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Oxidative stress in Alzheimer's disease

by

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Abbreviations:
8 OHdG, 8-hydroxy-2'-deoxyguanosine
Aβ, β-amyloid peptide
AD, Alzheimer's disease
AGEs, advanced glycation end products
APP, amyloid precursor proteins,
BPN, N-*t-butyl*-phenylnitrone
CK, cretine kinase
CNS, central nervous system
CPA, cis-parinaric acid
ELISA, enzyme-linked immunosorbent assay
EPR, electron paramagnetic resonance
GS, glutathione syethetase
LDH, lactic dehyrogenase
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide
NFTs, neurofibrillary tangles
PBN, *N-Tert*-butyl-α-phenylnitrone
ROS, reactive oxygen species
TdT, terminal deoxynucleotidyl transferase

Outline

1. Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with a deadly outcome. AD is the leading cause of senile dementia and although the pathogenesis of this disorder is not known, various hypotheses have been developed based on experimental data accumulated. Most approaches to explain the pathogenesis of AD focus on its two histo-pathological hallmarks, the β amyloid peptide- (Aβ-) loaded senile plaques and the neuro-fibrillary tangles (NFTs), which consist of the filament protein tau. Various evidence support a central role of Aβ in the pathogenesis of AD and an increasing number of studies show that Aβ may be one molecular link between oxidative stress and AD-associated neuronal cell death. Aβ itself can be neurotoxic and can induce oxidative stress in cultivated neurons. Consequently, antioxidant approaches for the prevention and therapy of AD are of central interest. Experimental as well as clinical data show that lipophilic antioxidants such as vitamin E are neuroprotective and may help patients suffering from AD.

This paper will focus on Aβ and its induced oxidative stress in pathogenesis of AD and the antioxidant therapy.

2. Introduction

Alzheimer's disease (AD) is a common, complex and challenging neurodegenerative disease. It is estimated to affect approximately 15 million people worldwide, and the incidence increases from 0.5% per year at age 65 years to 8% per year at age 85 years [1,2]. As more people live to old age, AD is becoming a greater medical and social problem. AD is characterized by progressive decline in memory, language and other cognitive functions accompanied by concomitant behavioral, emotional, interpersonal and social deterioration. It is the most common form of dementia and may coexist with other causes of cognitive decline, particularly vascular dementia [3].

The primary cause of AD remains unknown. Despite this, there have been significant advances in the understanding of the disorder, as a result of an enormous amount of basic science, animal and human neuropathological, and applied clinical research. The neuropathological hallmarks of AD are the accumulation of extracellular amyloid plaque containing β-amyloid and intracellular neurofibrillary tangles containing polymerized and hyperphosphorylated tau protein [4]. It is likely that, from a molecular perspective, AD is more than one disorder, and several genetic associations have been identified (Table 1) [5]. The initiating event or events leading to AD are unknown.

Table 1. Known genetic associations with Alzheimer's disease*

Gene	Chromosome	Typical age of onset (years)
Presenilin 2	1	30-40
Presenilin 1	14	50-65
Apolipoprotein E	19	Over 60
Amyloid precursor protein	21	40-50

* Adapted from [5].

Its pathophysiology is complex and likely involves multiple over-lapping and perhaps redundant pathways of neuronal damage, and one of the pathways of neuronal damage and death in AD is mediated by free radical injury.

Free radicals are formed during normal metabolism, and free radical injury occurs within living cells when the generation of reactive oxygen species (ROS) exceeds intrinsic antioxidant ability, which is referred to as oxidative stress. In recent years, much attention has focused on free radical injury as a fundamental pathophysiologic mediator of tissue injury in human disease. This article will focus on AD and discuss in detail the evidence for the role of oxidative stress as an initiating or propagating pathway in disease

pathophysiology, and also the possible role of neuroproptection with putative antioxidant compounds in AD.

3. Pathology of AD

Senile dementia of the Alzheimer's type is the major cause of dementia. It can be categorized into two groups: 1) late onset, after about age 60, 90-95% of cases, largely non-familial, i.e., sporadic, and 2) early onset, before about age 60, 5-10% of cases, almost all are familial. The pathologic lesions in both early and late onset are the same and have the same distribution pattern in the brain [6].

Two of the pathological features of Alzheimer-type dementia, first reported by the German psychiatrist Alois Alzheimer in 1906, are the presence of neurofibrillary tangles and senile plaques in the brain (Figure 1).

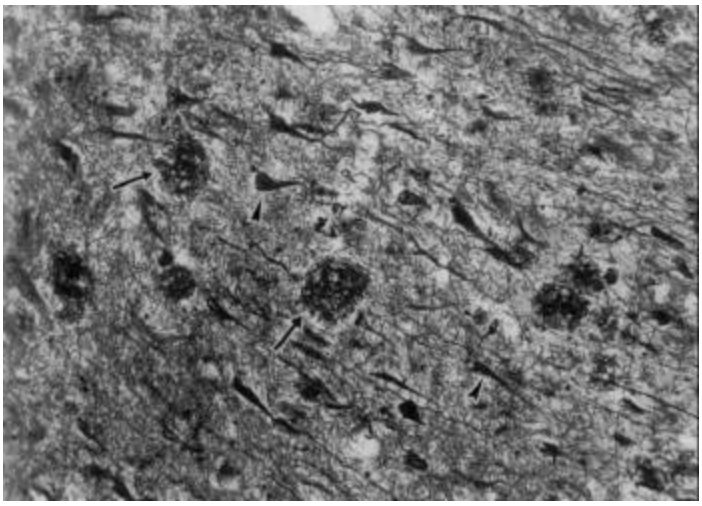

Figure 1. Section of brain from a patient with AD. Senile plaques (arrows) and NFTs (arrowheads) are shown. Holmes staining (silver impregnation) x 250. Senile plaques are round or oval, with diameters ranging from 1.5 to 20 nm: this is the "classical plaque" of AD. Also present are diffuse deposits of Aβ proteins, often called "diffuse" or "pre-amyloid" plaques, not accompanied by neurites or glia. Adapted from [7].

Several brain regions, especially the cortex, suffer massive neuronal loss. The tangles are fibrous masses inside affected neurons in several brain regions. They largely consist of pairs

of filaments, each about 10 nm in diameter, twisted around each other with a cross-over roughly every 80 nm (paired helical filaments). They contain the microtubule-associated protein tao (τ) phosphorylated to an abnormally high extent and with bound ubiquitin. The normal function of tau seems to be to help maintain the microtubular structure [7].

Senile plaques are extracellular localized areas of degenerating and frequently swollen axons, neuritis and glia surrounding a core of amyloid. Many of the neuritis contain the paired helical filaments described above. Plaques are most common in the amygdala, hippocampus, and neocortex, but they can also occur elsewhere. Amyloid protein is additionally deposited in blood vessel walls in the Alzheimer brain; this protein seems identical with the amyloid of plaque cores. NFTs and plaques of the Alzheimer type are also found in young adults with Down's syndrome and in cases of Guam dementia [7].

4. Oxidative stress in AD

4.1. Oxidative stress

Under normal conditions, damage by oxygen radicals is kept in check by an efficient array of antioxidant systems that display extensive redundancy (*e.g.* the simultaneous metabolism of H_2O_2 by catalase and glutathione peroxidase). However, during pathological conditions, the oxidant versus antioxidant balance is necessarily altered, either primarily or secondarily. Oxidative damage occurs when the oxidative balance is disturbed such that reactive oxygen production exceeds cellular antioxidant defenses, including increased generation of ROS, which is termed oxidative stress.

4.2. Sources of oxidative stress in AD

In AD, there are a number of contributory sources that are thought to play an important role in free radical production.

(1) Iron, in a redox-active state, is increased in NFTs as well as in Aβ deposits [8]. Iron catalyzes the formation of $^{\bullet}OH$ from H_2O_2 as well as the formation of advanced glycation end products (AGEs). Furthermore, aluminum, which also accumulates in NFT-containing neurons, stimulates iron-induced lipid peroxidation.

(2) Activated microglai, such as those that surround most senile plaques, are a source of $^{\bullet}NO$ and $O_2^{\bullet-}$ that can react to form peroxynitrite, leaving nitrotyrosine as an identifiable marker [9]

(3) Aβ, the central constituent in senile plaques in AD brain, form highly reactive free radicals that damage and eventually kill neuronal and gilia cells.

(4) AGEs in the presence of transition metals can undergo redox cycling with consequent production of reactive oxygen [10].

(5) Abnormalities in the mitochondrial genome or deficiencies in key metabolic enzyme suggest that metabolic abnormalities affecting mitochondria may be the major, and possibly initiating, source of reactive oxygen in AD [11, 12].

Additionally, AGEs, as well as Aβ, activate specific receptors, such as the receptor for AGEs (RAGE) and the class A scavenger-receptor, to increase reactive oxygen production [13].

4.3. Genetics and oxidative stress

Mutations in the human presenilin genes 1 and 2 are genetic factors linked to early onset of AD. Although their pathogenic mechanisms are not fully understood at this point, a role for oxidative stress has been suggested. Increased presenilin 2 expression increases DNA fragmentation and produces apoptotic changes, which are both important consequences of oxidative damage [14]. Apolipoprotein E is a protein that has been found to confer increased susceptibility when the ApoE4 allele is present. ApoE has been shown to be

adducted with the highly reactive lipid peroxidation product, hydroxynonenal, in AD brains and cerebrospinal fluid [15]. Furthermore, ApoE is a strong chelator of copper and iron, both of which are important redox-active transition metals. Another suggested genetic risk factor, bleomycin hydrolase genogype, is also associated with alterations in redox homeostasis [16].

5. Amyloid beta-protein toxicity and Ab-induced oxidative stress in AD

Different lines of evidence support a causative role of Aβ and APP in the pathogenesis of AD. Therefore, suggesting the deposition of Aβ as the central disease-causing and disease-promoting event (amyloid-cascade-hypothesis) (Figure 2) [17].

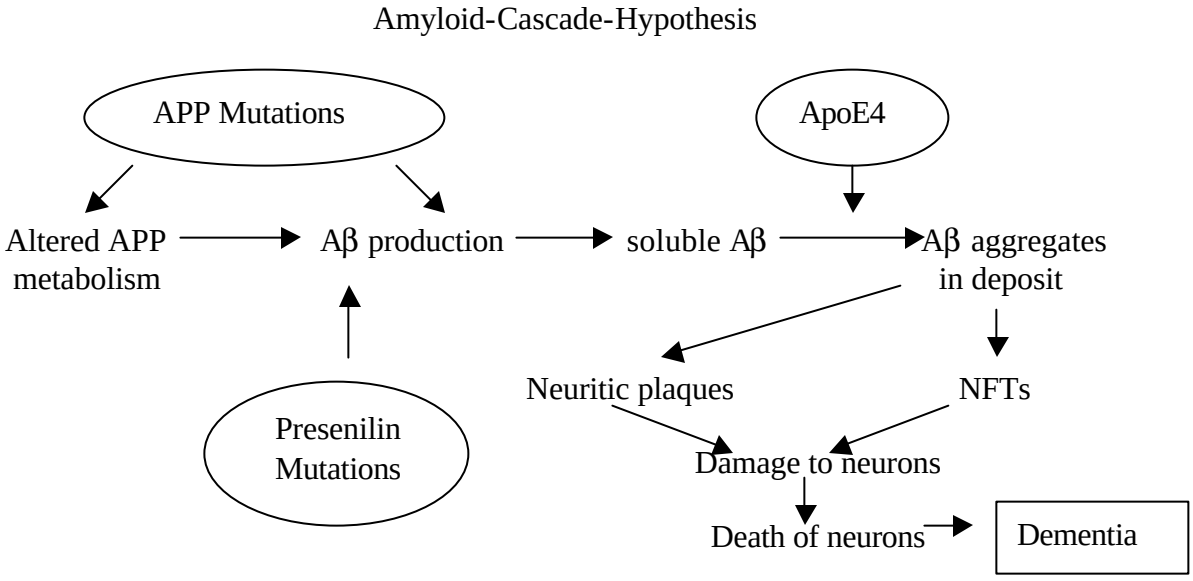

Figure 2. The amyloid-cascade-hypothesis assigns the production and deposition of Aβ a central role in AD pathogenesis. Various mutations in the APP gene lead to an overproduction of Aβ. An increase burden of potentially neurotoxic Aβ may lead to detrimental cellular changes and, ultimately neuronal cell death. Adapted from [17].

5.1. Nature of amyloid in AD

Amyloid protein deposits in AD contain truncated products (amyloid β-peptides) of a family of much larger proteins (amyloid precursor proteins, or APPs). These precursor proteins are transmembrane proteins, expressed in all cells, with a short intracellular C-terminus and a longer extracullular N-terminus. They are encoded by a gene on chromosome

21 in humans and form a set of polypeptides from 563 to 770 residues in length. The most abundant of these, APP695, is predominantly expressed in neurons. APPs are made (and secreted) by many cell types and several functions have been ascribed to them; in neurons, they may facilitate survival and growth during brain development as well as neuronal responses to excitatory neurotransmitters. The normal secretion of APP involves proteolytic processing (by α-secretase) to release the N-terminal sequence and leave behind a small fragment in the membrane. By contrast, release of β-peptide is said to involve γ- and β-secretase enzymes (Figure 3) [7].

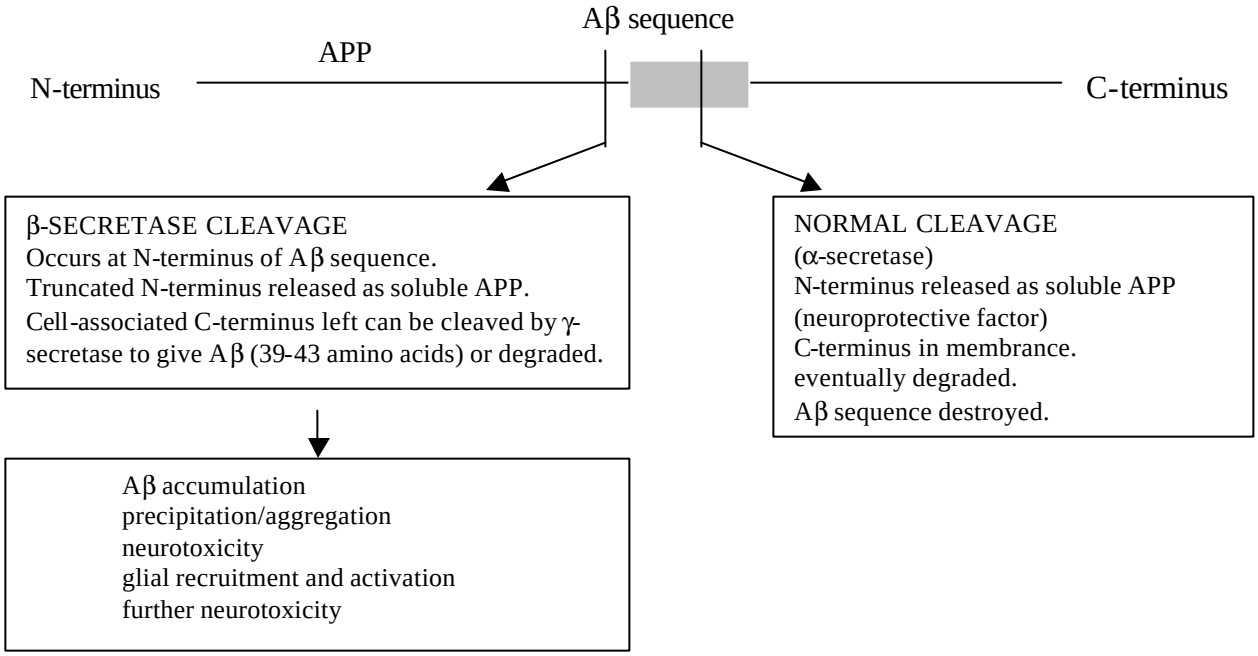

Figure 3. Processing of the APPs. The chemical identity of the secretases is as yet uncertain. APP is made in the endoplasmic reticulum and passes through the Golgi apparatus to reach the plasma membrane, undergoing glycosylation and other modifications during its passage. At the cell surface a minority of APP molecules are attacked by α-secretase to release soluble APP into the extracellular environment. Uncleared APP can be recycled by endocytosis and either completely degraded or recycled to the cell surface associated with β-secretase cleavage. Adapted from [7].

5.2 Toxicity of Aβ in AD

5.2.1 Toxicity of Aβ aggregates *in vitro*

Amyloid is a group of unrelated peptides that share the ability to aggregate as insoluble fibrils under physiological conditions. They share the common structural feature that all amyloid fibrils form non-branching filaments that are 6-10 nm wide. The proteins within the fibril structure are arranged in cross-β-pleated sheet conformation, and this fibril structure is a prerequisite for Aβ toxicity [18].

Aβs that contain the hydrophobic amino acids 29-35 form stable aggregates only very slowly and become neurotoxic only after "aging" in solution. One important exception is the 25-35 fragment of Aβ. $A\beta_{25-35}$ aggregates immediately upon dissolution and also exerts its toxicity immediately, and its toxic effect is highly reproducible [19]. Pathomechanism report by Schubert and colleagues suggested that different cytotoxic amyloid peptides share not only a common fibril structure but also a common mechanism of cytotoxicity [20].

5.2.2. Toxicity of Aβ *in vivo* and animal models of AD

To investigate Aβ's potential toxicity *in vivo,* several approaches have been selected. First of all microinjections of Aβ into adult rat brains have been performed. Although some studies demonstrated a direct neurotoxicity after Aβ injection [21, 22], other approaches did not lead to neuronal cell death or long-term effects after Aβ injections [23, 24]. This variability might be due to the physical and chemical state of Aβ or the mode of application. The aggregation state of Aβ appears to be relevant for the *in vivo* toxicity, as studies showed that Aβ injected into rat brain can be toxic after the formation of fibril deposits [25]. These injection models may induce some AD-related pathological events, but unfortunately with high variability.

5.3. Oxidative stress induced by Aβ

A potent inducer of oxidative stress is Aβ itself. Membrane damage due to exposure of neurons to Aβ was found in an electron-microscopy study, showing that high concentrations of Aβ added to rat pheochromocytoma PC12 cells or to rat primary cortical neurons can induce rapid membrane disintegration and the breakdown of membraneous structure [26]. Using both primary CNS cultures and clonal cell lines it was later shown that: (1) antioxidants and the H_2O_2-detoxifying enzyme catalase are neuroprotective against Aβ toxicity. Figure 4 shows that for each cell type catalase significantly protects cells from Aβ toxicity. The protection by catalase is dependent upon Aβ concentration, suggesting that H_2O_2 generating system is activated in an Aβ dose-dependent manner. Aβ itself does not modify catalase activity [27].

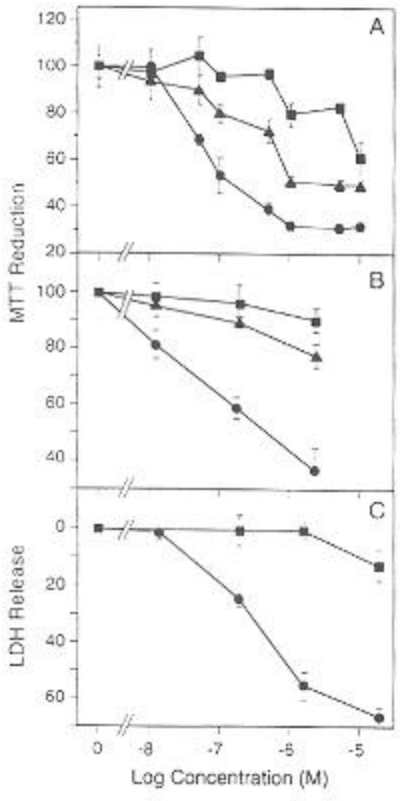

Figure 4. Catalase protects cells from Aβ toxicity. Catalase was added to culture cells at the indicated fixed concentrations; and 15 min later increasing amounts of Aβ were added. After 24 hr, cytotoxicity was monitored by the MTT assay (A and B) or LDH release (C). (A) PC 12 cells: •, $Aβ_{25-35}$ alone; ? , catalase (1 mg/ml) plus Aβ; ¦ , catalase (4 mg/ml) plus Aβ. (B) B12 cells: •, $Aβ_{25-35}$ alone; ¦ , catalase (1 mg/ml) plus Aβ; ? , catalase (500 µg/ml) plus Aβ. (C) CNS primary cultures: •, $Aβ_{1-40}$ alone; ¦ , catalase (400 µg/ml) plus Aβ. Catalase alone had no effect on cell viability in the absence of Aβ in any of the assays. Adapted from [27].

(2) An elevation of intracellular H_2O_2 correlates with Aβ neurotoxicity. Figure 5 shows that the production of peroxides is highly correlated with LDH release at several concentrations of Aβ [27].

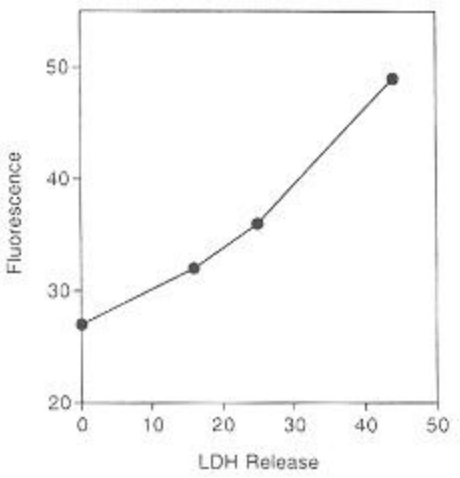

Figure 5. Aβ concentration dependence of peroxide production and toxicity. $A\beta_{25-35}$ was added to exponentially dividing suspension B12 cells at four concentration (0 M, 10^{-7} M, 10^{-6} M, 10^{-5} M; shown left to right), the relative increase in H_2O_2 generated fluorescence was determined after 24 hr, and the amount of LDH release was determined after 48 hr. The fluorescence units are arbitrary, with 28 U being the control value, and the LDH data are percent of LDH release after total cell lysis with detergent. Adapted from [27].

Aβ resistant clones of PC12 cells were selected by growth in the presence of 20 μM Aβ, a concentration that is toxic to the wild-type cell. After several months, clones of PC12 cells that are able to divide in 20 μM $A\beta_{25-35}$ were isolated (Table 2). They are all resistant to high levels of $A\beta_{25-35}$ as well as $A\beta_{1-40}$. When these cells are exposed overnight to 125 μM and 250 μM H_2O_2, they are markedly more resistant to H_2O_2 toxicity than their wild-type parental cell line (Table 2). These data support that H_2O_2 is involved in Aβ toxicity [27].

Table 2. H_2O_2 toxicity in Aβ-resistant PC12 clones *

Cell lines	$A\beta_{25-35}$ (20 μM)	$A\beta_{1-40}$ (20 μM)	H_2O_2 (125 μM)	H_2O_2 (250 μM)
Wild type	40.8 ± 0.6	51.1 ± 5.3	69.3 ± 5.7	33.9 ± 10.8
βArC11	103.5 ± 6.4	102.7 ± 5.4	97.3 ± 5.0	85.3 ± 4.5
βArC14	103.1 ± 3.7	102.1 ± 3.4	99.0 ± 5.5	84.4 ± 1.5
βArC17	101.5 ± 5.5	103.4 ± 2.3	100.9 ± 6.7	97.0 ± 1.8

* Adapted from [27].

(3) Lipid peroxidation occurs as the final step in these cellular systems. A sensitive fluorimetric assay was used that involves cis-parinaric acid (CPA), a polyunsaturated fatty acid that becomes incorporated into membranes and is subject to peroxidation. Table 3 shows that within the viable cell population there is a 15-16% decrease in CPA fluorescence between hour 1 and 2 after loading the cells with CPA. This large shift in the mean value was not observed in B12 control cultures. Aβ treatment therefore induces the decay of CPA fluorescence due to enhanced lipid peroxidation. Vitamin C and BPN reagents can suppress the decrease of CPA fluorescence [27].

Table 3. Aβ-induced lipid peroxidation *

Reagent	Concentration	Percent decrease in fluorescence of CPA	Reagent	Concentration	Percent decrease in fluorescence of CPA
Experiment 1			Experiment 2		
Control	------	4 ± 2	Control	------	1 ± 3
Aβ-alone	10 μM	15 ± 3	Aβ-alone	10 μM	16 ± 3
Vit. E alone	230 μM	2 ± 3	Vit. E alone	230 μM	0 ± 2
Vit. E plus Aβ	230 μM	1 ± 3	Vit. E plus Aβ	230 μM	0 ± 3
BPN alone	50 μM	0 ± 2	BPN alone	50 μM	0 ± 2
BPN plus Aβ	50 μM	0 ± 3	BPN plus Aβ	50 μM	0 ± 3

* Adapted from [27].

Consistent with these findings, an increased peroxidation of membrane lipids can be found in AD brains *in vivo* [28]. Therefore, it was hypothesized that Aβ-aggregates can induce the accumulation of H_2O_2 in neurons, probably caused by the induction of superoxide generating enzyme systems which are sensitive to flavin-containing oxidase inhibitors. But H_2O_2 not only can be a source for the generation of hydroxyl radicals, but also can function as an inducer of the activity of the transcription factor NF-κB, which has been postulated to

be involved in neurological disorders [29]. The model of induction of oxidative stress by aggregated Aβ is shown in Figure 6 [30].

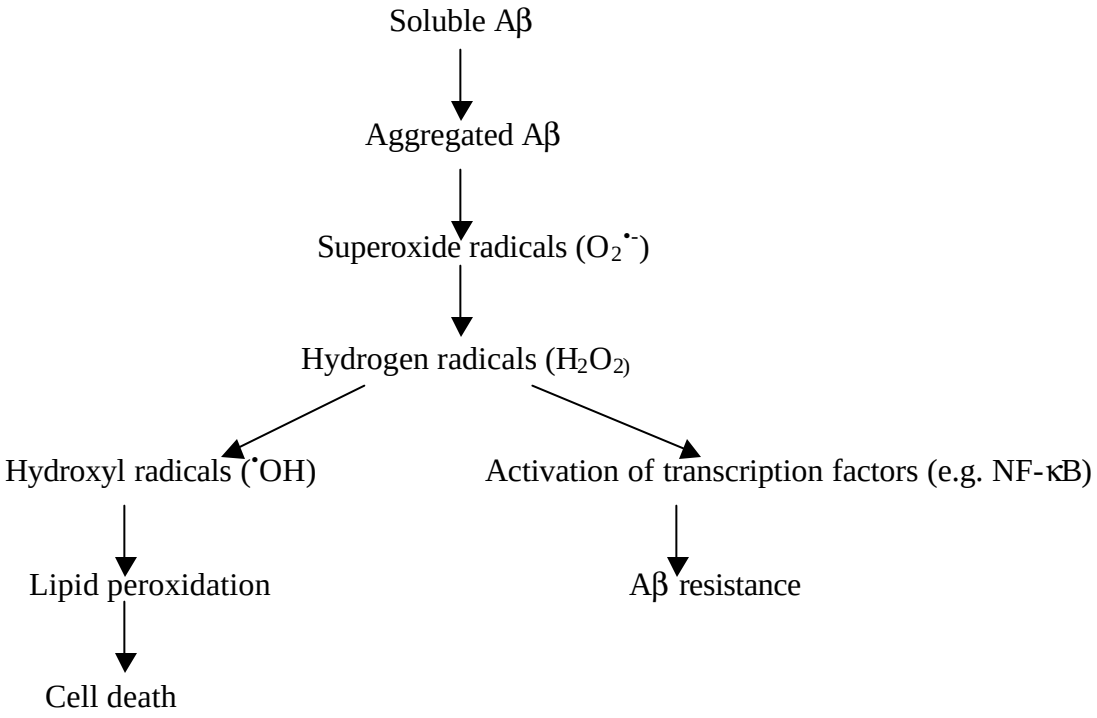

Figure 6. Model for the induction of oxidative stress by aggregated Aβ. Hydrogen peroxide as the mediator of Aβ toxicity and as an inducer of a stress response. Aggregated Aβ can cause the intracellular accumulation of hydrogen peroxide and related peroxides. This may lead to the formation of hydroxyl radicals *via* the Fenton reaction and ultimately to the peroxidaiton of membrane lipids and cell death. In addition, hydrogen peroxide can induce the activation of transcription factors such as NF-κB, which can induce cellular defense programs. This may ultimately lead to the resistance to oxidative stress. Adapted from [30].

6. Free radical injury in AD

The brain may be particularly vulnerable to oxidative damage, because it has high energy requirements and a high oxygen consumption rate. It is rich in peroxidizable fatty acids; contains high levels of transition metals, which may catalyze the formation of the reactive hydroxyl radical; and has a relative deficit of antioxidant enzymes, such as catalase, SOD and glutathione peroxidase to combat elevated levels of ROS, compared with other

organs [31]. There is a growing number of studies on oxidative injury is involved in the pathogenesis of AD. Table 4 shows the histopathologic evidence of oxidative stress in brain with AD.

Table 4. Histopathologic evidence for oxidant stress in brain with AD *

Oxidation type	Evidence
Lipid peroxidation	4-Hydroxynonenal
Protein oxidation	Protein carbonyls, Nitrotyrosine
DNA oxidation	TdT end-labeling, 8-OHdG
Glycooxidation	Advanced glycation end products

* Adapted from [5].

6.1. Lipid peroxidation

Oxidative stress to the CNS predominantly manifests as lipid peroxidation because of its high lipid content and unusually high concentration of polyunsaturated fatty acids that are particularly susceptible to oxidation. Several studies suggest that lipid peroxidation is a major cause of membrane dysfunction and subsequent cell death in AD. Postmortem studies have shown that patients who had AD had a significantly reduced amount of membrane phospholipids and cholesterol in the white matter of their brain tissue [32], hippocampus [33] and superior temporal gyrus [34]. Further studies have indicated that lipid peroxidation was localized to the cortex in AD [35]. These studies, taken together, support the role of lipid peroxidation in the pathophysiology of AD.

6.2. Protein oxidation

ROS can attack amino acid residues (particularly histidine, arginien, and lysine) to produce carbonyl ($\rangle C = O$) functions that can be measured after reaction with 2,4-dinitro-phenylhydralazine [36]. Thus, the "carbonyl assay" has become the most widely used protocol to measure protein oxidation. Smith *et al* found that brain carbonyl level were

increased with age, but no difference was observed between aged and AD brains [37]. The presence of protein carbonyls in neurofibrillary tangles, nontangle bearing neurons, and glia has been detected by using immunocytochemic techniques with *in situ* 2,4 dinitrophenyl-hydralazine labeling in AD but not in controls [38].

6.3. DNA oxidation

Free radical attack on DNA can damage all four DNA bases, and analyses of all these modified bases have been developed. One of the most studied oxidatively modified nucleoside is 8-hydroxy-2'-deoxyguanosine (8-OHdG), which derives from hydroxyl attack on deoxyguanosine. Studies showed that a two-fold higher number of DNA single strand breaks in brain nuclei samples from AD compared with controls [39]. Levels of 8-OHdG measured by HPLC have been reported to be elevated in mitochondrial DNA of patients with AD compared with controls [40].

6.4. Glyco-oxidation

Monosaccharides can induce irreversible modification of proteins by forming free radicals that will form reactive carbonyls, or by nonenzymatic glycation, *via* the Mallard reaction resulting in the formation of AGEs. Using a competitive ELISA technique, plaque fractions of brains with AD were found to contain three-fold more AGEs per milligram of protein than preparations from controls [41].

7. Antioxidant as neuroprotectants

According to the oxidative stress hypothesis of AD, numerous approaches for an effective antioxidant neuroprotection have been developed. Antioxidant therapy is discussed for AD as well as for a variety of other neurodegenerative disorders, such as Parkinson's disease and ischemia. Numerous free radical scavengers have been used in experimental

paradigms of neuronal cell death *in vitro* and *in vivo*. Clinical studies of antioxidants in AD mainly focus on vitamin E.

7.1. Preclinical studies of antioxidants in AD

Vitamin E (α-tocopherol), the pineal hormone melatonin, the lazaroids (21-amino-steroids) or mifepristone (RU486) have been tested in experiments and all of these compounds showed antioxidative potential.

Among those antioxidants, vitamin E was most widely studied by different groups with different methods. Studies *in vitro* showed that it could block hydrogen peroxide production and the resulting cytotoxicity. Vitamin E reduces Aβ induced cell death in rat hippocampal cell cultures [42] and PC 12 cells [27]. Another study showed that vitamin E inhibited Aβ induced lipid peroxidation, protein oxidation, loss of glutamine synthetase activity, and neuronal cell death [43]. Furthermore, vitamin E and other antioxidants effectively improve cognitive performance in aged animals and prevent oxidative damage in animal models of AD. Socci *et al* found that aged rats treated with antioxidants, including vitamin E, have greater memory retention than do placebo-treated rats [44]. In another study, vitamin E supplementation was shown to protect against the deterioration in passive avoidance response seen with aging in rats [45]. Vitamin E also protects against impaired water maze performance resulting from treatment with a neurotoxin (AF64A) that induces oxidative stress in cholinergic neurons [46].

7.2. Clinical studies of antioxidant in AD

A placebo-controlled, clinical trail of vitamin E in patients with moderately advanced AD was conducted by the Alzheimer's Disease Cooperative Study. Subjects in the vitamin E group were treated with 2000 IU (1342 α-tocopherol equivalents) vitamin E per day. The results indicated that vitamin E might slow functional deterioration leading to nursing home

placement. A new clinical trial is planned that will examine whether vitamin E can delay or prevent a clinical diagnosis of AD in elderly persons with mild cognitive impairment [47].

8. Proposal of further studies

8.1. Pathogenesis studies

From the review above, we can see that currently many researchers are focusing on the role of the AD–associated Aβ, and the amyloid-cascade-hypothesis suggests the deposition and aggregation of Aβ as the central disease-causing and disease-promoting event in AD, and one of the important mechanisms of Aβ toxicity in AD is Aβ produces oxidative stress. In order to prove this hypothesis further, following experiments are designed:

1. Aβ neurotoxicity test

Experiment 1. Ab toxicity in CNS cell

The sensitivity of different cell lines to Aβ was found to vary considerably. The sensitivity of various rat and human cell types of neuronal origin to the $A\beta_{25-35}$ was assessed by monitoring MTT reduction in these cells following a single application of $A\beta_{25-35}$, and it was shown that rat phaeochromocytoma PC 12 was the most sensitive cell line [48].

In this experiment, PC 12 cells are exposed to $A\beta_{25-35}$ for 20-24 hr. The toxicity of Aβ can be measured by MTT assay, which measure the ability of MTT to be reduced by electrons flowing through the mitochondrial electron transport chain and therefore reflects early redox change within the cell.

2. Oxidative stress test

(1) Direct evidence in generating of free radicals

Experiment 2. Detection of amyloid radicals and neurotoxicity

In this experiment the relationship between free-radical production by Aβ and neuronal toxicity will be examined.

The embryonic rat hippocampal cell is chosen for this experiment as shown in previous literature [48]. Cells are cultured with $A\beta_{1-40}$ for different time: 0 hr, 6 hr, 24 hr and 48 hr. Electron paramagnetic resonance (EPR) with spin-trapping *N-Tert*-butyl-α-phenylnitrone (BPN) is used to test the free radicals generated.

To examine the time course of radicalization in relation to the time course of toxicity, we will choose primary rat hippocampal cultures and treat them with $A\beta_{1-40}$. For this experiment, $A\beta_{1-40}$ is added to the cultures for 48 hr. Neuronal damage will be evaluated by morphological criteria that correlate well with vital dye staining methods [49].

(2) Detection of oxidation

Two experiments are designed to test the oxidation caused by Aβ.

Experiment 3. Detection of oxidation by fluorescence microscopy

Oxidative damage to proteins produces an increase in the carbonyl content of the protein due to oxidation of sensitive amino acids such as histidine, proline, arginine and lysine [50]. Oxidative modification of individual amino acids in proteins results in inactivation of key metabolic enzymes such as glutathione synthetase (GS) and cretine kinase (CK) [51]. It is reported that there is an increase in carbonyl content and decrease in oxidation sensitive GS and CK enzymes in AD [36]. Therefore, we can use carbonyl content as a readout for the oxidative damage to protein.

A technique that allows sensitive detection of carbonyl groups by fluorescence microscopy will be used [52]. The method utilizes a biotin-conjugated hydrazide to bind carbonyl groups followed by standard fluorescein-labeled streptavidin visualization technique.

The cell line used in this experiment is embryonic rat hippocampal cell, the same as in the experiment 2, and the cells are preincubated with $A\beta_{1\text{-}40}$ for 48 hr, 24 hr, 12 hr, 6 hr, 3 hr and 0 hr. The carbonyl content in cells will be tested by fluorescence microscopy. An increase in fluorescence (oxidation) is expected to be seen when neurons are treated with $A\beta_{1\text{-}40}$.

Experiment 4. Intracellular ROS

This experiment is designed to observe if there is an increase in intracellular ROS production induced by Aβ. The cell line used and treatment are the same as in experiment 3. ROS is detected by conversion of 2,7-dichlorofluorescin to 2,7-dichlorofluorescein (DCF).

(3) Antioxidant enzyme activity assay

Experiment 5. Antioxidant enzyme activity assay

To investigate the change of antioxidant enzymes induced by Aβ in AD, a transgenic mice model will be used.

Research showed that transgenic mice overexpressing the 695-amino acid isoform of human Alzheimer APP containing a $Lys^{670} \rightarrow Asn, Met^{671} \rightarrow$ Leu mutation had a five-fold increase in $A\beta_{1\text{-}40}$ and a 14-fold increase in $A\beta_{1\text{-}42/43}$ accompanied by the appearance of normal learning and memory deficits [53].

We will take the brains of the transgenic mice of 9-10 months of age when the damages are showed, and test the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the frontal, temporal and cerebellar cortexes of transgenic mice. We will also test the protein and gene expression of these enzymes.

8.2. Antioxidant therapy studies

Experiment 6. Effect of Vitamin E and Vitamin C as neuroprotecdant in AD

There are many antioxidant compounds and some of them have been tested as neuro-protective drugs. Vitamin E has been studied for its antioxidant function in AD not only preclinically, but also clinically as reviewed in section 7. Since vitamin C may help to recycle vitamin E, the two antioxidant vitamins may best be coadministered together. In this experiment three groups of antioxidants are tested *in vitro* and *in vivo*.: vitamin E, vitamin C and vitamin E plus vitamin C.

In the studies *in vitro* we will repeat experiments 1-4 by adding drugs to the incubated cells. In studies *in vivo*, the transgenic mice models in experiment 5 will be administrated the antioxidants. Besides testing antioxidant enzymes activity and gene expression in experiment 5, we will also carry out behavior tests, including general behavioral tests, spontaneous alternation in a Y maze, and water-maze task.

9. Summary

AD is a complex disorder of the nervous system with many clinical facets. The pathogenesis of this devastating disease is not really understood, a fact that currently prevents an effective treatment. Many researchers are now focusing on the role of the AD-associated Aβ and its induced oxidative stress, and a plausible scenario can be constructed to rationalize the role of free radicals in AD pathology. This may bring a prospect of using antioxidants as one of the major therapies in AD treatment.

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