

Role of Metals in Neuronal Apoptosis: Challenges Associated with Neurodegeneration

Bharathi*, R. Ravid and K.S. Jagannathan Rao*

*Department of Biochemistry and Nutrition, *Central Food Technological Research Institute, Mysore, India; Netherlands Brain Bank, Amsterdam, The Netherlands*

Abstract: Apoptosis is a tightly controlled process in which cell death is executed through activation of specific signalling pathways. Within cells, there are positive and negative regulatory pathways of apoptosis, hence it is targeted as 'Double-edged sword', the balance between these pathways dictates the cell fate. The past decade has seen intense focus on the mechanisms of apoptosis. Many important observations on the various signalling pathways mediating apoptotic cell death have been made and our understanding of the importance of apoptosis in both normal growth and development and pathophysiology has greatly increased. In addition, mechanisms of metal-induced toxicity continue to be of interest given the ubiquitous nature of these contaminants. The purpose of this review is to summarize our current understanding of the apoptotic pathways that are initiated by metals in Alzheimer's disease. Increased understanding of metal-induced (direct) and metal-amyloid- (indirect) linked neuronal cell death through the formation of reactive oxygen species (ROS) is critical to illuminate mechanisms of metal-induced cell death, as well as the potential role of metal speciation in neurodegeneration.

Keywords: Apoptosis, metals, amyloid- , Alzheimer's disease, oxidative stress, reactive oxygen species.

INTRODUCTION

Apoptosis is a morphologically and biochemically well characterized phenomenon to eliminate damaged cells in various situations to maintain a balance between cell proliferation and cell death [1-5]. Several mechanisms are under discussion on programmed cell death (PCD) v/s passive cell death (apoptosis v/s necrosis) and/ or autophagy [6, 7]. Normally the term apoptosis is often equated or used as a misnomer with programmed cell death (PCD), but they are not exactly same, since apoptosis is only one morphologic form of programmed cell death which is characterized by nuclear fragmentation, intranuclear clumping of chromatin with DNA fragmentation/ laddering and breaking up of the cell into membrane bound ultra-structurally well preserved intracellular organelles and lack of inflammatory reaction [1, 2, 8]. Progressive cell loss in specific neuronal population is a pathological hallmark of neurodegenerative disorders, but its mechanism still remains unresolved. It has been understood recently that any alterations in neuron survival or any event which triggers the neuronal apoptotic machinery contributes to the pathogenesis of neurodegenerative disorders. Elucidating the mechanism of neurodegeneration in AD, PD etc has led to an increased understanding of the basic mechanisms of nerve cell death. In general, cell death can follow two basic pathways. Apoptosis/ PCD is an active form of cell degeneration and is executed by enzymes like caspases. Necrosis, the other type of cell death, is frequently occurs during acute insults and is characterized by rapid cell lysis. Both the processes of cellular degeneration are frequently accompanied by an overshooting generation of free radicals and other cellular events leading to oxidative stress [10-13].

In contrast, the neurons in our brain or any other organism have a limited capacity for self-renewal, and most neurons survive for the life of the person, unless affected with any neurodegenerative disorder. The decision of a neuron to undergo apoptosis can be influenced by a wide variety of extrinsic as well as intrinsic stimuli. Different studies reported both necrotic and apoptotic mechanisms for metal-mediated and A β -mediated neurotoxicity [12, 14, 15]. In particular, oxidative mediated DNA damage, with a pattern indicative of apoptosis, was found in AD brain [16, 17], which is consistent with several lines of experimental evidences linking oxidative stress and neuronal apoptosis. Neuronal cell death in the above chronic neurodegenerative diseases assumed as a result of mutation in one or several genes. This genetic alteration in turn changes the function of the gene product in a way that will have detrimental effect on the cell [18-20]. While the various molecular players in apoptotic cell death are becoming increasingly well known, whether or not they are involved or may interact with one another in neuronal cell death cascade, in AD [21, 22]. Environmental factors like metals have also been incriminated in neurodegeneration, but the cause remains still elusive. Hence in the present review, we have tried to summarize the current understanding of the apoptotic pathways that are initiated by metals.

ROLE OF METALS IN AD

There is substantial evidence that accumulation of non-essential trace elements such as Aluminium (Al) lead (Pb), Cadmium (Cd) and Mercury (Hg) [23-26] are reported to be associated with AD, Not only non-essential but the essential, redox-active transition metals such as copper (Cu), iron (Fe) are known to be involved in AD, PD and Amyotrophic Lateral Sclerosis (ALS), if accumulated in excess [27, 28]. How

*Address correspondence to this author at the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore-570020, India; Fax: 091-821-2517233; E-mail: kjr4n@yahoo.com

these elements affect the brain is complicated due to the heterogeneity of AD. Our lab has shown that paramagnetic elemental levels are significantly altered in hippocampus and frontal cortex of AD brain and also in cerebrospinal fluid (CSF) [29-31]. The distribution of major divalent metals in normal and AD brain hippocampus and frontal cortex [30, 31] has been represented in (Fig. 1). The elemental concentration of normal CSF was compared with that of AD CSF as shown in (Table 1). Accumulation of these redox-active metals leads to the overload of reactive oxygen species (ROS) by these metals, in turn leading to "Oxidative Imbalance", a major culprit in AD [32]. If there is any perturbation in the metal homeostasis, it leads to the abnormalities like cellular dysfunction/cell death.

Redox-Imbalance-A Gaze

Several lines of evidence implicate that redox imbalance attributed by overproduction of reactive oxygen species (ROS) or reactive nitrogen species (RNS) that overwhelm the protective defense mechanism of cell. Factors such as transition metals under certain circumstances contribute to the formation of oxygen species such as hydrogen peroxide (H₂O₂), superoxide radicals and hydroxyl radicals and react with reactive nitrogen species such as NO or peroxynitrite and in turn leads to neuronal cell death.

Formation of Hydrogen Peroxide/ Hydroxyl Radical by Metals

In reaction [1], the superoxide radical is converted to hydrogen peroxide by dismutation of O₂^{•-} catalyzed by superoxide

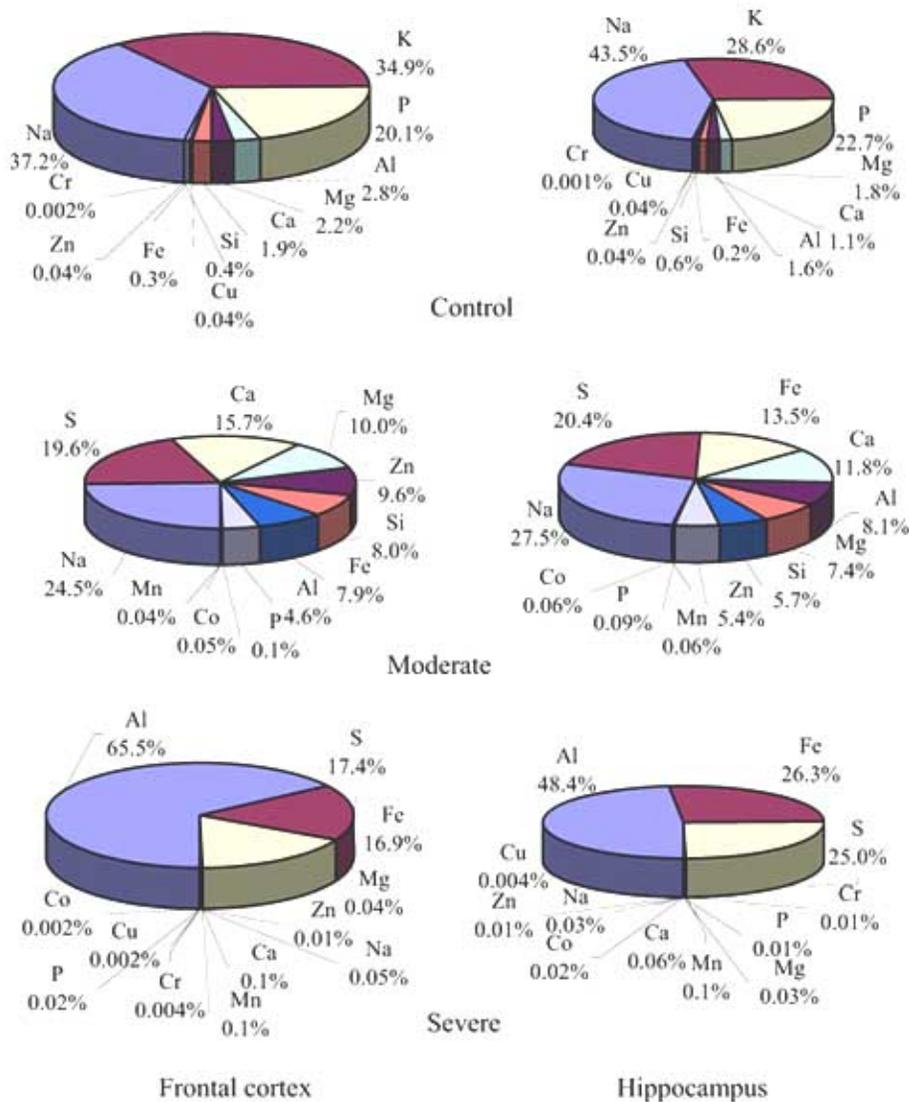
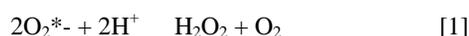


Fig. (1). Comparison of relative mole % of trace elements in frontal and hippocampal regions of normal and AD human brain. Fe and S are elevated more in moderately affected AD, while Fe and Al are co-elevated in severely affected AD. Figure with permission- Reference [31].

Table 1. Trace Elemental Distribution in Control and AD CSF [With Permission, Reference 29]

Elements	Conc. in micromole/ml (Average)	
	Control (n=6)	AD (n=6)
Ca	1.9	2.05
Al	1.42	1.46
Mg	1.15	1.13
Zn	0.014	0.01
Fe	0.012	0.006
Cu	0.003	0.001

dismutase (SOD), which is in varying concentration in neural cells. Thus the conversion removes $O_2^{\bullet-}$ and prevents its direct toxic action as well as its interaction with metal ions to increase the production of hydroxyl radical, a most potent reactive metabolite produced in brain.



In reaction [2] H_2O_2 is directly reduced by O_2 to produce OH^- , O_2 and OH^{\bullet} .



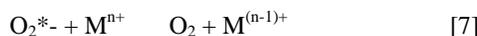
Hydrogen peroxide decompose into the highly reactive hydroxyl radical (OH^{\bullet}) and the hydroxyl ion (OH^-), according to equation [3] and [4].



The formation of OH^{\bullet} thus requires reduced form of metal ions such as Fe^{2+} and Cu^+ . So that superoxide radical $O_2^{\bullet-}$ can give rise to Fe^{3+} and Cu^{2+} as revealed in equation [5] and [6] respectively



Metal ions generally depicted as M^{n+} accelerate reaction [2] in two intermediate steps, [7] and [8].



In reaction [9] $O_2^{\bullet-}$ reduces Fe^{3+} and Cu^{2+} and generates singlet oxygen. The reduced form of the metal then reacts with H_2O_2 to produce oxidized form of metal, the hydroxide ion, and the hydroxyl radical.



Later the H_2O_2 formed is decomposed by catalase, reaction [10], the levels of catalase are high in hypothalamus and the substantia nigra compared to other brain regions (levels/conc). But catalase and its expression seems to decrease with age



Apart from the above reactions H_2O_2 is also formed during the deamination of monoamines reaction [11] catalyzed by monoamine oxidase, an enzyme associated with outer mitochondrial membrane



The above equations are taken from *Benzi and Moretti* [33].

Major Centres of ROS Formation

The major centres of ROS formation is depicted in (Fig. 2). In particular, activated microglia, and aberrant protein interaction with metals, proteasomal dysfunction and mitochondrial abnormalities leads to the formation of ROS in turn enhances oxidative stress in the cellular environment, finally leading to programmed cell death.

Cause or Effect of ROS

Metal-Protein Interaction

Metals reflect the physiological factors that can influence the protein structure. It is now becoming clear that one of the major factors involved is the direct binding of certain metal ions to the protein [34-36]. Crucially, the peptide-metal ion complex reduces oxygen to hydrogen peroxide (presumably via superoxide). H_2O_2 is freely permeable across membranes and so readily inflicts oxidative damage to cells. Hydrogen peroxide is also readily reduced by Fe (II) and Cu (II) to liberate the highly reactive and unselective hydroxyl radical, which is capable of inflicting severe oxidative damage [34-36]. This, in general outline, is what we suspect is the cause of neuronal cell death in several different neurodegenerative disorders. However, many questions remain unanswered, precisely which form of the protein is associated with the disease (eg. monomeric, oligomeric, protofibrils, mature fibrils) are responsible for the generation of ROS?. Alternatively, is the generation of ROS actually a 'by-product' of the aggregation process itself?. Do aggregating proteins other than A β , α -synuclein and Prp, including those found outside of the brain also generate ROS?. How can this proposition explain the selective vulnerability of different areas of the brain in different neurodegenerative disease?. A proposed mechanism on A β -metal interaction leading to neuronal cell loss is as shown in (Fig. 3).

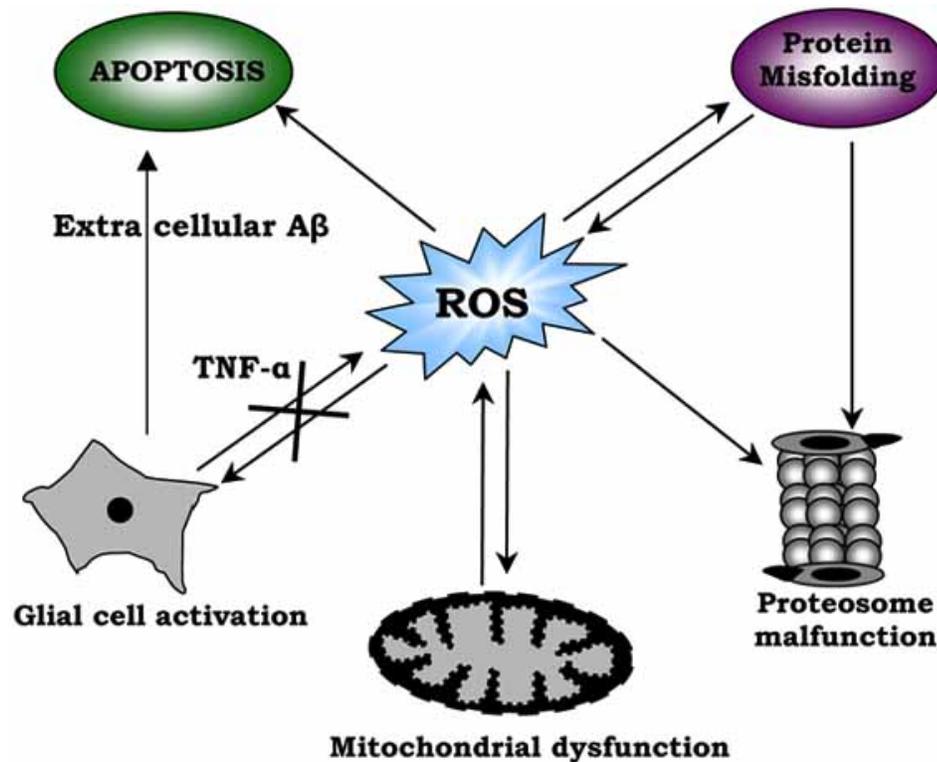


Fig. (2). ROS production as a major player in the cycle of events that leads to apoptosis; The important events that events leads to the ROS generation, through mitochondrial dysfunction, proteasomal malfunction, microglial activation, aberrant protein folding.

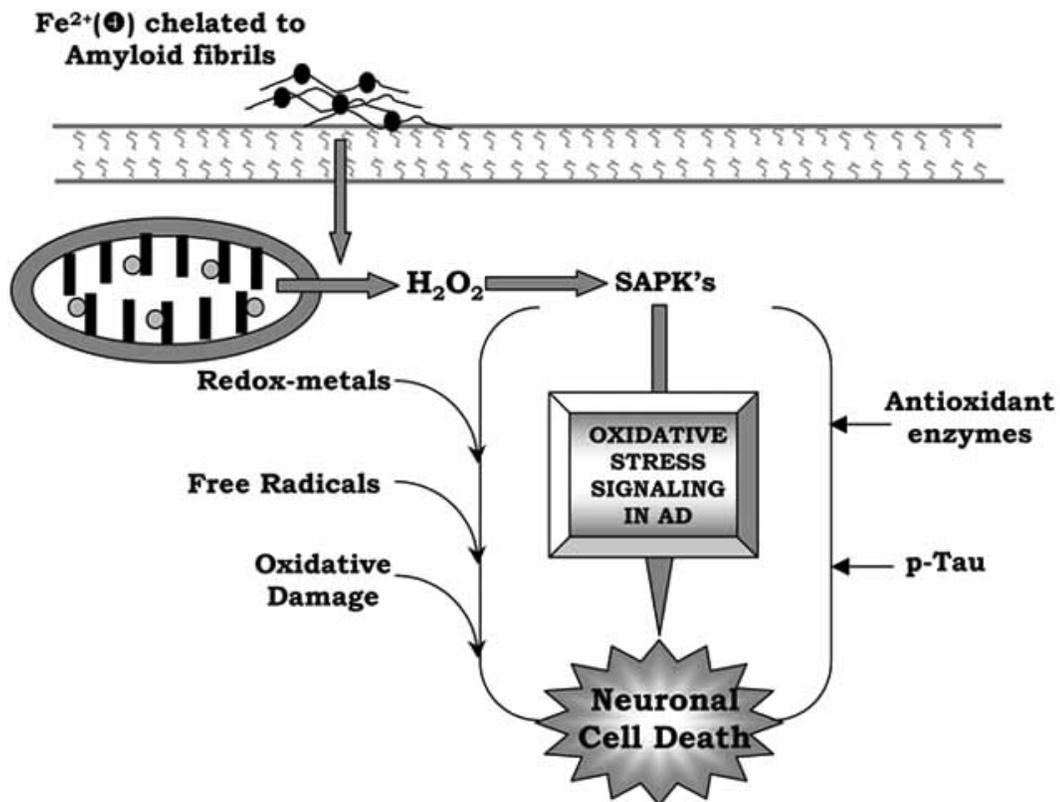


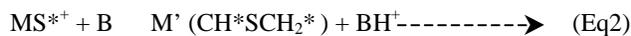
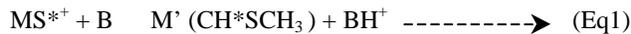
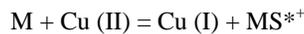
Fig. (3). Schematic illustration of oxidative stress signaling leading to neuronal cell death in AD. Release of H₂O₂, dysfunctional mitochondria propagate a series of interactions between redox metals and oxidative stress response elements. A in the presence of redox-active ion, generate excessive H₂O₂ which possess a great threat on neuronal cells by damaging important macromolecules, compensatory responses provoked by H₂O₂ via the activation of SAPK pathways and downstream adaptations in AD.

Protein Oxidation-Carbonyls Deposition

Protein carbonyls are generated due to oxidation of sensitive amino acids such as lysine, histidine, proline and arginine [37]. In tissues from AD patients, but not in controls, carbonyls are increased in neurons and glia indicates enhanced oxidative stress in AD [38]. The evidence indicates that carbonyl residues and protein nitration in AD brain may come from the peroxy nitrite, a powerful oxidant [39]. Furthermore, peroxy nitrite-related damage is extended beyond AD lesions, suggesting a generalized oxidative brain damage [39, 40]. Thereby it leads to enhanced accumulation of protein carbonyls, oxidized protein deposits, enhancing loss of activity in the brain cellular metabolism.

Eg 1: ROS leads to A oxidation through M³⁵

We have cited an example to depict how methionine oxidation in A linked to metal through ROS plays a key role in the pathogenesis of AD. Methionine (M) residue at position 35 in its C-terminal end, which is critical for neurotoxicity, aggregation and free radical/ ROS formation initiated by the peptide [41, 42].



The oxidation M-35 by ROS provides a great manifold of possible reaction mechanisms, controlled by the nature of the oxidizing species [43] lead to the accumulation of irreversible protein damage which in turn leads to ROS formation.

Eg 2 ROS and RNS-- Synuclein Oxidation by T¹²⁵

-synuclein, a major component of Lewy bodies in Parkinson's disease, dementia with Lewy bodies, and glial cytoplasmic inclusions in multiple system atrophy. Increasing evidence suggests that the oxidation and nitration of tyrosine residues in synuclein induced by oxidative injury is involved in the formation of inclusions characteristic to these synucleinopathies. Exposure of synuclein to peroxy nitrite induces nitration of tyrosine residues, thereby forming synuclein oligomers, ROS formation [44].

DNA/ RNA Oxidation by ROS

DNA molecule is dynamic and polymorphic in nature. It has been proposed that DNA topology has a crucial role in DNA functional aspects like replication, transcription etc. The major question is, does the initiation of apoptotic events lead to DNA structural change through DNA damage?. Anitha et al [45] proposed that there could be a link between apoptosis and DNA structural change with reference to AD.

The DNA as one with RNA are susceptible for oxidative modification, rendering hydroxylated products of its bases. There is evidence of increased oxidative damage to cytoplasmic RNA [46], nuclear [47] and mitochondrial DNA in AD. Further, redox factor-1 involved in DNA repair [48] and the DNA excision-repair-cross-complementing proteins p80 and p89 [49] were found to be over expressed in brain regions of patients in AD. Although there is evidence for an impairment in DNA repair system, by a considerable increase of the predominant marker of DNA damage 8-

hydroxy-2'-deoxyguanosine (8-OH dG) in intact DNA and a decrease in free 8-OH dG in cerebral fluid [50]. Thus there is a definite imbalance between the DNA damage mechanism and the DNA repair system- which leads to disturbed cellular homeostasis.

RNA Oxidation

Some of the recent evidences suggest that oxidative modification of cytoplasmic RNA in vulnerable neurons is an important, well documented feature of the pathophysiology of AD. Honda *et al.* [51, 52] reported that RNA-bound iron plays a pivotal role for RNA oxidation in vulnerable neurons in AD brain. The cytoplasm of hippocampal neurons showed significantly higher redox activity and Fe (II) staining than age-matched controls. Notably both were susceptible to RNase, suggesting a physical association of Fe (II) with RNA [51, 52]. Further the ribosomes purified from AD hippocampus contained significantly higher levels of RNase-sensitive iron. In conclusion, it is shown that the ribosomes, rRNA provides a binding site for redox-active iron and serves as a redox center in generation of ROS within cytoplasm of vulnerable neurons in AD [53].

Lipid Peroxidation by ROS

Lipid peroxidation is an important event, which can be either the cause or the effect of ROS formation. Lipids are modified by ROS and there is a strong regional correlation between lipid peroxides visualized as Thiobarbituric acid, antioxidant enzymes, the presence of senile plaques and neurofibrillary tangles in AD brain [54]. The A induces lipid peroxidation of membranes [55, 56] and lipid peroxidation products are involved in modification of proteins by covalent binding. 4-hydroxynonenal (4-HNE) an aldehydic product of membrane lipid peroxidation [57], generated following exposure of neuronal membranes to A peptide [58, 59], binds directly to tau, a microtubule-associated protein, inhibiting its dephosphorylation. This fact suggests that 4-HNE may be involved in tau phosphorylation found in degenerating neurons of AD individuals [60], although there is evidence that oxidative stress generated by H₂O₂ induces dephosphorylation of tau [61]. It has been reported that 4-HNE can damage and kill primary hippocampal neurons [58] and can induce cross-linking of cytoskeletal proteins in cultured neuroglial cells [62]. Free 4-HNE can reach micromolar levels within several hours, after hippocampal and cortical cells have been exposed to A [58]. Moreover 4-HNE were significantly elevated in the ventricular fluid of AD patients compared with the control subjects. These are the few evidences suggesting that lipid peroxidation in AD brain has a role for 4-HNE, effects the neurodegenerative events [63].

Oxidative Stress and AD

The term 'oxidative stress' is used when the body's natural defense mechanisms are exceeded by the production of deleterious ROS, resulting in damage to susceptible cell components such as DNA, proteins and lipids. Classically oxidative stress is described as an imbalance between generation and elimination of ROS and RNS. These reactive species are originally considered to be exclusively detrimental to cells [64]. It is now recognized that redox regula-

tion involving ROS, is key to the modulation of critical cellular functions, ion transport, calcium mobilization, and apoptosis program activation. When oxidative stress occurs, cells functions to counteract the resulting oxidant effect and to restore the redox balance. Among mechanisms of neuronal death, apoptosis and excitotoxicity play a pivotal role [65, 66]. ROS and RNS are involved in both apoptosis and excitotoxicity [67, 68].

Oxidative stress has been implicated as an important mechanism in AD. A balance between the production of reactive oxygen radicals (ROS) and antioxidant defenses is essential to avoid oxidative stress. Under normal conditions, damage by oxygen radicals is kept in check by an array of antioxidant systems which display extensive redundancy (eg: the simultaneous metabolism of H₂O₂ by catalase and glutathione peroxidase). However the oxidant versus antioxidant balance is altered during degenerative conditions, as it is associated with ageing either primarily or secondarily. But fortunately the cells have a variety of mechanisms designed to compensate during periods of increased oxidative stress. In AD, evidence suggests that susceptible neurons recruit multiple regulatory mechanisms that involve a complex interplay between proteins, DNA and metals. Hence it is highly important to maintain a balance between them. Antioxidant therapies might prove promising avenue to certain extent for treatment, However, if the treatments have to be successful the initial source or the triggering factor for this has to be characterized. Neither the upregulation, nor subsequent deposition of A β , nor the protein modifications found in NFT seem to be the cause of the oxidative stress, But ROS is termed to be the initial trigger and the major contributing factor for the 'Oxidative Imbalance', including the redox-active transition metals and abnormalities in the mitochondrial metabolism.

Many researchers believe that one of the strong theories involved in the etiology of AD might be the oxidative stress hypothesis. The A β peptide, a hallmark in the pathogenesis of AD and the main component of senile plaques, generates free radicals in a metal-catalyzed reaction inducing neuronal cell death by a reactive oxygen species. It is well known that wide number of enzymatic and non-enzymatic oxygen free radical generating systems are able to catalyze the oxidative modification of proteins when Fe (II) or Cu (II) are in the presence of O₂ and an appropriate electron donor [37, 69]. In fact the conversion of superoxide radicals (O₂^{•-}) and H₂O₂ to the highly cytotoxic hydroxyl radical (HO) can only take place when catalytic concentrations of transition metals are present [70]. Moreover, In AD it has been reported an increased content in brain Al [71, 72] and Fe [73], two metals capable of stimulating free radical generation. An elevation in the amounts of total iron and ferric iron has also been found in frontal cortex and cerebellum from autopsies of brains from AD patients [74]. Since it is the loosely bound iron, the one that is responsible for free radical reactions *in vivo* [75]. A β plays a direct role in oxidative damage, it is able to potentiate the free-radical generating capacity of metal ions such as Fe, Cu and Al *in vitro* [76], and recently Huang *et al.* [77, 78] showed that the A β peptide is capable of generating H₂O₂ by metal ion reduction. Cu, Fe and Zn are highly concentrated within the core and periphery of plaque deposits [79]. The transition metals accumulated in

plaques may be a direct source of ROS associated to the oxidative stress observed in AD [80]. In fact, transgenic mice that over-express β -APP present amyloid deposition associated to redox-active iron and oxidative damage markers [81]. These metals may exert their toxic effects not only catalyzing the generation of ROS, but also by direct stimulation of senile plaque formation, as suggested by the finding that copper induces aggregation of A β in a pH dependent form [82] and that low concentrations of Fe, Al, and Zn are required to stimulate A β aggregation *in vitro* by 100±1000 fold [83, 84] This evidence indicates that perturbation of metal homeostasis may play a role in the pathogenesis of AD [85, 86] leading to neuronal cell loss.

Oxidative Stress Signalling, Elicit Apoptosis

Oxidative stress, as one of the earliest events in AD pathogenesis, plays a significant role in the formation of AD pathology [80]. Changes in gene expression and enzyme activities induced by oxidative stress are mediated through the interplay of multiple signalling pathways. Among these are the stress activated protein kinase (SAPK) pathways, JNK-SAPK and p38-SAPK2 [87-91], which are the central mediators that propagate stress signals from the membrane to the nucleus. In neuronal cells, potentially deleterious stimuli, such as oxidative stress, provoke an intracellular stress response that either leads to defensive-protective adaptations or apoptosis [91]. The complex nature and genesis of oxidative damage and responses in AD can perhaps now be partly answered by mitochondrial abnormalities that can initiate oxidative stress. By releasing excess levels of H₂O₂, dysfunctional mitochondria propagate a series of interactions between redox metals and oxidative stress response elements (Fig. 4). Adding to the complexity is that A β , in the presence of redox-active ion, may also generate excessive H₂O₂ [92]. Although the formation of highly reactive hydroxyl radicals by interaction between H₂O₂ and metal ions poses a great threat on neuronal cells by damaging important macromolecules, compensatory responses provoked by H₂O₂ via the activation of SAPK pathways and downstream adaptations [93, 94] such as induction of anti-oxidant enzymes, tau phosphorylation and NFT formation may provide some protective mechanisms to ensure neuronal cells do not succumb to such oxidative insults [95, 96]. A shift in homeostasis may be achieved via the dynamic balance between oxidative damage and compensatory responses which is likely the case in AD.

A Two-Hit Hypothesis on Cell Death in AD: A Debate?

Cell death in aging and AD might differ in the early stages but at later stages they converge. They either adopt necrotic or apoptotic pathway or both. But how the cell faces the apoptotic insult through oxidative stress, what are the possible triggering factors for apoptotic events still remains elusive. Hence we have put forth an understanding of the cellular events that underlie between normal mitotic cell death and neurodegenerative through oxidative insults. Oxidative stress and aberrant mitotic signalling have roles early in the pathogenesis of AD, but their temporal relation to each other is unclear. ERK activation is generally thought of as a response to mitotic signalling and is specifically linked to markers that affect cell cycle control such as phosphorylation

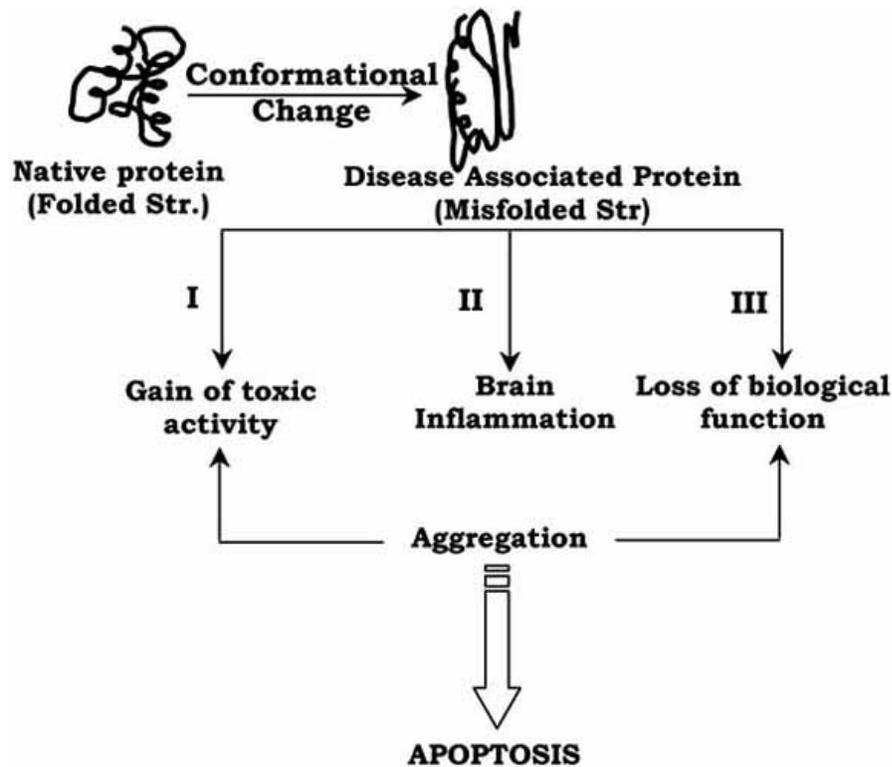


Fig. (4). A schematic representation of neuronal cell death associated with protein misfolding. In this context three models have been proposed. I) Gain of Toxicity; II) Brain inflammation hypothesis; III) Loss of biological function. Conformational change of normal protein seems to be the hallmark event in AD. Protein misfolding may be associated to disease by either the absence of biological activity of the folded protein or by a gain of toxic activity by the misfolded protein. Aggregation of the misfolded protein may also contribute to the disease pathogenesis. Although the beginning and the end of the process are the same in three hypothesis, the events that induce neuronal death are different.

of c-Myc and expression of p16INK4a in AD, whereas the activation of JNK-SAPK and p38 [87] is associated with a response to oxidative stress. However, what is interesting is the simultaneous activation of ERK and JNK-SAPK during Braak I-II stages, which shows that both processes are evident at the earliest pathological stages of disease [97, 98]. However, in Braak stage 0 (ie, before the onset of the disease), although either ERK or JNK-SAPK activation commonly occur, we never see them coexisting in the same cell. Therefore, conversion from pre-AD to the very earliest stage of AD requires both aberrant mitotic signalling and oxidative stress. In other words, although both mitotic signalling and oxidative stress can exist individually and represent a "single hit", only after a "second hit" does the disease process start. Given that MAPK pathways are tightly regulated, the persistent activation of JNK-SAPK and ERK throughout the disease process further indicates that these same "two-hits" are necessary for further disease progression [97, 98]. By way of example we now highlight the plight of a neuron subject to two hits an oxidative injury followed by a mitotic insult. However, the reverse mitotic followed by oxidative insult is also possible. Oxidative stress is a pervasive feature in AD at all stages. However, what is striking is that few neurons (less than one in 10 000 at any given time) show signs of apoptosis [99, 100] therefore, we suspect that a uniquely chronic, tolerable exposure of neurons to oxidative stress provides an explanation for the low amounts of neu-

ronal apoptosis in AD as well as for the abnormally sustained activation of SAPK pathways [91]. Tolerable amounts of oxidative stress provoke compensatory changes that lead to a shift in neuronal homeostasis which is initially reversible if the oxidative stress is acute. However, with persistent oxidative stress as is seen in prodromal AD and AD it is not only likely, but essential that, after a certain threshold (in terms of severity and chronicity of oxidative stress), most neurons make permanent adaptive changes [101, 102]. In this new steady state, neurons still function normally or even in a slightly compromised fashion in a pro-oxidant environment. This state that we call "oxidative steady state" is where neurons can efficiently function for decades [103-105]. In fact, because oxidative stress is much higher in prodromal AD than in normal ageing [106, 107], it is likely that neurons at oxidative steady state devote much of their compensatory potential to prevent oxidative stress. Hence understanding the complete mechanism of cell death through apoptosis in AD still remains hard to pin down.

Mitochondrial Dysfunction/ Abnormalities

Mitochondria plays a central role in both cell life and death [108] by regulating intracellular Ca^{2+} homeostasis and reactive oxygen species (ROS). In contrast mitochondria play a key role in controlling pathways that lead to apoptosis. Levels of Ca^{2+} plays a key role in mitochondrial function. Defects of mitochondrial function leads to excessive pro-

duction of ROS, release of small proteins that trigger the initiation of apoptosis such as cytochrome c and apoptosis inducing factor (AIF), from the mitochondria space to cytoplasm. More evidence for mitochondrial dysfunction in AD comes from several reports of cytochrome c oxidase deficiency in AD brain [109]. Furthermore *In situ* hybridization studies also show decreased levels of the mitochondrial DNA encoded subunit II in AD brain, elevated levels of ROS and reduced ATP levels. Besides the above current evidence indicates A β may cause mitochondrial dysfunction, resulting in oxidative stress through metals and caspase activation [110].

Mitochondrial abnormalities are examined in vulnerable neurons by mitochondrial DNA (mtDNA), mitochondrial protein and mitochondrial number in AD [111, 112]. In vulnerable neurons the mitochondrial degradation products are significantly high. These components are damaged because of the hydroxynonenal (HNE) adducts to lipoic acid and ROS generation [113]. But the question arises what is happening in non-neuronal cells? A study from Takuma et al [114] found that there is significant reduction in mitochondrial density showed that mitochondrial abnormalities are also seen in fibroblasts and other cells from patients with AD [115]. It is tempting to consider the range of oxidative balance abnormalities noted in AD stem from a fundamental mitochondrial deficiency [116, 117], but clearly more work is necessary to establish such a relationship. In this pretext mitochondrial heredity may be important but AD is restricted to only vulnerable neurons, rather involving all cell types, hence mitochondrial inheritance may not be the responsible factor. In summary, mitochondrial dysfunction/ abnormalities and the resulting energy deficit may trigger the onset of neuronal apoptosis in AD [110, 114, 118].

Role of Endoplasmic Reticulum in Neuronal Cell Death

Recent studies have suggested that neuronal death in AD or ischemia could arise from dysfunction of the endoplasmic reticulum (ER) [119]. Inhibition of protein glycosylation, perturbation of calcium homeostasis, and reduction of disulfide bonds provoke accumulation of unfolded protein in the ER, and are called 'ER stress' [119]. Normal cells respond to ER stress by increasing transcription of genes encoding ER-resident chaperones such as GRP78/BiP, to facilitate protein folding or by suppressing the mRNA translation to synthesize proteins [120, 121]. Recent studies suggests that the ER also plays an important role in regulating cell death. The ER is an important subcellular site, since it is the major storage location for calcium and contains members of the Bcl-2 family of proteins, Bcl-2 and Bcl-xL [120]. The stress induced by metals like Al in the ER has also been shown to result in a specific type of apoptosis mediated by caspase-12 and is independent of mitochondrial-targeted apoptotic signals [122]. It was clarified what molecules related to cell death are activated in the case of AD it was discovered that caspase-4 plays a key role in ER stress-induced apoptosis. Caspase-4 also seems to act upstream of the A β induced ER stress pathway, suggesting that activation of caspase-4 might mediate neuronal cell death in AD [122-124].

Apoptosis is initiated through intracellular mechanisms that often involve alterations in mitochondria or endoplasmic

reticulum and by signalling through cell membrane death receptors- the so called Intrinsic and Extrinsic apoptotic pathways.

Extrinsic Pathway

In extrinsic apoptosis, external signals or ligands interact with a receptor at the plasma membrane, initiating a cascade of events that leads to apoptosis. Here both mitochondria and ER is involved [114, 119, 120]. The death receptor family induces tumor necrosis factor (TNF), Fas-associated death domain like interleukin 1-converting enzyme inhibitory proteins, death receptors and decoy receptor. Direct binding of A β or A β oligomers to death receptors remains to be shown, but the activation of the downstream caspases (caspase 2 and caspase 8) supports the involvement of the extrinsic pathway in A β -mediated apoptotic processes. Alternatively the intracellular A β produced in the ER might lead to the ER stress, or binding of A β to the mitochondrial alcohol dehydrogenase may lead to the mitochondrial stress [125, 126].

Intrinsic Pathway

Oxidative stress induced or enhanced by A β and metals activates mitochondria and leads to its dysfunction [114]. Eg: Decrease in mitochondrial membrane potential and depletion of ATP. Cytochrome c is released by dysfunctional mitochondria and activates caspase 9, whereas in ER due to the unfolded protein response in particular A β activates caspase 4 and 12 which in turn activates the executioner caspase 3 and 7 [114, 115], capable of cleaving tau protein which favor formation of NFT's. Here both pathways (Extrinsic and Intrinsic) converge (Fig. 5).

Is it Precise to Employ Necroptosis in AD?

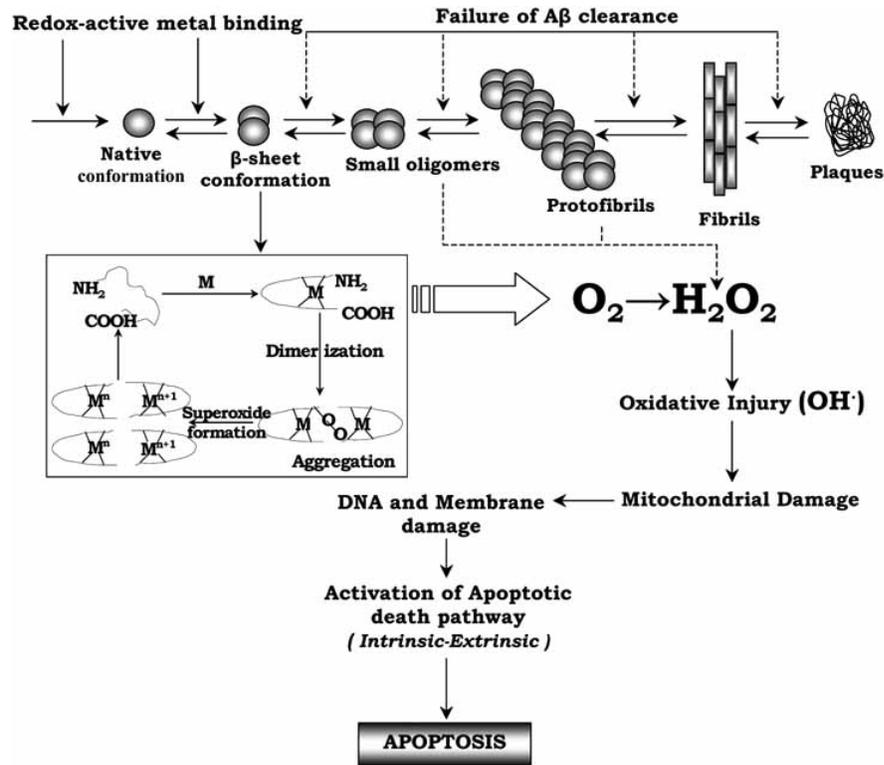
In AD the question arises whether apoptosis and necrosis overlaps. The dividing line that separates necrosis from apoptosis has been emphasized for years owing to the clear distinct features that classifies both events. However, death in neurons can be biphasic, beginning with necrosis and then showing delayed apoptosis. Hence it could be precise use the term "Necroptosis" in AD.

INDIRECT PATHWAY

Neuronal cell loss in AD occurs by programmed cell death or apoptosis. At least three hypothesis have been proposed to explain how protein undergo misfolding and aggregation might be associated with neuronal apoptosis.

i. Loss of Function Hypothesis: In this view, AD is caused by the loss of normal activity of the protein, which is depleted during misfolding and aggregation [127]. The biochemical pathway leading to loss of function in AD through binding to metals. Free radicals play are thought to play a critical role in A β -mediated neurotoxicity [128, 129]. This neurotoxicity is mediated *via* the generation of oxygen free radicals and the accumulation of H₂O₂ with its fatal oxidative consequences. Metal binding to A β leads to imbalance in metal homeostasis, indicating that an abnormal reaction between a protein and a redox-active metal ion might promote the formation of reactive oxygen species or radicalization and lead to cell death [128].

A)



B)

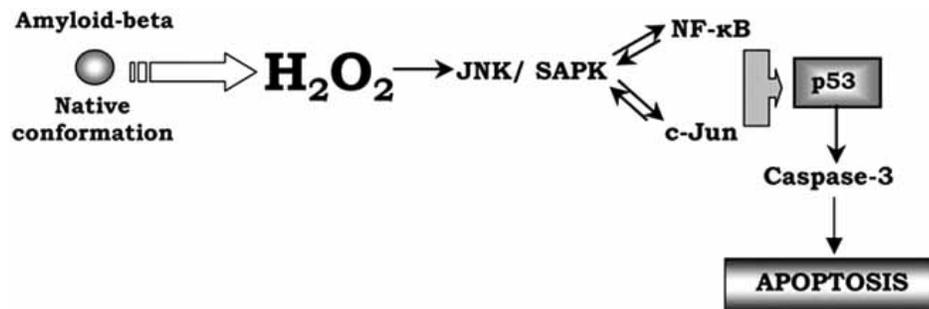


Fig. (5A and 5B). Schematic model of A β and metal induced apoptosis by an oxidative stress mechanism. Both metals and generate H₂O₂ (5A) and A β alone (5B) or in combination, activates JNK/SAPK kinases pathway which in turn activate in parallel both NF- κ B and c-Jun transcription factors. NF- κ B subsequently activate the transcriptional factor p53 and subsequently it may activate the pro-apoptotic Bax protein inturn activates caspase-3 leading to cell death by apoptosis. Alternatively H₂O₂ formed by metal-protein interaction reacts with the free metals to produce highly reactive oxygen species (hydroxyl radicals by Fenton reaction), which damage nuclear DNA, activates the apoptotic pathway (extrinsic-intrinsic) and provoke plasma membrane damage. We strongly believe that failure membrane binding preprties is hall mark in neuronal dysfunction.

ii. Gain of Toxic Activity: The most widely accepted theory of brain degeneration in neurodegenerative diseases proposes that misfolding and aggregation results in the acquisition of a neurotoxic function by the misfolded protein [130, 131]. This concept is based on direct induction of neuronal apoptosis by aggregates of misfolded A β . Additional support for this hypothesis comes from experiments with transgenic animals in which incorporation of the human mutated gene encoding the misfolded protein trigger neu-

rodegeneration. Misfolding and aggregation (protofibrils and oligomeric species) of A β results in inherent toxicity [130, 131]. For eg: Extracellular aggregates might activate a signal transduction pathway that leads to apoptosis by interacting with specific cellular receptors in particular RAGE (receptor for advanced glycation end products), a multi-ligand immunoglobulin super-family cell surface molecule binds A β fibrils, thereby inducing cellular stress and activates NF- κ B [132, 133]. Intracellular aggregates damage cells by recruit-

ing factors that are essential for cell viability into fibrillar aggregates. This concept might provide a unifying mechanism of cell death in neurodegeneration.

iii. Brain Inflammation Hypothesis: The final target of A β , that is important in AD is the immune system. In this hypothesis abnormal A β aggregates acts as irritants and cause a chronic inflammatory reaction in the brain that leads to neuronal cell death and synaptic changes [134, 135]. A β present in neuritic senile plaques apparently activates microglia possibly by binding type-2 scavenger receptors. Activated microglia produce large amounts of free radicals and might contribute significantly to the free radical burden in AD. Besides the generation of ROS (by NADPH oxidase) activated microglia also generates nitric oxide, excitotoxins, Interleukin-I and cytokines such as tumor necrosis factor- α , transcription growth factor- β and the neurotrophic basic fibroblast growth factor [135]. These events promotes oxidative stress thus leads to neuronal cell loss (Fig. 6).

- The pathway of A β -linked neuronal cell death is as summarized below.
- Non-specific intercalation of aggregated forms of A β into membranes [136].

The spontaneous fragmentation of A β to give highly reactive peptidyl radicals [137].

- The direct production of hydrogen peroxide from A β [138].
- The impulsive generation of ROS from A β in the presence of metals [139].
- Interaction between A β and a specific cell surface receptor, such as the RAGE (receptor for advanced glycation products) [140-142]
- Interaction between A β and a intracellular target molecule such as ERAB (endoplasmic reticulum A β binding protein) [143].
- Activation of p53, NF- κ B, caspase-3 and increased expression of bax protein.

A β -Metal Linked Neuronal Cell Death

The biochemical pathways leading to neurotoxicity and inturn leads to cellular dysfunction is a highly contentious issue [144]. Free radicals are thought to play a critical role in A β mediated neurotoxicity. The formation of A β as aggregates and its deposition in senile plaques are believed to be a

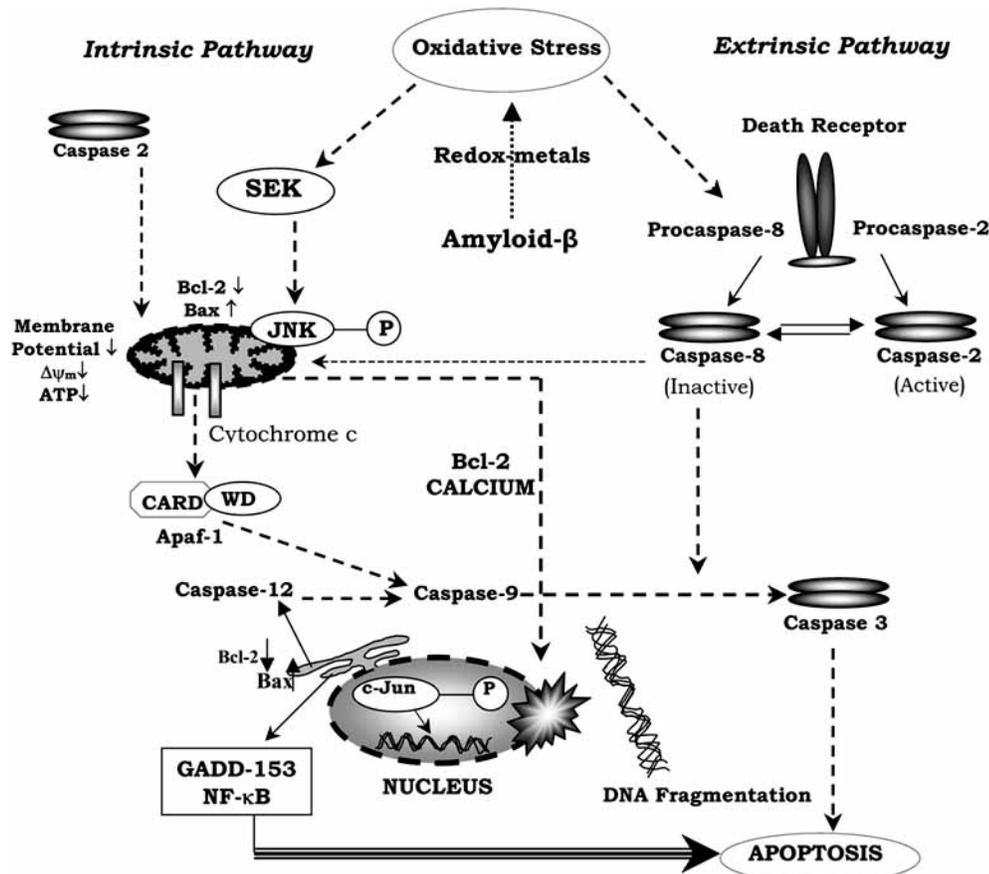


Fig. (6). A proposed model of apoptotic biochemical cascades occurring in AD: a sequence of events leading to caspase activation. Two parallel pathways, activated by A β mediated through metals may operate within individual neurons. Oxidative stress induced or enhanced by A β through redox-active metals leading to mitochondrial dysfunction and triggering both Extrinsic and Intrinsic pathways. Cytochrome c is released by dysfunctional mitochondria and activates caspase 9. Later Intrinsic pathway such as those initiated by cell stress, induce activation of caspase 2 for permeabilization of mitochondria. Both the pathways may then converge by activating caspase 3, and the execution of cell death.

central step in the pathogenesis of AD [144]. This neurotoxicity is mediated via the generation of oxygen free radicals and the accumulation of hydrogen peroxide (H_2O_2) with its fatal oxidative consequences. A β seems capable of inducing free radical production through multiple pathways, one such is binding metals, such as Cu, Fe and Zn. Binding to metals seems to stimulate aggregation of A β and have been shown to prevent A β aggregation. Metals are important because they catalyze the conversion of H_2O_2 to hydroxyl radicals (OH^*) through the Fenton reaction [145]. The ability of A β has important consequences, because as A β accumulates so do metals. Thus as A β and metals accumulate in neuritic senile plaques, OH production increases, these evidences has to be addressed in detail.

A classical example of A β 25–35 in presence of redox-active iron triggering the apoptotic events is explained in detail. The A β deposition in the neuritic plaques is one of the major neuropathological hallmarks of the AD. Studies *in vitro* have demonstrated that the A β 25–35 fragment, which contains the cytotoxic functional sequence of the amyloid peptide, induces neurotoxicity and cell death by apoptosis [145]. Despite intense investigations, a complete picture of the precise molecular cascade leading to cell death in a single cellular model is still lacking. Studies by Pardo et al [145] provide evidence that A β 25–35 induce apoptosis either alone or in presence of iron in peripheral blood lymphocytes cells (PBL) in a concentration-dependent fashion by an oxidative stress mechanism [144]. A β 25–35/ H_2O_2 generation precede the apoptotic process and that once H_2O_2 is generated, it is able to trigger a specific cell death signalization. These consequences may contribute to explain the importance of A β alone or in the presence of redox-available iron in association with A β plaques (and neurofibrillary tangles) in AD brains and the significant role played by H_2O_2 as a second messenger of death signal in some degenerative diseases linked to oxidative stress stimuli [146]. Moreover, because most studies have looked at a given pathway in isolation, the potential interactions between pathways and A β -metal linked neuronal cell death have often not been addressed in detail.

Metal-Mediated Apoptosis

Consequently metal induced apoptotic mechanism is elucidated in detail. An example citing Al-maltolate induced apoptosis in aged rabbits is elicited in brief. Al affects both mitochondria and endoplasmic reticulum (ER), integrity and functionality. In addition the stress that Al causes to the ER leads to the activation of caspase-12 [147], thus leading to endoplasmic mediated cell death [148]. In contrast to Bax, the anti-apoptotic Bcl-2 has the ability to block the release of cytochrome c from mitochondria by mechanisms such as a direct blockade of the MTP opening, or by functioning as a docking protein [149, 150]. Al has been demonstrated to accumulate in neurons following cell depolarization, where it inhibits Na^+/Ca^{2+} exchange and thereby induces an excessive accumulation of mitochondrial Ca^{2+} [147, 148]. Increases in intra mitochondrial Ca^{2+} levels lead to an opening of the MTP with cytochrome c release and subsequent apoptosis resulting from activation of the caspase family of proteases [148]. We have shown that the intracisternal administration of Al results in cytoplasmic cytochrome c translocation, Bcl-

2 down-regulation and Bax up-regulation, as well as caspase-3 activation. These results indicate that Al targets the mitochondria. Furthermore, the fact that we can demonstrate is the release of cytochrome c, which is inhibited by cyclosporin A, a specific inhibitor of the MTP opening, implicates opening of the mitochondrial transition pore as the process by which cytochrome c translocates to the cytoplasmic space from mitochondria [148, 151]. The use of pharmacological agents that prevent or reverse the apoptotic effects of Al can provide valuable mechanistic information on the effects of Al on cellular protein targets. Ghribi et al [151] demonstrated that the glial cell-line derived neurotrophic factor (GDNF) protects hippocampus from the neurotoxic effect of Al, but does not prevent the release of cytochrome c as the sole trigger of Al-induced apoptosis, at least in this animal model system. However, GDNF treatment increases the level of the anti-apoptotic protein, Bcl- x_L , which when over-expressed, has the ability to sequester apaf-1, and thereby to inhibit Apaf-1 dependent caspase-9 activation. Recent studies from Savory et al showed that chronic treatment of rabbits with lithium in the drinking water results in inhibition of the Al-induced cytochrome c release, enhances levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL, prevents the redistribution of the pro-apoptotic protein bax levels and inhibits caspase-3 activation and DNA fragmentation [151].

Although mitochondrial alterations may represent an important step in the mechanisms underlying neuronal cell death, ER a multifaceted organelle regulates protein synthesis, protein folding, cellular responses to stress and intracellular Ca^{2+} , Al^{3+} , Cu^{2+} , induces a redistribution of the apoptosis-regulatory proteins, with Bax being present at higher levels in the ER than in the cytosol and with decreased amounts of Bcl-2 in the ER [152]. It is also been reported that metals induces stress in the ER, as demonstrated by the activation of gadd 153 and its translocation into the nucleus. The gadd 153 gene is specifically activated by agents that disturb ER function. Although it has been demonstrated the effect of Al on ER function, it remains unclear which signalling mechanisms lead to perturbation of ER homeostasis by Al. Al may disturb the Ca^{2+} homeostasis or protein processing in the ER. Severe insult results in sustained depletion of Ca^{2+} stores might be the one of the causes for apoptotic cell death [153, 154].

Do Neurons Have a Choice on Death in AD?

Apoptosis is a program to remove cells detrimental to higher level organization in development, neoplasia, or after irreparable damage. That so many signs of apoptosis are activated in neurons in AD can be reconciled if we suspend reductionism and accept that one of the pathways by which cells deal with stress is activation of various proteolytic and structural changes [100, 101]. Unlike homogeneous organs, where death of damaged cells could be desirable, in the adult brain, conservation of brain cell topography is critical. Therefore, it is likely that evolution has promoted neuronal survival at all costs so that, in the context of a human brain, a multiplicity of responses will work to promote neuronal survival through factors not seen *in vitro* and may be not even in *in vivo* models that have non-physiological homeostatic balances [155, 156]. An instance of this is A PP-

overexpressing mice, which show A β deposits and other changes of AD. In these mice, A β is being inappropriately recruited to a scene where it is neither called for nor wanted, and its presence may initiate changes coming from it, as well as the changes that normally precede it [157]. A β and phosphorylated tau may instead be critical to homeostasis in AD [158] and not with the decreased activity of hippocampal neurons which is not related to the presence of NFT's [159]. Increases in both during injury at any age, as well as the central tenet of biology of the preservation of higher order, argue for a homeostatic function of responses induced in disease rather than an etiological role [156]. A protein's role may be absent or aberrant with genetic mutation, an argument certainly supported by the abnormalities in mice overexpressing normal A β PP or normal tau but promoted by mutations in either. With such strong pressure to live, as they do throughout normal aging, it is not unexpected that neurons, and possibly other post-mitotic cells, have unusual redundancy in their ability to respond to insult by inducing anti-apoptotic signalling, and this balance is what defines neuronal homeostasis in AD [157]. Instead of succumbing at death's door, neurons select other portals. It is these choices, we argue, that define neuronal longevity in normal aging as well as neuronal persistence in AD. Hence understanding neuronal cell death or apoptosis in AD still remains as a mystery.

Abortosis or Abortive Apoptosis - Resolving Cell Death in AD, a Debate!

Abortosis is a novel phenomenon that represents an inhibition of apoptosis at the post initiator stage in neurons that survive in AD. Although much effort has been expended on elucidating pathways leading to the death of susceptible neurons in AD, precious little is agreed upon in terms of nature of cell death in AD. A wide variety of apoptogenic promoters such as reactive oxygen species [160], amyloid- β , energy failure and HNE oxidants [160] act alone or synergistically. Many studies assume neuronal death in AD that cell death program finally results in apoptotic phenotype [161]. However a major complicating matter is the nature and time course of neuronal cell death in AD [161]. Therefore, while evidence supporting apoptosis as a central mechanism of cell death is accumulating, questions surfaced about how plausible this claim is?. In this regard, apoptosis needs only 16-24 hours of completion, and in a chronic disease like AD, which has an average duration of approximately 10 years, less than 1 in about 4000 cells should be undergoing apoptosis at a given time. However, if all the neurons that are presumably vulnerable, namely those that shows possible DNA cleavage, actually underwent apoptosis, the brain would in rapid fashion be stripped of neurons will take months together. However, this scenario is far from the picture that is seen in AD [162, 163]. Therefore, it is not surprising that the morphological stereotype that constitutes the end stages of this cell death program such as chromatin condensation, nuclear segmentation, blebbing and apoptotic bodies are not seen in AD, although many have attempted to look for these evidences. The initiator phase of apoptosis is seen in AD, but lacks the effective apoptotic signal propagation to the distal effectors [162, 163]. The study of all the above processes occurring in the human brain will be carried out on autopsy

brain material. It is a misconception certain times with the data published on autopsy brain material. Agonal state effects the stability of brain compounds and causes brain hypoxia which also leads to cell death, hence delusion might occur with respect to neurochemical changes as observed in apoptosis of neurodegenerative disorders [164, 165]. It is for these fundamental reasons that it is utter failure to detect apoptosis is quite puzzling phenomenon based on the morphology.

Antioxidants: How Far Effective in Preventing Neuronal Cell Death?

A number of antioxidant therapies have been tested in clinical trials and have shown to affect the onset as well as the progression of AD, but some of them have pleiotropic effects [166, 167]. For example the non-steroidal anti-inflammatory drugs NSAIDs have been demonstrated to reduce the incidence of AD by 50%, Vitamin E plays a neuroprotective role and combats to the overproduction of ROS in AD [167]. At this point it is also difficult to say whether it will be most effective for long term treatment and prevention of AD. Oxidative stress and ROS generation also takes place parallelly in the activated microglia, primary factor for inflammation, Hence anti-inflammatory drugs might have a higher therapeutic value [167]. Although AD concomitantly demonstrates oxidative stress response as a mechanism that links oxidative damage and cell death, But the cells undergoing oxidative stress lacks death. In summary, while several antioxidants and anti-inflammatory are therapeutic in AD, future antioxidants should focus on the earliest, most specific and reversible oxidative changes of AD [167, 168].

Metal Chelation Therapy: Is it Promising?

Current research approaches have focused on drug development for AD. The main approaches attempt is to prevent A β production (secretase inhibitors) or to clear A β (vaccine). However there is compelling evidence that A β does not spontaneously aggregate, but that there is an age-dependent reaction with the excess brain metal Cu or Fe, induces the production of H₂O₂, which may mediate conspicuous oxidative damage to the brain. Hence in this perspective it is important to develop metal-binding compounds that inhibit the invitro generation of H₂O₂ [169]. Although Zn, Cu, Fe, induce rapid aggregation of A β in postmortem brain tissue of AD patients [170, 171] there is not much detailed information available on the mechanism of aggregation. *Recent Developments:* Clioquinol (CQ) was identified as a prototype metal-protein-attenuating compound (MPAC) [172]. A recent report has shown that oral treatment of CQ, Cu/Zn chelator to transgenic mice APP 2567 inhibits A β accumulation significantly [170]. CQ, a derivative of 8-hydroxy quinoline is as such not a specific chelator of Cu and Zn, as the stability constants for binding to metals is as follows. Fe>Cu>>Ni>Al> Zn and the affinity of CQ to Cu is much less than (K₁ for Cu= 8.9; K₁ for Zn= 7.0) the attomolar levels reported by Atwood et al [173]. Lipophilic chelators have been designed that may be capable of crossing the blood-brain barrier, a property lacking in desferrioxamine (DFO), a chelator in widespread clinical use [174], the therapeutic use of lipophilic Fe chelators remains a potential strategy that requires investigation. Certain compounds have

to be looked for that can block the adverse generation of H₂O₂ and in turn inhibiting aggregation phenomenon may permit greater insights into the significance of metal-protein interactions across AD. However the clinical role metal chelators need to addressed further.

SUMMARY

In summary, apoptosis mediated through ROS are complex phases in neurodegeneration. The potential role for ROS, the mitochondria, ER and the activation of several signalling pathways (MAPK and p53) has been established for metals through oxidative phenomenon. Interestingly, there appears to be both common and metal-specific pathways. In addition, a paucity of data on the *in vivo* induction of apoptosis by these metals exists. Thus a number of issues remain to be addressed including the cell specific responses, the relevance of the *in vitro* findings to the *in vivo* situation. Further, detailed study on apoptotic processes in the AD brain are necessary before definite conclusions can be drawn. Elucidating the mechanisms in detail will aid in better understanding of neuronal cell death events.

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