

Methemoglobinemia—A biomarker and a link to ferric iron accumulation in Alzheimer's disease

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ABSTRACT

Understanding the mechanism of oxidative stress is likely to yield new insights regarding the pathogenesis of Alzheimer's disease (AD). Our earlier work focused on the difference between hemoglobin and methemoglobin degradation, respectively leading to ferrous (Fe^{2+}) iron, or ferric (Fe^{3+}) iron. Methemoglobin has the role of carrier, the donor of cytotoxic and redox-active ferric (Fe^{3+}) iron, which can directly accumulate and increase the rate of capillary endothelial cell apoptosis, and may cross into the brain parenchyma, to the astrocytes, glia, neurons, and other neuronal cells (neurovascular unit). This supposition helps us to understand the transport and neuronal accumulation process of ferric iron, and determine how iron is transported and accumulated intracellularly, identifiable as “Brain rust”. Earlier research found that the incidences of neonatal jaundice ($p = 0.034$), heart murmur ($p = 0.011$) and disorders such as dyslalia and learning/memory impairments ($p = 0.002$) were significantly higher in those children born from mothers with methemoglobinemia. Our hypothesis suggests that prenatal iron abnormalities could lead to greater neuronal death, the disease ageing process, and neurodegenerative disorders such as AD and other neurodegenerative diseases.

KEYWORDS

Alzheimer's Disease (AD); Apoptosis; Blood-Brain

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Barrier (BBB); Brain Capillary Ferric Iron Deposition; Hemoglobin and Methemoglobin Catabolism; Neurodegenerative Brain Disease; SIDS

1. INTRODUCTION

The mechanisms responsible for redox-active iron accumulation in some regions of the brain in Alzheimer's disease (AD) are unknown, nor if it is an initial event that causes neuronal death or a consequence of the disease process. However, little is still known about the chemical form of iron associated with neurodegenerative diseases, its role in neurodegeneration (if any) and its origin. Our goal is to understand iron-induced oxidative stress and point out the deleterious effects of redox-active ferric iron as a final product from methemoglobin and heme degradation. Ferric iron has a direct impact on capillary brain endothelial structure and function and in consequence of apoptosis brain parenchyma atrophy finally follows, so pinpointing specific clinical determinants for the onset and progression of AD.

Ferric-Iron Brain Accumulation as a Cause of Neurodegenerative Brain Disease: A New Insight in Understanding the Mechanism of Iron Transport

Continuously inhaling strong oxidants such as NO_x (NO and NO_2) reversibly oxidizes oxyhemoglobin (Fe^{2+}) to methemoglobin (Fe^{3+}), and irreversible methemoglobinemia results as a disorder from the impacts of the me-

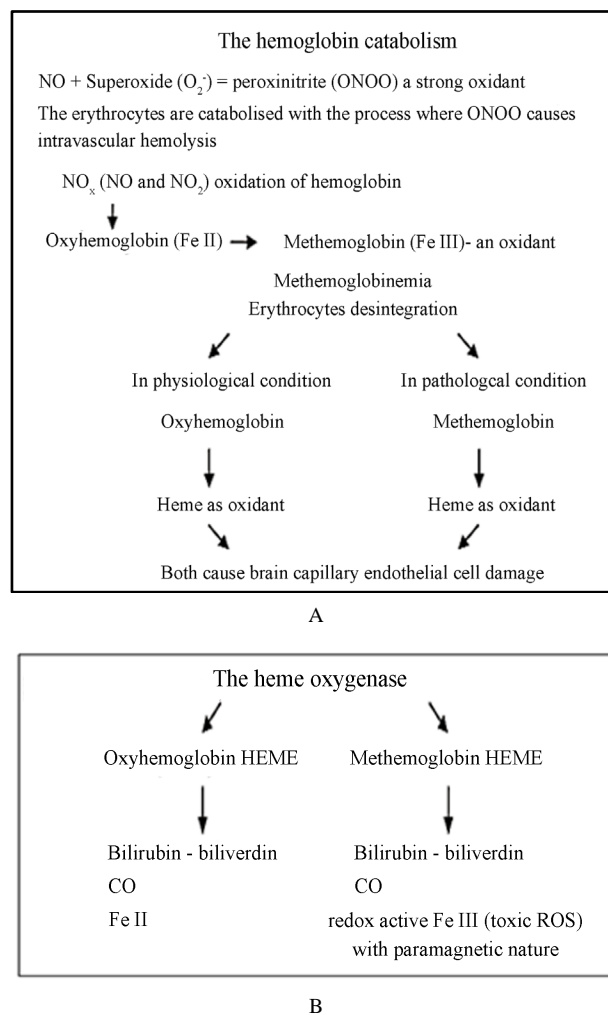
tabolic synergy of nitrogen oxides such as oxidants (RNS) and sulfur dioxide metabolites (RSS) as inhibitors of thiol antioxidants [1].

Methemoglobin by itself, and heme, have prooxidant properties and induce structural and functional changes in the vascular endothelium [2,3]. These changes include cell growth arrest, senescence, morphological alterations and cell apoptosis, and they lead to both vessel thrombosis and endothelial cell denudation under the influence of redox-active ferric iron (Fe^{3+}), as a product of heme-oxygenase, responsible for methemoglobin-heme degradation [4].

There is a striking difference between physiological hemoglobin catabolism, and pathological methemoglobin catabolism, because of the different final products, respectively ferrous and ferric iron, with distinct characteristics (Figures 1A and B). Ferrous iron form has the potential for catalyzing and generating highly cytotoxic hydroxyl radicals shown as Fenton reactions ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^\bullet + \text{OH}^- + \text{Fe}^{3+}$). The substantial difference of intracellular ferric iron originates from the physiological Fenton reaction, and ferric iron originates from pathological methemoglobin catabolism occurring as the level of methemoglobin cellular uptake increases, and the resulting methemoglobinemia causes ferric iron-induced oxidative stress injury. We consider that the final product of ferric iron from methemoglobin catabolism is a significant added source to Fenton reaction derived from ferric iron, whose continuous formation impacts the brain and confirms the statement that iron and iron-induced oxidative stress constitutes a common mechanism in the development of neurodegeneration.

Our former research results suggest that methemoglobin plays a particularly important role as a carrier and donor of cytotoxic redox-active ferric (Fe^{3+}) iron, and determines how iron is transported intracellularly. Neuroscience has traditionally focused on the neurons of the central and peripheral nervous systems, and it is now becoming clear that neurons, glia and microvessels are organized into a well-structured **neurovascular unit**, and recent studies have highlighted the importance of brain endothelial cells in this modular organization [5].

Leung and Moody [6] have demonstrated that ferric heme is significantly more pro-oxidant than ferrous heme. In tandem, this evidence suggests that the MR imaging-detected T1 high signal intensity within the vessel wall is an endogenous biomarker of an intraplaque environment that is highly pro-oxidant and proatherogenic. MR imaging measures showed a T1 relaxivity that was 10 times higher for ferric than for ferrous forms of hemoglobin. Their results support the hypothesis that ferric methemoglobin-generated T1 high signal intensity reflects a pro-oxidant environment that, in the setting of vessel wall disease, might be proatherogenic. This justifies the study of the oxidant effect of methemoglobin catabolic



Source: Balla J. *et al.* Proc Natl Acad Sci USA 1993, 90:9285-9; Denicola A. *et al.* Proc Natl Acad Sci USA 1998, 95:3566-71; Jeney V. *et al.* Blood 2002; Umbreit J. Am J Hematol 2007, 100:879-87.

Figure 1. Difference between physiological hemoglobin catabolism and pathological methemoglobin catabolism.

products on vital organs and the CNS, resulting in their dysfunction. The research MRI data showed that extracellular methemoglobin generates significantly more lipid oxidation than intracellular products. However methemoglobin in both these environments has similar measures of r1. Therefore, the T1 high signal intensity due to methemoglobin is not solely restricted to an environment that causes lipid oxidation. Thus, the ability of this high signal intensity to reflect at-risk plaque may be diminished. However, it is known that in the absence of any chemical modifications, ferric heme substantially degrades the integrity of red blood cells (RBC) and the membrane, and the eventual fate of static RBC is lysis. Thus, intracellular methemoglobin is destined to rapidly become extracellular, thereby adding to oxidative drive [7]. The cellular and intercellular iron transport mechanisms in the CNS are still poorly understood, while accu-

mulating evidence suggests that impaired iron metabolism is an initial cause of neurodegeneration [8]. Brar *et al.* [9] concluded that the development of parkinsonism during the course of AD appears to be associated with the accumulation of iron, which in turn may contribute to the pathogenesis of neurologic decline.

2. METHODS AND RESULTS

The present standpoint derived from literature and proper research results showed that continuous exposure to inhaled toxic substances in addition to oxidants by food, water, drugs and their associated cumulative effects, causes increased oxidative stress. It postulates the role of environmental toxic factors on the endothelial small vessels of the brain, increasing the rate of endothelial cell apoptosis and making possible the transport of methemoglobin and heme derived ferric iron across the blood-brain-barrier (BBB), and its accumulation in the brain parenchyma.

Our results point out the consequence of toxic environmental oxidants, caused by brain damage with a view to the role of methemoglobin catabolism in pregnancy as the source of ferric (Fe^{3+}) iron concentrated in various brain regions.

Methemoglobin and hemolysis both occur as a result of oxidative stress, but the prevalent difference between them is that methemoglobin is a reversible phenomenon (oxidant-antioxidant balance) whereas hemolysis, which occurs as a result of oxidative stress on the erythrocyte membrane, is an irreversible event. Methemoglobinemia can additionally exacerbate an existing anemia, stimulating hypoxia that may be additionally dangerous. Our prospective study of methemoglobin in pregnancy, revealed a significant rise in the level of methemoglobin $>1.5 \text{ g/L}$ ($r = 0.72$, $p < 0.01$) in the air-polluted exposure period, which can be explained on the basis of an oxidant-antioxidant imbalance, resulting in methemoglobinemia [10]. Methemoglobinemia and stillbirth recorded throughout the exposure period are significantly higher than those recorded in the control period ($p = 0.0205$) and the frequencies of reproductive loss were significantly lower in the *control* than in the *exposure* period ($p < 0.05$) [11].

As we have found no evidence of the consequences of maternal methemoglobinemia on the fetus, the second objective was to direct attention to methemoglobin. As oxidants possess the capacity to cross the damaged fetomaternal placental barrier, for instance from the methemoglobin catabolism ferric iron placental chorionic deposition appearance (Figure 2), “fetal preeclampsia” is an expected manifestation. Under these adverse conditions, ferric iron positivity in capillary endothelial cells of the BBB in the fetus rises, resulting in preterm birth, stillbirth or early neonatal death (Figure 3). As an early

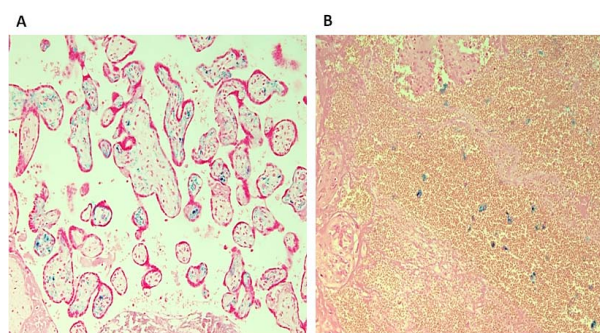


Figure 2. Positive ferric iron deposits in an intervillous hemorrhage (A) and in placental chorionic villi stroma (B). Placenta from a stillbirth after 38 weeks of gestation. Mother MetHb $> 1\%$. Staining: Perl's Prussian Blue reaction for ferric iron—Magnification: (A) 4 \times ; (B) 20 \times .

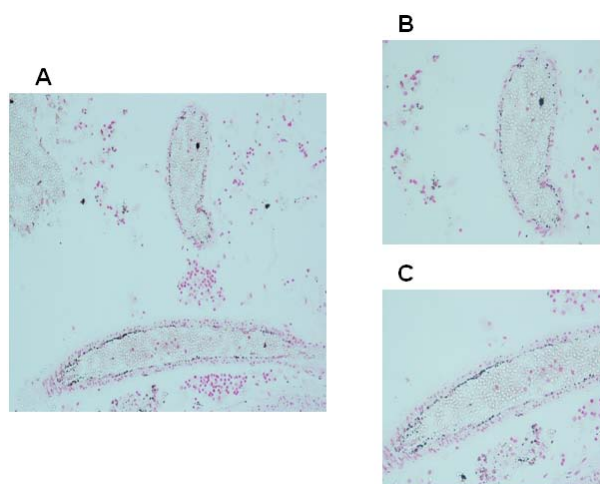


Figure 3. Iron positivity in capillary endothelial cells of the blood-brain barrier in a victim of sudden intrauterine unexplained death (SIUD) aged 36 gestational weeks. Staining: Perl's Prussian Blue reaction for ferric iron—magnification: A) 10 \times ; B) and C) 20 \times

biomarker of environmental toxic and oxidative stress effects, this puts pregnancy at risk and may later impair the health of newborns, children and adolescents. Obtained proper research found an incidence of neonatal jaundice ($p = 0.034$), heart murmur at a later age ($p = 0.011$), as well as child and adult mild disorders such as dyslalia and learning/memory impairments ($p = 0.002$) significantly higher than in the children and adults of control mothers without pregnancy methemoglobinemia [12]. Lavezzi, Mohorovic *et al.* recently presented findings, confirmed by pathohistological techniques, that under adverse conditions, ferric iron positivity in capillary endothelial cells of the BBB in the fetus rises, also resulting in preterm birth, stillbirth or early neonatal death [13]. The application of the Blue Prussian method highlighted accumulations of blue granulations, indicative of nonheme Fe^{3+} -positive reactions in the brainstem

and cerebellum of 12 (33%) of the 36 sudden intrauterine unexplained death (SIUD) and sudden infant death syndrome (SIDS) victims and in none of the control group. In positive cases, iron deposits were widespread in the brain parenchyma or localized in specific areas showing variable extent and intensity (Figures 4 and 5). The positivity of free iron in the cerebral parenchyma of a patient with Alzheimer's disease is represented in Figure 6. In the same cases of their previous study [13], Lavezzi, Mohorovic *et al.*, by applying the TUNEL method, highlighted in the same cases showing iron deposition in the capillary endothelial cells of the BBB and in the brain parenchyma, high percentages of apoptotic cells in the ventricular barriers, and precisely in the ependyma and in choroid plexus epithelial cells (Figures 7 and 8).

3. DISCUSSION

Proper research results suggest that methemoglobin plays an important role as a biomarker, a carrier and a donor of cytotoxic and redox-active ferric iron, and determines how iron is transported and accumulated intracellularly, identifiable as "Brain rust".

We highlight the role of methemoglobin and heme catabolism to produce ferric iron with cytotoxic and paramagnetic properties. We propose that ferric iron and ferric iron-induced oxidative stress constitutes a common mechanism in the development of neurodegeneration, and also suggests an initial cause of neuronal death. The experiments showed that ferric and ferrous iron can enter cells via different pathways; they do not indicate which pathway is dominant in humans [14].

Smith *et al.* suggest that iron is able to participate in "*in situ*" oxidation and readily catalyzes an H_2O_2 -dependent oxidation, and indicate that iron accumulation could be an important contributor to the oxidative damage of Alzheimer's disease [15]. Our work, from our standpoint, supports the above statement about the importance of disturbed heme metabolism. Heme oxygenase-1, an en-

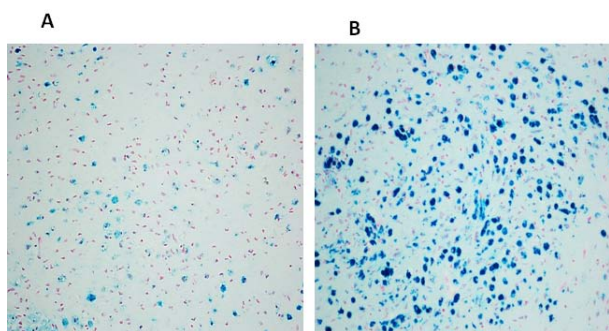


Figure 4. Free iron positivity in the medullary parenchyma of a 7 month-old victim of SIDS. (A) widespread distribution of positive neurons. (B) accumulation of strong iron-positive neurons. Staining: Perl's Prussian Blue reaction for ferric iron—Magnification: (A) and (B) 20 \times .

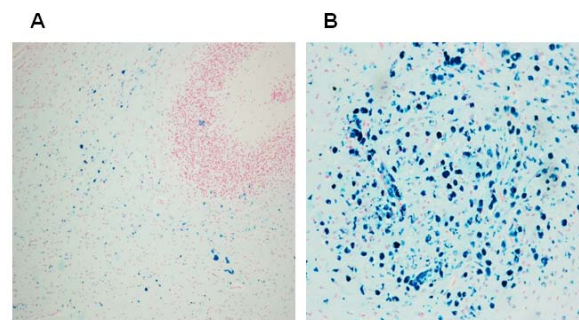


Figure 5. Free iron positivity in the subcortical region (A) and parenchyma (B) of the cerebellum of a 6 month-old victim of SIDS (case no.9). Staining: Perl's Prussian Blue reaction for ferric iron—Magnification 20 \times .

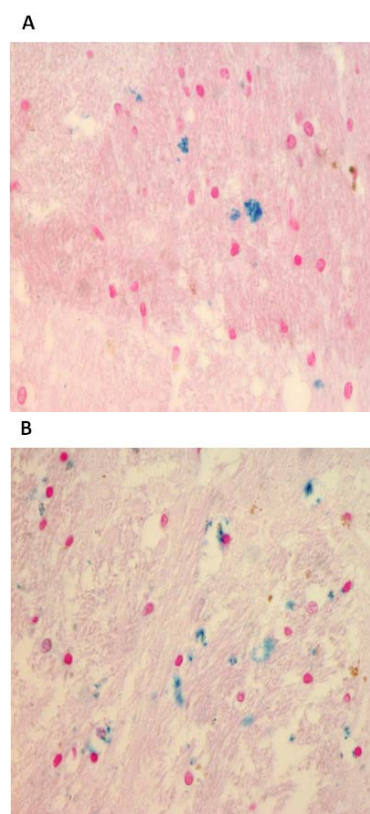


Figure 6. Free iron positivity in the cerebral parenchyma of a 77 year old patient with Alzheimer's disease A) Focal distribution of positive glia and neurons; B) accumulation of strong iron-positive perivascular regions. Staining: Perl's Prussian Blue reaction for ferric iron—Magnification: 40 \times .

zyme that catalyzes the conversion of methemoglobin and heme to ferric iron, is increased in Alzheimer's disease, suggesting heme turnover as a source of redox-active iron. Perry *et al.* have found that while mitochondrial DNA and cytochrome C oxidase protein are increased in Alzheimer's disease, the number of mitochon-

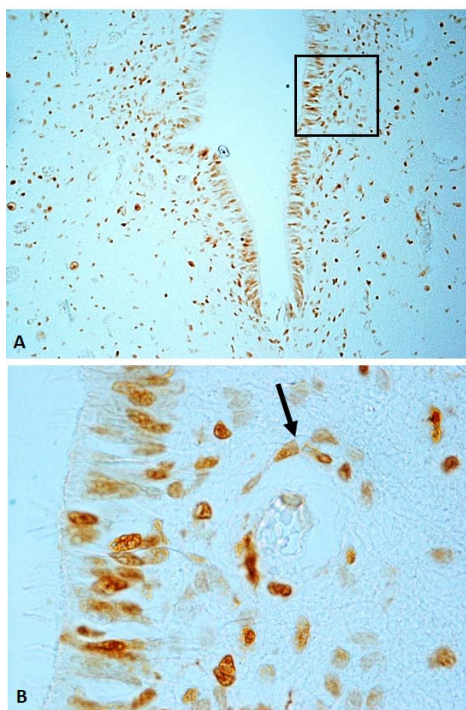


Figure 7. Floor of the fourth ventricle showing high expression of apoptosis in the ependyma and subependymal parenchyma. The framed area in (A) is represented at higher magnification in (B). The arrow indicates apoptotic endothelial cells of a blood vessel. Immunostaining: TUNEL method. Magnification: (A) 10 \times ; (B) 100 \times .

dria is decreased, indicating accelerated mitochondria turnover, and suggests mitochondrial dysfunction as a potentially inseparable component of the initiation and progression of Alzheimer's disease [16]. It was also found that oxidative damage occurs primarily within the cytoplasm rather than in mitochondria. From this originates the hypothesis that mitochondria, as a source of hydrogen peroxide, an intermediate, once in the cytoplasm, will be converted into highly reactive hydroxyl radicals through the Fenton reaction in the presence of metal ion, causing damage to the cytoplasm [17,18]. Various pathologies can result from oxidative stress-induced apoptotic signaling, consequently leading to ROS increases and/or antioxidant decreases, the disruption of intracellular redox homeostasis, and an irreversible impact on oxidative modifications of lipid, protein, or DNA [19]. Furthermore, iron participates in different pathological processes through the Fenton reaction. To test the hypothesis that this reaction accelerates apoptosis, Jacob *et al.* used human umbilical vein endothelial cells (HUVECs) as surrogates for the microvasculature *in vivo*. HUVECs were loaded with Fe [III] (ferric chloride and ferric ammonium citrate), causing apoptosis after heat shock stimulus [20]. Accordingly, in our studies on vic-

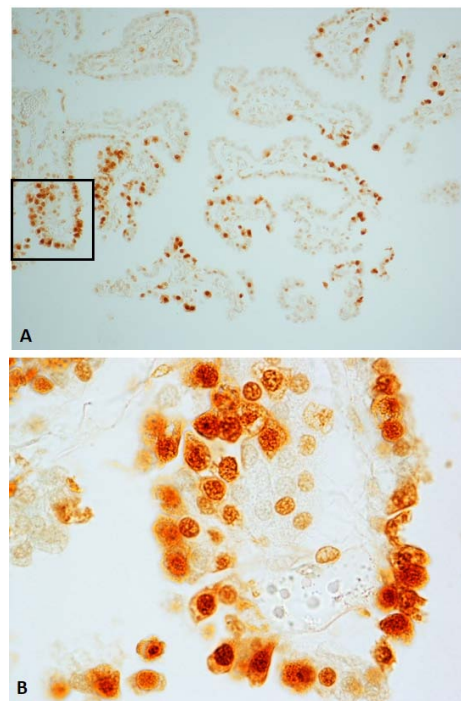


Figure 8. Choroid plexus of the fourth ventricle showing high expression of apoptosis in the epithelial cells. The framed area in (A) is represented at higher magnification in (B). Immunostaining: TUNEL method. Magnification: (A) 10 \times ; (B) 100 \times .

tims of sudden perinatal death (SIUD and SIDS), we observed that, under adverse hypoxic conditions, iron deposition was associated to apoptotic degeneration of the brainstem lining. Brain iron is a major contributor to the MRI contrast in normal gray matter. Non-heme brain iron is present mainly in the form of ferritin. The quantitation of non-heme brain ferric iron indicated by MRI helps in the diagnosis and monitoring of different neurological diseases [21]. Most brain non-heme iron is believed to be present as a storage pool, consisting of ferritin or hemosiderin, and also as a product of methemoglobin catabolism [22]. This fact suggests considering the role of methemoglobin catabolism as the source of ferric iron (FeIII) form concentrated in various brain regions. Nowadays, non-heme-bound Fe³⁺ is quantified using MRI, thanks to its paramagnetic properties. It is believed that most non-heme-bound iron is deposited in the form of ferritin, haemosiderin, or methemoglobin catabolic products, whereas transferrin-bound iron concentration is always low and can not be detected by MRI [23]. Recent research results indicate a ferrihydrite-magnetite core-shell ferritin structure. It was also found that magnetite in the ferritin iron core is not a source of free toxic ferrous iron, as previously believed. Therefore, the presence of magnetite in the ferritin cores of patients with Alzheimer's disease is not a cause of their increased brain ferr-

ous iron(II) concentration [24].

4. CONCLUSION

A proper statement should emphasize the role of methemoglobin and heme which by themselves have prooxidant properties. An abundant source of cytotoxic and redox-active ferric (Fe^{3+}) iron, without ferrous-ferric inversions “*in situ*”, as a cause of iron-induced oxidative stress has a direct and specific impact on the endothelial small vessels of the brain, and increases the rate of endothelial cell apoptosis, making it possible to cross over from methemoglobin and heme derived ferric iron and accumulate in the brain parenchyma. In conclusion we point out the importance of methemoglobinemia not only as a biomarker and precursor of environmental oxidants’ noticeable effects, but also as a carrier and donor of redox-active ferric iron form. We point out ferric iron as an originator of brain parenchyma accumulation, having an important role in crossing the brain microvessels to the neurons (**neurovascular unit**), causing neuronal death, continuous ageing process, and leading finally to hard neurodegenerative disorders such as AD, parenchyma and other diseases. Nevertheless our findings in relation to environmental oxidants and the pathogenesis of neurodegenerative diseases need further research confirmation.

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