that a polyclonal antibody against pro-caspase-3 may have high affinity, so that no difference in the level of pro-caspase-3 may be detected. Present results also showed that the generation of cleaved caspase-3 was found in some extent in undifferentiated cells. The role of caspase-3 activity in promoting neuronal differentiation was demonstrated across a broad spectrum of cell lineages including olfactory sensory neurons, neural stem cell, and PC12 cells [33]. Unlike these cells, previous studies of our and other laboratories revealed that the generation of cleaved caspase-3 is observed in undifferentiated NSC-34 cells as well as in differentiated forms [22, 34]. Therefore, it seems that activation of caspase-3 is not responsible for the neuronal differentiation in NSC-34 cells. Similar to our present results, it has also been reported that the number of active caspase-3-positive cells is dramatically increased by several oxidative stress inducers such as hydrogen peroxide, tumor necrosis factor-α, and high doses of glutamate in differentiated NSC-34 cells [34]. On this basis, we conclude that PGE2 exerts apoptosis-inducing neurotoxicity via activation of the caspase-3 cascade, and that production of ROS acts upstream of the caspase-3 cascade to participate in the mechanism of cell death in motor neuron-like NSC-34 cells.

It has been shown that the pathological and physiological effects of PGE2 are mediated via four functionally related GPCRs, designated EP1–EP4 [28, 35]. Previous studies from our laboratory have demonstrated that EP2 and EP3 are highly expressed in differentiated NSC-34 cells as well as motor neurons in the mouse spinal cord, suggesting that differentiated NSC-34 cell is a suitable model for assessing the response to PGE2 in motor neurons [10, 21]. We have also shown that EP2 plays a key role in PGE2-induced cell death in differentiated NSC-34 cells [21]. Here, we further investigated the effects of well-characterized EP agonists and antagonists to clarify the mechanisms underlying PGE2-induced ROS production in differentiated NSC-34 cells. As in the case of PGE2, treatment of these cells with butaprost, a selective EP2 agonist, caused a time-dependent increase of ROS production. In contrast, application of an EP1/EP3 agonist, sulprostone, transiently decreased the levels of intracellular ROS. We also showed that an EP2-selective antagonist (PF-04418948) but not an EP3-selective antagonist (L-798,106) suppressed the PGE2-induced production of intracellular ROS. More importantly, generation of ROS was crucial to the actions of PGE2 and butaprost, as the antioxidant NAC (3 mM) suppressed the increase in the

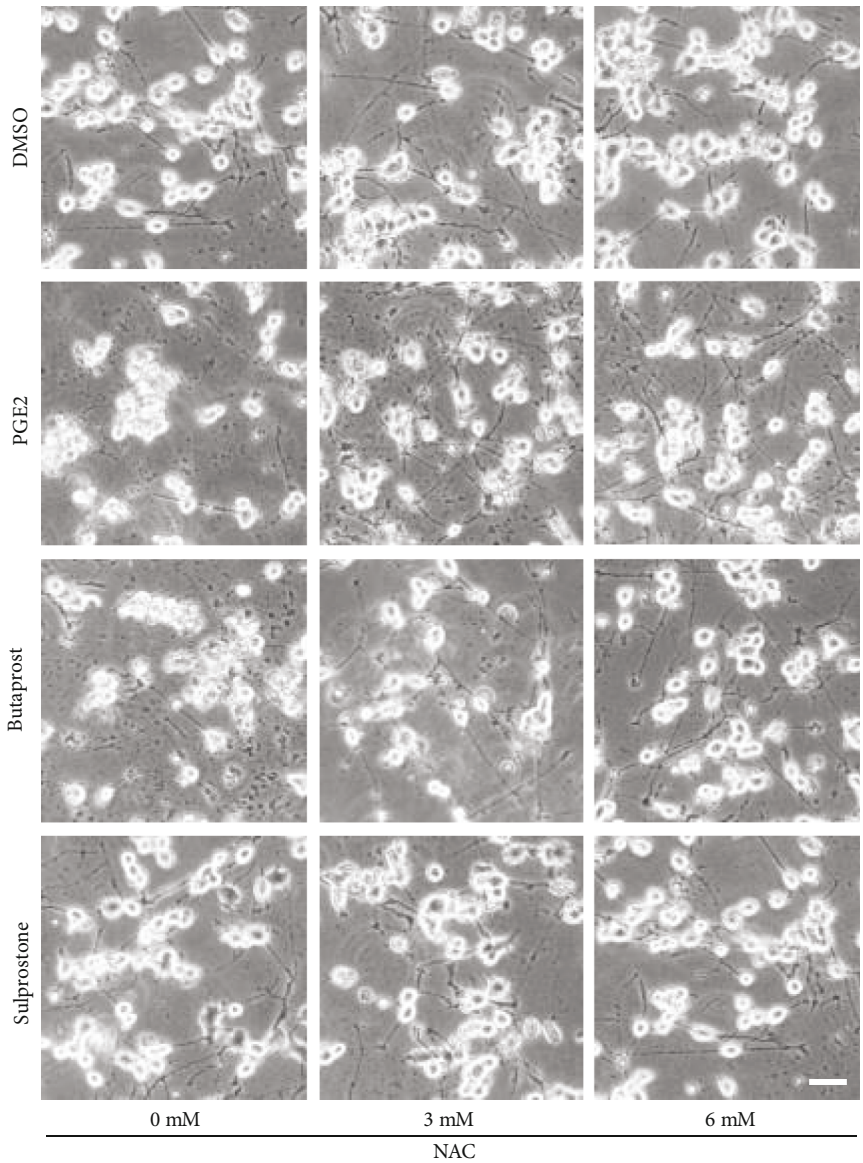

Figure 5: Phase-contrast photomicrographs of differentiated NSC-34 cells treated with PGE2 and EP agonists. Differentiated NSC-34 cells were treated with vehicle (0.15% DMSO), 80 μM PGE2, 40 μM butaprost (an EP2 agonist), or 40 μM sulprostone (an EP1/3 agonist) in the presence or absence of NAC for 48 h. Cell morphology was observed using a phase-contrast microscope (IX71, Olympus, Tokyo, Japan). Photographs show typical phase-contrast microscopy images in each treatment group. Scale bar indicates 50 μm.

level of cleaved caspase-3 expression and the cell death induced by PGE2 and butaprost. These results suggest that PGE2-induced intracellular ROS production is mostly attributable to the activation of EP2, and not EP3, in differentiated NSC-34 cells.

Classically, EP2 has been shown to couple to Gs proteins and activate adenylate cyclase, leading to intracellular generation of cAMP and activation of cAMP-dependent protein kinases [28, 35]. It has been reported that the cAMP-dependent protein kinase A (PKA) signaling pathway is activated under hypoxic conditions and exacerbates hypoxia-induced ROS formation in PC-12 cells [36]. In contrast, activation of EP2 by butaprost has been reported to protect dopaminergic neurons against 6-hydroxydopamine (6-OHDA)-mediated oxidative stress in primary cultured neurons prepared from embryonic rat midbrain [37]. This neuroprotective effect of butaprost was also conferred by cAMP analogs and was blocked by PKA inhibitors, suggesting that the neuroprotection afforded by EP2 activation is mediated through cAMP-dependent PKA activity in these cells [37]. Recently, we demonstrated that PGE2 promotes the conversion of undifferentiated NSC-34 cells to motor neuronal cells by activating the EP2 subtype, and that an exogenously applied cAMP analog, dbcAMP, facilitates neurite outgrowth with no effect on cell proliferation in undifferentiated NSC-34 cells [22]. Unlike the situation in undifferentiated cells, the results of the present study showed that dbcAMP partially mimicked PGE2- and butaprost-induced intracellular ROS generation and cell death in differentiated NSC-34 cells. These results suggest that the cAMP signaling pathway is at least

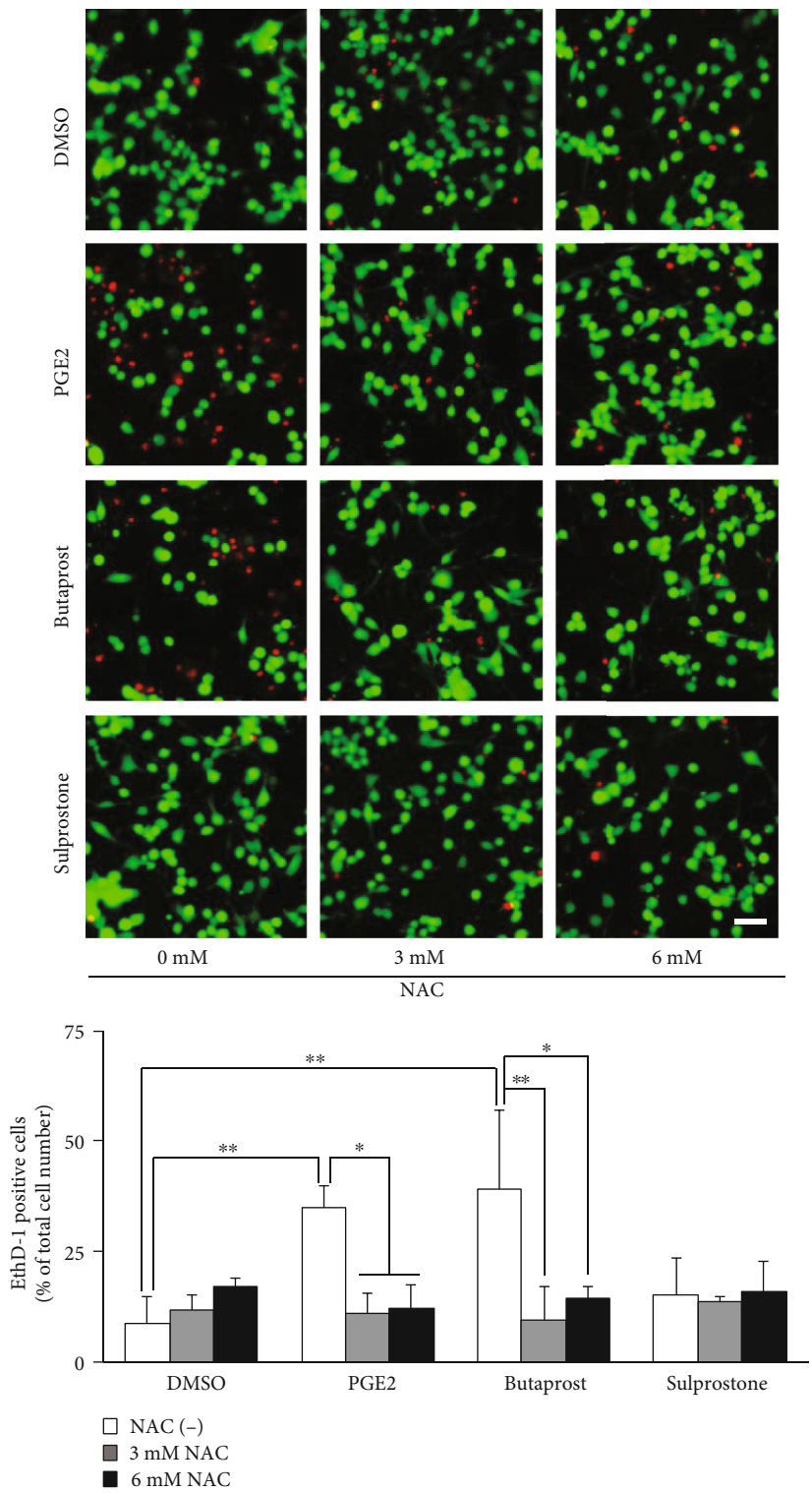

Figure 6: LIVE/DEAD staining of differentiated NSC-34 cells treated with PGE2 and EP agonists. Differentiated NSC-34 cells were treated with vehicle (0.15% DMSO), 80 μM PGE2, 40 μM butaprost (an EP2 agonist), or 40 μM sulprostone (an EP1/3 agonist) in the presence or absence of NAC for 48 h. Photographs show typical fluorescence images of calcein-AM (green, live cells) and EthD-1 (red, dead cells) double staining in each treatment group. Scale bar indicates 50 μm. Graphs show the percentage of EthD-1-positive dead cells in these cells. Each value represents the mean ± S.D. for three different experiments. $^{**}p < 0.01$, $^{*}p < 0.05$.

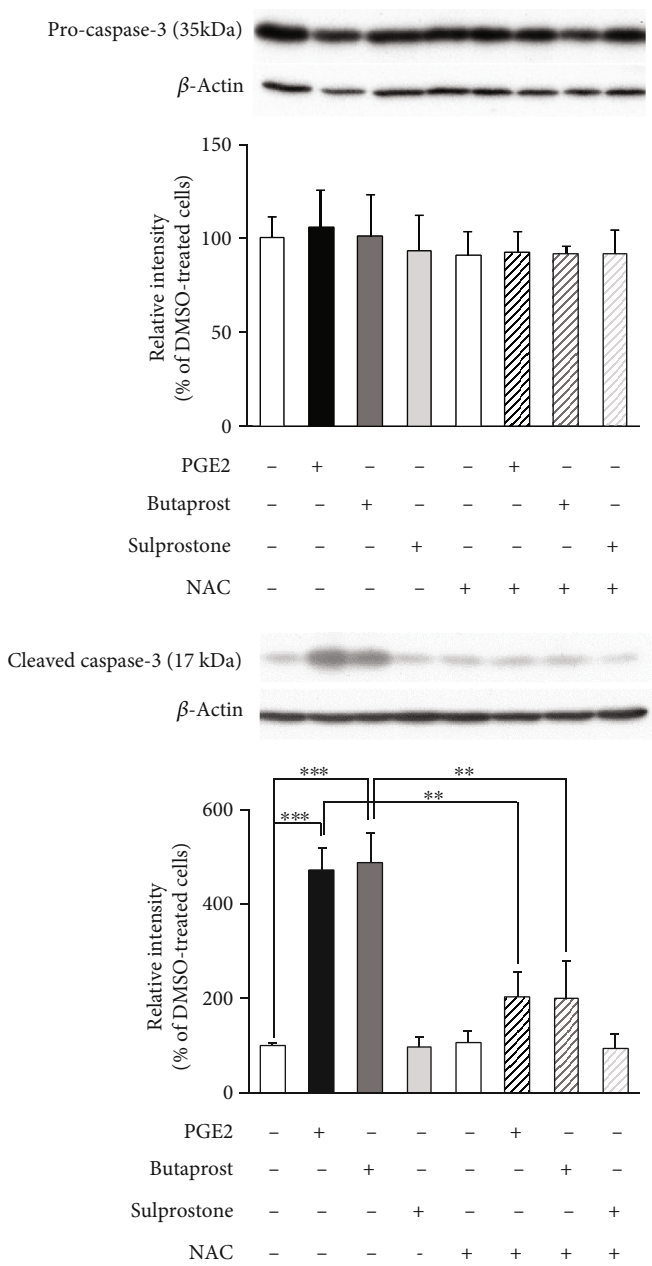

Figure 7: Effects of NAC pretreatment on PGE2- and EP agonist-induced caspase-3 cleavage in differentiated NSC-34 cells. Differentiated NSC-34 cells were treated with vehicle (0.15% DMSO), 80 μM PGE2, 40 μM butaprost (an EP2 agonist), or 40 μM sulprostone (an EP1/3 agonist) in the presence or absence of 3 mM NAC for 8 h. Equal amounts of cell lysate (10 μg) were analyzed by Western blotting using anti-pro-caspase-3 antibody, anti-cleaved caspase-3 antibody, and also with anti-β-actin antibody as an internal control. The level of pro-caspase-3 (35 kDa) and cleaved caspase-3 (17 kDa) was assessed by densitometric analysis, and quantitative results were expressed as the ratio of the band intensity of pro-caspase-3 or cleaved caspase-3 to the band intensity of β-actin. Each value represents the mean ± S.D. for four different experiments. N.D.: not detected. $^{***}p < 0.001$, $^{*}p < 0.05$.

partly involved in PGE2-induced cytotoxicity in differentiated NSC-34 cells, and that CNS region, cell maturity, and differentiation could all be important in determining whether PGE2 causes salutary or detrimental effects in individual neurons. Although further study will be needed to identify the downstream mechanisms of EP2 activation operating in the cytotoxic effects of PGE2 on differentiated NSC-34 cells, the present study has newly revealed that activation of Gs-linked GPCRs evokes intracellular ROS generation in motor neuron-like cells.

The EP3 receptor has multiple isoforms generated through alternative mRNA splicing in the carboxyl tail of the EP3 receptor gene. Three mRNA splice variants of the EP3 receptor have so far been identified in the mouse:

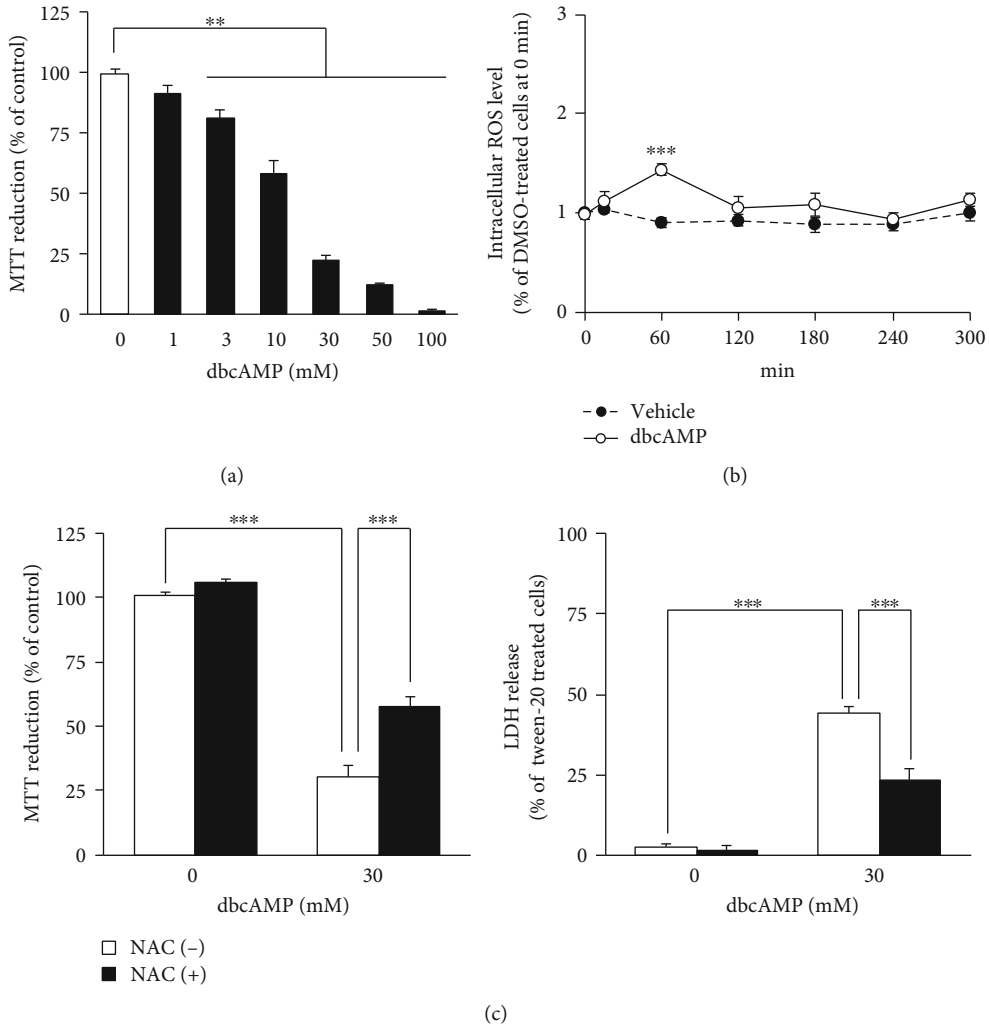

Figure 8: Exogenous application of cAMP analog induces cell death and facilitates intracellular ROS generation in differentiated NSC-34 cells. (a) Differentiated NSC-34 cells were incubated with various concentrations of dbcAMP or vehicle (distilled water) for 48 h. The viability of the cells was assessed by the MTT reduction assay. Graph shows effect of dbcAMP on MTT reduction activity in these cells. The results are expressed as a percentage relative to the control (nontreated) cells. Values represent means ± S.E.M. for four separate experiments. $^{**}p < 0.01$. (b) The intracellular ROS levels were measured over time by monitoring DCF fluorescence intensity in differentiated NSC-34 cells incubated in vehicle (distilled water) or in the presence of dbcAMP (30 mM). Graph shows time courses of DCF fluorescence intensity in the treated cells. Values represent means ± S.E.M. for four separate experiments. $^{***}p < 0.001$ compared to vehicle-treated cells at each time point. (c) Differentiated NSC-34 cells were exposed to vehicle (distilled water) or 3 mM NAC for 4 h, and then the cells were treated with vehicle (distilled water) or 30 mM dbcAMP for 48 h. The viability of the cells was assessed by the MTT reduction assay and LDH assay. Graphs show effect of dbcAMP on MTT reduction activity (left) and LDH release (right) in these cells. Results are expressed as a percentage relative to the control (nontreated) cells. Values represent means ± S.E.M. for four separate experiments. $^{***}p < 0.001$.

EP3α, EP3β, and EP3γ [28]. Previously, we have confirmed that EP3γ is the major expression isoform in the motor neurons of mice [26]. As is the case for the mRNA profile in mouse motor neurons, the present study showed that EP3γ is dominantly expressed in differentiated NSC-34 cells. Although the EP3α and EP3β isoforms couple exclusively to Gi protein, the EP3γ isoform couples to both Gs and Gi proteins [28]. Consistent with previous results from our laboratory [21], treatment of differentiated NSC-34 cells with sulprostone did not affect cell survival. We also found that intracellular ROS levels were slightly but significantly decreased in sulprostone-treated differentiated NSC-34 cells, and that PGE2-induced ROS production was not affected by the presence of L-798,106, an EP3-selective antagonist, in these cells. Thus, EP3 appeared not to play a role in PGE2-induced neurotoxicity in the cells.

Numerous studies have found evidence of increased oxidative stress in the pathogenesis of many neurodegenerative diseases, including ALS, Parkinson's disease, Alzheimer's disease, and Huntington disease [38]. We recently reported that the spinal cord PGE2 levels in the G93A mutant SOD1 transgenic mouse model of ALS at the early symptomatic stage

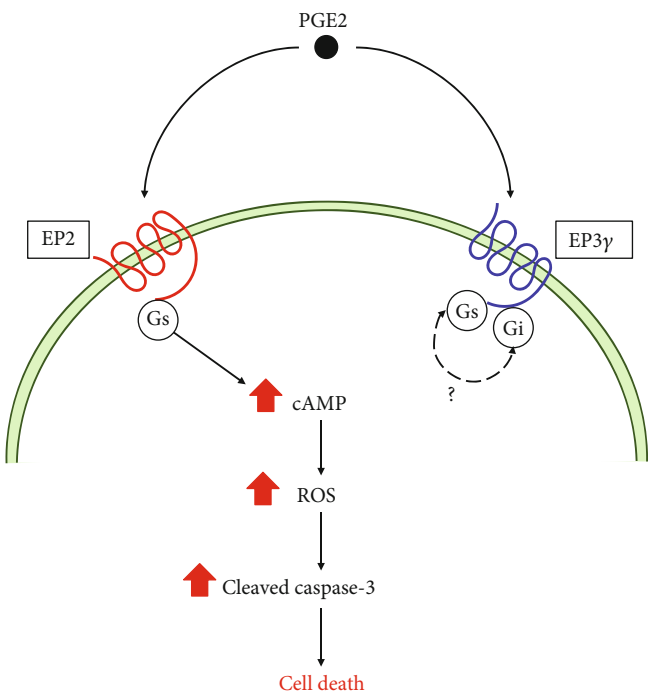

Figure 9: Proposed mechanism underlying PGE2-induced neurotoxicity in motor neuron-like NSC-34 cells. PGE2: prostaglandin E2; EP2: E-prostanoid receptor-2; EP3: E-prostanoid receptor-3; Gs: Gs protein; Gi: Gi protein; cAMP: cyclic adenosine monophosphate; ROS: reactive oxygen species.

tended to be increased relative to those in control mice [7]. Our present findings suggest that PGE2 may act as an inducer of oxidative stress in ALS-associated neuronal damage. In addition, we have previously revealed that the protein expression of mPGES-1, an inducible terminal synthase in PGE2 biosynthesis, is increased in the motor neurons of SOD1 mutant mice at the presymptomatic and early symptomatic stages, suggesting that PGE2 might be locally generated around motor neurons at 11 weeks of age [8]. Further studies are conducted to confirm the effects of PGE2 in the primary cultured motor neurons from mouse spinal cord and are needed to clarify the role of localized PGE2 in motor neuron oxidative stress in ALS model mice. Consistent with the important role of PGE2 suggested by our present findings, one recent study has shed new light on the relationship between PGE2 and oxidative stress using primary cultured mesencephalic neurons from mPGES-1-knockout mice [39]. Genetic deletion of mPGES-1 has been found to prevent dopaminergic neurodegeneration caused by 6-OHDA-induced oxidative stress and to inhibit 6-OHDA-induced PGE2 production both *in vitro* and *in vivo* [39]. Interestingly, exogenous application of PGE2 to mPGES-1-knockout neurons compensated for the deficiency of 6-OHDA-induced PGE2 production and abrogated 6-OHDA toxicity to almost the same extent as that seen in WT neurons [39]. These results suggest that mPGES-1 exacerbates 6-OHDA-induced dopaminergic neuronal death by enhancing oxidative stress via PGE2 production, thus increasing the vulnerability of neurons to oxidative stress through intracellular ROS generation in neurodegenerative diseases.

5. Conclusion

In conclusion, the present study has demonstrated for the first time that PGE2 is an endogenous inducer of intracellular ROS, and that production of ROS induced by PGE2-EP2 receptor signaling is coupled to the caspase-3 cascade, the major pathway of apoptosis, in motor neuron-like NSC-34 cells, as shown in a summarized schema in Figure 9. These findings suggest that PGE2 is a lipid mediator with key links to inflammation and oxidative stress. Understanding this novel effect of PGE2 on oxidative stress in motor neuron-like cells may provide a potential target for the treatment of motor neuronal diseases such as ALS.

Abbreviations

aCSF: Artificial cerebrospinal fluid
cAMP: Cyclic adenosine monophosphate
ALS: Amyotrophic lateral sclerosis
dbcAMP: Dibutyryl-cAMP
DCF: Dichlorofluorescein
DCFH-DA: 2′,7′-Dichlorodihydrofluorescin diacetate
DMSO: Dimethyl sulfoxide
DP: D-prostanoid receptor
ECL: Enhanced chemiluminescence
EP: E-prostanoid receptor
EthD-1: Ethidium homodimer-1
FP: F-prostanoid receptor
FBS: Fetal bovine serum
GPCRs: G-protein-coupled receptors
IP: I-prostanoid receptor
LDH: Lactate dehydrogenase
mPGES-1: Microsomal PGE synthase-1
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
NAC: N-acetyl-L-cysteine
6-OHDA: 6-Hydroxydopamine
PGD2: Prostaglandin D2
PGE2: Prostaglandin E2
PKA: cAMP-dependent protein kinase A
PVDF: Polyvinylidene difluoride
ROS: Reactive oxygen species
SDS: Sodium dodecyl sulfate
TP: Thromboxane-prostanoid receptors.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yasuhiro Kosuge and Hiroshi Nango contributed equally to this work.

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