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STROKE (JF MESCHIA, SECTION EDITOR)

Rationale and Current Evidence for Testing Iron Chelators for Treating Stroke

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Abstract

Purpose of Review To discuss the mechanisms of iron regulation in the brain and the pathophysiological role of deregulation of iron homeostasis following a stroke, and to review existing evidence supporting the potential role of iron chelators in the treatment of ischemic and hemorrhagic stroke.

Recent Findings In recent years, accumulating evidence has highlighted the role of neuroinflammation in neurological injury after ischemic and hemorrhagic stroke, and that free iron is central to this process. Via the Fenton reaction, free iron catalyzes the conversion of superoxide ion and hydrogen peroxide into hydroxyl radicals, which promote oxidative stress.

Summary Advances in our understanding of changes in brain iron metabolism and its relationship to neuronal injury in stroke could provide new therapeutic strategies to improve the outcome of stroke patients. Pharmacological agents targeting brain iron regulation hold promise as potentially effective treatments in both ischemic and hemorrhagic stroke.

Keywords Iron · Chelators · Ischemic stroke · Cerebral hemorrhage · Inflammation · Neuroprotection

Introduction

Iron is essential for multiple biological functions. It plays a critical role in the electron transport chain for aerobic respiration, as well as oxygen transport in hemoglobin. In the brain, iron homeostasis is critical. Free iron, released in a variety of neurotoxic settings including ischemic and hemorrhagic stroke, results in deregulation of brain iron homeostasis and drives the pathophysiology of neurological injury seen in these conditions. This review will focus on the role iron dysregulation plays in stroke and emerging data supporting a potential role for iron-modifying drugs as therapies to improve clinical outcomes after ischemic and hemorrhagic stroke.

This article is part of the Topical Collection on *Stroke*

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Iron Homeostasis in the Brain

The body has about 5 g of iron in total, and despite the fact that only 2% of this resides within the brain, homeostasis of iron is essential for virtually all of the normal cerebral physiological functions of the brain, ranging from enzymes required for aerobic respiration and cerebral autoregulation to the production of neurotransmitters [1]. Brain iron is comprised of heme-bound and non-heme bound forms. Under physiological conditions, iron is normally bound by transport proteins. The majority of iron is found in the ferrous (Fe2+) state complexed by porphyrin ring to form the heme moiety in hemoglobin. The remainder of "non-heme iron" is bound to circulating transferrin and intracellular storage proteins, mostly ferritin [2]. About one-third to three-quarters of the iron in the brain is stored in the form of ferritin, predominantly in glial cells [3]. When red blood cells are phagocytosed and degraded by macrophages, the free hemoglobin complexes with haptoglobin, and further degradation releases free heme. There are receptors for every stage of red blood cell degradation; CD 163 is the hemoglobin-haptoglobin receptor, and toll-like receptor 4

(TLR4) is one of the heme receptors. The expression of both CD163 and TLR4 is upregulated after hemorrhagic stroke, but also in some models of ischemic stroke. Both receptors are involved in phagocytosis of their respective red blood cell ligands as well as upstream of inflammatory pathways [4–8]. Since the vast majority of iron is contained within heme in red blood cells, some understanding of iron uptake for red blood cell genesis (erythropoiesis) is necessary.

Role of Iron in Erythropoiesis Erythrocytes contain hemoglobins, and every mole of hemoglobin contains four heme moieties that can each bind 1 mol of oxygen. Considering the amount of red blood cells contained within the body, the uptake of iron needs to be efficient to maintain this level of erythropoiesis. Dietary iron is absorbed in the duodenum and transported in the circulation via complex with transferrin. Transferrin-bound iron in the plasma gains access into brain cells via a receptor-mediated endocytosis from transferrin [9]. Transferrin receptors are present on the endothelial cells of brain barrier capillaries [10]. As the transport of transferrin through the blood-brain barrier is restricted, some of the transferrin-bound iron is released into the brain as transferrin-unbound iron [11]. In the brain, transferrin is produced by oligodendrocytes and choroids plexus cells [12]. Neuronal uptake of iron also involves transferrin receptors, which are present in oligodendrocytes, on blood vessels, and are mostly neuronal [13–15]. Therefore, transferrin is important for proper mobilization and distribution of iron to and inside the brain.

Role of Hepcidin The grand orchestrator of all this iron hemostasis is hepcidin, a hepatically produced protein. Hepcidin controls the expression of ferritin, transferrin, hemosiderin, and heme. When erythrocytes are degraded by macrophages and stored as hemosiderin, some of the heme is recycled and exported for use by other cells by a protein channel known as ferroportin (FPN), contributing a large portion of necessary heme [16]. Furthermore, the final stages of heme synthesis require transport across the mitochondrial membranes. The export of completed heme out of the mitochondria is accomplished by feline leukemia virus subgroup C receptor 1 (FLVCR1), and specifically isoform 1b to leave the heme in the cytosol for incorporation into hemoglobin if occurring in a red blood cell [17]. Heme not required by the red blood cell is exported across the plasma membrane by FLVCR1a.

Toxic Effects of Iron and Ferroptosis

Tight regulation of iron homeostasis is required to prevent toxicity. Free iron plays an important role in the production of reactive oxygen species (ROS) as it can participate in the Fenton and Haber-Weiss reactions to yield superoxide anion and the highly reactive hydroxyl radical [18••]. Furthermore, reactions involving superoxide anion and nitric oxide can yield peroxynitrite, a type of reactive nitrogen species (RNS). Both RNS and ROS are involved in lipid peroxidation and nitration, as well as direct protein modification of the very enzymes that are responsible for the tight iron homeostasis: hepcidin, ferritin, FPN, and FLVCR1. By modifying these critical iron regulatory proteins, the iron-initiated inflammation is propagated and ferroptosis can occur [19]. Ferroptosis is a form of regulated cell death characterized by the iron-dependent accumulation of lipid hydroperoxides to lethal cellular levels. That is cells have actually evolved to deal with iron overload and some lipid damage via glutathione-mediated reduction of glutathione peroxidase 4 (GPX4) [19]. GPX4 converts potentially toxic lipid hydroperoxides (L-OOH) to non-toxic lipid alcohols (L-OH). Free iron, via the Fenton reactions, inhibits the regeneration of glutathione, which in turn results in the inactivation of GPX4 and ferroptosis. The iron-initiated inflammation then results in the release of pro-inflammatory cytokines by members of the innate immune system, specifically circulating monocytes and macrophages, which are then driven to erythrophagocytosis. This results in an anemia of inflammation as well as further generation of free iron to propagate the inflammation.

Iron-Mediated Inflammation in Ischemic Stroke

In states of hypoxia, even normal amounts of free iron can catalyze the pathologic Fenton reactions. In one study, peroxynitrite, an RNS derived from Fenton reactions involving superoxide anion and nitric oxide, was critical to stroke pathology in an in vivo middle cerebral artery occlusion (MCAO) model and in vitro oxygen-glucose deprivation (OGD) model on murine blood vessels [20]. The peroxynitrite directly nitrated smooth muscle proteins resulting in loss of vascular tone with subsequent reduction in perfusion pressures and worsening infarct volume. This suggests that targeting peroxynitration could be helpful to mitigate iron-mediated injury following cerebral ischemia. However, targeting ROS and RNS using scavengers has proven challenging.

Another study using a neonatal hypoxia model implicated the NMDA receptor, subunit 2B as another potential target for Fenton reactions [21]. In mice where a tyrosine in NMDA-2B was substituted by phenylalanine, an amino acid that cannot be phosphorylated, reduced expression of NADPH oxidase subunit gp91phox and p47phox and superoxide production, lower activity of proteases implicated in necrotic and apoptotic cell death, and less brain damage were noted compared with controls. The src family kinases (SFKs), specifically Fyn, have been implicated in the phosphorylation of NMDA-2B. From these data, a pathway for Fenton-induced inflammation can start to be constructed where Fyn is

activated, followed by the phosphorylation of NMDA-2B, which then leads to further generation of ROS and RNS [21, 22]. Given that iron chelators have been shown to inhibit SFKs and nitration, further studies investigating these non-canonical mechanisms of action may offer some novel pharmacological targets to ameliorate Fenton-induced brain damage in ischemic stroke [23, 24].

As the majority of patients with ischemic stroke tend to be older, Tou et al. [25] studied the relationship between tau proteins, iron transport, and ferroptosis after ischemic stroke. In a wild-type MCAO model, they found that ischemia resulted in decreased tau expression, decreased iron export from cells, and increased ferroptosis. When this experiment was repeated in a young tau-knockout mouse, the infarct volume was reduced, presumably because the young mice had found other means to address iron homeostasis in the absence of tau and were better prepared for ischemia. On the contrary, when similar experiments were performed in old tau-knockout mice, no attenuation of infarct volume was seen. Furthermore, iron stores and ferroptosis were equivalent to that seen in wild-type mice. These unique findings provide a plausible novel mechanism for age to be invoked in ischemic stroke.

Taken together, iron plays a critical role in the pathology of ischemic stroke even when there is not an abundance of red blood cell breakdown products as there are in hemorrhagic stroke. In the event of ischemia, there is so much iron in the cell already that release or lack of export of iron can catalyze significant productions of ROS and RNS via Fenton reactions. Therefore, targeting the source of the radicals by removing free, non-heme iron may serve an important neuroprotective role in ischemic stroke.

Iron-Mediated Inflammation in Hemorrhagic Stroke

In contrast to ischemic stroke where iron plays a less director role in the initiation of inflammation, all types of iron, heme-bound and non-heme bound, have necessary roles in the subsequent inflammation and ferroptosis in hemorrhagic stroke, including both intracerebral (ICH) and subarachnoid (SAH) hemorrhage. There are many similarities between the iron-mediated toxicity and inflammatory cascades seen in both conditions.

In ICH rodent models, red blood cell lysis and the release of heme-bound iron can occur as early as 1–2 days after injection of autologous blood, and when lysed erythrocytes are injected, edema formation, blood-brain barrier breakdown, and expression of heat shock proteins can be detected only hours later, thus providing evidence of the toxicity of erythrocyte contents [26••]. The contents of lysed erythrocytes, hemoglobin, and eventually heme can illicit specific inflammatory signal transduction pathways. The hemoglobin-haptoglobin (Hp)/complement of differentiation (CD) 163 (Hp-CD163) plays a dominant role in the transportation of heme-bound iron. The majority of CD163 is found on macrophages and other antigen-presenting cells (APCs), which are responsible for phagocytosing an ectopic heme burden [4]. In CD163-knockout mice, a biphasic response was seen after autologous blood injection. Initially, CD163 appeared to have a deleterious effect with the knockout mice showing reduced hematoma volumes and decreased neuronal damage at 3 days after injury. However, 10 days after injury, the expected protective effects of CD163 were observed with mice lacking CD163 having larger hematoma volumes and worsened cognitive outcomes [27].

In an ICH piglet model, CD163 was upregulated after ICH induction, and an initial reduction in red blood cell diameter, causing clot retraction, occurred over the first 3 days. Erythrophagocytosis associated with CD163-positive macrophages/microglia was observed, as well as the accumulation of these cells within the clot over time [28]. Similarly, when CD163 expression on neurons was studied, it seemed that CD163 was a marker of inflammation and had deleterious effects that were ameliorated by the iron chelator, deferoxamine, which decreased CD163 expression [29].

The degradation of hemoglobin to heme also results in a unique signaling initiated by heme oxygenase (HO). HO has two isozymes, where HO-1 is the inducible form and HO-2 is the constitutive form. Both forms of HO are mainly expressed in APC type cells, as these are the cells most responsible for erythrocyte degradation. HO catalyzes the breakdown of heme into ferrous iron, biliverdin, and carbon monoxide (CO) [30].

Deletion or overexpression of HO in ICH seems to have mixed results. In one study, the deletion of HO-2 resulted in improved functional outcomes and less microglia in the perihematomal area [31]. On the other hand, selective HO-1 overexpression in astrocytes reduces mortality, blood-brain barrier disruption, perihematomal cell injury, and neurological deficits [32].

Recent work demonstrates that neutrophils, in addition to inflammatory mediators, also release lactoferrin and interleukin-27. Lactoferrin is an iron-scavenging protein, and IL-27 suppresses neutrophil production of pro-inflammatory products and increases the expression of lactoferrin. In rodent ICH model, administration of IL-27 or lactoferrin reduced edema, enhanced hematoma clearance, and improved neurological outcomes [33].

The toll-like receptors (TLRs) are ubiquitously expressed receptors that can sense damaged proteins and are critical to the heme-induced cerebral inflammatory response after SAH. We previously demonstrated that heme and its breakdown products utilize TLR4 to initiate ferroptosis in an autologous blood injection model of SAH model. We found that TLR4 knockouts, compared with controls, had less neuronal apoptosis and cognitive dysfunction [6].

Using a conditional knockout of HO-1 in microglia, we found that these mice had more neuronal apoptosis, larger

hematoma burdens, and worse cognitive function compared with wild-type mice after experimental SAH. Similar results were seen when we used a semi-selective pharmacological inhibitor of HO-1. To determine how microglial HO-1 was conferring neuroprotection, we studied microglia in vitro and found that the lack of HO-1 leads to ineffective erythrophagocytosis [34].

Collectively, the CD163 and HO pathways, along with virtually all of the neuroglia and microglia, have necessary roles to play in inflammation after ICH and SAH. Iron-modifying agents may quell the inflammatory response at the top of the ferroptotic cascade. Figure 1 illustrates the major inflammatory mediators/pathways after hemorrhagic stroke.

Role of Iron Chelators to Mitigate Inflammation in Stroke

Preclinical Data Substantial preclinical evidence exists to support the use of iron chelating agents in all types of stroke, with the majority of these studies using deferoxamine mesylate (DFO) [24, 35–40]. Although the protective effects of iron chelators are theorized to involve the removal of non-heme iron and thereby reduce radical formation and lipid peroxidation, there is evidence that iron chelators may have other off-target beneficial effects. For example, DFO preconditioning protected against cerebral ischemia in rats by inducing the expression of hypoxia-inducible factor 1α and erythropoietin [41]. In a murine SAH model, the addition DFO to primary microglial cell or intrathecally resulted in increased expression of HO-1 [36]. We also found that microglial HO-1 was sufficient and necessary for DFO-induced neuroprotection in in vivo model of SAH [36]. Similarly, in piglet model of ICH, DFO may mediate neuroprotective effects by reducing the expression of CD163 on microglial and invading macrophages within the clot and increasing the "don't eat me," CD47 signal, indicating that erythrophagocytosis may actually be part of the sterile inflammation that results in neuronal damage, and DFO might exert protective effect by slowing down this process [28]. Furthermore, DFO was found to attenuate death rate, hemorrhagic transformation, infarct volume, and brain swelling in a rat transient focal ischemia with hyperglycemia model suggesting that it could have potential application to minimize the risk of hemorrhagic transformation in ischemic stroke [40]. In other studies, preconditioning of mesenchymal stem cells with DFO increased the production of pro-angiogenic, neuroprotective, and anti-inflammatory factors, thus expanding its therapeutic potential to the field of stem cell therapy [42].

Clinical Data Despite the preponderance of data demonstrating the toxicity of free iron and the potential neuroprotective effects of iron chelators in animal models of ischemic and hemorrhagic stroke, there have been very few translational studies examining

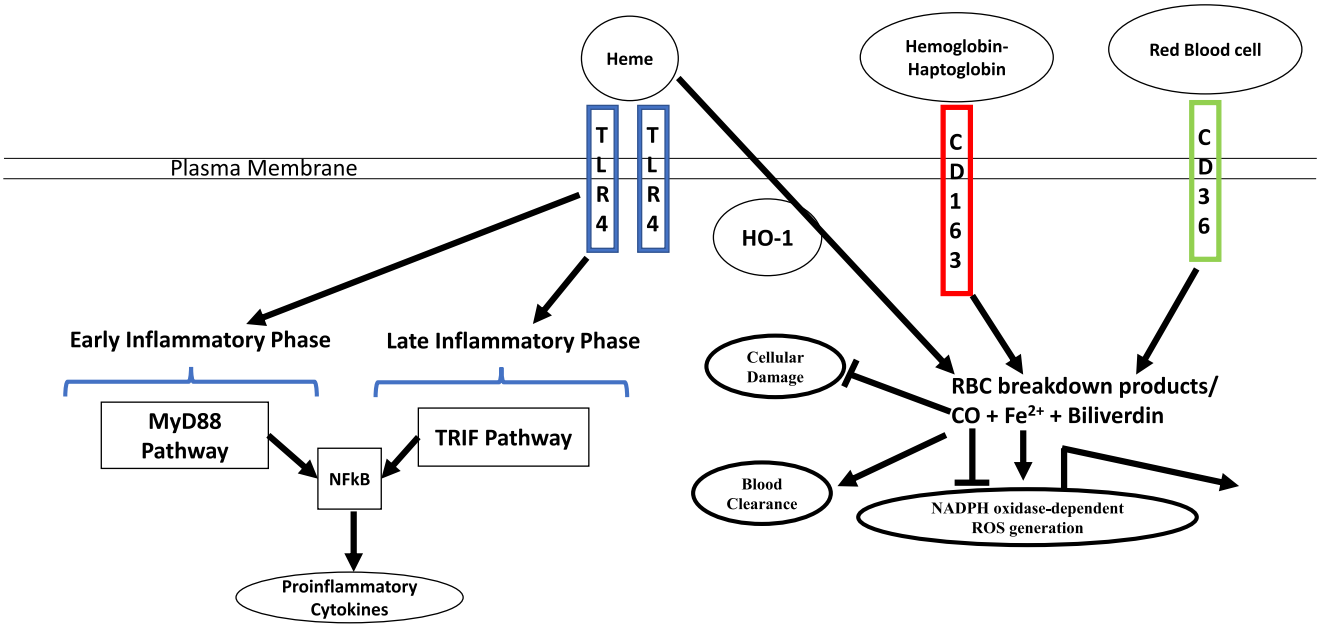

Fig. 1 A summary of neuroinflammatory signal transduction in hemorrhagic stroke. While not all inclusive, this diagram summarizes the 3 major cell surface receptors involved in mediating neuroinflammation after hemorrhagic stroke. TLR4 has been shown to be directly activated by heme in in vitro studies, CD 163 by the hemoglobin-haptoglobin complex, and red blood cells are recognized by the CD 36 scavenger receptor, among others. Inflammation from the TLR pathways has been best elucidated, but the breakdown of heme by HO-1 yields significant ROS-induced inflammation via the creation of free iron and the Fenton reaction. MyD88 myeloid differentiation protein 88, TLR4 toll-like receptor 4, TRIF TLR interaction region-domain-containing adapter-inducing interferon-β, HO-1 heme oxygenase 1, NF-kB nuclear factor kappa beta, NADPH nicotinamide adenine dinucleotide phosphate, and ROS reactive oxygen species

the role of iron toxicity and the use of iron chelators in stroke patients. An exploratory study in four patients with ischemic stroke and five with hemorrhagic stroke, treatment with 500 mg of DFO for 3 days reduced serum levels of hydroperoxides and lipoperoxides and increased total radical-trapping antioxidant capacity levels [43], providing a preliminary proof-of-concept that DFO can exert potential antioxidant neuroprotective effects in stroke patients. A study in 23 patients with ICH reported a positive correlation between serum ferritin and relative perihematomal edema volume on day 3 to 4 after ICH [44]. In another pilot study, non-transferrin bound iron levels in the cerebrospinal fluid correlated with the development of delayed cerebral ischemia in 12 patients with aneurysmal SAH [45]. Recent findings from a non-randomized prospective study in patients with traumatic ICH (47 DFO-treated (20 mg/kg/day for 5 days) patients vs. 47 controls) using a propensity score-matched analysis suggested that treatment with DFO may accelerate hematoma absorption and reduce the formation of edema [46]. While there was no difference in hematoma volume at presentation between DFO and control groups (12.6 ± 7.8 vs. 12.8 ± 6.4 mL; $p = 0.896$), the hematoma volume on day 7 was smaller in the DFO group (5.2 ± 4.8 vs. 9.4 ± 7.2 mL; $p = 0.001$). Similarly, the relative perihematomal edema volumes were comparable between the two groups on day 1, but higher in the control group on days 3 and 7. A prospective, multi-center, open-label, phase I, safety, and dose-finding study of DFO in spontaneous ICH has been successfully completed [47]. Twenty subjects were enrolled into 5 dose tiers, starting with 7 mg/kg per day for three consecutive days and ending with 62 mg/kg per day as the maximum tolerated dose. Consecutive daily infusions of DFO after ICH were feasible, well-tolerated, and not associated with excessive serious adverse events or mortality. However, a subsequent phase II, placebo-controlled, futility-design trial (ClinicalTrials.gov # NCT01662895) was terminated prematurely after enrollment of 42 subjects due to six cases of adult respiratory distress syndrome among 21 participants who received DFO at 62 mg/kg/day for 5 days, suggesting pulmonary toxicity of the drug. The protocol was amended to reduce DFO dose to 32 mg/kg/day for 3 days (ClinicalTrials.gov # NCT02175225), and this trial recently completed recruitment of 294 subjects. The objectives of this trial are to assess the safety of DFO in a larger cohort of ICH patients and to determine whether DFO has sufficient promise based on modified Rankin Scale scores 0–2 at 90 days to embark on a subsequent large-scale phase III efficacy trial. We anxiously await the results from this trial, which should help to shape future investigations of iron chelating agents in stroke.

Conclusions and Future Directions

Advances in our knowledge and understanding of iron-mediated neurotoxicity and its signaling pathways coupled with advances in iron imaging and iron chelation pharmacotherapy usher in a tremendous promise to develop novel and effective therapies to improve outcomes after stroke. Iron chelating agents, in particular DFO, have universally shown neuroprotective effects in preclinical models by a variety of mechanisms. Their utility in hemorrhagic stroke seems almost unquestionable from a preclinical perspective. The recent availability of orally effective chelators, deferasirox, and deferiprone, and new routes for DFO administration provide an opportunity to develop a multi-modal neuroprotective approach, rather than simply chelating free iron, and should improve the feasibility of future clinical trials.

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Compliance with Ethical Standards

Conflict of Interest Khalid A. Hanafy, Joao A. Gomes, and Magdy Selim declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This is a review article so we have referenced studies done in humans and animals, but these studies were not done as part of this article, and they are just referenced here. For those studies performed by the authors, all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. And all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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