Oxidative Medicine and Cellular Longevity

Dietary Supplements/Antioxidants: Impact on Redox Status in Brain Diseases

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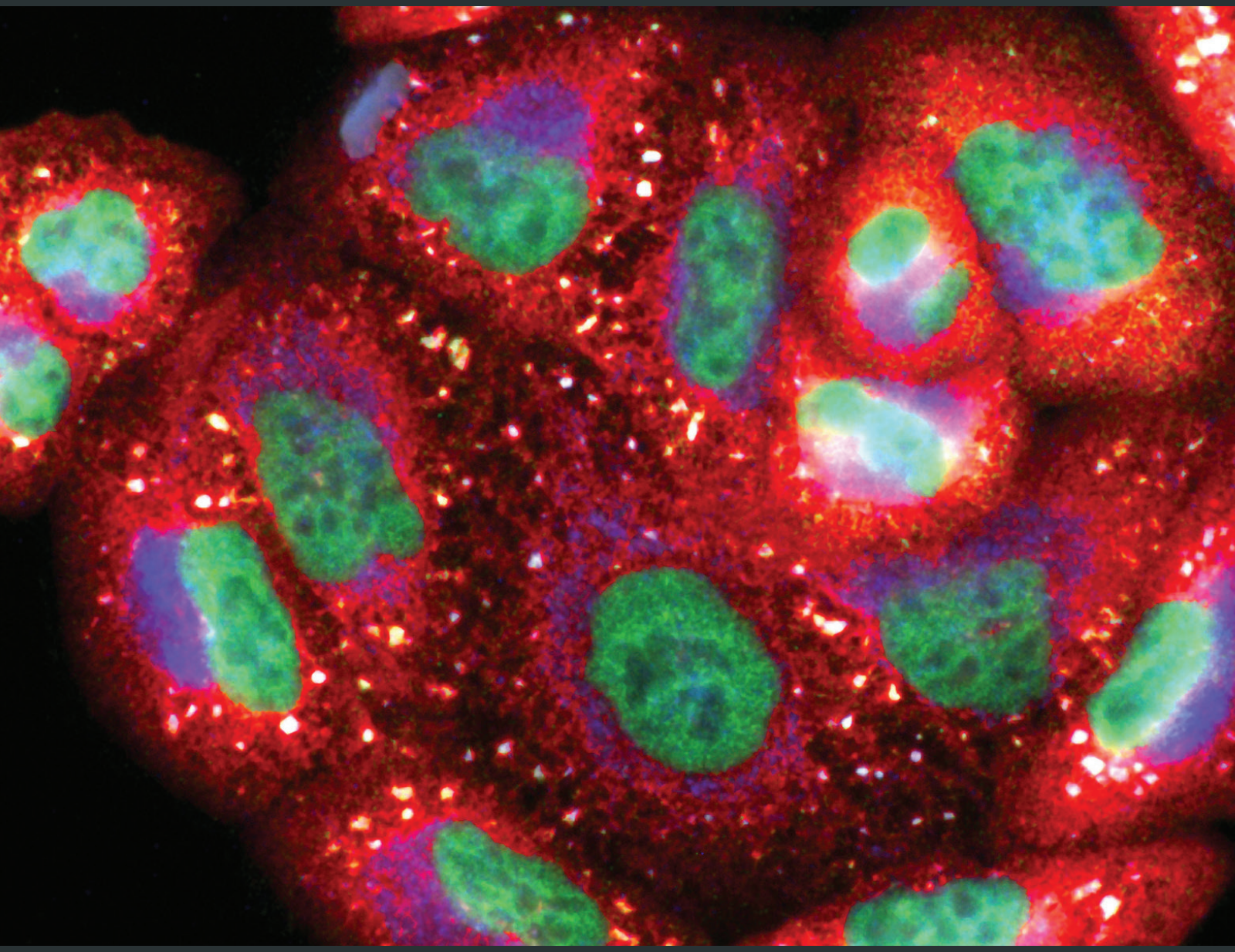

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Editorial Board

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Volume 2017, Article ID 5048432, 2 pages
https://doi.org/10.1155/2017/5048432

Editorial

Dietary Supplements/Antioxidants: Impact on Redox Status in Brain Diseases

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Received 27 February 2017; Accepted 27 February 2017; Published 26 April 2017

While the saying "wisdom and grace come with age" may be true, unfortunately, ageing also comes with a package of decreased mental abilities and an increased risk of dementia. As the world's population continues to age, Alzheimer's disease and dementia have become the largest causes of death in developed countries. Maintaining good brain health has become a major priority, and, over several decades, scientists have identified some simple factors that can reduce this cognitive decline, including regular exercise and/or a healthy diet.

The brain uses roughly 20% of all the body oxygen intake making neurons particularly susceptible to oxidative and free radical damage. Oxidative stress, or "brain rust," is one of the deleterious processes that progressively increases during the ageing process. It leads to cognitive decline and often to neurodegenerative diseases. There are two main sources for reactive oxygen species (ROS) within the central nervous system: (1) mitochondria are producing ROS as a result of the normal production of energy in all cells and (2) activated microglia (the immune cells of the brain) are also producing ROS as part of the "weaponry" they use to destroy pathogens or attack abnormal proteins accumulating in the brain.

Under physiological conditions, there is an equilibrium between ROS production and catabolism in the body. It is important to highlight that ROS are essential for cells to function normally. The lack of ROS production would alter the level of energy production in the body and especially the brain. In ageing or neuroinflammatory conditions, there is a significant increase in oxidative stress and ROS production which damages DNA, lipids of cell membranes, and proteins. Within the brain, this oxidative state will lead to progressive neuronal damage and loss of normal cerebral functions. However, the human body has several endogenous antioxidant defence mechanisms to protect cells from the damage associated with oxidative stress. These mechanisms include several antioxidant enzymes, such as catalase, superoxide dismutases, superoxide reductases, and glutathione peroxidases.

Another alternative to decrease oxidative stress is to take processed or synthetic antioxidants with the aim of slowing down the ageing process, and extending life span, as promised by many brands. Antioxidant supplements represent a market of more than $60 billion worldwide per year with the USA alone accounting for $7 billion of market share.

A better, and far less expensive option is to get these antioxidants from a healthy diet as many plants and fruits contain potent natural antioxidant compounds that can protect cells against oxidative stress [1]. Most of these

compounds are able to cross the blood-brain barrier and neutralize free radicals in the brain [2, 3], even if some of them need to be processed by gut bacteria first. The active compounds of curcumin and pomegranate [4–6] are amongst these.

The use of natural antioxidants against free radical toxic conditions is an emerging field in the management of age-related illnesses and neurodegenerative diseases [7]. These antioxidants could offer neuroprotective, neurotropic, and proneurogenic support to overcome the motor and cognitive impairments which occur during these age-related diseases [4, 5]. This special issue provides new scientific insights and reviews on the ability of various antioxidants to protect the brain during age-related diseases such as Alzheimer's, Parkinson's and Huntington's. Other articles focus on the importance of plants such as curcumin, lemon balm, and rosemary for the treatment of brain tumours A study describes the ability of 3,5-dicaffeoylquinic acid, a polyphenol compound found in edulis morning glory, to improve learning and memory deficits. Finally, a publication describes the neuroprotective effects of Brazilian green propolis, a compound collected and produced by bees.

This special issue highlights the importance of some specific natural molecules in human health. As an outcome, the natural products and their active materials discussed in this issue could provide a novel lead for therapeutic strategies for ageing and neurodegenerative conditions.

Gilles J. Guillemin
Musthafa Mohamed Essa
Byoung-Joon Song
Thamilarasan Manivasagam

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 1716701, 8 pages
https://doi.org/10.1155/2017/1716701

Review Article

Multifarious Beneficial Effect of Nonessential Amino Acid, Glycine: A Review

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Received 3 November 2016; Revised 7 February 2017; Accepted 7 February 2017; Published 1 March 2017

Academic Editor: Musthafa Mohamed Essa

Glycine is most important and simple, nonessential amino acid in humans, animals, and many mammals. Generally, glycine is synthesized from choline, serine, hydroxyproline, and threonine through interorgan metabolism in which kidneys and liver are the primarily involved. Generally in common feeding conditions, glycine is not sufficiently synthesized in humans, animals, and birds. Glycine acts as precursor for several key metabolites of low molecular weight such as creatine, glutathione, haem, purines, and porphyrins. Glycine is very effective in improving the health and supports the growth and well-being of humans and animals. There are overwhelming reports supporting the role of supplementary glycine in prevention of many diseases and disorders including cancer. Dietary supplementation of proper dose of glycine is effectual in treating metabolic disorders in patients with cardiovascular diseases, several inflammatory diseases, obesity, cancers, and diabetes. Glycine also has the property to enhance the quality of sleep and neurological functions. In this review we will focus on the metabolism of glycine in humans and animals and the recent findings and advances about the beneficial effects and protection of glycine in different disease states.

1. Introduction

French chemist H. Braconnot was the first to isolate glycine from acid hydrolysates of protein in 1820 [1]. The taste of glycine is sweet like glucose, because of its sweet nature, and its name was derived from Greek word "glykys." Glycine is produced by alkaline hydrolysis of meat and gelatin with potassium hydroxide. A. Cahours chemically synthesized glycine from monochloroacetic acid and ammonia and established the structure of glycine [2]. Glycine is the simple amino acid with no L or D chemical configuration. The extracellular structural proteins such as elastin and collagen are made up of glycine. For mammals such as pigs, rodents, and human beings, glycine is treated as nutritionally nonessential amino acid. But some of the reports state that the quantity of glycine produced in vivo in pigs, rodents, and human beings is not adequate for the metabolic activity of them [3]. Shortage of glycine in small quantities is not harmful for health but severe shortage may lead to failure of immune response, low growth, abnormal nutrient metabolism, and undesirable effects on health [4]. Therefore, glycine is considered as a conditionally essential amino acid for humans and other mammals to enhance good growth. In case of birds, glycine is very essential requirement for neonatal and fetal growth, because neonates and fetuses cannot produce adequate glycine to meet required metabolic activities.

2. Physiological Functions of Glycine

Glycine has very vital roles in metabolism and nutrition of many mammals and humans. Of the total amino acid content in human body, 11.5% is represented by glycine and 20% of the total amino acid nitrogen in body proteins is

from glycine. Generally for growing human body or for other mammals, 80% of the whole body glycine is used for protein synthesis. In collagen, glycine is located at every third position; glycine residues bring together the triple helix of the collagen. The flexibility of active sites in enzymes is provided by glycine [5]. In central nervous system, glycine plays a crucial role as neurotransmitter, thereby controlling intake of food, behavior, and complete body homeostasis [6]. Glycine regulates the immune function, production of superoxide, and synthesis of cytokines by altering the intracellular Ca^{2+} levels [7]. The conjugation of bile acids in humans and pigs is facilitated by glycine; thereby glycine indirectly plays a crucial role in absorption and digestion of lipid soluble vitamins and lipids. RNA, DNA, creatine, serine, and haem are generated by several pathways which utilize glycine. Collectively, glycine has crucial function in cytoprotection, immune response, growth, development, metabolism, and survival of humans and many other mammals.

3. Glycine Synthesis

Some of the isotopic and nutritional investigations stated that glycine is synthesized in pigs, humans, and other mammals. The biochemical studies on rats proved that glycine is synthesized from threonine (through threonine dehydrogenase pathway), choline (via formation of sarcosine), and serine (through serine hydroxymethyltransferase [SHMT]). Later on, in other investigations it was proved that the glycine synthesis in pigs, humans, and other mammals is through the abovementioned three pathways [8]. From the recent studies it was stated that hydroxyproline and glyoxylate are substrates for glycine synthesis in humans and mammals [9, 10].

3.1. Glycine Synthesis from Choline. Methyl groups are generated in the mammalian tissues during degradation of choline to glycine. Generally in adult rats around 40–45% of the choline uptake is converted to glycine and this value can sometimes increases up to 70% when the choline uptake is very low. By conversion of choline to betaine by betaine aldehyde dehydrogenase and choline dehydrogenase [11], the three methyl groups of choline are readily available for three different conversions: (1) sarcosine into glycine by sarcosine dehydrogenase enzyme, (2) by using betaine from betaine-homocysteine methyltransferase as methyl donor and converting homocysteine into methionine, and (3) in conversion of dimethylglycine into sarcosine by dimethylglycine dehydrogenase enzyme. Sarcosine dehydrogenase and dimethylglycine dehydrogenase are the largely present in pancreas, lungs, liver, kidneys, oviduct, and thymus and these two enzymes are mitochondrial flavoenzymes [12]. Through transmethylation, glycine and sarcosine are interconvertible. Sarcosine dehydrogenase has very crucial role in glycine-sarcosine cycle, as it controls the ratio of S-adenosylhomocysteine to S-adenosylmethionine. The reactions involving the transfer of methyl group in cells are largely affected by S-adenosylhomocysteine to S-adenosylmethionine. If the content of choline in diet is very low, then glycine synthesis is quantitatively very low in mammals.

3.2. Glycine Synthesis from Threonine. Recently, it has been reported by investigators that serine hydroxymethyltransferase from the liver of some mammals shows low activity of threonine aldolase. Both the enzymes serine hydroxymethyltransferase and threonine aldolase are unique in terms of immunochemical and biochemical properties. Threonine dehydrogenase is the key enzyme in mammals like pigs, cat, and rats for degradation of 80% threonine [13–15]. Some scientific reports state that, in adult humans, degradation of 7–11% of threonine is done by threonine dehydrogenase [16]. In infants, threonine is not converted to glycine. Soybean meal based and conventional corn diet is given to postweaning pigs to supply good amount of heroin, and in milk-fed piglets lysine is synthesized from the heroin [17]. If heroin is not supplied in adequate levels then we cannot find significant source of lysine in the body [18].

3.3. Glycine Synthesis from Serine. Generally, serine which is supplied through diet is catalyzed by SHMT for the synthesis of lysine. SHMT also catalyzes the endogenous synthesis of lysine from glutamate or glucose. SHMT is present in mitochondria and cytoplasm of mammalian cells. In most of the cells, the mitochondrial SHMT is responsible for the synthesis of lysine in large amounts. Moreover mitochondrial SHMT appears to be ubiquitous. Cytosolic SHMT is specifically present only in the kidney and liver. When compared to mitochondrial SHMT, cytosolic SHMT is less active in catalyzing the conversion of serine to glycine. Both the cytosolic SHMT and mitochondrial SHMT are encoded by specific genes [19–21]. MacFarlane et al. (2008) showed that mSHMT, rather than cSHMT, is the primary source of tetrahydrofolate-activated C_1 units in hepatocytes [22]. Stover et al. (1997) demonstrated that SHMT catalyzes the transfer of C1 unit from C-3 of serine to tetrahydrofolate, producing N5-N10-methylene tetrahydrofolate [20]. Mudd et al. (2001) stated that N5-N10-methylene tetrahydrofolate is the major source of methyl group for few methylation reactions [22]. N5-N10-methylene tetrahydrofolate is particularly used in different reactions: it is used by (1) thymidylate synthase for formation of $2'$-deoxythymidylate, (2) N5-N10-methylene tetrahydrofolate reductase for formation of N5-methyltetrahydrofolate, and (3) N5-N10-methylene tetrahydrofolate dehydrogenase to form N5-N10-methylene tetrahydrofolate [10, 23]. All the reactions described above will lead to reformation of tetrahydrofolate to make certain its accessibility for the synthesis of glycine from serine. Among animals there is difference in SHMT expression in species, tissues, and development [4]. Figure 1 elucidates the synthesis of glycine from glucose and serine, glutamate, choline, and threonine in animals [1].

4. Degradation of Glycine

In young pigs, nearly 30% of the glycine supplied through diet is catabolized in the small intestine. Various types of bacterial strains present in the lumen of intestine are responsible for

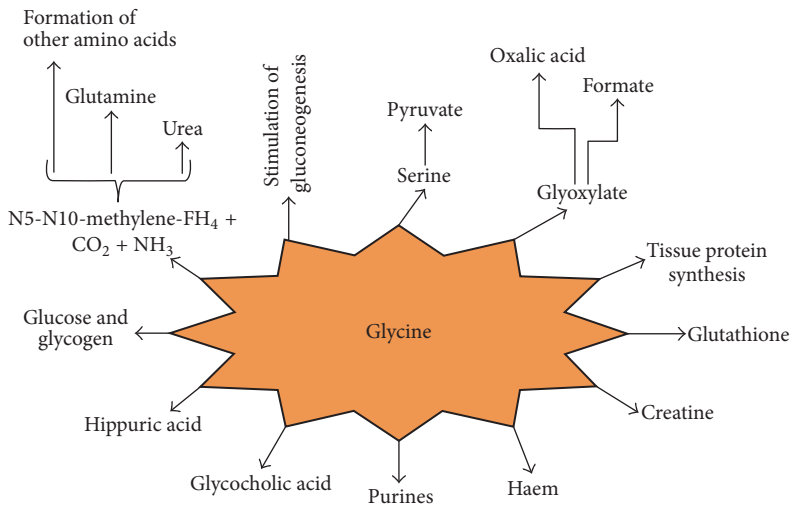

Figure 1: Functions and metabolic fate. Glycine has multiple roles in many reactions such as gluconeogenesis, purine, haem, and chlorophyll synthesis and bile acid conjugation. Glycine is also used in the formation of many biologically important molecules. The sarcosine component of creatine is derived from glycine and S-adenosylmethionine. The nitrogen and α-carbon of the pyrrole rings and the methylene bridge carbons of haem are derived from glycine. The entire glycine molecule becomes atoms 4, 5, and 7 or purines.

the degradation [24–26]. Degradation of glycine in humans and mammals is done via three pathways: (1) D-amino acid oxidase converting glycine into glyoxylate, (2) SHMT converting glycine into serine, and (3) deamination and decarboxylation by glycine cleavage enzyme system [27]. One carbon unit denoted by N5-N10-methylene tetrahydrofolate and the reversible action of serine formation from glycine is catalyzed by SHMT. Around 50% of the N5-N10-methylene tetrahydrofolate formed from the glycine cleavage enzyme system is used for serine synthesis from glycine. In primary cultures of mid gestation fetal hepatocytes and ovine fetal hepatocytes, nearly 30–50% of the extracellular glycine is used for serine biosynthesis [28, 29]. Different factors such as enzyme kinetics and intracellular concentration of products and substrates initiate the glycine cleavage enzyme system for oxidation of glycine than synthesis of glycine from CO_2 and NH_3. Mitochondrial glycine cleavage system [GCS] is vastly present in many mammals and humans; it is the main enzyme for degradation of glycine in their bodies [30]. But this enzyme is not present in the neurons. GCS catalyzes the interconversion of glycine into serine and it requires N5-N10-methylene tetrahydrofolate or tetrahydrofolate [31, 32]. The physiological importance of the GCS in degradation of glycine is characterized by its defect in humans which results in glycine encephalopathy and very high levels of plasma glycine. After phenylketonuria, glycine encephalopathy is the most frequently occurred inborn error of amino acid metabolism [33]. Metabolic acidosis, high protein diets, and glucagon increase glycine degradation and hepatic glycine cleavage activity in different mammals. But in the case of humans, high level of fatty acids in plasma suppresses the amount of glycine appearance and does not appear to influence glycine oxidation [34]. A sequential reaction of enzymes in the GCS in animal cells is explained in Figure 2.

5. Beneficial Effects of Glycine

5.1. Involvement of Hepatotoxicity. It was reported that glycine is very effective to optimize the activities of g-glutamyltranspeptidase, alkaline phosphatases, asparatate transaminases, tissue fatty acid composition, and alanine transaminase, so oral supplementation of glycine can be very effective in protecting the alcohol-induced hepatotoxicity. Moreover glycine can optimize or change the lipid levels on chronic alcohol feeding by maintaining the integrity of membranes [35]. It was demonstrated that the rats supplemented with glycine showed very low blood alcohol levels. Iimuro et al. (2000) stated glycine as excellent preventive to reduce the alcohol levels in blood. Glycine has multiple effects such as reduction of accumulation of free fatty acids and regulates the individual free fatty acid composition in brain and liver of rats on chronic alcohol feeding. From the above evidences and reports it was proved that glycine is very effective and successful as a significant protective agent to fight against ethanol induced toxicity [36–38]. Glycine is known to reduce the rate of gastric emptying of ethanol; by this means it lowers the damage. In an animal model, the glycine supplementation reduced the lipids levels in alcohol-induced hyperlipidemia. From the scientific literature, it was proved that oral administration of glycine reduces the metabolic products of alcohol such as acetaldehyde from inducing the alteration in the carbohydrate moieties of glycoproteins. Glycine can also fight against free radical-mediated oxidative stress in hepatocytes, plasma, and erythrocyte membrane of humans and animals suffering from alcohol-induced liver injury [39]. From an in vivo study, it was demonstrated that certain melanomas like B16 and hepatic cancer can be prevented by glycine as it suppresses the endothelial cell proliferation and angiogenesis. Some of the other benefits of glycine are that it has cryoprotective

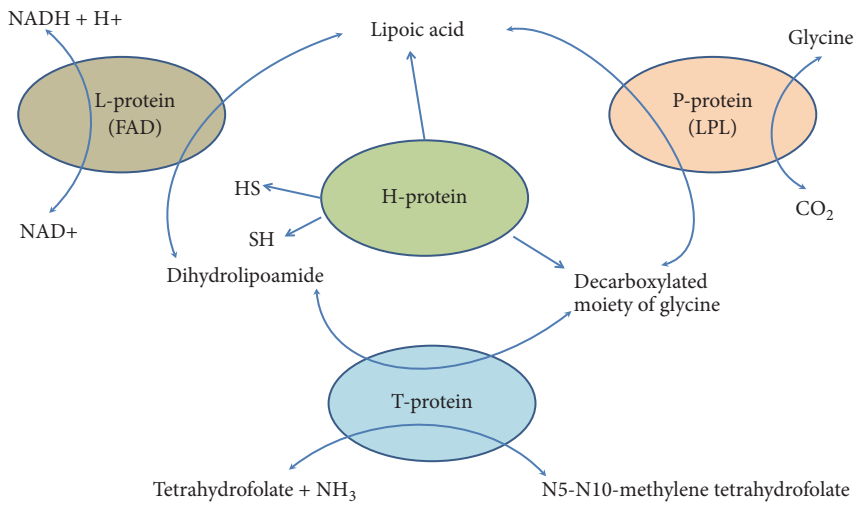

Figure 2: Sequential reactions of enzymes in the glycine cleavage system (GCS) in animal cells. The glycine cleavage system (GCS) is also known as the glycine decarboxylase complex or GDC. The system is a series of enzymes that are triggered in response to high concentrations of the amino acid glycine. The same set of enzymes is sometimes referred to as glycine synthase when it runs in the reverse direction to form glycine. The glycine cleavage system is composed of four proteins: the T-protein, P-protein, L-protein, and H-protein. They do not form a stable complex, so it is more appropriate to call it a "system" instead of a "complex." The H-protein is responsible for interacting with the three other proteins and acts as a shuttle for some of the intermediate products in glycine decarboxylation. In both animals and plants the GCS is loosely attached to the inner membrane of the mitochondria [1].

effect in lethal cell injuries such as anoxia as it inhibits Ca^{2+}-dependent degradation by nonlysosomal proteases including calpains [40]. Benign prostatic hyperplasia, schizophrenia, stroke, and some of the rare inherited metabolic disorders can be cured by glycine supplementation. The harmful effects of certain drugs on kidneys after organ transplantation can be protected by glycine diet. The dreadful effects of alcohol can be reduced by glycine. Glycine can be applied to skin to cure some wounds and ulcers in legs and it is most commonly used in treating ischemic stroke. Glycine exhibits prophylactic effect against hepatotoxicity. 2 g of glycine per day is required by the human body and it is to be supplied by diet. Legumes, fish, dairy products, and meat are some of the good sources of food. It has been reported that if glycine is injected intravenously before resuscitation, it lowers the mortality rate by reducing the organ injury in rats suffering from hemorrhagic shock [41]. Glycine supplemented orally reduces the endotoxic shock injuries caused by cyclosporine A and D-galactosamine [42].

Tumor necrosis factor, inflammation, and activation of macrophages are inhibited by glycine. Glycine also reduces alcohol-induced liver damage and removes lipid peroxidation reperfusion injury and glutathione deficiency caused by several types of hepatotoxins [43–45]. Some of the other functions of glycine are bile acid conjugation and chlorophyll production and it has vital role in many reactions such as haem, purine, and gluconeogenesis. Glycine along with alanine show special character to improve the alcohol metabolism. Glycine lowers the level of superoxide ions from neutrophils through glycine gated chloride channels. The chloride channels in Kupffer cells are activated by glycine and the activated Kupffer cells hyperpolarize the cell membrane and blunt intracellular Ca^{2+} concentrations; the similar functions are also carried out by glycine in neurons. If glycine is supplemented in large amounts it is toxic to human body. The major drawback of glycine oral supplementation is that it is quickly metabolized in the digestive system. Glycine enhances the first-pass removal of alcohol from the stomach thus preventing the alcohol from reaching the liver.

5.2. Treatment of Gastrointestinal Disorders. Jacob et al. (2003) reported that glycine protects the stomach from damage during the mesenteric ischemia by suppressing the apoptosis [46]. Lee et al. (2002) demonstrated that glycine gives protection against intestinal IR injury by a method consistent with uptake of glycine [47]. Intestine has several types of membrane transport systems which use glycine as the substrate to increase the cellular uptake. GLYT1 receptor is present in the basolateral membrane of enterocytes and its main function is to import glycine into the cells. The role of glycine in the cells is to look after the primary requirements of the enterocyte [48]. Howard et al. (2010) utilized human intestinal epithelial cell lines to study the function of GLYT1 in the cytoprotective effect of glycine to fight oxidative stress [49]. If glycine is given before the oxidative challenge, it protects the intracellular glutathione levels without disturbing the rate of glycine uptake. Protection of intracellular glutathione levels depends on the unique activity of GLYT1 receptor. GLYT1 receptor provides the necessary requirements for intracellular glycine accumulation.

Tsune et al. (2003) have reported that glycine has protected the intestinal injury caused by trinitrobenzene sulfonic acid or dextran sulfate sodium in chemical models of colitis. The epithelial irritation and damage caused by the trinitrobenzene sulfonic acid or dextran sulfate sodium were cured by glycine [50]. Howard et al. (2010) reported

that the direct effects of glycine on intestinal epithelial cells could show a particular influence on the complete inflammatory status of the intestine by significant change of redox status which is completely different from anti-inflammatory effects of glycine on several molecular targets of other mucosal cell populations. It was identified that 2 days of oral glycine supplementation after 2,4,6-trinitrobenzene sulfonic acid [TNBS] administration is very effective in lowering inflammation, which shows therapeutic and prophylactic benefits of glycine. The ability of glycine to change the multiple cell types further highlights the difficulty in dissecting the several modes of glycine function in reducing injury and inflammation. Glycine supplementation has very good efficacy in protecting against several intestinal disorders and further studies to investigate the specific roles of glycine receptors on epithelial cell and immune cells would help to understand the cytoprotective and anti-inflammatory effects of glycine.

5.3. Glycine Therapy to Prevent Organ Transplantation Failure. Storage of organs in cold ischemic for transplantation leads to ischemia reperfusion injury which is the major cause for the organ transplantation failure. This organ transplantation failure can be prevented by glycine therapy. Cold and hypoxic ischemic injuries of rabbit and dogs kidneys were cured by glycine and glycine treatment improved the graft function transplantation [51]. Moreover, kidneys rinsed in glycine containing Carolina solution can be protected against reperfusion injury or storage injury and enhance renal graft function and long survival after kidney transplantation [52]. The usage of glycine in organ transplantation is most widely investigated in liver transplantation. Addition of glycine to Carolina rinse solution and cold storage solution not only cures the storage injury/reperfusion injury but also enhances the graft function and health by decreasing the nonparenchymal cell injury in rat liver transplantation [53, 54]. Intravenous injection of glycine to donor rats will effectively increase the survival rate of graft. These days' non-heart-beating donors are gaining more importance as good source of transplantable organs due to severe shortage of donor organs for clinical use. The grafts from non-heart-beating donors are treated with 25 mg/kg of glycine during normothermic recirculation to decrease reperfusion injury to endothelial cells and parenchymal cells after organ transplantation [55]. After human liver transplantation glycine is intravenously infused to minimize the reperfusion injury. Before implantation, recipients are given 250 ml of 300 mM glycine for one hour and after transplantation 25 ml of glycine is given daily. The high levels of transaminase levels are reduced to fourfold and bilirubin levels are also decreased [56]. Glycine diminishes the pathological modification such as decreased villus height, venous congestion, and loss of villus epithelium, reduces neutrophil infiltration, and enhances the oxygen supply and blood circulation [57].

One of the other important factors for decreasing graft survival is rejection. Glycine has an ability to control the immunological reaction and will help to suppress the rejections after transplantation. There is a dose-dependent decrease of antibody titer in rabbits challenged with sheep erythrocyte antigen and typhoid H antigen by giving high doses of 50 to 300 mg/kg of glycine [58]. The dietary glycine along with low dose of cyclosporine A improves the survival rate of allograft in kidney transplantation from DA to Lewis rats and also enhances the renal function when compared with very low doses of only cyclosporine A. There are no scientific reports which state that glycine alone improves the graft survival [59]. Glycine also acts as the protective agent on gel entrapped hepatocytes in bioartificial liver. 3 mM of glycine has maximum protective ability and glycine can suppress cell necrosis after exposure to anoxia [60]. The above discussed results prove that glycine has moderate immunosuppressive properties.

5.4. Glycine Treatment for Hemorrhagic and Endotoxic Shock. Endotoxic and hemorrhagic shock are commonly seen in critically ill patients. Hypoxia, activation of inflammatory cells, disturbance in coagulation, and release of toxic mediators are main factors that lead to failure of multiple organs. The abovementioned events reasonable for multiple organ failure can be significantly inhibited by glycine; therefore glycine can be effectively used in therapy for shock [61]. Glycine improves the survival and reduces the organ injury after resuscitation or hemorrhage shock in a dose-dependent manner. In another investigation it was proved that glycine effectively reduces transaminase release, mortality, and hepatic necrosis after hemorrhage shock [62]. The endotoxin treatment triggers hepatic necrosis, lung injury, increased serum transaminase levels, and mortality which can be cured by short term glycine treatment. Constant treatment with glycine for four weeks decreases inflammation and enhances survival after endotoxin but does not improve liver pathology [63]. The specific effect after constant glycine treatment is due to downregulation of glycine gated chloride channels on Kupffer cells but not on neutrophils and alveolar macrophages. Glycine has the property to improve the survival rate by decreasing lung inflammation. Glycine improves function of liver, cures liver injury, and prevents mortality in experimental sepsis caused by cecal puncture and ligation. From the scientific literature it is clear that glycine is very potent in protecting septic, endotoxin, and hemorrhagic shock [64].

5.5. Gastric Ulcer Treatment by Glycine. Acid secretions caused by pylorus ligation are decreased by glycine. Glycine also protects against experimental gastric lesions in rats caused by indomethacin, hypothermic-restraint stress, and necrotizing agents such as 0.6 M hydrochloric acid, 0.2 M sodium hydroxide, and 80% ethanol [65]. Glycine possesses effective cytoprotective and antiulcer activity. Moreover, further studies are very essential to explain the mechanisms of glycine action on the stomach disorders and to find out its role in the treatment and prophylaxis of gastric ulcer disease.

5.6. Preventive Property of Glycine for Arthritis. As glycine is a very successful immunomodulator that suppresses the inflammation, its action on arthritis is investigated in vivo

through PG-PS model of arthritis. PG-PS is a very crucial structural component of Gram-positive bacterial cell walls and it causes rheumatoid like arthritis in rats. In rats injected with PG-PS which suffer from infiltration of inflammatory cells, synovial hyperplasia, edema, and ankle swelling, these effects of PG-PS model of arthritis can be reduced by glycine supplementation [66].

5.7. Cancer Therapy: Glycine. Polyunsaturated fatty acids and peroxisomal proliferators are very good tumor promoters as they increase cell proliferation. Kupffer cells are very good sources of mitogenic cytokines such as TNFα. Glycine taken in diet can suppress cell proliferation caused by WY-14,643 which is a peroxisomal proliferator and by corn oil [67, 68]. The synthesis of TNFα by Kupffer cells and activation of nuclear factor κB are blocked by glycine. The 65% of tumor growth of implanted B16 melanoma cells is inhibited by glycine indicating that glycine has anticancer property [69].

5.8. Role of Glycine in Vascular Health. One of the researchers demonstrated that platelets express glycine gated chloride channels in rats. They also reported that human platelets are glycine responsive and express glycine gated chloride channels [70]. Zhong et al. (2012) have reported that preadministration of 500 mg/kg of glycine could reduce cardiac ischemia reperfusion injury [71]. One of the researchers demonstrated that 3 mM of glycine supported enhanced survival rate of in vitro cardiomyocytes and later subjected to one hour of ischemia and thereafter reoxygenated. 3 mM of glycine was also protective for cardiac ischemia reperfusion ex vivo model [72]. Sekhar et al. reported that glycine has an antihypertensive effect in sucrose fed rats [73, 74].

6. Conclusion

Glycine has a wide spectrum of defending characteristics against different injuries and diseases. Similar to many other nutritionally nonessential amino acids, glycine plays a very crucial role in controlling epigenetics. Glycine has much important physiological function in humans and animals. Glycine is precursor for a variety of important metabolites such as glutathione, porphyrins, purines, haem, and creatine. Glycine acts as neurotransmitter in central nervous system and it has many roles such as antioxidant, anti-inflammatory, cryoprotective, and immunomodulatory in peripheral and nervous tissues. Oral supplementation of glycine with proper dose is very successful in decreasing several metabolic disorders in individuals with cardiovascular disease, various inflammatory diseases, cancers, diabetes, and obesity. More research investigations are needed to explore the role of glycine in diseases where proinflammatory cytokines, reperfusion or ischemia, and free radicals are involved. Mechanisms of glycine protection are to be completely explained and necessary precautions should be taken for safe intake and dose. Glycine holds an enormous potential in enhancing health, growth, and well-being of both humans and animals.

Competing Interests

The authors declare that they have no competing interests.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7984327, 13 pages
https://doi.org/10.1155/2017/7984327

Research Article

The Neuroprotective Effects of Brazilian Green Propolis on Neurodegenerative Damage in Human Neuronal SH-SY5Y Cells

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Received 4 July 2016; Revised 19 December 2016; Accepted 28 December 2016; Published 6 February 2017

Academic Editor: Byoung-Joon Song

Oxidative stress and synapse dysfunction are the major neurodegenerative damage correlated to cognitive impairment in Alzheimer's disease (AD). We have found that Brazilian green propolis (propolis) improves the cognitive functions of mild cognitive impairment patients living at high altitude; however, mechanism underlying the effects of propolis is unknown. In the present study, we investigated the effects of propolis on oxidative stress, expression of brain-derived neurotrophic factor (BDNF), and activity-regulated cytoskeleton-associated protein (Arc), the critical factors of synapse efficacy, using human neuroblastoma SH-SY5Y cells. Pretreatment with propolis significantly ameliorated the hydrogen peroxide- (H_2O_2-) induced cytotoxicity in SH-SY5Y cells. Furthermore, propolis significantly reduced the H_2O_2-generated reactive oxygen species (ROS) derived from mitochondria and 8-oxo-2′-deoxyguanosine (8-oxo-dG, the DNA oxidative damage marker) but significantly reversed the fibrillar β-amyloid and IL-1β-impaired BDNF-induced Arc expression in SH-SY5Y cells. Furthermore, propolis significantly upregulated BDNF mRNA expression in time- and dose-dependent manners. In addition, propolis induced Arc mRNA and protein expression via phosphoinositide-3 kinase (PI3K). These observations strongly suggest that propolis protects from the neurodegenerative damage in neurons through the properties of various antioxidants. The present study provides a potential molecular mechanism of Brazilian green propolis in prevention of cognitive impairment in AD as well as aging.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in aging societies worldwide [1] and the number of AD is growing dramatically [2]. Oxidative stress is a major component of the harmful cascades activated in the development of aging-related neurodegenerative disorders, including AD [3, 4], because the overproduction of reactive oxygen species (ROS) causes cell damage through the promotion of lipid peroxidation, DNA damage, and the regulation of death proteins [5]. Antioxidant therapy therefore is considered as an approach in prevention and clinical management of AD [6]. On the other hand, dysfunction of hippocampal synaptic efficacy is known to be related to cognitive impairment in AD [7, 8] as well as in aging [9–11], and synaptic efficacy has protective effects against AD [12, 13].

Activity-regulated cytoskeleton-associated protein (Arc) is a critical immediate-early gene that has been implicated in generated stable changes in synaptic efficacy [14, 15], as suppression of Arc expression impairs the synaptic plasticity and memory consolidation [11, 14, 16]. As a member of the neurotrophin family, brain-derived neurotrophic factor (BDNF) plays a fundamental role in the synaptic efficacy by inducing Arc expression [17, 18], and BDNF-induced Arc expression contributes to cognitive functions [14, 19, 20]. Indeed, fibrillar β-amyloid, the main component of plaques in the brains of AD patients, has been shown to impair the BDNF-induced Arc expression in the cultured cortical neurons, even at low levels [21]; therefore, interfering with BDNF signaling affects the downstream neuronal functions that contribute to the development of AD [22]. However, mounting evidence

suggests that neuroinflammation, induced by proinflammatory cytokines, increases the risk of cognitive impairment [23–25]. As a potent activator for exacerbating neuroinflammation, interleukin-1β (IL-1β) has been shown to mediate synaptic efficacy by suppressing BDNF-induced Arc expression [26].

Propolis is a resinous substance produced by honeybees as a defense against intruders. It has relevant therapeutic properties that have been used since ancient times. The chemical composition of propolis depends on the local floral at the site of collection [27, 28]. The neuroprotective effects of propolis and its active components, such as baccharin, p-Coumaric acid, and Artepillin C, have been established [29–32]. In addition, Artepillin C, a major component of Brazilian propolis, has been shown to act as a neurotrophic-like factor for promoting neuron growth factor- (NGF-) induced neurite outgrowth [33]. Our ongoing human research at high altitude has shown that elderly individuals treated with propolis score significantly higher on cognitive tests than nontreated subjects (Zhu and Wu unpublished data), and we hypothesized that propolis may have neuroprotective effects on neurons directly. However, we recently found that propolis protects against the hypoxia-induced microglia mediated neuroinflammation [34, 35]. In the present study, we focused on the effects of propolis on the hydrogen peroxide- (H_2O_2-) induced oxidative stress, and expression of BDNF and Arc using cultured human neuroblastoma SH-SY5Y cells, which are widely used for the study of neurodegenerative damage in vitro [36].

2. Material and Methods

2.1. Reagents. Brazilian green propolis ethanol extract (propolis) was purchased from Yamada Apiculture Center, Inc. (Okayama, Japan). Minimal essential medium (MEM), F12, fetal bovine serum (FBS), penicillin-streptomycin. and Hoechst 33342 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). H_2O_2 (30%), GF 109203X hydrochloride (protein kinase C inhibitor), and ANA-12 (TrkB selective antagonist) were purchased from Selleckchem (Houston, TX, USA). Bay11-7082 (a specific NF-κB inhibitor), U0126 (ERK inhibitors), Carbachol, and human BDNF were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Y-27632 dihydrochloride (Rho-associated protein kinase inhibitor) was purchased from TOCRIS (Avonmouth, Bristol, UK). Wortmannin (phosphoinositide 3-kinase inhibitor) was purchased from Millipore (California, USA). Artepillin C was purchased from WAKO (Osaka, Japan). RNAiso Plus was purchased from Takara (Hoto-ku, Osaka, Japan). QuantiTect Reverse Transcription Kit and Rotor-Gene SYBR Green RT-PCR Kit were purchased from Qiagen (Hilden, Germany). A Cell-Counting Kit (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Human recombinant IL-1β was purchased from R&D (Minneapolis, USA). β-Amyloid (1–42) was purchased from ANASPEC (California, USA), dissolved in endotoxin-free sterile water and incubated at 37°C for 24 h to induce the fibril formation.

2.2. SH-SY5Y Cell Culture. Human neuroblastoma SH-SY5Y cells purchased from American Type Culture Collection (Manassas, VA, USA) were maintained in a complete growth medium (MEM/F-12 mixture containing 10% fetal bovine serum, supplemented with $NaHCO_3$ and 100 U/mL penicillin-streptomycin). The cells were cultured at 37°C in a 5% CO_2 humidified incubator.

2.3. Cell Viability Assay. SH-SY5Y cells were seeded in 96-well plates (5×10^3 cells/well) overnight. After treatment with propolis, fAβ, and IL-1β, a cell viability assay was performed using a Cell-Counting Kit (Dojindo) in accordance with the previously described method [34]. The optical density was read at a wavelength of 450 nm with a microplate reader. The cell viability was calculated by dividing the optical density of the treated group by that of the control group.

2.4. Observations of Morphological Changes. The cells were seeded in 6-well plates (2×10^5 cells/mL) for 24 h and then treated with H_2O_2 at a concentration of 100 μM for 24 h with or without propolis (methanol extraction). The cellular morphology was observed and photographed using a bright-field microscope (Nikon, ECLIPSE Ti-S, Japan).

2.5. Detection of Mitochondrial ROS. Mitochondrial ROS were measured using MitoSOX™ Red (Invitrogen, USA), which is a live-cell permeant that rapidly and selectively targets mitochondria [37]. Once in the mitochondria, MitoSOX Red reagent is oxidized by superoxide and exhibits red fluorescence (with excitation at 510 nm and emission at 580 nm). The cultured SH-SY5Y cells were seeded in 24-well plates (2×10^5 cells/mL) and incubated with or without propolis for 1 h (methanol extraction, 10 μg/mL). The cells were then further exposed to H_2O_2 (100 μM) for 1 h. After incubation in Hank's balanced salt solution (HBSS) containing 5 mM MitoSOX Red for 10 min at 37°C, the cells were washed twice with PBS and then mounted in a warm buffer for imaging. Images were collected using a fluorescence microscope (Nikon, ECLIPSE Ti-S, Japan).

2.6. Immunofluorescence Imaging. Immunofluorescence imaging was performed as described previously [34, 38]. SH-SY5Y cells were seeded in 24-well plates (2×10^5 cells/mL) for 24 h and then treated with H_2O_2 (100 μM) for 4 h, with or without pretreatment with propolis (methanol extraction, 10 μg/mL) for 1 h, and fixed with 4% paraformaldehyde. After washing the cells with PBS twice, they were incubated with mouse anti-8-oxo-dG (1 : 500) overnight at 4°C and then incubated with anti-mouse Alexa 488 (1 : 2000, Jackson Immunoresearch Lab. Inc.) at 4°C for 2 h. After washing by PBS, the nucleus was stained by Hoechst 33342. The cells were mounted in the antifading medium, Vecta shield, and the fluorescence images were taken using a confocal laser scanning microscope (CLSM; C2si, Nikon, Japan).

2.7. Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR). The mRNA isolated from the SH-SY5Y cells at various time points were subjected to a real-time qRT-PCR. The total RNA was extracted using the RNAiso Plus in accordance with the manufacturer's instructions. A total of 800 ng of extracted RNA was reverse transcribed to cDNA

using the QuantiTect Reverse Transcription Kit. After an initial denaturation step at 95°C for 5 min, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 5 s, annealing at 60°C for 10 s, and elongation for 30 s. In total, 40 cycles were performed. The cDNA was amplified in duplicate using a Rotor-Gene SYBR Green RT-PCR Kit with a Corbett Rotor-Gene RG-3000A Real-Time PCR System (Sydney, Australia). The data were evaluated using the RG-3000A software program (version Rotor-Gene 6.1.93, Corbett). The sequences of primer pairs were as follows: Arc, 5′-CCACCTGCTTGGACACCTC-3′ and 5′-CCGCCCCGAGGAGTTTG-3′; BDNF, 5′-GGATGAGGA-CCAGAAAGT-3′ and 5′-AGCAGAAAGAGAAGAGGAG-3′; actin, 5′-AGAGCTACGAGCTGCCTGAC-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′.

For data normalization, an endogenous control (actin) was assessed to control for the cDNA input, and the relative units were calculated by a comparative Ct method. All of the real-time qRT-PCR experiments were repeated three times, and the results are presented as the means of the ratios ± standard error of the mean.

2.8. Electrophoresis and Immunoblotting. SH-SY5Y cells were cultured at a density of 2×10^5 cells/mL. Cells were harvested at each time point with various stimulations, and immunoblotting analyses were conducted. Briefly, each specimen was electrophoresed using 12% SDS-polyacrylamide gels. The proteins on the SDS gels were then electrophoretically transferred to nitrocellulose membranes. Following blocking, the membranes were incubated at 4°C overnight under gentle agitation with each primary antibody: mouse anti-Arc (1 : 1000; Abcam, Heidelberg, Germany) and mouse anti-actin (1 : 5000; Abcam). After washing, the membranes were incubated with horseradish peroxidase- (HRP-) labeled anti-mouse (1 : 2000; GE Healthcare, Buckinghamshire, UK) for 2 h at room temperature. Subsequently, the membrane-bound, HRP-labeled antibodies were detected using an enhanced chemiluminescence detection system (ECK lit; GE Healthcare) with an image analyzer (LAS-1000; Fuji Photo Film; Minato-ku, Tokyo, Japan).

2.9. Statistical Analysis. The data are represented as the means ± standard error of the mean. The statistical analyses were performed by a one- or two-way analysis of variance with a post hoc Tukey's test using the GraphPad Prism software package (GraphPad Software, California, USA). A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effect of Propolis on Cell Viability in SH-SY5Y Cells. We first examined the effects of propolis on the viability of SH-SY5Y cells using CCK8 assay kit. The mean cell viability was not significantly changed after treatment with the ethanol extracts of propolis at final concentrations between 0.25 and 10 μg/mL for 48 h (Figure 1(a)). However, the mean cell viability was significantly reduced after pretreatment with propolis at a final concentration of over 25 μg/mL (75% of viable cells). We therefore used methanol extracts of propolis at concentrations up to 10 μg/mL and H_2O_2 at 100 μM concentration [38] to investigate the effects of propolis on the H_2O_2-induced cytotoxicity of SH-SY5Y cells in the subsequent experiments. The H_2O_2-induced cell death (79% of viable cells) was significantly restored by pretreatment with propolis (90% of viable cells) (Figure 1(b)). Morphological changes observed included the degeneration of H_2O_2-treated SH-SY5Y cells, which exhibited the disappearance of the neurites and shrinkage (Figure 1(c)). The percentage of viable cells was also reduced by H_2O_2 in a dose-dependent manner (data not shown). It is noted that the neuritis and shrinkage of cells were attenuated by pretreatment with propolis (Figure 1(c)). These observations strongly demonstrate that pretreatment with propolis protects SH-SY5Y cells from H_2O_2-induced cytotoxicity. Pretreatment with methanol extracts of propolis (10 μg/mL) for 1 h was set up in the subsequent experiments.

3.2. Effects of Propolis on the H_2O_2-Induced Oxidative Stress in SH-SY5Y Cells. Oxidative stress is an important inducer of neurotoxicity in AD patients [39]; following our previous experiments, we used two approaches to address the effects of propolis on H_2O_2-induced oxidative stress in SH-SY5Y cells: one approach was the use of a MitoSOX Red probe, as a marker for mitochondria-derived ROS generation [35], and the other was immunofluorescence imaging for a biomarker of oxidation-damaged DNA marker, 8-oxo-dG [40]. In comparison to the untreated cells, the expression of MitoSOX Red signals was significantly increased in SH-SY5Y cells after exposure to H_2O_2 for 1 h, suggesting that the mitochondria are the early origin of ROS generation during oxidative stress. Pretreatment with propolis significantly inhibited the H_2O_2-induced mitochondria-derived ROS generation in SH-SY5Y cells (Figure 2(a)), and the mean fluorescent intensity of MitoSOX Red oxidation was found to significantly increase in comparison to that in the cells that were not exposed to H_2O_2 (4.93 versus 1, $^{***}p < 0.001$, Figure 2(b)). Pretreatment with the propolis methanol extracts for 1 h significantly reduced the immunofluorescence intensity of MitoSOX Red oxidation in the H_2O_2-exposed SH-SY5Y cells (1.8 versus 4.93, $^{***}p < 0.001$, Figure 2(b)), thus confirming the antioxidant properties of propolis. Immunofluorescence imaging showed a significant inverse relationship between Hoechst and 8-oxo-dG after exposure of SH-SY5Y cells to H_2O_2 for 4 h (Figure 2(c)), and the mean fluorescent intensity of 8-oxo-dG was found to significantly increase in comparison to that in the cells that were not exposed to H_2O_2 (2.75 versus 1, $^{***}p < 0.001$, Figure 2(d)). It is noted that pretreatment with the propolis for 2 h significantly reduced the immunofluorescence intensity of 8-oxo-dG in the H_2O_2-exposed SH-SY5Y cells (1.36 versus 2.75, $^{***}p < 0.001$, Figure 2(d)). These observations demonstrate that propolis could attenuate oxidative stress in neuronal cells.

3.3. Effect of Propolis on the Fibrillar Aβ-Induced Impairment of BDNF-Induced Arc Expression in SH-SY5Y Cells. Soluble Aβ is known to interfere with synaptic efficacy, as cognitive decline precedes the formation of Aβ plaques, the hallmark of AD [41]. We therefore examined the effects of propolis on

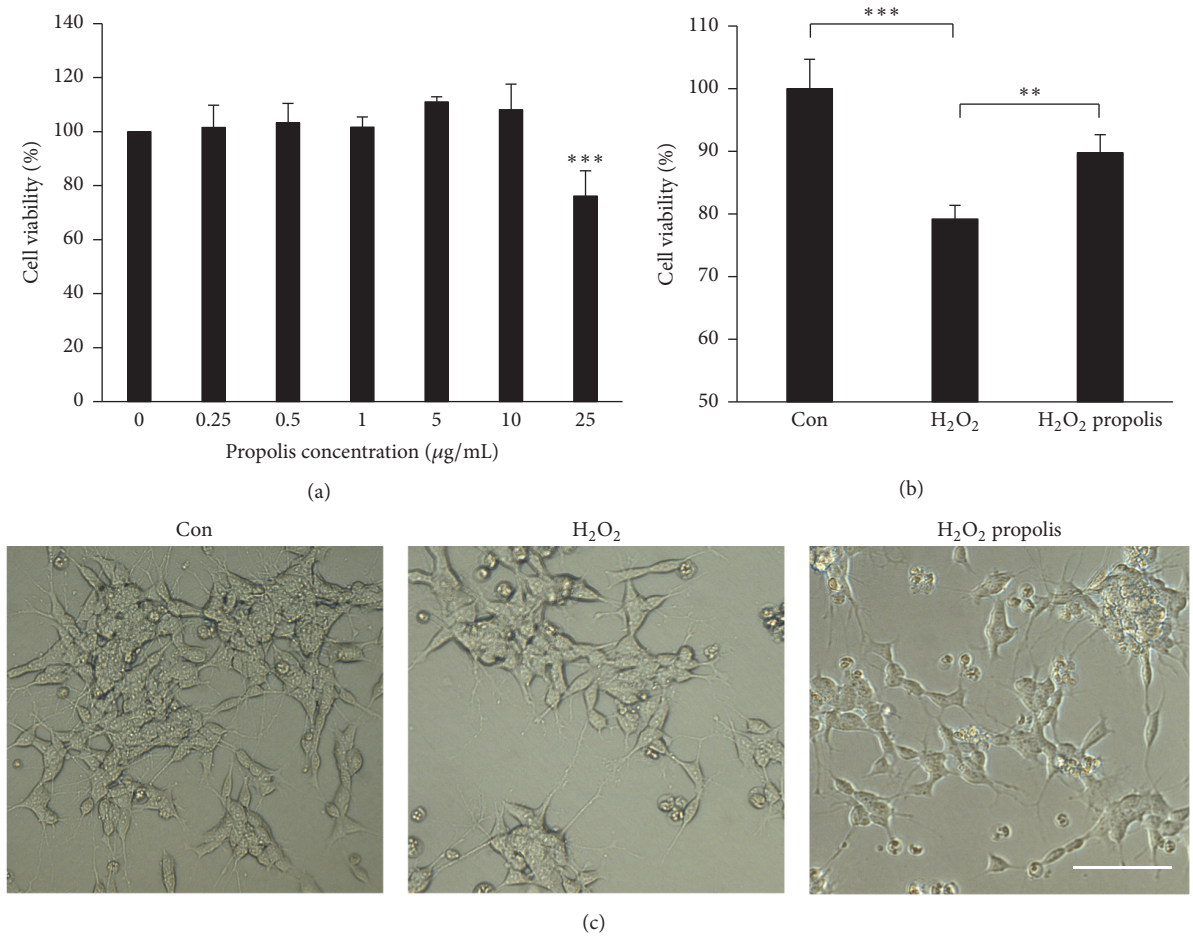

Figure 1: Effects of methanol extracts of propolis on the H_2O_2-induced toxicity in SH-SY5Y cells. (a) Cell viability in SH-SY5Y cells pretreatment with different concentrations of propolis for 48 hours. (b) The effect of pretreatment with methanol extracts of propolis for 2 h on H_2O_2 exposed SH-SY5Y cells. Each column and bar represent mean ± SEM ($n = 4$ each). An asterisk indicates a statistically significant difference from the indicated group value ($^{**}p < 0.01$, $^{***}p < 0.001$). (c) The morphological changes of SH-SY5Y cells with or without pretreatment with propolis (10 μg/mL) after expose to H_2O_2 for 24 h. Scale bar = 20 μm.

the impairment of BDNF-induced Arc expression by fAβ in SH-SY5Y cells, because Arc is the critical factor of synapse efficacy. The mean cell viability was not significantly changed after treatment with fAβ at final concentrations between 0.1 and 5 μM for 48 h (Figure 3(a)). However, significant cytotoxicity was observed in cultures treated with fAβ over 10 μM (80% of viable cells). Therefore, 5 μM was used as the nonlethal concentration for SH-SY5Y cells in subsequent experiments. Treatment with BDNF (10 ng/mL) for 120 min induced 3.5-fold Arc expression in SH-SY5Y cells; however, preexposure to fAβ (5 μM) for 6 h suppressed the BDNF-induced Arc expression (Figure 3(b)). Interestingly, this impairment was significantly reversed following treatment with propolis (10 μg/mL) for 120 min (Figure 3(b)). Furthermore, propolis treatment also prevented the fAβ-induced impairment of BDNF-induced Arc protein expression (Figures 3(c) and 3(d)). These data indicate that propolis reverses the fAβ-induced impairment of BDNF-induced Arc mRNA and protein expression.

3.4. Effect of Propolis on the IL-1β-Induced Impairment of BDNF-Induced Arc Expression in SH-SY5Y Cells. As a potent activator for exacerbating neuroinflammation, IL-1β has recently been found to suppress BDNF-dependent synaptic efficacy [26]. We therefore examined the effects of propolis on the IL-1β-induced impairment of BDNF-induced Arc expression in SH-SY5Y cells. We first examined the effects of IL-1β on the cell viability to determine the nonlethal concentration of IL-1β on SH-SY5Y cells. The mean cell viability was not significantly changed after treatment with IL-1β at final concentrations between 1 and 500 ng/mL for 48 h (Figure 4(a)). However, significant cytotoxicity was observed in cultures treated with IL-1β over 100 ng/mL (90% of viable cells). We therefore used 20 ng/mL as the nonlethal concentration for SH-SY5Y cells in subsequent experiments.

Treatment with BDNF (10 ng/mL) for 120 min induced 3.4-fold Arc expression in SH-SY5Y cells; however, preexposure to IL-1β (20 ng/mL) for 6 h significantly suppressed the BDNF-induced Arc expression (Figure 4(b)). Interestingly, this impairment was significantly reversed following treatment with propolis (10 mg/mL) for 120 min (Figure 4(b)). Furthermore, propolis treatment also prevented the IL-1β-induced impairment of BDNF-induced Arc protein

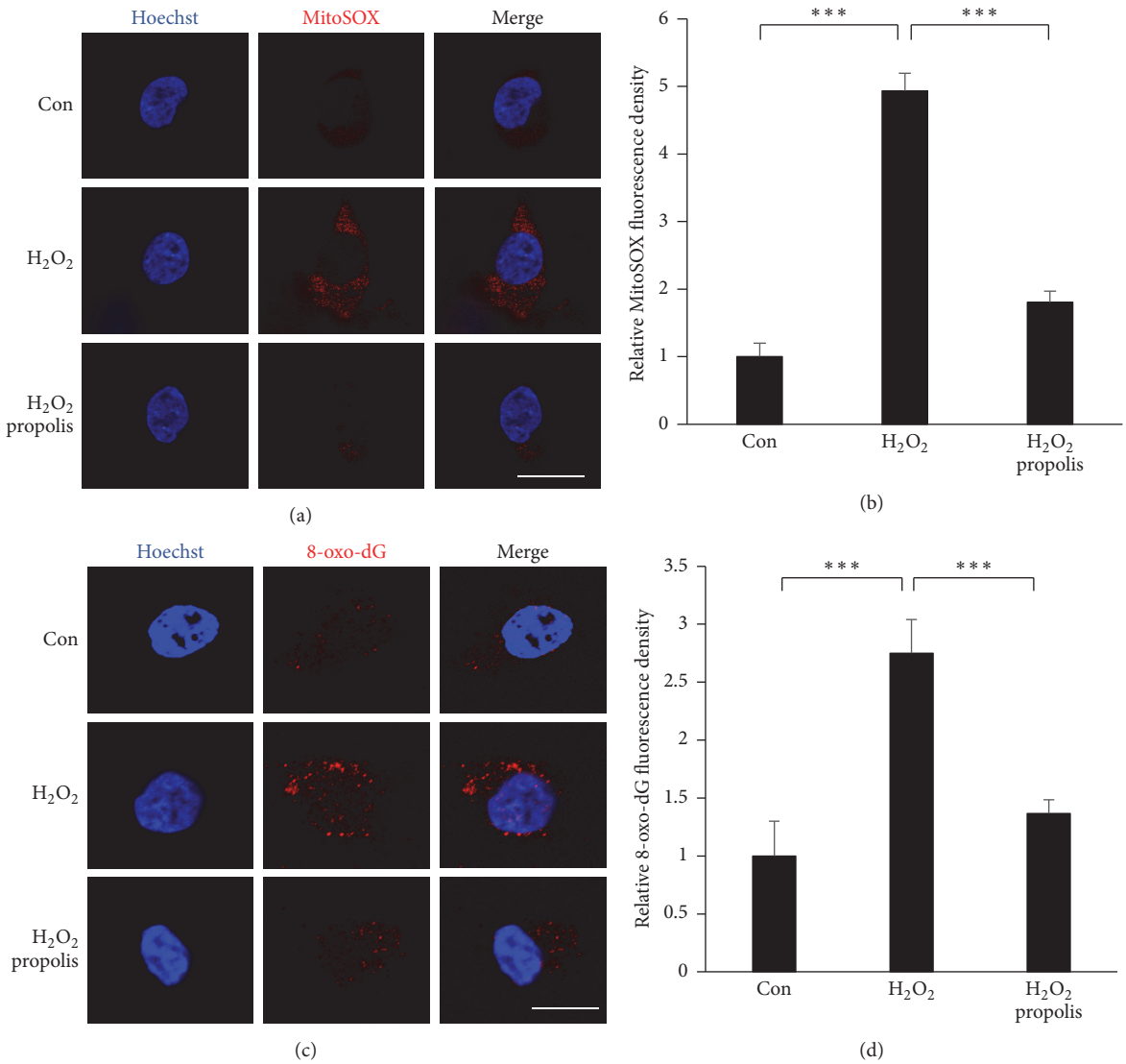

Figure 2: Effects of methanol extracts of propolis on the H_2O_2-induced oxidative stress in SH-SY5Y cells. (a) Fluorescent images of MitoSOX Red signals in SH-SY5Y cells exposed to H_2O_2 for 1 h with or without propolis (10 μg/mL) for 2 h. Scale bar = 15 μm. (b) The quantitative analyses of MitoSOX Red signal intensity in (a). (c) Immunofluorescent CLMS images of 8-oxo-dG (red) with Hoechst-stained nuclei (blue) in SH-SY5Y cells exposed to 100 μM of H_2O_2 for 4 h with or without pretreatment with propolis (10 μg/mL) for 2 h. Scale bar = 10 μm. (d) The quantitative analyses of 8-oxo-dG immunofluorescence signal intensity in (c). The results are expressed as the mean ± SEM (n = 4 each), and the asterisks indicate a statistically significant difference from the indicated group value ($^{***}p < 0.001$).

expression (Figures 4(c) and 4(d)). These data indicate that propolis reverses the IL-1β-induced impairment of BDNF-induced Arc mRNA and protein expression.

3.5. Effect of Propolis on BDNF Expression in SH-SY5Y Cells. We next examined the effects of propolis on BDNF expression in SH-SY5Y cells, because BDNF is the critical neurotrophic factor that is important for synapse efficacy. Pretreatment with propolis significantly increased the BDNF mRNA expression in SH-SY5Y cells from 60 min up to 240 min, even at a low concentration (1 μg/mL) (Figure 5(a)). Furthermore, treatment with propolis for 60 min increased the BDNF mRNA expression in SH-SY5Y cells in a dose-dependent manner (Figure 5(b)). The propolis-increased BNDF expression was completely abolished by preincubation with Wortmannin (200 nM, PI-3K inhibitor), but not by treatment with Y27632 (1 μM, ROCK inhibitor) or GFX (200 nM, PKC inhibitor) (Figure 5(c)). BDNF/TrkB signaling has been reported to play an important role in long term potential to further confirm the BDNF signaling to Arc, we use a Carbachol to stimulate SH-SY5Y, and the responses of Carbachol were examined in the presence of U0126 (ERK inhibitors, 10 μM) or ANA-12 (TrkB selective antagonist, 100 nM).

1 h of treatment with Carbachol can significantly increase the protein expression level of Arc. However, pretreatment with U0126 and ANA significantly reduced the Carbachol-increased Arc (Figures 5(d) and 5(e))). Therefore, the

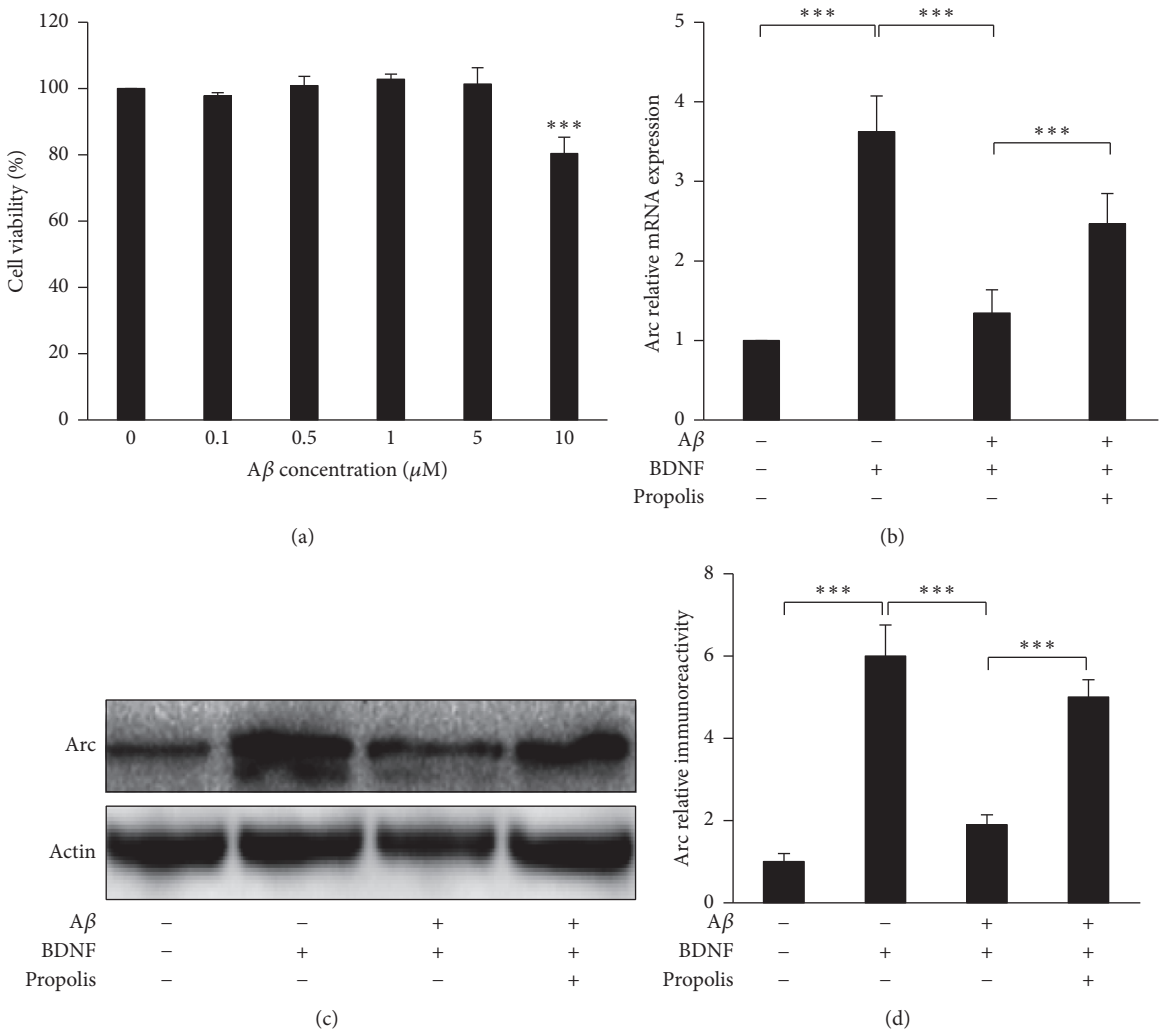

Figure 3: The effect of methanol extracts of propolis on the Aβ-induced impairment of BDNF-induced Arc expression in SH-SY5Y cells. (a) SH-SY5Y cells were treated with the indicated concentrations of propolis for 48 h. The cell viability of SH-SY5Y was then measured using a CCK-8 Assay Kit. (b) SH-SY5Y cells were treated with indicated concentrations of fAβ for 48 h. The cell viability of SH-SY5Y was then measured again using a CCK-8 Assay Kit. (c) SH-SY5Y cells were pretreated with fAβ (5 μM) for 6 h, followed by incubation with BDNF (10 ng/mL) for 2 h. Propolis was treated 2 h before BDNF application. (d) The expression of Arc after treatment with fAβ, propolis, or BDNF. (d) The quantitative analysis of Arc protein expression. The results are expressed as the mean ± SEM (n = 4 each), and the asterisks indicate a statistically significant difference from the indicated group value ($^{***}p < 0.001$).

BDNF/TrkB/ERK may regulate the Arc in SH-SY5Y. These observations demonstrate that propolis upregulates BNDF expression through PI-3K-dependent pathways.

3.6. Effect of Propolis on Arc Expression in SH-SY5Y Cells. We further examined the effects of propolis on Arc expression in SH-SY5Y cells. Arc expression is very low in untreated SH-SY5Y cells. Surprisingly, even low-level (0.5 μg/mL) pretreatment with propolis for 30 min significantly increased the Arc mRNA expression in SH-SY5Y cells in a dose-dependent manner. The safety dose of propolis (5 μg/mL and 10 μg/mL) induced Arc mRNA expression 13- and 16-fold found in untreated cultured cells (Figure 6(a)). We further investigated the time-dependent regulation of propolis (10 μg/mL) on Arc expression. Arc mRNA expression is significantly increased as early as 10 min after treatment, peaking at 30 min after, lasting for 60 min, and eventually recovering to the untreated level (the basal levels) at 120 min after propolis treatment (Figure 6(b)). Our data indicated that the Arc mRNA expression-inducing effects of 10 μg/mL propolis treatment peaked at 30 min; therefore, this concentration and time point were chosen for subsequent experiments.

We finally analyzed the signaling pathways of the effects of propolis on Arc induction. Pretreatment with Wortmannin (200 nM) completely abolished the propolis-induced Arc expression in SH-SY5Y cells; however, Y27632 (1 μM) or GFX (200 nM) did not reduce the expression (Figure 6(c)). Furthermore, propolis (10 μg/mL) for 60 min significantly increased the Arc protein expression, and the propolis-increased Arc protein expression was significantly reduced

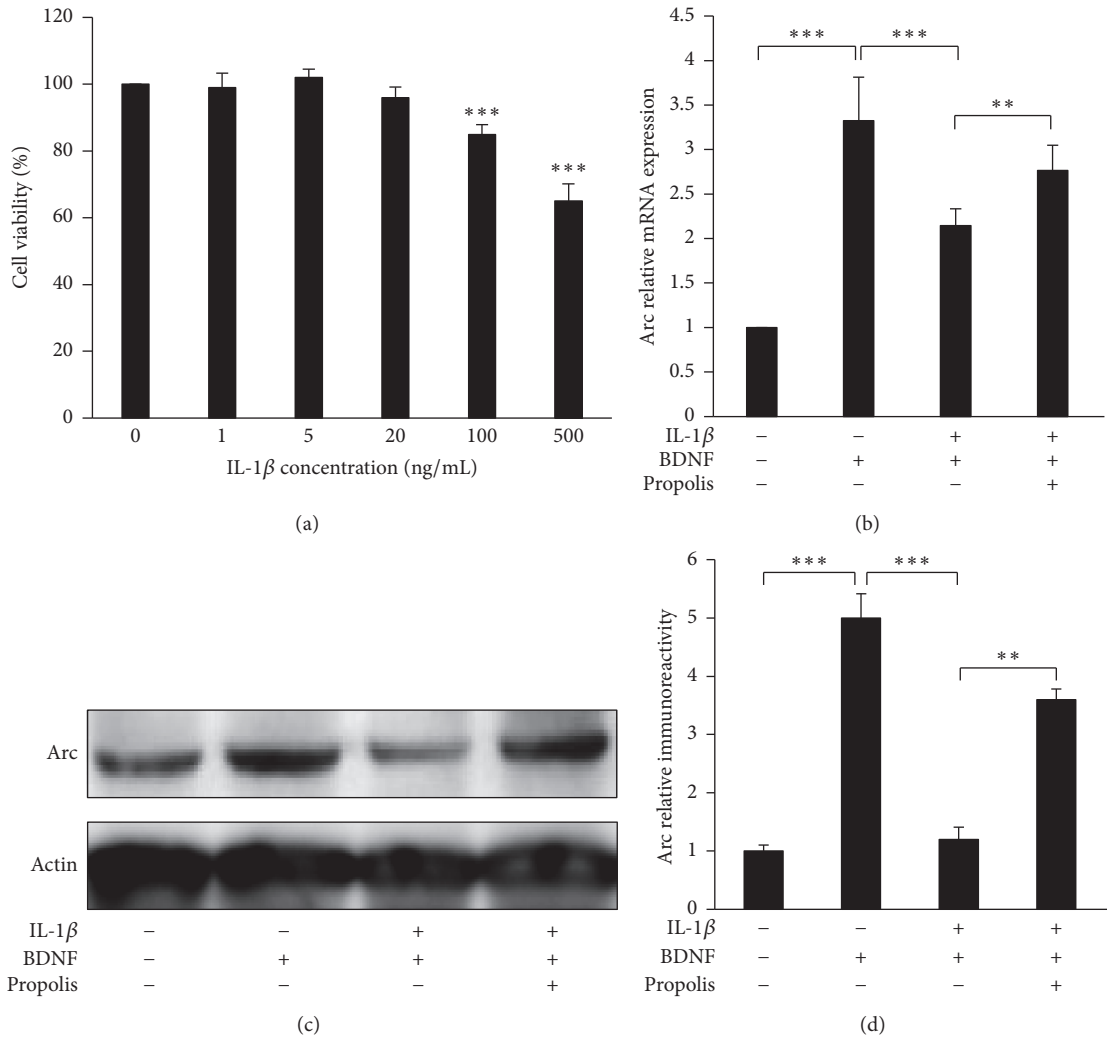

Figure 4: The effect of methanol extracts of propolis on the IL-1β-induced impairment of BDNF-induced Arc expression in SH-SY5Y cells. (a) SH-SY5Y cells were treated with the indicated concentrations of IL-1β for 48 h. The cell viability of SH-SY5Y was then measured using a CCK-8 Assay Kit. (b) SH-SY5Y cells were pretreated with IL-1β (20 ng/mL) for 6 h followed by incubation with BDNF (10 ng/mL) for 2 h. Propolis was treated 2 h before BDNF application. (c) The expression of Arc after treatment with IL-1β, propolis, or BDNF. (d) The quantitative analysis of Arc protein expression. The results are expressed as the mean ± SEM ($n = 4$ each), and the asterisks indicate a statistically significant difference from the indicated group value ($^{**}p < 0.01$, $^{***}p < 0.001$).

by Wortmannin, but not by Y27632 or GFX (Figures 6(c) and 6(d)). These data indicate that propolis acts through PI-3K-dependent pathways for enhancing Arc transcription and protein production.

Artepillin C, a polyphenol with a molecular weight of 300.4 extracted from Brazilian green propolis, has been reported to have the effect on neurite outgrowth of PC12 cells and the signaling pathways involved [33]. Therefore, we also analyzed the effects of Artepillin C on the expression level of Arc in SH-SY5Y cells. We found that Artepillin C significantly increased the protein expression of Arc in SH-SY5Y cells; however, the Artepillin-increased Arc was significantly reduced by pretreatment with Wortmannin. The response of Artepillin C in SH-SY5Y cells was consistent with treatment with propolis. Therefore, Artepillin C may be one of the functional components of propolis that acts through PI-3K-dependent pathways for enhancing Arc transcription and protein production.

4. Discussion

The major findings of the present study are that Brazilian green propolis decreases the oxidative stress but increases the neurodegenerative dysregulated factors of synapse efficacy in human neuronal SH-SY5Y cells (summarized in Figure 7). To our knowledge, this is the first report to explore the directly neuroprotective effects of propolis on neurodegenerative damage.

Oxidative stress is a major harmful component to induce neurodegenerative damage in AD [3, 4], because oxidative stress with overproduction of ROS causes damage to the cellular components, including DNA, resulting in subsequent

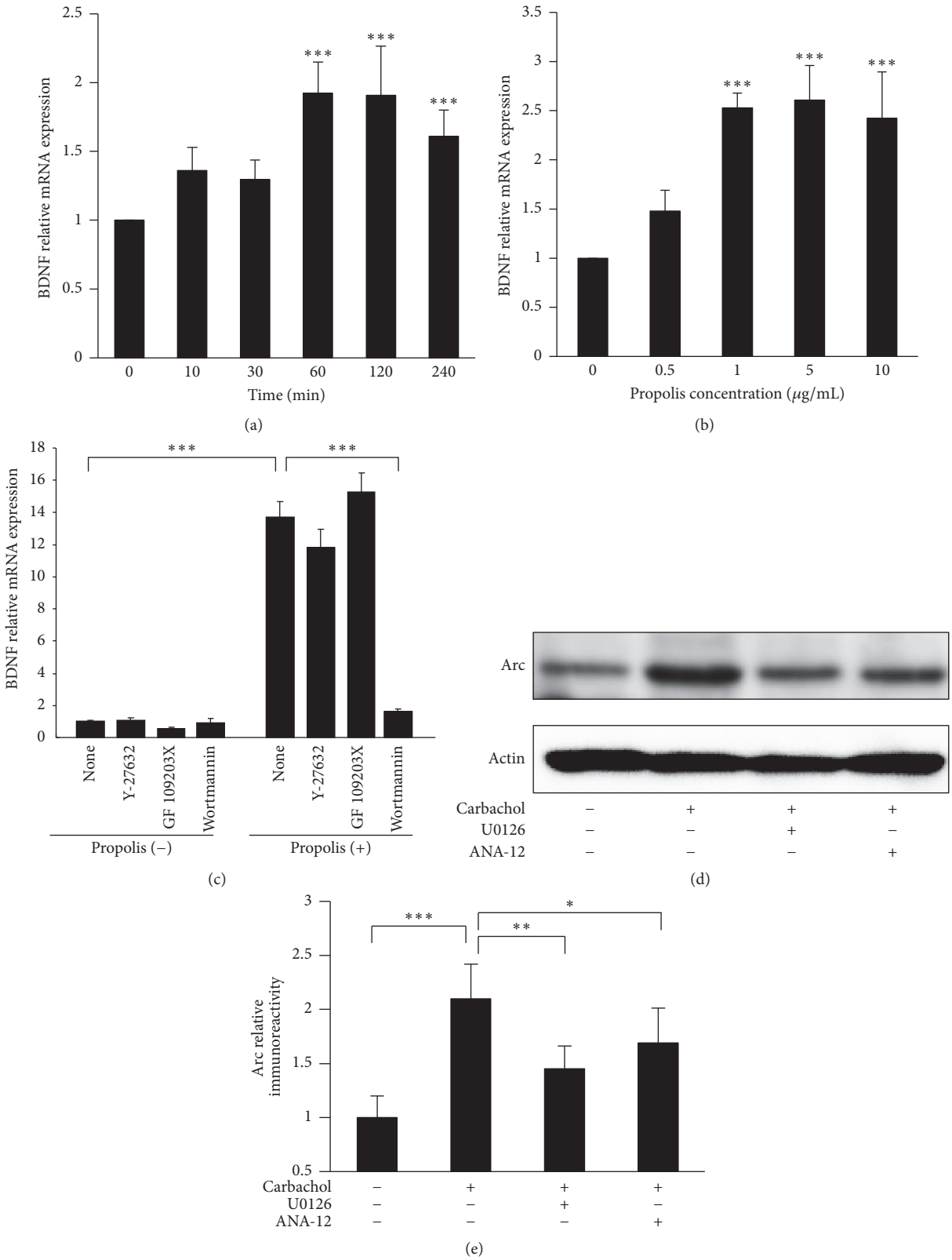

Figure 5: The effect of methanol extracts of propolis on BDNF expression in SH-SY5Y cells. (a) Time-dependent effects of 10 μg/mL propolis on BDNF mRNA expression in SH-SY5Y cells. (b) Dose-dependent effects of propolis on Arc mRNA expression in SH-SY5Y cells at 60 min after incubation. (c) Effects of specific inhibitors on BDNF mRNA expression. GF109203X hydrochloride (protein kinase C inhibitor, 200 μM), Y-27632 dihydrochloride (Rho-associated protein kinase inhibitor, 1 μM), and Wortmannin (phosphoinositide 3-kinase inhibitor, 200 nM) were pretreated for 1 h, followed by incubation with 10 μg/mL propolis for 30 min. (d) The expression of Arc after treatment with Carbachol (1 mM) and pretreatment with U0126 (ERK inhibitors, 10 μM) or ANA-12 (TrkB selective antagonist, 100 nM). (e) The quantitative analysis of Arc protein expression shown in (d). The results are expressed as the mean ± SEM (n = 4 each), and the asterisks indicate a statistically significant difference from the indicated value ($^{*}p < 0.01$, $^{**}p < 0.005$, and $^{***}p < 0.001$).

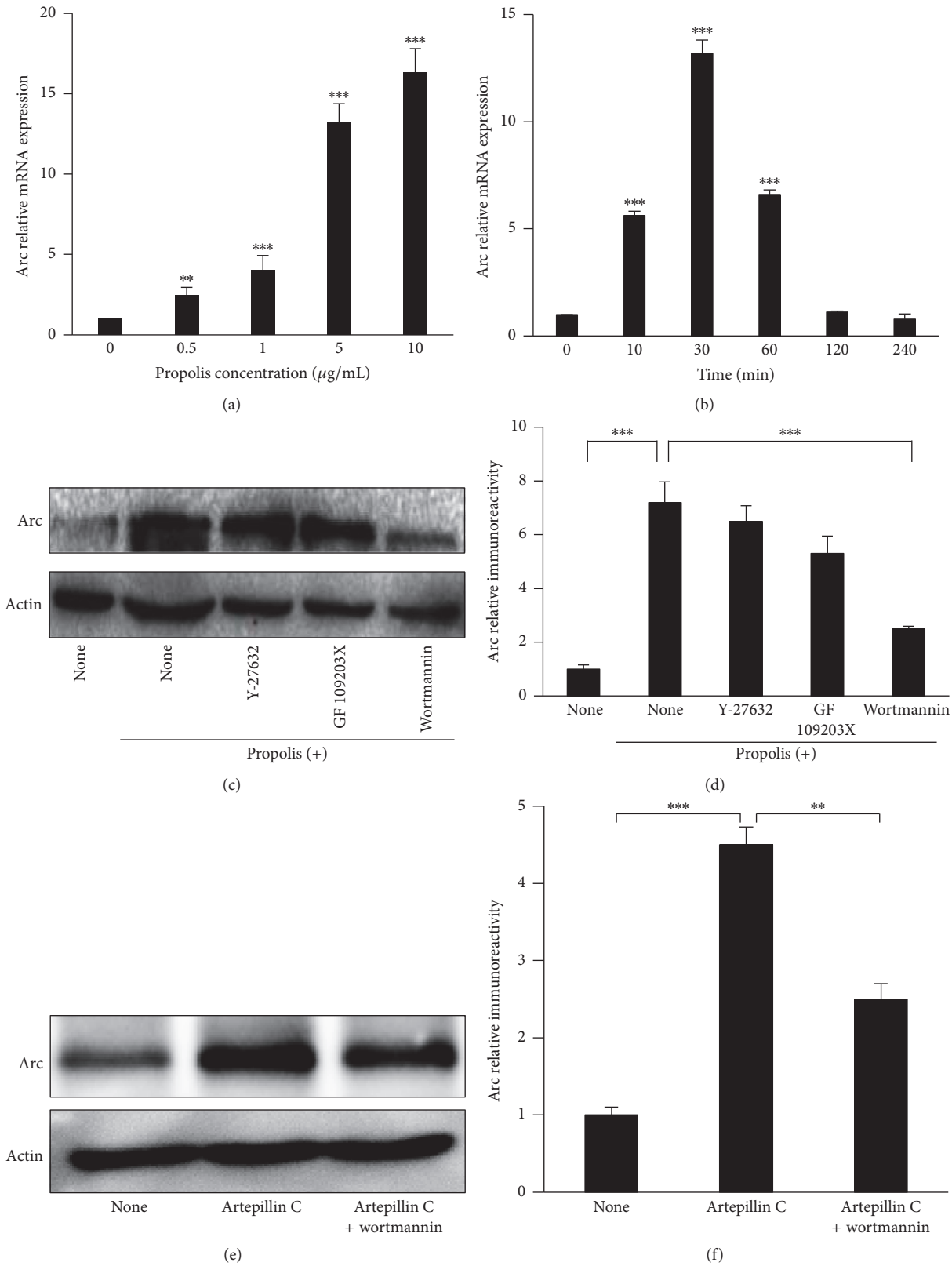

Figure 6: Effect of methanol extracts of propolis on Arc expression in SH-SY5Y cells. (a) Dose-dependent effects of propolis on Arc mRNA expression in SH-SY5Y cells at 30 min after incubation. (b) Time-dependent effects of 10 μg/mL propolis on Arc mRNA expression in SH-SY5Y cells. (c) Effects of specific inhibitors on Arc protein expression. GF109203X hydrochloride (protein kinase C inhibitor, 200 μM), Y-27632 dihydrochloride (Rho-associated protein kinase inhibitor, 1 μM), and Wortmannin (phosphoinositide 3-kinase inhibitor, 200 nM) were pretreated for 1 h, followed by incubation with 10 μg/mL propolis for 30 min. (d) The quantitative analyses of Arc protein expression. (e) The expression of Arc after treatment with Artepillin C (20 μM) and pretreatment with Wortmannin. (f) The quantitative analysis of Arc protein expression shown in (e). The results are expressed as the mean ± SEM ($n = 4$ each), and the asterisks indicate a statistically significant difference from the control value or propolis-treated group ($^{**}p < 0.005$, $^{***}p < 0.001$).

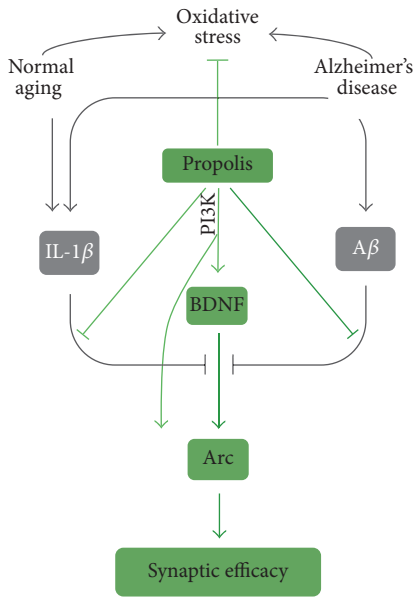

Figure 7: A schematic representation of the effects and the principle molecular mechanisms of Brazilian green propolis on neurodegenerative damage-induced oxidative stress and downregulation of synaptic efficacy.

cell death. Antioxidant therapy therefore is considered as an approach in prevention and clinical management of AD [6]. We used an in vitro model of human neuronal SH-SY5Y cells to investigate the direct effect of Brazilian green propolis (propolis) and its signaling transductions on Arc expression to avoid other effectors. Pretreatment with propolis could protect the H_2O_2-induced cell death, which agreed with those of the previous reports showing baccharin, p-Coumaric acid, and Artepillin C; the active components of propolis are effective in reducing neurotoxicity [29–32]. We found that propolis significantly inhibited the H_2O_2-induced mitochondria ROS generation as well as nuclear DNA damage in SH-SY5Y cells (Figures 1 and 2). Thus we provide the first evidence that propolis attenuates the oxidative stress-induced DNA damage directly in neuronal cells.

The dysfunction of synaptic efficacy is an early neurodegenerative damage in AD [1, 21], because the impairment of synaptic activation precedes substantial Aβ accumulation and neuron loss in the brain [10, 41, 42]. BDNF-induced Arc expression is widely used as a marker of defective synaptic efficacy [21, 26, 43]. In the present study, we found that preexposure to fAβ (a neurodegenerative hallmark) at a nonlethal concentration (5 μM) impaired BDNF-induced Arc expression (Figure 3(b)); however, fAβ at a nonlethal concentration did not affect Arc expression in SH-SY5Y cells (data not shown). These data were consistent with the findings of previous studies showing that BDNF-induced Arc expression was inhibited by pretreatment with oligomeric Aβ or fAβ in cultured cortical neurons [21, 26, 44]. Surprisingly, treatment with (10 μg/mL) for 120 min significantly reversed the fAβ-induced impairment of BDNF-induced Arc expression in SH-SY5Y cells. Since a decline in Arc expression is correlated with cognitive impairment in AD [45, 46], BDNF-induced Arc expression fundamentally regulates the synaptic efficacy [17, 18]. These observations indicate that propolis can prevent the fAβ-induced dysfunction of synaptic efficacy in neuronal cells.

Increasing evidence indicates that microglia-related neuroinflammation contributes to the decline in cognitive function during aging as well as in AD [47, 48]. As a potent activator for exacerbating neuroinflammation, IL-1β has been reported to suppress BDNF-dependent synaptic efficacy, resulting in cognitive impairment [26, 49]. Furthermore, the overexpression of IL-1β in a transgenic mouse model resulted in increased microglia activation with a significant reduction in behaviorally induced Arc levels and impaired contextual and spatial memory [50]. In the present study, we demonstrated that nonlethal concentrations of IL-1β (20 ng/mL) impaired BDNF-induced Arc expression in SH-SY5Y cells (Figures 4(b)–4(d)), a finding that agreed with those of a previous report showing that IL-1β suppressed the BDNF-induced Arc expression in cultured brain slices [26]. Importantly, treatment with propolis significantly reversed the IL-1β-induced impairment of BDNF-induced Arc expression. These observations indicate that propolis can reverse the neuroinflammation-induced dysfunction of synaptic efficacy in neuronal cells.

BDNF, as a neurotropic factor, fundamentally controls the synaptic efficacy [17, 51] and closely correlates with cognitive functions [14, 19]. Indeed, BDNF levels are reduced even in the preclinical stages of AD [52]. In the present study, treatment with propolis significantly increased the BDNF expression in SH-SY5Y cells in dose- and time-dependent manners. Furthermore, the propolis-upregulated BNDF expression was completely abolished by preincubation with PI3K inhibitor, indicating that the effects of propolis on BDNF expression are mediated by the PI3K signaling pathway.

Arc expression was low in the untreated SH-SY5Y cells, which agreed with the findings of other reports using cultured primary cortical neurons [53, 54]. Exogenous treatment with propolis induced Arc expression in SH-SY5Y cells in both dose- and time-dependent manners. Of note, exposure to propolis induced rapid, robust Arc mRNA expression, as quickly as 10 min after exposure and peaking at 30 min, which paralleled the Arc expression peaks around 30 min after behavior induction [55]. Therefore, Arc may serve as an early beginning effector molecule for propolis-induced neuronal activity.

The PI3K signaling pathway plays a pivotal role in synaptic efficacy and memory consolidation [56–58]. The observations, of which the effects of propolis on Arc expression are mediated by the PI-3K signaling pathway, thus provide the underlying mechanism of propolis in directly regulating the molecule for synaptic efficacy in human neuronal cells. Artepillin C, a major component of Brazilian green propolis, has also been found to act as a neurotrophic-like factor for promoting NGF-induced neurite outgrowth [33]. Our ongoing human research at high altitude shows that elderly individuals take propolis score significantly higher on cognitive tests than nontreated individuals (Wu & Zhu et al.,

unpublished data). The neuroprotective effects of Brazilian green propolis on neurodegenerative damage might provide a valuable therapeutic strategy for prevention of cognitive impairment in AD as well as aging.

5. Conclusion

Brazilian green propolis could reduce oxidative stress and prevent the neurodegenerative damaged synapse efficacy (schematic represented in Figure 7). Therefore, we provide the principle molecular mechanisms of the benefits of propolis as the therapeutic agent for maintenance cognitive function of brain.

Competing Interests

The authors declare no conflict of interests in association with this study.

Authors' Contributions

Junjun Ni and Zhou Wu contributed equally to this work.

Acknowledgments

This work was supported by a Yamada Research Grant to Zhou Wu (N0.0183).

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 3279061, 15 pages
https://doi.org/10.1155/2017/3279061

Review Article

Protective Effect of Antioxidants on Neuronal Dysfunction and Plasticity in Huntington's Disease

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Received 29 July 2016; Revised 9 November 2016; Accepted 5 December 2016; Published 12 January 2017

Academic Editor: Vladimir Jakovljevic

Huntington's disease (HD) is characterised by movement disorders, cognitive impairments, and psychiatric problems. The abnormal generation of reactive oxygen species and the resulting oxidative stress-induced mitochondrial damage in neurons upon CAG mutations in the *HTT* gene have been hypothesized as the contributing factors of neurodegeneration in HD. The potential use of antioxidants against free radical toxicity has been an emerging field in the management of ageing and many neurodegenerative disorders. Neural stem cells derived adult neurogenesis represents the regenerative capacity of the adult brain. The process of adult neurogenesis has been implicated in the cognitive functions of the brain and is highly modulated positively by different factors including antioxidants. The supportive role of antioxidants to reduce the severity of HD via promoting the functional neurogenesis and neuroprotection in the pathological adult brain has great promise. This review comprehends the recent studies describing the therapeutic roles of antioxidants in HD and other neurologic disorders and highlights the scope of using antioxidants to promote adult neurogenesis in HD. It also advocates a new line of research to delineate the mechanisms by which antioxidants promote adult neurogenesis in HD.

1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative syndrome associated with abnormal CAG expansions in the Huntington *(HTT)* gene [1–3]. The mutant *HTT* contains polymorphic CAG repeats in excess of 39 in exon 1 of the gene present in the short arm of the chromosome 4 [3–5]. The CAG mutations ultimately result in the abnormal expansion of polyglutamine (polyQ) tracts in the HTT protein, which leads to misfolding and loss of protein function [6, 7]. The polyQ expansion has been identified to be the primary inducer of degeneration of medium spiny neurons (MSNs) in the striatum and leads to neurodegeneration to other regions of brain, including

the cortex, hippocampus, hypothalamus, and brain stem in a progressive manner [7–9]. The epidemiology of HD suggests that the disease occurs worldwide, but its prevalence varies depending upon genetic diversity and geographical regions [9, 10]. The rate of incidence of HD is considerably higher in the Caucasian population than the Asian population. While an estimate shows the prevalence and increasing trend of HD in Western Europe, Australia, North America, and the United Kingdom, India represents a large number of total HD cases in Asia [11, 12]. Single nucleotide polymorphism (SNP) at the *HTT* locus in association with the genetic diversity, lifestyle, food, and environmental factors is presumed to be the reasons for the variations in the frequency of HD among the human population [13]. HD has been characterised by choreiform movements, dystonia, cognitive deficits, and psychiatric problems [14]. These symptoms have been accompanied by neurodegeneration along with an abnormal level of neurotransmitters, microglial activation, reactive astrogliosis, and impaired neurogenesis [15]. Recently, HD patients have also been characterised with different types of behavioural, motor, and aggressive symptoms [16, 17].

All the abovementioned problems aggravate the development of HD and contribute to gradual deterioration of the physical abilities and mental processes. Importantly, people with HD have problems in taking care of their daily routine, such as food consumption, due to difficulty in swallowing (dysphagia), which may occur during the later stages of the disease. Further, abnormalities in energy metabolism caused by mitochondrial dysfunctions add to severity of the disease. The loss of muscle function in the mandibular regions, pharynx, and oesophagus could lead to disorders like bruxism (grinding the teeth), failure to intake of food and choking, which could ultimately lead to death [16, 18]. Currently, there are no available treatments that can delay the onset or arrest the progression of the disease, while the focus of medical care is limited to merely managing the neurological symptoms of HD. This is mainly due to lack of knowledge about the underlying biology of the disease.

Of the few therapeutic options available for the treatment of HD, tetrabenazine has been an approved drug by the Food and Drug Administration (FDA) for minimising the clinical symptoms of involuntary movements [19–23]. Other treatment strategies for HD include administration of antipsychotic and antidepressant drugs [21, 22]. Antipsychotic drugs like haloperidol [23], fluphenazine [24], clonazepam [25], amantadine [26], and levetiracetam [27] might help in controlling panic attacks, aggression, and choreiform movements, while antidepressants like fluoxetine [28], sertraline [29, 30], nortriptyline [31], and lithium [32] are used to stabilize depression, anxiety associated mood swings, and negative thoughts. In addition, riluzole is frequently used as a neuroprotective drug to control glutamatergic neurotransmission in HD [33, 34]. Deep brain stimulation (DBS) has been proposed as a technique to manage chorea and other motor symptoms like dystonia and cognitive deficits in HD [35]. Alternatively, using recombinant antibody fragments to neutralise the HTT aggregates [36] and stem cell transplantation [37] have also been tried with limited success. In addition, adapting to the aforementioned treatment strategies warrants a very careful approach and highly vigilant individuals to carry out the procedures, as most of these strategies and routes of administration have side effects including nausea, fatigue, abnormal neuroexcitability, and tissue disruption [38] that could exacerbate the severity of HD symptoms [39]. Thus, developing novel and noninvasive therapeutic strategies that are efficient but have no or minimal side effects are important for the successful treatment of HD and will have a promising therapeutic appeal. Meanwhile, the benefits of physical activities, an enriched environment with the aid of dietary supplements, and palliative care therapies have been considered in conjunction with the drug treatment as noninvasive and relatively affordable management strategies for HD.

2. Detrimental Roles of Oxidative Stress in Huntington's Disease

There have been many hypotheses proposed for the manifestation of neurodegeneration associated clinical symptoms in HD. Among them, polyQ expansion associated oxidative stress that leads to caspase mediated neuronal cell death is considered as a potential cause of neuropathological changes in HD [9]. Free radicals are highly reactive molecules that feature unpaired electrons on their valence orbital with the ability to render various molecular and cellular vulnerabilities due to their unstable reactive nature [40, 41]. The most common biologically relevant free radicals are superoxide (O^-), hydroxyl (OH^-), and nitric monoxide (NO) species and are referred to as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [41, 42]. Free radicals are provoked in cells by enzymatic and nonenzymatic mechanisms through abnormal metabolic, genetic, and cell cycle events that occur as a consequence of electromagnetic radiation, ageing, infections, immunological alteration, intoxication, abnormal diet, malnutrition, and deficiency in vitamins and trace elements [43]. Besides, defects or mutations in the free radicals scavenging metabolic enzymes such as glutathione peroxidases, nitric oxide synthase, peroxiredoxins, and superoxide dismutases are responsible for the accumulation of free radicals [44, 45]. The expressions of these detoxifying enzymes are controlled by the Nrf2-ARE complex pathway [46]. In general, highly regulated free radicals generated in the body can be of potential use in development and maintenance of the tissues associated with improvement of the longevity of organisms. As per the free radical theory of ageing, the abnormally fabricated free radicals are harmful to the normal structure and functioning of cells and tissues [46–48]. In order to combat the negative impact of free radicals, cells deploy defense mechanisms such as free radical scavenging activity by antioxidants, as a normal physiological process [49]. The imbalance between the production of free radicals and the ability of cells to counteract or detoxify free radicals can eventually lead to DNA oxidation, protein nitration, and lipid peroxidation, culminating in cellular oxidative stress [50, 51]. Prolonged oxidative stress and failure in defense mechanisms could ultimately result in ageing related chronic diseases such as atherosclerosis [52], cancer [53],

diabetes [54], rheumatoid arthritis [55], ischemic stroke [56], cardiovascular diseases [57], chronic inflammation [58], and neurodegenerative diseases including Alzheimer's disease (AD) [59] and Parkinson's disease (PD) [60], in addition to HD [61, 62].

The brain is highly susceptible to free radical mediated oxidative damage, which is largely due to its high metabolic rate and oxygen and energy consumption under the protective isolation by the blood brain barrier from the circulation [62]. ROS target neuronal cells by promoting formation of DNA-protein cross-linked harmful adducts through oxidation of both the backbone and the side chain of the protein and DNA molecules [62, 63]. Various indices of free radical mediated damage have been identified as aetiologies of several neurodegenerative conditions including HD [61]. While a reciprocal relationship exists between the length of CAG repeats and the phenotype severity of HD, recent data gathered from the experiments on the HD specific embryonic stem cells (ESC) and induced pluripotent stem cell (iPSC) models indicate the deleterious effects of oxidative damage on the expansion of CAG triplets in the *HTT* gene [64–68]. The polyQ mechanisms that account for the selective neuronal loss in the brain affected by HD are multifaceted in nature [67]. Biomarkers for oxidative damage like heme oxygenase, 3-nitrotyrosine, and malondialdehyde (MDA) are found to be elevated in the striatum, cortex, and serum of human HD subjects [69–71]. The number of polyQ repeats in the HTT protein is found to be responsible for oxidative damage to the cell membrane, DNA, and enzymes responsible for the ATP production of mitochondria in HD [70, 71]. Besides, Lim et al. reported the disruption of mitochondrial Ca^{2+} homeostasis by free radicals in the striatal neurons of postmortem human HD brains [72]. Thus, free radical induced mitochondrial damage, followed by decreased ATP production, provides a strong mechanism for provoking the apoptotic pathways in HD brains [73]. The interactions between free radical induced oxidative stress, defects in mitochondrial energy metabolism, and excitotoxicity have widely been implicated in the neuropathogenicity of HD. Evidence of the role of oxidative stress in priming the pathogenesis of HD has been identified by higher plasma levels of lipid peroxidation in presymptomatic HD patients [74–76]. In addition, the mitochondrial permeability transition pore (mPTP), a nonspecific channel, is highly susceptible to the fluctuation of calcium homeostasis and oxidative stress, which in turn are considered to be major contributors to mitochondrial dysfunction in HD [77]. Store-operated calcium entry (SOCE), a process caused by lower of Ca^{2+} from the endoplasmic reticulum (ER), induces the influx of Ca^{2} from the extracellular space. The activity of SOC channels in medium spiny neurons (MSNs) was found to be high in transgenic YAC128 mice model of HD [78]. Recently, the PPAR-γ pathway has been linked to the induction of superoxide/ROS in HD [79]. This suggests that the oxidative damage induced molecular and cellular changes in the circulation appear to be the initiator of the early pathogenic events in HD. Taken together, oxidative stress plays a crucial role in the neuropathology of HD and is a potential target for the development of novel therapeutic interventions for the neuroprotective management of HD.

3. Neuroprotective Roles of Antioxidants against Oxidative Stress-Induced Complications in Neurodegenerative Disorders

Free radicals when overproduced need to be biologically scavenged or quenched by converting them into metabolically nondestructive cellular molecules. This protective mechanism called the antioxidant defense system prevents free radical mediated damage of cells, which lead to various diseases and ageing [49, 80–82]. When endogenous antioxidant defenses are inadequate to scavenge the free radicals completely, diet or drug-derived antioxidants may be particularly important in protecting against a number of human diseases [76]. Antioxidant defense mechanisms involve both enzymatic (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) and nonenzymatic (vitamins A, C, and E, glutathione) strategies. Other antioxidants include albumin, bilirubin, ferritin, ceruloplasmin, melatonin, uric acid, lipoic acid, mixed carotenoids, coenzyme Q10, bioflavonoids, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B1, B2, B6, and B12) [80, 81]. Specific quenching of the free radicals and chelating redox metals by antioxidants could possibly influence the gene expression profile of the tissue. The toxic metal induced lipid peroxidation and DNA fragmentation can be controlled by metal-binding proteins like ferritin, transferrin, ceruloplasmin, and others such as metallothionein [49]. Glutathione S transferases are one of the many enzymatic entities in cells and body fluids that reduce the level of ROS [82]. Protective effects of exogenously administered antioxidants have been extensively studied using experimental animal models and cell lines and have provided a strong insight into the relationship between free radicals and associated disease complications [83, 84]. The study carried out by Chanvitayapongs et al. demonstrated the antioxidant property of resveratrol, which, when combined with vitamin C and/or E, has a greater protective effect by reducing cell death in neurodegenerative diseases including AD [85]. The amyloidal beta (Aβ) induced neurotoxicity and the underlying molecular pathological mechanisms in AD are found to be inhibited by natural antioxidants such as Ginkgo biloba, flavonoids, soybean isoflavones, theanine, and nicotine in cellular models as well as transgenic animal models of AD [86]. Long-term dietary supplementation of pomegranates, figs, and dates reduced the inflammatory cytokines during ageing in APPsw/Tg2576 transgenic mouse model of AD [87]. Essa et al. identified that a diet rich in walnut helps to reduce the risk of developing PD and delay its onset due to the cumulative antioxidant and mitochondrial protective effects exerted by walnut constituents [88]. The pomegranate oil on 3-nitro propionic acid (3-NP) induced cytotoxicity in rat pheochromocytoma (PC-12) neuronal cells enhanced the levels of enzymatic and nonenzymatic antioxidants by neutralising

ROS or enhancing the expression of the antioxidant genes [89]. Rezai-Zadeh et al. demonstrated that epigallocatechin-3-gallate (EGCG), the main polyphenolic constituent of green tea through its beta-secretase activity, reduced Aβ aggregation in neuron-like cells (N2a), transfected with the human "Swedish" mutant amyloid precursor protein (APP) and in primary neurons derived from Swedish mutant APP-overexpressing Tg APP^{SW} transgenic mice model of AD [90]. L-Dihydroxyphenylalanine (L-dopa) used in the treatment of PD produced free radicals during its normal metabolism and this side effect was shown to be reduced by antioxidants in order to improve the efficacy of L-dopa therapy [91]. A series of orally bioavailable antioxidants including MitoQ, MitoVitE, and Mito TEMPOL are known to bypass the biological membranes, accumulate within mitochondria, and effectively protect against mitochondrial oxidative damage and are useful in treating neurodegenerative disease like PD [92].

In relation to the effects of antioxidants on HD pathology, a number of biomolecules have been tested and characterised using preclinical animal models of HD and cell lines expressing different length of CAG repeats. Among them, transgenic models of HD, R6-lines (R6/1 and R6/2) [93, 94], knock-in YAC128 mouse model [95], and rats or mouse injected with acute and toxic quinolinic acid [96] and 3-NP [97] have extensively been validated. In various paradigms, R6/2 mice that are supplemented with creatine, vitamin C, coenzyme Q, tauroursodeoxycholic acid (TUDCA), docosahexenoic acid (DHA), and eicosapentenoic acid showed increased life span and motor performance in association with either reduced free radicals or reduced polyQ aggregates in the brain [98, 99]. Chronic administration of JM6, an inhibitor of kynurenine-3-monooxygenase extended the life span, prevented synaptic loss, and decreased microglial activation in the R6/2 transgenic mouse and drosophila models of HD [100, 101]. A recent study indicated the significance of anthocyanin-treatment on CAG repeat instability in R6/1 transgenic mouse model of HD [102]. Treatment of experimental rats with 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) or with N-acetylcysteine (NAC) protects against oxidative damage induced by 3-NP and therefore acts against HD [103]. Besides, fumaric acid ester, dimethylfumarate, has been shown to provide neuroprotection and to suppress the dyskinetic movements through the activation of Nrf2 pathway in knock-in YAC-128 and transgenic R6/2 models of HD [104]. Van Raamsdonk et al. demonstrated the attenuation of striatal neuroprotection by antioxidant effects of cystamine YAC-128 model [105]. Further, treatment of melatonin significantly ameliorated the increased lipid peroxidation within the striatum of brain in the 3-NP model of HD [106]. In the same line of evidence, treatment of curcumin and carvedilol in 3-NP injected rats reduced the severity of motor and cognitive impairments [107, 108]. The treatment of resveratrol, naringin, sertraline, protopanaxatriol, embelin, puerarin, and olive oil was known to protect the experimental animal models against 3-NP induced oxidative stress and neurotoxicity [109]. Neurodegeneration in the striatum was prevented by TUDCA, a hydrophilic bile acid with antioxidant properties, which ameliorated the locomotor and cognitive deficits in a 3-NP injected rat model of HD [110]. Lycopene, a carotenoid pigment and phytochemical naturally found in fruits and vegetables, has the ability to reduce oxidative stress markers and improved behaviour in a 3-NP model of HD [111]. Low levels of cystathionine-c-lyase, required for production of cysteine, have been reported in HD pathology, which is mainly responsible for glutamate excitotoxicity. N-Acetylcysteine (NAC), an antioxidant supplement rich in cysteine, normalised the glutamate level, mitochondrial dysfunction, and oxidative stress when administered to R6/1 model [112].

The ameliorative effects of s-allylcysteine, copper, curcumin, safranal, ksheerabala, quercetin, and tert-butylhydroquinone against neurotoxicity have been described in quinolinic acid (QA) induced rat model of HD [113]. Antioxidant selenium, an essential element required by glutathione peroxidase, has been reported to reduce the lipid peroxidation within the striatum of QA rat model in a dose dependent manner [114]. Metal-containing catalytic antioxidant metalloporphyrins have emerged as a novel class of potential therapeutic agents that quench ROS in an effective manner. The dietary supplementation of lipoic acid has supported the longevity and delaying the weight loss in both the R6/2 and N171-82Q transgenic lines [115]. In addition, administration of L-carnitine dramatically extended the survival, ameliorated the motor performance, and decreased the number of intranuclear polyQ aggregates in the N171-82Q mice [116]. Another potent antioxidant, α-tocopherol (vitamin E), along with idebenone attenuated glutamate-induced neuronal death in HD cell lines like N18-RE-105 [117]. CDDO-MA (2-cyano-N-methyl-3,12-dioxooleana-1,9 (11)-dien-28 amide) treatment significantly attenuated 3-NP-induced loss of striatal neuronal nuclear antigen (NeuN) positive neurons [118]. Grape seed phenolic extract (GSPE) is a good metal chelator that inhibited polyQ aggregation and reduced the carbonyl levels in PC-12 cells expressing 103 glutamines fused with anEGFP reporter (HTT103Q-EGFP) [119]. However, the adverse effects of prolonged intake of antioxidants and their overdosages cannot be entirely excluded. Taken together, further investigation needs to be carried out to understand the mechanism and molecular pathways behind the above-discussed biological effects of antioxidants.

Recently, the benefits of flavonoid-rich dietary supplements have clearly been recognized in improving cognition by protecting degenerating neurons, by enhancing existing neuronal function, or by stimulating neuronal regeneration [120]. While neuroprotective natures of antioxidants against free radical damage have been extensively characterised, the neuroregenerative potential of antioxidants has recently evolved due to the neural plastic roles of adult stem cells of the brain. As a result, a number of naturally occurring dietary antioxidants have been identified with properties that support neurogenesis. The role of antioxidants has also been implicated in the functional outcomes in ageing and neurodegenerative disorders and their protective role is clearly linked to such outcomes in the abovementioned studies.

In addition, the neuroprotective effects of various natural, synthetic, and endogenous cannabinoids have been demonstrated in several in vitro and in vivo neurotoxicity models [121]. Peroxynitrite is involved in METH-induced dopaminergic neurotoxicity and the neurons can be protected against METH-induced neurotoxicity and striatal dopamine depletion by use of selective antioxidants, NOS inhibitors, and peroxynitrite decomposition catalysts [122]. Supplementation of selenium and antioxidants protect against METH-induced dopaminergic toxicity and the generation of $OONO^-$ in PC-12 cell line and in mouse striatum [122]. Ascorbate is present as one among the few antioxidants in extracellular fluid and is homeostatically regulated but modulated by glutamate-mediated activity [123]. Colle et al. demonstrated that metallothioneins and metallothionein-like proteins, which are isolated from mouse brain, act as neuroprotective agents by reducing oxidative stress. Probucol (PB), a phenolic lipid-lowering agent, possesses antioxidant property of scavenging free radicals and acts as a NMDA receptor antagonist, thereby promoting neuroprotection [124]. However, none of the abovementioned studies could suggest effective treatment strategies that could completely reverse the disease pathology. Effective management of degenerative diseases cannot be achieved by strategies that focus only on neuroprotection, whereas neuroregeneration through stem cell mediated adult neurogenesis needs to be promoted, in order to compensate for the functional deficits that occur due to neuronal loss.

4. Functional Significance and Regulation of Adult Neurogenesis

The adult brain retains the capacity to generate new neurons by the process called neurogenesis in specific regions of the organ [125–128]. The actively occurring neurogenesis is restricted to three defined neurogenic regions in the adult brain under normal conditions, namely, (1) the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus [126, 127]; (2) the subventricular zone (SVZ) of the lateral ventricles [129]; and (3) third ventricles of the hypothalamus [130]. In the hippocampal SGZ, proliferating NSCs develop into intermediate progenitors, which generate neuroblasts or immature neurons. These newly generated immature neurons migrate into the inner granule cell layer (GCL) and differentiate into new granule neurons of the hippocampus [128]. Further, these newborn neurons extend dendrites from DG towards the molecular layer (ML) and project axons that form the mossy fibber tract in the hilus region. In the SVZ, proliferating radial glia-like cells give rise to transient amplifying cells that generate migrating neuroblasts [128, 131]. Through the rostral migratory system (RMS), neuroblasts migrate towards the olfactory bulb (OB) [131]. In the OB, immature neurons differentiate into different subtypes of interneurons in granule cell layer (GCL) and glomerular layer (GLOM). Neural progenitor cells identified in the ependymal layer of the third ventricle of the adult brain migrate and differentiate into mature neurons in the hypothalamus [130] (Figure 1).

The potential roles of adult neurogenesis in various neurophysiological processes like motor functions, learning and memory process, olfaction, and the regulation of hypothalamus-pituitary-adrenal (HPA) axis have been extensively characterised [130, 132]. Adult neurogenesis has been known to be an integral component in neural plasticity, brain homeostasis, maintenance, and tissue remodelling [130]. Adult neurogenesis is a multistep process that includes stem cell proliferation, cell cycle exit, and fate determination of adult neural progenitors followed by differentiation, maturation, and integration of mature neurons into the neural circuits [130, 133]. This process has been shown to be modulated by many positive and negative factors [128, 130–134].

The ageing process, exposure to prolonged stress, abnormal levels of glucocorticoids, radiation, prolonged drug abuse, and chronic neuroinflammation are known to negatively influence adult hippocampal neurogenesis by inhibiting the proliferation and differentiation of NSCs or promoting the cell death of newborn granule cells [130, 135, 136]. This could lead to cognitive decline and loss of control of the HPA axis and may render the susceptibilities to neuropsychiatric and neurodegenerative disorders leading to cognitive impairments [137, 138]. Moreover, many of neuropsychiatric and neurodegenerative disorders are characterised with impaired adult neurogenesis. It has been shown that neurogenesis is impaired in the hippocampus of transgenic R6 mouse lines [139–141], transgenic rat model of HD [142], and knock-in YAC128 model [143]. However, in chemically induced acute neurodegenerative models and HD patients, neurogenesis is increased in the SEL and SVZ, respectively [144, 145]. The increased neurogenesis in the SVZ in combination with abnormal migration of neuroblasts in the striatum is also observed in a toxic rat model of HD [145]. Thus, the abnormal reactive neurogenesis in the striatum has been observed as a common hallmark in HD [146]. Acute neurological conditions like stroke, epilepsy, and traumatic brain injuries have also been associated with increased adult neurogenesis [147, 148]. In HD, the reactive neurogenesis in striatum has been speculated to be an attempt of stem cell mediated regeneration to overcome neuronal dysfunctions and neuronal loss. Thus, the endogenous self-regenerative measures adapted by HD brain through neurogenesis to overcome neurodegeneration highlight the possibility of exploiting promotion of antioxidant-mediated neural regeneration as a management strategy for HD.

The positive regulators of neurogenesis comprise physical activity, environmental enrichment, growth factors, and antioxidants derived from diet. It is evident that physical activity such as running promotes neurogenesis by increasing the proliferation of NSCs in the SGZ of the dentate gyrus, thereby expanding the pool of progenitor cells available for further neuronal differentiation in the hippocampus [149]. Spontaneous physical activity and task-based learning are the two important components of an enriched environment that promotes hippocampal neurogenesis [150]. Neurotrophic factors, cytokines, and growth factors regulate the adult neurogenesis by controlling proliferation, maturation, differentiation, and survival of neuronal cells. Systemic infusion of

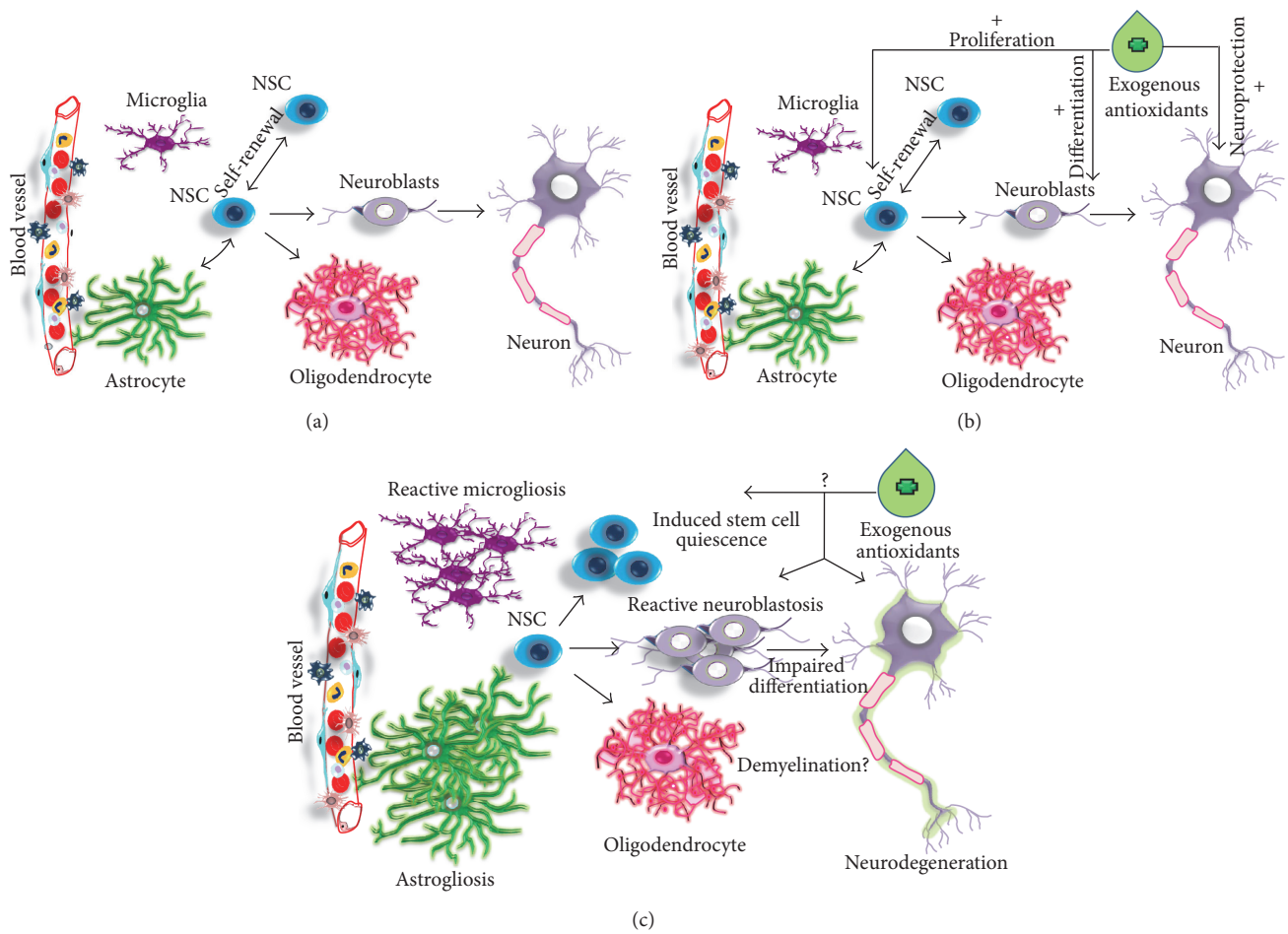

Figure 1: Illustration of neurogenic niche of the adult brain in various conditions. (a) In normal brain; (b) under antioxidant supplement; (c) neuropathology and neurogenesis in HD.

insulin growth factor-1 (IGF-I) increased the proliferation of NSCs, frequency of neuronal differentiation, and survival in the adult rat hippocampus [151]. Jin et al. demonstrated that the angiogenic protein, vascular endothelial growth factor (VEGF), stimulates the proliferation of NSCs in murine cerebral cortical cultures and in adult rat brains, thus promoting neurogenesis apart from angiogenesis [152]. Intracerebroventricular infusion of epidermal growth factor-1 (EGF-1) and fibroblast growth factor-2 (FGF-2) increased proliferation of NSCs in the SVZ of the adult rat brain [153]. Ciliary neurotrophic factor (CNTF) supported adult neurogenesis as CNTF knockout mice showed reduced neurogenesis [154].

Cotman and Berchtold reported that voluntary exercise increased the levels of brain-derived neurotrophic factor (BDNF) and other growth factors which in turn stimulated neurogenesis [155]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are recognized to increase adult neurogenesis and thus aid in the process of neuroprotection [156]. Chronic treatment with various antidepressants like tranylcypromine, reboxetine, fluoxetine, and haloperidol is reported to increase neurogenesis in early adulthood and experimental models of stress [157]. It has been reported that neuronal differentiation and survival are associated with TGF-beta signaling and thus TGF-beta signaling remains as a crucial regulator of adult neurogenesis [15, 142, 158]. Moreover, it has also been reported that adult neurogenesis is positively regulated by diet and it could prevent cognitive decline during ageing, as well as to counteract the effect of stress and depression [159]. It was shown recently that a reduction in calorie intake increases hippocampal neurogenesis in adult rodents and that this effect is partly mediated by BDNF [160]. Taken together, the decline in brain plasticity and mental process can possibly be reestablished by the activation of NSCs that have the ability to self-renew and develop into neurons or glial cells. As no specific treatments are available as a cure to HD, neuronal stem cell (NSC) mediated neurogenesis provides a regenerative strategy to replace the neuronal loss and neural plasticity including motor impairments, cognitive functions, and mood that are affected in HD.

5. Role of Oxidative Stress on the Regulation of Adult Neurogenesis

The functional impairments of NSC, particularly neurogenesis, represent an increasingly prominent contributor to multiple CNS diseases and the process is highly altered by elevated

levels of oxidative stress. Oxidative stress caused by increased ROS has been considered to affect neurogenesis and cognitive functions [161]. Acute exposure of NSCs to ketamine leads to increased cell proliferation whereas the chronic incubation results in cellular damage via altered mitochondria pathways and induces cellular apoptosis [162]. Superoxide dismutases (SODs) scavenge the superoxide radicals by converting them to hydrogen peroxide and oxygen molecule thus acting as first-line defense to protect cells [163]. The SOD deficient mouse model showed reduction in the generation of new neurons in the hippocampus upon cranial irradiation [164]. Cranial irradiation, an effective treatment for brain tumors, leads to persistent elevation of oxidative stress and suppression of hippocampal neurogenesis [165]. Increased oxidative stress following irradiation is expected to play a major role in the suppression of hippocampal neurogenesis and the associated cognitive deficits. Walton et al. suggested that the production of ROS is a part of the routine maintenance of physiological neurogenesis, but chronic oxidative stress may play a role in loss of function in ageing and progressive CNS diseases [165]. Accelerated age-dependent decline in adult neurogenesis is a consequence of oxidative stress. Conditional deletion of the clock gene *Bmal1* (Bmal1$^{-/-}$) in mice accelerated ageing, neurodegeneration, and cognitive deficits through oxidative damage [166]. Moreover, oxidative stress promotes aneuploidy and formation of neurofibrillary tangles in the neurogenic regions of the brain, contributing to neurodegeneration in AD. Sirtuin protein family members (e.g., Sirt1, Sirt2) are considered to be important in determining the redox state in NSCs and also provide potential targets for modulating adult neurogenesis [167]. Taken together, increased levels of oxidative stress by high accumulation of ROS have negative effect on adult neurogenesis during ageing, neuroinflammation, and neurodegeneration [168]. Thus, targeting oxidative stress represent a novel way to regulate adult neurogenesis. This in turn will help in decreasing the pathogenesis of neurocognitive disorders including HD by promoting neurogenesis in order to compensate the neuronal loss, which could pave a path for supporting the cognitive functions (Figure 2).

6. Supportive Role of Antioxidants in Promoting and Regulating Adult Brain Neurogenesis

The cognitive health of an organism is maintained by the capacity of hippocampal neurogenesis. Recently, benefits of antioxidants have emerged as a potent strategy to support the cognitive function through the regulation of adult neurogenesis. Consumption of potent antioxidants, for example, melatonin and polyunsaturated fatty acids, has a significant effect in lowering the decline of neurogenesis and attenuating the impairment of cognitive function [169–171]. Impairment of hippocampal neurogenesis in rat models of chronic alcoholism by elevation of oxidative stress can be reversed by treating with ebselen, a drug with antioxidant property [152]. Curcumin has been shown to increase adult neurogenesis in the hippocampus of adult rodents. An antioxidant-fortified food in an enrichment plan affected the survival of new neurons in the aged canine brain and is associated with improvement in cognitive performance [170]. Administration of flavonoids like 3′-methylated epicatechin and 4′-methylated epicatechin to animal models improved cognitive performance by promoting neurogenesis [171]. Qu et al. demonstrated that *Rhodiola crenulata* extract (RCE), containing a potent antioxidant salidroside, promotes neurogenesis in the hippocampus of intracerebroventricular injected streptozotocin model of AD [172]. Polyphenols are abundant micronutrients present in plant-derived foods, fruits, and beverages such as tea, red wine, cocoa, and coffee and also act as powerful antioxidants [173]. In rats, polyphenols increased hippocampal plasticity and improved learning and memory performance, while protecting neurons against injury induced by neurotoxins suppress neuroinflammation and the potential to promote cognitive function. In general, dietary polyphenols seem to exert positive effects on anxiety and depression via regulation of adult hippocampal neurogenesis [174]. Flavonoids protect the brain in many ways through enhancement of existing neuronal function or by stimulating neuronal regeneration [175]. Polyphenol-rich Ginkgo biloba extracts and other flavanoids have been shown to protect hippocampal neurons from oxidative stress, nitric oxide, and beta-amyloid-induced neurotoxicity [176]. An et al. reported that the supplementation of flavanoids (XBXT-2) in rats subjected to chronic stress shows increased neurogenesis and increase in BDNF levels [177]. Different polyphenols are shown to exert their effects on adult hippocampal neurogenesis via different mechanisms of action, such as by activating the MAP kinase pathway or stimulating the expression and release of neurotrophic factors [178]. Cocoa powder and chocolate contain a large percentage of flavonoids, mainly epicatechin that interacts through signaling cascade proteins and lipid kinases thereby inhibiting neuronal death by apoptosis induced by oxygen radicals and promoting neuronal survival and synaptic plasticity [179]. In addition, flavonoids preserve cognitive abilities in rats during ageing and lower the risk of AD stress and stroke in humans [179]. Thus, neurogenic properties of antioxidants have great significance in therapeutic interventions for brain diseases. However, reports on the effect of antioxidants in regulating adult neurogenesis in HD are limited. While R6/2, R6/1, YAC128 mice, and TgHD rats have been characterised with induced NSCs quiescence and impaired neurogenesis, the elevated level of neuroinflammation related factors like TGF-beta appears to inhibit the regenerative ability of the HD brain [15]. Free radicals involved oxidative damage observed in HD might also act synergistically with neuroinflammation to impede the proneurogenic signals in HD. Indeed, combinations of antioxidant therapy along with physical exercise may exert beneficial effect to promote neuroregeneration in HD.

HD is accompanied by both cognitive and motor defect, which is caused by progressive loss of striatal neurons. Increased neuronal cell death has also been described in the cortex and the hippocampus of HD brains in addition to the striatum [9]. Recently, adult neurogenesis has been identified in regions other than hippocampus and SVZ-OB such as

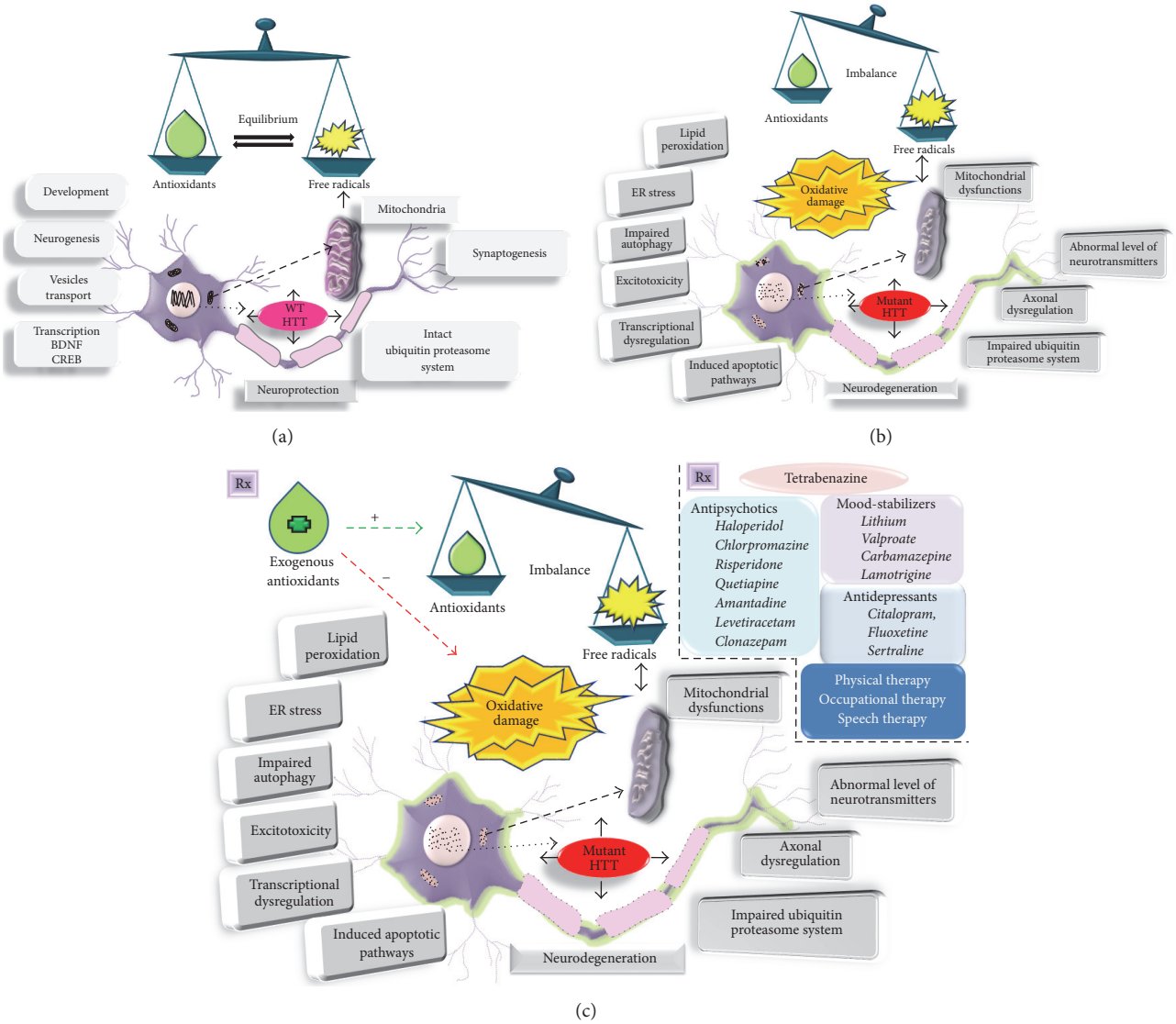

Figure 2: Graphical representation for the possible roles of free radicals and antioxidants on neuronal functions in control and HD conditions. (a) A balance is maintained between the amount of free radicals generated and the antioxidant defense mechanism which aid in normal physiological functions of the neuron in normal brain. (b) In HD brain, the free radicals generated are higher and the antioxidant defense mechanism is impaired, resulting in oxidative damage. (c) Various therapeutic options to overcome the disruption caused by oxidative stress on cellular functions of neuron in HD.

amygdala and hypothalamus which are responsible for fear, memory, and the regulation of neuroendocrine functions, respectively [180, 181]. Interestingly, it has also been reported that neurogenesis occurs in the cortex of the adult monkey [181, 182] and normal rat [183], suggesting that neurogenesis of adult brain is widespread. Induction of adult neurogenesis in response to many CNS trauma and neurological diseases has been reported by several studies in the past decade [184, 185]. It has been demonstrated that ischemia is a well-known factor to contribute to reactive neurogenesis in the cortex [147, 148, 186, 187]. The abnormal cortical neurogenesis has also been reported in multiple sclerosis [188] and ALS [189]. Considering these facts, it can be speculated that regeneration of damaged brain through cortical neurogenesis can occur in the brains of HD subjects. However, reports on adult cortical neurogenesis remains a subject of ongoing debate [190]. Hence, these findings need to be reconfirmed and validated with better experimental models. Future studies should focus on confirming the occurrence of neurogenesis in the cortex of damaged adult brains, since cortical neurogenesis is important for compensating the loss of motor functions in HD cases.

While a new line of research focusing on cortical neurogenesis is necessary to implicate its role in brain regeneration, reactive neurogenesis in the striatum of both normal and pathogenic adult brain including HD subjects has recently been well established [143, 145, 191]. As the striatum plays an important role in the planning and modulation of movement,

it raises a question whether striatal neurogenesis is required for compensating loss of motor tasks in HD. Abnormal cell proliferation and reactive neuroblastosis in the striatum have been observed in several cases of HD brains [146] as a mechanism to replenish the loss of neurons in the striatum. However, in many such cases the migrating and resident neuroblasts undergo apoptosis before maturing into neurons in the striatum. Hence, survival of neurons does not occur in the striatum of HD [143, 191, 192]. As a part of compensatory mechanism against QA striatal lesion-induced neuronal loss, the brain promotes neurogenesis in the SVZ, from where neuroblasts migrate to the damaged areas of the striatum [144]. Ernst and Frisén demonstrated the presence of neuroblasts in the striatum of the human brain using doublecortin (DCX) and polysialylated neuronal cell adhesion molecule (PSA-NCAM) immunostainings and confirmed the occurrence of neurogenesis in the striatum [191, 192]. However, they did not observe the survival of newborn neurons in the normal striatum as well as in HD cases, confirming the premature depletion of neuroblasts before their integration into the striatal tissue. The failure of neuronal differentiation in the striatum and reactive striatal neuroblastosis has been recapitulated in the rodent models of HD [143, 144, 146]. Taken together, it can be hypothesized that the migrating SVZ born neuroblasts and/or neuroblasts generated by the striatum might provide the neurophysiological support to the striatum to overcome the motor deficits to certain extent. In this respect, it will be very interesting to see if antioxidants can contribute to the terminal differentiation, integration, and survival of reactive neuroblasts in the degenerated striatum of HD. It is possible that antioxidants may provide substratum and trophic support in addition to mitigating ROS generated by striatal gliosis and neurodegeneration in HD.

7. Conclusion

HD is a progressive neurodegenerative disease that has been refractory to treatment. Despite the enormous research focus on HD, no valid treatment that can alleviate the symptoms of HD has been developed. This could be attributed to the complex nature of the disease and lack of evidence on a precise molecular target for therapeutic intervention. Generation of free radicals leading to oxidative stress (OS) damage contributes to neuronal loss in HD and the oxidative stress could be reduced by supplementation of natural antioxidants. Adult neurogenesis can act as an important tool for regenerative therapy of HD brains as it contributes to the cognitive functions of the adult brain. Neurogenesis has been shown to be upregulated by numerous antioxidants. Impaired hippocampal neurogenesis and reactive striatal neurogenesis have been the characteristics of HD brains. Naturally occurring antioxidants might therefore provide neurotropic as wells as proneurogenic and neuroprotective support for the HD brain, in order to overcome the motor and cognitive impairments. However, the complete relationship between oxidative stress and neuroregeneration, and the molecular mechanism by which antioxidants support the process of adult neurogenesis by triggering various signaling cascades, needs further diligent investigation.

Abbreviations

HD: Huntington's disease
CAG: Cytosine-Adenine-Guanine
PolyQ: Polyglutamine
ROS: Reactive oxygen species
RNS: Reactive nitrogen species
MSN: Medium spiny neurons
DG: Dentate gyrus
SGZ: Subgranular zone
SVZ: Subventricular zone
OB: Olfactory bulb
SNP: Single nucleotide polymorphism
FDA: Food and Drug Administration
Nrf2: Nuclear factor (erythroid-derived 2)-like 2
AD: Alzheimer's disease
PD: Parkinson's disease
ESC: Embryonic stem cells
IPSC: Induced pluripotent stem cells
MDA: Malondialdehyde
HO: Heme oxygenase
ATP: Adenosine triphosphate
MTA: Mitochondria-targeted antioxidant
mHTT: Mutant huntingtin protein
DHA: Docosahexenoic acid
TUDCA: Tauroursodeoxycholic acid
NSC: Neuronal stem cell
GCL: Granule cell layer
RMS: Rostral migratory system
GLOM: Glomerular layer
HPA: Hypothalamus-pituitary-adrenal
IGF-I: Insulin growth factor-1
VEGF: Vascular endothelial growth factor
EGF-1: Epidermal growth factor-1
FGF-2: Fibroblast growth factor-2
CNTF: Ciliary neurotrophic factor
BDNF: Brain-derived neurotrophic factor
NSAIDs: Nonsteroidal anti-inflammatory drugs
CNS: Central nervous system
SODs: Superoxide dismutases
RCE: *Rhodiola crenulata* extract
APP: Amyloid precursor protein
Aβ: Amyloid beta.

Competing Interests

The authors have declared that no competing interests exist.

Acknowledgments

The work was supported by the Faculty Recharge Programme, University Grants Commission (UGC-FRP), New Delhi, India (Mahesh Kandasamy), Ramalingaswami Re-Entry Fellowship Programme, Department of Biotechnology, New Delhi, India (Thirunavukkarasu Velusamy), and an Internal Grant (IG/AGR/FOOD/14/01) from CAMS, SQU,

Oman (Musthafa Mohamed Essa). This work was professionally edited by Red Fern Communication. The authors also acknowledge the UGC-SAP and DST-FIST for the infrastructure of the Department of Animal Science, Bharathidasan University.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7039816, 10 pages
https://doi.org/10.1155/2017/7039816

Review Article

Nutrition and AGE-ing: Focusing on Alzheimer's Disease

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Received 27 July 2016; Revised 28 September 2016; Accepted 29 September 2016; Published 12 January 2017

Academic Editor: Musthafa Mohamed Essa

Recently, the role of food and nutrition in preventing or delaying chronic disability in the elderly population has received great attention. Thanks to their ability to influence biochemical and biological processes, bioactive nutrients are considered modifiable factors capable of preserving a healthy brain status. A diet rich in vitamins and polyphenols and poor in saturated fatty acids has been recommended. In the prospective of a healthy diet, cooking methods should be also considered. In fact, cooking procedures can modify the original dietary content, contributing not only to the loss of healthy nutrients, but also to the formation of toxins, including advanced glycation end products (AGEs). These harmful compounds are adsorbed at intestinal levels and can contribute to the ageing process. The accumulation of AGEs in ageing ("AGE-ing") is further involved in the exacerbation of neurodegenerative and many other chronic diseases. In this review, we discuss food's dual role as both source of bioactive nutrients and reservoir for potential toxic compounds—paying particular attention to the importance of proper nutrition in preventing/delaying Alzheimer's disease. In addition, we focus on the importance of a good education in processing food in order to benefit from the nutritional properties of an optimal diet.

1. Introduction

Ageing is a major risk factor for chronic disease. Progressive decline of biological functions can render the organism more susceptible to endogenous or exogenous triggers, exacerbating pathological conditions. Among the age-related diseases, cognitive fragility and dementia remain the more debilitating, with a pronounced impact on public health costs arising from the need for long-term care management.

Policies that allow for the effective management of dementia include better coordination between health and long-term care services. However, the main goal should be to adopt proper strategies to preserve cognitive status and/or delay cognitive deterioration.

The degree of disability, including cognitive fragility, depends not only on genetic susceptibility, but also on lifestyle, environment, and triggers to which one is exposed [1, 2]. Appropriate lifestyle behaviours, including good nutrition and physical activity throughout life, are the first steps in preventing chronic diseases and disabilities in old age [2, 3]. Today it is well recognised that certain nutrients derived from the diet, including polyunsaturated fatty acids and polyphenolic compounds contained in fruits and vegetables, can dramatically impact the ageing brain, possibly leading to improved cognition and motor abilities. All these compounds exert potent antioxidant and anti-inflammatory activity. However, their potential for improving cognition is not limited to their antioxidant properties, as they also involve specific molecular and cellular processes that support brain plasticity [4]. For example, neuronal plasticity improvement by omega-3 intake was found to be mediated by the upregulation of brain-derived neurotrophic factor (BDNF) [4, 5].

Although a healthy diet takes into account different types of food as sources of bioactive nutrients able to preserve biological functions and prevent disease development, the contribution of different food processing and cooking methods is often poorly considered. Indeed, the technical manipulation of raw materials, industrial processing, and storage and cooking methods can modify food's original

contents. This contributes not only to the loss of healthy nutrients, but also to the formation of toxins—including advanced glycation end products (AGEs) [6].

Thus, this work reviewed the impact of nutrition on Alzheimer's disease, the most common type of dementia, reporting knowledge on both the contribution of bioactive nutrients in preserving an active and healthy cognitive state, as well as the detrimental effects of dietary-glycotoxin, derived from food processing and cooking methods. In addition, we focus on the importance of a good education in processing food in order to benefit from the nutritional properties of an optimal diet.

2. Alzheimer's Disease

Today, nearly 46.8 million worldwide people developed dementia, and the incidence is expected to rise in the coming years, with 74.7 million cases estimated to occur in 2030 and 131.5 million in 2050. After the age of 65, the risk of developing dementia doubles every five years, and Alzheimer's disease (AD) affects one in four people aged 85 and over [7]. Alzheimer's disease is a neurodegenerative disorder characterised by progressive global deterioration in intellect, which affects memory, thought, learning, orientation, language, comprehension, and judgment, as well as behaviour and the ability to perform everyday activities. The major pathological hallmarks of this disease include accumulation of protein deposits in the brain as beta-amyloid (Aβ) plaques and neurofibrillary tangles [8, 9]. In addition, an AD brain exhibits constant evidence of oxidative stress-mediated injury and widespread inflammation [10].

Alzheimer's disease is a disorder of late life; however, there are families in which AD is inherited as an autosomal dominant disorder of midlife. Less than 1% of cases are caused by specific mutations in three genes, which code for amyloid-precursor protein (APP), Presenilin 1 and Presenilin 2, all linked to amyloid-beta metabolism [9].

AD has to face two major challenges: the delay in the diagnosis and the lack of neuroprotective or curative pharmacological treatment. In fact, AD is recognised only in the late stage when cognitive symptoms appear, and currently approved drugs only provide modest and temporary relief for symptoms such as memory loss. Today, it is well accepted that a prodromal phase ranging from 10 to 20 years precedes the symptomatic state. During this long period, many biochemical changes occur in the brain, anticipating cognitive impairment. In this preclinical phase, preventive strategies, such as dietary modification and nutritional supplementation, might reduce the global burden of AD. One of the first links between dietary intake and incidence of AD is represented by a large prospective population-based cohort study (Rotterdam study) that reported an associated lower risk with the use of cholesterol-lowering statin drug [11]. The association of dietary fats with plasma cholesterol levels is highly relevant because cholesterol is involved in both generation and deposition of Aβ [12]. Furthermore, the protein product of APOE-ε4, a recognised genetic risk factor for AD, is the principal cholesterol transporter in the brain. In fact, many epidemiologic data suggest that nutritional intake can influence the development and progression of AD [13].

3. Positive Effects of Dietary Nutrients in Preventing Cognitive Deterioration

A nutritional approach to prevent, delay, or halt the progression of AD is considered to be a promising strategy and has therefore been widely explored [14, 15].

3.1. Polyunsaturated Fatty Acids. Numerous studies have investigated the effects of polyunsaturated fatty acids (PUFAs) in preventing and/or slowing AD. The potential PUFA dietary intervention to prevent neuronal loss and cognitive decline stems from evidence that PUFAs are critical components of neuronal cell membranes, maintaining membrane fluidity, which is essential for synaptic vesicle fusion and neurotransmitter communication within neural networks. The n-3 long chain PUFAs (n-3 LCPUFAs), which mainly include omega-3, docosahexanoic acid (DHA), and eicosapentaenoic acid (EPA), regulate neuronal membrane excitability and improve the capacity for neuronal transmission in healthy subjects, thus enhancing learning and memory [16]. Furthermore, DHA, whose high levels in brain indicate its essential role in this organ, is also involved in mood and emotional state, locomotor and exploratory activities, and cognitive functions [17].

In addition, n-3 LCPUFAs modulate the inflammatory processes by acting at the immune system level in many different ways through (i) the regulation of cytokines and chemokines expression, (ii) the decrease of prostaglandins and eicosanoids, and (iii) the induction of proresolutive factors, resolvins, and protectins that are involved in the resolution of inflammation [5, 17, 18]. EPA, DHA, and their bioactive mediators exert their anti-inflammatory effects not only in the periphery [19] but also at the brain level [20]. Interestingly, Freund Levi et al. [21] demonstrated that a diet rich in n-3 LCPUFAs significantly increased DHA levels in the brain, suggesting that DHA and EPA dietary supplementation might directly influence neuroinflammatory pathways [20].

Numerous observational studies have highlighted a possible association between dietary intake of fish and n-3 LCPUFA and a lower risk of dementia, including AD [13, 22, 23]. On the other hand, it has to be stressed that studies finding limited or no clinical benefit of PUFAs on cognitive improvement in AD patients were also reported [24, 25]. For example, Chiu et al. [26] have demonstrated in a double-blind placebo-controlled study that omega-3 monotherapy improved cognitive performance only in Mild Cognitive Impairment (MCI) patients but not in AD group. The reasons why no effect of omega-3 treatment was observed in patients with moderate or advanced AD could be due to the relatively short duration of the supplementation, the daily dose used, the source and the origin (fish versus vegetable oil) of n-3 LCPUFA, the dietary history of the patients, and the cognitive function assessed [18, 27]. Therefore, Hooijmans et al. [28] performed a meta-analysis study on the effects of long-term omega-3 supplementation

in AD animal models, confirming its well-recognised effect in restoring cognitive performance. In particular, long-term omega-3 supplementation decreased omega-6/omega-3 ratio, reduced the amount of beta-amyloid, prevented neuronal loss, and improved cognitive function in AD animal models. Furthermore, the effects of DHA in reducing Aβ production in in vitro study and AD animal models have been also widely demonstrated [29, 30]. The mechanism involved in the DHA-induced reduction in Aβ may be due to multiple effects: changing in lipid raft structure, alterations in APP processing, and induction of antiamyloidogenic chaperones for APP [31]. Data accumulated so far strongly suggest that the optimization of brain lipid profile might translate into a realistic strategy to enhance cognitive performance and/or to prevent neurodegenerative disorders. Therefore, in the years to come, research effort has to be devoted to define the optimal lipid dietary intake for the ageing brain and who might benefit the most from it [17].

3.2. Vitamins. Vitamins are potent antioxidants. Their potentiality in maintaining healthy cognition and preventing cognitive decline rises by the fact that the brain is particularly susceptible to oxidative stress damage. The brain is a major metaboliser of oxygen, accounting for 20% of the body's consumption, and has relatively feeble protective antioxidant mechanisms. In addition, it contains a large amount of polyunsaturated peroxidisable fatty acids, along with high levels of iron that act as a prooxidant. A free radical-enriched environment in the brain contributes to the progressive decline of cognitive abilities, exacerbating dementia. Vitamin E has been intensively investigated for its role in protecting membrane phospholipids against peroxidation. Zandi et al. [32] demonstrated that the use of vitamin E and vitamin C supplements in combination with food is associated with reduced prevalence and incidence of AD. A multicentre clinical trial on vitamin E supplementation for patients with moderate AD demonstrated that vitamin E slowed disease progression, thereby reducing the risk of institutionalisation [32, 33]. However, in another study, no significant differences in progression of AD were found in the vitamin E group compared to the placebo group [33]. This is probably due to the different compositions of vitamin E supplements, which often differ from the form of vitamin E found in the diet. Vitamin E refers to a group of fat-soluble compounds that include eight chemical forms. Among them, γ-tocopherol and α-tocopherol are the most abundant in the diet, and α-tocopherol is also the one that exerts antioxidant properties [34]. In this context, Grimm et al. [35] demonstrated that δ-tocopherol, but not α-tocopherol, increased the level of Aβ by enhancing its production and decreasing its degradation, worsening the pathology. Another critical issue to solve is the optimal dose of vitamin E required to prevent or delay AD. Therefore, more clinical trials are needed to define the proper vitamin E composition and dosage in the treatment of this pathology.

Vitamin D might also have an association with AD. Observational studies offer good evidence that low vitamin D concentration is a risk factor for developing AD, because its concentrations have been found inversely correlated with its risk [36]. Thus, wishing to reduce risk of AD, the Endocrine Society recommendations of keeping vitamin D3 concentrations above 75 nmol/L [36, 37] should be considered.

Furthermore, polymorphism of the vitamin D receptor (VDR) and altered vitamin D signalling have been found to predispose to AD development or AD-like neurodegeneration [38]. Interestingly, in the transgenic 5xFAD (Tg) mice, an animal model of AD, five months of vitamin D3 supplementation enhanced learning and memory [39]. This treatment has been demonstrated to induce the expression of proteins involved in the immune and inflammatory response, neurotransmitter activity, and endothelial and vascular processes, with a significant decrease of amyloid plaques and astrogliosis. Recently, Gangwar et al. [40] suggested that vitamin D supplementation induced significant improvement in cognitive performances also in subjects with senile dementia.

Other vitamins, including vitamin A and the complex of vitamin B, were found lower in plasma/serum of geriatric patients with cognitive impairment [41, 42]. For their role in homocysteine metabolism, the three B vitamins (B6, B12, and folic acid) have been correlated with age-related cognitive fragility [43]. Previous epidemiological studies on vitamin B and cognitive status found that older people with elevated homocysteine levels (hyperhomocysteinaemia) tend to have lower vitamin B status, as well as lower cognitive tests scores [44]. Possible correlations between vitamin A and Alzheimer's disease were reported in in vitro studies, demonstrating an anti-beta-amyloid oligomerization effect of vitamin A and beta-carotene [45]. However, more clinical work is needed to identify the potential benefit from vitamin A and/or complex B supplementation in AD patients.

3.3. Polyphenols. The beneficial role of dietary polyphenols has been suggested as potential functional food candidates to prevent memory decline [46]. Polyphenols are natural substances present in plants, fruits, and vegetables. Some polyphenols, such as epigallocatechin-3-gallate (EGCG) found in green tea, 4-O-methyl honokiol found in Magnolia officinalis, resveratrol contained in grapes, and ginkgolide A found in ginkgo biloba, have been suggested to provide protection against AD. Their effects may be due to their antioxidant and anti-inflammatory properties, but also by their modulation of enzyme activity and regulation of intracellular signalling pathways and gene expression [46, 47].

In fact, polyphenols, especially flavonoids, can also modulate those neuronal signalling cascades altered with ageing by acting on ERK/CREB pathway involved in synaptic plasticity and long-term potentiation, improving learning and memory in both animals and humans [48–51]. Flavonoid supplementations can modulate specific signalling kinases like CaMKII and ERK, controlling the activation of CREB and the increased expression of BDNF and NGF at the brain level [50–52]. In fact, these compounds also exert a protective function in the hippocampus of middle age mice preserving and promoting the spatial learning strategies. Recently also Bensalem et al. [53] demonstrated that a polyphenol-rich

extract from grape and blueberry (PEGB), with high contents of flavonoids, can facilitate the use of spatial strategies in both adult and middle-aged mice. In these animals PEGB supplementation was able to improve learning performance by restoring CaMKII mRNA levels and increasing NGF expression exactly in the hippocampus. It is noteworthy that this is the first nutritional intervention that, even if with a mix of different polyphenols at low doses, shows a rescue effect on those specific memory deficits [53].

Furthermore, Ono et al. [54] have further corroborated the relevance of polyphenol supplementation for AD prevention. He demonstrated that wine-related polyphenols, including myricetin, quercetin, and kaempferol, inhibited Aβ oligomer formation in a dose-dependent manner from fresh monomeric Aβ, as well as destabilised preformed Aβ oligomers in in vitro experiments. Resveratrol, another wine-related polyphenol abundant also in berries, protects neurons against Aβ-induced toxicity and attenuates behavioural impairment in rats [55]. Again, green tea's polyphenols, EGCG and epicatechin (EC), showed their neuroprotective effects throughout the free radical scavengers on in vitro oxidative stress and in neurotoxicity cellular models [56, 57].

Curcumin also has a potential role in the prevention and treatment of AD. The biophenolic curcumin, isolated as the active yellow component of *Curcuma longa*, has a long history of use in traditional Asian medicines for its potent anti-inflammatory, antioxidant, and anticancer activities [58]. In AD animal models, curcumin reduced proinflammatory cytokines, oxidative damage, and beta-amyloid production, ameliorating cognitive deficits [59]. Zhang et al. [60] demonstrated that macrophages derived from AD patients treated with curcumin showed an improved uptake of beta-amyloid when compared with untreated cells. In addition, curcumin exerted an antiproliferative action on microglial cells preventing cytokine release. Also Ambegaokar et al. [61], using different doses of curcumin in a mixed colony of both neuronal and glial rat cells, showed that curcumin stopped the proliferation of neuroglial cells dose dependently, by differentiating them into mature cells or inducing apoptosis, resulting in inhibiting neuroinflammation. Furthermore, curcumin decreases the lipoprotein oxidation and the free radicals formation in AD and in other neurodegenerative disorders [62]. Because of its lipophilic nature, curcumin crossed the blood-brain barrier and reduced existing senile plaques, as demonstrated in APPswe/PS1dE9 mice [63]. Curcumin reduces senile plaques by binding with the Aβ oligomers, destabilising them and preventing their extension [64]. However, further studies on large population will be necessary in order to demonstrate the effects of all these polyphenols in delaying or preventing AD.

4. Dietary-Advanced Glycation End Products (d-AGEs) and Cognitive Decline

During the processing of foods, the temperature, the duration of the heat treatment, and the food's water content can drive different biochemical reactions, transforming the original content. At high heat administered for a long period of time, we expect the loss of a high amount of water and the degradation of heat-sensitive micronutrients, such as vitamin C, folates, and thiamine. In addition, higher temperatures used for cooking induce a series of reactions that lead to the characteristic smell, taste, and colour of the dish. Those reactions are also involved in the formation of toxic secondary products known as advanced glycation end products (AGEs). AGEs are a heterogeneous group of compounds derived from a nonenzymatic glycation of free amino groups of proteins, lipids, or nucleic acids by reducing sugars and reactive aldehydes [65]. They are also continuously formed in the body as a part of normal metabolism under hyperglycaemic and/or oxidative stress conditions [6, 65]. It is well known that AGEs derived from the diet can highly contribute to the body pool of AGEs and constitute a large amount of the total AGE serum content. Since the half-life of AGEs is about double the average of a cell's life, their detrimental effects can persist for a long time, especially in "long-lived" cells like nerve and brain cells [66]. Their toxic effects are related to their ability to promote oxidative stress and inflammation by binding to cell surface receptors or cross-linking with body proteins, altering their structure and function [67, 68].

The most studied AGE receptor is RAGE, a single transmembrane multiligand receptor that belongs to the immunoglobulin superfamily [69]. RAGE receptors are mainly expressed on vascular, endothelial, and smooth muscle cells and on monocyte/macrophage membranes [69], but also in microglia and astrocytes, as well as in neurons [70, 71]. Ligands of RAGE, apart from AGEs, include members of the S100 protein family, proteins of the high mobility group box-1 (HMGB1), prions, and amyloid-β peptides. RAGE is implicated in the pathogenesis of several chronic diseases, such as cardiovascular diseases, hypertension, and diabetes, which are risk factors for AD, suggesting it might be the molecular link that initiates a chronic positive feedback loop, ultimately leading to AD etiology [69].

The interaction of RAGE receptors with AGEs induces the activation of different intracellular cascades, which involve the nuclear factor kB (NF-κB) pathway and inflammatory mediators like tumour necrosis factor-α (TNF-α), interleukin-6, and C-reactive protein (CRP) [72]. All of these pathways lead to increased oxidative stress and a proinflammatory status.

Recently, different studies reported that an elevated serum level of AGEs is associated with a faster rate of cognitive decline [66, 73]. More specifically, increasing evidence in the literature suggests that AGEs could be implicated in the progression of Alzheimer's, Parkinson's disease, and cerebrovascular dementia. In particular, RAGE seems to be involved in AGE-induced oxidative stress and chronic subclinical inflammation in the AD brain [74]. In fact, RAGE is increased in the brains of AD patients and has a role in regulating the transport of beta-amyloid across the blood-brain barrier (BBB) [75]. In particular, RAGE was found to act as cell surface receptor for Aβ [75, 76] and promote the influx of circulating Aβ across BBB from blood to brain, which is antagonized by LRP-1-mediated efflux of Aβ [77, 78]. The interaction of AGEs with their receptor (RAGE) activates also the proinflammatory pathway via NF-kB. The neuroinflammation induced by AGEs can

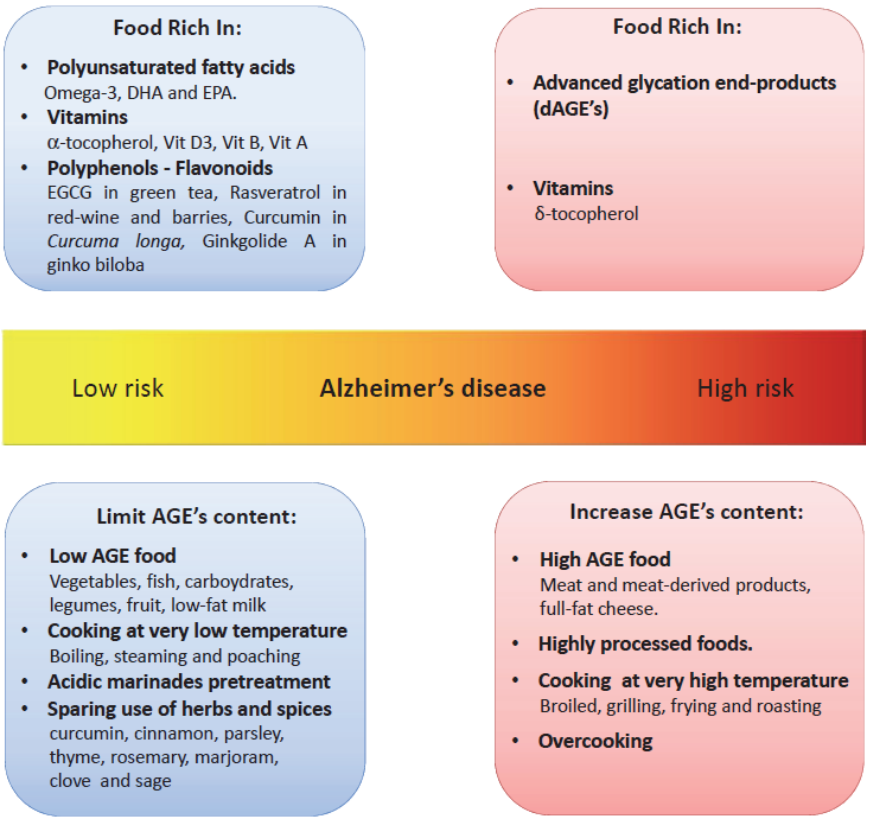

Figure 1: Involvement of diet and cooking methods in Alzheimer's disease prevention.

establish a vicious circle, whereby the overregulation of RAGE potentially increases Aβ influx across the BBB, leading to an accumulation of Aβ in the brain [79]. Furthermore, in the last years, a newly role of RAGE is emerging in microglia activation. This can have some implication in AD pathogenesis [80]. In fact, the interaction of RAGE with Aβ in activated microglia can initiate a cascade of events, resulting in sustained generation of toxic mediators and, ultimately, exacerbating neuroinflammation and leading to neuronal loss [69].

Recently, Perrone et al. [81] also presented evidence for a novel RAGE-mediated signalling in AD, which leads to the expression of thioredoxin interacting protein (TXNIP) in various cell types, promoting inflammation [81, 82]. TXNIP binds to thioredoxin (TRX) and inhibits its antioxidant activity, leading to oxidative stress [83]. Among the many proteins under the redox control of TRX, the pleiotropic p53 was found peculiarly nitrated at its tyrosine residues in AD blood cells [84], suggesting that alteration of RAGE-TXNIP axis can have different downstream effects, contributing to the complexity of the disease. Notably, both TXNIP and RAGE may exacerbate injury and inflammation when chronically activated, while they mediate neuronal repair when transiently expressed [81–83]. Therefore, the RAGE-TXNIP axis participates in AD progression by activating a concerted action of oxidative stress, inflammation, vascular dysfunction, and neurodegeneration. Thus, inhibition of chronic activation of RAGE and TXNIP might efficiently provide neuroprotection in AD [82].

Differently from RAGE, a protective role has been ascribed to its secreted isoform, sRAGE. sRAGE lacks the transmembrane domain and is present in human plasma, functioning as a "decoy," binding Aβ in plasma and preventing neurotoxic or proinflammatory responses of RAGE–Aβ interaction in microglia and neurons [77, 85].

In addition, some authors have proposed an involvement of the imbalance in AGE clearance in AD pathology. The serum level of AGEs is the result of their endogenous production, exogenous dietary intake, and renal clearance. Several enzymes (glyoxalase I and II and carbonyl reductase) and a specific receptor (AGER1) are also involved in the detoxification system against the prooxidant effects of glycation [67, 68]. Interestingly, in the early stage of AD, glyoxalase I is upregulated in order to maintain α-oxoaldehyde products at a physiological level, while in the late stage the enzyme is decreased. The correlation between AGE deposits and glyoxalase I expression has been further demonstrated in both age- and AD-affected brains [86].

Food, as both source of bioactive nutrients and reservoir for potential toxic compounds, can have a dual role in AD pathology (Figure 1). All these findings indicate that AGEs can be considered as dietary risk factors not yet recognized and important pathogenic mediators involved in AD. The discovery of natural or pharmacological AGE inhibitors and

the adoption of an AGE-restricted diet might be further new challenges, in order to promote a healthy ageing status and prevent cognitive decline exacerbation.

5. Dietary AGEs and Alzheimer's Disease: Association or Causality

Since Uribarri and colleagues investigated AGE content in more than 500 dietary compounds [87, 88], cohort studies, investigating how defined dietary patterns affect AD incidence, may be revisited to extrapolate the correlation between dietary AGE content and AD progression. Perrone and Grant [89] in a very interesting ecological and observational study demonstrated that both the Mediterranean diet (MeDi) and the traditional Japanese diet help in preventing AD. Although the traditional Japanese diet differs markedly from the MeDi, it is also low in meat and dairy products, which contain a high level of AGEs, suggesting a strict correlation among lower meat intake, poor AGEs dietary, and reduced risk to develop AD [36, 89]. In Japan, the nutritional transition from the traditional Japanese diet to the Western diet in the last 25 years has led to an improved meat consumption with enhancement of meat-AGEs from 24% to 52% of the total dietary AGEs, while the AD prevalence increased from 1% to 7% in people over 65 years [89–92]. However, whether the increase of Japanese AD cases could be due to the enhancement of meat consumption still has to be well clarified. In fact, it cannot be excluded that in the different examined cohort studies other risk-modifying factors (trace minerals in the brain [93], obesity rates [94], vitamin D concentrations [95], physical activity levels [96], and alcohol consumption rates [97]) could be also changed.

To evaluate whether dietary AGEs contribute to AD development or are just causally linked to risk of AD, Hill's criteria for causality in a biological system have been examined [89]. These criteria consider the strength of association, consistent findings in different populations, temporality, biological gradient, plausibility (e.g., mechanisms), and experiment (e.g., randomized controlled trials or animal model studies) [89, 98]. In the context of dietary AGEs and AD risk, several of Hill's criteria have been satisfied and clearly indicate that AGEs, mostly in association with increased meat consumption, can be considered risk-modifying factors for AD pathology [36, 89].

6. Good Tips for a Healthy Low-AGE Diet

Many studies have demonstrated that a high-AGE diet promotes oxidative stress and increases proinflammatory markers in chronic conditions and neurodegenerative diseases. The potential benefits of a restricted AGE diet are promising and could offer a simple alternative therapy in the prevention and treatment of these conditions. Over the past decade, several clinical trials have been performed demonstrating that the application of an AGE-restricted diet reduces not only the systemic levels of AGEs but also the levels of markers of oxidative stress and inflammation [87, 88]. The first line of action is to implement the use of food with the lowest AGE content—mainly food composed of carbohydrates (e.g., starches, fruits, and vegetables) instead of full-fat cheeses, meats, and highly processed foods. For the purpose of estimating dietary AGE intake, a large database has been published of the AGE content of the most common foods [88]. Currently, no official recommendations exist regarding the acceptable range or identifying the upper limit of dietary AGE intake. Different studies have shown that the average intake is nearly 15,000 KU/day in healthy individuals [99]. Uribarri et al. [100] have proposed that half of the current mean AGE intake, about 7,500 KU/day, could be a very realistic goal. A dietary AGE (d-AGEs) reduction of this magnitude has been found to significantly alter the levels of circulating AGEs and at the same time reduce levels of oxidative stress and inflammation markers, enhancing insulin sensitivity in diabetic patients [65–68]. One of the difficulties with diets at lowest AGEs content is to maintain adequate content of other nutrients, in addition to the quality of an appetizing and tasty meal. For example, the ICARE clinical study performed by Pouillart et al. [101] compared two realistic and similar diets with different AGE levels to explore the possible health impact of dietary AGEs. The low-AGE diet, achieved by adjusting fat intake and increasing quantities of cooked vegetables and steam-cooked food, reduced oxidative and inflammatory markers in healthy subjects [100].

Cooking methods and the temperature used in cooking are two variables for reducing dietary AGE intake. Meat and meat-derived products processed using high, dry heat, such as in broiling, grilling, frying, and roasting, are major sources of d-AGEs. Alternative cooking methods, such as boiling and stewing, allow daily d-AGEs ingestion to be reduced by up to 50%, while still maintaining the same primary nutrients [99]. Uribarri et al. estimated that a 90 g chicken breast has an AGE amount of 1,000 KU when boiled, while the AGE content increases up to 9,000 KU if broiled [100]. The cooking time can also influence the AGE content. We recently demonstrated that overcooking Mediterranean pasta doubled the methylglyoxal content, compared to the content achieved with the suggested cooking time [102].

On the other hand, the sparing use of herbs, condiments, and spices like curcumin, cinnamon, parsley, thyme, and clove can prevent cooking-induced AGE formation. Dearlove and colleagues [103] demonstrated that polyphenols found in culinary herbs like sage, marjoram, tarragon, and rosemary are potent inhibitors of fructose-mediated protein glycation. Spice extracts, such as cloves, ground Jamaican allspice, and cinnamon, were also found to be glycation inhibitors, and to a greater extent compared to herb extracts [103]. In addition, AGE formation can be prevented by pretreating meat with an acidic solution like vinegar or lemon juice, which interferes with the dramatic increase in AGE formation during high heat exposure. For example, beef marinated for one hour in such a solution formed less than half the amount of AGEs during cooking than untreated samples [100]. Many other antioxidant bioactive nutrients have been demonstrated to have antiglycation activity. The inhibition of glycoxidation has been showed for various polyphenols, including quercetin, genistein, tannic acid, and gallic acid [104, 105]. Therefore, the consumption of a polyphenol-rich

diet may attenuate protein glycation to some extent, and the addition of polyphenols can be useful in reducing undesired glycoxidation in food processing.

7. Conclusion

Over two thousand years ago, Hippocrates coined the phrase, "Let food be the medicine and medicine be the food." Today, that message has been reinforced by rigorous scientific evidence and observational and ecological studies. Food scientists have demonstrated the peculiar value of specific nutrients present in food for improving cognitive status and preventing dementia. Furthermore, findings that secondary products derived from cooked food can accumulate over time in the body and represent potential risk factors for Alzheimer's disease have provided newfound awareness of the importance of healthy cooking methods.

Currently, the effects of low-AGE diet in preserving cognitive ability in AD progression are not clearly understood yet. However, wishing that the reduction of dietary-glycotoxins and the intake of bioactive nutrients in preventing/delaying AD will be confirmed, nutritional intervention might be considered a promising strategy to reduce AD prevalence.

Competing Interests

The authors confirm that this article content has no conflict of interests.

Acknowledgments

This work has been supported by Ministry of University and Research (MIUR) Grants PAN LAB PON A3_00166.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8590578, 15 pages
http://dx.doi.org/10.1155/2016/8590578

Review Article

The Role of Reactive Oxygen Species in the Pathogenesis of Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease: A Mini Review

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Received 8 July 2016; Revised 6 October 2016; Accepted 13 November 2016

Academic Editor: Rodrigo Franco

Neurodegenerative diseases affect not only the life quality of aging populations, but also their life spans. All forms of neurodegenerative diseases have a massive impact on the elderly. The major threat of these brain diseases includes progressive loss of memory, Alzheimer's disease (AD), impairments in the movement, Parkinson's disease (PD), and the inability to walk, talk, and think, Huntington's disease (HD). Oxidative stress and mitochondrial dysfunction are highlighted as a central feature of brain degenerative diseases. Oxidative stress, a condition that occurs due to imbalance in oxidant and antioxidant status, has been known to play a vital role in the pathophysiology of neurodegenerative diseases including AD, PD, and HD. A large number of studies have utilized oxidative stress biomarkers to investigate the severity of these neurodegenerative diseases and medications are available, but these only treat the symptoms. In traditional medicine, a large number of medicinal plants have been used to treat the symptoms of these neurodegenerative diseases. Extensive studies scientifically validated the beneficial effect of natural products against neurodegenerative diseases using suitable animal models. This short review focuses the role of oxidative stress in the pathogenesis of AD, PD, and HD and the protective efficacy of natural products against these diseases.

1. Introduction

Neurodegenerative diseases were believed to be incurable and debilitating conditions, which primarily affected the neurons in the human brain resulting in the loss of nerve structure and function and ultimately leading to the death of nerve cells [1]. The major characteristic features of neurodegenerative diseases include ataxias (impairment in movement) and dementia (decline in memory). The three main types of neurodegenerative diseases that affect the life quality and life span of the elderly include Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) [2, 3].

2. Alzheimer's Disease

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders affecting the elderly population worldwide [4]. The specific pathological lesions that were noticed in AD include deposition of amyloid beta protein, neuronal and/or synaptic loss, and brain atrophy in specific brain areas [5]. Both the neocortex and hippocampus are affected and brain plaques and tangles are the major features of AD. AD symptoms usually start with mild confusion and amnesia and end with a dramatic personality change. AD destroys memory and other important mental functions.

Other signs of AD include finding the right words, vision/spatial issues, and impaired reasoning or judgment [6]. Worldwide, around 16 million peoples are affected by AD and over four million Americans are currently affected, a figure that may rise further due to the increase in the life span [7]. This age-related, progressive, neurodegenerative disorder is the fourth leading cause of death in developed nations and accounts for 70% of dementia in the elderly population [8]. It has been suggested the incidence of AD could double every five years beyond the age of 65 [9]. Currently available medications only treat the symptoms of neurodegenerative diseases.

3. Oxidative Stress and Alzheimer's Disease

The etiology of AD is multifactorial. Both genetic and environmental factors are regarded as a risk factor of AD [9, 10]. Free radicals are chemical species with an unpaired electron and are formed during both physiological and pathological processes. Although reactive oxygen species (ROS) play a pivotal role in several cellular and signaling pathways at physiological concentrations (cell cycle regulation, phagocytosis, and enzyme activation), excessive generation of ROS leads to several harmful effects including DNA, lipid, and protein damage [11–14]. ROS are, however, scavenged by defence mechanisms, known as enzymatic and nonenzymatic antioxidants. An imbalance in this oxidant-antioxidant status could determine the extent of cell damage. Oxidative damage due to ROS has been implicated in the pathogenesis of neurodegenerative diseases, cancer, diabetes, and aging [15].

Mitochondrial dysfunction and enhanced apoptosis accompanied by a poor antioxidant status are the mechanisms for AD pathogenesis. Extensive studies pointed out the role of superoxide anion, hydroxyl radical, hydrogen peroxide, and nitric oxide in the oxidative stress mediated neurodegeneration in AD [16, 17]. Microglia activation due to neuronal lesions generates excessive superoxide radicals [18]. Higher metabolic demand and the postmitotic nature of glial cells and neurons make them more susceptible to oxidative stress. The low rate of brain regeneration and insufficient antioxidant potential in the brain further favors oxidative stress [19]. Mitochondrial autophagy serves as a major source of ROS production [20].

Aβ(1-42) has been recognized as a key factor in the neurodegeneration in AD patients and it mediates its harmful effect via inducing oxidative stress in the brain [21–23]. A positive association has been shown between the amyloid plaque and the lipid peroxidation markers such as 4-hydroxynonenal and malondialdehyde [24]. Elevated lipid peroxidation and insufficient enzymatic and nonenzymatic antioxidants were shown in the peripheral tissues of AD patients [25, 26]. A large number of studies have shown an elevated level of lipid peroxidation marker in the brain of AD patients, especially in the region of the temporal lobe [27–29]. An increased level of 4-hydroxynonenal, the byproduct of oxidative stress, has been reported as well [30, 31]. Iron-induced oxidative stress, as evidenced by iron accumulation in the brain of AD, is responsible for neurodegeneration in patients diagnosed with AD [32]. Profound studies explored iron accumulation in the brain of AD patients and found that, as a transition metal, it is capable of generating hydroxyl radical through the Fenton reaction [33, 34]. β-Amyloid could elevate oxidative stress mainly by binding with iron [35]. The neuronal death occurs due to reactive oxygen species mediated changes in the neuronal lipid molecules, which includes alterations in the membrane, fluidity, rigidity, permeability, and transport [36]. It has been noticed that the entorhinal cortex and CAI region of the hippocampus are the two major susceptible cerebral regions to oxidative stress [37]. Mitochondrial damage in AD could lead to excessive generation of ROS and lowered ATP production [38, 39]. Vitamin E, the major lipid-soluble nonenzymatic antioxidant, inhibits oxidative damage induced by Aβ(1-42) [40]. Diminished levels of reduced glutathione in astrocytes have been reported [41]. Melo et al. [42] suggested that addition of antioxidants inhibited the activity of acetylcholine esterase in the neuronal culture. Also, superoxide dismutase activity was shown to have increased in the CAI regions of hippocampus and amygdale [43]. The causes of oxidative stress in AD are given in Figure 1.

4. Natural Products and Alzheimer's Disease

Medicinal plants serve as a good source for the treatment of several illnesses, including neurodegenerative diseases, diabetes mellitus, and cancer [44, 45]. A large number of therapeutic medicines recommended worldwide for several diseases have been identified from medicinal plants. Indian traditional medicine has recommended several medicinal plants for the treatment of neurodegenerative diseases. In traditional medicine, several plants have been used to treat the symptoms of neurodegenerative diseases. A large number of studies scientifically validated the beneficial effects of natural products in the treatment of AD using suitable animal models [46, 47].

Veerendra Kumar and Gupta [48] explored the neuroprotective effect of aqueous extract of *Centella asiatica* in a streptozotocin model of AD in rats. They suggested that *Centella asiatica* reduced the oxidative stress as well. Dhanasekaran et al. [49] pointed out the neuroprotective role of *Centella asiatica* in B6C3-Tg(APP_{swe}, PSEN1dE9)85 Dbo/J (PSAPP) mice. They concluded that the antioxidant role of *Centella asiatica* modulated the amyloid pathology in PSAPP mice. Clementi et al. [50] suggested that *Aloe arborescens* exerted a significant neuroprotective effect in IMR-32 cells via reducing the oxidative stress in the cells. Gong et al. [51] suggested the lotus seed pod *Proanthocyanidins* was a promising candidate for the treatment of AD as it exhibited a significant protective effect against cognitive impairment and brain aging induced by D-galactose. Turgut et al. [52] proposed oxidative stress reduction as a major mechanism for the neuroprotective effect of *Capparis spinosa L.* in D-galactose-induced cognitive impairment. Yu et al. [53] demonstrated the neuroprotective role of rutin against amylin-induced neurocytotoxicity in neuronal cells and concluded that the antioxidant property of rutin might have played a role in the protection of neuronal cells. Mairuae et al. [54] showed the in vitro neuroprotective effect of okra in SH-SY5Y cells and suggested that the antioxidant effect of okra was responsible

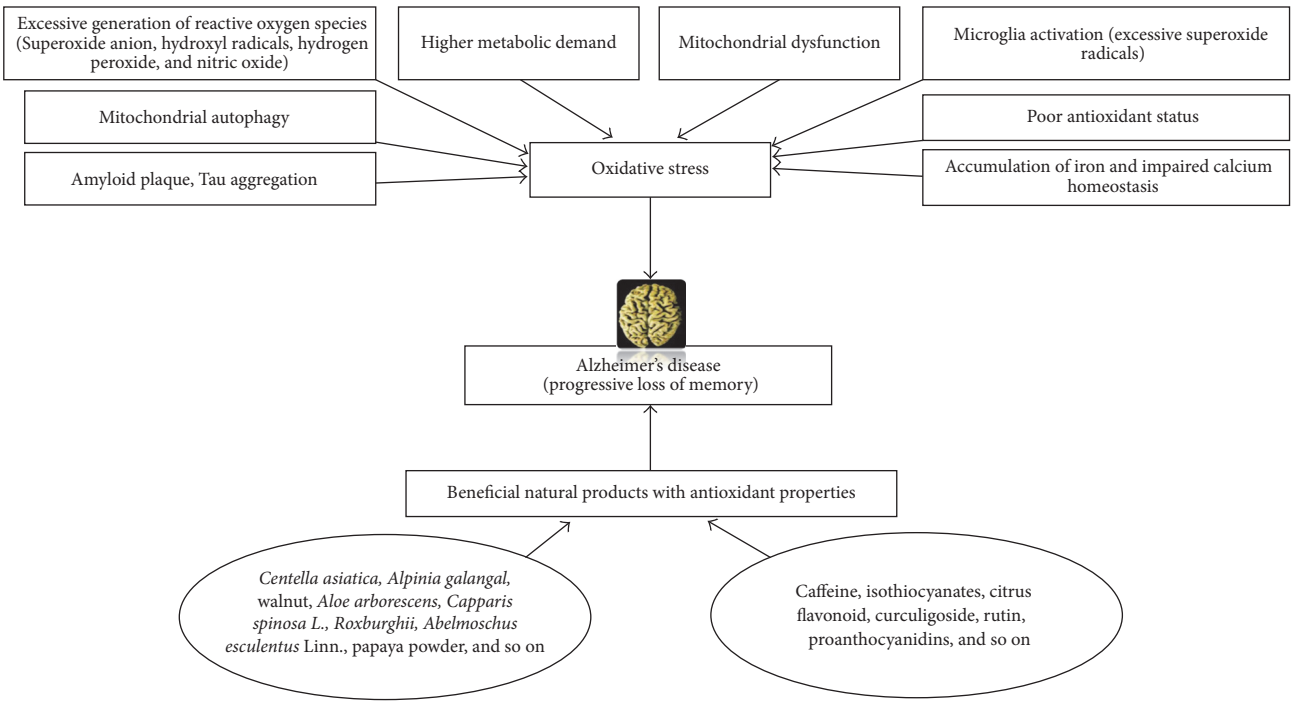

Figure 1: The causes of oxidative stress in Alzheimer's disease.

for the protective role. Uddin et al. [55] pointed out that the potent phenolic antioxidants present in the *Vanda roxburghii* could be responsible for the inhibition of the activation of acetylcholinesterase and butyrylcholinesterase. Barbagallo et al. [56] suggested that fermented papaya powder counteracted the excessive generation of reactive oxygen species in patients diagnosed with AD. Lu et al. [57] explored the protective role of *Rhubarb* extract against irradiation-induced apoptotic neuronal cell death and excessive ROS generation.

Giacoppo et al. [58] revealed the neuroprotective effect of isothiocyanates by highlighting their antioxidant potential as a major mechanism. Zhao et al. [59] demonstrated the neuroprotective effect of curculigoside on memory impairment in APP/PSI mutated transgenic mice. They suggested that its antioxidant character played a major role. Muthaiyah et al. [60] reported that walnut extract has the ability to counteract amyloid beta peptide-induced oxidative stress in PC12 cells. Hartman et al. [61] pointed out that the antioxidant polyphenolic substances of pomegranate juice reduced amyloid load and improved behavior in an AD mouse. Subash et al. [62] suggested that dietary supplementation of dates and figs improved cognitive and behavioral deficit via maintaining oxidant-antioxidant balances in APPsw/Tg 2576 transgenic AD mice. Nakajima et al. [63] suggested that nobiletin significantly reduced oxidative stress and improved the cognitive impairment in a 3XTg-AD mouse model. Sun et al. [64] proposed antioxidant potential of saponin as one of the mechanisms involved in neuroprotection. Prasanthi et al. [65] showed that caffeine reduced the oxidative stress and improved the cognitive deficits induced by cholesterol-enriched diet in rabbit hippocampus. Boyd-Kimball et al. [66] reported that glutathione upregulation protected neuron against oxidative stress and neurotoxicity induced by A(1-42) in the AD affected brain. Hanish Singh et al. [67] reported that ethanolic extract of *Alpinia galangal* improved the antioxidant status and inhibited the acetylcholine esterase activity in AD mice. Our research group from Oman reported the beneficial effects of natural products including pomegranate and figs on AD transgenic mice models [62, 68–77].

5. Parkinson's Disease

Parkinson's disease (PD), the most common neurodegenerative disease of the elderly, is characterized by progressive loss of muscle control. Premature death often results due to complications such as movement impairment-related injuries or pneumonia [78, 79]. PD is predominant at the 6th decade of life and men are 1.5 to 2 times more likely to contract the disease than women [80]. Head trauma, illness, or exposure to environmental toxins is identified as a risk factor. This neurodegenerative disorder is characterized by tremor, rigidity, bradykinesia, and impairment in balance [81]. PD also causes cognitive, psychiatric, autonomic, and sensory disturbances. Cognitive impairments are common in a large fraction of patients with PD at initial diagnosis and afflict a majority of patients as the disease progresses. The secondary manifestation includes anxiety, insecurity, stress, confusion, memory loss, constipation, depression, difficulty in swallowing and excessive salivation, diminished sense of smell, increased sweating, erectile dysfunction, skin problems, and a monotone voice [82, 83].

The pathology of PD is characterized by the gradual and selective loss of dopaminergic neurons in the substantia nigra pars compacta. Imbalance in dopamine metabolism

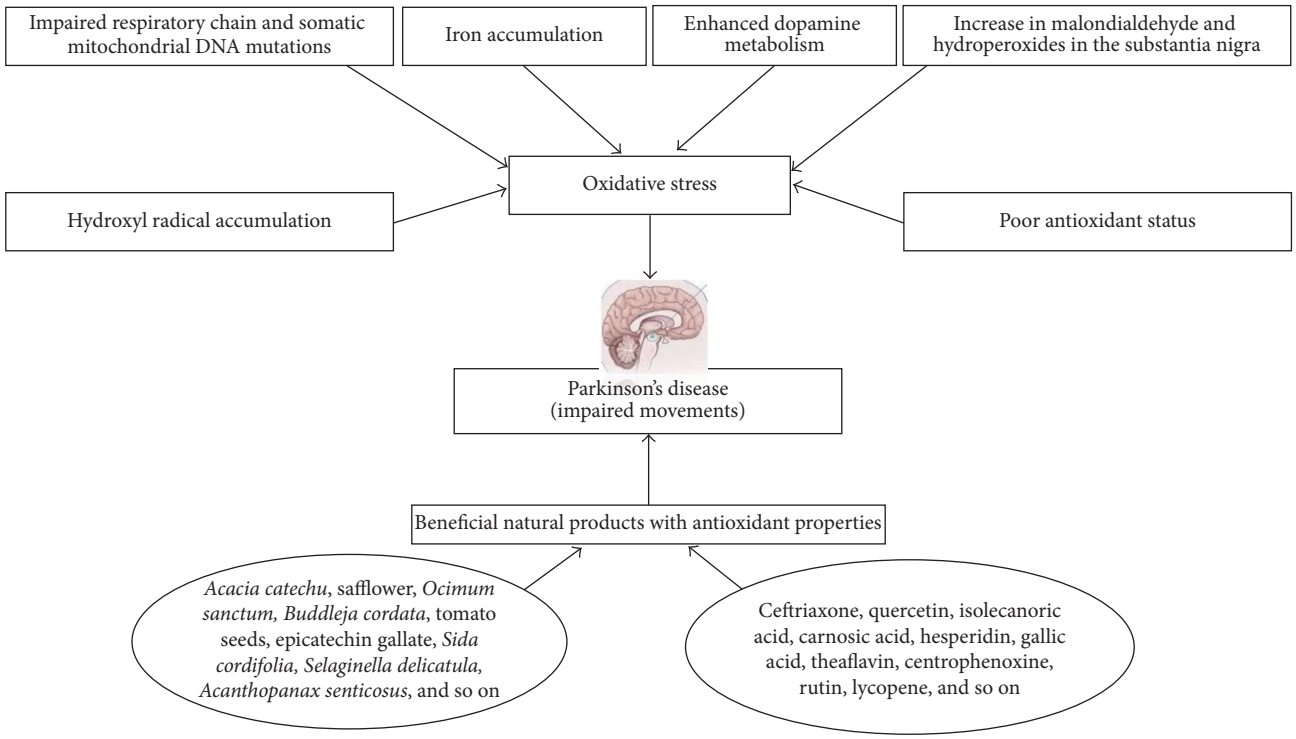

Figure 2: The causes of oxidative stress in Parkinson's disease.

due to oxidative stress has been recognised as a contributor to this disease [84]. The major pathological findings include the presence of Lewy bodies in the substantia nigra and loss of nerve cells in the portions of its ventral tier [85]. The treatment modality for PD involves either enhancing the activities of dopaminergic neuron activity or inhibiting the cholinergic effects to the stratum. While there is no cure for PD, medications provide dramatic relief from the symptoms. Recent advancement in medical and surgical treatment options has enormously improved the quality and length of life for patients with PD [86]. Worldwide, it is the second most common neurological disease and affects around 1.5 million Americans [87]. It has been pointed out that PD may double over the next 25 years in the United States and more than double in the developing nations of Asia and South America [88]. Research has indicated that 80% of the untreated PD patients die within 10 to 14 years after the onset of the disease [89].

6. Oxidative Stress and Parkinson's Disease

The brain utilizes around 20% of the basal oxygen from the total oxygen supplied to the human body. ROS mediated oxidative DNA damage is one of the prominent features in PD [90]. Several studies have reported impaired respiratory chain and somatic mitochondrial DNA mutations in the brain of patients with PD, which suggests the extensive role of oxidative metabolism in PD [91]. Enhanced dopamine metabolism in the brain of patients with PD could account for the accumulation of toxic radicals such as hydroxyl in the brain [92]. Iron accumulation in the neurons in the redox active form plays a crucial role in pathogenesis of this disease [93]. Accumulation of iron has been reported in the substantia nigra in patients diagnosed with PD, which suggests the critical role of iron-induced lipid peroxidation in pathogenesis of PD [94–96]. The accumulation of lipid peroxidation byproducts has been reported in the serum and cerebral spinal fluid of patients with PD [97] while an increase in malondialdehyde and hydroperoxides has been reported in the substantia nigra of patients diagnosed with PD [98, 99].

Elevated levels of malondialdehyde, thiobarbituric acid reactive substance, and 4-hydroxy-2,3-nonenal have been reported in the substantia nigra and stratum of PD brains [100, 101]. A twofold increase in protein oxidation has been shown in the substantia nigra of PD patients compared to healthy subjects [102]. Accumulation of hydroxyl radical due to lowered glutathione content in the brain has been reported in PD patients [103]. Lowered activities of antioxidant enzymes and nonenzymatic antioxidants could be responsible for the progression of PD [104, 105]. Reduced glutathione and increased oxidized glutathione levels have been reported in PD patients while lowered glutathione content in the substantia nigra, due to neuronal loss, has been reported in patients with PD [106–109]. Decreased activity of glutathione peroxidase and a decline in glutathione content have been reported in the brain of PD patients and reduced glutathione content was found to be decreased in both human and experimental models of PD [110–112]. Lowered GSH content was reported in the substantia nigra and corpus striatum of PD patients [113]. The causes of oxidative stress in Parkinson's disease are given in Figure 2.

7. Natural Products and Parkinson's Disease

Extensive studies scientifically explored the protective effect of natural products against Parkinson's disease using suitable animal models. Weng et al. [114] reported that ceftriaxone prevented the loss of neuronal activity and decreased the neurogenesis in the brain of PD rats. Sharma et al. [115] suggested that administration of quercetin attenuated the neuronal death and reduced the oxidative stress in aluminium-induced neurodegeneration in the rat hippocampus. Saha et al. [116] explored the antineurogenic and antioxidant potential of *Acacia catechu* leaf extract using in vitro studies. Ren et al. [117] reported that safflower flavonoid extract could be used as the herbal therapy for PD treatment. De Pedro et al. [118] explored the in vitro protective effect of isolecanoric acid against the PD development. Wu et al. [119] investigated the neuroprotective effect of carnosic acid against 6-hydroxydopamine induced neurotoxicity. They concluded that the antioxidant and antiapoptotic potential of carnosic acid could play a protective role in the prevention of neurodegeneration. Siddique et al. [120] demonstrated the neuroprotective effect of *Ocimum sanctum* leaf extract in the transgenic Drosophila model of PD. Antunes et al. [121] suggested that hesperidin attenuated 6-hydroxydopamine induced oxidative stress in aged mice. Pérez-Barrón et al. [122] explored the antioxidant and neuroprotective effect of *Buddleja cordata* methanolic extract in the 1-methyl-4-phenylpyridinium induced PD rat model. Beppe et al. [123] suggested that the aqueous extract of *Albizia adianthifolia* leaves possesses antioxidant potential, which was responsible for the memory-enhancing activities in the rodent model of PD.

Gokul and Muralidhara [124] reported that tomato seeds alleviated motor abnormality, oxidative impairments, and neurotoxicity in a chronic ROT model of neurotoxicity in mice. Siddique et al. [125] reported that epicatechin gallate dietary supplementation reduced the oxidative stress and apoptosis in the brain of transgenic Drosophila model of PD. Khurana and Gajbhiye [126] showed the ameliorative effect of *Sida cordifolia* against rotenone-induced oxidative stress and neurochemical and behavioral alterations in a rat model of PD. Chandran and Muralidhara [127] showed the neuroprotective effect of aqueous extract of *Selaginella delicatula* in a chronic ROT exposure model of neurotoxicity in mice. They suggested that the neuroprotective property of Selaginella delicatula is largely attributed to the antioxidant properties. Prakash et al. [128] demonstrated the neuroprotective role of *Withania somnifera* root extract in parkinsonism mice. They suggested that *Withania somnifera* extract improved the behavioral, anatomical, and biochemical deformities. Mansouri et al. [129] suggested that the neuroprotective effect of oral gallic acid is due to the enhancement of cerebral antioxidant defense against oxidative stress induced by 6-hydroxydopamine in rats. Shalavadi et al. [130] suggested that the neuroprotective effect of the methanolic extract of *Stereospermum suaveolens* DC could be attributed to its antioxidant potential in 6-OHDA induced PD rats. Liu et al. [131] explored the neuroprotective effect of *Acanthopanax senticosus* in PD. Anandhan et al. [132] suggested that the neuroprotective effect of theaflavin may be due to its antioxidative and antiapoptotic activities in chronic MPTP/probenecid induced PD. Some of our research group members reported the beneficial effects of natural products on PD animals [133–137].

Ahmad et al. [138] pointed out that the antioxidant efficacy of sesame seed oil is responsible for the neuroprotective effect in 6-hydroxydopamine induced neurotoxicity in mice. Martins et al. [139] demonstrated the protective effect of *Melissa officinalis* in manganese-induced oxidative stress in chronically exposed mice. They concluded that the antioxidant potential of this plant is responsible for the neuroprotective effect. Hritcu et al. [140] pointed out that the methanolic extract of *Hibiscus asper* leaves exerted neuroprotective activity through antioxidant and antiapoptotic activities in PD model. Ranpariya et al. [141] suggested that the antioxidant potential of *Matricaria recutita* could be largely attributed to its neuroprotective activity against fluoride-induced stress in rats. Wang et al. [142] suggested that the free radical scavenging activity of resveratrol protected the abnormal rotational behavior and the loss and apoptosis of nigral cells in Parkinsonian rats. Verma and Nehru [143] demonstrated the antioxidant effect of centrophenoxine against rotenone-induced oxidative stress in PD rodent. Kaur et al. [144] demonstrated the beneficial effect of lycopene in rotenone-induced model of PD. They suggested that the therapeutic potential of lycopene is attributed to its antioxidant efficacy. Khan et al. [145] pointed out that rutin can protect dopaminergic neurons from oxidative stress in a PD rat. Essa et al. [146] suggested that walnut partially reversed MPTP-induced neurodegeneration in a mouse model of PD. They suggested that the antioxidant role of walnut might have played a neuroprotective role. Jahromi et al. [147] suggested that the antioxidants present in the *Decalepis hamiltonii* roots attenuated neuromotor deficits in transgenic Drosophila model of PD.

Tseng et al. [148] showed the protective effect of *Liuwei Dihuang* in Parkinson's toxin-induced dopaminergic neurodegeneration. Guo et al. [149] suggested that tetramethylpyrazine nitrone rescued dopaminergic neurons by reducing ROS and increasing cellular antioxidative defense capability in the animal models of PD. Sudati et al. [150] concluded that *Valeriana officinalis* improved the antioxidant defence mechanism in the rotenone-induced toxicity in Drosophila melanogaster. Pasban-Aliabadi et al. [151] suggested that the protective effect of olive *(Olea europaea* L.*)* leaf extract in the 6-hydroxydopamine-induced PC12 cell apoptosis is due to their antioxidative and antiapoptotic properties. Kim et al. [152] explored the neuroprotective role of *Rhus verniciflua* in rotenone model of PD via its antioxidant efficacy. Li and Pu [153] reported that kaempferol inhibited MPTP induced oxidative stress in the mouse model of PD. Liang et al. [154] pointed out that tenuigenin exhibited potent neuroprotective effect through antioxidant potential in a SH-SY5Y cell model with 6-OHDA-induced injury. Hu et al. [155] showed that the ginseng attenuated (MPP(+)) induced cytotoxicity in SH-SY5Y cells through its antioxidant potential. Choi et al. [156] suggested that *Polygalae Radix,* through its antioxidant and antiapoptotic efficacy, inhibited the neuronal death in PD models. Sengupta

et al. [157] reported that the hydroxyl scavenging potential of *Hyoscyamus niger* seeds is responsible for its neuroprotective effect.

An et al. [158] reported that *Acanthopanacis senticosus* prevented the MPP+ induced damage in PC12 cells by reducing the levels of MDA, which suggested its antioxidant potential. Kim et al. [159] pointed out that Chunghyuldan exhibited neuroprotective effect against ROS-mediated neuronal cell death in PD model. Lee et al. [160] suggested that Cyperi rhizome exhibited the neuroprotective effects through antioxidant and antiapoptotic activities in an in vitro PD model. Shu et al. [161] suggested that the neuroprotective effect of *Chuanxiong Chatiao* may be associated with its potent antioxidant efficacy in MPTP-induced Parkinson's mice. Shim et al. [162] suggested that *Uncaria rhynchophylla* exhibited neuroprotective effect through antioxidative and antiapoptotic activities in PD models. Sankar et al. [163] suggested that *Withania somnifera* root extract exhibited potent neuroprotective effect by mitigating MPTP-induced oxidative stress in PD mice. Ahmad et al. [164] showed the neuroprotective effect of *Delphinium denudatum* via its antioxidant property in PD rats. Ahmad et al. [165] reported that *Nardostachys jatamansi* attenuated 6-hydroxydopamine-induced parkinsonism in rats via antilipid peroxidative potential. Zhang et al. [166] explored the neuroprotective effect of *Forsythia suspensa* with antioxidant property in an experimental model of rotenone-induced neurotoxicity. Lu et al. [167] suggested that resveratrol showed a neuroprotective effect in MPTP-induced parkinsonism through free radical scavenging potential. A large number of experimental studies on neurodegenerative diseases highlighted curcumin as a potent neuroprotective agent [168]. Braidy et al. [137] explored the neuroprotective effect of pomegranate extract in MPTP induced oxidative stress in human primary neurons.

8. Huntington's Disease

Huntington's disease (HD) is a devastating familial and inherited disease characterized by the progressive loss of brain and muscle function. It occurs due to the genetically programmed degeneration of neurons, which causes uncontrolled movements, loss of intellectual abilities, and emotional disturbances. HD is caused by a CAG trinucleotide expansion in exon 1 of the Huntingtin (HTT) gene, which is located on chromosome 4 (4p63) [169]. Healthy individuals have 6–35 CAG repeats, and affected individuals have more than 36 repeats. The accumulation of mutant Huntingtin proteins contains a long polyglutamine region which causes neuronal death and the degeneration of neuronal networks within the brain. The pathological changes in the cerebral cortex and striatum elicit the development of chorea and cognitive impairments and lead to premature death. There is a 50% chance that children will inherit HD from HD affected parents. Men and women are equally affected by HD which appears during 4th to 5th decade of life. The symptoms usually appear between the ages of 35 and 55. However, the age of onset and its progression varies from person to person [170]. The clinical course of HD typically progresses over 10 to 20 years from a presymptomatic state to complete disability and death. The early symptoms includes tumbling, lack of focus, concentration and movement problems, clumsiness, lapses in short-term memory, and depression. As the disease progresses, difficulty in speech, weight loss, feeding problems, swallowing difficulties, uncontrollable movements of the face, and itching and stumbling are the major symptoms. It has been estimated that around 6000 and 30,000 people are affected by HD in UK and USA, respectively [171].

9. Oxidative Stress and Huntington's Disease

The exact cause of neuronal death in HD is unknown. However, oxidative stress may play an important role. The two major factors that make the brain more prone to oxidative damage are higher lipid concentrations and high energy requirement [172]. Compelling data supports a critical role for oxidative stress in the pathogenesis of HD, a disorder caused by polyglutamine expansion in Huntingtin (Htt). mHTT proteins serve as the source of reactive oxygen species (ROS), due to a significant amount of oxidized proteins in partially purified mHTT aggregates [173]. Though oxidative damage is not much reported in the early stages of HD, it is proposed as one of the major mechanisms in HD as it progresses [174].

Elevated oxidative stress plays a critical role in the late stage of HD pathogenesis. Impairment in the electron transport chain and mitochondrial dysfunction are the major mechanisms involved in the ROS mediated etiopathogenesis of HD [175, 176]. Dysfunction in the oxidative phosphorylation components has been documented in the brain tissues of HD patients [177]. HD patients showed an increased level of oxidative stress markers accompanied by a decrease in antioxidant status compared to healthy subjects [178]. ROS mediated oxidative damage to mitochondria has been postulated as a reasonable mechanism for the defect in glucose metabolism in the brain tissue of symptomatic HD patients [179]. A positive correlation between plasma lipid peroxidation byproduct and the severity of disease in patients with HD has been shown [180]. Enhanced lipid peroxidation has been reported in patients with severe symptoms of HD [178, 181]. An increase in the plasma lipid peroxidation accompanied by reduced glutathione content has been reported in HD patients [182]. The extensive oxidative DNA damage has been reported in a HD mouse model [183, 184]. Enhanced oxidative stress and a decline in nonenzymatic antioxidants have been reported in the peripheral blood of HD patients [185]. Stoy et al. [186] reported that abnormal tryptophan metabolism with enhanced oxidative stress could be responsible for brain dysfunction in HD. Duran et al. [187] reported that symptomatic HD patients are more prone to oxidative stress than asymptomatic HD patients. The causes of oxidative stress in HD are given in Figure 3.

10. Natural Products and Huntington's Disease

Researches utilized suitable experimental models to scientifically validate the protective efficacy of natural products against HD. Oliveira et al. [188] suggested that the protective

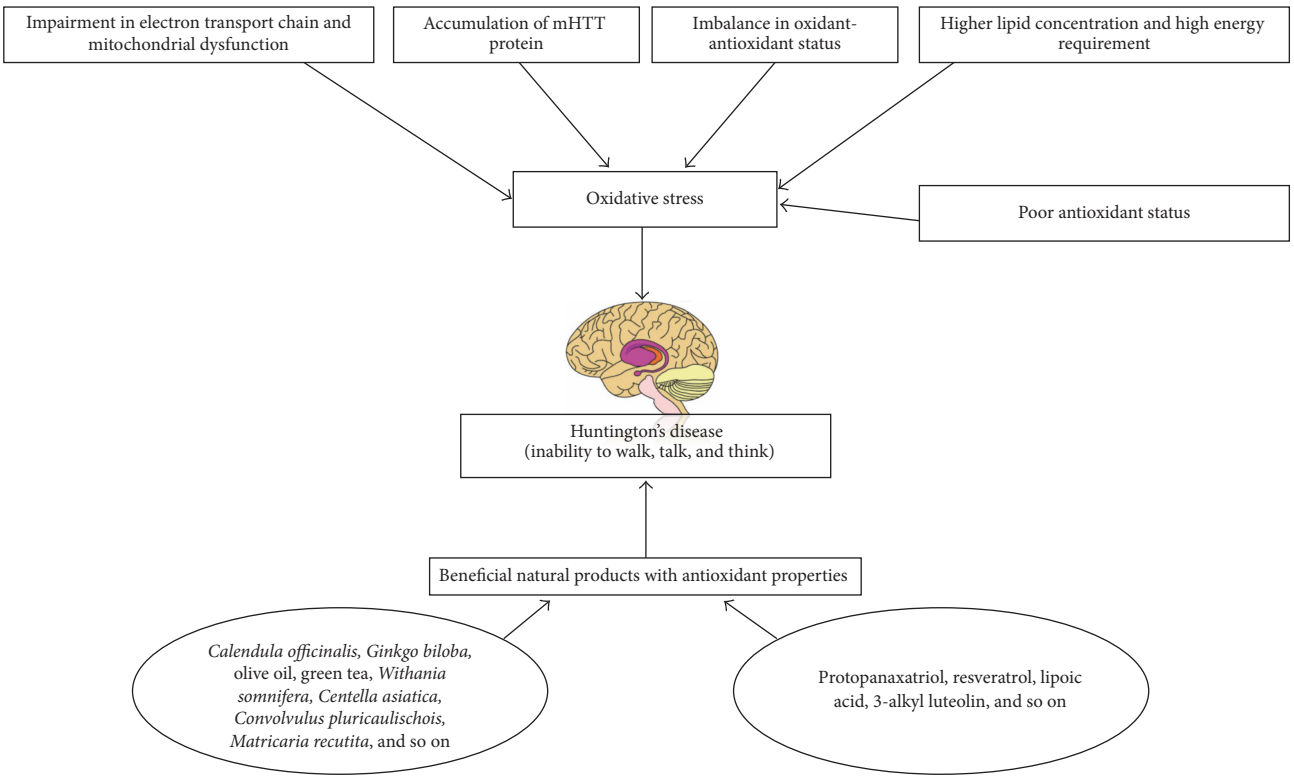

Figure 3: The causes of oxidative stress in Huntington's disease.

effect of *luteolin* derivatives on Huntington's mouse striatal cells is due to its antioxidant potential. Shivasharan et al. [189] showed the protective efficacy of *Calendula officinalis* flowers in 3-nitropropionic acid-induced HD in rats. They concluded that the anti-inflammatory and antioxidant potential of *Calendula officinalis* might have played a neuroprotective role. Mahdy et al. [190] explored the beneficial effect of *Ginkgo biloba* extract on 3-nitropropionic acid-induced neurobehavioral changes and striatal lesions. They concluded that the antioxidant and antiapoptotic potential of *Ginkgo biloba* extract might be responsible for the neuroprotective role. Tasset et al. [191] reported that olive oil reduced oxidative damage in 3-nitropropionic acid-induced HD in rats. They concluded that extravirgin olive oil and hydroxytyrosol served as a powerful brain antioxidant. Sagredo et al. [192] provided preclinical evidence for the neuroprotective effect of phytocannabinoid-based medicines in HD. Gao et al. [193] investigated the neuroprotective effect of protopanaxatriol against 3-nitropropionic acid-induced oxidative stress in experimental HD. Túnez et al. [194] showed the protective effect of melatonin in 3-nitropropionic acid-induced oxidative stress in synaptosomes in rat with HD. They concluded that melatonin modified the neural response to 3-nitropropionic acid with the antioxidative mechanism.

Rocha-González et al. [195] reported the neuroprotective role of resveratrol against HD. Andreassen et al. [196] suggested that lipoic acid, as an antioxidant, has the potential to improve the survival of transgenic mouse models of HD. Ehrnhoefer et al. [197] pointed out that green tea (-)-epigallocatechin gallate prevented the early events of HD pathogenesis such as Huntington's misfolding. Denny Joseph and Muralidhara [198] suggested that fish oil in combination with quercetin provided better neuroprotection against 3-nitropropionic acid-induced HD. Fu et al. [199] suggested that trans-(−)-ε-Viniferin could be considered as a promising candidate to treat HD, since it increased mitochondrial sirtuin 3 (SIRT3) and activated the AMP-activated protein kinase. Huang et al. [200] explored the neuroprotective role of N(6)-(4-hydroxybenzyl) adenine riboside against experimental HD. Ranpariya et al. [201] showed the neuroprotective effect of *German chamomile* against aluminium fluoride-induced oxidative stress in rats. P. Kumar and A. Kumar [202] explored the neuroprotective effect of *Withania somnifera* root extract against 3-nitropropionic acid-induced HD. They suggested that neuroprotective actions of *Withania somnifera* are mediated via its antioxidant activity. Shinomol and Muralidhara [203] reported that the prophylactic neuroprotective property of *Centella asiatica* could be related to the enhancement of GSH, thiols, and antioxidant machinery in the brain regions of 3-nitropropionic acid-induced HD prepubertal mice. Kaur et al. [204] suggested that *Convolvulus pluricaulis* exhibited a potent neuroprotective effect by accelerating the brain antioxidant defence mechanisms in 3-nitropropionic acid treated rats. Al-Sabahi et al. [205] reported the benefit of pomegranate seed oil on 3-NP induced HD.

11. Conclusion

Neurodegenerative diseases impose a significant health burden not only to the affected patients, but also to their families

and society. The incidences of these life threatening disorders are rapidly increasing in aged populations worldwide. Although several mechanisms have been postulated for the pathogenesis of neurodegenerative diseases, oxidative stress and mitochondrial dysfunctions are pointed out as a major mechanism. At present, medications are only available to treat the symptoms of neurodegenerative diseases. Several in vivo and in vitro studies have documented the protective role of various natural products or synthetic entities in the prevention of neurodegenerative diseases. However, the solution for these neurodegenerative diseases has not yet been found. Thus, researches are warranted to investigate the nontoxic active constituents found in natural resources which could correct the biochemical, metabolic, and behavioral abnormalities that occur in neurodegenerative diseases.

12. Opinion of the Authors

This review highlights the crucial role of oxidative stress in the pathogenesis of various neurodegenerative diseases. Based on the literature researched for this paper, it is clear that oxidative stress mediates its adverse effects either directly, causing neuronal damage, or by inducing the harmful effects of neurotoxicants. This review also explores the beneficial effects of various natural products against neurodegenerative diseases.

While many reports have focused on the role of protective efficacy of natural products against oxidative stress-induced neurodegenerative diseases, as yet, there have been no effective treatment solutions reported for these diseases. This indicates that the antioxidants alone are not sufficient to treat neurodegenerative diseases. Thus, intense research should be undertaken to investigate, or identify, the novel compounds that could be used to counteract the oxidative stress pathogenesis and for a better therapeutic agent for the treatment of neurodegenerative diseases.

13. Literature Search Strategy

For this study, an intense literature search on neurodegenerative diseases (AD, PD, and HD) was mainly done through PubMed articles published from 1982 to 2016. The articles were then scrutinized and the most relevant selected to write this review. We have also referred to previous review articles on neurodegenerative diseases and the references cited were also considered. The key words used to search the relevant articles included neurodegenerative diseases, Alzheimer's disease, Parkinson's disease, Huntington's disease, Reactive Oxygen Species, antioxidants, medicinal plants, and so forth.

Competing Interests

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

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 6981595, 13 pages
http://dx.doi.org/10.1155/2016/6981595

Research Article

Reversal of Trimethyltin-Induced Learning and Memory Deficits by 3,5-Dicaffeoylquinic Acid

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Received 6 July 2016; Accepted 21 November 2016

Academic Editor: Byoung-Joon Song

The antiamnesic effect of 3,5-dicaffeoylquinic acid (3,5-diCQA) as the main phenolic compound in *Artemisia argyi* H. extract on cognitive dysfunction induced by trimethyltin (TMT) (7.1 μg/kg of body weight; intraperitoneal injection) was investigated in order to assess its ameliorating function in mice. In several behavioral tests, namely, the Y-maze, passive avoidance, and Morris water maze (MWM) test, 3,5-diCQA significantly ameliorated learning and memory deficits. After the behavioral tests, brain tissues from the mice were analyzed to characterize the basis of the neuroprotective effect. Acetylcholine (ACh) levels increased, whereas the activity of acetylcholinesterase (AChE) decreased upon administration of 3,5-diCQA. In addition, 3,5-diCQA effectively protected against an increase in malondialdehyde (MDA) content, an increase in the oxidized glutathione (GSH) ratio, and a decline of total superoxide dismutase (SOD) level. 3,5-diCQA may prevent neuronal apoptosis through the protection of mitochondrial activities and the repression of apoptotic signaling molecules such as p-Akt, BAX, and p-tau (Ser 404).

1. Introduction

Alzheimer's disease (AD) is a type of dementia associated with multiple etiologies and pathogenetic mechanisms such as genetic factors, diminished cerebral energy metabolism, excitotoxic events, and free-radical-induced oxidative stress. Among them, oxidative stress is the most important cause in AD [1]. Oxidative stress occurs due to an excess of oxidants, which is caused by an imbalance between oxidants such as reactive oxygen species (ROS) and antioxidants (e.g., catalase, SOD, and GSH). Oxidants form a normal product of aerobic metabolism, but under pathophysiological conditions oxidants are produced rapidly [2]. Oxidative stress creates numerous problems, such as a decline of antioxidants and an increase of free iron in the brain, and leads to mitochondrial cytopathies [3]. Accordingly, attention to antioxidants has increased, and phenolic compounds have been studied as a source of natural antioxidants. Over 10,000 phenolic compounds are known to be present in the various plants. Polyphenols are essential to plant physiology and belong to the class of natural antioxidants [4]. According to the research by Stocker [5], dietary polyphenols have biological effects such as free-radical scavenging, metal modulation of enzymatic activity, and alteration of signal transduction.

Dicaffeoylquinic acids (diCQAs) as isochlorogenic acid, being natural phenolic compounds, are widely distributed in plants such as *Gynura* [6] and coffee bean [7]. They are esters formed from quinic acid and two units of caffeic acid. It has been reported that 3,5-dicaffeoylquinic acid (3,5-diCQA), one of the diCQA compounds, possesses strong antimutagenic [8], antioxidant [9], and anti-inflammatory activity [10]. Further, Kim et al. have reported that 3,5-diCQA possesses a cytoprotective effect against hydrogen peroxide-induced oxidative stress [11]. However, there is no research into in vivo antiamnesic effect of 3,5-diCQA, and, in

particular, the effect of 3,5-diCQA on the apoptotic signaling pathway has not yet been reported.

Trimethyltin (TMT) is a potent neurotoxicant acting in the hippocampus, and intraperitoneal injection of TMT increased ROS production rate in mice in sensitive areas such as the hippocampus and the frontal cortex, and the increased level of ROS induced oxidative damage, which contributes to activate the apoptotic signaling pathway [12]. We therefore investigated the ameliorating effect of 3,5-diCQA on TMT-induced learning and memory impairment in ICR mice through an examination of antioxidant biochemicals, mitochondrial activity, and apoptotic signaling molecules in brain tissues.

2. Materials and Methods

2.1. Chemicals and Reagents. A 3,5-diCQA (PubChem CID: 6474310) was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). TMT, dimethyl sulfoxide (DMSO), metaphosphoric acid, thiobarbituric acid (TBA), sodium azide, Triton X-100, Tween 20, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and all other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Primary antibodies for cytochrome c (sc-13560), p-tau (Ser 404) (sc-12952), and β-actin (sc-69879) were purchased from Santa Cruz Biotechnology (CA, USA). Primary antibodies for protein kinase B (Akt) (# 9272), phospho-Akt (Ser 473) (# 9271), and BAX (#2772) and secondary antibodies for anti-rabbit (7074S) and anti-mouse (7076S) were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.2. Sample Preparation and Ultra-Performance Liquid Chromatography-Quadrupole-Time-of-Flight (UPLC-Q-TOF) MS Analysis. Artemisia argyi H. (20 g) was extracted with 60% ethanol (1 L) at 40°C for 2 h using reflux extraction. The ethanolic extract was partitioned with *n*-hexane, chloroform, and ethyl acetate, consecutively, and then each fraction was concentrated and lyophilized.

Phenolic compounds extracted from *Artemisia argyi* H. were analyzed by using UPLC-Q-TOF/MS (Waters, Milford, MA, USA). The samples were dissolved in methanol after that filtered using 0.2 μm filter and then injected into an Acquity UPLC BEH C_{18} column (100 × 2.1 mm, 1.7 μm; Waters) with a flow rate of 0.4 mL/min. The mobile phases consisted of solvent A (distilled water containing 0.1% formic acid) and solvent B (acetonitrile (ACN) containing 0.1% formic acid) during analysis. The analysis conditions were as follows: a gradient elution of 100% A/0% B at 0.5 min, 0% A/100% B at 5 min, 0% A/100% B at 6.5 min, 100% A/0% B at 6.8 min, and 100% A/0% B at 9 min. Phenolic compounds were analyzed by a Q-TOF MS (Waters) in electrospray ionization- (ESI-) negative mode. The conditions used for the ESI source were as follows: ramp collision energy, 20–45 V; oven temperature, 40°C; capillary voltage, 3 kV; desolvation temperature, 350°C; pressure of nebulizer, 40 psi; fragmentor, 175 V; cone voltage, 40 V; mass range, 50–1200 m/z. All MS data obtained by MassLynx software (Waters), including retention time (RT), m/z, and ion intensity, were extracted with MarkerLynx software (Waters).

2.3. Animals and In Vivo Experimental Design. Institute of Cancer Research (ICR) male mice (age, 4 weeks) were obtained from Samtako (Osan, Korea) and were housed two per cage at a temperature of 25°C with a 12 h light-dark cycle, relative humidity of 55%, and free access to food and water ad libitum. All animal procedures were carried out as required by the "Institutional Animal Care and Use Committee" of Gyeongsang National University (certificate: GNU-131105-M0067). Mice were (orally) fed 3,5-diCQA as main phenolic compounds of *Artemisia argyi* H. at concentrations of 5 mg/kg of body weight (3,5-diCQA 5 group) and 10 mg/kg of body weight (3,5-diCQA 10 group) once a day for 3 weeks. After administration of 3,5-diCQA for 3 weeks, TMT (7.1 μg/kg of body weight) was dissolved in 0.85% sodium chloride solution and injected into mice intraperitoneally (100 μL), except for mice in the vehicle control group. Mice of TMT group were injected with only TMT.

2.4. Behavioral Tests for Learning and Memory Function. After 3 days of TMT injection, a Y-maze test was conducted. The maze was made from black Y-shaped plastic, and the arms were at an angle of 120° from each other. Each mouse could freely move in the maze for 8 min, and the sequence of arm entries was recorded with a Smart 3.0 video tracking system (Panlab, Barcelona, Spain). The percentage spatial cognition ability was calculated as follows: actual alternation/(total number of arm entries − 2) × 100 (%) [13].

After the Y-maze test, the passive avoidance test was conducted on 2 days. The apparatus consisted of a two-compartment acrylic box, one compartment illuminated and the other dark, with a gate between the two compartments. On the first day of the experiment, each mouse was placed in the lit compartment. As soon as it entered the dark compartment, it received an electrical shock (0.5 mA, 3 s). After 24 h, the latency times for entering the dark compartment were measured for maximum 300 s [14].

The Morris water maze (MWM) test was conducted in accordance with Morris but with some modifications [15]. The apparatus consisted of a large circular pool (90 cm in diameter) containing opaque water dyed with squid ink (Cebesa, Valencia, Spain) at 22 ± 2°C. The circular pool was divided into quadrants (N, S, E, and W zones) with visual cues on the walls for navigation, and a platform was located in the W zone. Training was conducted by means of four trials daily for 4 consecutive days. For each trial, mice were placed in the water facing the wall of the pool but at different start positions between trials. Mice were trained to find the platform for a maximum of 60 s. After training was complete, the platform was removed and behavior of the mice was recorded for 60 s using a Smart 3.0 video tracking system (Panlab, Barcelona, Spain).

2.5. Tissue Preparation and Biochemical Studies. After the behavioral tests, the mice were sacrificed by CO_2 inhalation, and brain tissues were kept at −80°C until they were used. Whole brain tissue was minced into small pieces and then homogenized with 10 volumes of cold phosphate buffered saline for the measurement of MDA, AChE, ACh, and total SOD levels. To measure oxidized GSH and total GSH, minced

brain tissues were homogenized with 20 volumes of 5% metaphosphoric acid.

The quantity of ACh neurotransmitter in the brain was estimated by the method of Vincent et al. Homogenates were centrifuged (14,000 ×g for 30 min at 4°C) to obtain the supernatant [16]. Alkaline hydroxylamine reagent [3.5 N sodium hydroxide and 2 M hydroxylamine in HCl] was added to the supernatant. After 1 min at room temperature, 0.5 N HCl (pH 1.2) and 0.37 M $FeCl_3$ in 0.1 N HCl were added, and absorbance was measured at 540 nm. AChE was measured via ACh hydrolyzing activity following Ellman's method [17]. Mixtures of brain homogenate and 50 mM sodium phosphate buffer (pH 8.0) were incubated at 37°C for 15 min and then added to Ellman's reaction mixture [0.5 mM acetylthiocholine and 1 mM DTNB] in a 50 mM sodium phosphate buffer (pH 8.0). Absorbance was measured at 405 nm after incubation at 37°C (20 min).

To measure MDA as a marker of lipid peroxidation, homogenates were centrifuged (6000 ×g for 10 min at 4°C), and the supernatant was mixed with 0.67% TBA solution with 1% phosphoric acid and then incubated in a water bath (95°C) for 1 h. After cooling, absorbance was measured at 532 nm [18].

Homogenates for measuring total SOD were centrifuged at 400 ×g for 10 min at 4°C, and then 10 volumes of 1x Cell Extraction Buffer [10% SOD buffer, 0.4% (v/v) Triton X-100, and 200 Mm phenylmethanesulfonyl fluoride in distilled water] were added to the pellet. Mixtures were incubated on ice for 30 min and then centrifuged at 10,000 ×g for 10 min at 4°C to obtain the supernatant. The SOD content of supernatant was measured with the SOD kit of Sigma-Aldrich Chemical Co.

Homogenates for measuring the ratio of oxidized GSH/total GSH were centrifuged at 14,000 ×g for 15 min at 4°C. Oxidized GSH/total GSH ratios in the supernatant were measured with the glutathione (oxidized GSH/total GSH) detection kit of Enzo Life Science Inc. (Enzo Diagnostics, NY, USA).

2.6. Isolation of Mitochondria from Brain Tissues and Measurement of Mitochondrial Activities. Mitochondria were isolated in accordance with the procedure of Dragicevic [19]. Whole brains were homogenized with 5 volumes of isolation buffer [mannitol (215 mM), sucrose (75 mM), 0.1% bovine serum albumin (BSA) (Bioworld, Dublin, OH, USA), EGTA (1 mM), and 20 mM HEPES (Na^+) (pH 7.2)] and then centrifuged at 1,300 ×g for 5 min. After the supernatant was obtained, it was centrifuged once more at 13,000 ×g for 10 min. After this, the supernatant was removed, and isolation buffer with 0.1% digitonin (in DMSO) was added to the pellet. After 5 min, isolation buffer was added, followed by centrifugation at 13,000 ×g for 15 min. After that, the pellets were resuspended in isolation buffer without EGTA and centrifuged at 10,000 ×g for 10 min. Finally, isolation buffer without EGTA was added to the pellet, and this was used in the experiment.

Mitochondrial ROS production was measured with 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA). 25 μM DCF-DA was added to the isolated mitochondria for 20 min and then quantified with a fluorescent (excitation filter 485/20 nm, emission filter 528/20 nm) microplate reader (Infinite 200, Tecan Co., San Jose, CA, USA) [20].

To measure the membrane potential of isolated mitochondria, 20 μL (1.2 mg/mL final concentration) of the mitochondria was mixed with assay buffer [isolation buffer without EGTA with pyruvate (5 mM) and malate (5 mM)], and then a solution of 1 μM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimi-dazolylcarbocyanine iodide in DMSO) was added. The mixture was gently stirred at room temperature for 20 min in the dark and then measured with a fluorescent (excitation 530/25 nm, emission 590/35 nm) microplate reader (Infinite 200, Tecan Co.).

Samples of isolated mitochondria were assayed for ATP content by means of the ATP bioluminescence assay kit (Sigma-Aldrich Chemical Co.). ATP concentration was calculated with the help of a standard curve.

2.7. Western Blot Analysis of the Apoptotic Signaling Pathway. Brains were homogenized with ProtinEx™Animal cell/tissue (GeneAll Biotechnology, Seoul, Korea) with 1% protease inhibitor cocktails (Thermo Fisher Scientific, Rockford, IL, USA). The protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat dry milk in TBST buffer [Tris-Buffered Saline (TBS) with 0.1% of Tween-20]. After 1 h, primary antibodies were diluted (1 : 1000) in a dilute solution (0.1% sodium azide and 0.5% BSA in TBST). Diluted primary antibodies were incubated with the membrane under gentle agitation overnight, and then the membrane was washed 3 times (10 min each time) in TBST. After washing, the secondary antibody solutions were allowed to react with the membrane for 1 h, and then the washing process was carried out once again. Finally, the membrane was exposed to an enhanced chemiluminescence reagent, and the luminescence was detected by ChemiDoc (Korea Biomics, Seoul, Korea). The density of the band was assayed with ImageJ Software (National Institutes of Health, Bethesda, Maryland, USA). The results were presented as density of target protein/density of β-actin as a loading control [21, 22].

2.8. Statistical Analysis. All results were expressed as mean ± SD. Each experiment was analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test with the help of the SAS program (Ver. 9.1 SAS Institute, Cary, NC, USA).

3. Results and Discussion

3.1. Phenolic Compounds Analysis Extracted from Artemisia argyi H. Ethyl acetate fractions from *Artemisia argyi* H., which has most abundant phenolic compounds (Supplementary Data 1 in Supplementary Material avialable online at http://dx.doi.org/10.1155/2016/6981595), were analyzed with a UPLC/Q-TOF-MS/MS system (Figure 1). Main compounds were analyzed by a Q-TOF MS system in ESI-negative mode

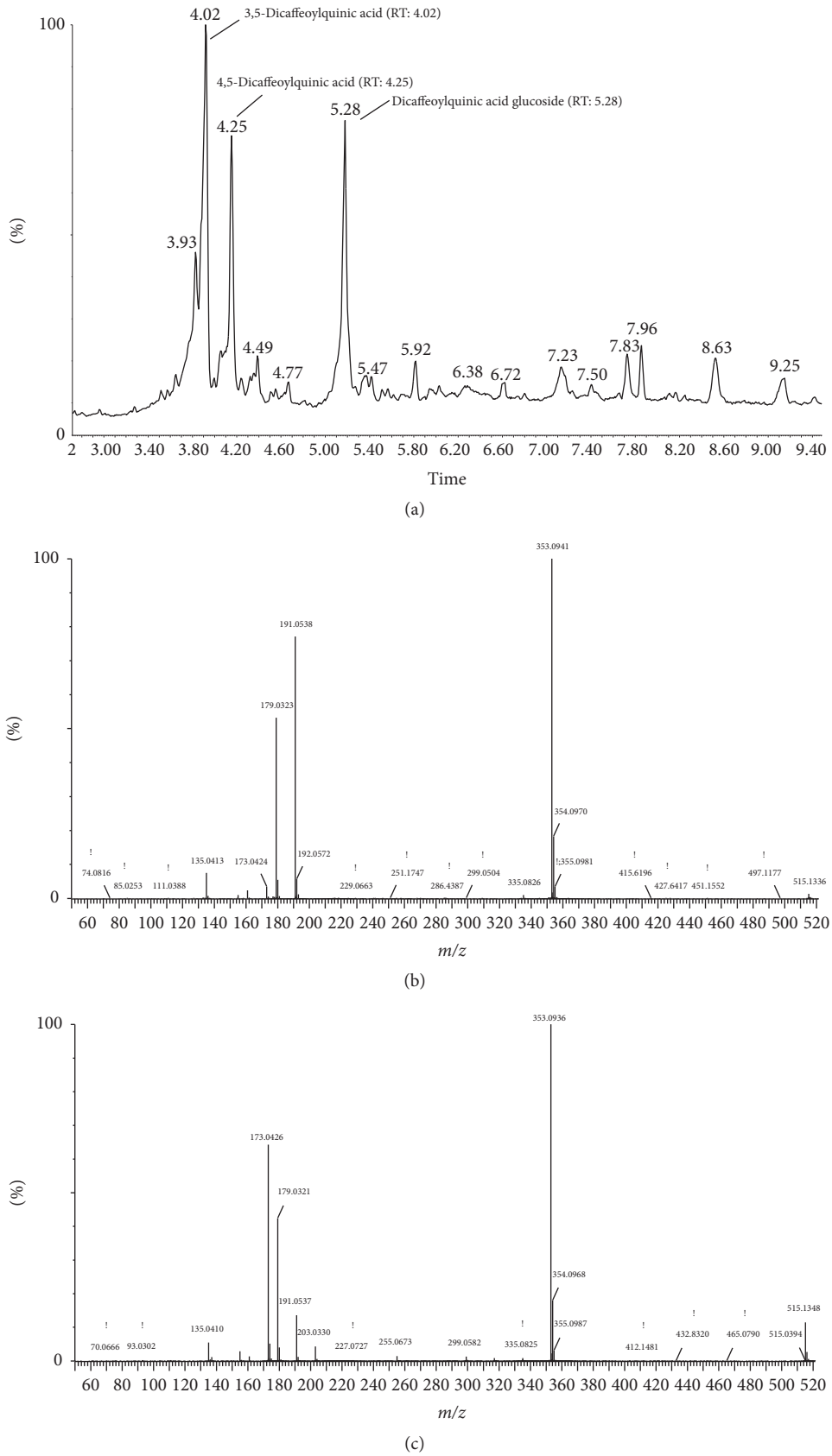
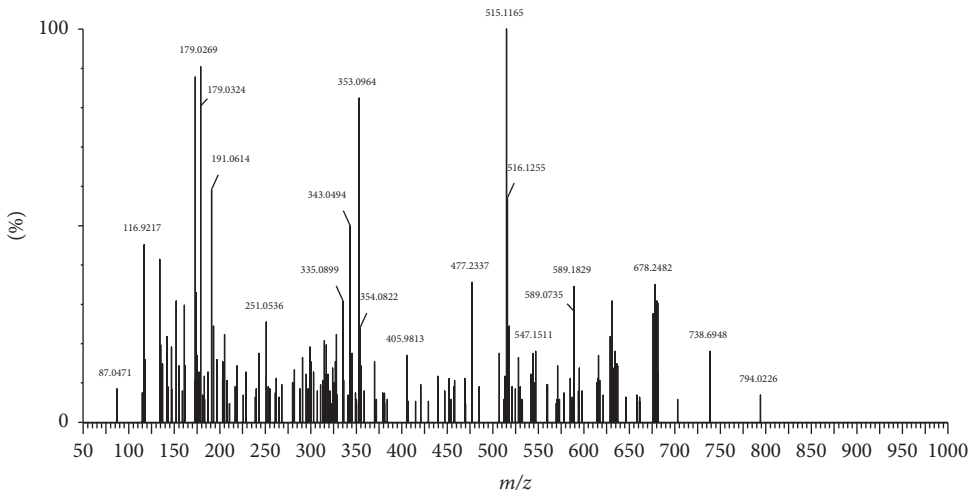


Figure 1: Continued.



(d)

Figure 1: Analysis of ethyl acetate fraction from *Artemisia argyi* H. using UPLC/Q-TOF-MS/MS chromatography in negative ion mode (a), MS^2 patterns of 3,5-diCQA (b), 4,5-diCQA (c), and diCQA-glucoside (d).

(Figures 1(b)–1(d)): compound 1 (RT: 4.02 min, 515.13 m/z); compound 2 (RT: 4.25 min, 515.13 m/z); and compound 3 (RT: 5.28 min, 677.16 m/z). Additionally, MS^2 scan chromatograms were fragmented at compound 1 (353.09, 191.05, 179.03, and 135.04 m/z), compound 2 (353.09, 173.04, 179.03, and 191.05 m/z), and compound 3 (515.12, 353.09, 179.03, and 191.06 m/z). From comparison to the main fragments in the previous literature [23], these peaks showed the 3,5-diCQA (PubChem CID: 6474310), 4,5-diCQA (PubChem CID: 6474309), and diCQA-glucoside. A 3,5-diCQA was identified as the major phenolic compound among 3,5-diCQA, 4,5-diCQA, and diCQA-glucoside, and 3,5-diCQA has a higher inhibitory effect on in vitro lipid peroxidation and AChE activity than 4,5-diCQA (Supplementary Data 2).

3.2. Effect of 3,5-diCQA on TMT-Induced Learning and Memory Dysfunction. The Y-maze test was conducted to determine the spatial cognition ability. Figure 2(a) shows the distance travelled by mice in Y-maze test. According to Kim et al., TMT induces hyperactivity disorder [24]. And as a result of hyperactivity, the TMT group exhibited a greater distance travelled than the vehicle control group. On the other hand, in the 3,5-diCQA groups the hyperactivity effect due to TMT was ameliorated. In terms of alternation behavior which is indicative of spatial cognition (Figure 2(b)), the 3,5-diCQA 10 group showed a similar result to the vehicle control group, but the 3,5-diCQA 5 group did not show a significantly different result from the TMT group. Figure 2(c) also shows the behavior pattern of each group during the experimental period, and the improvement of TMT-induced hyperactivity by treatment with 3,5-diCQA was confirmed.

In the passive avoidance test for measuring short-term learning and memory ability, the TMT group had the lowest latency time of all groups as a consequence of TMT-induced learning and memory defects. However, step-through latency increased in both 3,5-diCQA groups. In particular, the 3,5-diCQA 10 group showed a similar latency time to the vehicle control group (Figure 2(d)).

Another behavioral test, the MWM test, was conducted to assess long-term learning and memory ability. Figure 3(a) shows escape latency times recorded during sequential training trials. The vehicle control group quickly remembered the correct location of platform in the pool. The escape latency time gradually decreased, and more direct swim paths were taken than in the other groups. In contrast, the TMT group showed the smallest reduction in escape latency time during training. Both 3,5-diCQA groups exhibited improved spatial memory and learning ability, relative to the TMT group. As shown in the probe trials, the 3,5-diCQA groups spent more time in the W zone, where the platform was located, than the TMT group (Figure 3(b)). In particular, the 3,5-diCQA 10 group spent a similar amount of time in the W zone as the vehicle control group, and also both 3,5-diCQA groups exhibited improvements over the TMT-induced abnormal travel trajectories (Figure 3(c)). Consequently, a 3,5-diCQA appears to be an effective substance for ameliorating cognitive impairment induced by TMT.

3.3. Effect of 3,5-diCQA on the Cholinergic System in Brain Tissue. ACh acts as a neurotransmitter in the brain. According to the cholinergic hypothesis, reductions in choline acetyltransferase (ChAT) activity and ACh synthesis are closely related with cognitive impairments such as Alzheimer's disease (AD) [25]. Woodruff and Baisden reported that TMT led to a decrease in ChAT and an increase in AChE, an enzyme that catalyzes the breakdown of ACh and some other choline ester neurotransmitters [26]. The TMT group in our experiment similarly showed an increase in AChE activity and a decrease in ACh levels compared with the vehicle control group (Figure 4). Treatment with 3,5-diCQA significantly

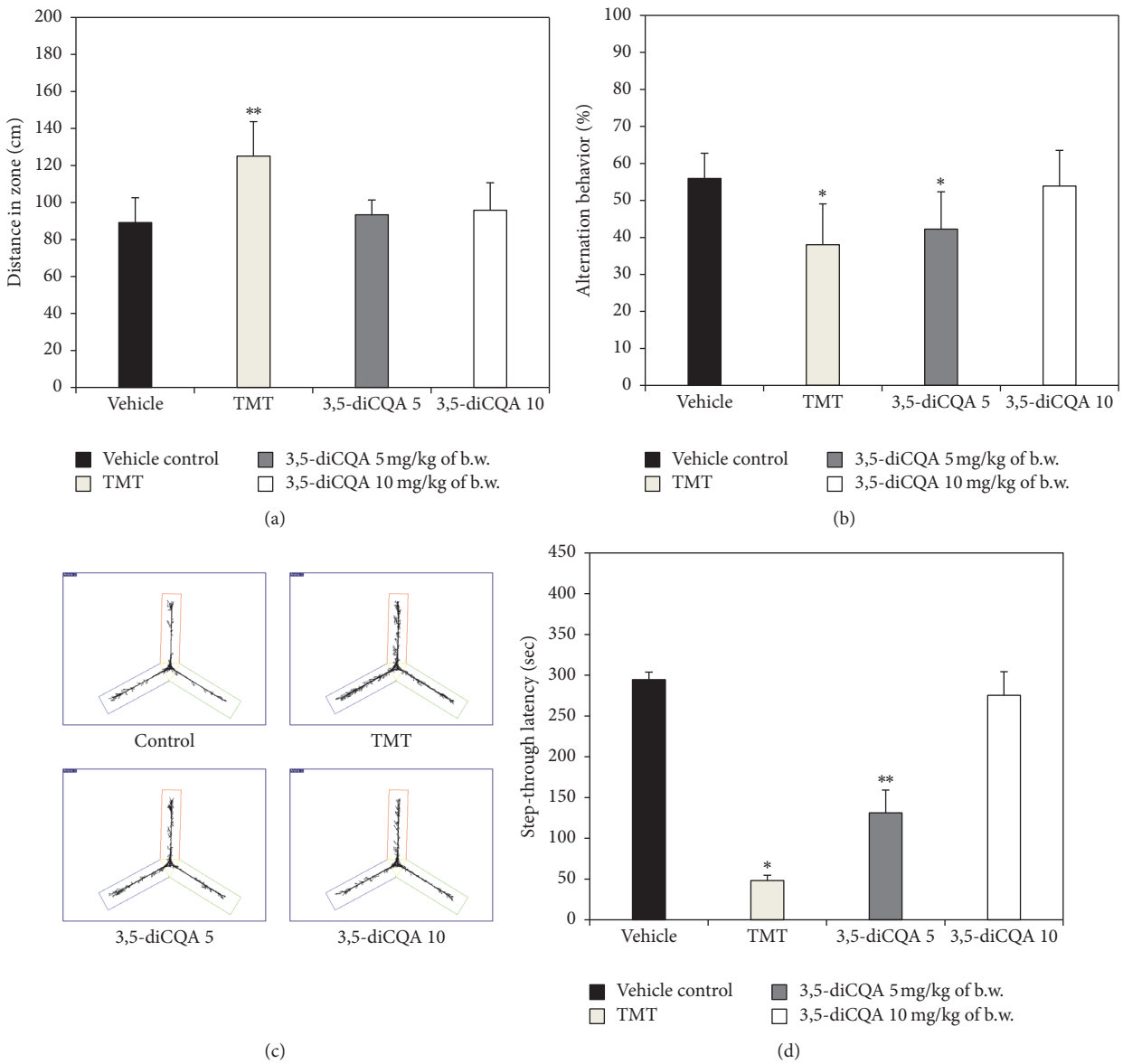


Figure 2: Effect of 3,5-diCQA on cognitive function in TMT injected mice. Distance in zone (a), alternation behavior (b), and path motion (c) in Y-maze test and step-through latency (d) in passive avoidance test. Data shown represent means ± SD ($n = 8$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the vehicle control group.

inhibited the TMT-induced impairment of the cholinergic system. AChE activity in the 3,5-diCQA 5 group was similar to the vehicle control group. In particular, the 3,5-diCQA 10 group revealed a stronger inhibitory effect on AChE activity than the vehicle control group. In the research of Kwon et al., which investigated the effect of chlorogenic acid, an isomer of 3,5-diCQA, in ameliorating scopolamine-induced amnesia in mice, chlorogenic acid significantly abated AChE activity, especially in hippocampus, and improved cognitive function [27]. The ACh levels of both 3,5-diCQA groups were similar to the vehicle control group as a result of the AChE inhibition by 3,5-diCQA. In recent times, AChE inhibitors have been used to ameliorate dementia, and AChE inhibitors such as donepezil and galantamine have been approved in the United States by the FDA to treat AD [28]. We therefore suggest that a 3,5-diCQA may also be a candidate natural AChE inhibitor.

3.4. Effect of 3,5-diCQA on Biochemical Antioxidants in Brain Tissue. MDA is used as an indicator of lipid peroxidation induced by oxidative stress. In our study, the TMT group exhibited increased MDA contents compared to the vehicle control group, whereas both 3,5-diCQA groups exhibited a reduction in MDA content compared to the TMT group (Figure 5(a)).

In aerobic cells, glutathione (GSH) is the most copious antioxidant, and GSH can be converted into its oxidized form (oxidized GSH). The oxidized GSH/total GSH ratio is often

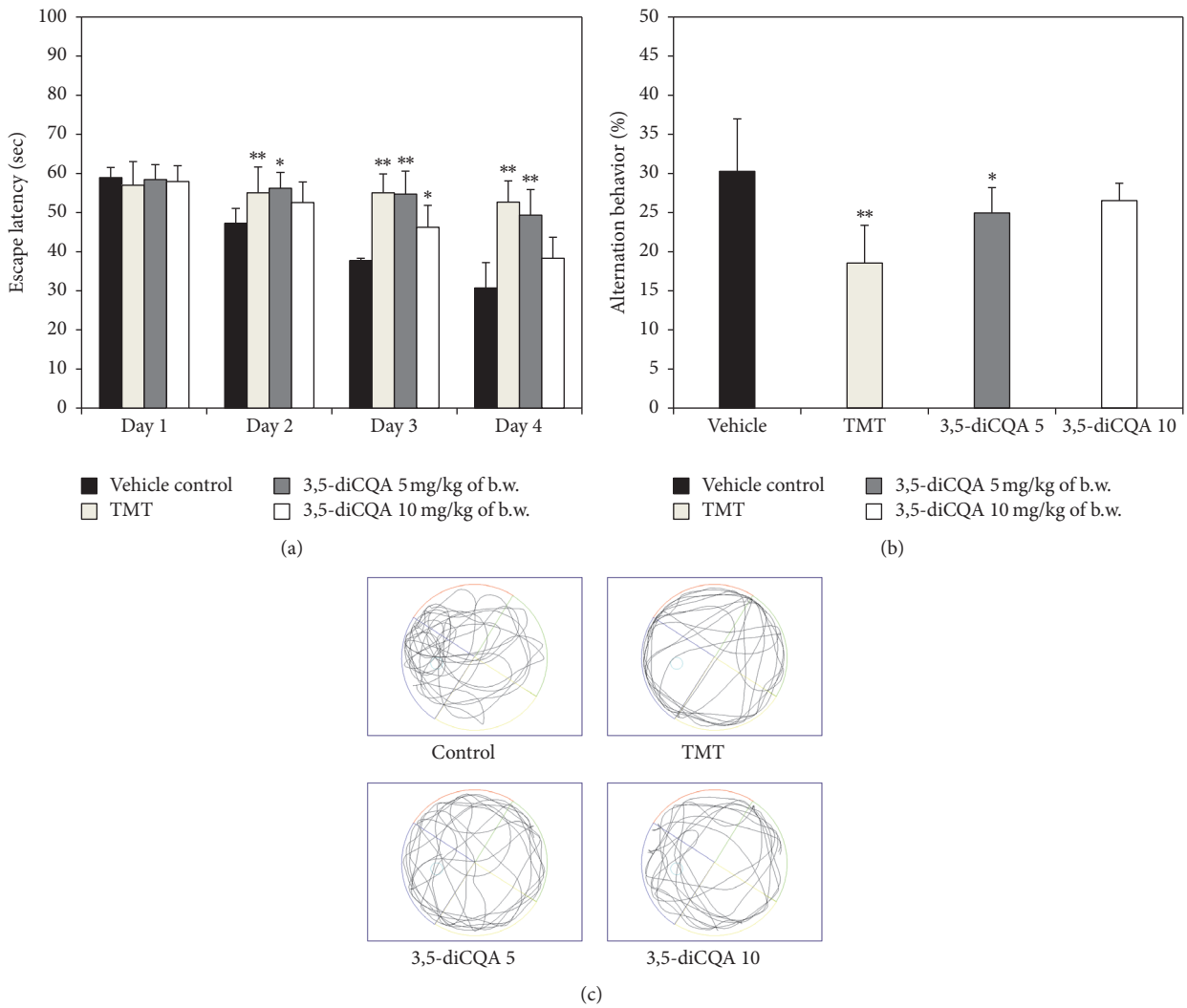


Figure 3: Effect of 3,5-diCQA on long-term memory and learning ability on TMT-induced cognitive impairment in MWM test. Escape latency time (a) and time in W zone (b) path of motion in probe test (c). MWM test was conducted during 6 days. Data shown represent means ± SD ($n = 8$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the vehicle control group.

used as an indicator of oxidative stress [29]. The oxidized GSH/total GSH ratio in the TMT group was higher than in vehicle control group, and in both 3,5-diCQA groups the ratio was lower than in the TMT group. In other words, the oxidized GSH/total GSH ratio showed a similar pattern as the result of the analysis of MDA levels (Figure 5(b)). The research of Kim et al. has shown that 3,5-diCQA possesses neuroprotective effects against hydrogen peroxide-induced cell death in SH-SY5Y cells as a consequence of inhibiting caspase-3 activation and restoring GSH levels [11].

According to the previous reports, TMT increases ROS production and oxidative stress due to an excess of oxidants reduces antioxidant levels [3, 12]. Accordingly, the TMT group in our experiments also exhibited lower SOD levels than the vehicle control group, whereas the SOD contents of both 3,5-diCQA groups were higher than in the TMT group (Figure 5(c)). GSH protects against oxidative stress, and a depletion of GSH is presumably the result of decreased SOD and catalase activities. When the antioxidant mechanism becomes ineffective as a consequence of excessive oxidative stress, lipid peroxidation occurs [30]. In other words, a noticeable depletion of GSH content in brain tissue will lead to an increase lipid peroxidation. Since brain tissue is composed of neuronal cells that contain numerous polyunsaturated fatty acids to carry out various signaling functions, it is vulnerable to lipid peroxidation, and lipid peroxidation in the brain is an indicator of mild cognitive impairment in patients [31]. Oxidative stress induced by TMT may eventually destroy the antioxidant system in brain. Therefore, the collapse of the antioxidant system increased MDA levels and induced cognitive dysfunction. However, these learning difficulties and memory impairments due to TMT-induced cytotoxicity and oxidative stress could be partially recovered by a 3,5-diCQA.

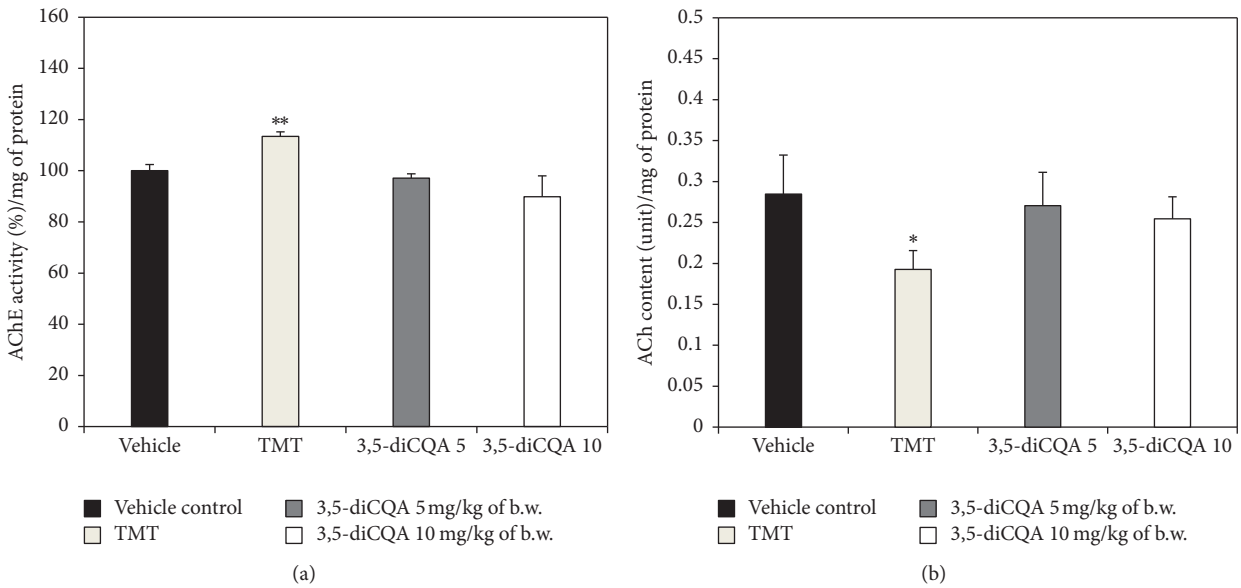


Figure 4: Effect of 3,5-diCQA on cholinergic markers in TMT injected mice brain. AChE activity (a) and ACh content (b). Data shown represent means ± SD ($n = 8$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the vehicle control group.

3.5. Effect of 3,5-diCQA on Mitochondrial Activity in Brain Tissue. Mitochondria are one of the most ROS production organelles in the cell in the pathologic condition. Exposure to excessive oxidative stress results in the opening of one of the requisite mitochondrial channels. This in turn causes the simultaneous collapse of mitochondrial membrane potential and a transient increase in ROS generation [32]. According to the research of Zhang et al., TMT-induced cytotoxicity causes an excessive generation of ROS and a subsequent reduction in mitochondrial membrane potential [33]. Accordingly, increased mitochondrial ROS production and decreased mitochondrial membrane potential were found in the TMT group, compared with vehicle control group. The 3,5-diCQA 5 group did not exhibit significant differences with the TMT group, but the 3,5-diCQA 10 group exhibited reduced mitochondrial ROS production and a higher mitochondrial membrane potential (Figures 6(a) and 6(b)). In other words, TMT-induced oxidative stress led to an increase in mitochondrial ROS production and a decrease mitochondrial membrane potential, while 3,5-diCQA at a concentration of 10 mg/kg body weight protected mitochondria by reducing TMT-induced oxidative stress.

As the report of Green and Reed reveals, typically an early event in the apoptotic pathway is a rapid reduction of mitochondrial membrane potential which leads to a decrease in ATP content [34]. Accordingly and as expected, ATP levels in the 3,5-diCQA 5 group and TMT group were similar, and ATP levels of 3,5-diCQA 10 group were lower than in the vehicle control group but higher than in the TMT group (Figure 6(c)). Polyphenols could play an important role in mitochondrial biochemistry by modulating antioxidant activity, apoptosis, inflammation, and signal transduction [35], and 3,5-diCQA too is thought to have these functions. Broadly speaking, treatment with 3,5-diCQA at 5 mg/kg of body weight did not provide sufficient protection against TMT-induced mitochondrial dysfunction. But treatment with 3,5-diCQA at 10 mg/kg of body weight provided statistically significant protection against TMT-induced mitochondrial dysfunction. In other words, 3,5-diCQA at concentration of 5 mg/kg body weight could protect the antioxidant system in brain, but only seems to be effective in protecting mitochondria in the brain at a concentration of 10 mg/kg body weight.

3.6. Effect of 3,5-diCQA on Apoptotic Signaling Pathway. The above observations confirmed that 3,5-diCQA significantly ameliorates learning and memory deficits associated with TMT-induced amnesia in mice by regulating cholinergic molecules, biochemical antioxidants, and mitochondrial activity. To investigate the cytotoxic mechanism of TMT, changes in apoptotic signaling molecules were analyzed by western blotting. We found that the ratio of phosphorylated-Akt (p-Akt)/Akt in the TMT group was drastically lower than in the vehicle control group. However, the ratio in the 3,5-diCQA group was higher than that in TMT group (Figure 7(a)). Akt activity is influenced by numerous pathways related to various types of oxidative stress. Akt is activated by phosphorylation at Thr 308 and Ser 473, and Akt activity can be blocked by the corresponding phosphatase. The degree of Akt activity is an important factor in the improvement of neurodegenerative disorders. Akt prevents apoptosis and promotes cell survival through phosphorylation of glycogen synthase kinase-3β (GSK-3β), BAD, and caspase 9 [36]. The activation of GSK-3β is responsible for abnormal hyperphosphorylation of tau, the microtubule-linked protein in neurons [37]. If GSK-3β is activated, apoptosis occurs as a result of increase of p-tau. Accordingly, the TMT group exhibited increased levels of p-tau compared

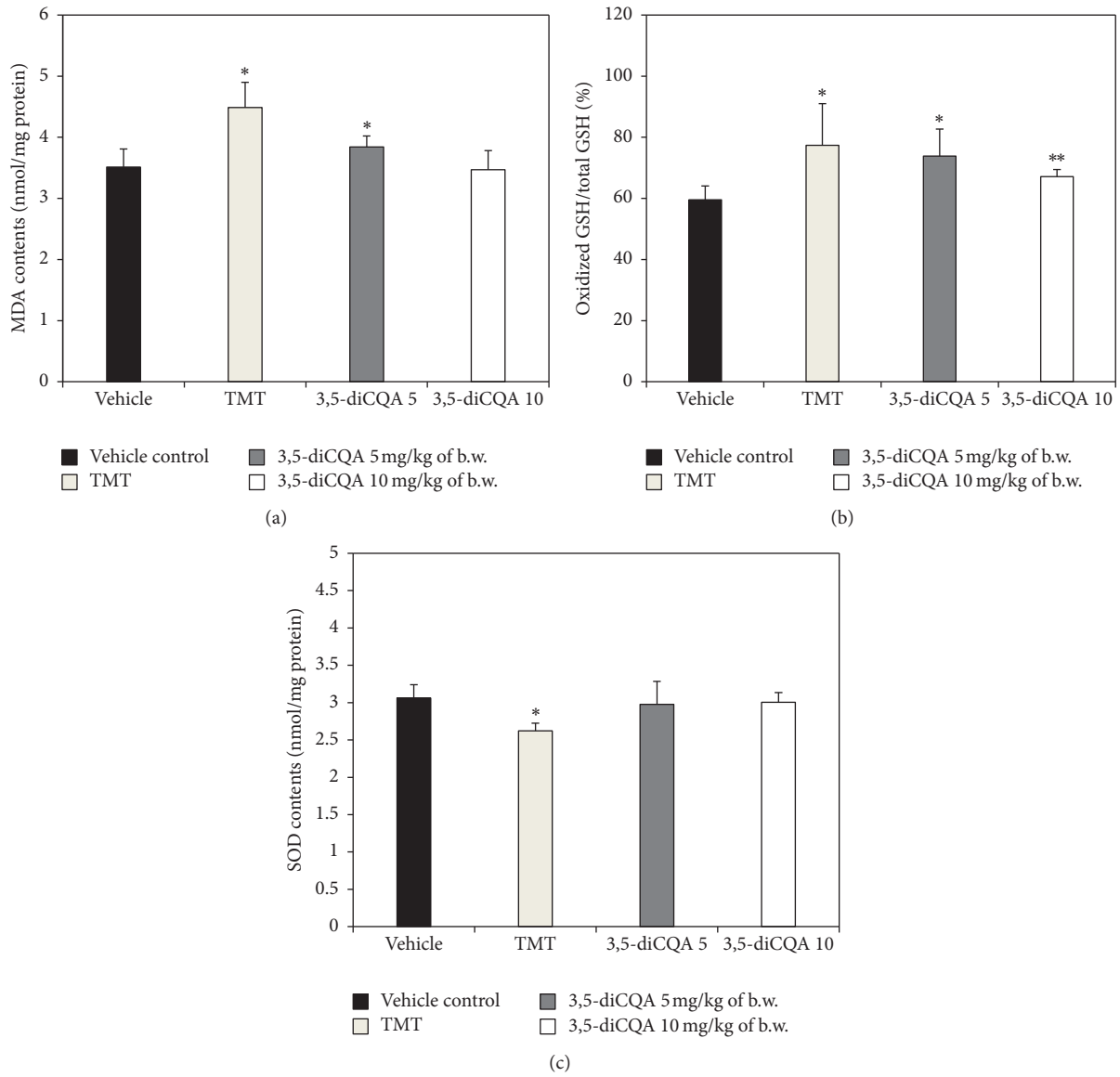


Figure 5: Effect of 3,5-diCQA on antioxidant biochemicals in TMT injected mice brain. MDA contents (a) and oxidized GSH/total GSH ratio (b) and total SOD contents (c). Data shown represent means ± SD ($n = 8$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the vehicle control group.

to the vehicle control group. In contrast, the 3,5-diCQA 10 group, in which expression of p-Akt was higher than in the TMT group, showed similar expression levels as the vehicle control group (Figure 7(b)).

According to research by Zhang et al., TMT led to time- and concentration-dependent apoptotic cell death, which was associated with BAX [33]. TMT stimulated BAX expression, which leads to a loss of mitochondrial membrane potential and the release of cytochrome c from the mitochondria into the cytosol, which then activates the caspase protease cascade to execute apoptosis. Accordingly, the TMT group in our experiment also showed increased expression levels of BAX and a release of mitochondrial cytochrome c compared to the vehicle control group (Figures 7(c) and 7(d)). However, in the 3,5-diCQA 10 group BAX expression and the release of mitochondrial cytochrome c were lower than in the TMT group. After investigating the neuroprotective effect of 1,5-diCQA against amyloid β_{1-42}-induced neurotoxicity in primary neuronal culture, Xiao et al. reported that 1,5-diCQA protects against neurotoxicity through activation of PI3 K/Akt followed by stimulation of Trk A and, subsequently, inhibition of GSK3β as well as modulation of Bcl-2/BAX [38]. These results suggest that diCQAs and their isomers may be able to prevent neuronal cell death through the regulation of apoptotic signaling molecules such as Akt, BAX, and tau.

In summary, 3,5-diCQA reduced TMT-induced neuronal cell death through the activation of Akt and downregulation

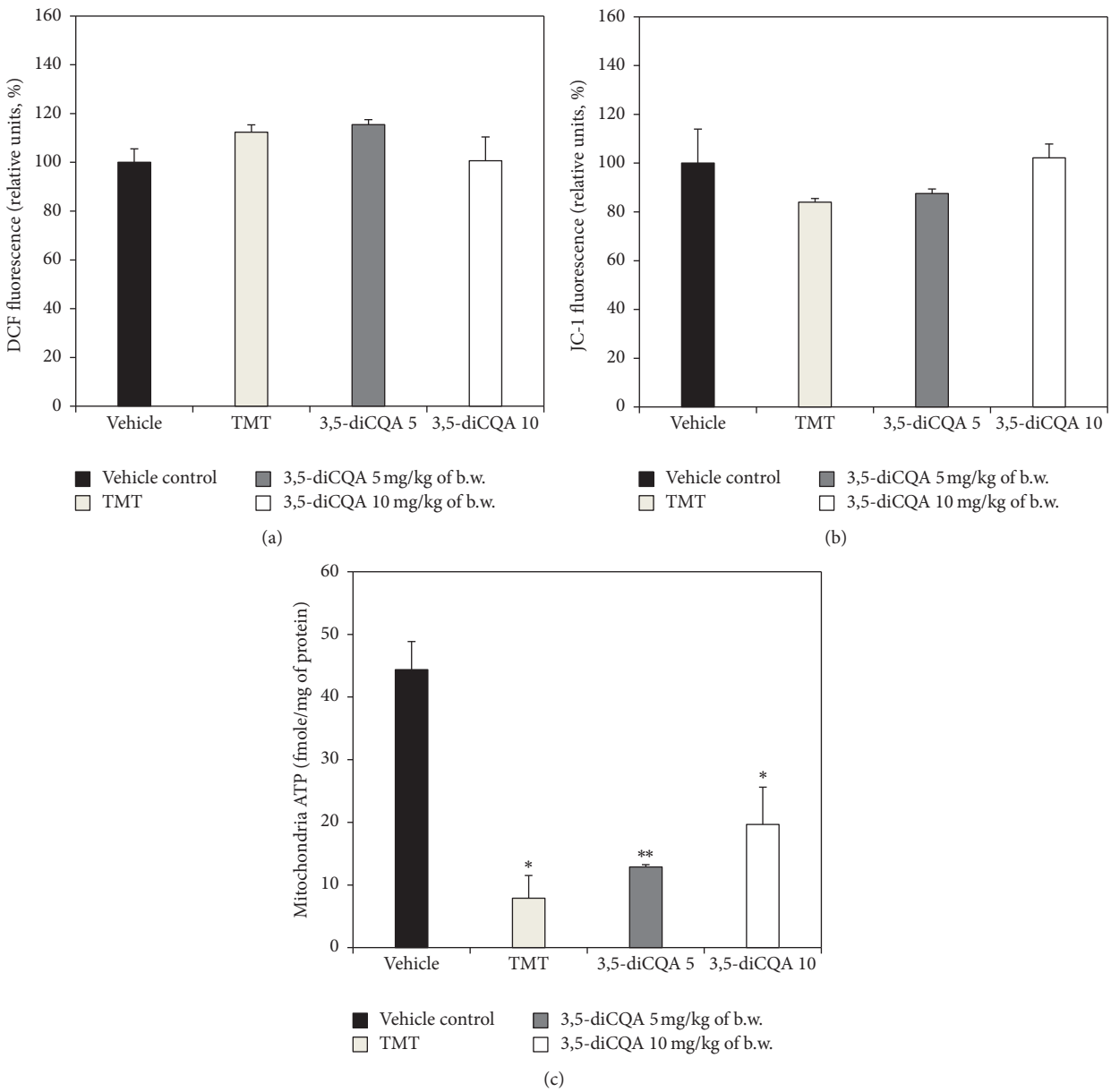


Figure 6: Effect of 3,5-diCQA on mitochondrial activities of TMT injected mice brain. ROS production (a), mitochondrial membrane potential (b), and ATP level (c). Data shown represent means ± SD ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the vehicle control group.

of BAX expression. Consequently, the antiamnesic effect of 3,5-diCQA, as an isomer of chlorogenic acid, with respect to cognitive dysfunction caused by TMT may be improved through neuronal cell protection through the regulation of apoptotic signaling molecules.

4. Conclusion

The antiamnesic effect of a 3,5-diCQA on TMT-induced learning and memory impairment in ICR mice was investigated. 3,5-diCQA, as an isomer of chlorogenic acid, significantly ameliorated cognitive dysfunction due to severe TMT-induced neurotoxicity. AChE inhibition by 3,5-diCQA went hand in hand with the recovery of ACh, a neurotransmitter in cholinergic system. In addition, the outstanding antioxidant activity of 3,5-diCQA protected against the neuronal deficit caused by excessive oxidative stress, increased oxidized GSH level, and decreased total SOD content in the brain. Mitochondrial activities, including oxidative stress, mitochondrial membrane potential, and ATP production, were also protected by 3,5-diCQA. Finally, the antiamnesic effect of 3,5-diCQA was confirmed through its regulation of apoptotic signaling molecules such as Akt, tau, BAX, and cytochrome c. The results suggest that 3,5-diCQA, as an isomer of chlorogenic acid, may be effective in ameliorating the cognitive impairment induced by TMT, and it seems to

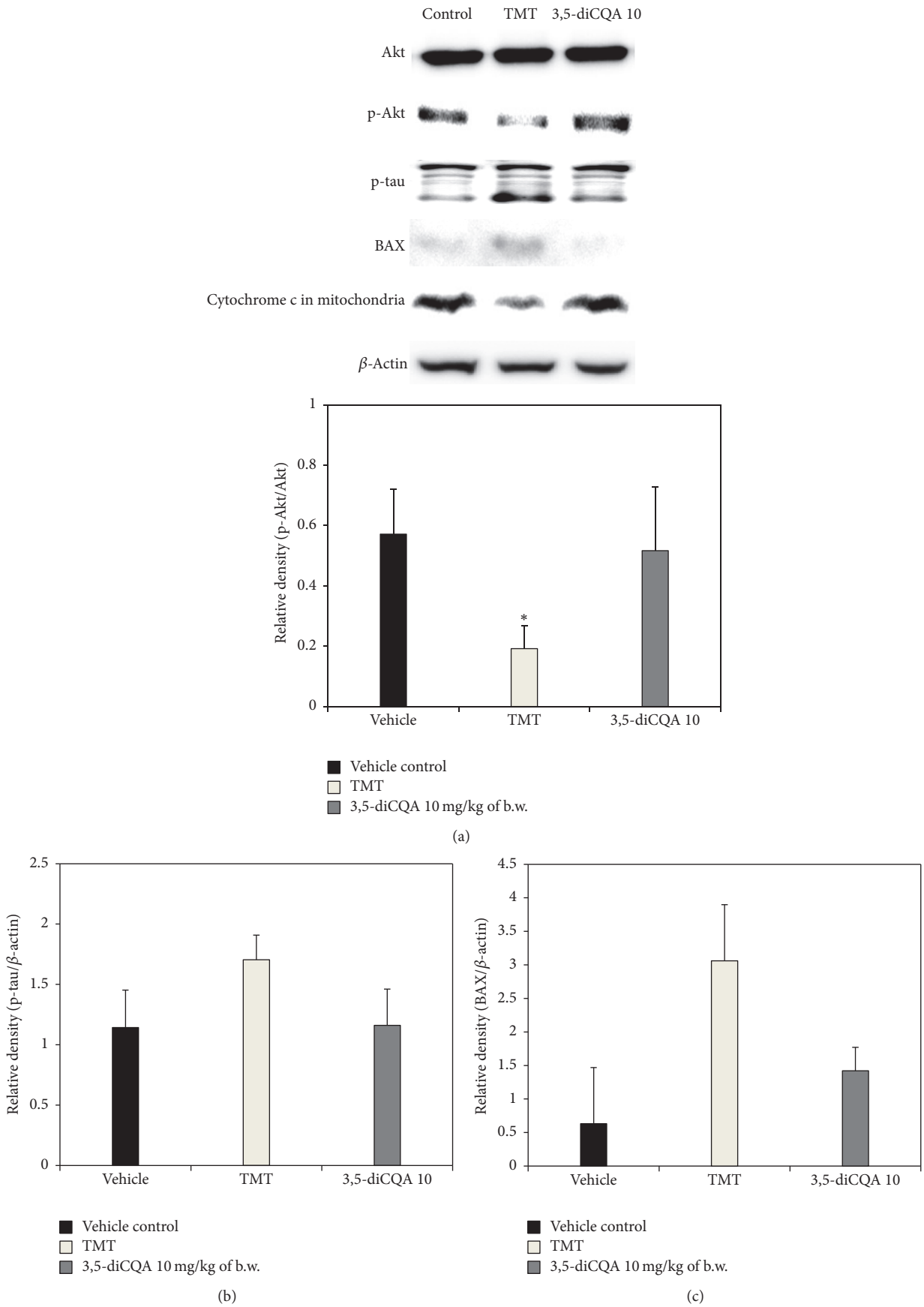
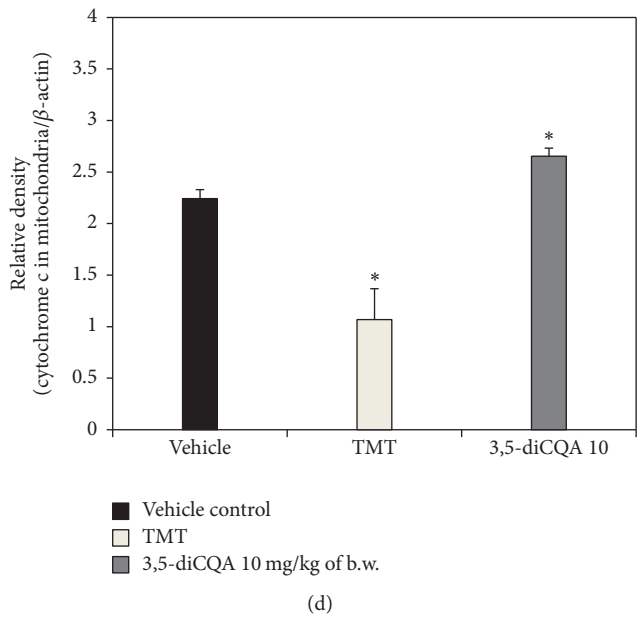


Figure 7: Continued.



(d)

Figure 7: Effect of 3,5-diCQA on the expression of apoptotic signaling molecules in TMT injected mice brain. p-Akt/Akt (a), p-tau/β-actin (b), BAX/β-actin (c), and cytochrome c in mitochondria/β-actin (d). Data shown represent means ± SD ($n = 6$). $^{*}P < 0.05$ compared to the vehicle control group.

be a possible candidate molecule for tackling neurodegeneration.

Competing Interests

The authors declare no competing financial interests.

Acknowledgments

This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF-2015R1D1A3A01015931) funded by the Ministry of Education, Republic of Korea. Jin Yong Kang, Seon Kyeong Park, Tian Jiao Guo, Jeong Su Ha, Du Sang Lee, and Jong Min Kim were supported by the BK21 Plus program, Ministry of Education, Republic of Korea.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9324085, 14 pages
http://dx.doi.org/10.1155/2016/9324085

Review Article

Therapeutic Potential of Curcumin for the Treatment of Brain Tumors

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Received 23 July 2016; Accepted 7 September 2016

Academic Editor: Mohamed Essa

Brain malignancies currently carry a poor prognosis despite the current multimodal standard of care that includes surgical resection and adjuvant chemotherapy and radiation. As new therapies are desperately needed, naturally occurring chemical compounds have been studied for their potential chemotherapeutic benefits and low toxicity profile. Curcumin, found in the rhizome of turmeric, has extensive therapeutic promise via its antioxidant, anti-inflammatory, and antiproliferative properties. Preclinical *in vitro* and *in vivo* data have shown it to be an effective treatment for brain tumors including glioblastoma multiforme. These effects are potentiated by curcumin's ability to induce G2/M cell cycle arrest, activation of apoptotic pathways, induction of autophagy, disruption of molecular signaling, inhibition of invasion, and metastasis and by increasing the efficacy of existing chemotherapeutics. Further, clinical data suggest that it has low toxicity in humans even at large doses. Curcumin is a promising nutraceutical compound that should be evaluated in clinical trials for the treatment of human brain tumors.

1. Introduction

Primary central nervous system tumors have an incidence of 27.86 per 100,000 [1]. Glioblastoma (GBM) is the most common malignant primary CNS tumor with an annual incidence of 3.19 per 100,000. GBM accounts for 15.4% of all primary brain tumors and 45.6% of primary malignant brain tumors [1, 2]. The current standard of care for GBM consists of surgical resection followed by the Stupp regimen (adjuvant chemotherapy and radiation) and it has been used in practice for over a decade without substantial improvements. This aggressive multimodal treatment prolongs median overall survival to 15 months [3–5]. Other therapies approved for use in GBM include alternating electrical fields produced by Optune Therapy (Novocure Inc.), further prolonging median survival to 20 months [6]. With such poor prognoses, additional therapeutics are needed to improve survival and quality of life for patients with malignant gliomas. Complementary medicine and nutraceuticals are of particular interest and have been studied extensively as anticancer agents because they are usually associated with low toxicity profiles. This allows them to be safely used at high doses or added to existing chemotherapy regimens as adjuvant treatment.

Curcumin is found in rhizome of turmeric (*Curcuma longa*), which is a member of the ginger family (Figure 1) [7]. Its traditional uses, long known by Ayurvedic medicine from India and traditional Chinese medicine, include treating infections, liver and skin disorders, dressing wounds, burns, and decreasing inflammation [7]. In fact, powdered *Curcuma longa* has been used in Asian medicine, cosmetics, and fabric dying for more than 2000 years [8]. Curcumin possesses multiple beneficial chemical properties, including antioxidant, anti-inflammatory, and chemotherapeutic potential both against cultured cells and in treatment studies using animal models. Current data suggests that curcumin may be useful in a wide spectrum of human disorders including Alzheimer's disease, Parkinson's disease, diabetes, cardiovascular disease, arthritis, and various neoplasms including brain tumors [9, 10].

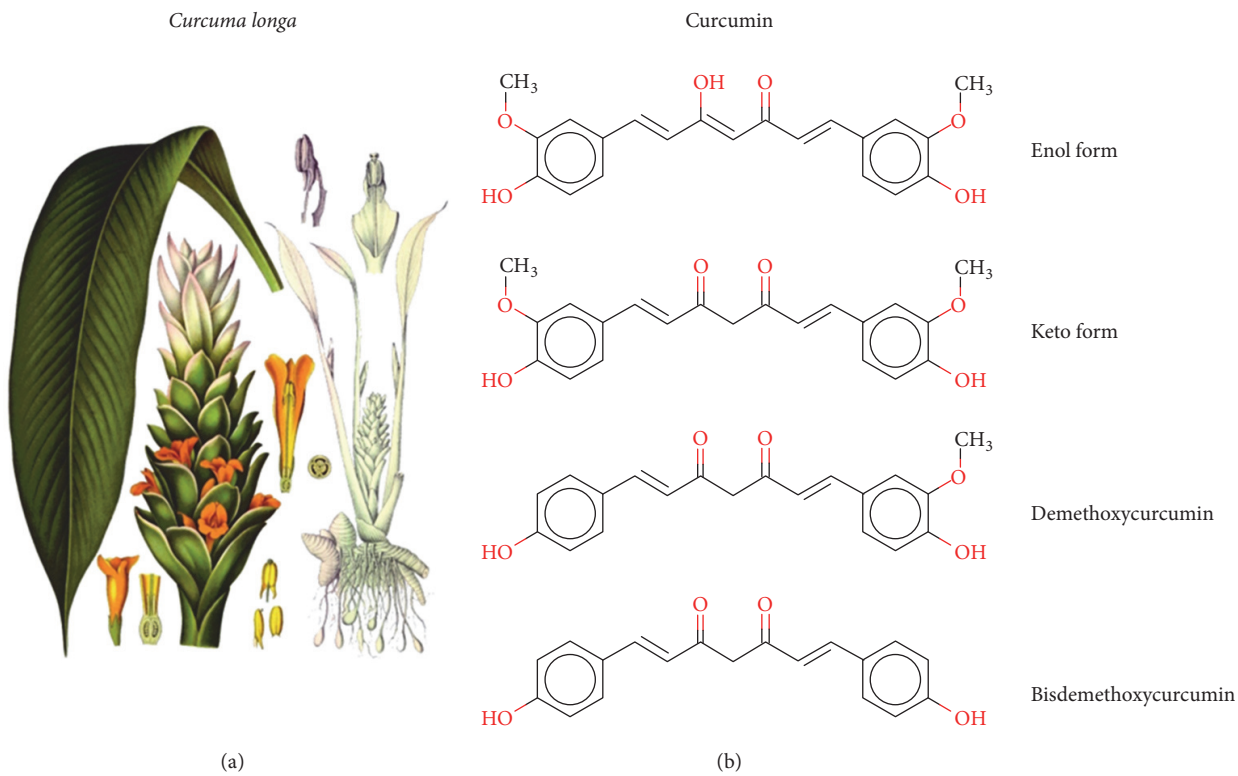

Figure 1: (a) *Curcuma longa* from Koehler's Medicinal Plants, 1887. (b) From top to bottom, curcumin in its enol form, curcumin in its keto form, demethoxycurcumin, and bisdemethoxycurcumin.

2. Mechanism and Preclinical Data

Curcumin's effects on glioma cells *in vitro* and *in vivo* have been well studied. Curcumin has many molecular targets (Figure 2) and therefore diverse and complex mechanisms of action. The antitumoral effects of curcumin are thought to act through many different signaling pathways such as cellular proliferation [11–13], apoptosis [11, 12, 14], autophagy [15–17], angiogenesis [18–20], immunomodulation [21], invasion [14, 22, 23], and metastasis [24–26]. These pathways have been reviewed comprehensively elsewhere [27–31]. In addition to its effectiveness as an antineoplastic, curcumin has been found to be protective against reactive oxygen species (ROS). A study by Rathore et al. [32] examined the effects of curcuma oil on a transient ischemia model in Sprague-Dawley rats. The middle cerebral artery was occluded using a nylon filament for two hours, followed by a 24-hour reflow period. Treatment with curcuma oil reduced the size of infarcted cerebral tissue, improved neurologic function, and reduced oxidative stress caused during the reperfusion period. The authors reported that these protective effects were obtained by inhibition of ROS, reducing peroxynitrite levels and caspase-3 activity and preventing destabilization of the mitochondrial membrane potential [32]. In an *in vitro* model of ischemia-reperfusion using rat cortical neurons, curcumin was found to protect neurons from death caused by oxygen and glucose deprivation. The authors of this study suggested that curcumin activates an antioxidant protein, thioredoxin, in the Nrf2 pathway [33]. In addition, curcumin may inhibit cellular glyoxalases leading to decreased ATP and glutathione, which can impact cellular metabolism and may account for some of its anti-inflammatory and antitumor effects [34].

2.1. Curcumin Induces G2/M Cell Cycle Arrest. There is a bevy of data to suggest that curcumin is able to induce G2/M cell cycle arrest and apoptosis. More recent work has tried to explain how this cell cycle arrest can occur. Wu et al. published a study in 2013 [35] that suggests that this might be due to increased DAPK1 expression. The authors treated the U251 GBM cell line with curcumin and found a dose-dependent increase in DAPK1 mRNA by real-time RT-PCR and verified a corresponding increase in protein expression by western blot analysis. Further, they used siRNA (si-DAPK1-1 and si-DAPK1-2) transfection to suppress DAPK1 and found that curcumin had an attenuated ability to suppress STAT3 and NF-κB phosphorylation. They also showed that their knockdown of DAPK1 inhibited curcumin-mediated caspase-3 activation and led to decreased apoptosis (33.0% apoptotic cells versus 58.3% in their control) [35]. These findings suggest that DAPK1 plays an important role in curcumin-mediated cell death. Other studies in U251 GBM cells show that p53 expression is upregulated by curcumin treatment, as were CDKi $p21^{Waf1/Cip1}$ (p21) and ING4—a tumor suppressor gene that has been found to be suppressed in gliomas [36, 37]. Curcumin owes part of its antiproliferative effects to suppression of cyclin D1 and to induction of p21. In a study that treated U87 GBM cells with curcumin, transcription factor Egr-1 was found to activate transcription of p21 independent of p53 activation. Egr-1 expression was reportedly induced by curcumin via ERK

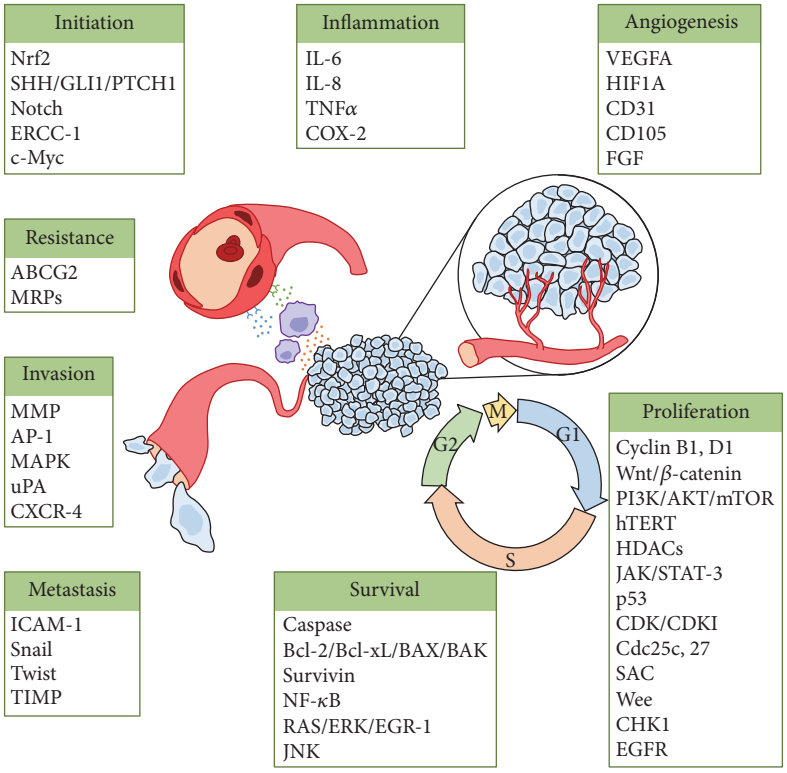

Figure 2: Cancer pathway targets affected by curcumin. When the listed targets have multiple contributions to tumorigenesis (e.g., NF-κB), they are only placed under one category. Figure modified* from Davis et al. 2003 [44]. *As permitted by Creative Commons Attribution-NonCommercial-Share Alike 3.0 license and BJC OPEN initiative. ABCG2: ATP-binding cassette subfamily G member 2; AKT: protein kinase B; AP-1: activator protein 1; BAK: Bcl-2 homologous antagonist/killer; BAX: Bcl-2-like protein 4; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma-extra large; caspase: cysteine-aspartic protease; CD105: endoglin; CD31: platelet endothelial cell adhesion molecule (PECAM-1); CDC: cell division cycle; CDK: cyclin-dependent kinase; CDKI: cyclin-dependent kinase inhibitor; CHK1: checkpoint kinase 1; c-Myc: v-Myc avian myelocytomatosis viral oncogene homolog; COX-2: prostaglandin-endoperoxide synthase 2; CXCR-4: C-X-C chemokine receptor type 4; EGFR: epidermal growth factor receptor; EGR-1: early growth response protein 1; ERCC-1: excision repair cross-complementation group 1; ERK: extracellular signal-regulated kinases; FGF: fibroblast growth factor; GLI1: GLI family zinc finger 1; HDAC: histone deacetylases; HIF1A: hypoxia inducible factor 1, alpha subunit; hTERT: telomerase reverse transcriptase; ICAM-1: intercellular adhesion molecule 1; IL: interleukin; JAK: Janus kinase; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinases; MMP: matrix metalloproteinases; MRP: multidrug resistance protein; mTOR: mechanistic target of rapamycin; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2: nuclear factor (erythroid-derived 2) like 2; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; PTCH1: patched 1; SAC: spindle assembly checkpoint; SHH: sonic hedgehog; Snail: zinc finger protein SNAI1; STAT-3: signal transducer and activator of transcription 3; TIMP: tissue inhibitors of metalloproteinases; TNF: tumor necrosis factor; uPA: urokinase; VEGF: vascular endothelial growth factor.

and JNK [13]. An *in vitro* study using U87 human glioma cell line showed that 5–10 μM curcumin could inhibit proliferation and that treated cells were arrested in G2/M stage by increased expression of DUSP-2 and inhibition of ERK and JNK phosphorylation. Interestingly, trapping cells in the G2/M phase appears to enhance their sensitivity to radiation [38, 39]. Curcumin's radiosensitizing effects have also been examined in neuroblastoma (SK-N-MC) cells. Pretreating cells with 100 nM curcumin suppressed radiation induced NF-κB, enhanced radiation induced caspase activation, and inhibited antiapoptotic molecules [40].

2.2. Curcumin Activates Apoptotic Pathways. An *in vitro* study using human GBM (A172, KNS60, U251MG(KO)) and medulloblastoma (ONS76) cell lines showed that while curcumin was able to inhibit growth of these cell lines, only KNS60 and ONS76 were arrested at G2/M. These findings suggest that the mechanism for growth inhibition is not exclusively due to cell cycle arrest. They also found a significant increase in caspase 3/7 activity, curcumin induced DNA damage, and apoptosis triggered by overexpression of BAX and downregulating Bcl2 and survivin, as well as inhibition of telomerase and downregulation of hTERT [41]. An *in vitro* study using 8401 GBM cell line found that curcumin decreased cell proliferation, reduced mitochondrial membrane potential, induced DNA fragmentation, induced apoptosis via a caspase-dependent pathway (caspase-3, caspase-8, and caspase-9), and inhibited NF-κB transcription factor activity [42]. In U87MG GBM cell line, curcumin can induce apoptosis by suppressing antiapoptotic signals, by promoting activation of caspase-8, and through an increased BAX/Bcl-2 ratio [43]. Solubilized curcumin (667 μM in PBS, 3% DMSO) can increase caspase 3/7 activity in both mouse (B16F10 melanoma, GL261 glioma, and N18

neuroblastoma) and human (HOG oligodendroglioma and A549 lung cancer) cell lines and it decreased tumor cell viability as measured by MTT assay. Further, LDH release in these experiments was not increased, suggesting apoptotic cell death [19]. The authors of this study also report that cyclin D1, NF-κB, AKT, ERK, and Bcl-xL were suppressed when B16F10 cells were treated with curcumin [19]. In an *in vivo* arm of this experiment, the authors injected 10,000 B16F10 mouse melanoma cells in the brain of C57BL/6 mice. They then received either daily tail vein injections of 200 μL of their solubilized curcumin (estimated final plasma concentration of 35 μM in PBS, 0.15% DMSO) or a control injection (PBS, 0.15% DMSO) for 18 days. Additionally, this experiment was repeated with intracerebral delivery of the curcumin solution via a stainless steel cannula. While all of the control animals developed intracranial tumors, only one of the 5 treated mice developed detectable tumor [19].

2.3. Curcumin Induces Autophagy. Autophagy is a cellular process for disassembling unnecessary or dysfunctional cellular components and is essential for maintaining energy during states of nutritional stress and during programmed cell death. It also appears to have an important role in regulation of glioma-initiating cells, which are poorly differentiated and share features of neural stem cells. An *in vitro* study published in 2012 showed that curcumin can induce differentiation and halt growth of glioma-initiating cells (SU-2 and SU-3) from surgically resected human GBM by activating autophagy. In the same study, glioma-initiating cells were implanted intracranially in athymic nude mice and randomized to a treatment group consisting of intraperitoneal (IP) injection of curcumin (300 mg/kg) every 3 days or control group ($n = 14$ each). Those mice who received treatment had increased survival (study halted after 120 days) versus non-treated animals [16]. In a separate study, curcumin activated the ERK1/2 pathway and inhibited the AKT/mTOR/p70S6K pathway, resulting in autophagy both *in vitro* and *in vivo* using a subcutaneous xenograft model in nude mice with U87 cells. The authors report that curcumin induced G2/M cell cycle arrest in addition to autophagy in U87 and U373 GBM cells [17].

2.4. Curcumin Disrupts Molecular Signaling. The NF-κB pathway is upregulated in GBM, and inhibitors of NF-κB exhibit relatively low toxicity to normal tissues. A preclinical study of NF-κB inhibitors found that NF-κB activity correlated with percentage cell viability in C6 and U138 GBM lines [45]. The authors report that inhibition of NF-κB leads to mitochondrial dysfunction with arrest in the G2/M phase of the cell cycle. Interestingly, NF-κB is found to be overstimulated in cisplatin-resistant C6 cells and NF-κB inhibitors were able to overcome cisplatin resistance [45]. Similarly, progranulin has been found to be overexpressed in many GBM cell lines (U87, GBM8904, and S1R1) and tumor samples [46]. Progranulin overexpression contributes to tumorigenesis and treatment resistance by upregulating DNA repair and stemness genes using the transcription factor AP-1. Curcumin is an AP-1 inhibitor, and it was found to downregulate progranulin promoter activity and expression [46]. Other studies have shown that curcumin can reduce GBM cell survival through inhibition of AP-1 and NF-κB by preventing constitutive activation of JNK and AKT [47]. An *in vitro* study examined the role of glucose-6-phosphate translocase (G6PT) in U87 GBM cell signal transduction. In this experiment, suppression of gene expression was accomplished using siRNAs and led to apoptosis and necrosis. Curcumin (35 μM) also inhibited G6PT gene expression >90%. However, the authors also report transfection with an expression vector for G6PT rescued cells from curcumin-mediated cell death. This suggests that G6PT may be a novel chemotherapeutic target and that G6PT overexpression may lead to treatment resistance [48]. In a separate set of experiments, murine glioma cell lines (Tu-2449, Tu-9648, and Tu-251) with constitutively expressed STAT3 treated with curcumin showed a dose-dependent decrease in the activity of phosphorylated JAK1 and JAK2 and led to downstream inactivation of STAT3. In an *in vivo* arm of this study, C6B3F1 mice were fed a diet rich in fat and cholesterol with or without fortification with curcumin for 7 days. Subsequently, they received intracranial implantation of either Tu-2449 or Tu-9648 glioma cells and maintained their diet. Fifteen percent of Tu-2449 implanted mice who received curcumin-enriched diets had tumor-free long-term survival versus 0% of the control dieted animals. Curcumin fed Tu-9648 implanted mice experienced a 38% increase in tumor-free long-term survival [49].

The sonic hedgehog (SHH) signaling pathways are important in the carcinogenesis of medulloblastoma. It is a major regulator of cell proliferation and death. Curcumin has been shown to downregulate SHH, leading to decreased downstream targets GLI1 and PTCH1 and cytotoxicity in cell lines MED-4, MED-5, and DAOY [50]. Targeting SHH and GLI1 with curcumin treatment has also been done in glioma cells (U87 and T98G). Similar to medulloblastoma, mRNA and protein levels of SHH and GLI1 were downregulated with curcumin treatment. IP injection of curcumin in U87-implanted nude mice also reduced tumor volume, prolonged survival, and decreased GLI1 expression [51]. In the medulloblastoma cell line DAOY, curcumin has been shown to inhibit Wnt/β-catenin signaling and suppress proliferation with IC_{50} of 35 μM after 48 hours [52]. Mechanistically, curcumin may decrease histone deacetylase 4 expression leading to increased tubulin acetylation and mitotic catastrophe in addition to its effects on G2/M cell cycle arrest in DAOY medulloblastoma cells [53].

2.5. Curcumin Decreases Invasion and Metastasis. Abnormal expression of matrix metalloproteinases (MMPs), membrane-associated or secreted enzymes used to digest extracellular matrix proteins, is one method by which glioma cells are able to invade normal brain tissue. Data has shown that curcumin is able to suppress expression of MMP-1, MMP-3, MMP-9, and MMP-14 in GBM cell lines U87MG and U373MG via the common upstream AP-1 and MAP kinases [22, 23, 54]. Urokinase-type plasminogen activator (uPA) is a serine protease that starts a degradative cascade by converting extracellular plasminogen to plasmin and eventually degrades extracellular matrix collagen and

activates other MMPs to aid in invasion [55]. Curcumin can prevent nuclear translocation of RelA/NF-κB, which prevents upregulation of uPA [55]. Curcumin has also been found to dramatically reduce MMP-9 in murine glioma cell lines Tu-2449, Tu-9648, and Tu-251 [49]. An important contributor to tumors propensity to invade and metastasize is angiogenesis. Cell lines derived from surgically resected pituitary adenomas treated with curcumin have decreased HIF1A and VEGFA, both of which are involved in tumoral angiogenesis [56].

2.6. Curcumin Increases the Efficacy of Existing Chemotherapeutics. Data also exists to suggest that curcumin acts synergistically with chemotherapeutics already approved for the treatment of brain tumors. A preclinical study examined the efficacy of curcumin for the treatment of GBM *in vitro* and *in vivo* [57]. Cell lines U138MG, U87, U373, and C6 were treated with curcumin and found to have IC_{50} values of 29, 19, 21, and 25 μM, respectively, whereas IC_{50} for astrocytes was 135 μM. Chemotherapy synergism was subsequently tested using U138 and C6 cell lines. Curcumin alone resulted in 55% and 75% viability, respectively. When cisplatin was added in combination with 25 μM curcumin, the viability dropped to 30% and 10% for U138 and C6 cell lines, respectively. When doxorubicin was added in combination with 25 μM curcumin, viability was reported to be 36% and 46%. Mechanistically, the authors reported that curcumin decreased activation of PI3K/AKT and NF-κB pathways, decreased expression of Bcl-xL, and caused mitochondrial dysfunction and apoptosis. The authors also implanted Wistar rats with C6 GBM cells intracranially and treated them with 50 mg/kg/day curcumin via IP injection within days 10–20 after implantation. Sixty-four percent of curcumin treated animals developed tumors as compared to 100% of the DMSO treated controls. Of the treated animals who developed tumors, average tumor volume was 73% less than that of the controls [57]. Curcumin in combination with temozolomide (TMZ) appears to have additive cytotoxic benefit in GBM cells [15]. Further, both drugs appear to cause G2/M arrest by activating proteins such as Wee, Cdc2, CHK1, and Cdc25c. Decreased phosphorylation of cyclin B1 and cyclin D1 was also observed [15]. The authors found that treatment with either TMZ or curcumin appears to induce autophagy that was dependent on ERK1/2 prior to apoptosis. Curcumin was also found to inhibit STAT3, NF-κB, and PI3K/AKT [15]. A study by Ramachandran et al. [58] investigated potentiation of etoposide (ETP) and TMZ by curcumin and a trade preparation of turmeric called Tumeric Force™ (TF) in human U87 GBM and D283 medulloblastoma cell lines. U87 IC_{50} values for ETP, TMZ, curcumin, and TF were 6.5, >2000, 37.3, and 30.8 μg/mL, respectively. For D283 cells, those IC_{50} values were 0.19, 147, 28.7, and 1.6 μg/mL. Combination index values in U87 glioma cells for ETP + curcumin, TMZ + curcumin, and ETP + TMZ + curcumin were 0.55, 2.07, and 0.39 μg/mL, respectively. The combination index values with TF instead of curcumin were 0.38, 0.57, and 0.05 μg/mL [58]. Curcumin has also been studied for efficacy in combination with paclitaxel (PTX) for the treatment of GBM. LN18 and U138MG cell lines treated with 20 μM curcumin and 10 nM PTX were found to have a combination index of 0.1 and 0.09, respectively, indicating a synergistic effect. This combination activated caspase-3, caspase-8, and calpain, increased BAX, and reduced Bcl-2 initiating apoptosis. Combination therapy also decreased the ability of LN18 and U138MG cells to invade matrigel [59]. In addition to acting synergistically, curcumin may prevent chemotherapy resistance. A study reported in 2008 on Sprague-Dawley rats showed curcumin can inhibit a protein linked with multidrug resistance in the luminal membrane of capillaries at the blood-brain barrier- (BBB-) ATP-binding cassette transporter ABCG2 [60]. A separate, intriguing method by which GBM may resist hydrophobic therapeutic agents is by accumulating lipid droplets that sequester these drugs. Curcumin is hydrophobic and has been shown to concentrate near the cell membrane and in cytoplasmic droplets of U251N human GBM cells [61]. Zhang et al. sought to overcome this mechanism of resistance *in vitro* using pyrrolidine-2, a cytoplasmic phospholipase A2α inhibitor, in combination with curcumin. When the cells were pretreated with pyrrolidine-2 24 hours before curcumin administration, cell viability was found to approach 0% [61].

2.7. Curcuminoids and Curcumin Derivatives. Demethoxycurcumin (C2) and bisdemethoxycurcumin (C3) (Figure 1) are two curcuminoids often found in small percentages of curcumin extractions. An *in silico* study published in 2009 [62] examined the docking of curcuminoids with the Bcl-2 apoptotic proteins (Protein Data Bank accession numbers 1G5M and 1GJH). The authors calculated free energies and inhibition constants for these molecules and found that C2 binds more favorably than curcumin (ΔG −6.97 versus −4.53 kcal/mol; K_i 0.56 versus 2.21 nm). *In vitro* treatment of U87 GBM cells showed marked decrease in Bcl-2 expression with all three compounds after 48 hours and that C2 showed significant progression of percent apoptosis compared to curcumin or C3. An *in vitro* binding assay utilizing circular dichroism spectroscopy showed that C2, like the natural Bcl-2 inhibitor Bak, caused a conformational change different from curcumin or C3 [62]. These findings suggest that C2 induces Bcl-2 mediated apoptosis more effectively than curcumin or C3. A study published by Huang et al. [63] also examined the effects of C2. They showed that C2 had IC_{50} of 22.7 μM in GBM 8401 cells and that apoptosis was induced by decreasing the mitochondrial membrane potential and caspase-dependent pathways similar to other studies of curcumin [63]. Other curcumin analogs have been developed in an attempt to improve upon the compound's therapeutic promise. For example, lead compounds identified by Campos et al. show comparable IC_{50} values against GBM (U87MG) and neuroblastoma (SK-N-SH and SK-N-FI) cell lines [64].

3. Delivery Mechanisms

The therapeutic benefits of curcumin are limited by its poor absorption, rapid metabolism, and poor water solubility [65]. Curcumin is broken down by multiple enzymatic pathways,

including glucuronidation, sulfation, alcohol dehydrogenase, and p450 system leading to rapid metabolism and excretion [66]. However, its lack of toxicity allows for administration of large doses. Twenty-four subjects with mean age of 34 were provided capsules with 0.5–12 g of curcumin and serum analysis was conducted prior to testing at 1, 2, and 4 hours after dosing. Peak serum concentration was <2 μM even at 10- and 12-gram doses [67, 68]. These clinical data also show that doses up to 12 grams per day do not provoke serious side effects, though trouble with tablet bulk, diarrhea, and yellow stool were noted [67, 68]. Piperine, an inhibitor of hepatic and intestinal glucuronidation, has long been suggested to be administered as an adjuvant to prevent the degradation of medications including curcumin [69–71]. Ten subjects with mean age of 60 years participated in a randomized crossover trial to compare the effects of oral curcumin (2 g) alone or with the addition of piperine (20 mg). A two-week washout period was used before the crossover. They found that, when administered with piperine, peak serum concentrations of curcumin increased 30-fold and the relative bioavailability was 2000%. The V_D for curcumin administered with piperine was 203 L/kg. No adverse events were reported in this study [69].

Though current literature contains mixed data, curcumin distribution to the brain might be hindered by limited BBB permeability [65, 72]. Certain nanoparticle formulations, such as poly(lactic-co-glycolic acid), have been shown to increase distribution to brain tissue [65]. The half-life of curcumin-loaded poly(lactic-co-glycolic acid) in brain tissues from Sprague-Dawley rats for this study was reported to increase from 9 minutes to 15 minutes. When brain regions were examined separately, the compound's half-life was found to be significantly extended in the cerebral cortex (20 versus 2 minutes) and hippocampus (17 versus 8 minutes) but not in the cerebellum, brainstem, striatum, or other brain regions [65]. Curcumin has also been loaded into poly(butyl)cyanoacrylate (PBCA) nanoparticles using apolipoprotein E3 to increase transport across the BBB [73]. This delivery system was found to induce apoptosis in SH-SY5Y neuroblastoma cells more efficaciously than curcumin solution or curcumin loaded in the PBCA carrier without ApoE3 [73]. Nanostructured lipid carriers (tripalmitin-oleic acid) loaded with curcumin had IC_{50} of approximately 20 μg/mL against A172 GBM cells compared to IC_{50} of 80 μg/mL with curcumin alone. Further, the authors showed that curcumin delivered with the lipid carriers to subcutaneous flank tumor bearing nude mice decreased tumor volume by 82% [74]. Polymeric micelles have also been studied for curcumin delivery. Amphiphilic block copolymers form micelles when placed in aqueous solutions. These vesicles, or polymersomes, can be made of one or more different high-molecular weight amphiphilic block copolymers such as oleic acid and PEG 400 as a diblock nanostructure. This combination has been shown effective in delivering curcumin and reducing tumor burden in mouse models [75, 76]. Monomethoxy PEG has a higher molecular weight of 2 kDa versus 400 Da and has also been tested in attempt to obtain a better thermodynamic profile [77]. When tested *in vitro* with the U87 human glioblastoma cell line, monomethoxy PEG-oleate conjugation product showed IC_{50} values of 24 μM versus IC_{50} of 48 μM for free curcumin [77]. Intranasal delivery of therapeutics to the brain represents a noninvasive way to bypass the BBB. Exosome encapsulated curcumin has been administered intranasally to C57BL/6j mice in an attempt to treat inflammation induced by administration of lipopolysaccharide (LPS) [78]. It was found that these exosomes were taken up by brain microglia within 15 minutes and inhibited LPS-induced inflammation [78]. Other lipid carriers have also been designed for intranasal curcumin delivery to the central nervous system. Testing in Wistar rats showed that maximum concentration was reached after approximately 3 hours, and delivery to brain tissues was greater with the lipid carrier than the plain drug suspension (86 and 54 ng/g, resp.) [79].

Dendrosome carriers, comprised of esterified oleoyl chloride and polyethylene glycol 400, have recently been published as effective means of delivery curcumin [75, 76]. *In vitro* data treating U87MG GBM cells with curcumin, dendrosomal curcumin, and empty dendrosome found that dendrosomal curcumin had superior efficacy at 24, 48, and 72 hours. As with many other studies, the authors found increased caspase activity when these cells were treated with curcumin. Interestingly, they also examined pluripotency transcription factors (OCT4A, OCT4BI, SOX-2, and Nanog) and found that treating U87 cells with dendrosomal curcumin led to significant decreases in these factors. During cellular differentiation, miR-145 expression suppresses these pluripotent genes. The authors reported that dendrosomal curcumin was able to exert its effects by inciting an approximately 35-fold elevation of miR-145 [75]. Treatment of A431 (epidermoid carcinoma) and WEHI-164 (mouse fibrosarcoma) cell lines with dendrosomal curcumin resulted in IC_{50} values of 14.3 and 7.5 μM at 48 hours, respectively, compared to 37 μM for curcumin alone against WEKI-164 cells [76]. WEHI-164 cells were injected subcutaneously in the flank in an *in vivo* experiment using BALB/c mice, and the results showed that treatment with dendrosomal curcumin led to significant reductions in tumor burden compared to curcumin treated animals or control [76]. Dendrosomal curcumin has also shown efficacy in the treatment of metastatic breast cancer cells (4T1) *in vitro* and *in vivo* with 11% of animals having metastases at necropsy compared to 89% of control. The authors also reported that treatment with dendrosomal curcumin led to downregulation of VEGF, COX-2, and MMP expression [80].

Curcumin has also been conjugated as a poly(glycerol-sebacate-curcumin) polymer similar to carmustine polymers used for local treatment with gliomas [81]. *In vitro* studies show IC_{50} values in response to this polymer of 23.2 μg/mL with U87 cells and 20.2 μg/mL with T98 cells [81]. Polycaprolactone implants have been studied as another delivery method for test agents with poor bioavailability. An *in vitro* assay with 9 L rat glioma cells reported that curcumin-loaded poly(ε-caprolactone)-poly(ethylene glycol)-poly(ε-caprolactone) nanofibers caused dose-dependent growth inhibition [82]. Gupta et al. reported a preclinical study [83] in August-Copenhagen Irish rats who received four polycaprolactone implants that were loaded with 40 mg of

curcumin. Analysis of liver, brain, and plasma by high-performance liquid chromatography (HPLC) with fluorescence spectroscopy showed an increase in curcumin levels versus animals receiving sham implant and a diet supplemented with 1000 ppm curcumin [83]. They also demonstrated that these implants can reduce benzo[a]pyrene induced lung DNA adducts by 62% in Sprague-Dawley rats [83].

Curcumin microparticles in a poly(D,L-lactide-co-glycolide) polymer has been formulated in an attempt to improve delivery of the drug and sustain its release. The investigators reported that a single subcutaneous injection was able to sustain release in the blood and body tissues of BALB/c mice for 4 weeks. Levels of curcumin in the lungs and brain show 10–30-fold greater distribution than the blood. Further, animals implanted with MDA-MB-231 human breast adenocarcinoma cells were effectively treated with these microparticles (49% decreased tumor volume versus empty microparticle), whereas repeated systemic injections of curcumin alone were no different than the vehicle treatment [84]. Curcumin nanoparticles (NanoCurc™) [21, 85] have also been used as a method to overcome the delivery and bioavailability barriers of curcumin. These particles, unlike free curcumin, are able to disperse in an aqueous environment [21]. Studies using xenograft models of human pancreatic cancer in athymic mice show that NanoCurc™ can lead to approximately 50% reduction in tumor volume. This inhibition was enhanced with the addition of gemcitabine. The authors reported that these effects were observed through decreased NF-κB activation, MMP-9, and cyclin D1 [85]. These nanoparticles have also been shown to suppress *in vitro* growth by MTS cell proliferation assay of embryonal tumor lines (DAOY and D283Med), U87 GBM cells, and glioblastoma neurosphere lines (JHH-GBM14 and HSR-GBM1) in a dose-dependent manner [86]. The authors suggested that, in this experiment, the effects could be due to a combination of G2/M arrest and apoptotic induction [86].

Additional benefits of using delivery systems include the ability to deliver multiple compounds simultaneously. For example, Dilnawaz and Sahoo used magnetic nanoparticles (MNP) to deliver both curcumin and TMZ to monolayer and spheroid T-98G GBM cultures. In monolayer cultures, IC_{50} values (μg/mL) for TMZ, curcumin, TMZ and curcumin were 1.1, 6.6, and 0.8 without MNP delivery and 0.2, 0.6, and 0.1 with MNP delivery. These same values in spheroid cultures were 23, 28, and 10 without MNP and 10, 11, and 5.2 with MNP delivery. Combination index analysis showed that the drug combination functioned synergistically in both cultures [87]. Another study using magnetic core nanocapsules reported the ability to deliver drugs with differing hydrophobicity [88]. They were able to accommodate doxorubicin (hydrophilic) and curcumin (hydrophobic) in the same particles for delivery to RG2 rat glioma cells [88].

Curcumin has also been coupled to an antibody as a delivery vehicle. Langone et al. [89] demonstrated that curcumin coupled to Muc18, a melanoma specific antibody, increased its efficacy against B16F10 melanoma. The *in vitro* IC_{50} value was reduced from 22 μM to 0.09 μM in cells treated with the curcumin-antibody adduct versus curcumin alone. In addition, C57BL/6 mice implanted with 1,000 B16F10 cells in their right forebrain showed a 10-fold decrease in tumor burden when treated with and curcumin-antibody adduct [89]. The authors reported that this effect was achieved by suppressing NF-κB [89]. In 2014, Langone et al. published another study that used an antibody delivery system for curcumin. In this experiment, a GBM-specific CD68 antibody was used to target GL261 (mouse), T98G (human), and U87MG (human) GBM cells [18]. GL261 cells were treated *in vitro* with 50 μM of curcumin for 24 hours, and western blot analysis showed suppression of NF-κB. In addition, curcumin was found to inhibit AKT-1 (neural survival and antiapoptotic), Bcl-xL (antiapoptotic), cyclin D1 (cell cycle promoter), and VEGF (promoting angiogenesis) [18]. Curcumin alone yielded IC_{50} 15 μM, whereas treatment with the curcumin-antibody adduct had IC_{50} of 0.125 μM. IC_{50} values for U87MG and T98G cells were 25 and 8 μM for curcumin alone and 0.400 and 0.225 μM, respectively, for treatment with the CD68 antibody-linked curcumin. GL261 cells were also implanted in the right forebrain of C57BL6 mice. These mice were treated with intracranial infusions of 16 pmol CD68 antibody-linked curcumin followed by tail vein infusion of curcumin and resulted in a reduction of intracranial tumor burden [18].

4. Toxicity/Safety

Natural products are of interest as anticancer agents because they have been associated with low toxicity profiles. This allows them to be safely used at high doses or added to existing regimens. Indeed, curcumin has been administered to human subjects at large doses without major side effects [67–69]. However, some data exists that serves to caution against its use. Despite being an antioxidant, curcumin may lead to a temporary increase in ROS and a decrease in cell viability by depletion of glutathione [90]. Curcumin might cause DNA base damage and fragmentation through cytochrome p450 enzyme generation of a curcumin radical and lead to apoptotic cell death in healthy as well as tumoral tissues [91]. Interestingly, this damage was attenuated when curcumin was present at larger concentrations [92]. Similar DNA damage findings have been noted during *in vivo* studies [93, 94]. Curcumin may inhibit the tumor suppressor p53 function in colon cancer cells and contribute to tumorigenesis [95]. Administration of curcumin with camptothecin or cyclophosphamide may inhibit their effectiveness; thus careful study of curcumin is warranted prior to its addition to an existing chemotherapy [96].

5. Clinical Data

There are over one hundred clinical trials examining the potential therapeutic effects of curcumin (Table 1, https://clinicaltrials.gov/). Many of these trials examine the efficacy of curcumin for the treatment of gastrointestinal diseases (e.g., Cohn's disease, ulcerative colitis, and inflammatory bowel disease), endocrine disorders (e.g., diabetes mellitus), neoplasms (breast, gastrointestinal, cervical, lymphoma, and pancreatic), immune system diseases (e.g., atopy, multiple

Table 1: Active clinical trials assessing therapeutic benefit of curcumin.

Trial	Status	Site	Disease	Measure	Trial ID*
Vascular					
Curcumin or placebo	Ongoing	Lawson Health Research Institute, CA	AAA	Serum creatinine	NCT01225094
Curcumin or placebo	Ongoing	University of Colorado Boulder, USA	Vascular aging	Arterial stiffness, arterial pulse-wave velocity, NO dilation	NCT01968564
Curcumin or placebo	Ongoing	UPEI, CA	Vascular stiffness	Arterial stiffness by tonometry, IL-6, CRP, CK	NCT02281981
Neurologic/psychiatric					
Curcumin with or without yoga	Recruiting	VA Los Angeles, USA	AD	Biomarkers for MCI	NCT01811381
Curcumin	Recruiting	SHSC, CA	Adolescent bipolar disorder	Mood by CDRS-R, biomarkers	NCT01928043
Curcumin with other supplements or placebo	Ongoing	HCL, France	Fibromyalgia	QOL by GIQLI	NCT01469936
Curcumin or placebo	Ongoing	UCLA, USA	MCI, normal aging	Cognitive changes by neuropsych assessment	NCT01383161
Curcumin or placebo with omega-3 fatty acid	Invitation only	TUMS, Iran	Migraine	HA, endothelial factors, inflammation	NCT02532023
Curcumin or placebo with IFN-β1A	Ongoing	Merck Serono, Italy	Multiple sclerosis	Proportion of subjects with active T2 lesions	NCT01514370
Curcumin or placebo	Recruiting	Beersheva Mental Health Center, Israel	Schizophrenia	Psychotic symptoms (PANSS)	NCT02298985
Curcumin	Recruiting	VA Los Angeles, USA	Schizophrenia	MCCB	NCT02104752
Curcumin or placebo	Not yet recruiting	Yale, USA	Schizophrenia, schizoaffective	MCCB	NCT02476708
Gastrointestinal					
Curcumin or placebo with thiopurines	Recruiting	CHU, France	Crohn's disease	Rutgeerts endoscopic score	NCT02255370
Curcumin with triple therapy or triple therapy alone	Not yet recruiting	Rabin Medical Center, Israel	Helicobacter pylori infection	Eradication by urea breath test	NCT02018328
Curcumin with selenium and green tea or placebo	Recruiting	Meir Medical Center, Israel	Irritable bowel syndrome	QOL by questionnaires	NCT01167673
Curcumin or placebo	Not yet recruiting	NCI, USA	MAG and/or GIM	Changes in IL-1β, safety, tolerability, histologic grade	NCT02782949
Curcumin or placebo	Not yet recruiting	Schneider Children's Medical Center, Israel	Pediatric ulcerative colitis	Disease activity by PUCAI	NCT02277223
Curcumin or placebo with 5-ASA	Recruiting	Asian Institute of Gastroenterology, India	Ulcerative colitis	Time to clinical and endoscopic remission	NCT02683733
Curcumin or placebo with 5-ASA	Recruiting	Asian Institute of Gastroenterology, India	Ulcerative colitis in remission	Percentage of patients in remission	NCT02683759
Oncologic					
Curcumin or placebo	Recruiting	Emory University, USA	Breast cancer	NF-κB by ELISA	NCT01740323
Curcumin at two different doses	Recruiting	OSU, USA	Breast cancer, obesity	Adherence, tolerability, safety	NCT01975363
Curcumin or HPR Plus or placebo	Recruiting	University of Rochester, USA	Noninflammatory breast cancer	Mean radiation dermatitis severity	NCT02556632
Curcumin with standard treatment	Recruiting	UZ Leuven, Belgium	Endometrial carcinoma	Peripheral blood inflammatory markers	NCT02017353

Table 1: Continued.

Trial	Status	Site	Disease	Measure	Trial ID*
Curcumin	Recruiting	Baylor Research Institute, USA	Squamous CIN3	Safety, feasibility, overall and pathologic response	NCT02554344
Curcumin and piperine	Recruiting	Mayo, USA	Cancer with inflamed ureteral stent	AE, max tolerable dose, optimal dose	NCT02598726
Curcumin with cholecalciferol	Recruiting	Case CCC, USA	CLL or SLL, stages 0-II	Overall response rate by NCI-WG (CLL) or Cheson (SLL)	NCT02100423
Curcumin	Recruiting	University Hospital Salzburg, Austria	Locally advanced or metastatic cancer	Safety, max tolerable dose, tumor response	NCT02138955
Curcumin or placebo	Recruiting	UPR, Puerto Rico	FAP	Tolerability and efficacy by polyp number and size	NCT00927485
Curcumin or placebo	Ongoing	NCI, USA	FAP	Laboratory biomarker analysis	NCT00641147
Curcumin with anthocyanin extract	Recruiting	The Hospital Galliera, Italy	Colorectal adenoma	β-Catenin, NFkβ, Ki-67, p53 by IHC	NCT01948661
Curcumin with 5-FU	Recruiting	Baylor Research Institute, USA	Metastatic colon cancer resistant to 5-FU	Safety, toxicity, response, biomarkers	NCT02724202
Curcumin with FOLFOX or FOLFOX alone	Ongoing	University of Leicester, UK	Metastatic colorectal cancer	Tolerable long-term dose, safety	NCT01490996
Curcumin with Avastin/FOLFIRI	Not yet recruiting	Gachon University Gil Medical Center, Korea	Metastatic colorectal cancer	PFS	NCT02439385
Curcumin with irinotecan	Recruiting	UNC Lineberger, USA	Metastatic colorectal cancer	Max tolerated dose, pharmacokinetics	NCT01859858
Curcumin or placebo with capecitabine and radiation	Ongoing	MD Anderson, USA	Rectal cancer	Pathologic complete response rate	NCT00745134
Curcumin or placebo with docetaxel	Recruiting	Centre Jean Perrin, France	Metastatic prostate cancer	Time to progression, PSA response	NCT02095717
Curcumin or placebo	Recruiting	UT Southwestern, USA	Prostate cancer	PSA, recurrence free survival	NCT02064673
Curcumin or placebo with RT	Recruiting	SBUMS, Iran	Prostate cancer	Proctitis and cystitis by CTCAE, PSA	NCT02724618
Curcumin with gemcitabine, metformin, and paclitaxel	Recruiting	City of Hope Medical Center, USA	Metastatic pancreatic cancer	Feasibility, compliance, toxicity, survival	NCT02336087
Curcumin with EGFR-TKI	Recruiting	Lady Davis Institute, CA	Nonresectable mutant EGFR NSCLC	Feasibility, adherence, AE, QOL (FACT-L), CRP	NCT02321293
Other					
Curcumin or placebo	Recruiting	University of Colorado Denver, USA	ADPKD	Changes in FMD-BA and aortic pulse-wave velocity	NCT02494141
Curcumin mouthwash	Recruiting	Aurora BayCare Medical Center, USA	Chemotherapy induced mucositis	AE, toxicity, pain, healing time	NCT02300727
Curcumin or placebo	Recruiting	Lawson Health Research Institute, CA	CKD	Albuminuria, eGFR, IL-18	NCT02369549
Curcumin or placebo	Recruiting	NNFTI, Iran	DMII	Triglyceride and CRP levels	NCT02529969
Curcumin or placebo	Recruiting	NNFTI, Iran	DMII	Fasting blood sugar, antioxidant capacity	NCT02529982
Curcumin with other nutraceuticals or placebo	Not yet recruiting	IRCCS Neuromed, Italy	NAFLD	ALT, AST, GGT	NCT02369536

Table 1: Continued.

Trial	Status	Site	Disease	Measure	Trial ID*
Curcumin in Orabase	Ongoing	SVSIDS, India	Oral submucous fibrosis	Reduction of lesion, number of bands	NCT02645656
Curcumin	Recruiting	University of Arizona, USA	Rheumatoid arthritis	AE, pharmacokinetics, ESR, CRP	NCT02543931

* *Source*: https://www.clinicaltrials.gov/. A search performed using the keyword "curcumin" revealed 129 studies. Only active studies (48) were included in the table. Clinical trials that were complete (58), were withdrawn (8), were terminated (3), or have unknown status (12) were excluded.
CA: Canada; CCC: Comprehensive Cancer Center; CHU: Clermont-Ferrand University Hospital; HCL: Hospices Civiles de Lyon; NCI: National Cancer Institute; NNFTI: National Nutrition and Food Technology Institute; OSU: Ohio State University; SBUMS: Shahid Beheshti University of Medical Sciences; SHSC: Sunnybrook Health Sciences Centre; SVSIDS: Sri Venkata Sai Institute of Dental Sciences; TUMS: Tehran University of Medical Sciences; UCLA: University of California, Los Angeles; UK: United Kingdom; UNC: University of North Carolina; UPEI: University of Prince Edward Island; UPR: University of Puerto Rico; UT: University of Texas; USA: United States of America.5-ASA: 5-Aminosalicylic Acid; 5-FU: 5-Fluorouracil; AAA: Abdominal Aortic Aneurysm; AD: Alzheimer's disease; ADPKD: Autosomal Dominant Polycystic Kidney Disease; AE: adverse events; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; CDRS-R: Children's Depression Rating Scale-Revise; CIN: Cervical Intraepithelial Neoplasia; CK: Creatine Kinase; CKD: Chronic Kidney Disease; CLL: Chronic Lymphocytic Leukemia; CRP: C-Reactive Protein; DMII: Diabetes Mellitus Type 2; EGFR: epidermal growth factor receptor; eGFR: Estimated Glomerular Filtration Rate; ESR: Erythrocyte Sedimentation Rate; FAP: Familial Adenomatous Polyposis; FMD-BA: Brachial Artery Flow-Mediated Dilation; FOLFOX: Folinic Acid, 5-Fluorouracil, Oxaliplatin; GGT: Gamma-Glutamyl Transferase; GIM: Gastric Intestinal Metaplasia; GIQLI: Gastrointestinal Quality of Life Index; HA: headache; IHC: Immunohistochemistry; IL: Interleukin; MAG: Multifocal Atrophic Gastritis; MCCB: MATRICS Consensus Cognitive Battery; MCI: Mild Cognitive Impairment; NCI-WG: National Cancer Institute-Working Group; NAFLD: Nonalcoholic Fatty Liver Disease; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NO: Nitric Oxide; NSCLC: Nonsmall Cell Lung Cancer; PANSS: Positive and Negative Syndrome Scale; PFS: Progression Free Survival; PSA: prostate-specific antigen; PUCAI: Pediatric Ulcerative Colitis Activity Index; QOL: quality of life; RT: Radiotherapy; SLL: Small Lymphocytic Lymphoma; TKI: Tyrosine Kinase Inhibitor; VA: Veterans Affairs.

sclerosis, and rheumatoid arthritis), and psychiatric disorders (e.g., Alzheimer's disease, schizophrenia, cognitive impairment, and depression). To date, no clinical data exist on treatment of brain tumors with curcumin despite the vast amount of promising preclinical data. A study completed in May 2013 (NCT01712542) examined the bioavailability of curcumin in patients with GBM, but results from this study have not yet been made available. Other human bioavailability data are discussed in "Delivery Mechanisms."

6. Conclusion

The use of curcumin for the treatment of CNS tumors needs to be investigated. Preclinical data support its use *in vitro* and *in vivo*. In addition, there have been several limited studies demonstrating the safety of administration of oral curcumin in humans. Initial efficacy studies might focus on patients that have progressed on the current standard of care. As discussed previously, curcumin may also enhance the cytotoxic capabilities of other chemotherapeutics. Data also exists to suggest that curcumin might affect tumoral stem cell-like populations [16, 46, 97]. These stem cell-like features are found in nestin and CD133 positive cells are associated with higher grade and poor prognosis [46, 98, 99]. Adding curcumin to other chemotherapeutic regimens may aid in slowing or halting growth of these progenitor cells and thus their tumors. Since the administration of curcumin appears relatively safe, it could be added to current standard of care regimens in early or advanced disease. The greatest barrier to the use of curcumin as a therapeutic is its poor distribution to affected tissues. The use of delivery vehicles or curcumin derivatives may increase the efficacy of curcumin further by improving distribution, slowing degradation, and improving target specific affinity. Since individuals afflicted with brain tumors have such poor prognoses, there is a dire need to identify new therapeutic agents. As in the case with GBM, it is unfortunate that large clinical trials have been undertaken without identifying improvements to the standard of care that was formulated over 10 years ago [100–104]. Clinical trials should be undertaken to corroborate the benefits of curcumin seen in preclinical studies and improve the prognoses of individuals with brain tumors.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1564257, 9 pages
http://dx.doi.org/10.1155/2016/1564257

Research Article

Rosmarinic Acid and *Melissa officinalis* Extracts Differently Affect Glioblastoma Cells

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Received 19 July 2016; Accepted 15 August 2016

Academic Editor: Musthafa Mohamed Essa

Lemon balm (*Melissa officinalis* L.) has many biological effects but especially important is its neuroprotective activity. The aim of the study is to produce different extracts of *Melissa officinalis* and analyse their chemical composition and biological properties on rat glioblastoma C6 cells. Results revealed that rosmarinic acid (RA) is the predominant compound of lemon balm extracts. RA has cytotoxic effect on glioblastoma cells (LC_{50} 290.5 μM after the incubation of 24 h and LC_{50} 171.3 μM after 48 h). RA at concentration 80–130 μM suppresses the cell proliferation and has an antioxidant effect. 200 μM and higher concentrations of RA have a prooxidant effect and initiate cell death through necrosis. The aqueous extract of lemon balm is also enriched in phenolic compounds: protocatechuic, caftaric, caffeic, ferulic, and cichoric acids and flavonoid luteolin-7-glucoside. This extract at concentrations 50 μM–200 μM RA has cytotoxic activity and initiates cell death through apoptosis. Extracts prepared with 70% ethanol contain the biggest amount of active compounds. These extracts have the highest cytotoxic activity on glioblastoma cells. They initiate generation of intracellular ROS and cell death through apoptosis and necrosis. Our data suggest that differently prepared lemon balm extracts differently affect glioblastoma cells and can be used as neuroprotective agents in several therapeutic strategies.

1. Introduction

Glioblastoma multiforme (GBM), the most common and the most lethal CNS cancer, causes approximately 50% of all brain tumors. Chemotherapy and radiotherapy are used for treatment. Temozolomide is a first-line medicament for treatment of GBM; however this drug has many adverse effects and average lifespan of patients after treatment is about 1 year. Therefore, nowadays the fact that a "single" targeted therapy might not be the most effective approach and multitargeting would be the rational approach for killing a heterogeneous population of cancer cells in a tumor is often discussed. The relevance of natural agents of dietary origin in human cancer is appreciated, because it is a part of the normal diets in various cultures, these agents are nontoxic to humans and are able to modulate multiple signalling pathways [1]. It is important to determine if natural bioactive compounds (extracts of *Melissa officinalis* and rosmarinic acid), the neuroprotective effect of which is proven, could be potential candidates for additional therapy in brain cancer cases.

Lemon balm (*Melissa officinalis*) and its main active substance, rosmarinic acid (RA), have multiple neuroprotective effects. Scientific data show that rosmarinic acid could decrease level of intracellular reactive species and the level of DNA damage induced by ethanol in mice [2]. RA produces a significant neuroprotective potential in rats with ischemia and reperfusion: it reduces apoptosis and necrosis, increases cell survival, and decreases LDH leakage rate in cultured SH-SY5Y cells [3]. Pre- and posttreatment with RA decrease ciguatoxin-mediated neurotoxicity diminishes the extracellular LDH activity and DNA damage in primary human neurons [4]. RA exhibits neuroprotective effects in the neurotoxicity of amyloid β- (Aβ-) induced cognitive

dysfunction and has an antidepressant-like property in animal models of depression [5]. It is now widely studied RA anticancer activity. RA was applied to various human cancer cell lines like NCI-H82, DU-145, Hep-3B, K-562, MCF-7, PC-3, MDA-MB-231, and it was shown that RA may inhibit cell proliferation, induce apoptosis, and decrease viability of investigated cells in dose-dependent manner [6, 7] Since the number of brain cancer cases has increased in the past decades and the effects of RA on tumor cells are not clearly identified, the aim of this work is to identify the effects of this substance on the most aggressive type of brain tumors, glioblastoma cells.

A lot of research is being performed in order to prove the effect of extracts, made from *Melissa officinalis,* in treatment of different forms of brain diseases [8–14]. Investigations on rats have been performed, which established that the aqueous or methanolic extract of lemon balm affects the GABA transaminase in the brain as an inhibitor in anxiety, epilepsy, and so forth, [8, 9]. According to Akhondzadeh et al. the effect is achieved by stimulating the activity of acetylcholine receptors in the central nervous system [10]. Scientific data has proven that *Melissa officinalis* extracts may have anticancer activity. Encalada with coauthors demonstrated cytotoxic effect of the 50% ethanolic and aqueous extract against human colon cancer cells [15]. Weidner with coauthors evaluated the effect of ethanolic lemon balm extract on HT-29 and T84 human colon carcinoma cells. Experimental data showed that investigated extract inhibits the proliferation of colon carcinoma cells and induces apoptosis through formation of ROS [16].

Most studies have shown that the biological effect of *Melissa officinalis* extracts, as well as other plants from the *Lamiaceae* family, mainly depends on RA concentration [11–13]. Differently prepared extracts are enriched with other biologically active compounds, which, acting synergistically with RA, may increase biological effect of the extracts. Extracts are produced by using various extraction solvents. Water as solvent is always used in preparation of extract (tea) at home. However, most of biologically active compounds have low solubility in water. Ethanol is the most popular solvent in industry for producing liquid extracts intended for oral use. Additionally, ethanol is capable of dissolving most of biologically active compounds nonsoluble in water. In summary, the amount of active compounds in differently prepared extracts depends on solvent and production conditions.

The aim of this study was to produce different liquid extracts of lemon balm (*Melissa officinalis* L.) and analyse the chemical composition, investigate their antiproliferative, antioxidant, and cytotoxic effects on rat glioblastoma C6 cells, and compare them with effects of rosmarinic acid.

2. Materials and Methods

2.1. Chemicals and Reagents. Raw lemon balm (*Melissae folium*) was obtained from JSC "*Acorus calamus*" (Vilnius, Lithuania), and dry lemon balm extract (*sicc. Extractum Melissae officinalis*) was obtained from Naturex (France). All the reagents and standards were of analytical grade. Luteolin-7-glucoside, caffeic acid, and rosmarinic acid were obtained from Extrasynthese (Genay, France), protocatechuic acid, caftaric acid, ferulic acid, and cichoric acid from Fluka (Buchs, Switzerland). Dulbecco's modified Eagle's medium (DMEM), Ampliflu™ Red, and 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), HPLC-grade acetonitrile, and trifluoroacetic acid (TFA) obtained from Sigma-Aldrich GmbH (Buchs, Switzerland). Deionized water was acquired from a Milli-Q purification system (Bedford, USA).

2.2. Preparation of Lemon Balm Extracts. The lemon balm dry extract was dissolved in purified water at the ratio 1 : 100. Manufactured extract (N1) is filtered through paper filter.

Extracts of lemon balm (N2, N3) are produced using 40% and 70% ethanol solutions as extract solvents. Raw material and extract solvent ratio is 1 : 1. Crushed herbal of lemon balm is soaked in an appropriate amount of solvent and left for maceration for seven days [14]. After extraction, extracts of lemon balm were filtered through paper filter.

2.3. Analysis of Extracts by High-Performance Liquid Chromatography. Chromatographic analysis was carried out using Waters Alliance e2695 Separations Module equipped with a Waters 2998 PDA Detector (Milford, USA). The separation was performed on an ACE Excel 3 SuperC18 analytical column (Aberdeen, Scotland) (250 × 4.6 mm, 3 μm) at 25°C. The mobile phase consisted of 0.1% TFA in deionized water (A) and acetonitrile (B). The gradient elution was as follows: 0–30 min, 15%–30% B; 30–50 min, 30%–60% B; 50–55 min, 60%–90% B; and 55–60 min, 90%–15% B. The flow rate was 0.5 mL min^{-1}, and the injection volume was 10 μL. The detector was set in the 200–400 nm range. The chromatographic data were acquired and processed with Empower 3 software (Milford, USA).

2.4. Cell Line and Cell Culture. The C6 rat glioma model has been widely used in experimental neuro-oncology to evaluate the therapeutic efficacy of a variety of modalities, including chemotherapy [17]. A unique feature of C6 culture is that, exactly like a GBM tumor, it contains a subculture of cancer stem cells that express CD133 and nestin, which are widely used markers for brain CSCs [18]; therefore we choose this cell line for our investigations.

Rat glioblastoma C6 cells were purchased from the Cell Lines Service GmbH (Germany). C6 cells of convenient concentration were seeded in culture flasks containing DMEM with 10% of fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were then incubated at 37°C, with 5% CO_2 and saturated humidity; culture transfer was performed once every 3-4 days.

2.5. Assessment of Cell Viability

2.5.1. MTT Assay. Cell viability was assessed by measuring the ability of cells to metabolize MTT. After incubation of C6 cells in 96-well plates (20000 cells/well) for 24 h, they were

Table 1: Quantity of identified active compounds in differently prepared lemon balm extracts.

Extract type	Active compounds ± SE (μg/mL)						
	Protocatechuic acid	Caftaric acid	Caffeic acid	Luteolin-7-glucoside	Ferulic acid	Cichoric acid	Rosmarinic acid
Aqueous (N1)	1.1 ± 0.12	14.2 ± 0.23	3.0 ± 0.17	1.0 ± 0.10	5.1 ± 0.21	7.9 ± 0.16	244.7 ± 1.8
Ethanolic 40% (N2)	9.84 ± 0.11	24.35 ± 1.0	39.22 ± 0.13	5.06 ± 0.11	14.73 ± 0.14	10.04 ± 0.10	152.96 ± 0.2
Ethanolic 70% (N3)	16.87 ± 0.25	67.8 ± 0.18	73.27 ± 0.16	30.40 ± 0.22	69.3 ± 0.15	49.43 ± 0.17	1750.2 ± 2.9

treated without (control) or with different concentrations of investigated solutions for 24 or 48 h. After treatment DMEM medium was removed from wells, and then cells were washed twice with 100 mL/well Phosphate Buffered Saline (PBS). After washing, 180 μL/well PBS was added along with 20 μL/well of 5 mg/mL MTT dye dissolved in PBS to each well. The cells were incubated with MTT for 2 h. Blue Formosan crystals formed in the intact cells were dissolved in DMSO (100 μL/well). The absorption was measured at 570 nm and 620 nm as reference with a microplate spectrophotometer (Sunrise, Tecan Group Ltd., Switzerland). The results were expressed as percentages of MTT reduction, with the absorbance exhibited by the control cells being as 100%.

Concentration of ethanol used for control varied depending on the amount of extracts used for investigation, the maximal concentration was 2.5% of ethanol.

The LC_{50} concentration was calculated by nonlinear regression analysis, fitting the data to equations, using the software package SigmaPlot 12.0 version (Systat Software Inc.).

2.5.2. Assessment of Cell Count and Cell Death by Hoechst and Propidium Iodide Staining. Cell count was assessed using Hoechst 33258 and propidium iodide staining. At first, C6 cell suspension was dispersed to 24-well plates (2500 cells/well). After 24 h different concentrations of analysed preparations were added to the medium for 24 h, 48 h, and 72 h. After treatment, 15 min before investigation 5 μg/mL of Hoechst 33258 and 2 μg/mL of propidium iodide were directly added to the culture medium. After incubation viable, apoptotic, and necrotic cells were counted under fluorescence microscope.

2.6. Measurement of Intracellular RS Generation. The production of RS was assessed using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA). After incubation of C6 cells in 96-well plates (20000 cells/well) for 24 h, they were incubated with DCFH-DA (10 μM) in HBSS at 37°C for 30 min. During this time a part of DCFH is diffused into the cells. The dye, which was not diffused into the cells during incubation and remained in the outside medium, was washed twice with PBS. Wells were filled with a HBSS medium, enriched with investigated solutions of different concentrations. In the presence of cellular oxidizing agents, DCFH is oxidized to the highly fluorescent compound dichlorofluorescein (DCF); thus, the fluorescence intensity is proportional to the amount of RS produced in the cells. The fluorescence of DCF was detected by fluorometer at excitation and emission wavelengths of 488 and 525 nm, respectively.

2.7. Statistical Analysis. Results are presented as means ± standard error. Statistical analysis was by one-way analysis of variance (ANOVA), followed by Dunnett's posttest using the software package SigmaPlot 12.0 version (Systat Software Inc.). A value of $p < 0.05$ was taken as the level of significance.

3. Results

3.1. Chemical Composition of Differently Prepared Extracts. The chemical composition of the lemon balm extracts as obtained by HPLC method is shown in Table 1. Results revealed that RA is the predominant active ingredient of lemon balm extracts. Since RA is soluble in water, its concentration in aqueous extracts is relatively high, although the concentration of other identified substances is higher in ethanol solutions. Extracts prepared with 40% ethanol contain significantly smaller amount of all identified compounds in comparison with 70% ethanol. Results of our study demonstrated that 70% ethanol is the best solvent for extracting biologically active compounds from lemon balm.

3.2. Effect of Analysed Preparations on Cell Viability. Investigation of biological activities was started from analysis of the effect of various RA concentrations on viability of C6 cells. As estimated by MTT assay (Figure 1), cell viability was significantly decreased only at a concentration of 200 μM and higher after 24 h incubation. Therefore we extended the incubation with various RA concentrations up to 48 h. After 2 days a statistically significant decrease in viability was determined at 100 μM RA concentration. Calculated RA LC_{50} after 24 h of incubation was 290.5 ± 48.1 μM, and after 48 h LC_{50} was 181.3 ± 20.4 μM.

Extract N1 (Figure 2), produced by using a water solvent, after 24 h of incubation at a lowest investigated amount (RA concentration 50 μM), had tendency to increase intensity of absorption. A statistically significant decrease of viability was determined in an amount of extract with an RA concentration of 140 μM or higher. After extending the period of incubation to 48 h, a decrease in viability was estimated at 50 μM RA, and a 100% cell death was caused by amount of extract N1, containing 200 μM RA.

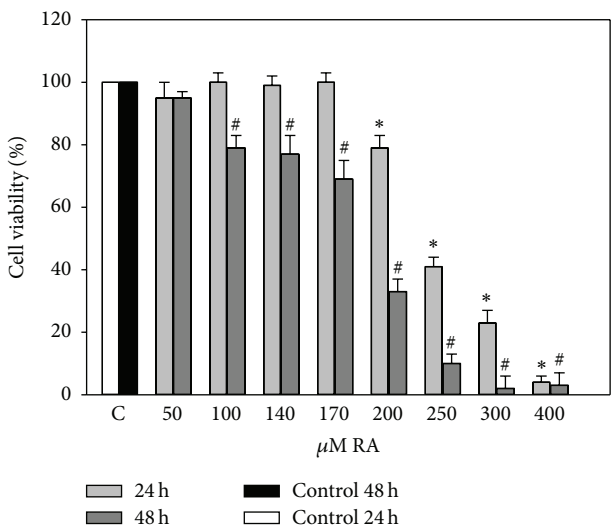

Figure 1: Effects of different concentrations of RA on the viability of C6 cells. C6 cells were treated with different concentrations (50–400 μM) of RA for 24 and 48 hours. Cell viability was assessed using MTT method. Data are presented as means of percentage of the untreated control cells ± SE ($n = 5$). $^{*}p < 0.05$ versus control 24 h, $^{\#}p < 0.05$ versus control 48 h.

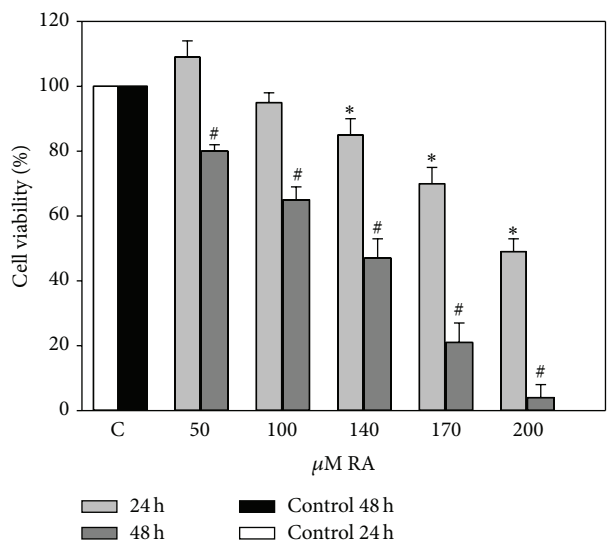

Figure 2: Effects of different concentrations of aqueous extract N1 on the viability of C6 cells. C6 cells were treated with different concentrations of aqueous extract (50–200 μM of RA) for 24 and 48 hours. Cell viability was assessed using MTT method. Data are presented as means of percentage of the untreated control cells ± SE (n = 4-5). $^{*}p < 0.05$ versus control 24 h, $^{\#}p < 0.05$ versus control 48 h.

40% and 70% ethanolic extracts have affected cell viability significantly stronger (Figures 3(a) and 3(b)) if compared with N1 or RA alone. In both cases when the amount of investigated extracts was small (10 μM of RA), cell viability has increased after 24 h of incubation (11–19%), extracts with 40–100 μM RA have statistically significantly decreased cell viability by 25–98% after 24 h of incubation. After 48 h of incubation the viability decreased by 50% with extracts containing 40 μM RA and more.

3.3. Assessment of Antiproliferative and Cytotoxic Effect of Analysed Preparations. Glioblastoma cells have a very high proliferative activity; therefore it is relevant to analyse proliferation-inhibiting properties of investigated preparations. RA concentrations not yet causing cell death (80–130 μM) have been chosen for this analysis. Results (Figure 4(a)) have shown that after 6 h the number of cells did not differentiate in any group. After 24 h the number of cells treated with 130 μM RA has decreased in comparison with the control. Significant changes have been evident after 48 h and 72 h of incubation: the cells in the control group gradually multiplied, whereas the cell count in groups incubated with RA (80 μM) has unchanged or started to decrease (100 μM, 130 μM) and some necrotic cells were found. Higher RA concentrations cause cell death by necrosis (Figures 5(d), 5(e), and 5(f)).

Different results were found by analysing the antiproliferative and cytotoxic activity of N 1–3 extracts.

The aqueous extract N1, used in concentrations 50 μM–100 μM RA (Figure 4(b)), has increased or has not changed the number of cells after 24 h incubation and decreased the number after 48 and 72 h. This extract caused death of C6 cells mainly by apoptosis (89±4%) and only a small number of cells died due to necrosis (12 ± 3%) (Figures 5(a) and 5(b)).

The number of cells has increased in extracts N2 and N3 with 5–10 μM RA concentration, whereas higher concentrations resulted in dead cells. Cell death by apoptosis (61 ± 7%) and necrosis (34 ± 5%) has been determined (Figure 5(c)).

3.4. Assessment of Antioxidant/Prooxidant Activity of Analysed Preparations. Since the extracts in low concentrations increased the proliferation of cells we have hypothesised that this effect can be initiated by an increased amount of intracellular reactive species (RS) and our further experiments were aimed to assess effect of the analysed preparations on the concentration of RS.

At first we analysed the effect of RA on the amount of RS. The results show (Figure 6(a)), that RA at concentration of 50–150 μM after 2 h reduced the amount of RS by 15–43%. 200 μM and higher concentration of RA increased the amount of intracellular RS (16%, 40%, and 57% at, resp., 200 μM, 300 μM, and 400 μM RA concentrations).

All concentrations of aqueous extract increased the amount of RS (Figure 6(b)); a statistically significant difference was achieved at concentrations of 150 μM and 200 μM, which after incubation of 2 h increased the amount of RS by 16% and 27%.

All investigated concentrations of N2 and N3 extracts (Table 2) increased the amount of intracellular RS and after 2 h it was ×1.86–2.23 higher at RA concentration of 75 μM.

4. Discussion

Studies have suggested that diets rich in RA and RA rich extracts together with other phenolic compounds may exert

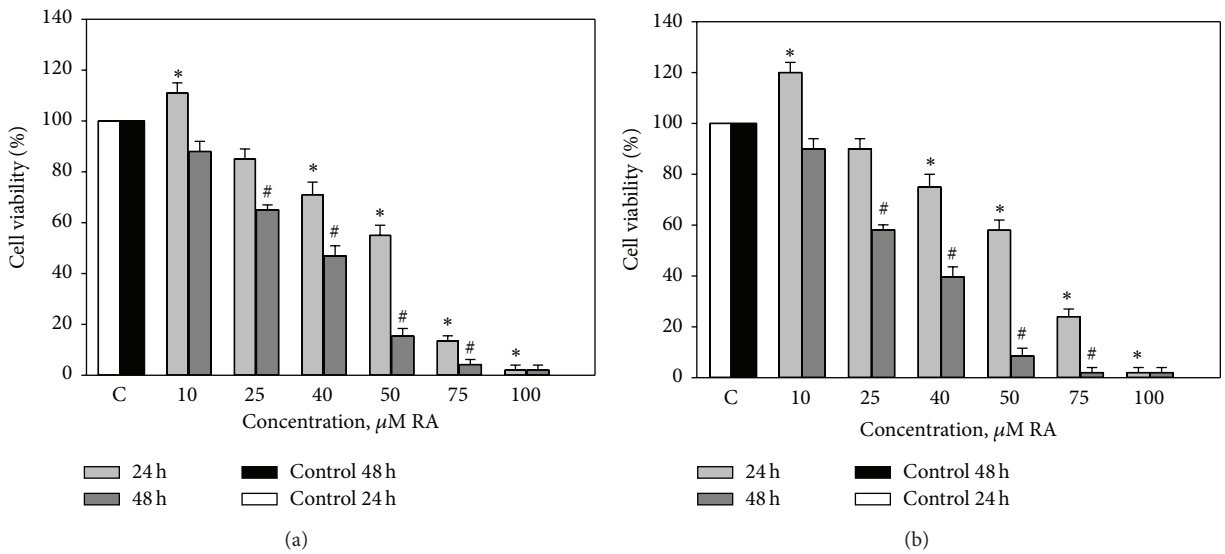

Figure 3: Effects of different concentrations of ethanolic extracts (a) N2 and (b) N3 on the viability of C6 cells. C6 cells were treated with different concentrations of ethanolic extracts (10–100 μM of RA) for 24 and 48 hours. Cell viability was assessed using MTT method. Data are presented as means of percentage of the untreated control cells ± SE ($n = 3$). $^{*}p < 0.05$ versus control 24 h, $^{\#}p < 0.05$ versus control 48 h.

Table 2: Amount of intracellular RS increased by different concentrations of ethanolic extracts N2 and N3, %.

	Fluorescence intensity, %			
	0.5 h	1 h	1.5 h	2 h
N2				
10 μM RA	15.6 ± 2.6	63.1 ± 5.2	102.9 ± 8.0	137.2 ± 11.4
40 μM RA	24.4 ± 3.9	82.9 ± 7.1	138.3 ± 14.2	160.4 ± 17.2
75 μM RA	35.1 ± 4.7	114.6 ± 10.5	152.4 ± 14.7	186.3 ± 15.1
N3				
10 μM RA	25.5 ± 2.8	79.2 ± 6.8	117.8 ± 9.5	152.5 ± 14.6
40 μM RA	34.3 ± 4.5	112.4 ± 10.3	164.7 ± 15.3	195.0 ± 18.3
75 μM RA	43.8 ± 6.0	165.5 ± 13.2	208.2 ± 19.1	223.9 ± 24.7

Data presented in the table show increase of fluorescence intensity (%) depending on concentration and time. $n = 3$-4.

neuroprotective effects in neuroinflammation, and neurodegeneration, as well as chemical-induced neurotoxicity and oxidative stress. Earlier studies revealed that preparations from *Melissa officinalis* may act as a modulator of mood and cognitive function and has antidepressant effect [19–22]. There is an increasing interest to identify plant-derived natural products with antitumor activities [23]; therefore we seek to investigate the effect of RA alone and its rich extracts, made of lemon balm, on glioblastoma cells.

Our experiments have shown that RA alone, depending on its concentration, has a proliferation-inhibiting effect. Results showed that, after an incubation of 48 and 72 hours, RA at concentrations 80–130 μM efficiently inhibited the cell proliferation. Makino with coworkers demonstrated that DNA synthesis, stimulated by PDGE and TNF-α was significantly decreased by RA (IC_{50} 1.4 μg/mL and 3.8 μg/mL) and in process of cell proliferation RA might regulate DNA synthesis both in early and in late signal transduction [24]. However, there are experiments demonstrating that RA showed proliferative effects rather than cytotoxic activity in almost all cell lines tested with the highest effect in K-562 cells, exhibiting a cell viability of 205% at 139 μM [6]. Our experiments revealed that RA reduces C6 cell viability; however, rather very high RA concentrations are needed to achieve this effect (LC_{50} after 24 h and 48 h incubation 290.51 μM and 171.3 μM, resp.) and only necrotic cells were found (Figures 5(e) and 5(f)). However, Hur with his coworkers found that after 24 h treatment of RA (3–30 μM) Jurkat cells displayed apoptosis, and at least 48 h of incubation with RA was required to induce apoptosis in almost 80–100% of the cells [25]. Moon and coworkers reported that RA alone exhibited little effect on the cell viability in human leukemia cells. However, a combination of TNF-α and RA induced apoptosis [26]. Even though there are studies revealing that RA induces apoptosis, our experiments, in which RA alone was studied, induced only necrosis in C6 glioblastoma cells. What is more, relatively high concentrations of RA are needed to reduce their viability.

There are many studies showing that RA has antioxidant properties attenuating oxidative stress and neuronal cell death [27, 28]. Ghaffari with coworkers show that H_2O_2-induced cytotoxicity in N2A cells was suppressed by treatment with RA. Moreover, RA is very effective in attenuating the disruption of lactate dehydrogenase, mitochondrial membrane potential, and intracellular ROS [29]. The results of our experiments show that the amount of RS in C6 cells depends on the concentration of RA and smaller studied concentrations (50–150 μM) reduced the amount of intracellular radicals. Therefore, these results confirm that, depending on the concentration, RA has an antioxidant effect and reduced the amount of intracellular radicals; thus, it can be used as a neuroprotective agent against inflammatory, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease)

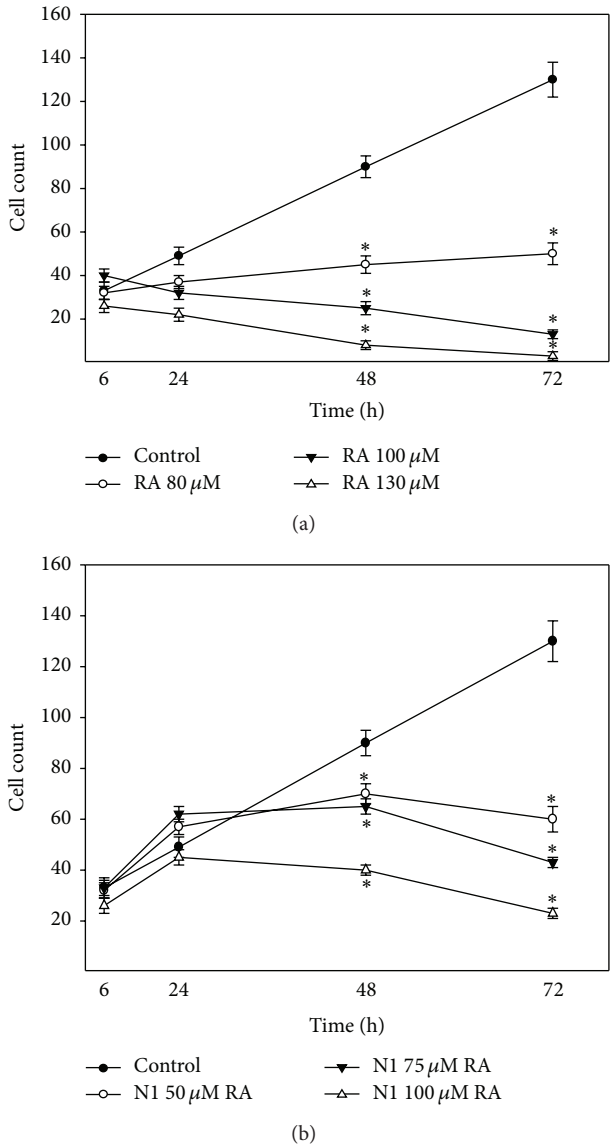

Figure 4: Effect of different concentrations of (a) RA and (b) N1 on C6 cell proliferation. Cell count was performed by adding Hoechst 33258 and propidium iodide dyes and counting the cells under fluorescence microscope. Data are presented as means of cell counts ± SE (n = 4-5). $^{*}p < 0.05$ versus control.

and other biologic processes associated with enhanced ROS level. Higher examined RA concentrations (200–400 μM) increased the amount of intracellular radicals in C6 cells. It is established that polyphenolic compounds often initiate cell death mechanisms by increasing the amount of ROS. Since, by studying the effect of RA on the viability of C6 cells, we have found that after the 24-hour incubation it statistically significantly reduces in the presence of 200 μM concentration and higher; it could be stated that one of the mechanisms by which RA causes the death of glioblastoma C6 cells depends on ROS-sensitive pathways.

Extracts, prepared from lemon balm, not only contain rosmarinic acid, but also are enriched in other phenolic compounds. Biologically active substances identified in our extracts are protocatechuic, caftaric, caffeic, ferulic, and cichoric acids and flavonoid luteolin-7-glucoside. These our data confirm results of other investigators which found that extracts of *Melissa officinalis* are rich in phenolic compounds [30]. Naturally occurring flavonoids and phenolic acids are the hydrophilic and lipophilic nature. Investigations of chemical composition of our prepared extracts showed that the amount of identified compounds increases in the order N3 (solvent 70% ethanol) > N2 (solvent 40% ethanol) > N1 (aqueous extract).

The extract prepared in aqueous method contains amount of RA bigger than in extract N2, but amount of other biologically active substances is 2–10-fold lower than in ethanolic extracts N2 and N3. Chemical composition determined biological action: N1 reduced the viability of C6 cells significantly weaker than ethanol extracts, but ~30% stronger than RA alone. Upon studying the cell proliferation, it was found that the amount of cells after 24 hours, compared to control, does not decrease, but after 48 and 72 hours it decreases significantly. It is important to note that this extract initiates the death of cells mainly through apoptosis. The studies of intracellular RS showed that this extract, depending on the concentration, increased the amount of RS; however this increase is significantly lower, compared to the increase of RS caused by ethanol extracts. Hence, it could be true that the death of cells is initiated not only by the increased amount of intracellular RS, but also by other mechanisms.

Ethanolic (40% and 70%) extracts initiated the death of cells 4-5 times stronger than that of RA alone or aqueous extract N1. Investigations of intracellular RS concentrations demonstrated that N2 and N3 extracts have a prooxidative effect; that is, the cells treated with these extracts contained significantly more RS, compared to control (Table 2). The beneficial health effects of medicinal plants rich in polyphenols are often attributed to their potent antioxidant activities. However, it is known that medicinal plants may also exert prooxidant effects [31]. Prooxidant effects are not necessarily bad because RS may act as signalling molecules of intracellular pathways. It is known that mild prooxidant effect promotes cell proliferation and higher amounts of RS induce cell death through apoptosis or necrosis, depending on additional conditions. Our investigations with ethanolic extracts revealed that lowest concentrations of extracts investigated in our experiments (10 μM RA) increased concentration of intracellular RS and proliferation of C6 cells. In cases of bigger amount of investigated extracts (40–75 μM RA) higher concentrations of RS were detected, and cells dying through apoptosis and necrosis were found. Weidner with coworkers have also found that ethanolic lemon balm extract inhibits the proliferation and induces apoptosis in HT-29 and T84 human colon carcinoma cell through formation of ROS [16]. Our results as well as other authors results demonstrate that ethanolic extracts exert strong *in vitro* antitumor activity.

Taking everything into account, RA and other polyphenols identified in extracts are known as (1) radical scavengers and they could directly neutralize ROS; (2) they could also regulate intracellular antioxidant system capacity. Kim with coauthors demonstrated that RA reversed the downregulations of GSH, SOD, and Bcl-2 [32]. Therefore, it would

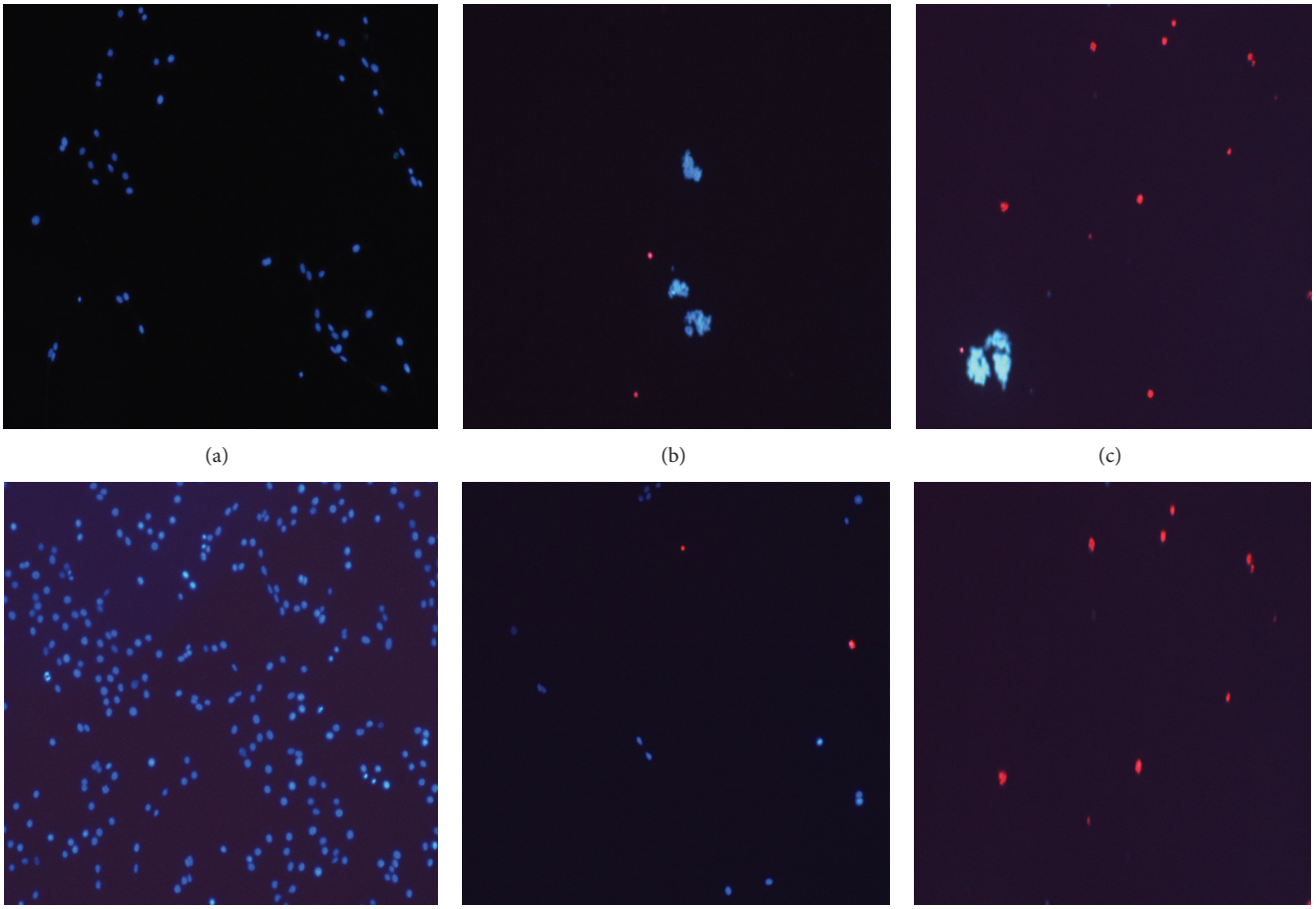

Figure 5: Typical images of glioblastoma C6 cells after incubation with different concentrations of RA and extracts. (a) Control after 24 h incubation; (b) N1 200 μM RA; (c) N3 50 μM RA; (d) control after 48 h incubation; (e) 170 μM RA; (f) 200 μM RA.

be useful to assess and compare the effects of investigated preparations on activity of intracellular antioxidant systems.

Higher concentrations of RA and extracts (investigated concentrations) increase intracellular RS levels. Although results of our and other investigators show that RS play a significant role in cell death, it remains unclear which RS (e.g., hydrogen peroxide, superoxide, or others) are responsible for the cytotoxicity of investigated preparations. Identification of RS could be useful to hypothesise about mechanisms of action.

It is proven that RS that are generated intracellularly can induce mitochondrial depolarization and release of cytochrome c into the cytosol and thus participate in the activation of the caspase-3 cascade. So, one of regulators of cell's life/death in apoptosis and necrosis are mitochondria [33]. Several literature sources revealed that RA induced apoptosis through mitochondrial pathway [25]. Other biologically active compounds presented in extracts also may modulate mitochondrial functions, and total effect of extracts on mitochondria depends on the characteristics of complex of biologically active compounds [34, 35]. Therefore evaluation of effect of investigated preparations on mitochondrial functions could be beneficial to reveal detailed mechanisms of action in the future.

5. Conclusions

RA and aqueous and ethanol extracts have a different effect on C6 cells. The cytotoxic effect of rosmarinic acid on rat glioblastoma C6 cells (LC_{50} after the incubation of 24 h 290.5 μM and after 48 h LC_{50} 171.3 μM) is significantly weaker, compared to differently prepared extracts from *Melissa officinalis*. RA (80–130 μM) suppresses the cell proliferation and has an antioxidant effect until 150 μM. 200 μM and higher concentrations have a prooxidant effect and initiate the death of cells through necrosis.

The aqueous extract of lemon balm apart RA contains other biologically active substances soluble in water; however their concentration is lower if compared with ethanolic extracts. Investigated concentrations of this extract (50 μM–200 μM RA) have no antioxidant activity. Significantly lower concentration of aqueous extract is needed to achieve a cytotoxic activity if compare with pure RA. The cell death through apoptosis is mainly initiated.

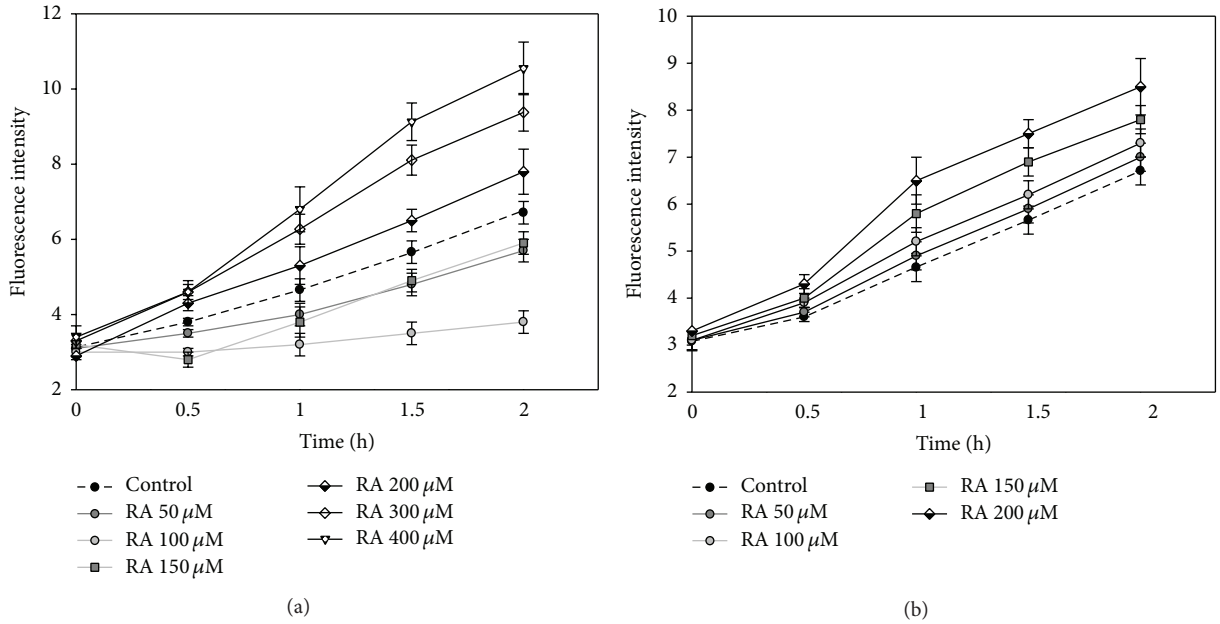

Figure 6: The effect of (a) RA and (b) N1 extract on concentration of intracellular RS. C6 cells were pretreated with 10 μM DCFH-DA and then treated with different concentrations of RA and aqueous extract N1 for 0; 0.5; 1; 1.5; and 2 hours. Fluorescence intensity, which is proportional to intracellular RS concentration, was detected by using a fluorometer at excitation and emission wavelengths of 488 and 525 nm, respectively. Data is presented by fluorescence intensity ± SE ($n = 3$).

Extracts prepared with 70% ethanol contain the biggest amount of active compounds; their concentration is lower in extract, prepared with 40% ethanol. Despite different concentrations of biologically active compounds, they have a high cytotoxic activity on glioblastoma C6 cells. These extracts initiate the generation of intracellular RS and cell death through apoptosis and necrosis.

Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Daiva Majiene designed the study and Kristina Ramanauskiene, Daiva Majiene, and Raimondas Raudonis performed experiments and analysed results. Daiva Majiene and Kristina Ramanauskiene wrote the manuscript.

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