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A review of specific dietary antioxidants and the effects on biochemical mechanisms related to neurodegenerative processes

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Abstract

Aging is a major risk factor for neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). An unbalanced overproduction of reactive oxygen species (ROS) may give rise to oxidative stress which can induce neuronal damage, ultimately leading to neuronal death by apoptosis or necrosis. A large body of evidence indicates that oxidative stress is involved in the pathogenesis of AD, PD, and ALS. An increasing number of studies show that nutritional antioxidants (especially Vitamin E and polyphenols) can block neuronal death in vitro, and may have therapeutic properties in animal models of neurodegenerative diseases including AD, PD, and ALS. Moreover, clinical data suggest that nutritional antioxidants might exert some protective effect against AD, PD, and ALS. In this paper, the biochemical mechanisms by which nutritional antioxidants can reduce or block neuronal death occurring in neurodegenerative disorders are reviewed. Particular emphasis will be given to the role played by the nuclear transcription factor-κB (NF-κB) in apoptosis, and in the pathogenesis of neurodegenerative disorders, such as AD, PD, and ALS. The effects of ROS and antioxidants on NF-κB function and their relevance in the pathophysiology of neurodegenerative diseases will also be examined.

Keywords: Neurodegeneration; Alzheimer's disease; Parkinson's disease; Amyotrophic lateral sclerosis; Oxidative stress; Nuclear factor κB; Antioxidants; Polyphenols; Neuroprotection; Nutritional

1. Introduction

Among the most common neurologic diseases are neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). As the elderly population increases, the prevalence of these age-related diseases is likely to increase. The cause of these diseases is not known and, with the possible exception of PD, there is no treatment that alters significantly the progression of any of these disorders. Of the few risk factors that have been identified for these diseases, increased age is the only one that is common to AD, PD, and ALS. For AD, the incidence and prevalence of the disease increase dramatically after age 60; one study showed a 47% prevalence for patients over age 85 [66]. In addition to the possible involvement in aging, mitochondrial dysfunction and oxidative damage may play important roles in the slowly progressive neuronal death that is characteristic of several different neurodegenerative disorders including AD, PD, and ALS [33,124,125,127,197].

There is substantial evidence that the brain, which consumes large amounts of oxygen, is particularly vulnerable to oxidative damage. Free radicals are normal products of cellular metabolism [100]. The predominant cellular free radicals are the superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) species [127,254]. Other molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), although not themselves free radicals, can lead to the generation of free radicals through various chemical reactions. Thus H_2O_2, in the presence of reduced metal, forms the highly reactive $OH^{\bullet}$ via the Fenton reaction [254]. Peroxynitrite ($ONOO^-$), formed by the reaction of nitric oxide ($NO^{\bullet}$) with $O_2^{\bullet -}$, is a highly reactive molecule that also breaks down to form $OH^{\bullet}$. Together, these molecules are referred to as reactive oxygen species (ROS) to signify their ability to lead to oxidative changes within the cell [254]. Problems occur when the production of ROS exceeds the ability of cells to defend themselves against these substances. This imbalance between cellular production of ROS and the ability

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of cells to defend themselves against them is referred to as oxidative stress [254]. Oxidative stress can cause cellular damage and ROS oxidize critical cellular components such as membrane lipids, proteins, and DNA, thereby inducing apoptosis or necrosis [99,141,176,245,246]. Necrosis is characterized by a loss of plasma membrane integrity, the formation of large vacuoles, and cell swelling, whereas typical features of apoptotic cells are nuclear changes that include chromatin margination and condensation, DNA fragmentation, membrane blebbing, and cell shrinkage [233]. There is a large scientific literature regarding the relation between ROS production, the induction of apoptosis (or necrosis) and the pathogenesis of neurodegenerative disorders [16–18,40,75,120,233,278,289]. Although this subject is still a matter of debate, increasing evidence supports the hypothesis that neuronal death may occur primarily by apoptotic mechanisms in AD, PD and ALS [129,178,190,193,263]. Thus, clinical evidence shows signs of apoptosis in patients with AD, PD and ALS [8,193,263,264].

Cells normally have a number of mechanisms to resist against damage induced by free radicals [100]. The major antioxidant defenses consist of antioxidant scavengers such as glutathione (GSH), Vitamin C (ascorbic acid), Vitamin E (α-tocopherol), carotenoids, flavonoids, polyphenols, and antioxidant enzymes. Severe depletion of GSH in mice by administration of buthionine sulphoximine, which inhibits GSH synthesis, causes neuronal damage and mitochondrial degeneration [121]. There is a high concentration of ascorbic acid in the gray and white matter of the central nervous system in all species that have been examined [220]. Indeed, the brain, spinal cord and adrenal glands have the highest ascorbate concentrations of all the tissues in the body [220]. Ascorbate is a broad spectrum radical scavenger that is effective against peroxyl and hydroxyl radicals, superoxide, singlet oxygen, and peroxynitrite [220]. Also the lipid-soluble chain breaking antioxidant Vitamin E exerts a very important protective function against oxidative stress in the brain [99], and interacts with ascorbate enhancing its antioxidant activity [183]. Little information is available on the levels of carotenoids and flavonoids in the human brain. The antioxidant enzymes in the brain include Cu/Zn superoxide dismutase (SOD-1) and Mn superoxide dismutase (SOD-2) which catalyze the conversion of $O_2^{\bullet -}$ to H_2O_2 [73]. H_2O_2 is then converted to H_2O by either catalase or glutathione peroxidase (GSH-Px). Antioxidant defense mechanisms can be upregulated in response to increased ROS or peroxide production [42]. Although upregulating antioxidant defense systems may confer protection against ROS, they are not completely effective in preventing oxidative damage. Moreover, the efficiency of gene expression may decline with aging or become defective as oxidative damage to the genome increases. As already mentioned, the brain is especially vulnerable to ROS damage because of its high oxygen consumption rate, abundant lipid content, and relative paucity of antioxidant enzymes compared with other organs [45]. If the increased demand on the cell's capacity to detoxify ROS is not met, alterations, such as aldehydes or isoprostanes from lipid peroxidation, protein carbonyls from protein oxidation, and oxidized base adducts from DNA oxidation may occur [99]. Oxidation of polyunsaturated fatty acids (PUFA) results in the production of multiple aldehydes with different carbon chain lengths including propanal, butanal, pentanal, hexanal and 4-hydroxy-2-*trans*-nonenal (4-HNE). There is evidence that 4-HNE is capable of inducing apoptosis in PC12 cells and cultured rat hippocampal neurons suggesting that it is a mediator of oxidative stress-induced apoptosis [145]. These findings suggest that in addition to direct ROS damage to phospholipid membranes, there is an indirect mechanism involving 4-HNE, which may also be involved in neuronal death. In this regard, it noteworthy that 4-HNE has been suggested to be involved in the pathogenesis of PD [247,287]. Oxidative damage to proteins can be revealed by measuring protein carbonyl content [262], which was found to be elevated in AD and ALS patients [108]. Another indication of protein oxidation is the formation of nitrotyrosine by peroxynitrite. This might represent a useful clinical parameter of the occurrence of oxidative stress in neurodegenerative diseases, in as much as increased levels of nitrotyrosine have been found in AD, PD and ALS [1,18,84,85,109,257,265]. The most useful marker of DNA oxidation is 8-hydroxy-2′-deoxyguanosine (8-OHdG) which is elevated in patients with AD, PD and ALS [5,69,74,185,291].

Another index of oxidative stress is the activation of the transcriptional factor, nuclear factor-kappa B (NF-κB). Thus, a large body of evidences indicate that ROS can act as second messengers mediating intracellular responses, including NF-κB activation [50,70,159,180,211]. In turn, activated NF-κB can influence the expression of a large number of genes, including SOD-2 [50,179,180]. Hence, NF-κB activation can be considered as the executive branch of a feed-back mechanism that operates to regulate the intracellular concentration of ROS, trying to dampen an excessive accumulation of ROS which can be dangerous for the cell. Moreover, NF-κB induces the expression of the so-called inhibitor-of apoptosis proteins (IAPs), Bcl-2, and calbindins [179,270]. All these biochemical actions of NF-κB indicate that this transcription factor can exert an antiapoptotic effect, thereby protecting neurons against degeneration [179,180]. As we will discuss below, these data are consistent with clinical findings showing increased levels of NF-κB in vulnerable regions of the central nervous system of AD, PD and ALS [117,133,189].

Although the available data are still limited, epidemiological studies indicate that dietary habits can influence the incidence of neurodegenerative disorders such as dementia (including AD) and PD [54,106,155,201,223]. For example, incidence data from the so-called Personnes Agees Quid (PAQUID) study showed that people drinking three to four glasses of wine per day had an 80% decreased incidence of dementia and AD 3 years later, compared to those

who drank less or did not drink at all [51,155,201]. This protective effect was still highly significant after adjusting the data for potential confounding factors such as age, sex, education, occupation, and baseline Mini-Mental State Examination (MMSE). However, although in another study moderate wine consumption was found to be associated with a four-fold reduction of the risk for AD, this effect disappeared when institutionalization was taken into account [154]. These protective effects are most likely due to the presence of antioxidants in food and beverages [54,201], in as much as it has been found that wine drinking and the consumption of other foods and drinks which are rich in polyphenols can increase the antioxidant activity in serum [37,72,182]. Epidemiological studies have also found an inverse association between high intake of dietary Vitamin E (but not flavonoids or Vitamin C) and the occurrence of PD [82,223]. However, these data were not confirmed by other studies [106,167], although Hellenbrand et al. [106] reported a significant statistical trend toward protective effect by Vitamin C in PD. The clinical findings indicating a protective effect of dietary antioxidants against neurodegenerative disease are supported by data obtained in laboratory animals showing that diet supplementation containing fruits and vegetables rich in antioxidants (bluberries, strawberries and spinachs) can have beneficial effects on age-related decline of neuronal and cognitive function in old rats [128].

This review will focus on the actions of in vitro application of natural nutritional antioxidants in experimental models of neurodegenerative disorders. The capability of these compounds to counteract the damaging effects of ROS, and the relevance of this biochemical effect in their putative neuroprotective action will be examined. Among the numerous biochemical effects of ROS and antioxidants, particular emphasis will be given to their interference with NF-κB function, whose role in the pathophysiology of neurodegenerative disorders is gaining increasing attention. Finally, the effects of the administration of "pharmacological" doses of nutritional antioxidants in animal models and in patients with AD, PD, and ALS will be reviewed.

2. Natural dietary antioxidants

Natural dietary antioxidants include Vitamin A, C, and E, carotenoids, flavonoids, and polyphenols. Vitamin C (ascorbate) and Vitamin E (α-tocopherol) are absorbed from the gut. Ascorbate is rapidly distributed to all tissues, whereas α-tocopherol is incorporated into lipoproteins in the liver, and is then secreted together with them into plasma [99]. Ascorbate can scavenge many reactive species including $O_2^{\bullet -}$, $OH^{\bullet}$ and lipid hydroperoxides [220], and may stabilize catecholamines from forming ROS. α-Tocopherol is a powerful chain-breaking antioxidant that inhibits lipid peroxidation [183]. Carotenoids can scavenge singlet oxygen and a range of other ROS in vitro, but there is still little evidence that they contribute significantly to the antioxidant defense system in the central nervous system [99]. Flavonoids belong to a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, flowers, tea, and wine [188]. More than 4000 varieties of flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruits, and leaves [51]. Flavonoids represent the single, most widely occurring group of phenolic phytochemicals [221]. They can be divided into various classes on the basis of their molecular structure. The main four groups of flavonoids are the following: (a) flavones; (b) flavanones; (c) catechins; (d) anthocyanins. The flavones are characterized by a planar structure because of a double bond in the central ring. One of the best described flavonoids, quercetin, is a member of this group. Quercetin is found in abundance in onions, apples, broccoli, and berries. The second group is the flavanones, which are mainly found in citrus fruit. Flavonoids belonging to the catechins are mainly found in green and black tea and in red wine, whereas anthocyanins are found in strawberries and other berries, grapes, wine, and tea [51]. Another phenolic antioxidant is curcumin, a yellow curry spice derived from turmeric, which is used as a food preservative and herbal medicine in India. Most flavonoids are glycosylated in their natural dietary forms with the exception of the catechins [221].

Flavonoids can prevent injury caused by ROS in various ways [38]. One way is the direct scavenging of free radicals [101,229,230]. Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize ROS by reacting with the compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive, according to the following equation:

$$\text{Flavonoid(OH)} + R^{\bullet} \rightarrow \text{flavonoid}(O^{\bullet}) + RH$$

where $R^{\bullet}$ is a free radical and $O^{\bullet}$ is an oxygen free radical. Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen-derived radical peroxynitrite [31,194]. For example, flavanols are scavengers of superoxide anions [225], singlet oxygen [118], and lipid peroxy radicals [259], and they can sequester metal ions by chelation [269]. It has recently been shown that the flavonoid compounds caffeic acid and (+)-catechin can inhibit peroxynitrite-mediated oxidation of dopamine [138]. Moreover, it has been demonstrated that (−)-epicatechin, (−)-epicatechin gallate and quercetin serve as powerful antioxidants against lipid peroxidation when phospholipid bilayers are exposed to ROS in vitro [230,275]. There is also evidence that flavonoids can inhibit the activities of several enzymes, including lipoxygenase [115,149,216] cyclo-oxygenase [115,149], xanthine oxidase [43], phospholipase A_2 [78], and protein kinases [48]. These biological effects are believed to derive from the antioxidant properties of the related flavonoids [43].

In recent years, there has been a increasing interest in investigating the many positive pharmacological properties

of flavonoids. Much of this interest has been spurred by the dietary anomaly referred to as the "French paradox," the apparent compatibility of a high saturated fat diet with a low incidence of coronary atherosclerosis [219]. It was suggested that the polyphenolic substances such as flavonoids in red wine may provide protection against coronary heart disease. The natural phytoalexin resveratrol and the flavonoids quercetin and (+)-catechin have been invoked in order to explain the beneficial effects of moderate red wine consumption against coronary heart diseases [202,279]. In addition, epidemiological studies have shown that moderate wine consumption can be protective against neurological disorders such as age-related macular degeneration [195] and AD [201]. Moreover, in vitro and in vivo pre-clinical studies have shown the neuroprotective effect of lyophilized red wine [53], grape polyphenols [267], quercetin [249], *trans*-resveratrol [41,135,280], and (+)-catechin [119]. Taken together, these findings raise the possibility that red wine constituents may be beneficial in the prevention of age-related neurodegenerative disorders. There is also increasing interest for the role of tea (*Camellia sinensis*) in maintaining health and in treating disease. Although tea consists of several components, research has focused on polyphenols, especially those found in green tea. The green tea polyphenols include (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin-3-gallate (EGCG). Of these, EGCG generally accounts for greater than 40% of the total [102]. Green tea polyphenols are potent antioxidants [230]. EGCG usually has the greatest antioxidant activity, and is the most widely studied polyphenol for disease prevention [156,157]. Many of the putative health benefits of tea are presumed to be caused by its antioxidant effects.

The epidemiological evidence indicating the putative role of nutritional antioxidants in the prevention and attenuation of neurodegenerative disorders is receiving experimental confirmation in a number of laboratory studies. Thus, the polyphenol epicatechin was shown to attenuate neurotoxicity induced by oxidized low-density lipoprotein in mouse-derived striatal neurons [244]. Tea extracts and EGCG attenuated the neurotoxic action of 6-OHDA in rat PC12 cells, human neuroblastoma SH-SY5Y cells [156], and was shown to be neuroprotective in a mouse model of PD [157]. Moreover, recent reports have revealed that flavonoids may be neuroprotective in neuronal primary cell cultures. For example, the Ginkgo biloba extract, known to be enriched with flavonoids, has been shown to protect hippocampal neurons from nitric oxide or β-amyloid derived peptide-induced neurotoxicity [14,15]. In addition, the extract of Ginkgo biloba referred to as Egb 761 is one of the most popular plant extracts used in Europe to alleviate symptoms associated with a range of cognitive disorders [44,150]. The mechanism of action of Egb 761 in the central nervous system is only partially understood, but the main effects seem to be related to its antioxidant properties, which require the synergistic action of the flavonoids, the terpenoids (ginkgolides, bilobalide), and the organic acids, principal constituents of Egb [139]. These compounds to varying degrees act as scavengers of ROS, which have been considered the mediators of the excessive lipid peroxidation and cell damage observed in AD [205,213,256].

3. ROS, NF-κB and neurodegenerative disorders

The transcription factor NF-κB, originally studied in cells of the immune system wherein it regulates cell survival [9,10,253], is widely expressed in the nervous system and exists in neurons in both an inducible and a constitutively active form [130–132,200]. NF-κB resides in the cytoplasm in an inactive form consisting of three subunits: p65 and p50 and an inhibitory subunit called IκB [9,10,200,253]. When IκB is bound to p50/p65, it is inactive; signals that activate NF-κB cause dissociation of IκB releasing p50/p65, which then translocates to the nucleus and binds to specific κB DNA consensus sequences in the enhancer region of a variety of κB-responsive genes [9,10,32,179,180,253].

In neurons, NF-κB is activated by various intercellular signals, including cytokines, neurotrophic factors, and neurotransmitters [39,180,200]. Activation of glutamate receptors, and membrane depolarization lead to activation of NF-κB in hippocampal pyramidal neurons and cerebellar granule neurons in culture [91,131]. The mechanism whereby diverse stimulants lead to the activation of NF-κB has been a subject of intense research. Most work has focused on the p50/p65 dimer, the predominant form of NF-κB activated in many cells including neurons [179,180,253], and its association with IκBα. It is now known that upon stimulation with many NF-κB inducers, IκBα is rapidly phosphorylated on two serine residues (S32 and S36), which targets the inhibitor protein for ubiquitination and subsequent degradation by the 26 S proteasome [32]. Released NF-κB dimer can then translocate to the nucleus and activate target genes by binding with high affinity to κB elements in their promoters. The phosphorylation and degradation of IκBα are tightly coupled events, so it is always likely that agents that activate NF-κB do so by stimulating a specific IκB kinases, or alternatively by inactivating a particular phosphatase. Two IκB kinases (IKKs) termed IKKα and IKKβ have been described [32]. IKKα and β have been shown to be activated by important inducers of NF-κB such as IL-1 and TNF, to specifically phosphorylate S32 and S36 of IκBα, and to be crucial for NF-κB activation by these cytokines [32]. The IKKs are part of a larger multiprotein complex called the IKK signalsome. It appears that multiple pathways can regulate NF-κB, most of which lead to IκB phosphorylation via the IKK-containing signalsome [32]. A model has been proposed whereby diverse agents all activate NF-κB by causing oxidative stress [10,159,187]. This hypothesis is based on four main lines of evidence: (a) direct application of H_2O_2 to culture medium activates NF-κB in some cell lines [240–243]; (b) in some cell types

ROS have been shown to be increased in response to agents that also activate NF-κB [10,239–243]; (c) virtually all stimuli known to activate NF-κB can be blocked by antioxidants, including L-cysteine (a precursor of glutathione), *N*-acetyl-L-cysteine (NAC), caffeic acid phenethyl ester (CAPE), (−)-epigallocatechin-3-gallate, resveratrol, thiols, dithiocarbamates, Vitamin E and its derivatives, and thioredoxin (an important cellular protein oxidoreductase with antioxidant activity) [10,50,70,162,191,237,240,279,286]; (d) inhibition or overexpression of enzymes that affect the level of intracellular ROS has been shown to modulate the activation of NF-κB by some agents [173,239]. Ultimately, this theory led to the proposal of H_2O_2 as the central second messenger to NF-κB activation [239].

A large body of evidence indicates that NF-κB is involved in the control of cell survival. The great majority of the available data show that NF-κB exerts an anti-apoptotic action. Thus, activation of NF-κB can prevent cell death in various culture paradigms [19,180]. Moreover, increasing data suggest that NF-κB activation may transduce anti-cell death signals in neurons [180]. For example, TNFα protected cultured hippocampal neurons against death induced by metabolic, excitotoxic, and oxidative insults [12,180]. The involvement of NF-κB in such neuronal cell death paradigms is suggested by data showing that TNFα induces activation of NF-κB in cultured hippocampal neurons against excitotoxic and oxidative insults [12,13,87]. Moreover, in the PC12 neuronal cell line [268] and in primary sympathetic neurons [172], activated NF-κB has been found to mediate the anti-apoptotic effect of NGF (nerve growth factor). It has also been shown that the resistance of selected clones of PC12 cells to oxidative cell death induced by Aβ and H_2O_2 is mediated by NF-κB [158]. An inhibition of NF-κB potentiated Aβ peptide-mediated apoptotic damage in primary cultures of cerebellar granule cells [134], and increased the apoptotic death of PC12 induced by auto-oxidation of dopamine [153]. Similarly, a lack of p50 subunit increased the vulnerability of hippocampal neurons to excitotoxic injury [288]. Recent studies have shown that NF-κB is activated, and may play a protective role in neurodegenerative disorders such as AD [181], PD [117] and ALS [189] and severe epileptic seizures [288]. There is also evidence that NF-κB plays a pivotal role in the cell survival-promoting action of ADNF9, a nine amino acid activity-dependent neurotrophic factor (ADNF) peptide [80]. In addition, it has recently been reported that NF-κB is involved in the neuroprotective effect exerted by subtoxic concentration of NMDA, and can counteract low potassium-induced apoptosis in cultured cerebellar granule neurons [143,163]. Also preconditioning-induced neuroprotection in cultured hippocampal neurons seems to be mediated by activation of NF-κB [217]. The mechanism by which NF-κB can exert its anti-apoptotic effect is still unclear. One possible mechanism would be the transcription of genes encoding trophic factors, antioxidant enzymes, and calcium-regulating proteins. One of the first genes shown to be responsive to NF-κB was SOD-2, a mitochondrial antioxidant enzyme that protects cells against apoptosis [181]. Other genes induced by NF-κB include the cell adhesion molecules such as ICAM-1 [151], the inducible form of nitric oxide synthase [274], Bcl-2, Bcl-x, and the Bcl-2 homologue Bfl-1/A1 [79,270,292].

However, in some cases NF-κB can promote neuronal death [89,90,164]. Thus, the neuroprotective effect of acetylsalicylic acid is apparently mediated by inhibition of NF-κB [90]. More recently, it was found that NF-κB is essential for dopamine-induced apoptosis in PC12 cells [203]. Whether NF-κB inhibits or promotes apoptosis might depend on the cell type and the nature of the apoptosis-inducing stimulus [164]. However, the explanation for the conflicting results concerning an anti-apoptotic versus pro-apoptotic role of NF-κB activation is still not clear and has been described as “janus faces” of NF-κB [164].

4. Alzheimer’s disease, oxidative stress, NF-κB, and antioxidants

The estimated prevalence of senile dementia in Europe increases with age from 1% in man and women of 60 years of age to 44.7% in the population 90–95 years of age [112]. Alzheimer’s disease is the commonest form of dementia, with a prevalence of 0.4% in women and 0.3% in man aged 60–69 years [226]. A community based study has suggested that approximately 4 million persons in the United States have AD [66]. AD is a progressive dementing disorder characterized by selective neuronal loss in several areas of the central nervous system. In AD, the progressive memory deficits, cognitive impairments and personality changes are due to progressive dysfunction and death of the neocortex, limbic system, hippocampus and several of the subcortical regions of the brain. The majority of cases of AD are age-related and, indeed, age is the only reliable risk factor for the non-genetic sporadic forms (85% of all cases) and, therefore, for the majority of cases of this disorder [20]. The characteristic histopathologic alterations in AD are neuritic or senile plaques (SPs) composed largely of amyloid β-peptides (Aβ) and neuronal aggregates of abnormally phosphorylated cytoskeletal proteins [neurofibrillary tangles (NFTs)]. A number of data indicate that Aβ is responsible for the neuronal death in AD. Thus, aggregates of Aβ peptides are toxic to neurons in cultures [20–22,168] and can cause cell death by apoptosis [64,168,193,233,263], however, the exact mechanisms of Aβ-induced neurotoxicity are still unknown. Several lines of evidence suggest that the overproduction of ROS is implicated in Aβ neurotoxicity: (a) exposure of cultured neurons or neuronal cell lines to Aβ increases the intracellular levels of ROS [92–94,137,175,176] leading to the activation of NF-κB [133]; (b) markers of oxidative stress are found increased in a transgenic mouse model of AD [204,256]; (c) the neurotoxicity of Aβ is attenuated by antioxidants such as Vitamin E, the spin-trap compound PBN (α-phenyl-*tert*-butyl

nitrone), and lazaroids [20–22,86,103,171,176], and/or free radical scavengers [107]. Thus, in 1992 the protective effect of Vitamin E was first described on neurons in culture against Aβ-induced cell death [22]. Following these initial findings, a number of subsequent studies confirmed the role of oxidative stress in the neurotoxic effect of Aβ peptide. For example, Behl et al. [23] found that Aβ can induce the formation of H_2O_2 in hippocampal neurons which causes peroxidation of cell membranes and ultimately leads to neuronal death. Consistent with these findings, exposure of cultured hippocampal neurons to Aβ induced a significant increase in 4-HNE [174]. Moreover, it has recently been found that the phenolic antioxidant curcumin, which is largely used as a food preservative and herbal medicine in India, reduces oxidative damage and amyloid pathology in a transgenic mouse model of AD [161]. However, in another study, Aβ-induced neurotoxicity in rat hippocampal neurons in culture was not affected by several antioxidants [166]; nevertheless, pretreatment of cultures with Aβ significantly increased the sensitivity of neurons to H_2O_2, suggesting that Aβ can render neurons more susceptible to ROS damage [166].

In agreement with data obtained in experimental models, clinical findings indicate that oxidative stress occurs in AD, as indicated by the finding that higher than normal levels of lipid, protein, and DNA oxidation are found in the brains of AD patients [4,85,205,213,257]. Thus lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS) were found to be increased in various brain regions of AD patients [11,170,266]. Moreover, Mecocci et al. [185] found a significant three-fold increase in mitochondrial DNA oxidation in the parietal cortex of AD patients. In addition, immunohistochemical analysis of brain sections from AD patients using an antibody with selectivity for the activated nuclear form of p65 revealed that NF-κB was activated in neurons and astrocytes [133]. Cells with activated NF-κB were restricted to the close proximity of early plaque stages [133]. Thus, it is possible that Aβ-induced NF-κB activation contributes to the pathological changes observed in AD via the induction of proinflammatory and cytotoxic genes or, more likely, that Aβ-induced NF-κB activation is part of a cellular defense program.

Based on the preclinical and clinical data indicating the presence of oxidative stress in AD, clinical trials were carried out to test the effect of antioxidants in this pathological condition. Thus, a controlled clinical trial with D,L-α-tocopherol (synthetic form: 2000 IU/day) in patients with moderately severe impairment from AD showed some beneficial effects with respect to rate of deterioration of cognitive function [232]. In the same D,L-α-tocopherol clinical trial, selegiline (10 mg/day), a monoamine oxidase inhibitor, produced beneficial effects similar to that produced by D,L-α-tocopherol [232]. it is interesting to note that there was no significant difference in effect between the groups receiving a combination of D,L-α-tocopherol and selegiline and those receiving treatment with the individual agent [213,232]. Several possibilities were proposed to explain the lack of additive effect. One of them was that selegiline and Vitamin E may act by the same mechanism. Indeed, both reduce the levels of free radicals, although by different mechanisms. Vitamin E protects neurons by destroying formed ROS (“quenching”), whereas selegiline protects neurons by preventing the formation of ROS and by inhibiting oxidative metabolism of catecholamines. therefore, clinical studies involving Vitamin E and selegiline support the concept that ROS are one of the intermediary risk factors for the progression of neurodegeneration in AD [20].

5. Parkinson’s disease, oxidative stress, NF-κB, and antioxidants

Parkinson’s disease is a neurological syndrome manifested by any combination of tremor at rest, rigidity, bradykinesia, and loss of postural reflexes. The neuropathological hallmark of PD is the selective degeneration of dopamine (DA) neurons in the nigrostriatal system [122,238]. These neurons synthesize and release DA, and the loss of dopaminergic influence on other structure in the basal ganglia leads to the classic parkinsionian symptoms. Moreover, PD is characterized by degeneration of monoamine-containing neurons in the brain stem nuclei (predominantly the locus coeruleus) and is variably associated with pathology in non-nigral systems causing multiple neurotransmitter dysfunctions [63].

Although idiopathic PD is usually sporadic, it is now well established that there is a genetic component to the disease [81,199]. Approximately 5–10% of PD patients have a familial form of parkinsonism with an autosomal-dominant pattern of inheritance [199]. Case control studies have typically indicated a 2–14-fold increase in incidence in close relatives of PD patients [76] and although concordance rates between identical twins are low for overt expression of the disease, they are much higher when subclinical decline in striatal dopaminergic dysfunction is measured by positron emission tomography (PET) imaging (53% in monozygotic twins of PD patients, compared with 13% in dizygotic cases) [35]. Nevertheless, in sporadic PD, environmental factors have been emphasized [147]. Thus, epidemiological studies indicate that a number of factors may increase the risk of developing PD [272]. These include exposure to well water, herbicides, industrial chemicals, wood pulp mills, farming, and living in a rural environment. A number of exogenous toxins have been associated with the development of parkinsonism, including trace metals, cyanide, lacquer thinner, organic solvents, carbon monoxide, and carbon disulfide [199]. There has also been interest in the possible role of endogenous toxins such as tetrahydroisoquinolines and β-carbolines. However, no specific toxins has been found in the brain of PD patients. The most compelling evidence for an environmental factor in PD relates

to the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a byproduct of the illicit manufacture of a synthetic meperidine derivative. Some of the drug addicts who took MPTP developed a syndrome that strikingly resembled PD, both clinically and pathologically [114,148]. MPTP induces toxicity through its conversion in astrocytes to the pyridinium ion (MPP^+) in a reaction catalyzed by monoamine oxidase B (MAO-B) [255]. MPP^+ is then taken up by DA neurons and causes mitochondrial complex I defect similar to that found in PD [192]. This observation supports the possibility that an environmental factor might cause PD; however, no MPTP-like factor has been identified in PD patients to date.

The principal cytoskeletal pathology of PD is the Lewy body, which, in 85–100% of cases occur in many monoaminergic and other subcortical nuclei, spinal cord, sympathetic ganglia, and less frequently in cerebral cortex, myenteric plexuses, and adrenal medulla [71,113,122,123,169]. Lewy bodies are abnormal intracytoplasmatic neuronal inclusions that are considered to be a major anatomic hallmark of PD, although they are seen in pigmented nuclei in various disorders and in aging brain. In the majority of cases, the mechanisms involved in nigral degeneration in PD are unknown, but evidence from studies of post-mortem brain tissue suggests the involvement of ROS and oxidative stress [63,126,254]. Oxidative stress may arise from the metabolism of DA with the production of potentially harmful free radical species [56,126]. This may be important as surviving neurons increase DA turnover to compensate for diminishing synaptic transmission.

Circumstantial evidence exists that defects in mitochondrial energy metabolism may cause nigral neuronal degeneration in PD. Thus, MPTP produces dopaminergic neuronal degeneration and parkinsonian symptoms in humans and non-human primates [277]. 1-Methyl-4-phenylpyridinium (MPP^+), produced by the catabolism of MPTP by MAO-B in glia, is selectively taken up into dopaminergic by the DA transporter. Within dopaminergic neurons, MPP^+ is concentrated by the electrochemical gradient into mitochondria. MPP^+ selectively inhibits NADH CoQ reductase (complex I) of the mitochondrial electron transport chain and induces neuronal degeneration. Evidence exists that similar mitochondrial dysfunction may occur in idiopathic PD. Thus, a defect in complex I has been reported in the striatum of patients with PD [26,104,206,235]. Similar defects have been found in the platelets [144] but not muscles [61] of patients with PD. Reductions have been found in the substantia nigra but not in other regions of the brain, such as the globus pallidus or cerebral cortex [236]. Therefore, the specificity of mitochondrial impairment may play a role in the degeneration of nigrostriatal dopaminergic neurons. Interestingly, recent evidence indicate that exposure to complex I inhibitor rotenone can cause nigrostriatal dopaminergic degeneration associated with parkinsonian-like symptoms and accumulation of protein aggregates containing ubiquitin and α-synuclein [25].

Alterations in pro- and antioxidant molecules have been reported in post-mortem tissue from individuals with PD. Increased total iron has been found in the substantia nigra in PD [56,60,111,222]. Iron could increase oxidative stress by promoting the formation of $OH^\bullet$ from H_2O_2 via the Fenton reaction. Reductions in GSH levels in the substantia nigra have also been reported [208,210,222,250,251,258]. These reductions were not detected in other neurodegenerative diseases in which nigral cell loss occurs, suggesting they are specific to PD and not secondary to cell loss alone. Decreases in GSH have also been found in the substantia nigra in individuals with incidental Lewy bodies at post-mortem, a potential marker of preclinical PD, suggesting that alterations in GSH are an early event [59]. Reductions in GSH levels could promote or be a consequence of oxidative stress, or both. Because GSH is involved in the detoxification of H_2O_2, reductions in GSH could result from increased concentrations of H_2O_2 and in the presence of metals, the highly reactive $OH^\bullet$. The presence of lipid peroxidation and oxidative DNA damage further supports the existence of oxidative stress in PD [57,58,126,231].

As already mentioned, the hallmark of PD is a severe reduction of DA in all components of the basal ganglia. DA and its metabolites are depleted in the caudate nucleus, putamen, globus pallidus, nucleus accumbens, the ventral tegmental area, and the substantia nigra pars compacta and reticulata. Moderate losses of DA are found in the lateral hypothalamus, medial olfactory region and amygdaloid nucleus [290]. In early parkinsonism, there appears to be a compensatory increase in DA receptors to accommodate the initial loss of DA neurons [97,224]. As the disease progresses, the number of DA receptors decreases, apparently due to the concomitant degeneration of DA target sites on striatal neurons. In the remaining neurons in patients with PD, DA turnover seems greatly increased, judging from the concentrations of homovannilic acid [HVA] in the nerve terminals in the striatum and the cell bodies and dendrites in the substantia nigra [2], and the ROS production may very well increase in consequence. This hypothesis is strengthened by a study showing that the concentrations of GSH decrease when DA turnover increase after reserpine treatment in rats, indicating increased activity of the peroxide scavenging enzyme GSH-Px [261]. If the increase in ROS production due to increased DA turnover is not buffered by the scavenging enzymes (SOD, catalase, and GSH-Px), the compensatory hyperactivity of the dopaminergic neurons may become self-destructive. Chronic administration of L-DOPA would then only exacerbate the production of destructive ROS [186,260]. The administration of L-DOPA itself has been postulated to enhance the accumulation of ROS [88,282]. Hiramatsu et al. [110] by using electron spin resonance spectrometry have shown that 10 mM L-DOPA by itself was inactive, whereas it produced ROS in the presence of 10 mM Fe-diethylenetriamine-pentaacetic acid, and this effect was blocked by deprenyl, an inhibitor of MAO-B, which has been advocated as a symptomatic and protective

therapy in PD [83], as well as MPTP-induced parkinsonism [105]. Another index of oxidative stress in PD might be the evidence of a robust increase of NF-κB in the nuclei of dopaminergic neurons in the substantia nigra of PD patients [117]. This clinical finding is consistent with in vitro data showing that oxidative stress induced by C_2-ceramide treatment causes nuclear translocation of NF-κB in cultured mesencephalic neurons [117]. More recently, it has been shown that the neurotoxin 6-OHDA activates NF-κB in PC12 cells by enhancing intracellular ROS levels [27]. Interestingly, in this experimental model, NF-κB seems to sustain cell survival by stimulating the expression of the anti-apoptotic proteins Bcl-2 and Bfl-1 [27]. Moreover, as already mentioned, the potent green tea polyphenol antioxidant EGCG exerts a neuroprotective effect in a MPTP mouse model of PD [156].

When induced by the toxins 6-OHDA or MPTP in animal models of PD, nigral cell death seems to involve both necrotic and apoptotic processes. In human PD there has been some debate about whether key features of apoptosis could be demonstrated, at least when based on morphological features or terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end-labeling (TUNEL) alone [36,273]. However, the recent development of techniques involving double labeling with TUNEL to demonstrate DNA fragmentation in conjunction with cyanine dye that binds to DNA to provide structural details has demonstrated chromatin condensation and DNA fragmentation within the same nuclei in the substantia nigra in PD is greater than that seen in normal aging, consistent with the 10-fold higher rate of cell loss seen in patients with the disease [177,199].

The progressive nature of PD and the fact that neuronal degeneration in the substantia nigra is slow and protracted [68] present opportunities for therapeutic intervention aimed at blocking or slowing down the degenerative process. Recent neuroimaging and autopsy data indicated that there is a preclinical period of 4–5 years before symptoms appear, and that the rate of cell loss and decline of dopaminergic function in the striatum is likely to be in the order of 10% per year, with the disease progressing relatively more rapidly during the early phases than the more advanced stages of the disease [35,68]. Both PET and single-photon emission computed tomography (SPECT) imaging seem to be able to detect a decline in striatal dopamine function before clinical symptoms appear [35], which may make it possible to begin neuroprotective intervention during the preclinical phase.

The largest neuroprotective trial conducted to date, the Deprenyl and Tocopherol Antioxidant Therapy of Parkinsonism (DATATOP) study [207], involved two putative antioxidant agents, Vitamin E and deprenyl [248,276,285]. Vitamin E had no significant effect at the doses used, but deprenyl slowed the early progression of symptoms and delayed the emergence of disability by an average of 9 months. However, being an MAO-B inhibitor, this drug has symptomatic effects of its own, which has confounded interpretation of the results [248]. Interestingly, animal studies have suggested that the neuroprotective effect is not dependent on MAO-B inhibition per se, but rather on an antiapoptotic effect of the metabolite desmethyl-deprenyl, possibly acting on protein transcription [136,177]. Before the completion of the DATATOP large study ($n = 800$), an open trial with high dosages of α-tocopherol and ascorbate, administered to a small group of early PD patients ($n = 15$), found that this combination of natural antioxidants delayed by 2.5 years the time necessary to begin the therapy with L-DOPA [67]. There are many alternative antioxidative approaches that may be considered in future clinical trials, including free-radical scavengers, GSH, GSH enhancing agents, ion chelators and drugs that interfere with oxidative metabolism of DA. Interestingly, the classic directly acting DA receptor agonists may belong to the last group: by stimulating DA autoreceptors, these drugs reduce DA synthesis, turnover and release, so that less L-DOPA is needed [196]. In addition, some of these compounds have direct antioxidant effects [198,234a]. More recently, the DA receptor pramipexole has been used as a monotherapy for the treatment of PD, and it has been shown that it may have neuroprotective effects [234b].

6. Amyotrophic lateral sclerosis, oxidative stress, NF-κB, and antioxidants

Amyotrophic lateral sclerosis (ALS) is a fatal paralytic neurodegenerative disorder of unknown cause, mainly characterized by a progressive loss of motor neurons in the cerebral cortex, brainstem and spinal cord. ALS is a progressive disease that invariably leads to death within approximately 3–5 years from the onset of symptoms [228]. The annual worldwide annual incidence rates for ALS range between 0.4 and 1.8 per 100,000 population and the prevalence rates range between 4 and 6 per 100,000 population, with an overall male predominance [271]. Although most cases are sporadic, about 5–10% are familial, with inheritance following an autosomal dominant pattern. About 15–20% of patients with familial ALS (FALS), which is clinically indistinguishable from the more common sporadic ALS, carry mutations in the gene encoding for the free radical scavenging enzyme SOD-1 [47,52,227]. Over 50 different SOD-1 mutations have been documented in FALS patients [252]. Transgenic mice have been generated that express mutant forms of SOD-1 found in FALS cases, including $gly^{93} \rightarrow ala$ (G93A) [49,77,96] and $gly^{37} \rightarrow arg$ [284], which develop motor neuron disease and death within 4–6 months if the mutant enzyme is expressed at sufficient levels. Studies of FALS patients with mutations of SOD-1 indicates that SOD-1 activity is decreased 20–50% [34,52]. This suggested initially that the disease was due to ROS-induced damage resulting from structurally defective enzyme with reduced activity [52]. However, no deletions of SOD-1 gene have been found in FALS families, which implies that

expression of the mutant protein is required for pathogenesis. Studies in transgenic mice suggest that, rather than causing a loss of function, the mutations of SOD-1 in FALS patients cause a gain of function that results in neuronal degeneration [96,215]. Because transgenic mice expressing wild-type human SOD-1 with comparable elevation of brain SOD activity do not develop motor neuron disease [96,284] and in fact, show enhanced resistance to oxidative stress [152,214], disease is due to expression of the mutant protein and not to elevation of SOD activity in the brain [30,184,283]. Several investigators have found increased levels of ROS in animals models of ALS [29,98,165]. Consistent with animal data, a number of clinical studies indicate that oxidative stress may be involved in the pathology of ALS, as suggested by increased levels of oxidative damage products, such as protein carbonyls, 4-HNE, 8-OHdG, and nitrotyrosine [1,18,28,46,69,165,209]. In addition, fibroblasts from ALS patients were found to be more sensitive to oxidative stress [3]. Moreover, immunohistochemical studies have shown that NF-κB is strongly activated in astrocytes of the spinal cord of ALS patients, probably as a consequence of the oxidative stress [189]. Thus, the occurrence of oxidative stress and activation of NF-κB is a common characteristic of AD, PD, and ALS. In this regard, it is noteworthy that overlap syndromes with clinical and pathological features of dementia, ALS and PD have been described [116]. It is also important to mention that degeneration of midbrain DA neurons occurs in a mouse model of ALS [142].

Various drugs which can act by reducing oxidative stress have been used as potential therapeutic agents in transgenic mice expressing the mutated human SOD-1 enzyme. Thus, polyamine- or putrescine-modified catalase, an antioxidant enzyme that removes hydrogen peroxide and has good permeability at the blood–brain barrier, increases the survival of transgenic mice bearing the human mSOD-1^{G93A} [212,218]. Moreover, the copper chelator and thiol compound penicillamine, the copper chelator trientine, carboxyfullerenes, Vitamin E and *N*-acetylcysteine have been reported to increase the survival time in this mouse model and/or delay the onset of the disease to a small extent [6,7,62,95]. The drug riluzole, which inhibits glutamate release at presynaptic terminals, also extends lifespan slightly in human mSOD-1^{G93A} transgenic mice [95]. Interestingly, riluzole, which is used clinically in patients with ALS [24], has been shown to have direct antioxidative effect on cultured cortical neurons [140]. However, no clear evidence for a beneficial effect of α-tocopherol, selegiline, *N*-acetylcysteine or an antioxidant cocktail has been obtained in humans [55,146,281].

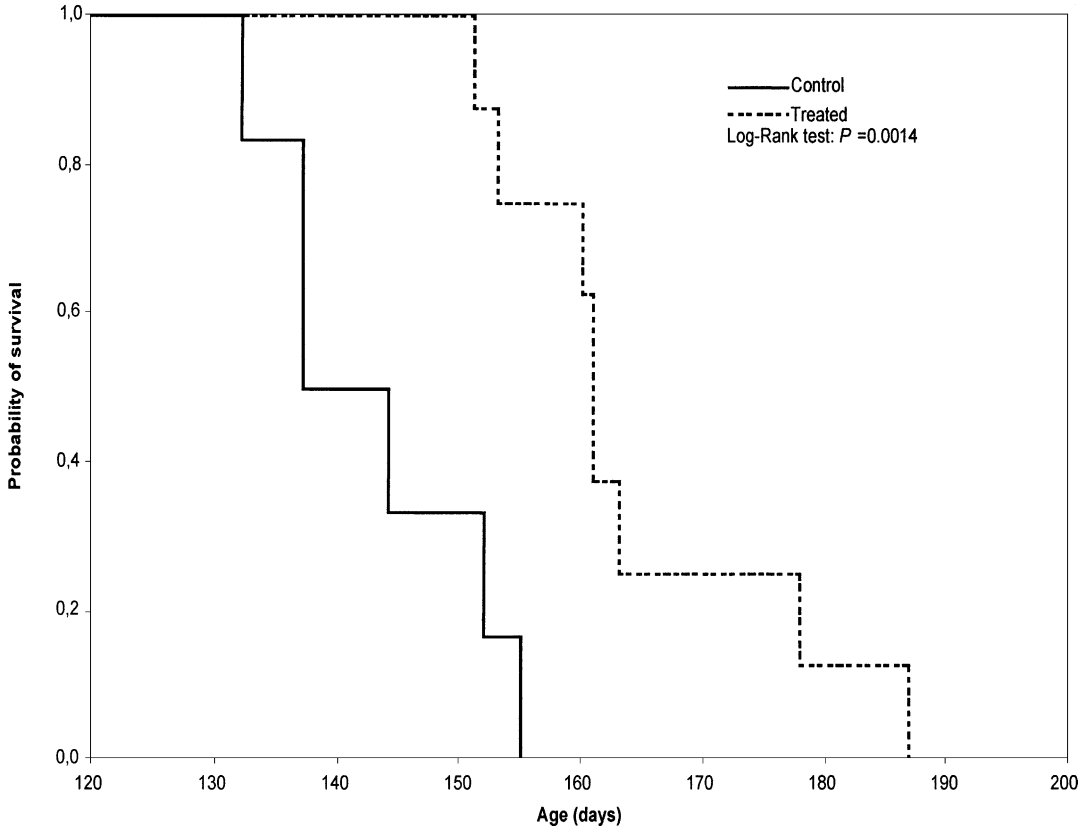

Fig. 1. Kaplan–Meier survival curve showing cumulative probability of survival in mSOD-1^{G93A} mice. Mean survival (days) ± S.E.M.: control = 143 ± 4.0 (n = 6); treated = 164 ± 4.5 (n = 8). Treatment with lyophilized red wine (dashed line) significantly prolonged survival (15%) when compared with littermates treated with drinking water (solid line). Onset of treatment: 30–40 days of age. Log-rank test, P = 0.0014.

Li et al. [160] have recently reported that blockade of caspase-1 and caspase-3 activity by *N*-benzyloxycarbonyl-Val-Asp-fluoromethylketone [zVAD-fmk], prolongs the survival of transgenic mice expressing the human mSOD-1^{G93A}, which begin to develop ALS symptoms at the mean age of about 3 months. These findings open new perspectives for the use of caspase inhibitors as potential therapeutic agents in the treatment of ALS and other neurodegenerative diseases. However, because of the low oral bioavailability and limited brain penetrance, zVAD-fmk was delivered by intracerebral administration. Thus, the physicochemical characteristics of zVAD-fmk might limit its clinical usefulness. Based on these findings and on the hypothesis that in transgenic mice expressing the human mSOD-1^{G93A} an increased formation of ROS occurs, we decided to treat them with lyophilized red wine (which is rich in antioxidant compounds), dissolved in the drinking water which was freely available to the animals. This treatment regimen caused a significant reduction in the overall mortality of the treated mice, as compared with control animals. Thus, lyophilized wine prolonged by 6% the survival of mSOD-1^{G93A} mice [65]. In the first series of experiments, the onset of treatment was variable, and ranging from 43 to 66 days of age [65]. We have recently repeated the experiments on mSOD-1^{G93A} mice which were treated with the same concentration of lyophilized red wine, but the treatment was started earlier, i.e., 30–40 days from birth. By using this protocol we have found that administration of lyophilized red wine significantly increased the mean survival time by 15%, as compared with control transgenic mice given drinking water only (Fig. 1). The calculated concentration of polyphenolic compounds, expressed as gallic acid equivalent (GAE), was 4824 mg/l. Considering that each mouse drank about 4 ml of liquid daily, it is possible to calculate the daily intake of GAE, which was about 20 mg per mouse. It is tempting to speculate that the mechanism of neuroprotection exerted by lyophilized red wine on mSOD-1^{G93A} mice might be due to its ability to inhibit caspase-3 activity. This hypothesis is based on in vitro experiments showing that lyophilized red wine (5 μg/ml) caused a significant inhibition of caspase-3 activity on primary cultures of rat cerebellar granule neurons [53]. However, it is presently impossible to establish whether the effect of lyophilized red wine on caspase-3 is direct or mediated by inhibition of ROS formation. Furthermore, ex vivo experiments aimed at investigating the inhibitory effect of lyophilized red wine on activated caspase-3 in mSOD-1^{G93A} mice are necessary to confirm our hypothesis.

7. Conclusions

There is growing evidence that oxidative stress may play an important role in the pathogenesis of AD, PD, and ALS. However, in spite of the large body of experimental data showing the protective effect of antioxidants in in vitro models of neurodegeneration and in some in vivo animal model, there is still limited evidence for a neuroprotective effect of antioxidants in the treatment of neurodegenerative disorders in humans. There may be several reasons for this discrepancy between pre-clinical and clinical data. Thus, it is conceivable that the therapeutic regimen used so far (e.g., one or two antioxidants) might not be sufficient to halt the neuropathologic process. As pointed out by others, a more efficient strategy would be the use of multiple antioxidants in the treatment of AD, PD, and ALS [213]. In this regard, it is important to point out that one possible advantage of the use of extracts of fruits, vegetables or beverages (such as red wine, green tea or Ginkgo biloba) in the treatment of neurodegenerative disorders, is that they often contain multiple antioxidant compounds which can potentiate each other. Particularly important would be the use of lyophilized red wine [65] which is provided with strong antioxidant capacity [37,72,182]. Moreover, one possible limitation of the neuroprotective strategy (including antioxidant administration) might be consequent to the fact that when overt symptomatology of AD, PD, and ALS occurs, a certain amount of neuronal death has already occurred. Thus, the neuroprotective agents (including antioxidants) can, at best, only rescue the surviving neurons, an effect which might not be sufficient to attenuate the neurologic symptomatology. It is therefore important to start the therapeutic intervention at an early stage of the disease process. In this regard, it is interesting to note that some epidemiological studies have shown that dietary habits can influence the incidence of neurodegenerative disorders. In particular, it was found that a diet rich in Vitamin E can reduce the risk for PD [82,223], and that moderate wine consumption may decrease the risk for AD [155,201]. However, there are still few and controversial [106,167] epidemiological data on this important point, which might be partly due to the intrinsic difficulties in performing epidemiological surveys regarding the dietary habits of large populations. Nevertheless, it is desirable that future studies aimed at investigating the relationship between dietary antioxidant intake and the relative risk for neurodegenerative disorders such as AD, PD, and ALS will throw more light on this very important aspect of public health.

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