

## Review

# Risk Factors for Alzheimer's Disease: Role of Multiple Antioxidants, Non-Steroidal Anti-inflammatory and Cholinergic Agents Alone or in Combination in Prevention and Treatment

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The etiology of Alzheimer's disease (AD) is not well understood. Etiologic factors, chronic inflammatory reactions, oxidative and nitrosylative stresses and high cholesterol levels are thought to be important for initiating and promoting neurodegenerative changes commonly found in AD brains. Even in familial AD, oxidative stress plays an important role in the early onset of the disease. Mitochondrial damage and proteasome inhibition represent early events in the pathogenesis of AD, whereas increased processing of amyloid precursor protein (APP) to  $\beta$ -amyloid ( $A\beta$ ) fragments ( $A\beta_{40}$  and  $A\beta_{42}$ ) and formation of senile plaques and neurofibrillary tangles (NFTs) represent late events. We propose a hypothesis that in idiopathic AD, epigenetic components of neurons such as mitochondria, proteasomes and post-translation protein modifications (processing of amyloid precursor protein to  $\beta$ -amyloid and hyperphosphorylation of tau), rather than nuclear genes, are the primary targets for the action of diverse groups of neurotoxins. Based on epidemiologic, laboratory and limited clinical studies, we propose that a combination of non steroidal anti-inflammatory drugs (NSAIDs) and appropriate levels and types of multiple micronutrients, including antioxidants, may be more effective than the individual agents in the prevention, and they, in combination with a cholinergic agent, may be more effective in the treatment of AD than the individual agents alone. In addition, agents, which can prevent formation of plaques or dissolve these plaques may further enhance the efficacy of our proposed treatment strategy.

### Key teaching points:

- Inflammatory reaction is one of the important etiologic factors in the development of Alzheimer's disease (AD).
- Oxidative and nitrosylative stresses are among the critical mediators of the diverse groups of neurotoxins that cause neurodegeneration in AD.
- Epigenetic components of neurons (mitochondria, proteasome, membranes, and post-translational protein modifications) rather than nuclear genes are the primary targets of diverse group of neurotoxins in AD.
- A combination of appropriate multiple micronutrients (including antioxidants) with NSAIDs may be more effective in the prevention, and as an adjunct to standard therapy in the treatment of AD than the individual agents.

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## Introduction

It is estimated that Alzheimer's disease (AD) affects about four to five million Americans and that greater than half of AD patients receive treatment and specialized care. The incidence of AD and other dementia doubles every five years beyond the age of 65, and about 50% of the U.S. population who are 85 years or older have symptoms of AD [1–3]. Only about 5% to 10% of AD is due to hereditary factors and appears at an early age; the remaining cases are considered to be idiopathic or sporadic and appear at a late age. In view of the fact that about 33 million Americans are of age 65 and older and this number is predicted to increase to 51 million by the year 2025 [1], Alzheimer's disease is a major medical concern. The annual economic cost of AD health care expenses and lost wages (for both AD patients and their caregivers) is estimated to be \$80 to \$100 billion [1]. In spite of extensive studies on the etiology and biology of AD, no scientifically-based, rational, comprehensive approach for the prevention and treatment of AD has been developed. Recent reviews have emphasized the importance of antioxidant supplements in its prevention and as an adjunct to standard therapy in the treatment of neurodegenerative diseases including AD [4,5].

The purpose of this review is to discuss the following issues: (a) various hypotheses with respect to etiology of AD, (b) our proposed hypothesis that epigenetic components of neurons are the primary targets for the action of diverse groups of neurotoxins, (c) our proposed sequence of cellular events that initiate and promote neurodegeneration and (d) epidemiologic, laboratory and clinical data that support our proposed strategy that a combination of multiple micronutrients, including certain antioxidants, and non-steroidal inflammatory drugs (NSAIDs) may be more effective for the prevention of AD. This combination, together with a cholinergic agent, may also be more effective for the treatment for AD than the individual agents alone.

## Neuropathology of AD

The diagnosis of AD is made by postmortem analysis of brains of patients with dementia. Intracellular neurofibrillary tangles (NFT) containing hyperphosphorylated tau protein and apolipoprotein E [6–8] and extracellular senile (neuritic) plaques containing many proteins, including  $\beta$ -amyloid ( $A\beta$ ),  $\alpha$ -synuclein, ubiquitin, apolipoprotein E, presenilins and alpha antichymotrypsin, are considered hallmarks of AD [9–15]. Interestingly, a recent study has shown that Lewy bodies are present in the brains of about 60% of AD cases [16]. The mechanisms of formation and dissolution of these cytoplasmic inclusions are under extensive investigation in order to develop novel drugs for the treatment of AD.

## Etiology of AD

Studies on the etiology of AD are important in order to identify targets for the development of new drugs for the prevention and the treatment of this disease. At present, two hypotheses regarding initiating factors, the inflammatory reaction hypothesis and the oxidative and nitrosylative damage hypothesis, are being extensively investigated. Additional hypotheses with respect to promoting factors, including the  $A\beta$ -induced neurotoxic hypothesis, the proteasome inhibition-induced neurotoxic hypothesis and the cholesterol hypothesis, are also being studied. Therefore, it is essential that each of these hypotheses is critically reviewed in order to establish their relative importance in the initiation and the progression of neurodegeneration.

**Inflammatory Reaction Hypothesis.** Evidence of inflammatory reactions in autopsied brains from patients with AD were first observed by Dr. Alois Alzheimer himself. This hypothesis is supported by the epidemiologic studies which show that rheumatoid arthritis patients, who were on high doses of NSAIDs, had a reduced incidence of AD [17–23]. However, this hypothesis became more attractive when it was demonstrated that the mediators and products of inflammatory reaction, such as cytokines [24,25], complement proteins [26–30], free radicals [31–35], adhesion molecules [36–38] and prostaglandins [39,40], were neurotoxic in experimental models of neurons. These products of inflammatory reactions may represent extracellular signals which initiate and promote neuronal degeneration in AD. Several intracellular signals, which mediate the actions of these extracellular signals, include  $\beta$ -amyloid [9–12], ubiquitin [14,15,41] and proteasome [42–44]. The inflammatory reaction hypothesis was further supported by clinical studies in which administration of NSAIDs reduced the rate of deterioration of cognitive function in moderate to advanced AD patients [45–48]. These drugs reduce the levels of neurotoxins that are released during inflammatory reactions and thereby protect neurons from further degeneration. However, they do not protect neurons from damage caused by free radicals that are generated by mechanisms other than inflammatory reactions. Agents which can initiate inflammatory reactions in normal brain include traumatic head injuries, infections and other cellular factors that activate resident microglial cells. In patients with AD, senile plaques serve as a continuous source of inflammatory reactions; therefore, they play an important role in progressive degeneration of neurons. Agents which can prevent the formation or can cause dissolution of these plaques may help remove one of the sources of inflammatory reactions and thereby may improve the efficacy of NSAIDs in the treatment of AD.

**Oxidative and Nitrosylative Damage Hypothesis.** According to this hypothesis, reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in the initiation and promotion of neurodegeneration in the brains of

patients with AD [31–35]. Some of these free radicals are released during inflammatory reactions, whereas others are formed during normal oxidative metabolism and auto-oxidation of certain neurotransmitters and by  $\beta$ -amyloid. Thus, the role of free radicals in the pathogenesis of AD should be considered, at least in part, independent of inflammatory reactions. Clinical studies showing the beneficial effects of high dose antioxidants such as vitamin E [49] and NADH [50] in the treatment of AD support the role of free radicals in progressive degeneration of neurons.

### Sources of Oxidative Stress in Normal Brain

The brain utilizes about 25% of respired oxygen even though it represents only 5% of the body weight. Free radicals are generated in the brain during the normal intake of oxygen, during infection and during normal oxidative metabolism of certain substrates. During normal aerobic respiration, the mitochondria of one rat nerve cell will process about  $10^{12}$  oxygen molecules and reduce them to water. During this process, superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl ( $OH^{\cdot}$ ) are produced. In addition, partially reduced oxygen, which represents about 2% of consumed oxygen, leaks out from the mitochondria and generates about 20 billion molecules of  $O_2^{\cdot-}$  and  $H_2O_2$  per cell per day [51,52]. During bacterial or viral infection, phagocytic cells generate high levels of nitric oxide (NO),  $O_2^{\cdot-}$  and  $H_2O_2$  in order to kill infective agents; however, these radicals can also damage normal cells [51]. During degradation of fatty acids and other molecules by peroxisomes,  $H_2O_2$  is produced as a byproduct. During oxidative metabolism of ingested toxins, free radicals are also generated.

Some brain enzymes such as monoamine oxidase (MAO), tyrosine hydroxylase and L-amino acid oxidase produce  $H_2O_2$  as a normal byproduct of their activity [53]. Furthermore, auto-oxidation of ascorbate and catecholamines generates  $H_2O_2$  [54]. Oxidative stress can also be generated by  $Ca^{2+}$ -mediated activation of glutamate receptors. The  $Ca^{2+}$ -dependent activation of phospholipase  $A_2$  by N-methyl-D-aspartate (NMDA) releases arachidonic acid, which then liberates  $O_2^{\cdot-}$  during the biosynthesis of eicosanoid [55]. Another radical, NO, is formed by nitric oxide synthase stimulated by  $Ca^{2+}$ . NO can react with  $O_2^{\cdot-}$  to form peroxynitrite anions that can form  $OH^{\cdot}$ , the highly reactive hydroxyl radical. NMDA receptor stimulation produces marked elevations in  $O_2^{\cdot-}$  and  $OH^{\cdot}$  levels [56]. Some enzymes such as xanthine oxidase and flavoprotein oxidase (e.g., aldehyde oxidase) also form superoxide anions during metabolism of their respective substrates. Oxidation of hydroquinone and thiol and synthesis of uric acid from purines form superoxide anions.

Certain external agents can increase oxidative stress. For example, cigarette smoking increases the level of NO by about 1000 ppm [57,58] and depletes antioxidant levels [59,60]. Free

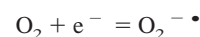
iron and copper can increase the levels of free radicals [61]. Some plants ingested as food contain large amounts of phenolic compounds such as chlorogenic and caffeic acid which can be oxidized to form radicals [62,63].

These studies suggest that the brain generates high levels of ROS and RNS every day. In addition, brain has the highest levels of unsaturated fatty acids which are easily oxidizable by free radicals. Paradoxically, the brain is least prepared to handle this excessive load of free radicals. It has low levels of both antioxidant enzyme systems and dietary antioxidants. These inherent biological features make brain very vulnerable to oxidative and nitrosylative stress [52,56,64,65]. Despite this, the risk of idiopathic AD becomes significant only after the age of 65 or more. This is due to the fact that neurons exhibit a high degree of plasticity in maintaining normal brain functions. The fact that clinical symptoms of neurological diseases including AD appear only when a significant number of neurons are lost supports the value of plasticity of the neurons in maintaining normal brain function.

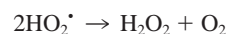
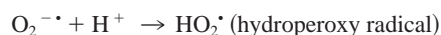
### Formation of Reactive Oxygen Species (ROS)

The brain utilizes of 3.5 mL oxygen/100 grams of brain tissue/minute [66]. About 2% of the oxygen consumed becomes reactive oxygen species (ROS) [52]. The formation of some of these ROS are described below.

When molecular oxygen ( $O_2$ ) acquires an electron, the superoxide anion ( $O_2^{\cdot-}$ ) is formed:



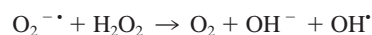
Superoxide dismutase (SOD) and  $H^+$  can react with  $O_2^{\cdot-}$  to form hydrogen peroxide,  $H_2O_2$ :



Ferric and ferrous forms of iron can react with superoxide anion and hydrogen peroxide to produce molecular oxygen and hydroxyl radical ( $OH^{\cdot}$ ), respectively:

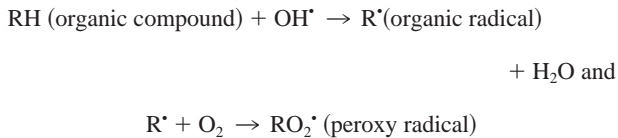


Hydroxyl radical can also be formed from superoxide anion by the Haber-Weiss reaction:

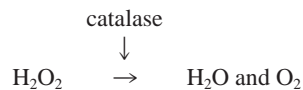


Both the Fenton and Haber-Weiss reactions require a transition metal such as copper or iron. Among ROS,  $OH^{\cdot}$  is the most damaging and very short-lived.

Hydroxyl radical is very reactive with a variety of organic compounds, leading to production of more radical compounds:



Catalase detoxifies hydrogen peroxide to form water and molecular oxygen:

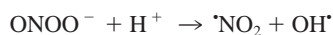


### Formation of Reactive Nitrogen Species (RNS)

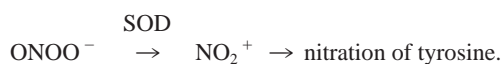
Reactive nitrogen species (RNS) are represented by nitric oxide (NO<sup>•</sup>), the quinone moiety of xenobiotics, the neurotoxin MPTP (N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine), and the herbicide, paraquat. NO<sup>•</sup> is synthesized by the enzyme nitric oxide synthase from L-arginine, and in the brain, it acts both as a neurotransmitter and, in excessive amounts, acts as a neurotoxin. NO<sup>•</sup> can combine with superoxide anion to form peroxynitrite, a powerful oxidant.



When protonated (likely at physiological pH), peroxynitrite spontaneously decomposes to reactive nitric dioxide and hydroxyl radicals:



Superoxide dismutase can also enhance the peroxynitrite-mediated nitration of tyrosine residues on critical proteins, presumably via species similar to the nitronium cation (NO<sub>2</sub><sup>+</sup>):



These data reveal that several different types of radicals are constantly formed in the brain. Their levels can be increased by enhanced turnover of catecholamines, increased levels of free iron, impaired mitochondrial functions, decreased glutathione levels, etc. Antioxidant enzymes which can protect cells against the damaging effects of these free radicals include catalase, superoxide dismutase and glutathione peroxidase. Therefore, decreased levels of catalase, glutathione peroxidase or superoxide dismutase can also enhance the amounts of free radicals. Natural dietary antioxidants include vitamin A, C, and E, carotenoids, flavanoids and polyphenols. Some biosynthetic antioxidants include co-enzyme Q<sub>10</sub>, α-lipoic acid, glutathione, NADH and urates. Consumption of a diet low in antioxidants may also increase the levels of free radicals. Thus, maintenance of a balance in the favor of antioxidants is essential for the protection of brain function. When this balance is shifted in favor of oxidants, the epigenetic components of neurons suffer

damage, and slow accumulation of such damage may initiate degeneration and eventually cause death of neurons.

### Evidence for the Involvement of Oxidative and Nitrosylative Stress in AD Brain

**Mitochondrial Damage Hypothesis.** The brain is particularly sensitive to oxidative stress due to increased levels of oxidative agents and decreased levels of antioxidants [52,64–65]. Indeed, increased oxidative stress has been implicated in the loss of neurons associated with AD [31–35]. Mitochondria may be one of the most sensitive primary targets of oxidative stress in adult neurons [67]. This may be due to the fact that mitochondrial DNA (mtDNA) does not encode for any repair enzymes, and, unlike nuclear DNA, it is not shielded by protective histones. In addition, mtDNA is in close proximity to the site where free radicals are generated during oxidative phosphorylation [68]. Indeed, an increased frequency of mutations in mtDNA has been found in autopsy samples of AD brains [69], and several studies have implicated mitochondrial defects in the pathogenesis of AD [64,65,67–69]. Because the onset of AD coincides with older age, it is reasonable to suggest that damaged mtDNA, which is normally removed during mitochondrial turnover, accumulates in neurons, due to slowing down of this process in older individuals. Thus, the number of defective mitochondria may accumulate with aging, and this could lead to reduced production of ATP that could then initiate slow degenerative processes in neurons. Reduced ATP levels result in decreased energy metabolism. For example, decreased glucose uptake coupled with reduced activity of cytochrome oxidase (complex IV) leads to increased production of ROS by mitochondria [64,65]. This could then constitute a continuous cycle of production of increased levels of free radicals and enhanced mitochondrial dysfunction. A defect in energy production may also increase the sensitivity of neurons to excitatory amino acids [70]. Impaired mitochondria may alter metabolism of APP, leading to decreased secretion of APP and increased generation of potentially amyloidogenic derivatives (11.5 kDa COOH-terminal derivative which contains the full length β-amyloid sequence) which are intermediate metabolites in the production of β-amyloid [71]. Excess of free Zn is found in the autopsied brain of AD [72,73], and increased free Zn can impair mitochondrial function [74]. Thus, mitochondria appear to be one of the major targets of oxidative damage that mediate neurodegeneration in AD. Increased oxidative stress may enhance intracellular accumulation of Aβ in neurons [75]. In addition, studies show that membrane containing oxidatively damaged phospholipids accumulated Aβ faster than membrane containing only unoxidized saturated phospholipids [76]. It has been proposed that one of the mechanisms of action in Aβ neurotoxicity is mediated by free radicals [77–79]. This is supported by the fact that vitamin E protects neuronal cells in culture against Aβ-induced toxicity [80]. It has been shown that methionine in the 35 position of Aβ may be responsible for

generating free radicals [81]. This was confirmed by a series of studies on substitutions of amino acid [81] and by prevention of A $\beta$ <sub>42</sub>-induced toxicity with vitamin E [80].

A number of other observations substantiate the presence of high levels of oxidative stress in AD brains. For example, (a) the serum levels of vitamins A, E and  $\beta$ -carotene were lower in patients with AD (who were well nourished) than in control patients [82]; (b) higher expression of heme oxygenase is found in the brains of AD patients [83,84]; (c) increased consumption of oxygen is found in AD patients [85]; (d) increased activity of glucose-6-phosphate dehydrogenase is found in the AD brain [86], and (e) activation of calcium-dependent neural proteinase (calpain) is found in AD brains [87] which may trigger events leading to the formation of free radicals [88]. The increased levels of lipofuscin formation in a small number of degenerating neurons probably results in a marked progressive increase in superoxide radicals and H<sub>2</sub>O<sub>2</sub> formation and reduced production of ATP, which overwhelms the endogenous systems which defend against free radical-induced damage [89]. The fibroblasts obtained from familial AD patients were more sensitive to oxidative stress than those obtained from age-matched normal controls [90]. Alpha-ketoglutarate dehydrogenase complex (KGDHC), a mitochondrial enzyme, is decreased in brains of AD patients [91]. It is interesting to note that, in AD patients who carry ApoE4 allele of ApoE gene, the clinical Dementia Rating (CDR) correlated better with KGDHC activity than with densities of neuritic plaques and NTFs; however, in patients without ApoE4, the CDR correlated better with plaques and NTFs than with KGDHC activity [91]. This suggests that mitochondrial dysfunctions may be more important for the development of AD in patients who carry ApoE4 allele than in those who do not.

Additional evidence for the increased oxidative stress in AD brain include the following: (a) homogenates of frontal cortex from AD brains obtained at autopsy revealed a 22% higher production of free radicals and, in the presence of iron, a 50% higher production of free radicals than those of age-matched normal controls [92]; (b) besides oxidative stress, nitrosylative stress, which is primarily mediated by peroxynitrites, can potentially exacerbate the pathogenesis of AD [93]; (c) increased neuronal nitric oxide synthase (nNOS) expression in reactive astrocytes correlated with apoptosis in hippocampal neurons of AD brains [94]; (d) glutamine synthetase, an enzyme highly sensitive to oxidative stress, showed decreased activity in AD brains [93]; (e) the level of glutathione transferase is decreased in ventricular CSF and in AD brains compared to brains from age-matched controls [95], and (f) increased levels of oxidized proteins are found in the blood of both AD patients and their relatives when compared with non-AD controls [96]. Excessive amounts of zinc (Zn) accumulation in brain tissue have been implicated in the pathogenesis of AD [72,73]. It has been shown that oxidative stress can elevate Zn in the brain [97]. Free zinc impairs mitochondrial functions [74] and causes

aggregation of A $\beta$  [98]. The fact that overexpression of glutathione peroxidase increases the resistance of neuronal cells (PC12 and rat embryonic cultured cortical neurons) to A $\beta$ -induced toxicity suggests the role of oxidative stress in the progression of AD [99]. In addition, evidence for oxidative and nitrosylative damage at autopsy in the brains of AD patients have been reported [100–102]. Taken together, these data suggest that oxidative- and nitrosylative-induced damage are involved in the pathogenesis of AD and that mitochondria are the primary targets for the action of diverse groups of neurotoxins including free radicals.

**A $\beta$ -Induced Neurotoxic Hypothesis.** It has been proposed that A $\beta$  fragments generated from APP play an important role in neurodegeneration [9–12]. A $\beta$  peptides are generated by two consecutive cleavage events:  $\beta$ -secretase cleaves the N-terminus of A $\beta$ , while  $\gamma$ -secretase cleaves the C-terminus [103]. Most peptides terminate at Val40 (A $\beta$ <sub>40</sub>) or Ala42 (A $\beta$ <sub>42</sub>). It has been shown that aggregates of A $\beta$  peptides are toxic to neurons in culture [77,104,105] and can cause cell death by apoptosis [106] or necrosis [107]. Several agents can enhance the aggregation of A $\beta$ . They include excess amounts of free Zn and Cu [98], iron and aluminum [108] and complement proteins [28]. The aggregated form of A $\beta$  participates in the formation of senile plaque, which can serve as a chronic source of inflammatory reactions, the products of which can enhance the progression of degeneration. In another study, A $\beta$ <sub>25–35</sub>-induced neurotoxicity in rat hippocampal neurons in culture was not affected by several antioxidants [109]; however, pretreatment of neural cultures with A $\beta$  significantly increased the sensitivity of neurons to H<sub>2</sub>O<sub>2</sub>. This suggests that A $\beta$  can make neurons more susceptible to free radical damage. Experiments on a transgenic mouse model of AD support the concept that A $\beta$ -induced neurotoxicity is mediated by oxidative stress. For example, it has been reported [110] that Cu/Zn superoxide dismutase (SOD) and hemoxygenase-1 (HO-1), markers of oxidative stress, were elevated in aged transgenic mice. This study was further confirmed in pheochromocytoma cells (PC-12). Both SOD and HO-1 levels were increased in PC-12 cells following treatment with A $\beta$  or hydrogen peroxide. Thus, both studies suggest that free radicals are involved in A $\beta$  neurotoxicity.

Acute brain injury in rats increases APP mRNA and APP protein levels [111,112]. Acute trimethyltin intoxication can increase APP mRNA in rat hippocampal neurons [113]. These studies suggest that increased levels of APP could be a stress-response like heat-shock protein. In AD brains there is no differential expression of APP mRNA [114]. Therefore, it is proposed that post-translational events in APP metabolism are important in amyloidogenesis and the pathogenesis of AD. The direct role of A $\beta$  in AD has been questioned by some studies. For example, levels of A $\beta$ <sub>42</sub> and A $\beta$ <sub>40</sub> that are generated from APP were higher in non-demented cases of AD and remained higher throughout progression of the disease [115]. A $\beta$ <sub>42</sub> levels were higher than A $\beta$ <sub>40</sub> levels in both non-demented and AD

cases. These studies suggest that  $A\beta_{40}$  and  $A\beta_{42}$  are not responsible for dementia. A similar view has been proposed in other studies [116–118]. Normally,  $A\beta$  fragments within a certain concentration range may not be toxic, but in excessive amounts, they may contribute directly or indirectly to neurodegeneration.

It has been shown that increase in  $A\beta_{40}$  and  $A\beta_{42}$  precede tau pathology (formation of NTFs) in the frontal cortex [115]. This suggests that tau pathology which requires at least two steps, hyperphosphorylation and then accumulation of hyperphosphorylated tau, occurs later than increased generation of  $A\beta$  fragments. Hyperphosphorylation of tau can result from increased PKA activity and/or decreased phosphatase activity. Proteasome inhibition may also reduce degradation of hyperphosphorylated tau proteins, causing them to accumulate slowly, and lead to the formation of NFTs within the cells.

#### **Proteasome Inhibition-Induced Neurotoxic Hypothesis.**

Proteasomes play an important role in regulating certain transcriptional factors by splicing inactive peptide fragments on to active ones. In addition, proteasomes also play a crucial role in the degradation of ubiquitin-conjugated abnormal proteins. Therefore, inhibition of proteasome in neurons can initiate and promote neurodegeneration. Indeed, the role of proteasome inhibition has been proposed for the degeneration of neurons in AD brains [42–44], and  $A\beta$  is one of the factors that could inhibit proteasome activity [44]. A defect in ubiquitin conjugate enzymes [41] or a mutation in ubiquitin (Ub) could also impair removal of unwanted proteins via proteasome. It has been reported that mutated ubiquitin is an efficient substrate for polyubiquitination *in vitro*. Such polyubiquitin chains become refractory to disassembly by deubiquitinating enzymes and their degradation by proteasomes [119]. In our preliminary study, inhibition of proteasome by lactacystin causes rapid degeneration of cAMP-induced differentiated neuroblastoma cells in culture [120]. Furthermore, we suggest that increased accumulation of ubiquitin [14,15] and hyperphosphorylated tau protein [6–8] in AD brains is a reflection of inhibition of proteasome activity. The exact mechanisms of proteasome inhibition in AD neurons are unknown, but they could involve more than one mechanism.

**Cholesterol-Induced Neurotoxic Hypothesis.** Recent epidemiologic and laboratory data suggest that cholesterol metabolism may be associated with the development of AD. Epidemiologic studies have found that hypercholesterolemia may be a risk factor in the development of AD [121,122]. This was confirmed in the transgenic animal model of AD [122]. This study revealed that high dietary cholesterol increases  $A\beta$  accumulation and thereby accelerates AD-related pathology in animals [123]. The accumulation of  $A\beta$  can be reversed by removing cholesterol from the rabbit's diet [123]. Inhibitors of HMG CoA reductase decrease production of  $A\beta$  in rabbit [122] and in fetal rat hippocampal neurons in culture [124]. A preliminary epidemiologic study has shown that lovastatin, an inhibitor of HMG CoA reductase, reduces the risk of AD in

hypercholesterolemic patients [125]. These results suggest that some of the effects of cholesterol are primarily mediated via  $A\beta$  rather than via poor circulation due to thickening of the arteries. Statins (cholesterol-lowering drugs) can be divided into two distinct groups, those with a closed-ring structure (lovastatin, simvastatin, mevastatin) and those with an open-ring structure (pravastatin and fluvastatin). Statins with a closed-ring structure are metabolized *in vivo* to an open-ring structure which then inhibits HMG CoA reductase activity. However, a small amount of the drug is maintained in a closed-ring structure which can inhibit proteasome activity [126]. Recently, we have demonstrated that mevastatin with a closed-ring structure caused rapid degeneration of differentiated neuroblastoma (NB) cells in culture, whereas pravastatin with an open-ring structure did not [127]. Mevastatin inhibited proteasome activity in differentiated NB, whereas pravastatin did not. Differentiated NB cells did not convert any portion of mevastatin into an open-ring structure. This is sharp contrast to the observation made *in vivo*, where most mevastatin is converted to an open-ring structure by the liver enzyme. These results suggest that mevastatin-induced degeneration of differentiated NB cells may be related to inhibition of proteasome activity [127]. The studies discussed in this section reveal that lowering cholesterol levels could reduce the risk of AD, whereas the presence of increased amounts of unmetabolized statins with a closed-ring structure could increase the risk of AD. A careful study of the effects of statins with a closed-ring and an open-ring structure on neuroprotection and neurodegeneration should be evaluated by laboratory experiments and epidemiologic studies before their relevance in AD can be determined.

#### **Our Proposed Hypothesis That Epigenetic Components of Neurons Are Primary Targets for the Action of Diverse Groups of Neurotoxins**

We have proposed a hypothesis that epigenetic components (mitochondria, proteasomes, post-translational modification of proteins) rather than nuclear genes are the primary targets for the action of diverse groups of neurotoxins in idiopathic AD [4,5]. Even in cases of familial AD, the products of mutated genes by themselves are not neurotoxic, rather they affect epigenetic components such as post-translational modification of proteins (increased processing of APP to  $A\beta_{40}$  and  $A\beta_{42}$ ) that could increase oxidative stress and/or make neurons more sensitive to oxidative stress. We also suggest that the shift in processing of APP to  $A\beta_{42}$  in AD brain is an example of post-translational modification of protein caused by certain neurotoxins. If our hypothesis is correct, novel drug development for the prevention and treatment of neurodegenerative diseases should be focused on lowering the levels of oxidative stress and maintaining the integrity and proper functioning of epigenetic components of neurons such as mitochondria, proteasome and normal proteolysis of APP.

There is no solid evidence for nuclear gene defects which increase the risk of idiopathic AD, although varying degrees of association between certain gene defects and onset of this disease exist. Several studies have suggested that persons who are homozygous for the apolipoprotein E (APOE), e4 allele, develop AD 10 to 20 years earlier than those who have e2 or e3 alleles [128–129]. Even persons who are heterozygous for e4 allele develop AD 5 to 10 years earlier than those who have e2 or e3 alleles [130]. About 40% of idiopathic AD is associated with the presence of e4 allele, and it is present in the senile plaque [6,130]. These data suggest that the presence of e4 allele could be an important risk factor for AD. However, it was shown that this allele is neither essential nor specific for the development of AD [130]. Thus, the role of this APOE allele in neurodegeneration is uncertain. It has been reported that e4 allele binds to neurofibrillary tangles and  $\beta$ -amyloid [6]. This property of APOE e4 is not sufficient to have any direct role in neurodegeneration associated with AD. However, a recent study has reported that in patients who carry ApoE4, the Clinical Dementia Rating was correlated better with decreased alpha-ketoglutarate dehydrogenase complex, a mitochondrial enzyme, than with plaques or NFTs [91]. This suggests that in some case of AD ApoE4 may have some role in the propagation of degenerative processes. A study has reported that mutation in alpha2-macroglobulin gene is present in about 30% of idiopathic AD [131]; however, another study found no such association between alpha<sub>2</sub>-macroglobulin mutation and risk of AD [132]. Recent studies have identified two gene defects in idiopathic AD. Mutation in the ubiquitin gene [119] and down-regulation of presenilin II [133–135] have been observed in AD brains. It is not certain whether they represent late events or early events. Their role in neurodegeneration remains to be defined.

In some familial AD, mutations (about seven) in the APP gene have been reported, all of which increase the production of  $\beta$ -amyloid [136]; however, this accounts for less than 1% of all familial AD. Mutations (about fifty) in presenilin-I gene have been found in about 50% of familial AD [136], whereas mutations in presenilin-II have been observed in less than 1% of familial AD [136,137]. Presenilin-I is present in senile plaques and NTFs of AD brains [136]. The nature of the interaction between APP and presenilins in causing neuronal damage is not well understood. It has been postulated [13] that APP interacts specifically and transcellularly with either presenilin I or presenilin II. This complex is incorporated into intracellular vesicles which fuse with multivesicular bodies that contain proteases.  $\beta$ -amyloid is then produced by proteolysis of APP and released by the usual intracellular traffic between the lysosomal compartment and the plasma membrane into the extracellular spaces where it forms senile (neuritic) plaques. It has been reported that mutations in presenilin-I may increase neuronal sensitivity to apoptosis by decreasing the levels of  $\beta$ -catenin, which is involved in regulation of apoptosis [138]. In addition, presenilin-I mutation may also impair proteolytic

release and nuclear translocation of Notch-1 intracellular domain, an essential step in activating Notch-1 signaling [139]. The significance of presenilin-I mutation-induced alteration in Notch-1 cleavage in neurodegeneration is unknown. These studies suggest that mutations in both APP and presenilins increase the rate of production of  $\beta$ -amyloid. Excessive production of  $A\beta$  can generate more free radicals, inhibit proteasome activity and contribute to the formation of senile plaques, all of which contribute to progressive neurodegeneration in AD brain.

It should be noted that, in spite of mutations in APP and presenilin genes, a minimum of about 30 years is needed for the development of familial AD. This suggests that the products of mutated genes by themselves are not toxic. It is possible that cells expressing these gene mutations may become more sensitive to neurotoxins including oxidative and nitrosylative stress. Indeed, we have shown that the expression of mutated  $\alpha$ -synuclein, that is associated with familial Parkinson's disease in dopamine neurons, makes them more vulnerable to oxidative stress [140]. There is no such direct evidence available for mutated APP or presenilins. Nevertheless, free radicals play an important role in the initiation and progression of neurodegenerative changes in familial AD brains. Therefore, quenching of free radicals by the appropriate antioxidants as early as possible may delay the early onset of AD. No clinical studies have been performed on this issue as of yet.

### **Proposed Sequence of Cellular Events in Degeneration of Neurons in Idiopathic AD**

Although several cellular defects in AD brain have been identified, the sequence by which these defects occur has not been adequately defined. Based on published data, it appears that inflammatory reactions represent one of the earliest events in the pathogenesis of AD. They can be initiated by acute brain trauma, chronic infection or other cellular factors that activate resident microglial cells. Products of inflammatory reactions, such as cytokines, complement proteins, adhesion molecules, prostaglandins and free radicals are neurotoxic. Free radicals are also produced independently of inflammatory reactions. They impair mitochondrial and proteasome functions. They also increase processing of APP to  $A\beta_{40}$  and  $A\beta_{42}$ . We propose that the products of inflammatory reaction and excessive amounts of free radicals that are generated by mechanisms which are independent of inflammatory reaction are some of the earliest events that initiate and promote neurodegeneration in patients with AD. Since mitochondria use oxygen to generate ATP, increased amounts of incompletely reduced oxygen leak out from impaired mitochondria generating a cascade of free radicals, including nitrogen derived free radicals. Reduced levels of ATP and increased production of free radicals may damage proteasome. The inhibition of proteasome can prevent degradation of ubiquitin-conjugated abnormal proteins that could initiate and promote neurodegeneration. The inhibition of

proteasomes can also cause accumulation of short-lived proteins that need to be removed by proteasomes in order to maintain cell viability and to maintain long-term memory. Certain proteins need to be removed by proteasomes in order to maintain cellular viability and function. For example, proteasome activation decreases apoptosis; therefore, inhibition of proteasome may increase apoptosis by activating caspase CPP32 [141]. Degradation of R1 subunit of protein kinase A is needed for long-term memory in *Aplysia*, and proteasome-ubiquitin pathway is responsible for hydrolysis of R1 subunit of PKA [142]. Therefore, inhibition of proteasome and/or lack of binding of R1 with ubiquitin may interfere with memory. It is not known whether R1 is involved in managing long-term memory in humans. We propose that the inhibition of proteasome may have a greater role in memory loss than other factors such as overproduction of  $A\beta$  or formation of senile plaques. The latter is supported by the fact that, in transgenic mice over expressing APP, neither the increased production of  $A\beta$  nor the formation of senile plaques causes loss of neurons or loss of memory [143]. The overproduction of  $A\beta$  may also inhibit proteasome activity [44]. Oxidative stress has been shown to increase the intracellular levels of  $A\beta$  [75]. Thus, after mitochondrial damage, proteasome inhibition appears to be the second most important event that initiates and promotes neurodegeneration.  $A\beta$  fragments become neurotoxic only when they form aggregates and when they participate in the formation of senile plaques. Although hyperphosphorylation of tau protein may occur earlier, NFTs are formed only subsequent to proteasome inhibition. Thus, like senile plaques, NFTs represent late-events which accelerate the rate of progression of degenerative changes in neurons. Thus, mitochondrial damage and inhibition of proteasome may be some of the early cellular damage which initiate and promote neurodegeneration. The shift in processing of APP to  $A\beta_{40}$  and  $A\beta_{42}$  and accumulation of hyperphosphorylated tau protein may occur subsequent to the above defects, and they contribute to the formation of senile plaques and NFTs, respectively. Therefore, novel targets for the prevention of AD should include protecting mitochondria and proteasomes against damage produced by free radicals and the products of inflammatory reactions. To reduce the progression of AD, it is not only necessary to protect mitochondria and proteasomes against further damage, but also prevent the formation and aggregation of  $A\beta_{40}$  and  $A\beta_{42}$ , as well as increase the dissolution of existing senile plaques. Defects in mitochondrial functions have been also observed in aging brain [144]; therefore, they by themselves can not be considered specific to AD.

### Analysis of Laboratory and Clinical Studies

Since the above studies show that ROS and NRS are important intermediary risk factors involved in the initiation and progression of neurodegeneration, it appears rational that antioxidants would be beneficial in the prevention of AD and as an adjunct to standard therapy in the treatment of AD. Indeed, a

controlled clinical trial with *dl*- $\alpha$  tocopherol (synthetic form, 2,000 IU/day) in patients with moderately severe impairment from AD showed some beneficial effects with respect to rate of deterioration of cognitive function [49]. Although this important clinical study supports the role of free radicals in the progression of AD, the use of a single antioxidant, vitamin E, and the administration regime (once a day) may not have been optimal for quenching all the various types of free radicals that are produced in the brain. For example, it has been reported that rat organs preferentially absorb the natural form of vitamin E [145]; therefore, the use of synthetic vitamin E in the above clinical study may not have produced an optimal effect. In addition, the  $\alpha$ -tocopherol form of vitamin E may not cross the blood-brain barrier as efficiently as *d*- $\alpha$  tocopheryl succinate ( $\alpha$ -TS), since  $\alpha$ -TS is more soluble in ethanol, and enters the mammalian cell more readily than  $\alpha$ -tocopherol [146]. In addition, the use of a single antioxidant may not be prudent for long-term therapy, because very high doses of a single antioxidant may be needed for a beneficial effect in AD patients; however, such high doses of individual antioxidants can cause toxicity. For example,  $\alpha$ -tocopherol at high doses (2,000 IU/day or more) over a long period of time can cause a clotting defect which can be reversed by vitamin K administration [147]. Furthermore, individual antioxidants, when oxidized, can be prooxidative, and that could be harmful over a long period of time. Because of these and other reasons to be discussed later, we recommend the use of multiple antioxidants at appropriate doses that have no known toxicity.

In a controlled clinical trial, selegiline (10 mg a day), a monoamine oxidase inhibitor, or *dl*- $\alpha$  tocopherol slowed the progression of disease in patients with moderately severe impairments from AD [49]. It was interesting to note that there was no significant difference in the effect between the groups receiving a combination of *dl*- $\alpha$ -tocopherol and selegiline and those receiving treatment with the individual agents [49]. In our opinion, this was expected because both selegiline and vitamin E reduce the levels of free radicals, although by different mechanisms. For example, vitamin E protects neurons by destroying formed free radicals ("quenching"), whereas selegiline protects neurons by preventing the formation of free radicals through inhibiting oxidative metabolism of catecholamines. Thus, clinical results using both vitamin E and selegiline support the concept that free radicals are important intermediary risk factors for the progression of neurodegeneration in AD.

The effect of antioxidants in the animal model of AD has not been investigated. *In vitro* experiments reveal that vitamin E protects neurons against known neurotoxins such as glutamate [148] and 6-hydroxydopamine [149] which are known to mediate their action in part by free radicals. Vitamin E protects rats against aggregated  $A\beta$ -induced behavioral impairments [150]. Lysosomes play a key role in preventing the formation of amyloid deposits and senile plaques, and vitamin C improves lysosomal functions of human brain astrocytes [151] and the

can thereby preserve cellular function. Thus, the use of antioxidants in the prevention of and as an adjunct to standard therapy in the treatment of AD has both laboratory and clinical support.

### **Rationale for Using Multiple Antioxidants for AD Prevention among High-Risk Populations**

High-risk populations include persons over age 65 or persons who have a family history of AD. Because of the potential for increased levels of oxidative stress and/or enhanced sensitivity of neurons to oxidative stress in the brains of patients of this population, oral supplementation with appropriate antioxidants appears to be one of the rational choices for the prevention and/or delayed onset of AD. Conventional experimental designs for prevention of AD have utilized only one or two antioxidants. These designs are not suitable for determining the maximal efficacy of antioxidant therapy due to the varied mechanisms of action of antioxidants, varied environments at the cellular and organ level (oxygenation, aqueous and lipid components) and the varied types of free radicals. We propose the use of multiple antioxidants for AD prevention trials. The biological rationale for using multiple antioxidants is described below.

Almost all antioxidants can act prooxidatively when oxidized. Therefore, the use of single antioxidants in clinical trials cannot be considered rational for improving disease outcome. For example, beta-carotene (BC) is more effective in quenching oxygen radicals than most other antioxidants [152]. BC can perform certain biological functions that cannot be produced by its metabolite vitamin A, and vice versa [153,154]. It has been reported that BC treatment enhances the expression of the connexin gene which codes for a gap junction protein in mammalian fibroblasts in culture, whereas vitamin A treatment does not produce such an effect [153]. Vitamin A can induce differentiation in certain normal and cancer cells, whereas BC and other carotenoids do not [155,156]. Thus, BC and vitamin A have, in part, different biological functions. The gradient of oxygen pressure varies within cells. Some antioxidants, such as vitamin E, are more effective as quenchers of free radicals in reduced oxygen pressure, whereas BC and vitamin A are more effective in higher atmospheric pressures [157]. Vitamin C is necessary to protect cellular components in aqueous environments, whereas carotenoids and vitamins A and E protect cellular components in lipid environments. In addition, vitamin C is necessary for the activity of tyrosine hydroxylase, which is the rate-limiting enzyme in the synthesis of catecholamines. Oxidized forms of vitamin C and vitamin E can also act as radicals; therefore, excessive amounts of any one of these forms, when used as a single agent, could be harmful over a long period of time. Vitamin C also plays an important role in maintaining cellular levels of vitamin E by recycling vitamin E radical (oxidized) to the reduced (antioxidant) form [158]. Also, oxidative DNA damage produced by levels of vitamin C could be protected by vitamin E.

The form of vitamin E used is also important in any clinical trial. It has also been established that d-alpha-tocopheryl succinate ( $\alpha$ -TS) is the most effective form of vitamin E both *in vitro* and *in vivo* [146,159]. This form of vitamin E is more soluble than  $\alpha$ -tocopherol and enters cells more readily [146]. Therefore, it is expected to cross the blood-brain barrier in greater amounts than  $\alpha$ -tocopherol. However, this has not yet been demonstrated in animals or humans. We have reported that oral ingestion of  $\alpha$ -TS (800 I.U./day) in humans increased plasma levels of not only  $\alpha$ -tocopherol, but also  $\alpha$ -TS, suggesting that  $\alpha$ -TS can be absorbed from the intestinal tract before hydrolysis to  $\alpha$ -tocopherol [146]. This observation is important because the conventional assumption based on rodents has been that esterified forms of vitamin E such as  $\alpha$ -tocopheryl acetate,  $\alpha$ -tocopheryl nicotinate and  $\alpha$ -TS can be absorbed from the intestinal tract only after they are hydrolyzed to  $\alpha$ -tocopherol. Our data show that this assumption may not be true for the absorption of  $\alpha$ -TS in humans.

Another antioxidant is glutathione which is effective in catabolizing  $H_2O_2$  and anions. However, oral supplementation with glutathione failed to increase plasma levels of glutathione significantly in human subjects [160], suggesting that this tripeptide is completely hydrolyzed in the G.I. tract. N-acetylcysteine and alpha-lipoic acid (glutathione-elevating agents by different mechanisms) can be used as an antioxidant in combination with others.

Besides well-characterized antioxidants such as vitamin A, carotenoids, vitamin C and vitamin E, other antioxidants such as coenzyme  $Q_{10}$ , NADH and glutathione have some potential therapeutic value in the treatment of certain neurodegenerative diseases. Since mitochondrial dysfunction is associated with AD and since coenzyme  $Q_{10}$  and nicotinamide adenine dinucleotide (NADH) are needed for the generation of ATP by mitochondria, it is essential to use these antioxidants for AD prevention among high-risk populations. A study has shown that ubiquinol (coenzyme  $Q_{10}$ ) scavenges peroxy radicals faster than  $\alpha$ -tocopherol [161] and, like vitamin C, can regenerate vitamin E in a redox cycle [162,163]. However, it is a weaker antioxidant than  $\alpha$ -tocopherol. Coenzyme  $Q_{10}$  administration has been shown to improve clinical symptoms in patients with mitochondrial encephalomyopathies [163]. NADH administration (10 mg/day before meal) has been beneficial in a pilot study of 17 AD patients [50]. Selenium is a co-factor of glutathione peroxidase, and Se-glutathione peroxidase also acts as an antioxidant. Therefore, selenium supplementation together with other antioxidants is also essential.

In addition to antioxidants, vitamin B-12 may have some role in the treatment of AD. In most studies the serum levels of vitamin B-12 in AD patients were significantly lower than controls, and this may partly contribute to degeneration of neurons [164,165]. Indeed, vitamin B-12 supplementation increased choline acetyl transferase activity in cholinergic neurons in cats [166] and improved cognitive functions in AD

patients [167]. Therefore, the inclusion of vitamin B-12 in multiple antioxidant preparations may be useful.

### **Recommended Antioxidant Supplements for Prevention of AD in High Risk Populations**

A base supplement for all risk groups would include a multiple antioxidant preparation containing vitamin A (retinyl palmitate, 5000 I.U./day), natural  $\beta$ -carotene (15 mg/day), vitamin E (d- $\alpha$ -TS, 100 I.U./day with d- $\alpha$ -tocopherol 100 I.U./day), vitamin C (calcium ascorbate, 500 mg/day), vitamin D (400 I.U./day), B vitamin doses twofold to threefold higher than RDA values, selenium (100  $\mu$ g/day), chromium (50  $\mu$ g/day) and zinc (15 mg/day). No iron, copper or manganese would be included because these trace minerals are known to interact with vitamin C to produce free radicals. In addition, increased iron stores have been linked to increased risk of several chronic diseases including AD [24,168]. In addition to the baseline product, we also recommend natural  $\beta$ -carotene (15 mg/day), d- $\alpha$ -tocopheryl succinate (400 I.U./day), vitamin C (calcium ascorbate, 1 g/day), selenium (100  $\mu$ g/day), N-acetylcysteine (250 mg/day), coenzyme Q<sub>10</sub> (60 mg/day), and NADH (5 mg/day). Doses of coenzyme Q<sub>10</sub>, NADH, vitamin E and vitamin C higher than those proposed here have been used in patients with neurodegenerative diseases and produced some beneficial effects when coenzyme Q<sub>10</sub> [169] or NADH [170] are used alone or when a combination of vitamin E and vitamin C is used [171]. Because of positive interactions between these micronutrients, lower doses of multiple antioxidants may be more effective than the single micronutrient at high doses. Although these proposed doses of multiple nutrients are somewhat arbitrary, they have been used in humans for several decades, and they should, therefore, be considered safe.

### **Rationale for Using Multiple Antioxidants in AD Patients**

Reactive oxygen species and reactive nitrogen species play an important role in the progression of neurodegeneration in AD. Therefore, multiple antioxidant supplements as an adjunct to standard therapy in the treatment of AD would be more useful than the individual agents alone. Antioxidant supplements recommended for early AD and for moderate to advanced AD are described separately.

### **Recommended Antioxidant Supplements in Patients with Early AD**

In addition to the base supplement of multiple antioxidants recommended for the high risk population, in patients with early AD, we recommend additional supplements of beta-carotene (30 mg/day), d-alpha-tocopheryl succinate (400 I.U./day), vitamin C (2 g/day), coenzyme Q<sub>10</sub> (90 mg/day), NADH (10 mg/day), N-acetylcysteine (250 mg/day), alpha-lipoic acid (30 mg/day) and selenium (200  $\mu$ g/day).

### **Recommended Antioxidant Supplements in Patients with Moderate to Advanced AD**

In addition to the base supplement of multiple antioxidants recommended for the high risk populations, in patients with active AD, we recommend additional supplements of natural  $\beta$ -carotene (30 mg/day), d- $\alpha$ -tocopheryl succinate (600 I.U./day), vitamin C (2 g/day), coenzyme Q<sub>10</sub> (120 mg/day), NADH (20 mg/day) and selenium (200  $\mu$ g/day). N-acetylcysteine (NAC) (1000 mg/day) and alpha lipoic acid (60 mg/day) at high doses are given only for a period of two months and then reduce them to lower doses NAC (500 mg/day) and alpha-lipoic acid (30 mg/day). In doses above 500 mg/day, N-acetylcysteine can act as a metal chelator. For example, at a dose of 800 mg/day for two weeks, N-acetylcysteine was shown to mobilize zinc stores and increase its urinary excretion [172]. Since increased levels of free Zn have been implicated in neurodegeneration [72,73], high doses of NAC and alpha lipoic acid for a two-month period may remove excessive amounts of free Zn and possibly other metals. The proposed doses of NAC (500 mg/day) and alpha lipoic acid (30 mg/mL) for a long-term consumption are unlikely to cause Zn deficiency. To reduce this possibility further, 15 mg of Zn and other minerals have been added to base line supplement. Although these doses are somewhat arbitrary, they have been used in humans for decades, therefore, they should be considered safe.

The recommended antioxidant supplements for all groups of patients should be taken orally and divided into two doses, half in the morning and the other half in the evening. This is because the biological half-life of most antioxidants varies from 6 to 12 hours. To maintain high levels of antioxidants in the brain, these antioxidants must be taken twice a day. Compliance rates should be determined by counting the pills and by determining the blood levels of antioxidants and appropriate minerals before supplementation, then once a year, for the entire period of a study.

### **Rationale for Using NSAIDs for Prevention and Treatment of AD**

Since inflammatory reactions represent one of the major factors that initiate and promote neurodegeneration in AD brain, the use of NSAIDs in the prevention and treatment of AD appears rational. Laboratory, epidemiological and clinical studies support this recommendation. Laboratory data have shown that products of inflammatory reactions such as prostaglandins [39,40], cytokines [24,25], complement proteins [26–30], adhesion molecules [36–38] and free radicals [31,34,89] are neurotoxic. Epidemiological studies have revealed that rheumatoid arthritis patients, who are on high doses of non-steroidal anti-inflammatory drugs (NSAIDs), have a reduced incidence of AD [17–23]. NSAIDs also reduce the rate of deterioration of cognitive functions in AD patients [45–48]. However, administration of prednisone, a powerful anti-inflammatory agent, was not useful in patients with AD [173]. Treatment with a

mixed cox-1/cox-2 inhibitor and a PGE2 analog failed to produce any significant benefit on cognitive function [174]. A specific inhibitor of cox-2 was also not useful in improving cognitive function [175]. Therefore, it was suggested that the cox-2 enzyme may not be the appropriate target for AD treatment [176]. This is supported by the following additional evidence: (a) the brains of non-demented elderly people taking NSAIDs had fewer activated microglia, suggesting reduced anti-inflammatory environment [177], and (b) chronic administration of ibuprofen reduced inflammation, dystrophic neurite formation and A $\beta$  deposition in a transgenic AD model [178]. Thus, the use of NSAIDs for prevention and reducing the progression of AD remains one of the viable options [4,176,179]. These drugs do not improve the function of surviving neurons or protect neurons from further damage caused by oxidative and nitrosylative stress that is generated by mechanisms other than inflammatory reactions.

### **Rationale for Using Cholinergic Agents in the Treatment of AD**

It has been proposed that the gradual loss of cognitive functions in AD is due to the loss of cholinergic neurons; therefore, cholinergic drugs are used to improve the function of surviving neurons in AD patients. However, these agents do not protect cholinergic neurons against the damaging effects of oxidative and nitrosylative stress and other neurotoxins. Consequently, neurons continue to die, and the beneficial effects of cholinergic drugs do not last long.

### **Rationale for Using Multiple Micronutrients in Combination with a NSAIDs in the Prevention of AD**

We propose that multiple micronutrients, including appropriate antioxidants, in combination with NSAIDs may be useful in the prevention of AD among high risk populations such as familial AD and persons 65 and over. High doses of antioxidants do not have known toxicity, but high doses of NSAIDs can cause toxicity including GI bleeding. A recent study has shown [180] that *d*-alpha-tocopheryl acetate in combination with aspirin inhibited lipopolysaccharide-induced prostaglandin E<sub>2</sub> formation and cyclooxygenase-2 protein and mRNA expression in murine macrophage cell line more than the individual agents alone. This study suggested that the dose requirement for NSAIDs may be reduced in the presence of antioxidants such as vitamin E and thereby may avoid the side effects of high doses of NSAIDs. Due to their potential toxicity, doses of NSAIDs must be monitored by a physician. The clinical efficacy of this combined treatment among the high risk AD patients has not yet been tested.

### **Rationale for Using Multiple Micronutrients in Combination with NSAIDs and a Cholinergic Agent in the Treatment of AD**

Based on the data presented in this review, we propose that multiple micronutrients, including appropriate antioxidants, in combination with NSAIDs and cholinergic agents would be more effective in the treatment of AD than the individual agents alone. Antioxidants may protect cholinergic neurons against damaging effects of free radicals. NSAIDs may attenuate the level of inflammatory reactions and reduce the release of potential neurotoxins, and cholinergic drugs may improve the function of surviving neurons. The clinical efficacy of this combined treatment in patients with AD has not yet been tested. In advanced AD patients who become completely unresponsive to all known therapeutic agents, the addition of antioxidant supplements may also become ineffective.

**Diet and Lifestyle Recommendations.** Even though there is no direct link between the diet and lifestyle related factors and the risk of AD or progression of AD, it is always useful to include a balanced diet that contains low fat and plenty of fruits and vegetables. Among fruits, blueberries and raspberries are particularly important because of their protective role against oxidative injuries in brain. Lifestyle recommendations include daily moderate exercise, reduced stress and no tobacco smoking.

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