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Inhibition of Early Upstream Events in Prodromal Alzheimer's Disease by Use of Targeted Antioxidants

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Abstract: A link between Alzheimer's disease (AD) and an excess presence of oxidant free radicals in the brain has frequently been reported. It is generally assumed that such oxidative stress and related cellular damage is caused by inflammatory changes in the brain and is consequent to amyloid deposition. This review makes the argument that elevated oxidative stress in AD is an early causal event in the initiation and advancement of this disease. Oxidative stress can be decreased by enhancing antioxidant enzymes through activation of the cytoplasmic transcriptional factor (Nrf2)/ARE (antioxidant response element) pathway, and by dietary and endogenous antioxidant chemicals. Reduction in the binding ability of Nrf2 to ARE lowers antioxidant enzyme levels. Decreased levels of Nrf2 and augmentation of oxidative stress in AD suggest that the ROS-dependent mechanism of activating the Nrf2/ARE pathway has become unresponsive. A combination of agents that can either activate the Nrf2-ARE pathway by ROS-independent mechanisms, or by acting directly as antioxidant chemicals, may be necessary to reduce oxidative stress in AD. Earlier shortcomings of using individual antioxidants may be due to consideration of antioxidants as pharmacological agents, ignoring the fact that individual antioxidants can be transmuted in the highly oxidant milieu that is present in AD. Interactions between various cellular compartments may require simultaneous examination of more than one agent. The clinical utility of such a more integrative method can reveal interactive effects such as those found in nutritional research and this can compensate for any mechanistic shortcomings of simultaneous testing of more than a single agent.

Keywords: Alzheimer's disease, antioxidants, free radicals, inflammation, neurodegenerative disease, oxidative stress.

1. INTRODUCTION

Alzheimer's disease (AD) involves gradual loss of intellectual function associated with degeneration and death of cerebral cortical neurons, and is the major cause of dementia. Individuals, who are 65 years or older, have risk of developing this neurodegenerative disease which sharply increases after that. Over 90 % of AD is idiopathic or sporadic and only about 5-10 % is clearly genetically derived. AD is the 5th leading cause of death among people age 65 years or older. Despite extensive research, it has hitherto not been possible to significantly reduce the incidence or the rate of progression of AD. During last few decades, several biochemical and genetic events that initiate and contribute to progressive degeneration and death of neurons have been identified in AD. These include: (a) increased oxidative stress [1-7], (b) mitochondrial dysfunction [8-12], (c) chronic inflammation [13-19], (d) Aß1-42 peptides produced from proteolysis of amyloid precursor protein (APP) [20, 21], (e) proteasome inhibition [22-24], (f) heritable mutations in APP, presenilin-1 and presenilin-2 genes [25-30] and (g) hyperphosphorylated tau protein [31-34]. Several of these biochemical and genetic events can lead to increased oxidative stress which is likely to play a key role in the commencement and development of AD. Therefore, reducing oxidative stress may be one of the logical approaches for inhibition and improved management of this disease. This can be accomplished by increasing the levels of antioxidant enzymes by way of the nuclear transcription factor (Nrf2)-ARE (antioxidant response element) and by addition of appropriate antioxidants to the diet. Several studies on the consequences of administration of individual antioxidants in isolation have been carried out using animal models of AD. A few human studies have utilized a single agent and these have yielded inconsistent results, varying from no effect to some beneficial effects on the symptoms of AD [35, 36]. No studies have been conducted with a combination of antioxidant chemicals and polyphenolic compounds which can reduce oxidative stress optimally by directly scavenging free radicals as well as by inducing antioxidant enzymes through activation of the Nrf2-ARE pathway. This could be due to the fact that the mechanistic investigations do not benefit from the simultaneous use of several intervening agents, which can cloud understanding of the effects of individual agents.

This review presents evidence in support of a hypothesis that (a) increased oxidative stress is one of the earliest biochemical defects which initiate neurodegeneration in AD, and that (b) increased oxidative stress together with other biochemical and genetic defects participate both in the progression of neurodegeneration and the final stage of neuronal death. In addition, a rationale is given for the administration in combination of several dietary antioxidants and specific polyphenolic compounds, in the reduction of oxidative

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stress. Together with standard therapy, this may result in a reduction in the incidence of AD. Such a tactic could also enhance the management of this disease.

2. EVIDENCE FOR INCREASED OXIDATIVE STRESS AS AN EARLY OCCURRENCE IN THE ONSET OF AD

It has been repeatedly reported that increased values of indices reflecting oxidative stress occur in the autopsied brain samples and in the vascular system of AD patients [37]. However, it is difficult to conclude whether increased oxidative stress is the cause or the consequence of the disease. Strongest support for the hypothesis of the initiating nature of oxidant events comes from three sources, the cell culture model of AD, the asymptomatic transgenic animal model of AD, and from asymptomatic individuals carrying AD specific mutated genes.

2.1. Studies on Cell Culture Models of AD

The concentrations of indicators of oxidative stress (4-hydroxynonenal and 3-nitrotyrosine) were elevated in nerve cells obtained from the transgenic AD mice expressing mutated APP or mutated presenlinin-1 compared to those found in nerve cells from the wild-type mice [38]. The relation between early oxidant status and later amyloidogenic change, is illustrated by the finding that application of hydrogen peroxide (H_2O_2) to isolated nerve cells of human origin significantly increased Aß production by enhancing the expression of β- and γ-secretases responsible for cleavage of APP to form Aß peptides [39]. Primary culture of neurons obtained from transgenic mice expressing both mutated APP and mutated preseninlin-1 exhibited increased levels of formation of free radicals. In addition, these nerve cells showed increased sensitivity to exogenous Aß1-42 and H_2O_2 leading to neuronal death [26]. These results also suggest that mutations in preseninlin-1 and APP may cause neurodegeneration in familial AD by further increasing oxidative events, which may progressively participate in the damage and ultimate loss of neurons in AD.

2.2. Studies on Animal Models of AD

Using a transgenic mouse model of AD (APP23 mice), increased protein oxidation and reduced energy metabolism occurred in the cortex of asymptomatic mice, suggesting that these biochemical markers of oxidative stress occur prior to the development of other biochemical defects and the amyloidogenic phenotype [40]. The early role of increased oxidative stress in neurodegeneration was further evidenced by pretreatment with an antioxidant (the SOD/catalase mimetic, EUK-207). This prevented cognitive dysfunction, reduced nucleic acid oxidation and lipid peroxidation, and also reduced levels of Aß1-42, tau and hyperphosphorylated tau, in the amygdala and hippocampus of transgenic AD mice (tau 3xTg) [1].

2.3. Studies on Asymptomatic Individuals Carrying Mutated AD Specific Genes

The presence of oxidatively damaged proteins was elevated in the blood of both AD patients and their family members relative to non-AD controls [41]. In a study comparing asymptomatic individuals carrying mutated preseninlin-1 or mutated APP with their relatives carrying no mutated genes, the plasma levels of oxidative markers, such as methionine sufoxide, a oxidation product of methionine, were found to be unusually high in persons carrying AD mutations in comparison to relatives with no mutated genes [42]. These studies indicate that increased oxidative stress occurs prior to other biochemical defects in the asymptomatic individuals carrying a mutated gene specific to AD.

2.4. Increased Oxidative Stress in an Early Phase of AD

The presence of mild cognitive impairment (MCI) can be considered as an early phase of AD. Several studies showed that increased oxidant activity occurred in patients with MCI, implying that increased oxidative stress is an early event, which also participates in the progression of AD. A significant increase in oxidized and nitrated biliverdin reductase -A (BVR-A) was found in the hippocampal region, but not in the cerebellum of patients with AD as well as in patients with MCI [43]. The analysis of serum concentrations of indicators of oxidative stress in 101 patients with AD, and 134 patients with MCI found that increased levels of serum hydroperoxides were linked with a heightened risk of developing MCI as well as AD, while low levels of total serum antioxidant capacity were associated with the increased risk of developing MCI [44]. In a clinical study on 33 individuals with MCI, 29 patients with early signs of AD and 26 healthy age-matched subjects, it was reported that plasma values for malondialdehyde were higher in persons with MCI and early AD than values in control subjects, whereas glutathione reductase activity in erythrocytes was lower in patients with MCI and AD than in control subjects [45]. The plasma levels of several antioxidants derived from the diet (vitamins A, C, E, several carotenoids, and of protective enzymes superoxide dismutase (SOD) and glutathione peroxidase were reduced in elderly subjects with early AD or with MCI relative to control subjects [46]. These results suggest that an elevation of oxidative is already present in patients with an early stage of AD.

2.5. Mitochondrial Dysfunction

Most free radicals are generated in the mitochondria, although some are also produced in the cytoplasm by oxidases. Mitochondria may be a susceptible primary focus of neuronal oxidative stress [12]. Increased pro-oxidant status induces mitochondrial dysfunction by inhibiting the activities of respiratory complexes [47] and inducing mutations in mitochondrial DNA (mtDNA). Mitochondrial DNA is very sensitive to increased oxidative stress because it does not contain genes for any repair enzymes, and, it is not safeguarded by protective histones. Furthermore, mtDNA is very close to the region where free radicals are produced as a consequence of oxidative phosphorylation [12]. An increased presence of mutations in mtDNA has been found in brain tissues derived from AD patients post mortem [11]. Other mitochondrial defects have also been found in such brains [9, 10]. In addition to becoming less efficient in energy production, damaged mitochondria release additional reactive oxygen species (ROS) as well as caspases which contribute to neurodegeneration. Reduced energy production can increase

the susceptibility of neurons to glutamate excitotoxicity [48]. Thus, mitochondrial dysfunction may further increase the extent of oxidant events in the neurons.

3. OXIDATIVE STRESS INCREASES PRODUCTION OF BETA AMYLOID (Aß1- 42 PEPTIDES)

Increased β-amyloid (Aß1-42) plays a key role in the pathogenesis of AD [21, 22]. Increased oxidant events are one of the elements that increase the production and accumulation of β amyloids. Indeed, it has been reported that increased oxidative events can accelerate the intracellular build up of β-amyloids in neurons [49]. Membranes containing oxidized phospholipids lead to accumulation of β-amyloids faster than those containing unoxidized saturated phospholipids [50]. The rate of cleavage of APP to Aß1-42 was increased in a transgenic AD mouse model lacking cytoplasmic superoxide dismutase-1 (SOD-1) relative to control mice, implying that increased oxidative events can promote the production of Aß1-42 peptides [51]. Furthermore, 4-hydroxynonenal (HNE), a product of lipid peroxidation increased γ-secretase and Aß1-42 assembly in neurons [52]. HNE modified the γ-secretase substrate receptor, nicastrin, in neurons from patients with AD. Such modification of nicastrin heightened its binding to the γ-secretase substrate APP. The levels of HNE-nicastrin were associated with increased γ-secretase activity and Aß plaque deposition [52].

4. Aß1-42 PEPTIDES CAUSE NEURONAL DEGENERATION BY INDUCING FREE RADICALS

Since vitamin E is protective against β-amyloid-induced injury of neuronal cells in culture [53], it may be that β -amyloid-induced neurotoxicity is facilitated by free radicals [50, 51, 53, 54]. Methionine in the 35 position of the beta-amyloid peptide may be a key site relating to the generation of free radicals [55, 56]. Binding of erythrocytes with Aß peptides triggers increased formation of oxidant free radicals that could impair delivery of oxygen to the brain tissue [57]. These data suggest that Aß-induced neuronal death may be partially mediated by way of free radicals. These studies together imply that elevated pro-oxidant events are likely to participate in the progression of AD.

5. MUTATIONS IN AD SPECIFIC GENES INCREASES THE PRODUCTION OF BETA-AMYLOIDS

Mutations in specific genes are associated with familial AD. Mutations in APP, presenilin-1, presenilin-2 and γ-secretase that increase production of beta-amyloids, lead to neuronal death associated with generation of excess free radicals. Mutation of the presenilin-1 gene increased the activity of γ-secretase leading to increased production of β-amyloids [29]. Mutation of the γ-secretase gene also increases the formation of β-amyloids [27]. Such mutations may cause neuronal damage at least in part by generating excessive amounts of free radicals *via* elevation of levels of β-amyloid.

6. INCREASED OXIDATIVE DAMAGE ELEVATES INDICES OF CHRONIC INFLAMMATION IN AD

Increased oxidative damage caused by free radicals initiates prolonged inflammation in AD. The role of extended inflammation in AD pathogenesis is suggested by epidemiological studies reporting that rheumatoid arthritis patients, who were using high doses of NSAIDs, had a decreased incidence of AD [58, 59]. The products of chronic inflammation, including cytokines ([60], complement proteins [61, 62], reactive oxidant species [63-65], adhesion molecules [66, 67], and prostaglandins [68] are damaging to neurons in isolated systems. High levels of pro-inflammatory cytokines including IL-1 β and TNF-α are present in post-mortem brains of victims of AD [18]. Beta- amyloid-induced toxicity is exacerbated by pro-inflammatory cytokines IL-1β and TNF-α [15]. Interferon-γ, IL-1β and TNF-α enhance production of beta-amyloid by increasing γ-secretase activity *via* JNK-dependent mitogen-activated protein kinase [69]. Beta-amyloids and the NMDA receptor agonist, glutamate tested separately, led to neuronal damage by way of enhancing free radical production and the application of β-amyloid and glutamate together was more effective than either agent alone in producing neuronal damage. IL-6, a pro-inflammatory cytokine alone could not directly cause neuronal damage but potentiated the effects of the paired β-amyloid and NMDA [70]. Thus increased oxidative stress together with pro-inflammatory cytokines may participate synergistically in promoting neuronal death in AD. The processes underlying this are gradually becoming better understood. The pathogenesis of AD has recently been reported to involve the oligomerization of NLRP3 in microglial inflammasomes which leads to breakdown of interleukin-1β and caspase-1 precursors and thence to formation of the active cytokine and apoptic protease [71].

7. OXIDATIVE STRESS INCREASES HYPERPHOSPHORYLATED TAU (P-TAU) PROTEIN IN AD

Tau is a microtubule-binding protein found within neurofibrillary tangles (NFT). Increased levels of β-amyloid in AD precedes the development tau pathology, namely the hyperphosphorylation of tau and formation of neurofibrillary tangles (NFTs) in the frontal cortex [33]. Since hyperphosphorylation of tau was prevented by high doses of antioxidants, increased oxidative stress appears to be a factor that contributes to hyperphosphorylation of tau in transgenic AD mice (Tg2576), [32]. Proteasome inhibition can diminish the rate of breakdown of hyperphosphorylated tau proteins leading to their gradual accumulation, and thus the appearance of NFTs within cells. It has been reported that intraneuronal tau inclusions appear decades before the deposition of Aß plaques. However, in cerebrospinal fluid, altered levels of Aß peptides occur before the elevation of phosphorylated tau, which only becomes apparent in the later progression of AD [31]. In addition to hyperphosphorylation of tau, acetylation of tau is also markedly elevated at the advanced stage of the disease and may also participate in the progression of AD [72].

8. PROTEASOME INHIBITION-INDUCED NEURODEGENERATION IN AD

A role of proteasome inhibition has been proposed for the neuronal degeneration found in AD brains [22, 23]. Inhibition of proteasome activity by lactacystin causes rapid death of isolated neuronal cells [24]. An increased oxidant

milieu and defects in ubiquitin conjugation enzymes [73], are factors which can inhibit proteasome activity. Proteasomal inhibition is considered a late event participating in the progression of neurodegeneration and death of neurons in AD brain.

9. THE CONTROL OF OXIDATIVE PROCESSES BY Nrf2

Oxidative stress in the body occurs when the antioxidant system fails to provide adequate protection against damage produced by free radicals (reactive oxygen species, ROS and reactive nitrogen species, RNS). Increased oxidative stress can be moderated by up-regulating antioxidant enzymes as well as use of dietary and endogenous antioxidant chemicals. Antioxidant enzymes reduce free radicals catalytically whereas dietary and endogenous antioxidant chemicals reduce free radicals by directly scavenging them. In response to reactive oxygen species, a nuclear transcriptional factor, Nrf2 is translocated from the cytoplasm to the nucleus where it binds with the antioxidant response element (ARE) which increases the content of antioxidant enzymes (glutathione peroxidase, glutathione reductase, and heme oxygenase-1), and phase 2 detoxifying enzymes; NADPH quinone oxidoreductase 1 and glutathione-S-transferase) [74-76]. However, existing levels of endogenous antioxidant chemicals cannot be elevated in response to oxidative stress without supplementation. Antioxidant enzymes are elevated by activation of Nrf2. In addition, elevated levels of antioxidant enzymes are also dependent upon the binding ability of Nrf2 with ARE in the nucleus.

9.1. ROS-dependent Regulation of Nrf2

Normally, Nrf2 is associated with Kelch-like ECH associated protein 1 (Keap1) protein which acts as an inhibitor of Nrf2 (INrf2) [77]. INrf2 protein serves as an adaptor to link Nrf2 to the ubiquitin ligase CuI-Rbx1 complex for degradation by proteasomes and maintains the steady levels of Nrf2 in the cytoplasm. INrf2 acts as a sensor for ROS/electrophilic stress. In response to increased ROS, Nrf2 dissociates itself from the iNrf2- CuI-Rbx1 complex and moves into the nucleus and combines with ARE leading to increased expression of antioxidant genes. Nrf2 regulates the transcription of INrf2, whereas INrf2 controls Nrf2 content by modulating its degradation by proteasomes [78].

9.2. ROS-independent Regulation of Nrf2

Antioxidants such as vitamin E, genistein (a flavonoid)[79], allicin, a major organosulfur compound found in garlic [80], sulforane, a organosulfur compound, found in cruciferous vegetables [81], kavalactones (methysticin, kavain and yangonin) [82] and dietary restriction [83] can activate Nrf2 by mechanisms not involving elevation of ROS.

9.3. Reduced Binding of Nrf2 with ARE

The age-related decline in antioxidant enzymes in the liver of older rats compared to that in younger rats has been attributed to reduction in the binding ability of Nrf2 with ARE. Treatment with alpha-lipoic acid reversed this defect, increased the levels of antioxidant enzymes and restored the loss of glutathione from the liver of old rats [84].

9.4. Differential Response of Nrf2 to ROS Generated During Acute and Chronic Oxidative Stress

Nrf2 seems to respond to ROS generated during acute and chronic oxidative stress differently. For example, acute oxidative stress during strenuous exercise translocates Nrf2 from the cytoplasm to the nucleus where it binds with ARE to up-regulate antioxidant genes. However, during chronic oxidative stress commonly observed in older individuals and in Parkinson's and Alzheimer's diseases, the Nrf2/ARE pathway becomes unresponsive to ROS.

Pretreatment of rats with N-acetyl cysteine (NAC) blocked thyroxin (a ROS donor)–induced activation of Nrf2 in the liver [85, 86]. This was interpreted to mean that supplementation with individual antioxidants may impair the normal Nrf2 response to ROS in reducing oxidative stress. We interpret these results differently. In response to ROS, such as observed after treatment with thyroxin or during strenuous exercise, NAC administration may directly scavenge directly all ROS, thereby blocking ROS-induced activation of Nrf2. In this manner NAC treatment may have prevented the normal Nrf2 response to ROS.

9.5. Nrf2 Regulation of ROS-inducing Effects

Exercise-induces acute transient oxidative stress by generating excessive amounts of ROS. Indeed, in wild-type mice, exercise activated Nrf2 and thus enhanced antioxidant enzymes through ARE, and reduced oxidative stress. However, in Nrf2 knockout mice (Nrf2-/- mice), exercise failed to increase antioxidant enzymes and reduce oxidative stress [87]. This suggests that the Nrf2/ARE pathway is responsive to ROS.

9.6. Nrf2 in Alzheimer's Disease (AD)

The levels of nuclear Nrf2 decreased in hippocampal neurons in AD despite increased oxidative stress [88]. This may account for the Nrf2/ARE pathway becoming unresponsive to ROS in AD. It is not known whether the defect in Nrf2 pathway occurs at the cytoplasm where Nrf2 forms complex with INrf2 or at the level of nucleus where it binds with ARE to up-regulate antioxidant genes.

9.7. Herbal Products and Antioxidants Promoting Activation of the Nrf System

Treatment of primary culture of hippocampal neurons with puerarin, a major flavonoid from the root of *Pueraria lobata*, significantly reduced β-amyloid-induced oxidative stress by activating Nrf2-ARE pathway [89]. Genistein, a flavonoid, and vitamin E reduced oxidative damage produced by β- amyloids (Aß25-35) in transformed cerebrovascular mouse endothelial cells in culture by activating Nrf2-regulated antioxidant genes [79].

A study on the aged mouse hippocampus revealed that supplementation with allicin, a major organosulfur compound found in garlic, which has an electrophilic center (electron deficient) prevented age-related decline in cogni-

tive function. This effect of allicin was due to enhancement of antioxidant enzymes *via* Nrf2-ARE pathway [80]. This study suggests that INrf2/Nrf2 complex and binding of Nrf2 with ARE, remain responsive to allicin.

Some agents can reduce oxidative stress by activating Nrf2-regulated antioxidant genes without ROS stimulation. Examples include organosulfur compound sulforaphane found in cruciferous vegetables, kavalactones, found in Kava shrubs, and puerarin, a major flavonoid from the root of *Pueraria lobata* [89], genistein and vitamin E [80]. Repeated administration of another organosulfur compound sulforaphane, found in cruciferous vegetables, simulated Nrf2-dependent increase of Nqo1 gene which codes for NAD(P)H:quinone oxidoreductase, and Hmox1 gene which codes for HO-1 enzyme in astrocytes in culture, and reduced oxidative damage [81]. It is also possible that sulforaphane-induced activation of Nrf2 does not require ROS stimulation. Indeed, kavalactone (methysticin, kavain and yangonin)-induced activation of Nrf2 is not dependent upon ROS stimulation in neuronal or astroglial cells in culture [82].

In a study on murine alveolar cells in culture, NAC, which directly scavenges free radicals *via* increasing intracellular glutathione levels, requires the presence of Nrf2 for an optimal reduction in oxidative stress [90]. For example, cigarette smoking produces greater damage in alveolar cells obtained Nrf2-deleted mice (Nrf2-/-) than in cells obtained from wild-type mice [91]. Pre-treatment of alveolar cells with NAC, reduced cigarette smoke-induced damage more the wild-type cells more than in those from Nrf2 deleted mice. In another study on rat liver, pretreatment with NAC prevented ROS-induced activation of Nrf2 [86]. Thus NAC appeared to scavenge sufficient ROS so as to prevent activation of the Nrf2/ARE pathway.

Some agents can reduce oxidative stress directly by scavenging free radicals, and can also act indirectly by activating Nrf2/ARE pathway. These include vitamin E [78], alpha-lipoic acid [84], curcumin [92], resveratrol [93], omega-3-fatty acids [94-96], and NAC [97].

9.8. Activation of Nrf2 by Dietary Restriction

Dietary restriction also reduces oxidative stress by activating Nrf2-ARE pathways [82]. It appears that Nrf2 activation induced by dietary restriction does not require ROS stimulation. However, prolonged activation of Nrf2 by dietary restriction can produce unacceptable serious side effects [98] and thus extended dietary restriction-induced reduction in oxidative stress may be impracticable is a treatment for AD.

10. THE USE OF INDIVIDUAL ANTIOXIDANTS IN AD TREATMENT

Research on animal and cells culture models of AD using single endogenous antioxidants and herbal products, has consistently shown protection of neurons against damage produced by oxidative stress. Individual antioxidants include alpha-lipoic acid which produced a beneficial effect [99] in improving cognitive function in animal models of AD.

Treatment with coenzyme Q10 reduced markers of oxidative stress in animal AD models [100, 101]. Vitamin A, vitamin E, β-carotene and pycnogenol when used individually inhibit biochemical aggregations *in vitro*, such as the creation of β-amyloid fibrils, and also promote degradation of existing β-amyloid fibrils [102]. In addition, these agents were able to decrease triggering of immune responses in of microglial and astrocytic cells, and to reduce onset of neuronal degeneration. In a mouse mutant modeling AD, such agents were able to improve spatial learning and memory [103]. Vitamin E treatment shielded synaptosomal membranes and hippocampal neurons against β-amyloid-induced toxicity [56]. Supplementation with vitamin E in the diet prevented Aß25-35 oligomer-induced memory deficits and reduced evidence of oxidative damage in the brain [104].

Human studies using individual dietary or endogenous antioxidants or herbal products have produced inconsistent results varying from no effect to some transient beneficial effects. Recent reports suggests that vitamin E in isolation might not be of utility in the prevention or improved management of AD [35, 105]. In contrast a clinical trial with *dl*-α- tocopherol (synthetic form; 2,000 IU/day) patients at an early stage of AD showing only moderate cognitive impairment, revealed a slowing of the rate of intellectual deterioration [36]. In a study of 43 patients with mild to moderate AD, the addition of alpha-lipoic acid to the treatment protocol reduced the rate of progression of the disease during a 2 year follow up period [105]. The maximum benefit was found in those patients where alpha-lipoic acid was introduced at a relatively early stage of AD. However, in another study, supplementation with vitamin C or vitamin E had no effect on the incidence of AD or dementia [106]. In a prospective cohort study conducted over 5.5 years supplementation with vitamin E and vitamin C individually or together did not reduce the incidence of AD or general dementia [107]. A further study involving elderly patients with dementia, treatment with vitamins C and E together was associated with reduced prevalence and incidence of AD [108]. Overall, such reports suggest that treatment with one or two dietary antioxidants-alone is not efficacious in reliably reducing the risk of AD in humans.

Several antioxidant vitamins, when in the oxidized form, can act as pro-oxidants [109,110]. The fact that many chronic diseases including AD are associated with excess levels of damage due to oxidant free radicals suggests that individual antioxidants may be subject to such damage and can then further contribute to a pro-oxidant milieu. Thus they may not produce beneficial clinical outcomes but rather may actually increase the risk of chronic diseases after long-term consumption. Supplementation with single antioxidants during training sprint training exercise reduced some of the beneficial effects of sprint training [111]. This could also be due to the oxidized forms of these agents acting as pro-oxidants that are toxic. B-carotene has been found to actually promote the onset of lung cancer in heavy smokers [112]. Thus high levels of antioxidant vitamins can potentially act in a pro-oxidant and thus a pro-inflammatory manner [113].

11. THE USE OF MULTIPLE MICRONUTRIENTS FOR TREATMENT OF AD

Most testing of the value of a therapeutic agent involves the use of that agent compared to an appropriate inert con-

trol. In a mechanistic study, whether clinical or animal-based, it is important to limit the number of variables in order to make precise inferences. This is critical in the development of new pharmaceutical agents. However, obviously nutritional as opposed to pharmacological studies may require the simultaneous testing of more than one substance, as the whole diet has to be considered as part of the study. The interaction of vitamins C and E in maintaining an effective redox balance in both lipid and aqueous cellular compartments illustrates this. The apparent harmfulness for heavy smokers, of β-carotene in the absence of vitamin C or other water-soluble antioxidant described above may be due to an imbalance between lipid and aqueous compartments. The alleviation of AD may be considered as a multi-factorial problem relating to overall cellular nutritional status rather than as a challenge to be solved by single point pharmacological intervention. The length of duration of this disease makes it resemble normal aging more closely, than it resembles an acute neurological event such as stroke. Thus, the simultaneous application of several agents designed to protect to redox wellbeing of the cell constitutes a valid approach. Obviously this is not the best means of dissecting out the molecular mechanisms underlying the progression of AD, but sometimes the design of optimal treatment must diverge from attempting to identify single key events contributing to the manner in which late stage AD presents. This article is based on understanding the need for such a divergence in creating a strategy for the prevention of, and intervention in the progression of AD.

12. INDIVIDUAL AGENTS CONSIDERED FOR INCLUSION IN A MULTIPLE MICRONUTRIENT SCHEDULE

12.1 Vitamin A and β-carotene

Beta-carotene is more potent in the quenching of oxygen radicals than are most other antioxidants [114]. It has specific biological functions that cannot be sustained by its metabolite vitamin A, and vice versa [115, 116]. For example, β-carotene augments the expression of the connexin gene coding for a gap junction protein in mammalian fibroblasts, but vitamin A cannot act as a substitute in producing this effect. On the other hand, vitamin A can induce differentiation in some types of normal cells and cancer cells, while β-carotene is not able to induce such differentiation [117, 118]. Thus β-carotene and vitamin A have different biological functions in the body.

12.2. Vitamins E and C

The gradient of oxygen pressure varies within cells. Some antioxidants, such as vitamin E, are more effective as quenchers of free radicals in conditions of reduced oxygen pressure, whereas β-carotene and vitamin A are more effective at higher oxygen levels pressures [119]. Vitamin C is necessary to protect cellular components in aqueous environments, whereas carotenoids and vitamins A and E protect cellular components in lipid environments. 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 [120].

The chemical type of vitamin E administered is also important. d-α-tocopheryl succinate (vitamin E succinate) appears to be the most effective derivative both in intact animals and in isolated systems [121]. This salt is more soluble and thus more amphiphilic than α-tocopherol and can cross the blood brain barrier and enter cells more readily.

12.3. N-acetyl Cysteine and Alpha-lipoic Acid

An endogenous antioxidant, glutathione, is effective in neutralizing the oxidant potential of H_2O_2. However, dietary glutathione does not significantly increase plasma levels of glutathione in human subjects, because this tripeptide is almost completely hydrolyzed in the G.I. tract. N-acetyl cysteine and alpha-lipoic acid can be absorbed across the intestinal wall and increase the cellular levels of glutathione [37].

12.4. Coenzyme Q

Since mitochondrial dysfunction is associated with AD and since coenzyme Q_{10} is needed for the generation of ATP by mitochondria, it may be helpful to add this agent to any multiple micronutrient preparation designed for AD treatment. Coenzyme Q_{10} scavenges peroxy radicals faster than α-tocopherol, and like vitamin C, can regenerate vitamin E in a redox cycle [120] Coenzyme Q_{10} can improve symptoms in patients with mitochondrial encephalomyopathies [122].

12.5. B- vitamins

Supplementation with vitamin B-12, folic acid and vitamin B6 when used individually or in combination produced no beneficial effect on cognitive function in individuals at relatively early stages of AD [123, 124]. However, supplementation with folate improved the effectiveness of a cholinesterase inhibitor in the treatment of AD [123]. Nicotinamide (vitamin B3) reduced oxidative stress-induced mitochondrial dysfunction and restored defective autophagy function in neurons in culture. Treatment of transgenic AD model mice (3xTg) with nicotinamide improved cognitive function and reduced the content of Aß and hyperphosphorylated tau and their associated neurodegeneration [124]. Nicotinamide a component of NAD, also attenuated glutamate-induced toxicity and sustained levels of NAD+ to maintain the activity of SIRT-1 [125] a regulator of mitochondrial biogenesis [126]. These studies suggest nicotinamide supplementation, is worthy of considering in a multi-agent approach to AD treatment. The addition of the remaining B-vitamins may also be useful because of their requirements for normal brain metabolism.

12.6. Selenium

Selenium is a component of glutathione peroxidase, which acts as an antioxidant by increasing ~~the~~ intracellular levels of glutathione. Selenium deficiency has been associated with elevated amyloid-β plaque deposition in Tg2576 transgenic mice [127]. Selenium may thus also be used in a multi-ingredient nutrient complex.

12.7. Curcumin

Curcumin is a natural yellow pigment of turmeric, which is widely used as a spice throughout Indian sub-

continent. It has antioxidant and anti-inflammatory activities. Curcumin inhibits aggregation of Aß peptides *in vitro*. In animal models of AD, it reduced aggregation of Aß peptides and tau phosphorylation. Curcumin prevented aluminum-induced aggregation of Aß peptides and its toxicity on isolated rat neuronal cells [128]. Preparation of curcumin-liposome was very effective in reducing the aggregation of Aß peptides and oligomeric Aß [129]. Curcumin treatment also inhibited deposition of β-amyloid fibrils and solubilized β-amyloid aggregates *in vitro* [100]. However, two clinical studies with curcumin performed in the USA and China revealed no beneficial effects on cognitive function in AD patients compared to those AD patients who received placebo [130].

12.8. Resveratrol

Treatment with resveratrol, a major polyphenolic constituent of red wine, diminished neuronal degeneration in an animal model of AD [131, 132] by reducing oxidative stress [133] and chronic inflammation [134], and increasing the proteasome degradation of β -amyloid [135]. Resveratrol can cross blood brain barrier in mice rats and gerbils [136-138]. Several epidemiological studies imply that the judicious consumption of red wine is associated with a reduced incidence of AD and dementia [139, 140]. In elderly individuals lacking the APOE epsilon-4-allele, consumption of three daily glasses of wine was accompanied by a lower risk of AD [141].

12.9. Carnitine

This chemical may reduce damage due to excess pro-oxidant activity by modulation of gene expression [142].

12.10. Omega-3- Fatty Acids

Supplementation with omega-3 fatty acids (1.7 g of docosahexaenoic acid and 0.6 g of eicosapentaenoic acid/day) in patients with relatively early AD did not delay the rate of cognitive decline but minimally beneficial changes were apparent in a small sub-group of patients with very early signs of AD [143]. While omega-3 fatty acids may slow down the rate cognitive decline in the elderly without dementia, it may be ineffective in reducing the incidence of AD or dementia [144]. In a Canadian study there was no association between omega-3 fatty acid intake and the risk of dementia [145]. In a randomized, double-blind, placebo-controlled trial of individuals with mild cognitive impairment, supplementation with omega-3 fatty acids showed significant improvement in Alzheimer's Disease Assessment scale compared to placebo control. However, there was no improvement noted in patients with fully developed AD [146]. The use of omega-3-fatty acids should be considered due to the finding that this class of compound reduces the appearance of AD-like pathology in the brains of AD animal models [147], and some clinical studies show benefits in patients in the elderly and those with very early signs of AD [146]. Such upstream intervention as that proposed here is likely to prove most fruitful in early prevention or delay of the onset of AD.

12.11. Non-steroidal Anti-inflammatory Drugs (NSAIDS)

Since increased chronic inflammatory processes have been proposed as a significant factor in the initiation and promotion of neurodegeneration, the use of NSAIDs in the treatment of AD may be beneficial. Many products of inflammatory reactions are neurotoxic. These include prostaglandins and cytokines [60, 68, 148], complement proteins [61, 62], adhesion molecules [67, 149] and free radicals [65, 150]. Rheumatoid arthritis patients, who habitually use high doses of non-steroidal anti-inflammatory drugs (NSAIDs), have been found to have a reduced incidence of AD [59]. Use of NSAIDs was associated with lower risk of AD and other types of dementia [151]. NSAIDs also reduce the rate of deterioration of cognitive functions in AD patients [14, 17]. However, use of prednisone, a potent anti-inflammatory agent, was not found beneficial in patients with AD [152].

Treatment with a Cox-1/Cox-2 inhibitor together with a PGE2 analog did not produce any benefit on cognitive function [153], neither was a specific inhibitor of Cox-2 of utility [154]. Thus Cox-2 enzyme may not be the appropriate target for AD treatment [155]. However, treatment with a selective Cox-1 inhibitor, SC-560, improved spatial learning and memory, and decreased Aß deposition and the extent of tau hyperphosphorylation in aged triple transgenic AD mice (3xTg). In addition, such treatment reduced glial activation and markers of chronic inflammation [89]. Administration of indomethacin-loaded lipid-core nanocapsules blocked Aß1-42- induced inflammation and suppressed glia and microglia activation [156]. Exposure of human neuronal cells to both vitamin C and aspirin inhibited inflammatory responses more than achieved by aspirin alone [157].

Several studies with non-steroidal anti-inflammatory drugs (NSAID), such as aspirin and ibuprofen reported beneficial effects in patients with idiopathic AD [14, 16, 59]. A more recent study with NSAID on patients with familial AD found no beneficial effects [158]. In familial AD, neurodegeneration may be due to excessive production of free radicals consequent to deposition of β -amyloid, rather than due to pro-inflammatory cytokines. Thus, the effects of prolonged inflammation may not be a major factor in patients with the familial form of AD.

The potential value of non-steroidal anti-inflammatory agents is also buttressed by the additional evidence: (a) the brains of non-demented elderly people taking NSAIDs have less activated microglia than in their peers taking no NSAIDS, implying lessened reduced anti-inflammatory activity [159]; (b) In a transgenic mouse model of AD, extended administration of ibuprofen reduced dystrophic neurite formation and β -amyloid deposition [160]. Thus, the use of NSAIDs in combination with anti-oxidants is a potential strategy for reducing the progression of AD.

12.12. Statins

Statins are commonly used in the prevention and treatment of heart disease. Treatment with statins atorvastatin and pitavastatin reduced senile plaques and inflammation marker TNF-α in transgenic AD model (App-Tg) mice, the mechanism apparently involving reduced oxidative stress and im-

proved insulin signaling pathways [161]. Once again, while this class of compound can clearly reduce plaque deposition and inflammatory responses in a genetic murine model of AD, the efficacy of these statins in human AD clinical trials, has yielded equivocal results [36, 162]. Statins are currently not recommended for those who are not at risk of developing heart disease.

12.14. Ginkgo Biloba

In an AD transgenic mouse model, long-term consumption of *Ginkgo biloba* extract through diet lowered levels of human APP levels by 50% compared to controls in much of the cortex but not in the hippocampal region [163]. However, in a double-blind, placebo-controlled clinical trial in those aged 75 years or more with normal cognition, administration of *Ginkgo biloba* was not effective in decreasing the rate of appearance of AD or overall dementia [164].

13. CAN ONSET OF FAMILIAL AD BE DELAYED?

It is often believed that the familial form of AD cannot be prevented or delayed by any pharmacological and/or physiological means. The gene HOP (TUM-1) is essential for the development of *Drosophila melanogaster* (fruit fly). A mutation in this gene markedly increases the risk of developing a leukemia-like tumor in female flies. Whole-body irradiation of these flies with proton radiation dramatically increased the incidence of cancer compared to that in un-irradiated flies [37]. Treatment with a mixture of antioxidants prior to and following irradiation totally prevented the radiation-induced cancer in fruit flies. This finding is of particular interest, because it is a demonstration of how disease with a strong genetic basis can be prevented by antioxidant treatment. It is unknown whether daily supplementation with antioxidants in children of parents who had heritable mutations that increases the risk of AD can prevent or suspend the onset of this disease.

14. EVALUATION OF MULTIPLE MICRONUTRIENTS AND PHENOLIC COMPOUNDS IN PATIENTS WITH EARLY PHASE AD

A clinical study with the listed micronutrients and phenolic compounds in combination with a low-dose aspirin may find use in patients with early phase AD in order to determine whether supplementation with micronutrients can reduce the progression of the disease. Low-dose aspirin may be of value because of its anti-inflammatory effect. In combination with vitamin E, aspirin produces a synergistic effect in reducing cyclooxygenase activity [165]. The combination of two agents may be more effective in reducing the levels of chronic inflammation. Use of vitamin E and vitamin C together with NSAIDs led to a reduction in the rate of cognitive decline in elderly individuals possessing an APOE-epsilon-4-allele [144].

15. THE USE OF MICRONUTRIENTS IN COMBINATION WITH STANDARD THERAPY IN PATIENTS WITH AD

Current treatments of AD are unsatisfactory, because they are based on the symptoms of the disease rather than on the underlying causes of the disease. These treatments have failed to stop the progression of the AD. Commonly prescribed drugs are cholinesterase inhibitors such as donepezil, galantamine and rivastigmine, and an N-methyl-D-aspartate antagonist (memantine).

The gradual loss of cognitive functions in AD may be due to the progressive loss of cholinergic neurons and cholinesterase inhibitors have used in an attempt to improve cognitive function by increasing the acetylcholine levels in remaining cholinergic neurons. The efficacy of these drugs depends upon the viability of surviving cholinergic neuron and so the effectiveness of cholinesterase inhibitors in improving the cognitive function lasts as long as cholinergic neurons are viable. In clinical trials, several acetylcholinesterase inhibitors were found to have greater effectiveness than placebo in maintaining cognitive function in cases of mild to moderate AD [166, 167]. None of these therapeutic approaches directly deal with underlying oxidative or nitrosylative stress or chronic inflammation. Neurons continue to die despite these treatments, which may have rather downstream targets. The use of a multi-component preparation and a low-dose aspirin in addition to standard therapy may enhance the valuable effects of current drugs in AD patients by protecting surviving neurons from damage caused by an elevated pro-oxidant and inflammatory setting.

16. SAFETY OF POTENTIAL THERAPEUTIC AGENTS

All ingredients and their doses that would be included in the formulation are safe and come under category of "Food Supplement", and therefore, do not require FDA approval for their use. Antioxidants at doses higher than those discussed here have been used by much of the US population for decades without significant toxicity. However, a few of them can produce harmful effects at higher doses in some individuals when consumed daily over an extended period. Vitamin A consumed by pregnant women at doses of 10,000 IU or more daily, can result in birth defects, and β-carotene at doses 50 mg or more can led to a reversible discoloration of the skin. Vitamin C at doses of over 10 grams or more daily, can lead to diarrhea. Vitamin E at doses of 2,000 IU or more daily for an extended period can result in defects in blood clotting. Vitamin B6 at 50 mg or more per day, can produce peripheral neuropathy, and 400 mcg or more per day of selenium can cause skin and liver toxicity after prolonged consumption. Coenzyme Q10 has no reported toxicity, and daily doses suggested are 30-400 mg. N-acetylcysteine doses of 250-1500 mg and alpha-lipoic acid doses of 600 mg are regularly used with no reported adverse effects. The potential interactions that may take place within a mixture of agents are not easily predicted but are more likely to be in a positive synergistic direction. This is illustrated by the multi-factorial "Mediterranean diet" which is rich in the antioxidants Vitamins C and E, polyunsaturated fatty acids and polyphenolic compounds, and is of value in reduction of rates of dementia [168].

CONCLUSION

The studies discussed in this review suggest that a prolonged period of excess pro-oxidant conditions may pre-

cede the overt development and progression of AD. Other events such as increased levels of chronic inflammation, generation of Aß1-42 peptides from APP, aggregation of Aß peptides, hyperphosphorylation and acetylation of tau, inhibition of proteasome and formation of extracellular senile plaques and intracellular NFT may occur subsequent to increased free radical activity. Increased oxidative events are likely to play a significant role in the onset and progression of AD-related damage. Therefore, inhibition of oxidative stress may reduce the risk of developing of AD, and together with conventional therapy, may improve the management of this disease. Because of the complexity of regulation of oxidative events in humans, it is probably not possible to reduce oxidative stress optimally by the use of a single antioxidant. Simplified diagrammatic representation of the various potential pathways of oxidative stress in causing neuronal death in during the pathogenesis of AD is shown in (Fig. **1**).

At present, there are no effective strategies to reduce the incidence of AD. It is suggested that a combination of agents that can activate Nrf2-ARE pathway by ROS-independent mechanisms and dietary and endogenous antioxidant chemicals that directly scavenge free radicals, may be necessary to modulate oxidative stress optimally in AD. Dietary and endogenous antioxidants, curcumin, together with resveratrol and omega-3-fatty acids may fulfill above requirements for reducing oxidative stress optimally. Thus, a preparation of the antioxidants described together with a low dose non-steroidal anti-inflammatory drugs (NSAIDs), may be of value in reducing the probability of dementia in high risk populations (those with a family history of AD or individuals 70 years or older). All the preventive agents described here are of low toxicity, which would allow their prolonged safe usage even among high-risk populations not expressing any symptoms of AD. Animal studies modeling AD often involve a defect at a single locus or a very limited number of loci. Since the human disease appears to involve layer upon layer of progressively broadening deficits, this is a serious limitation of animal and cell model of AD and probably accounts for the failure to make a successful therapeutic transition from the laboratory to humans. It is disappointing that while many trials on cellular or animal models of AD have yielded positive results, parallel human studies have been much less successful. It is thus necessary to consider a more poly-modal therapeutic approach. Since this gap cannot be currently bridged the need for more clinical trials is evident. While this review confines itself to describing some agents that may be of value in combinatorial trials, it does not attempt to precisely define the composition of an ideal combination. Those interested in the multiple micronutrient tactic in the prevention or improved control of AD may wish to implement these recommendations after appropriate consultation with physicians and other health professionals.

CONFLICT OF INTEREST

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

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PATIENT'S CONSENT

Declared None.

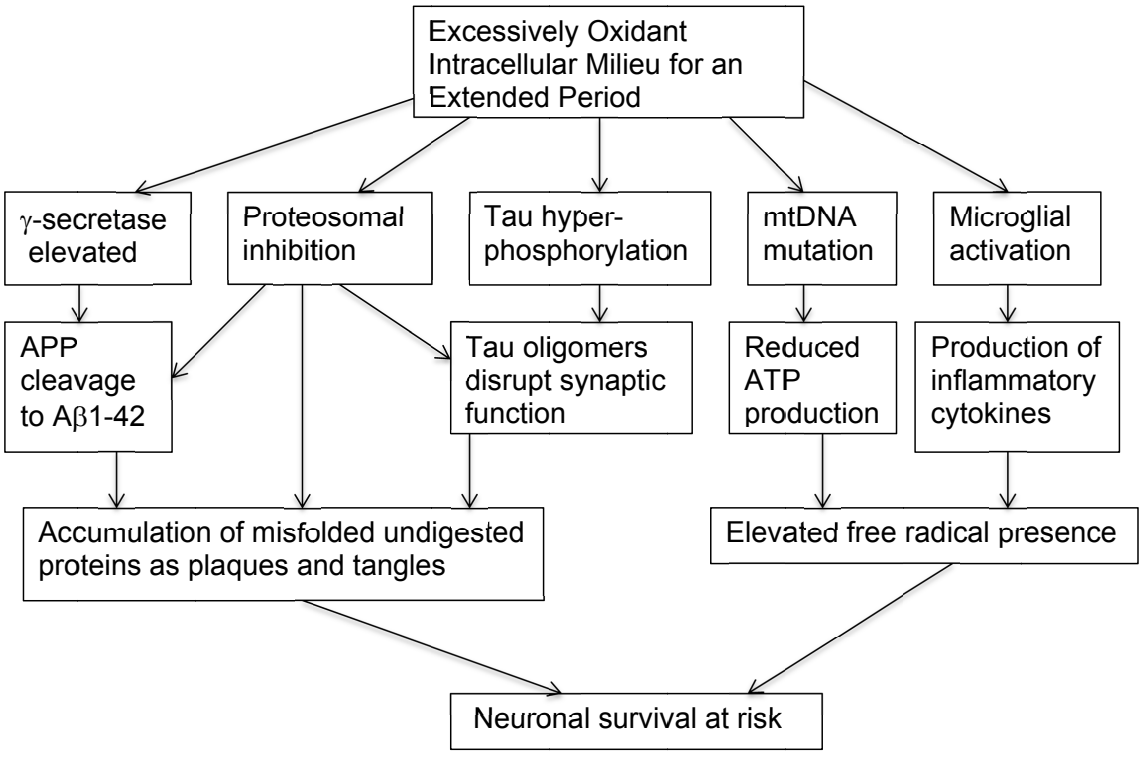

Fig. (1). Postulated relation between oxidative stress and neuronal death.

LIST OF ABBREVIATIONS

AD	=	Alzheimer's disease
APP	=	Amyloid precursor protein
MCI	=	Mild cognitive impairment
mtDNA	=	Mitochondrial DNA
NFT	=	Neurofibrillary tangles
NMDA	=	N-methyl-D-aspartate
NSAID	=	Non-steroidal anti-inflammatory drugs
ROS	=	Reactive oxygen species
SOD	=	Superoxide dismutase

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