Chapter 13

Involvement of Free Radicals in the Development and Progression of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a major dementia related to an overproduction of free radicals (FRs), which leads to the generation of oxidative stress in brain tissue. Amyloid beta-peptide of 42 amino acid residues ($A\beta_{1-42}$) is the main source of FRs in patients with AD. βA_{1-42} results from hydrolysis of the amyloid precursor protein by β-secretase in a process known as the amyloidogenic pathway. During βA_{1-42} aggregation, the peptide interacts with various transition metals to produce hydrogen peroxide (H_2O_2) by the Fenton reaction, generating the hydroxyl radical ($^{\bullet}OH$), which damages lipids, proteins, and nucleic acids, thereby contributing to neurodegeneration. In addition, βA_{1-42} is recognized by microglial receptors; it activates these cells, causing overproduction of superoxide anion ($O_2^{\bullet-}$) by NADPH oxidase; $O_2^{\bullet-}$ is also converted into H_2O_2 and finally to $^{\bullet}OH$ in the Fenton reaction. Other factors that contribute to oxidative stress during microglial activation are the overproduction of nitric oxide and interleukins and the overexpression of some enzymes, including cyclooxygenase and inducible nitric oxide synthase, all of which contribute to FR production. Currently, various models *in vitro* and *in vivo* exist that permit quantification of $O_2^{\bullet-}$ and H_2O_2 and determination of the effects of these reactive oxygen species.

Keywords: Amyloid beta, NADPH oxidase, free radicals, oxidative stress

1. Introduction

Alzheimer's disease (AD) is a chronic pathology, the development and progression of which has been related to free radical (FR) production such as occurs in diabetes, cancer, and other diseases secondary to molecular damage. AD is characterized by neuronal damage associated with an overproduction of free radicals (FRs). Although several hypotheses have been advanced to explain the memory loss that occurs in AD, the most accepted theory is that neuronal damage is associated with the presence of aggregates of the amyloid beta peptide of 42 residues ($A\beta_{1-42}$) that is related to FR production.

It is known that Aβ aggregation contributes to FR production because Aβ molecules are able to bind metals such as copper (cupric ion, Cu^{2+}) that are present at high concentrations in the brains of patients with AD. Cu^{2+} leads to the formation of hydrogen peroxide (H_2O_2), which is a reactive oxygen specie (ROS). In turn, H_2O_2 reacts with other metals such as iron (Fe^{2+}) through the Fenton reaction, producing the hydroxyl radical ($^{\bullet}OH$), which damages membrane lipids, proteins, and other biomolecules. Here, it is important to remember that ROS include not only FRs such as $^{\bullet}OH$, superoxide anion ($O_2^{\bullet-}$), and others but also non-FRs such as H_2O_2, ozone (O_3), and hypochlorous acid (HOCl). One hypothesis suggests that the ROS produced during AD hydrolyze a significant amount of acetylcholine (ACh), reducing cholinergic neurotransmission and thereby contributing to memory loss [1]. This has justified the use of acetylcholinesterase (AChE) inhibitors to treat AD; however, these drugs have shown limited clinical results [2].

Brain tissue is especially susceptible to oxidative stress due to its high aerobic metabolic activity and high lipid content. Oxidative stress is defined as the loss of cell homeostasis provoked by an imbalance between the production of prooxidant molecules (ROS) and the activity of antioxidant defense systems. Under physiological conditions, ROS are present at low concentrations in tissues, where they act as signaling molecules during cell growth, cell proliferation, redox homeostasis, and cellular signal transduction (activating tyrosine kinases, MAPKs (mitogen-activated protein kinases), or Ras protein) [3]. However, higher concentrations of ROS lead to a pathophysiological condition produced by an oxidative stress state.

It has been reported that AD is associated with a high level of oxidative stress and lowered antioxidant defenses. Thus, AD may be due to the presence of FRs that alter metal metabolism and result in Aβ aggregation toxicity [4]. In recent years, considerable research has focused on the amyloid fibrils that are produced in AD, and it has been shown that the structures of these aggregates are more complex than the linear addition of monomers to fibrils; in fact, a variety of Aβ aggregates have been described. Furthermore, the amyloid fibrils cause the formation of several toxic intermediates, including soluble oligomers that bind to hippocampal neurons to produce dysfunctions in synaptic plasticity and consequently contribute to the development of AD [5]. Hence, great efforts have been made to find ways to prevent Aβ aggregation because the oligomers and fibrils are also able to activate the NADPH oxidase in microglial cells, which are "like macrophage cells" in the brain. NADPH oxidase can produce great quantities of $O_2^{\bullet-}$, which is converted to H_2O_2; this, in turn, can participate in the Fenton reaction, producing $^{\bullet}OH$. Thus, the generation of

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FRs is important in AD because these have been related to its development, and Aβ and NADPH oxidase may be key targets for the treatment of this disease.

In this chapter, we describe the important implications of FRs in the development and progression of AD. First, we discuss some of the principal biomolecules involved in the production of FRs in AD, emphasizing the role of $A\beta_{1-42}$ due to its aggregation and its consequent implication in the formation of senile plaques when it reacts with metals to produce ROS. In addition, we explain how Aβ participates in microglial activation to produce more FRs due to the activity of NADPH oxidase. Subsequently, the reactions in which the ROS produced by Aβ and NADPH oxidase participate are described, and the relationship between FR production and the neuronal damage that occurs during AD is explained. Finally, we discuss how $^{\bullet}OH$ and H_2O_2 production can be determined using various experimental techniques *in vitro* and *in vivo*.

2. Biomolecules involved in free radical production in Alzheimer's disease

2.1. Amyloid beta formation

Aβ is the principal component of extracellular deposits called amyloid plaques that are present in the brains of patients with AD. According to the amyloid cascade hypothesis, which was first established in 1991, Aβ accumulation represents the critical step in the pathophysiology of AD [6]. Aβ originates from the processing of a large transmembrane glycoprotein, amyloid precursor protein (APP). APP is a single-pass transmembrane protein with a large extracellular domain. Alternate splicing of the APP transcript generates eight isoforms, the three most common of which are the 695-amino acid form, which is expressed predominantly in the central nervous system (CNS), and the 751- and 770-amino acid forms, which are more ubiquitously expressed [7].

The precise physiological function of APP is not known and remains one of the vexing issues in the field. In most studies, APP overexpression shows a positive effect on cell health and growth [8]. APP can be hydrolyzed following both the non-amyloidogenic and the amyloidogenic pathways, depending on the enzymes involved. In the non-amyloidogenic pathway, APP is hydrolyzed at amino acid residue 83 from the C-terminus by α-secretase (**Figure 1**). This cleavage produces a fragment of 83 amino acids (C83) and a large N-terminal ectodomain (sAPPα). C83 remains in the membrane, where it is hydrolyzed by the γ-secretase complex to produce p3, a short fragment, and the APP intracellular domain (AICD). In the amyloidogenic pathway, APP is hydrolyzed at amino acid residue 99 from the C-terminus by β-secretase to produce a fragment of 99 amino acids (C99) and an sAPPβ fragment. C99 remains in the membrane, where it is hydrolyzed by the γ-secretase complex, releasing the Aβ peptide, which consists of 42 amino acid residues ($A\beta_{1-42}$), and other peptides (**Figure 1**) [9]. The principal difference between these two pathways is that α-secretase cleavage occurs within the Aβ region, avoiding the formation of Aβ peptide, whereas β-secretase cleavage permits Aβ formation from APP.

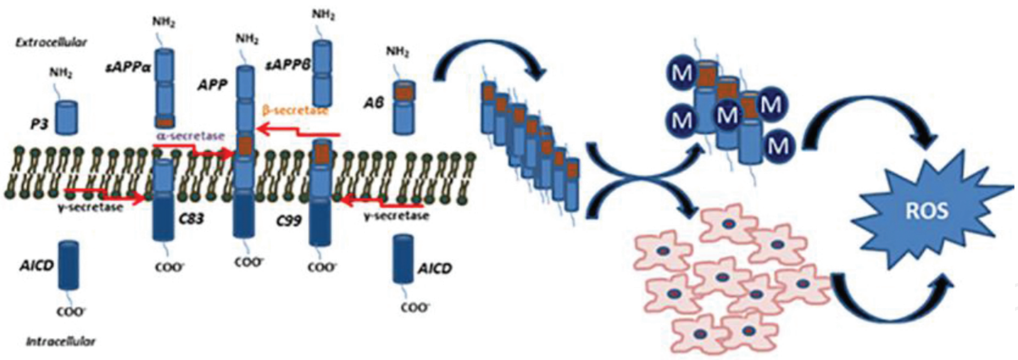

Figure 1. Hydrolysis of APP to produce the Aβ peptide. When $A\beta_{1-42}$ is released, it tends to aggregate to form oligomers and fibrils; these subsequently react with metals or with microglial cells and produce a large amount of ROS.

It is important to mention that under physiological conditions, both of these pathways occur; in fact, it has been demonstrated that Aβ is an enhancer of learning and memory and that low doses of Aβ produce presynaptic enhancement [10]. It was shown that concentrations of Aβ peptides in the picomolar-nanomolar range decrease the synthesis and release of ACh without causing neurotoxicity. The potency and reversible nature of this effect and the low concentrations of Aβ peptides found in normal brain cells suggest that Aβ-related peptides may act as modulators of cholinergic function under normal conditions [11]. However, during AD, the increase in the concentration of Aβ may be the result of an overproduction and/or a deficiency in its elimination, resulting in Aβ aggregation [12]. During the processing of APP by the amyloidogenic pathway, two principal Aβ species are produced: Aβ of 40 amino acid residues and Aβ of 42 amino acid residues ($A\beta_{1-40}$ and $A\beta_{1-42}$, respectively). Despite the difference of only two amino acids, the latter is more prone to aggregate; the additional amino acids give $A\beta_{1-42}$ distinct thermodynamic properties [13].

Two distinct mechanisms have been proposed to explain the formation of Aβ fibrils. The first invokes nucleated polymerization in which Aβ polymerization creates a nucleus to which monomers are added in an elongation process (**Figure 2**).

The second proposed mechanism is based on a nucleated conformational conversion in which oligomers are formed as intermediates; these intermediates then form protofibrils that subsequently assemble into fibrils [14]. Because Aβ oligomers have been implied in the pathophysiology of AD, it has been proposed that the second mechanism contributes more to the progression of the disease. However, although enormous efforts have been made to understand how Aβ aggregates, principally in the form of $A\beta_{1-42,}$ which is more cytotoxic than $A\beta_{1-40}$ [15], the mechanism by which $A\beta_{1-42}$ undergoes conformational changes to form oligomers and protofibrils remains unknown (**Figure 2**).

Recently, several experimental techniques such as nuclear magnetic resonance (NMR) (solid state), Fourier transform infrared spectroscopy (FTIR), cryo-electron microscopy (cryo-EM), single-touch atomic force microscopy (AFM), and fluorescence have allowed investigators to study the $A\beta_{1-42}$ fibril formation process in detail. The results suggest that a nucleated conformational conversion occurs when $A\beta_{1-42}$ is present at high concentrations (>20–30 μM). The predominant oligomers formed in the early step of aggregation are dimers, tetramers, pentamers, and hexamers, but their formation is temperature- and concentration dependent. At approximately 15°C and high Aβ_{1-42} concentration, the formation of protofibrils from oligomers occurs more rapidly. The principal conformational change is observed in the lateral association of oligomers to yield protofibrils; this conformation involves conversion from a random coil structure to a β-sheet via an antiparallel β-hairpin intermediate [16]. The antiparallel β-hairpin has intramolecular hydrogen bonds between two hydrophobic β-strands, one with an LVFF sequence and another with a GLMVG sequence at the C-terminus. However, conversion to a β-sheet involves the rotation of β-strands to form intermolecular hydrogen bonds with other monomers in the Aβ_{1-42} structure. It is known that the β-strands adopt a parallel orientation in the Aβ_{1-42} fibrils. The β-sheet is stabilized by intermolecular hydrogen bonds as well as by intramolecular and intermolecular interactions between the residue side chains in the β-strands. It has been confirmed that the formation of the antiparallel β-hairpin is a rate-determining step in fibril formation, with the interaction between aspartate 23 (Asp23) and lysine 28 (Lys28) being the most important.

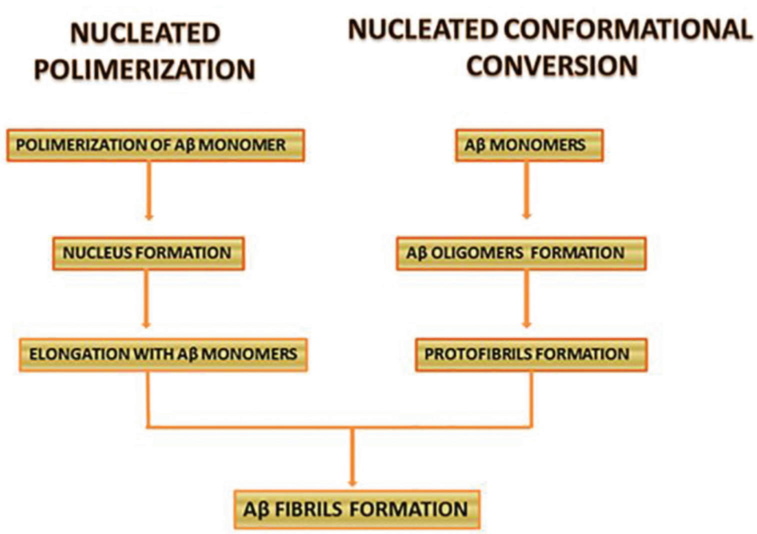

Figure 2. Proposed mechanisms of Aβ fibril formation. Left: nucleated polymerization at low Aβ concentrations. Right: nucleated conformational conversion at high Aβ concentrations. The latter mechanism is considered to be more related to the progression of AD because it produces a large amount of oligomers, which, together with the fibrils, are cytotoxic.

Other recent studies show that there are differences in Aβ_{1-42} and Aβ_{1-40} fibril formation [17]. One of these differences is that the Aβ_{1-42} fibril has a triple β-motif that consists of three β-sheets (β_1: 12–18; β_2: 24–33; β_3: 36–40); thus, this structure differs from the proposed β-loop-β motif structure for Aβ_{1-40} fibrils. Additionally, the reported structure of Aβ_{1-42} fibrils differs from that of Aβ_{1-40} fibrils from the brain, which have a U-shaped topology with Asp 23-Lys 28 forming a salt bridge and fewer β-regions [18]. Furthermore, in Aβ_{1-42} fibrils a salt bridge between Lys28 and the carboxylate of the C-terminal alanine (Ala42) was identified; this is important because it shows that Ala42 and not Asp 23, as had been proposed, stabilizes the salt-bridge interaction.

Although several models of $A\beta_{1-42}$ fibrils have been described, to date no $A\beta_{1-42}$ fibril structure has been obtained from the brain, and all that is known about the conformational structure of $A\beta_{1-42}$ fibrils has been obtained from synthetic $A\beta_{1-42}$. Therefore, all $A\beta_{1-42}$ models and observations are approximations that should be accepted with caution because $A\beta_{1-42}$ fibril formation may be influenced by temperature, pH, and other biochemical parameters that are not considered when the fibrils are formed *in vitro*. For example, it was recently reported that calcium (Ca^{2+}) interacts with glutamate 22 (Glu22) and the phospholipid bilayer to accelerate $A\beta_{1-42}$ aggregation [19]. Furthermore, this type of interaction between a cation and Glu22 could also be important in interactions of the peptide with metals such as Cu^{2+}, which at some concentrations favors $A\beta_{1-42}$ aggregation. Therefore, it is difficult to propose a definite and unique $A\beta_{1-42}$ fibril structure that could provide a basis for elucidating the steps involved in $A\beta_{1-42}$ aggregation.

As mentioned previously, the mechanism of $A\beta_{1-42}$ aggregation that has been proposed to contribute principally to the pathogenesis of AD is nucleated conformational change due to the formation of oligomers of $A\beta_{1-42}$ [20]. When the amyloid hypothesis was first proposed, it was postulated that only $A\beta_{1-42}$ fibrils were the toxic form of Aβ; however, it is now known that both oligomers and protofibrils are toxic species and that oligomers are more toxic than fibrils [21]. This has been generally accepted due to the finding that cognitive deficits are better correlated with the amount of soluble Aβ than with the number of amyloid plaques; thus, neurodegeneration is not a consequence of amyloid deposition [22]. This is consistent with the oxidative damage produced by the $A\beta_{1-42}$ oligomers. There are several hypotheses related to $A\beta_{1-42}$ aggregation and ROS production during AD development and progression. The results of a number of studies support the hypothesis that $A\beta_{1-42}$ genesis depends on ROS production, whereas other reports suggest that $A\beta_{1-42}$ is capable of forming ROS [23]. In addition, some previous evidence clearly shows an association between AD and the ROS produced by $A\beta_{1-42}$ oligomers and metals (**Figure 1**). Hence, some studies have focused on searching for strategies to avoid the oligomerization of $A\beta_{1-42}$ by inhibiting it or by decreasing ROS production through the design of multi-targeted compounds; this has resulted in a promising approach [8]. By targeting at this molecular level, it is possible to avoid $A\beta_{1-42}$ aggregate formation, which functions as a signal that activates microglial cells and initiates an innate immune response that results in the production of high levels of cytokines and ROS.

2.2. Microglial activation enhances NADPH oxidase activity

Due to their phagocytic activity, microglial cells represent the macrophages of the brain; for this reason, they are regarded as the predominant immune cells in the brain. In the healthy brain, these cells act as resting microglia, maintaining their ramified morphology and protecting the brain from pathogens by removing them by phagocytosis [24]. However, when microglial cells detect a sign such as a pathogen associated with molecular patterns (PAMPs) or damage associated with molecular patterns (DAMPs), the microglia are activated to acquire a wide range of phenotypes. Two classical phenotypes are the pro-inflammatory M1 phenotype (induced by pro-inflammatory cytokines and/or TLR activation (Toll-like receptor)) and the non-inflammatory M2 phenotype (induced by interleukin (IL)-4), according to the classification for macrophages outside the brain [25]. Aβ_{1-42} oligomers and fibrils interact with SCARA1, CD36, CD14, a6β1 integrin, CD47, TLR2, TLR4, TLR6, and TLR9 receptors on the microglia; when Aβ_{1-42} interacts with TLR or CD receptors, the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), tumor necrosis factor (TNF)-alpha, IL-1β, IL-6, etc. is induced, resulting in a dysregulated immune response that contributes to neurodegeneration [26].

Furthermore, it was found that low concentrations of Aβ_{1-42} induce microglial proliferation and cause release of H_2O_2 and $O_2^{\bullet-}$ to the extracellular space due to the activation of NADPH oxidase (**Figure 3A**) [27]. The fact that NADPH oxidase 2 (NOX2) is widely distributed in microglial cells and neurons has been corroborated in *in vitro* and *in vivo* models using microglia derived from NOX2 knockout mice and the NOX2 inhibitors diphenyleneiodonium (DPI) and apocynin [28]. In microglial cells, NOX2 is activated only after the binding of Aβ oligomers to the Mac receptor (Mac-1), an integrin receptor also known as CD11b/CD18, complement receptor 3 or aMβ2 that is important during reactive microgliosis and in neurodegeneration (**Figure 3B**) [29, 30].

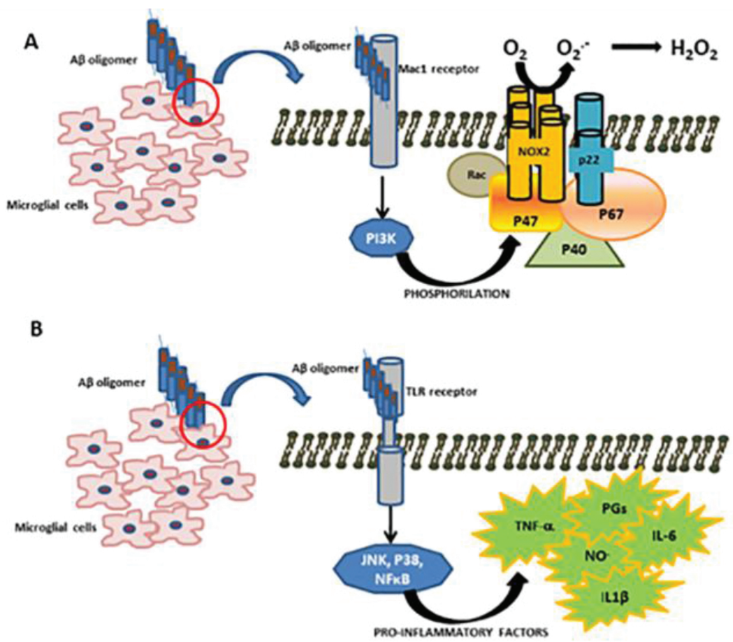

Figure 3. Pro-inflammatory factors produced by the interaction of Aβ_{1-42} oligomers with TLR or Mac-1 receptors. (A) Aβ_{1-42} oligomers induce the activation of NADPH oxidase. (B) Production of cytokines induced by the interaction of Aβ_{1-42} oligomers with the TLR receptor.

It is currently known that unique NADPH oxidase activity is associated with the generation of $O_2^{\bullet-}$ or H_2O_2, depending on the isoform. The confirmation of NADPH oxidase participation in microglial activation and the consequent production of ROS were obtained using cells from patients with chronic granulomatous disease (CGD). Because this disease is characterized by the inability of cells to produce H_2O_2 due to mutations in the genes that encode the subunits of NADPH oxidase, monocytes and neutrophils from CGD patients fail to produce ROS in response to fibrillary Aβ peptides [31, 32].

Park et al. assessed ROS production in the neocortex using hydroethidine fluoromicrography [29]. Fibrillar Aβ superfused through a cranial window increased ROS production in the neocortex. This effect could be abolished by the addition of a peptide inhibitor of the gp91phox subunit. These authors further demonstrated that ROS levels were increased in the Tg2576 mouse model of AD; however, no signs of ROS production were evident in a mouse model in which Tg2576 mice lacked the gp91phox gene.

NOX2 is an oligomeric protein composed of three cytosolic subunits (p60phox, p47phox, and p40phox) and two transmembrane subunits (p91phox and p22phox). For the production of $O_2^{\bullet-}$ by NOX2, p22phox must form a complex with p47phox. It has been demonstrated that, in primary microglial cells and monocytes exposed to fibrillar Aβ, p47phox and p67phox subunits are translocated from the cytosol to the membrane, favoring the enhanced activity of NADPH oxidase [33].

The production of $O_2^{\bullet-}$ together with the neurotoxic factors PGE2, IL-β1, TNF-alpha, H_2O_2, nitric oxide (NO), and peroxynitrite ($ONOO^-$) can result in neuronal death [34]. Subsequently, the $O_2^{\bullet-}$ produced by NOX2 reacts with NO generated by iNOS to form $ONOO^-$. In the presence of excessive amounts of NO, nitration and S-nitrosylation of several proteins, as well as dityrosine formation, occur. Tyrosine 10 of Aβ can undergo nitration, which in turn increases the probability of Aβ aggregation; this is shown by the fact that Aβ nitrotyrosine has been found in amyloid plaques [35].

During chronic neuroinflammation, microglia maintain the transcription of mRNAs coding for pro-inflammatory factors such as NOX2, iNOS, TNF-alpha, IL-1β, and COX2. The interaction between $A\beta_{1-42}$ oligomers and TLR receptors begins the phase of neurodegeneration (**Figure 3B**); as neuroinflammation progresses, the interaction between $A\beta_{1-42}$ oligomers and Mac-1 receptors results in the production of large amounts of FRs such as $O_2^{\bullet-}$, which is converted to H_2O_2 to maintain the neuroinflammation (**Figure 3A**).

$O_2^{\bullet-}$ dismutates spontaneously or by an enzymatic reaction catalyzed by superoxide dismutase (SOD), an enzyme that can scavenge $O_2^{\bullet-}$ and convert it into H_2O_2 [36]. Because $O_2^{\bullet-}$ is the primary ROS produced during the neuroinflammatory process, this is considered to play a key role in the activation of microglia and the activation of NADPH oxidase. However, $O_2^{\bullet-}$ can also be produced by xanthine oxidase and during mitochondrial respiration. The $O_2^{\bullet-}$ can reduce and liberate ferric ion (Fe^{3+}) from ferritin or ferrous ion (Fe^{2+}) from iron-sulfur clusters. This reaction is of great importance because Fe^{2+} participates in the Fenton reaction and produces $^{\bullet}OH$. $O_2^{\bullet-}$ contributes to the Fenton reaction via the Haber-Weiss reaction, in which $O_2^{\bullet-}$ reduces Fe^{3+} produced in the Fenton reaction to Fe^{2+} and maintains iron (II), thereby facilitating the Fenton reaction. The net Haber-Weiss reaction is as follows (1-3):

$$Fe^{3+} / Cu^{2+} + O_2^{\bullet-} \rightarrow Fe^{2+} / Cu^{+} + O_2 \quad (1)$$

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$$Fe^{2+} / Cu^{+} + H_2O_2 \rightarrow Fe^{3+} / Cu^{2+} + OH^{\bullet} + OH^{-} \quad (2)$$

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{-} \quad (3)$$

Numerous experimental studies have shown that Aβ oligomers are more toxic than Aβ fibrils, and that ROS are produced from the beginning of AD, playing a crucial role in neuroinflammation.

3. Biochemical and chemical reactions in Alzheimer's disease that yield free radicals

3.1. Metal dyshomeostasis during Alzheimer's disease

It is well known that certain transition metals are essential for neural function. The levels and transport of these metals are strictly regulated by the blood-brain barrier (BBB), and disruption of metal homeostasis in the brain is thought to play an important role in the pathogenesis of AD [37]. The principal areas of the brain in which metals tend to accumulate are the hippocampus, the amygdala, and the cerebrospinal fluid (CSF); in some of these areas, both senile plaques and neurofibrillary tangles are found (**Table 1**).

The mammalian brain contains an intrinsically high concentration of copper (Cu^{2+}), zinc (Zn^{2+}), and iron (Fe^{2+}) ions compared to other tissues due to its high requirement for numerous metal-dependent enzymes and metal-dependent metabolic processes [38]. Not only has dyshomeostasis of Cu^{2+}, Zn^{2+}, and Fe^{2+} been linked with AD but it has also been reported that senile plaques are related to high concentrations of these metals as well as of chrome (Cr^{3+}) and cadmium (Cd^{2+}) (**Table 1**) [39–41]. Furthermore, these metals are involved in FR production by their participation in the Fenton and Fenton-like reactions; importantly, it has been suggested that they may interact with biomolecules implicated in AD such as Aβ, AChE, and ACh [1], with deleterious results.

To clarify the functions and toxicities of various metals and their relationship to AD, specific information on each metal is provided as follows:

Iron: Divalent iron (Fe^{2+}) is the most abundant transition metal in the human brain. Iron is present *in vivo* in both the ferrous (Fe^{2+}) and ferric (Fe^{3+}) valence states. Fe^{2+} is crucial for neuronal processes such as myelination, synaptogenesis, and synaptic plasticity (SP). It has been well documented that Fe^{2+} deficiency can induce a series of neurochemical alterations that may eventually lead to cognitive deficits [42]. While essential for the maintenance of a healthy brain, Fe^{2+} can also play a toxic role. It exacerbates damage to brain tissue following processes such as stroke or trauma. A regional increase in Fe^{2+} within AD brains, compared with healthy controls, is considered a key factor in neuronal atrophy. Accumulations of Fe^{2+} occur in the cerebral cortex, the hippocampus, and the basal nucleus of Meynert, where they co-localize with lesions, neurofibrillary tangles, and plaques. These are particularly important areas in the clinical picture of AD because they are associated with the centers of memory and thought that are gradually lost as AD progresses [42]. Given that Fe^{2+} is highly reactive, an excess of this metal ion may result in the overproduction of reactive chemical species such as $^{\bullet}OH$. Thus, FRs are responsible for oxidative stress, which is considered a primary contributing factor in neurodegeneration [43].

Metal and concentration in AD brains (μM)	**Physiological functions in the brain**	**Brain areas where metal is accumulated**	**Relationship with AD**
Fe^{2+}, 669, 694	Formation and maintenance of the neuronal network and neurotransmitter synthesis.	Hippocampus (wet tissue), amygdala	Generation of an excess of reactive radical species leading to cell and tissue damage.
Cu^{2+}, 57.7, 53.2, 10–100	Cofactor and structural component of enzymes. Regulate synaptic function myelination, synaptogenesis, and synaptic plasticity.	Hippocampus (wet tissue), amygdala, cerebrospinal fluid	Copper in redox-active can catalyze the production of hydroxyl radicals ($^{\bullet}OH$) in a Fenton-like reaction. May influence clearance of Aβ from the brain at the level of the interface between the blood and cerebrovasculature in AD.
Zn^{2+}, 1000, 300	It is released from presynaptic nerve terminals into the synaptic cleft upon neuronal activation and has been shown to inhibit excitatory NMDA receptors.	Amyloid plaques, synaptic cleft (during neurotransmission)	Aggregation of the Aβ peptides to form oligomers and fibrils can be rapidly induced in the presence of zinc ions.
Cr^{3+}, 0.3, 0.4, 6.6	Carbohydrate metabolism and normal insulin sensitivity. Brain insulin signal transduction system.	Hippocampus (wet tissue), amygdala, cerebrospinal fluid	Reduction of the neuronal glucose and energy metabolism.
Cd^{2+}, 0.25–250, 50–500	It has not demonstrated a function of brain metabolism.	Parenchyma, cortical neurons	Increase of the blood-brain barrier permeability and oxidative damage.

Physiological functions, concentrations, brain areas of accumulation, and their relationship to AD.

Table 1. Principal metals involved in the development and progression of AD.

Copper: Copper in its divalent form (Cu^{2+}) is found in several enzymes involved in important biochemical pathways in neuronal and nonneuronal cells; these enzymes include SOD, cytochrome-C oxidase, ceruloplasmin, and tyrosinase. Following NMDA receptor activation, Cu^{2+} is released from neurons; the released Cu^{2+} regulates neuronal activation by limit-

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ing Ca^{2+} entry into cells [44]. Astrocytes express several Cu^{2+}-containing enzymes; however, excess Cu^{2+} in astrocytes results in damage due to the binding of Cu^{2+} to Aβ. This can catalyze the production of •OH in a Fenton-like reaction, favoring the establishment of oxidative stress and cell damage [45]. For these reasons, the increase in the distribution of brain Cu^{2+} that occurs in AD, producing concentrations ranging from 10 to 100 μM, could result in the establishment of oxidative stress in areas that are important for memory and learning such as the hippocampus and amygdala (**Table 1**).

The diet is the principal source of Cu^{2+}; in fact, studies by Sparks et al. show that the administration of trace amounts of this metal in drinking water may drive the accumulation of Aβ levels in the brain by altering the level of the interface between the blood and the cerebrovasculature in an AD rabbit model [46]. This suggests that dietary metals may promote Aβ accumulation [47].

Zinc: Under normal conditions, divalent zinc (Zn^{2+}) is concentrated in the neocortex; its concentration is closely regulated due to the potentially neurotoxic effects that occur under conditions of Zn^{2+} excess or deficiency. Zn^{2+} also has a neuromodulatory role in that it inhibits excitatory NMDA receptors, reaching concentrations of up to 300 μM [48, 49]. Religa et al. demonstrated that Zn^{2+} levels increase in parallel with tissue amyloid levels. Zn^{2+} levels were significantly elevated in the brains of the most severely demented patients with AD and in cases that displayed an amyloid burden. In fact, high concentrations of this metal ion (up to 1 mM) have also been found within amyloid plaques [50]. The formation of Aβ aggregates occurs rapidly in the presence of Zn^{2+} ions under physiological conditions *in vitro* [51]. Studies with synthetic Aβ show that chelation chemistry helps solubilize amyloid plaques and that it has a more marked effect on the extraction of Aβ than on the depletion of Cu^{2+} [52]. In addition, elevated Zn^{2+} levels have been found in AD postmortem neocortical samples (**Table 1**) [53].

Chromium: Trivalent chromium (Cr^{3+}) is essential for normal carbohydrate metabolism and normal insulin sensitivity. It has been reported that Cr^{3+} and Zn^{2+} are of importance to the brain's insulin signal transduction system. A Cr^{3+}-binding oligopeptide, which has been named chromodulin, has been reported. In the presence of insulin, chromodulin causes an eightfold stimulation of protein tyrosine kinase activity. Cr^{3+} ions increase insulin-stimulated tyrosine phosphorylation and thereby modulate cellular insulin signaling. Within the pathogenesis of AD, a reduction in neuronal glucose and energy metabolism is assumed. At the center of this lies the disruption of insulin-signaling mechanisms. The results of current biochemical studies indicate that Cr^{3+} and Zn^{2+} are important in the brain's insulin signal transduction system [54].

Cadmium: Divalent cadmium (Cd^{2+}) is a nonessential transition metal that is classified as a carcinogen due to its long biological half-life. Prolonged exposure to Cd^{2+} has toxic effects due to the accumulation of the metal in a variety of tissues, including the CNS. The principal effect of Cd^{2+} in the CNS is the induction of oxidative damage in cells. Increasing evidence has demonstrated that Cd^{2+} is a possible etiological factor for neurodegenerative diseases such as AD. Cerebral cortical neurons have been identified as targets of Cd^{2+}-mediated toxicity and Cd^{2+}-induced cell apoptosis [55].

3.2. Interaction of metals with amyloid beta and hydrogen peroxide production

Senile plaques are composed primarily of extraneuronal-aggregated Aβ, microglia, degenerated neurons, and relatively high amounts of redox-active metals such as Cu^{2+}, Fe^{2+}, and Zn^{2+}. Accurate determination of the redox potentials of Aβ and its metal complexes will certainly help unravel their roles in oxidative stress, metal homeostasis, detoxification, and Aβ aggregation/fibril formation. For these reasons, a number of techniques have been employed to determine the amino acids involved in the recognition of metals by Aβ. It is generally accepted that metal ions are bound to the histidine residues at positions 6, 13, and 14 [56]. Several studies have demonstrated that the interaction of Cu^{2+}, Zn^{2+}, Fe^{3+}, and Al^{3+} with Aβ is maintained by their coordination with His13-His14 of the peptide. The interaction is also maintained by a fourth element represented by a donor atom that can come from the aspartate at position 1 or the tyrosine at position 10, thus forming a tetragonal complex. In fact, marked inhibition of cortical amyloid accumulation by DP-109, a lipophilic metal chelator, has been shown [57].

An important aspect of the binding of Cu^{2+} to Aβ is that the complex retains its redox activity and is able to produce H_2O_2. As the principal ROS in living organisms, H_2O_2 acts as a second messenger in cellular signal transduction under physiological conditions. However, the overproduction of H_2O_2 results in the formation of high levels of $^{\bullet}OH$ and consequent oxidation of the peptide, which can be detected by the formation of carbonyl groups. It was demonstrated that this oxidation increases as the Cu^{2+}:peptide ratio increases and that it is accompanied by changes in the morphology of the aggregates as determined by AFM [58].

It has been shown that the coordination of Zn^{2+} with His13 of Aβ is critical to the metal ion-induced aggregation of Aβ [59]. NMR and circular dichroism (CD) studies of metal-Aβ complexes show that Zn^{2+} binding is dominated by intermolecular coordination and by the formation of polymeric species, including monomeric Zn^{2+}-Aβ and various Zn^{2+}-Aβ oligomeric complexes and aggregates. However, Zn^{2+}-Aβ complex formation is high only in brain areas containing synapses. There, the initial binding of Zn^{2+} to Aβ induces transformation of the peptide to an oligomeric or polymeric complex with increased Zn^{2+}-binding affinity, potentiating the effect of the metal on Aβ and possibly enabling Zn^{2+} to act as a seeding factor in amyloid plaque formation [60]. When aggregates are prepared with Cu^{2+} and Zn^{2+} ions, the ratio of Cu^{2+}:Zn^{2+} becomes an important factor in H_2O_2 generation, the formation of carbonyl groups in the peptide, and aggregate morphology. In fact, Aβ fibrils can hydrolyze H_2O_2 and generate damage by $^{\bullet}OH$ production [61].

Fe^{2+} is able to bind to Aβ, and increased amounts of redox-active iron that can generate an elevated amount of ROS have been found in the brains of AD patients; however, it is not clear how this redox-active Fe^{2+} is produced. It was postulated that Aβ may act by binding the Fe^{3+} and reducing it to pathological Fe^{2+} that is capable of inducing oxidative stress; this would suggest that Aβ possesses a strong reducing capacity for iron and that it acts as a metalloprotein capable of binding the metal ion. The interactions between iron and Aβ are governed by histidines 6, 13, and 14. These amino acid residues could coordinate a shared metal ion and generate a redox-active complex. An alternative explanation might be that an oxidative reaction that uses histidine as a substrate occurs in the presence of Aβ, thereby generating toxic oxygen species [62]. The contribution of each histidine residue to Aβ oligomerization and

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toxicity is different; it is thought that the His6 residue is important for beginning the Aβ dimerization process and that His13 and His14 are not. However, the latter residues could be important in producing the peptide conformations responsible for the Aβ-iron effects [63].

The reduction of metals (principally Cu^{2+}) by Aβ causes the oxidation of Met35, resulting in the production of H_2O_2 [64]. In addition, during the catalytic production of H_2O_2 by $A\beta_{1-42}$ and Cu^{2+}, the participation of Tyr10 is important because when this amino acid is substituted by alanine (Y10A) there is a significant decrease in the ability of Aβ to reduce Cu^{2+}. Here, it is important to note that the reduction of the metal and H_2O_2 production allow the formation of the $^{\bullet}OH$ radical by a Fenton-like reaction.

3.3. Fenton reaction

All the available evidence indicates that the Fenton reaction is important during Aβ aggregation and during metal dyshomeostasis in AD. This reaction was first described by H.J.H. Fenton as the strong oxidation effect of Fe^{2+}-H_2O_2 mixtures on organic compounds in a work entitled "Oxidation of tartaric acid in the presence of iron" [65]. Currently, the combination of Fe^{2+}-H_2O_2 is known as Fenton chemistry, the Fenton reaction, or Fenton reagent.

The Fenton reaction can be written as follows (4):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \quad (4)$$

During AD, the Fenton reaction occurs due to the presence of excessive levels of active redox metals and the generation of H_2O_2 by the reaction of $A\beta_{1-42}$ with the metals. Subsequently, $^{\bullet}OH$ are formed by the interaction of $A\beta_{1-42}$ and Fe^{3+} or Cu^{2+}. Several years ago, a speculative mechanism was proposed by which Aβ interaction with metals could produce ROS. In that mechanism, the binding of the metal is followed by the binding of oxygen to the metal via a peroxo bridge and $O_2^{\bullet-}$ production; the $O_2^{\bullet-}$ are then converted to H_2O_2, which reacts with metals and produces $^{\bullet}OH$ [66].

Furthermore, it has been proposed that during the Fenton reaction an intermediate such as ferryl ion $[Fe(IV){=}O]^{2+}$, a highly reactive oxidant that is able to undergo a reaction involving single-electron hydrogen abstraction and two-electron oxidation, is formed; however, this intermediate is not produced during $A\beta_{1-42}$ aggregation because it is formed during the reaction of Fe^{2+} complexes with H_2O_2 in the presence of organic substrates and a porphyrin complex. Therefore, $^{\bullet}OH$ are produced when aggregated $A\beta_{1-42}$ interacts with metals. However, several *in vitro* studies have shown that $^{\bullet}OH$ can be generated when Fe^{3+} is reduced in the presence of reducing agents such as ascorbic acid (5) or in the absence of redox agents in a reaction in which one electron from ^{-}OH (from the water self-ionization reaction) is transferred to Fe^{3+}, yielding Fe^{2+} and $^{\bullet}OH$ (6) [67]. This reaction also occurs in AD due to the presence of high levels of metals and the consequent production of $^{\bullet}OH$; this promotes $A\beta_{1-42}$ aggregation and consequently increases ROS production, creating a vicious cycle.

$$Fe^{3+} + Asc^{-} \rightarrow Fe^{2+} + Asc^{\bullet -} \quad (5)$$

$$Fe^{3+} + HO^{-} \rightarrow Fe^{2+} + HO^{\bullet} \quad (6)$$

The Fenton reaction can also occur in the presence of other metals via a Fenton reaction or a Fenton like-reaction, as shown below (7):

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO^{\bullet} + HO^{-} \quad (7)$$

where M is the metal (such as copper, which can also be reduced by $A\beta_{1-42}$) that is oxidized in the reaction. When M = Fe^{2+}, the reaction above is known as Fenton reaction; when M = any other metal, the reaction is known as Fenton-like reaction.

In AD, it has been suggested that $^{\bullet}OH$ formation damages biomolecules such as lipids, proteins, and nucleic acids due to the ability of $^{\bullet}OH$ to catalyze reactions such as hydrogen abstraction, addition reactions, and oxidation reactions. Hydrogen abstraction is one of the most important mechanisms because in this reaction the $^{\bullet}OH$ damages lipids in the brain and, as was mentioned previously, the brain has a high content of lipids. Lipoperoxidation (LPO) is the process by which $^{\bullet}OH$ abstract hydrogen from unsaturated fatty acids, forming alkyl radicals. The principal products of LPO are aldehydes as malondialdehyde (MDA) and propanal, hexanal and 4-hydroxynonenal (4-HNE). LPO of oleic acid in the brain occurs by abstraction of the hydrogens in the ninth and tenth positions; secondary reactions include hydrogen abstraction by alkoxy radicals ($RO^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) at the tertiary carbon atoms. Then, alkyl radicals ($R^{\bullet}$) and $ROO^{\bullet}$ are produced by ROS.

4. Damage produced by FRs during Alzheimer's disease

The brain is particularly vulnerable to oxidative stress because of its high metabolic rate, which utilizes 20% of the body's basal oxygen consumption. In addition, the brain has limited antioxidant defenses compared with other organs and high levels of transition metals, principally redox-active Cu^{2+} and Fe^{2+}; defective regulation of the levels of these metals can lead to reaction with O_2 and the production of ROS, resulting in cellular toxicity. Neurons are vulnerable to attack by FRs due to their lower glutathione content in comparison with other cells, their high proportion of polyunsaturated fatty acids susceptible to oxidation, and the fact that their metabolism requires substantial quantities of oxygen. The oxidation of biomolecules such as proteins, lipids, and DNA, and mitochondrial damage have consequences that are deleterious to neurons, including the loss of cell potential, the accumulation of excitotoxic glutamate, decreased glucose availability, decreased intracellular communication, and increased neurotoxicity [68].

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A large number of biological sources are thought to play important roles in FR production in AD. As mentioned above, some transition metals are increased in AD brains and are present in a redox-active state [69]. Fe^{2+} catalyzes the formation of $^{\bullet}OH$ from H_2O_2 by the Fenton reaction in the brains of patients with AD due to the imbalance in metal concentrations, and together with the H_2O_2 produced by Aβ aggregation it is possible to generate $^{\bullet}OH$, which results in the oxidation of lipids, proteins, and DNA [70]. Recent histochemical studies have demonstrated that the detection of redox activity in AD lesions is inhibited by prior exposure of tissue sections to Fe^{2+}- and Cu^{2+}-selective chelators. The activity can be reinstated following reexposure of the chelator-treated sections to either copper or iron salts, suggesting that the redox imbalance in AD is dependent on these metals. It is probable that the accumulation of Fe^{2+} and Cu^{2+} is a major source of the production of reactive oxygen, which is in turn responsible not only for the numerous oxidative stress markers that appear on senile plaques but also for the more global oxidative stress parameters observed in AD [71].

Activated microglia, such as those that surround most senile plaques [72] are a source of the reactive nitrogen species (RNS) NO and the ROS $O_2^{\bullet-}$, which can react to form $ONOO^-$, leaving nitrotyrosine as an identifiable marker [73], as shown in **Figure 4**.

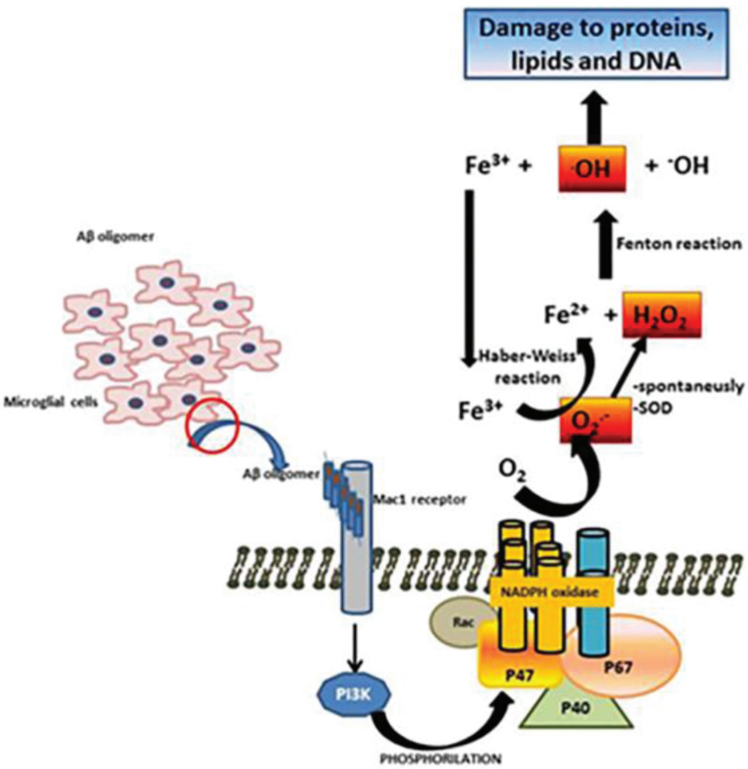

Figure 4. ROS and RNS produced after the activation of microglial cells by $A\beta_{1-42}$. ROS have effects on biomolecules such as lipids, proteins, and nucleic acids.

Several studies have reported that pro-inflammatory molecules and ROS secreted from fibrillar Aβ-stimulated microglia lead to neuronal apoptosis [74]. In addition, neurons, microglia, and astrocytes are capable of generating substantial amounts of NO through the iNOS [75]. Fibrillar

Aβ peptides stimulate iNOS and NO production through the NADPH-dependent oxidative deamination of L-arginine [76]. Microglial/neuronal coculture studies reveal that the NO released from Aβ-stimulated microglia causes neuronal cell death. In addition, iNOS has been reported to act synergistically to kill neurons through the formation of $ONOO^-$. This RNS is a potent oxidant with biological reactivity similar to that of •OH. $ONOO^-$ promotes the tyrosine nitration and nitrosylation of cysteines within cellular proteins. The addition of nitrite (NO_2^-) to tyrosine residues is extremely detrimental because it leads to protein and enzyme dysfunction and the eventual death of cultured neurons [77]. Taken together, these data suggest that Aβ-stimulated production of $ONOO^-$ plays an important role in the pathogenesis of oxidative damage in the AD brain.

The damage to lipids caused by FRs is evidenced by LPO, which has been demonstrated widely in all areas of the brain and shown to be higher in the hippocampus, the piriform cortex, the frontal lobe, and the occipital cortex [78]. Furthermore, LPO markers have been found in the cerebrospinal fluid (CSF) and urine of patients with AD, and their levels tend to increase with the progression of the disease [79]. Analysis of transgenic mice (Tg2576) that display oxidative damage similar to that found in the brains of AD patients revealed an elevation in oxidative stress markers preceding amyloid formation and increasing amyloid pathology [80]. Data from humans and transgenic mice indicate that elevated oxidative stress is an early event in AD pathogenesis.

Advanced glycation end products (AGEs) are involved in AD through several mechanisms. AGEs, which are produced by the interaction of carbohydrates and proteins, stimulate the production of ROS in the presence of transition metals by the establishment of redox cycling. In addition, both Aβ and AGEs activate receptors such as the receptor for advanced glycation end products (RAGEs) and the class A scavenger receptor and thereby increase ROS production [81].

Proteins damaged by ROS can be measured in plasma, serum, CSF, and brain tissue. Studies by Smith et al. have demonstrated an increase in the products of protein oxidation in the hippocampus of patients with AD, which showed neurodegenerative changes in comparison with normal and aged subjects [82].

The production of ROS through peptidyl radicals associated with Aβ contributes to Aβ aggregation; it was demonstrated that protein oxidation promotes the formation of protein aggregates. In addition, Aβ causes alterations in several transmembrane proteins present in neurons and glial cells, including ATPases, glutamate transporters, glucose transporters and guanosine triphosphate (GTP)-coupled transmembrane-signaling proteins, resulting in multiple changes in cellular physiology [83].

The type of damage found in macromolecules such as lipids, proteins, and carbohydrates in patients with AD has also been observed in DNA. Mecoccin et al. showed a 10-fold increase in the oxidation of mitochondria and nuclear DNA in brain samples from AD patients [84].

The formation of ROS by any of several possible mechanisms results in damage to neurons. The cholinergic system is the principal neurotransmission system that is affected by the production of oxidative stress. It was postulated that •OH may decrease the activity of AChE

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by modifying the amino acid residues, which form the anionic site that recognizes the natural substrate, ACh [85].

A large body of evidence implicates compromised antioxidant defense systems as a contributing factor in AD pathogenesis; however, studies of antioxidant enzymes in AD have not shown a consistent pattern. Glutathione (c-glutamyl-cysteinyl-glycine; GSH) is an abundant cellular antioxidant. Thiol-reduced GSH normally accounts for the majority (>98%) of total cellular glutathione, but it can also exist as oxidized glutathione disulfide (GSSG) or glutathione adducts. Glutathione peroxidase (GPx) catalyzes the oxidation of GSH to GSSG, whereas the reverse reaction is carried out by glutathione reductase (GR), which requires NADPH. Coupled to the oxidation of GSH, GPx can reduce H_2O_2, highlighting the importance of both GPx and GR in maintaining the cellular redox state. Indeed, the measurement of erythrocyte levels of GSH, expressed as the ratio of GSH/GSSG, provides a dynamic marker of oxidative stress *in vivo* [86, 87]. Lovell et al. found significantly elevated activity of GPx in the hippocampus, of GR in the hippocampus and amygdala, and of catalase (CAT) activity in the hippocampus and superior and middle temporal gyri in AD subjects compared with normal control subjects [88]. These changes were present in the medial temporal lobe structures where LPO was significantly increased, suggesting a compensatory rise in antioxidant activity in response to increased FR generation in these regions in AD. SOD levels were elevated in all brain regions in AD. CAT was elevated in the amygdala in AD in one study [88]. Marcus et al. demonstrated modifications in the activities of antioxidant enzymes in AD brains. The results showed a decrease in SOD activity in AD frontal and AD temporal cortex, whereas CAT activity decreased in AD temporal cortex. By contrast, these investigators found no differences in GPx activity. The results obtained in these studies show that alterations in the antioxidant enzymes in the brains of patients with AD are most significant in the temporal cortex [89]. For these reasons, the use of antioxidants represents a logical approach to the treatment of AD. This hypothesis is very attractive because most antioxidant compounds have a wide safety margin. The hypothesis has been evaluated under experimental and clinical conditions. Crapper McLachlan et al. [90] showed that the prolonged administration of an iron-chelating agent, desferrioxamine, slowed the development of the disease. Vitamin E, selegiline, and *Ginkgo biloba* extract were evaluated in clinical studies of AD and produced beneficial results [91]. These findings provide important evidence supporting the hypothesis that antioxidants may be capable of slowing the pathogenic process of AD. In addition, a decrease in the incidence of AD in patients treated chronically with non-steroidal anti-inflammatory drugs (NSAIDs) has been demonstrated; this could slow the progress of the disease by decreasing the production of prostaglandins [92].

5. Determination of free radicals in *in vivo* and *in vitro* models of Alzheimer's disease

As previously mentioned, the production of high levels of ROS is related to the establishment and progression of AD. Among these ROS are $O_2^{\bullet -}$, H_2O_2, $^{\bullet}OH$, NO, and $ONOO^-$, which can be produced by several mechanisms (direct ROS production by $A\beta_{1-42}$ oligomers, interaction of Aβ with metals, microglial activation, etc.). For these reasons, a variety of techniques have been employed to determine the species and amounts of ROS in biological samples of patients with AD and in samples from animal models.

5.1. Electronic paramagnetic resonance

Among ROS, $O_2^{\bullet-}$ and $^{\bullet}OH$ are molecules with unpaired electrons that react rapidly with various biomolecules. To quantify these molecules by electron paramagnetic resonance (EPR), it is necessary to employ compounds that increase the half-lives of the unpaired electrons. The most common compounds employed for this purpose are 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), N-tertiary-butyl-nitrone (PBN), and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), all of which react with the unpaired electron of a specific FR and form a complex that is sufficiently stable to be detected by EPR. This technique allows the quantification of FRs in a wide variety of samples obtained in *in vivo* and *in vitro* studies. The nitroxide MCP (3-methoxidecarnonyl-2,2,5,5,-tetramethylpyrrolidine-1-yloxy), which permeates the blood-brain barrier, has been used as a spin probe to noninvasively evaluate redox status in the brains of AD transgenic model mice (APdE9), allowing the measurement of the generation of FRs during the development of the disease [93]. In addition, with the use of DMPO an increase in the production of $^{\bullet}OH$ radicals in activated microglial cells in *in vitro* studies was demonstrated [94].

Although the EPR technique is of great help in identifying and quantifying FRs, its use is limited due to the fact that it requires an EPR spectrometer, which is expensive. If an EPR is not available, other techniques can be used to determine the amount of ROS produced; however, one disadvantage of these techniques is that they require samples from animals that must therefore be sacrificed.

5.2. Superoxide anion determinations

There are several techniques that permit the quantification of $O_2^{\bullet-}$ in biological samples; these include cytochrome C, WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt], lucigenin, luminol, and others. Several of these techniques are described below.

Cytochrome C. This technique has been used to quantify extracellular $O_2^{\bullet-}$ in cultures of microglial cells obtained from neonatal rats stimulated with lipopolysaccharide (LPS). The principle of the method is based on the reducing properties of $O_2^{\bullet-}$. $O_2^{\bullet-}$ donates an electron to ferricytochrome C, reducing ferrocytochrome C and increasing its absorbance at 550 nm. This method presents some limitations if the sample contains high amounts of $O_2^{\bullet-}$ because the cytochrome can be reduced by various molecules such as ascorbate, glutathione, and several reductases that are able to produce ferrocytochrome C [95].

Tetrazolium salt (WST-1). The reduction of WST-1 to a water-soluble yellow formazan by $O_2^{\bullet-}$ can be measured by spectrophotometry (**Figure 5A**). This method has been compared with the ferricytochrome C reduction method in which xanthine/xanthine oxidases are used to generate $O_2^{\bullet-}$; it was demonstrated that WST-1 generated an approximately twofold greater increase in absorbance than ferricytochrome C at their respective wavelengths [96].

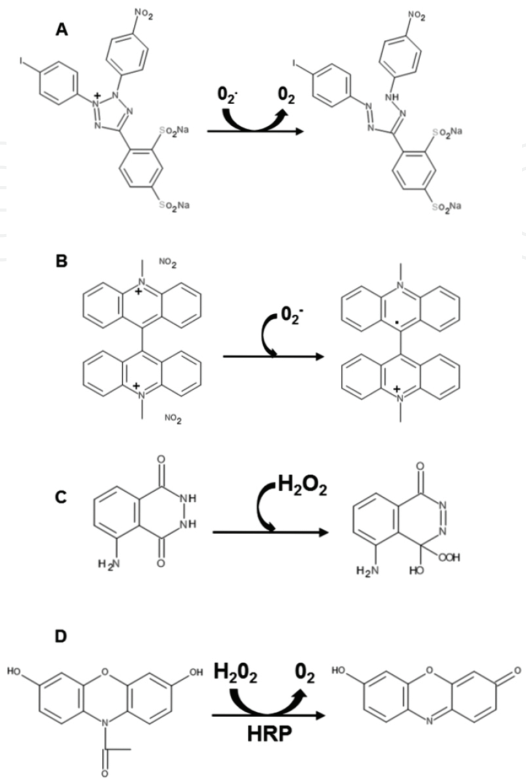

Figure 5. Reactions for the determination of $O_2^{\bullet-}$ and H_2O_2. (A) Reduction of WST-1 by $O_2^{\bullet-}$ to a water-soluble yellow formazan. (B) Reduction of lucigenin by $O_2^{\bullet-}$ to a lucigenin cation radical. (C) Oxidation of luminol by H_2O_2. (D) Oxidation of Amplex red by H_2O_2 in the presence of HRP to produce resorufin.

Lucigenin and luminol. These substances are selectively employed to determine the amounts of extracellular $O_2^{\bullet-}$ and intracellular $O_2^{\bullet-}$ and H_2O_2 by chemiluminescence. Lucigenin is selective for $O_2^{\bullet-}$, and luminol is selective for $O_2^{\bullet-}$ and H_2O_2. Lucigenin is reduced by $O_2^{\bullet-}$ to a lucigenin cation radical independently of peroxidase activity (**Figure 5B**), and luminol is oxidized using a peroxidase such as myeloperoxidase (MPO) or horseradish peroxidase (HRP) (**Figure 5C**).

5.3. Hydrogen peroxide determination

To determine the amount of H_2O_2, electrodes can be used, and the amount of H_2O_2 can then be determined polarigraphically. The sensitivity of the electrode allows precise and rapid measurement of extracellular H_2O_2. Other probes include the use of targets and are based on the ability of H_2O_2 to oxidize molecules such as Amplex red (N-acetyl-3,7-dihydroxyphenoxazine), scopoletin, and homovanillic acid in the presence of HRP. There are also many other techniques that allow the determination of the amount of H_2O_2, such as aryl-borate-based probes, peroxy Lucifer, and others; however, Amplex red is one of the most used. Amplex red is a non-fluorescent compound that is oxidized by H_2O_2 in the presence of HRP to produce resorufin, which is colored and highly fluorescent at 587 nm (**Figure 5D**). Amplex red has been used to measure H_2O_2 production by microglial cells and also directly *in vitro* to measure H_2O_2 production by Aβ during its interaction with metals such as copper [97].

6. Conclusion

It has been demonstrated that $A\beta_{1-42}$ is one of the principal biomolecules that contributes to the development and progression of AD due to its ability to generate ROS by its interaction with metals and also due to its ability to activate specific cells, producing neuroinflammation and consequently neurodegeneration. Therefore, therapeutic treatments to avoid Aβ production should be developed by the design of selective inhibitors of the β-secretase BACE-1.

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