

Body Iron Overload is a Determining Factor in Brain Damage in Acute Ischemic Stroke

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Introduction

Stroke is the second largest cause of death worldwide, with a world annual mortality incidence of about 5.5 million people, and it is also the leading cause of disability worldwide with 50% of survivors being chronically disabled [1]. It is estimated that one in four people will experience a stroke in their lifetime [2]. Strokes may be classified into two general types: ischemic and haemorrhagic. Ischemic stroke is caused by a reduction or an interruption of the blood supply to a certain region of the brain due to the obstruction of a blood vessel. In contrast, haemorrhagic stroke occurs due to the rupture of a blood vessel causing bleeding in the brain or in the subarachnoid space [3]. This short communication is centered on ischemic stroke, which is the most prevalent form of stroke worldwide [1].

Cerebral injury derived from ischemic stroke initially results from a transient reduction in local blood flow, which leads to a severe lack of oxygen and glucose in the infarcted area and is characterized by complex spatial and temporal events evolving over hours or even days [4]. The neuropathological analysis after local brain ischemia reveals two separate areas: the core of the infarction and the ischemic penumbra, i.e., its surrounding zone [5]. In the core area, the massive reduction in blood flow leads to a breakdown of metabolic processes causing a reduction in the cell energy supply and an imbalance in ion homeostasis. These processes involve a series of molecular pathways and signalling mechanisms that finally lead to the loss of cell integrity and cell death within minutes. Functional studies have identified the ischemic penumbra as a rim of tissue that is hypoperfused around the ischemic core [5]. Bordering blood vessels in the penumbra zone provide sufficient blood flow to maintain metabolism and structure of neurons but not their physiological functions. For example,

although the blood flow in the penumbra is enough to preserve the integrity and the activity of the ion channels in the cells, it is too low to maintain the neuron electrical activity [6]. The progression of the neurological deterioration seems to be associated with the evolution of the ischemic penumbra towards a definitive necrosis [7,8]. The pathophysiological mechanisms involved in cerebral ischemia are multifactorial and complex [5,7-9] and several factors have been associated with a greater brain injury following the stroke [5,10].

Iron Homeostasis and Ferritin

In the brain, the iron plays a key role in the synthesis of neurotransmitters and in the axon's myelination. The main attribute that makes iron essential for life is its redox activity: it readily accepts and donates electrons, alternating between Fe^{3+} and Fe^{2+} ions. Paradoxically, the same redox properties that allow iron to be a functional enzymatic cofactor also make it a key participant in oxygen-mediated toxicity [11,12]. Iron redox-active can generate reactive oxygen species (ROS), leading to oxidative stress and the initiation of signalling pathways crucial for cell survival and cell death [13]. To avoid this harmful effect, there is a tight regulation of the iron in the brain cells because excessive accumulation of free cytosolic ferrous iron increases the possibility of oxidative stress. Virtually all iron is tightly bound to proteins such as transferrin and ferritin, which control iron reactivity [14].

It is widely recognized that deregulation of brain iron homeostasis, generally associated with iron accumulation in the brain, acts as a mediator of neuronal damage in important neurodegenerative diseases including stroke [12,15-17]. Excess of iron results in tissue damage due to the generation of oxygen radicals, which are capable of damaging biomolecules such as lipids, carbohydrates, proteins and nucleic acids

[18]. Iron is involved in the mechanisms that underlie neurodegenerative diseases. Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, all of them are neuropathologies that differ in the causes that originate them, but all share oxidative stress as one of the main mechanisms that lead to the death of neurons. Iron accumulation in the brain is other common in this neuropathologies [17]. Iron is thought to contribute to cell death pathways through reactive oxygen species production [19]. Cell death by iron overload is thought to be the result of ROS generation, particularly in the lysosome. Iron-overloaded cells accumulate iron inside lysosomes as a consequence of the degradation of iron-loaded ferritin. Inside the lysosome, iron can cycle between Fe^{3+} and Fe^{2+} , generating ROS and leading to lysosomal membrane permeabilization and DNA damage. Cathepsin and other lysosomal proteases induce release of mitochondrial cytochrome c and other pro-apoptotic proteins, leading to downstream caspase activation and apoptosis [16].

The control of iron availability in the cells is largely dependent on ferritin [12], which is the main protein storing iron in the tissues [20]. Small amounts of ferritin are released from the cell, the amount secreted being strongly correlated with the quantity of intracellular iron, which makes serum ferritin concentrations an accurate indicator of body iron stores [21]. Ferritin is a 24-subunit protein shell that encloses a hollow interior in which large amounts of non-toxic soluble iron are stored to be available on demand. A ferritin molecule can store 4,500 Fe^{3+} ions, although it usually contains about 2,500 [20]. Ferritin consists of two types of polypeptide chains, light (L) and heavy (H), in varying amounts. Catalytically inactive subunit forms, called L-type subunits (19 kD) assemble with catalytically active H-type subunits (21 kD). H subunits have an iron-binding site which exhibits ferroxidase activity and can catalyze the oxidation of Fe^{2+} to Fe^{3+} . In contrast, L subunits are thought to function in the nucleation of the mineral core within the protein shell [22]. Many aspects of the biological function and the pathophysiology of ferritin remain unknown [20]. For example, little is known about the ferritin modifications affecting the protein shell and the core composition, and the possible influences that these modifications could have on ferritin function in the human brain and their implications in some neurological diseases.

Under normal physiological conditions the response to a cytoplasmatic increase of Fe^{2+} is a quick uptake in ferritin, where it is stored in a redox-inert form of iron oxyhydroxides containing only Fe^{3+} ions, thus protecting cells against oxidative damage [20,23]. This process of Fe^{2+} oxidation inside the ferritin molecule takes place in specific ferroxidase sites in the H subunit [24]. Comparative structural and chemical studies of ferritin cores point to the existence of ferritins in which the ferrous ion is also part of the core because of a defect in the ferroxidase activity of the H subunit. The presence of ferrous iron inside ferritin could mean that the mineralization process is perturbed, for example that the

enzymatic oxidation of ferrous iron inside ferritin is faulty [25]. The existence of these differences in the core of ferritins might be of biomedical interest due to different patterns found in physiological and pathological conditions.

Changes in the iron composition of the ferritin core (*pathological ferritin*) and its distribution in brain tissue have been described in the brain ferritin of patients with Alzheimer's (AD) and Parkinson's (PD) disease compared with *physiological ferritin*. Furthermore, the modification of the H to L chain proportion, reported in AD and PD, could be a cause of the alterations in the mineralization of the ferritin cores, and this fact may be associated with aging processes and especially with neurological diseases. The analysis of the core of pathological ferritins showed significant differences in the mineral composition compared to the physiological ones. In the brain of AD patients, a higher ferritin content and alterations in iron mineralization in the amygdala and hippocampus have been found compared with healthy aged subjects [25].

Ferritin and Oxidative Stress

Depending on the iron needs of the cells, ferritin can also release stored iron, acting as an important source of free iron. Iron release from ferritin is facilitated by the presence of superoxide radicals [26], nitric oxide [27] and acidic pH [28], all of which are increased in the ischemic region during a stroke [5]. In this environment, the presence of exogenous ferritin is decisive in the formation of reactive iron that can exacerbate ROS production and increase oxidative damage [29] making ferritin a powerful oxidizing agent. Oxidative stress is a major factor in the pathology of neurodegenerative disorders [5,7,30].

Stroke and Iron Overload

Several lines of research indicate that oxidative stress is a primary mediator of neurological injury following cerebral ischemia [30,31]. The relationship between oxidative stress and neuronal death has been extensively investigated and it has been found that oxidative stress opens the mitochondrial permeability transition pore which can, in turn, further stimulate ROS production, exacerbate energy failure, and release mitochondrial pro-apoptotic factors such as cytochrome c into the cytoplasm [32]. Although it is well established that iron plays a critical role in neuronal injuries caused by oxidative stress under ischemia, the exact mechanism is not well understood.

Clinical [33-35] experimental [28,36,37] and epidemiological [38,39] studies have shown that the worst outcomes after a stroke are associated with high contents of ferritin in plasma and in the cerebrospinal fluid (CSF). Initially, these results were attributed to an accumulation of iron in the brain. However, experimental studies in rats subjected to dietary

iron supplementation, which caused an overload of iron in the systemic tissues and high levels of ferritin in plasma, did not report a high iron content in the brain [36,37,40].

An explanation for these results may be that in mammals brain iron homeostasis differs from systemic iron homeostasis [12,41]. It is worth noting that the iron content of the brain appears to be independent of the systemic iron status. Under conditions of systemic iron overload induced by dietary iron supplementation [36,40,42] and in subjects with hemochromatosis (a disease characterized by an excessive accumulation of iron in systemic tissues and the presence of high levels of ferritin in the blood), an accumulation of iron in the brain or ferritin in the CSF has not been reported [43,44]. This indicates that the brain is well protected against body iron overload, which may be attributed to the fact that the central nervous system (CNS) is separated from the systemic circulation by the blood-brain barrier (BBB), at the capillary endothelium, and the blood-CSF barrier, at the choroid plexus epithelium [45,46]. In the brain vasculature there are tight junctions between the adjacent endothelial cells lining the vessel lumen. This junctional complex creates a strong barrier to water and solutes allowing the molecules that flow across the BBB to be regulated. The iron flux from blood into brain is controlled by the BBB and, to a lesser extent, by the blood-CSF barrier [47,48], since only a small proportion of the total transport into the brain occurs via the choroid plexus [49], the transferrin-transferrin receptor system in the endothelial cells of the BBB capillaries being the main mechanism by which iron flows from the blood to the brain. Thus, brain iron levels are highly regulated to ensure the normal function of the CNS [50] protecting the brain against iron overload. However, during stroke, integrity of the BBB is disrupted in the infarcted area [45,51].

The exogenous ferritin-bound iron is decisive in the formation of reactive iron that can exacerbate ROS production thereby increasing oxidative damage [26,29,52,53]. The exogenous ferritin increased the excitotoxic effect caused by excessive exogenous glutamate whereas extracellular apoferritin, the protein form without iron, does not increase the excitotoxicity of glutamate, which proves that iron was responsible for the neurotoxic effect of the exogenous ferritin. Extracellular ferritin iron exacerbates the neurotoxic effect induced by glutamate excitotoxicity and that the effect of ferritin iron is dependent of glutamate excitotoxicity [54].

These results suggest that, in conditions such as those found in the ischemic environment, the exogenous ferritin-bound iron may be a source of free iron that increases free radical production causing high levels of oxidative stress and provoking neuronal death after a stroke. Under conditions of body iron overload, the presence of high levels of serum ferritin leads to an accumulation of ferritin and free radicals in the endothelium of the cerebral capillaries and in the epithelium of the choroid plexus [45,37] without

an increase in iron in the brain. When a stroke occurs, once vessel occlusion is produced, the disruption in the blood flow decreases the delivery of oxygen and metabolic substrates to the neurons of the affected areas. Consequently, this lack of oxygen interrupts mitochondrial oxidative phosphorylation drastically, reducing ATP production. After several minutes, the inhibition of the Na⁺/K⁺-ATPase causes a profound loss of ionic gradients and the depolarization of the neuronal membranes, leading to an excessive release of excitatory amino acids (particularly glutamate) in the extracellular compartment. The presence of excessive amounts of glutamate in synaptic and extrasynaptic sites can eventually lead to neuronal death [4]. Excitotoxicity causes several deleterious consequences, including generation of free radicals and oxidative stress, mitochondrial damage, and activation of several transcription factors and their gene expression [55]. All these mechanisms acting additively or synergically can cause neuron death by apoptosis. Thus, oxidative stress induced by excitotoxicity is the main event leading to brain damage after stroke. In patients with body iron overload, the BBB disruption in the ischemic penumbra [45,51] enables that the ferritin accumulated in the microvasculature and in the plasma can reach the ischemic area where the presence of superoxide radicals [26], nitric oxide [27] and acidic pH [28] caused by excitotoxicity, favor the release of iron from ferritin [51,53]. This free iron immediately increases the generation of radical hydroxyl via the Fenton reaction, increasing oxidative stress and exacerbating the above-described cascade of events that lead to neuronal death and explaining why a greater neurological injury is present in stroke patients with body iron overload than in stroke patients without alterations in iron body metabolism.

Several pathways are thought to initiate BBB dysfunction, which include excitotoxicity, neutrophil recruitment, and mitochondrial alterations, all of them converging at the same point and causing increased production of ROS and oxidative stress. Interestingly, ROS also provide a common trigger for many downstream pathways that directly mediate BBB alteration, such as oxidative damage, tight junction modification, and activation of matrix metalloproteinases [56]. These observations suggest that ROS could be key mediators for BBB breakdown in stroke. A possible contribution of iron overload as a causative agent in the breakdown of the BBB requires the presence of free iron in the blood, participating in the formation of ROS. This would imply that iron overload could be a factor causing BBB dysfunction regardless of stroke, something that has not been demonstrated.

Although a deregulation in brain iron metabolism is known to be implicated in many neurodegenerative disorders [12,17,57], it is noteworthy that the higher neurotoxic effect caused by ferritin iron in stroke is due to iron overload in systemic tissues prior to stroke and not to an accumulation of iron in the brain [36,42].

Conclusion

This short communication reviews evidence supporting the idea that higher serum and CFS ferritin levels are associated with increased neurological damage caused by stroke. Stroke patients with previous increased serum ferritin concentration have a higher risk of poor clinical outcome. The greater early neurological deterioration observed in patients with high serum ferritin is not related to the different iron content in the brain tissue before the onset of stroke, iron being involved in the acceleration of neurological deterioration of systemic origin. The relationship between ferritin and stroke is mediated via iron metabolism: when stroke occurs, extracellular ferritin enhances excitotoxicity and oxidative stress, while apoferritin does not.

Due to its high prevalence, stroke poses a major public health challenge. Although there is not enough data on the proportion of stroke patients having high serum ferritin levels, the evidence reviewed in this short communication advises the need to include the measurement of ferritin levels in blood tests. An integrative clinical approach, including strategies for iron systemic control as well as the promotion of healthier lifestyle habits, would prevent iron overload. Since there are no physiological processes involved in the excretion of excess iron, once iron overload has occurred, phlebotomy and the use of iron chelators are possible ways to reduce iron overload. However, given the complex processes involved in the highly regulated iron homeostasis, future research should be aimed to clarify the determinants of serum ferritin and iron excess in the healthy adult population.

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