

Brain iron overload following intracranial haemorrhage

Thomas Garton, Richard F Keep, Ya Hua, Guohua Xi

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ABSTRACT

Intracranial haemorrhages, including intracerebral haemorrhage (ICH), intraventricular haemorrhage (IVH) and subarachnoid haemorrhage (SAH), are leading causes of morbidity and mortality worldwide. In addition, haemorrhage contributes to tissue damage in traumatic brain injury (TBI). To date, efforts to treat the long-term consequences of cerebral haemorrhage have been unsatisfactory. Incident rates and mortality have not showed significant improvement in recent years. In terms of secondary damage following haemorrhage, it is becoming increasingly apparent that blood components are of integral importance, with haemoglobin-derived iron playing a major role. However, the damage caused by iron is complex and varied, and therefore, increased investigation into the mechanisms by which iron causes brain injury is required. As ICH, IVH, SAH and TBI are related, this review will discuss the role of iron in each, so that similarities in injury pathologies can be more easily identified. It summarises important components of normal brain iron homeostasis and analyses the existing evidence on iron-related brain injury mechanisms. It further discusses treatment options of particular promise.

INTRODUCTION

Iron is a crucial nutrient for multiple biological functions, including, but not limited to, oxygen transport, electron transport, redox reactions, cell division, nucleotide synthesis and myelination.¹ In the brain, iron homeostasis is of critical importance, and dysregulation can lead to serious neurodegenerative diseases such as Alzheimer's disease,² Parkinson's disease³ and Hallerorden-Spatz syndrome.⁴ However, while much research has focused on understanding metal homeostasis in these diseases, the role of iron accumulation following intracranial haemorrhage (ICrH) and traumatic brain injury (TBI) has yet to be fully determined. ICrH is broadly defined as bleeding within the cranium, and has an incident rate of ~40 per 100 000 people/year.⁵ ICrH can be subdivided into intracerebral haemorrhage (ICH), intraventricular haemorrhage (IVH) or subarachnoid haemorrhage (SAH).

In addition, while TBI is not considered one of these subdivisions, it is often accompanied by ICrH. All forms of ICrH carry high mortality rates and poor prognosis. Injury following haemorrhage can be categorised into primary injury, sustained during the initial haemorrhage, and secondary injury, referring to subsequent and long-term damage due to other factors.

Over the past decade, interest in identifying the mechanisms of secondary injury after haemorrhage has spiked, and several specific blood components have been identified as being integral to this phase of damage.⁶ In particular, haemoglobin (Hb)-derived iron is thought to play an important role. Previous reviews have either generally discussed all blood components⁷ or specifically focused on one form of ICrH.⁸ This review focuses on the role iron accumulation plays in secondary damage following the entire spectrum of ICrH: ICH, SAH, IVH and TBI-induced haemorrhage, and assesses potential therapeutic options.

IRON HOMEOSTASIS IN NORMAL BRAIN

Because of its potential toxicity, iron content is tightly regulated in the brain. Little is found as the free ferric (Fe^{3+}) or ferrous (Fe^{2+}) ion. Some is bound to small organic molecules such as citrate, ATP or ascorbic acid.⁹ Iron is also an important component of many proteins.¹⁰ Thus, for example, it is an essential component of cytochromes a, b and c and cytochrome oxidase and other enzymes. There are also iron-sulphur clusters in Complex I and II of the electron transport chain. In some proteins, including neuroglobin and Hb, iron is bound in the form of haem. This section describes the regulation of these iron pools. Because of the importance of the latter in cerebral haemorrhage, regulation of haem iron will be reviewed separately.

Non-haem-bound iron

Brain iron homeostasis involves regulation of iron movement between blood and brain,



Department of Neurosurgery,
University of Michigan, Ann
Arbor, Michigan, USA

Correspondence to
Dr Guohua Xi;
guohuaxi@umich.edu

between brain intracellular and extracellular spaces and between different iron pools within such spaces. The movement of iron across cell membranes requires specific transport systems. Under normal conditions, the most important uptake mechanism is the transferrin–transferrin receptor system (Tf–TfR). Transferrin (Tf) is an 80 kDa glycoprotein with high affinity for iron,¹¹ with mRNA expression in oligodendrocytes, neurons and astrocytes. Once expressed, Tf scavenges free iron in the extracellular space. Tf binds to Fe³⁺ and, after binding to membrane TfR, undergoes endocytosis.¹² The endosome is then acidified, releasing the Fe³⁺ and reducing it to the Fe²⁺ state.¹³ Once reduced, the iron is released from the endosome into the cytoplasm by Divalent Metal Transporter 1 (DMT1), a protein that is widely expressed and which is capable of transporting a broad range of divalent and trivalent ions, including iron, zinc, manganese, cobalt, cadmium, copper, nickel and lead.¹² This cytosolic iron, also known as the labile iron pool, is largely contained within lysosomes and is in constant equilibrium with an iron-binding protein, ferritin (Ft).¹⁴ Ft is highly stable at a wide range of temperatures and acidities, and sequesters Fe²⁺ ions in ferroxidase centres of the Ft subunits. These ferroxidase centres have the important ability to consume all reagents of the radical Fenton reactions (see the Brain iron overload and toxicity section) and thereby inhibit iron-mediated oxidative stress.¹⁵ Ft is expressed in microglia and macrophages, but it is also found in some neurons.¹⁶ Should Ft levels become saturated, iron can be transported out into the cerebral interstitial fluid by ferroportin 1 (FP1). In conjunction with such transport, the toxic Fe²⁺ is oxidised to Fe³⁺ by the multicopper ferroxidase ceruloplasmin (CP) so that it can be bound more easily by transferrin once in the extracellular space. FP1 is stabilised by the amyloid precursor protein (APP), but the role of APP in iron transport is still a matter for debate.¹⁷ Duce *et al.*¹⁸ suggested that neuronal APP possesses ferroxidase capabilities stronger than even Ft. However, this group recently demonstrated that APP lacks ferroxidase capabilities but remains essential for FP1 persistence on the neuronal surface, thereby supporting iron export from neurons.¹⁷ The precise mechanism by which it stabilises FP1, however, is undetermined.

Other iron transport systems

The systems described above are not the only iron transport systems present in the brain. Iron is also a substrate for some zinc transporters, such as the Zip8 and Zip14 pathways.¹⁹ Other iron transporters include the lactoferrin–lactoferrin receptor system in neurons²⁰ and the melanotransferrin system, which is expressed in active microglia.²¹ However, these have not been the topic of much research in the realm of brain haemorrhage or TBI, with the exception of Terent *et al.*²²

Regulation of iron homeostasis

The Tf–TfR import system, the Ft sequestering system and the FP1–CP exporter system are relatively ubiquitous

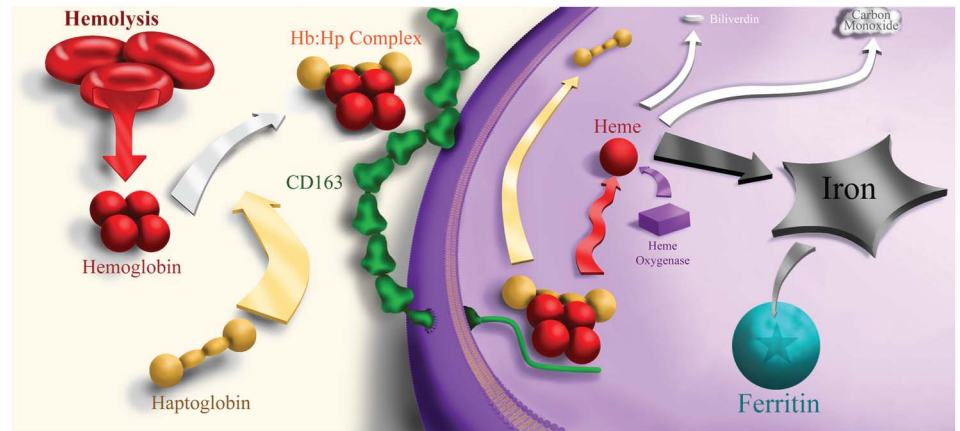
throughout the brain. Regulation of these systems is predominantly controlled by iron regulatory proteins, IRP-1 and IRP-2. These proteins bind to an iron-responsive element, a relatively conserved specific hairpin loop in the 5′-UTR of the mRNA coding region of a number of iron-related proteins, including Ft, aconitase, APP, FP1, HIF2 α and others.^{1–23} By binding to this hairpin loop, IRPs reduce mRNA expression.²⁴ The IRPs are themselves inhibited by iron; as such, elevated intracellular iron results in elevated Ft and FP1 transcription, allowing greater sequestering and export of iron.²⁵ Conversely, when iron levels are low, Ft and FP1 expression is inhibited, reducing the amount of energy wasted on unnecessary protein synthesis. In addition, IRPs bind to TfR and DMT1 mRNA, but in this case, they stabilise the mRNA and increase expression, thus allowing greater levels of iron influx during times of insufficient intracellular iron.^{1–26} The factors that play a role in this apparent dichotomy of IRP function have yet to be fully elucidated.

In addition to IRPs, the protein hepcidin is responsible for internalisation of FP1 and modulation of CP and DMT1 in the cerebral cortex and hippocampus.^{27–28} Therefore, it can cause iron accumulation within the cell when overexpressed.²⁹ Hepcidin expression is increased in response to cellular iron overload and inflammation.³⁰ It is therefore a potential therapeutic target in a variety of iron-related neurodegenerative disorders.

Haem-bound iron

In order to understand the posthaemorrhagic effects of iron, it is important to understand the iron homeostasis in normal brain. Brain iron is present in one of the two forms: haem-bound or non-haem-bound. Haem is the core moiety of Hb and refers to a protoporphyrin IX scaffold supporting an Fe²⁺ central atom. Haem-bound iron is transported via a variety of transportation pathways. Predominant among them is the Hb-haptoglobin (Hp)-CD163 pathway (figure 1). Hb, the oxygen-carrying protein, contains about 70% of whole-body iron in its haem cores.³¹ It is not limited to erythroid cells, but rather it can be expressed in a wide range of glial cells, macrophages and neurons within the central nervous system.^{32–35} Within neurons, Hb may act as an oxygen reservoir and control mitochondrial function.³⁶ While normally stored intracellularly, Hb is occasionally released from cells during haemolysis. This free extracellular Hb is subsequently scavenged by the protein Hp, an abundant plasma glycoprotein that binds to Hb with incredibly high affinity.³⁷ Hp is a tetrameric, $\alpha_2\beta_2$ serine protease, in which the α domain is responsible for dimer and multimer formations (Hp 1–1, 2–1 and 2–2 phenotypes), whereas the β subunit binds to Hb and Hp receptors after the Hb naturally dissociates into dimers.³⁸ This newly formed complex is redox-inactive, with Hp assisting in diffusing any radical chemistry that

Figure 1 Haemolysis and haemoglobin degradation after brain haemorrhages.



may occur following H_2O_2 attack (see the Mitochondrial damage section).³⁹

This complex has a high affinity for the membrane-bound receptor CD163, a 130 kDa transmembrane member of the scavenger receptor cysteine-rich domain-containing protein family. Binding of the Hb:Hp complex to CD163 results in endocytosis, preventing accumulation of Hb in the extracellular space.⁴⁰ The regulatory mechanism of CD163 in the brain is not well understood, though it is apparent that it is upregulated following vascular compromise, inflammation and by the presence of Hp:Hb complexes.^{41–42} CD163 is shed from the cell membrane by ADAM17, a membrane-bound serine protease responsible for a variety of cell signaling, including TNF- α and A β formation in Alzheimer's disease.^{43–45} This 'soluble' CD163 is composed of 94% of the protein's ectodomain and is an inflammatory biomarker. CD163 has long been viewed as a monocyte/macrophage marker, expressed exclusively within that cell line. However, recent *in vivo* findings demonstrated that following IVH, hippocampal neurons express CD163.⁴⁶ This finding has been corroborated and extended to cortical neurons by Chen-Roetling and Regan.⁴⁷

Following endocytosis, the Hb:Hp complex dissociates, allowing Hb to be degraded to haem inside endosomes^{48–49} (figure 1). Inside the cell, haem can be bound by one of several haem-binding proteins,⁵⁰ whose roles in ICrH have not yet been investigated, or it can be degraded by haem oxygenase (HO) proteins (the inducible HO-1 or the constitutively expressed HO-2). HO-1 mRNA expression is increased significantly by the presence of haem as well as a variety of other proinflammatory signal molecules.⁵¹ HO proteins degrade haem into carbon monoxide, biliverdin and Fe^{2+} .⁵² The biliverdin is converted to bilirubin (an antioxidant known to be neurotoxic in preterm neonates) by biliverdin reductase,^{53–54} while the iron is bound by Ft and sequestered.

INTRACEREBRAL HAEMORRHAGE

Introduction

ICH describes bleeding into the brain parenchyma due to the rupture of a cerebral blood vessel. Of the ICrH

subtypes, ICH is the most common, accounting for ~10–20% of all strokes.⁵⁵ ICH can either be secondary or primary. Secondary ICH refers to bleeding caused by a pre-existing condition, such as a tumour, whereas primary ICH is a vessel rupture without an underlying lesion, with hypertension being a major cause. ICH incidence has not altered in the past three decades, being stable at ~25 per 100 000 person-years.⁵⁶ Of note, Asian populations are at significantly higher risk of ICH than non-Asian populations. Prognosis is very poor, with death occurring within 1 month in over 40% of cases.⁵⁷ It is therefore of pressing concern to identify therapeutic targets for ICH. There is a substantial body of preclinical evidence that iron plays a role in ICH-induced injury⁸ and, in patients suffering from ICH, high-serum levels of Ft are independently associated with poor outcome.⁵⁸

Iron transport into the cell Haemoglobin

In ICH, erythrocytes are released into the brain parenchyma, where they lyse within hours/days, releasing their components, including Hb, into the extracellular space.⁵⁹ This Hb may be scavenged by Hp and endocytosed by CD163 into macrophages and monocytes as described above. The Hp is produced locally in the brain by oligodendrocytes.⁶⁰ However, recent findings suggest that Hp may also exacerbate neuronal vulnerability to Hb-mediated iron toxicity.⁴⁷ That study suggests that Hp increases the ability for haem-bound iron to gain access to neurons via CD163, thereby increasing neurotoxicity. In contrast to glial cells, neurons have low Ft levels and thus lack sufficient iron buffering to handle the amount of Hb-derived iron.^{47–61–62}

Haem

Hb that is not scavenged immediately degrades and releases haem.⁵⁹ Haem can react with lipid peroxide-generated hydrogen peroxides according to the Fenton reactions, leading to the formation of reactive oxygen species (ROS).^{63–64} To prevent this, extracellular haem is bound by haemopexin,⁶⁵ a 60 kDa glycoprotein that is normally not expressed in brain parenchyma,⁶⁶ but

which is induced after ICH.⁶⁷ The resultant haem-haemopexin complex can be taken up into cells via CD91 (also known as low-density lipoprotein receptor-related protein, LRP1) and metabolised by HO proteins.⁶⁸

CD91 is widely expressed in brain cell types, including in glial cells, macrophages and neurons.⁶⁹ The haemopexin-CD91 pathway is, therefore, another potential mechanism by which iron can access neuronal cells. After endocytosis, haemopexin is degraded within the lysosome, and consequently, decreases in plasma haemopexin concentration occur in severe haemolysis seen during ICH.^{70 71} Normally, the iron released by this degradation is stored in Ft and inhibits IRPs, thus stimulating a variety of iron-related proteins, including APP.⁷² However, excessive levels of intracellular haem can result in overuse and quick degradation of Ft, resulting in the formation of haemosiderin, an intracellular insoluble iron storage complex.^{73 74} Haemosiderin is a major location of iron accumulation and is used as a marker for haemorrhagic injury.⁷⁵ Haem-haemopexin complexes also increase the endogenous levels of holo-APP, as well as HO-1.⁷⁶ Deletion of the haemopexin gene aggravates injury following ICH.^{77 78}

Brain iron overload and toxicity

While the iron homeostatic mechanisms function well in normal physiological conditions, the amount of Hb released into the extracellular space following haemorrhage may overwhelm these iron-handling systems. Saturation of these systems, or iron overload, can result in the presence of iron in a form where it can participate in harmful reactions; for example, free radical production.

Mechanisms of iron toxicity

Iron toxicity is generally thought to result from the generation of free radicals via the Fenton reaction. In that reaction, ferrous iron reacts with hydrogen peroxide to form radical oxygen species according to the reaction below:



The resulting ferric iron can be reduced back to Fe²⁺ by a variety of reducing agents and thereby regenerate the starting reagents. In this way, the radical reaction cycle can begin again. Some cellular antioxidants like GSH and superoxide dismutase work to limit this damage, but these antioxidants have limited efficacy to combat the amount of oxidative stress during ICH.^{79 80}

Mitochondrial damage

The oxidative damage resulting from such iron overload targets the mitochondrial inner membrane.^{81 82} Iron overload in HT-22 hippocampal neurons induces mitochondrial fragmentation that is dependent on dephosphorylation of dynamin-related protein 1 (Drp1), a mitochondrially localised GTPase, which controls

mitochondrial fission.^{83 84} This mitochondrial fission is closely regulated by Calcineurin phosphatase signal pathways,^{85 86} which are also affected by iron overload.⁸³

Oedema

Iron overload also induces significant periaematoma oedema. Oedema has been repeatedly shown to be associated with poor outcome after ICH.^{87 88} In clinical studies, serum Ft is often used as an estimate of the body's iron load.^{89 90} Serum Ft levels are highly correlated to periaematoma oedema volumes following ICH;⁹¹⁻⁹⁴ while this is most likely because it reflects elevated iron levels, it has been proposed that serum Ft could play a role as an iron-independent proinflammatory signalling molecule in hepatic stellate cells.⁹⁵ There has been no research to assess whether it could play an equivalent role in the brain. Nevertheless, iron overload has been shown to increase brain water content following ICH.⁹⁶ Important to the formation of cerebral oedema is aquaporin 4 (AQP4), one of the most abundant water channel proteins in the brain.⁹⁷⁻⁹⁹ AQP4 is mostly expressed in astrocyte foot processes^{99 100} around blood vessels where it plays critical roles in brain water homeostasis. Increased expression of AQP4 is found in ICH,¹⁰¹ and there is a high correlation between iron accumulation and AQP4 expression.¹⁰² AQP4 is known to be regulated by metal ions such as mercury,¹⁰³ but it is unknown whether iron has similar effects.

Cell death

The above-described damage culminates in widespread glial and neuronal cell death. This cell death is therefore intrinsically linked to its iron source. The form of cell death is not purely necrotic. Ferrous citrate infusion (designed to simulate post-ICH iron overload) results in increased LC3-II expression and autophagic cell death, as assessed by BECN1 staining.¹⁰⁴ LC3-II is the activated conjugate of microtubule-associated protein 1A/1B light chain-3, and it is recruited to autophagosomal membranes, thereby being a key marker of autophagic cell death.¹⁰⁵ Iron-induced autophagy following ICH has been documented previously,¹⁰⁶ though the mechanism by which iron enhances autophagy is not fully understood. Autophagy is induced by oxidative stress, which has linked HO-1 (a marker for such stress) to this type of cell death following iron overload.¹⁰⁷ During ICH, it is possible that HO-1 promotes the intracellular release and capture of redox-reactive iron, thereby promoting autophagy.¹⁰⁸ Inhibition of autophagy leads to protection against iron-induced neurodegeneration.¹⁰⁹⁻¹¹¹

Apoptosis of neurons and astrocytes has also been identified after ICH.^{112 113} Haem alone can induce apoptotic cell death in neuronal cells (as identified by DNA fragmentation, caspase activation and mitochondrial membrane disruption).¹¹⁴ Such apoptosis is potentially related to a member of the high-temperature requirement (Htr) family, HtrA2, a mitochondrially

located serine protease which, on release into the cytoplasm, contributes to apoptosis via caspase-dependent and caspase-independent pathways. Haem incubation of PC12 neuroblastoma cells resulted in HtrA2-mediated apoptosis.¹¹⁵ A variety of other apoptotic pathways have been proposed following ICH, including SSTR1, bcl-2, CHMP4B, ESCRT-III, VCAM1 and c-Fos,^{116–119} but their links (if any) to iron have not yet been identified.

Another form of regulated cell death of note is ferroptosis, an iron-dependent form of cell death distinct from autophagy, apoptosis, necrosis and other forms of cell death.¹²⁰ On accumulation of lipid ROS, the RAS Selective Lethal proteins erastin and RSL3 mediate cell death that is critically dependent on iron (but not other divalent metals).¹²¹ However, research on ICH-induced ferroptosis has been significantly hindered by a lack of easily identifiable markers. Chang *et al.*¹²² found that ICH increases ferroptosis-related gene expression (including IRP2), and that this expression could be ameliorated by treatment with (-)-epicatechin, a scavenger of free radicals and other pro-oxidants, that activates the Nrf2 signalling pathway, a master regulator of antioxidant defence mechanisms. However, besides that study, there has been a lack of successful studies investigating the ability of ICH-induced iron overload to activate ferroptotic pathways. Given the emphasis on the iron-dependent nature of ICH and ferroptosis, this may be a fertile field of inquiry.

Vicious cycle of iron accumulation

In haemorrhagic transformation after cerebral ischaemia, iron overload may engage in a vicious cycle. Cerebral ischaemia causes a shift to glycolytic from oxidative metabolism, lactic acid production and marked decreases in brain pH. Iron can be released from Ft following exposure to decreased pH, superoxide radicals and ascorbate^{123–126} and it may then damage mitochondria further inhibiting oxidative metabolism and result in the production of free radicals. In turn, this may cause further release of iron, a vicious cycle. The extent to which this occurs in ICH, SAH and IVH is uncertain and merits investigation.

INTRAVENTRICULAR HAEMORRHAGE

Introduction

IVH is defined by the presence of bleeding into the brain ventricular system. It is particularly common in preterm neonates (in such cases, the bleeding often originates from the germinal matrix and therefore, IVH is sometimes referred to as germinal matrix haemorrhage) and carries with it high morbidity and mortality. Over 12 000 premature infants develop IVH every year in the USA,¹²⁷ and the incidence has increased over the last 20 years.⁷ IVH is also often a complication of other ICrH subtypes; 50% of patients with primary ICH and 45% of patients with SAH develop IVH.^{128 129} Despite this, IVH is probably the least studied and therefore least well-understood subtype of ICrH.

Iron in IVH

In IVH, blood can disperse within the ventricular system. Unlike in ICH, where iron has the greatest effects on perihematoma tissue, in IVH blood-derived Hb and iron can have effects distant from the initial bleed. A significant proportion of the iron within the ventricular cerebrospinal fluid (CSF) following IVH is free or non-protein-bound,¹³⁰ allowing it to engage in Fenton reactions. This iron eventually accumulates in the ependymal and subependymal regions as evidenced by the increase in Ft expression and iron deposition in those cells.^{131 132} In addition, after lysed red blood cell injection into the ventricles in a rat, periventricular HO-1 was upregulated, while iron injection resulted in ependymal cell injury with mitochondrial swelling and loss of cilia.¹³² The subventricular zone, a site of ongoing neuronal proliferation, became overloaded with iron following Hb injection in neonatal rats.¹³³ IVH has been shown to cause substantial damage to the bordering hippocampus in an iron-dependent fashion.¹³⁴ This damage has been identified as hydrocephalus-independent and possibly mediated by iron-activated c-Jun Kinase apoptotic pathways.⁴⁶

Choroid plexus

One potentially important site of damage following IVH is the choroid plexus epithelium. The choroid plexuses are present in the lateral, third and fourth ventricles of the brain. They form a unique blood–CSF barrier and are the predominant CSF-generating tissue. TfR is present on choroid plexus epithelial cells and iron is localised to these cells in healthy brains.^{135 136} These cells are highly responsive to ischaemic insult, and subsequent choroid plexus cell death results in increased permeability of the blood–CSF barrier to a variety of proinflammatory cytokines and leucocytes,¹³⁷ and decreased production of trophic factors.¹³⁸ Choroid plexus cells express AQP1, which is involved in CSF production.¹³⁹ However, following intraventricular injection of Hb to simulate IVH, AQP1 protein levels increased significantly and there was also an upregulation of AQP5, conveying a high degree of water permeability.¹⁴⁰ In addition, Hb intraventricular injection resulted in ultrastructural damage to the choroid plexus that was accompanied by oxidative stress, cellular activation and an inflammatory response.¹⁴¹ This damage to the blood–CSF barrier, in addition to the increase in AQP expression, suggests that iron may cause increased CSF production by the choroid plexus.

Posthaemorrhagic hydrocephalus

Posthaemorrhagic hydrocephalus (PHH) is a common complication of IVH and the role of iron in PHH has been a topic of interest recently. It has been demonstrated repeatedly that iron is intimately involved in PHH.^{132 133 142} The mechanism underlying this action is not well understood. Potential mechanisms involve an increase in choroid plexus production of CSF and

obstruction of CSF flow pathways. Meng *et al*¹⁴³ have proposed that iron-mediated upregulation of the Wnt signalling pathway, which regulates fibrosis, is involved. However, this is clearly an area in which additional investigation would have significant impact into the development of treatments for PHH.

SUBARACHNOID HAEMORRHAGE

Introduction

SAH is defined by the presence of blood in the CSF in the subarachnoid space between the arachnoid membrane and the pia mater. It is most frequently occurs following the rupture of an intracranial aneurysm.¹⁴⁴ Mortality rates are extremely high, estimated at between 40% and 50%,¹⁴⁵ with incident rates of up between 2 and 32/100 000.¹⁴⁶ SAH causes early and late brain injury, the latter being associated with delayed cerebral vasospasm.

Iron in SAH

SAH-induced damage within the first 72 hours of ictus is classified as early brain injury and iron plays an integral role in such injury. Following SAH, erythrocytes lyse and release their components into the subarachnoid space, exposing the brain to high concentrations of Hb. The Hb is promptly broken down to haem extracellularly. The haem-haemopexin scavenging has been demonstrated to be active and potentially harmful following SAH. CD91, the receptor for haemopexin, is positively correlated with iron deposition in brain tissue, which in turn is negatively correlated with neurological outcome.⁶⁸ This system may be responsible for intracellular accumulation of iron that results following SAH. Non-haem iron and Ft levels in the brain and CSF increase progressively for the first 72 hours.^{147 148} This increase is accompanied by a significant upregulation in HO-1 levels in microglia and neuronal death via oxidative DNA injury.¹⁴⁹ A pilot study in human patients suggested a causal relationship between free iron in the CSF following SAH and brain injury.¹⁵⁰ Indeed, suppression of hepcidin (and thus inhibition of FPI internalisation) attenuated brain damage following experimental SAH, suggesting the importance of intracellular iron accumulation.²⁹ This suggests that the initial inflammation caused by iron accumulation stimulates hepcidin expression, which in turn leads to a decreased efflux of iron from these cells. In this way, iron accumulation is a vicious cycle following SAH.

Additionally, a variety of other iron-handling proteins, such as Tf and TfR, are upregulated after SAH.¹⁵¹ Tf and TfR may also participate in intracellular iron accumulation. Once inside the cell, iron via the mitochondrial calcium uniporter can gain access to the mitochondrial matrix,^{152 153} where it can disrupt mitochondrial integrity, release caspase-3 and lead to neuronal degeneration. It is additionally possible that iron triggers apoptosis via cathepsins.¹⁵⁴

Vasospasm

Endothelins (ET) are potent vasoconstrictors that have a major role in SAH-induced vasospasm. Following stimulation by angiotensin, thrombin, cytokines or ROS, ET-1 is upregulated,¹⁵⁵ resulting in vasoconstriction. Such vasoconstriction is counteracted by nitric oxide (NO) that inhibits ET-1 release, resulting in vasodilation.¹⁵⁶ Iron-containing haem has been shown to cause vasoconstriction.¹⁵⁷ Indeed, reductions in free-iron levels ameliorate SAH-induced vasoconstriction.¹⁵⁸ The iron within haem binds to the vasodilator NO,¹⁵⁹ thereby reducing the ability of NO to inhibit ET-1 leading to vasoconstriction or vasospasm. Moreover, the formation of ROS by iron-mediated Fenton reactions is implicated in the generation of vasospasm, as the hydroxyl radicals also react with NO.^{160 161} Following vasospasm, ET also alters iron-regulatory proteins expression. Hepcidin is induced following ET-1 treatment/release, as is Ft and Tf/TfR.^{155 162} The increase in hepcidin expression may result in increased intracellular iron accumulation and, in turn, induce brain damage.

TRAUMATIC BRAIN INJURY

Introduction

TBI is the leading cause of death before the age of 40, with incident rates of ~200 per 100 000 person-years.¹⁶³ TBI accounts for about 40% of all deaths due to acute injury.¹⁶⁴ TBI is often associated with a single or multiple ICrHs which may contribute to brain injury.¹⁶⁵ As such, many of the findings specific to certain types of ICrH have significance to TBI. It is therefore important to include it in any discussion of ICrH-related research.

Iron accumulation in TBI

As in ICrH, damage from TBI is classified as primary or secondary, where primary injury refers to the immediate result of mechanical brain trauma, and secondary injury refers to the subsequent biomolecular and physiological responses to that injury. Iron has been frequently noted as a potential mediator for secondary injury following TBI due to its ability to form free radicals and induce oxidative stress.^{166 167} Iron levels in the brain are elevated following TBI.^{168–171} While haem-bound iron from erythrocyte lysis is a significant contributor to this, it appears that this iron is supplemented by a release of endogenous sources of iron, suggesting that, like in forms of haemorrhage, the secondary cell loss associated with TBI allows subsequent iron release and a vicious cycle is thus perpetrated.^{167 172} Repeated TBI results in chronically elevated iron levels, as is observed in chronic traumatic encephalopathy. Such iron deposition is positively correlated with cognitive impairment following TBI.¹⁷³

Mechanism of iron-induced injury in TBI

In mice, decreasing CP and APP expression inhibits iron export from neurons. Knockout of these proteins increased brain injury and iron accumulation following

TBI.¹⁷⁴ However, the neuroprotective role of APP in TBI is not fully established. A β deposition is seen in some patients with repeated TBI, and iron has been shown to increase the toxicity of A β .^{167 175 176} Iron is associated with increased brain oedema formation and blood-brain barrier (BBB) disruption after TBI.¹⁷⁷ Furthermore, there is evidence that iron can promote HO-1-mediated neurofibrillary tangle formation, which in turn results in tau phosphorylation as seen in Alzheimer's disease.¹⁷⁸ HO-1 expression has been shown to be upregulated significantly in response to TBI.¹⁷⁷ While it has been shown that iron further facilitates ROS-related damage to neuronal DNA,¹⁷⁹ more research must be performed to assess the nature of this facilitation.

TREATMENTS

Introduction

Preclinical evidence indicates that iron is a key player in brain injury after all forms of cerebral haemorrhage. To date, no existing neuroprotective therapy has been shown to improve morbidity or mortality following ICH in humans.¹⁸⁰ However, using animal models, investigators have identified therapeutic treatments, including but not limited to deferoxamine (DFX) and minocycline, which target iron-mediated damage.

Deferoxamine

Attenuation of brain injury

DFX is a ferric ion chelator that is used clinically for systemic iron overload. The efficacy of DFX in treating haemorrhage-induced brain damage in preclinical models has been reviewed previously.^{181–183} Evidence of efficacy in TBI has also been demonstrated.¹⁸⁴ A stratified meta-analysis showed DFX to be effective in experimental ICH, particularly when administered 2–4 hours after haemorrhage, and with a dosage of 10–50 mg/kg.¹⁸¹ DFX has been repeatedly demonstrated to ameliorate iron-induced oedema,¹⁸⁵ neuronal death,¹⁸⁶ hippocampal degeneration⁴⁶ and inflammation.^{185 187–189} During SAH, DFX treatment chelates free iron before it can form ROS, thus ameliorating vasospasm.¹⁵⁸

Attenuation of PHH

DFX significantly has been shown to reduce PHH.^{142 165} Gao *et al.*¹³² showed that hydrocephalus developed after intraventricular injection of lysed but not packed erythrocytes into adult rats, and that DFX coinjection reduced ventricular enlargement by 27%. Strahle *et al.*¹³³ also demonstrated that iron injection alone could cause hydrocephalus in neonatal rats, and that DFX reduced ventricular enlargement by 57%. Intriguingly, iron was singled out as a key component inducing hydrocephalus due to a comparison between haem and protoporphyrin IX (the latter being the haem scaffold without the iron core) which demonstrated that protoporphyrin IX

injection had no effect on ventricular volume compared with controls. Iron may induce PHH through free radical production and oxidative stress, but it is possible that it also activates the Wnt signalling pathway.¹⁴³ The Wnt signalling pathway is involved in fibrosis in a variety of tissues and, therefore, it may play a role in obstructive non-communicating hydrocephalus formation following haemorrhage. DFX reduced Wnt1/Wnt3a upregulation following IVH most likely via iron chelation.¹⁴³ These findings strongly implicated iron as a key player in PHH.

Clinical trials

In 2011, a Phase-I dose-finding study assessed DFX treatment in ICH and found it to be feasible and well tolerated.¹⁹⁰ That led to a Phase-II trial of DFX in human ICH patients, the High Dose Deferoxamine in Intracerebral Hemorrhage (HI-DEF) trial.¹⁹¹ In that trial, there were some concerns over the occurrence of acute respiratory distress syndrome and currently, a lower dose of DFX is being tested in the Intracerebral Hemorrhage Deferoxamine Trial (iDEF; NCT02175225). In addition, a recent 42 patients study investigating DFX treatment for ICH concluded that DFX may slow haematoma absorption and inhibit oedema formation.¹⁹² Aside from these trials, there have been no other large-scale attempts to implement this treatment into human patients in ICrH. In addition, there has been a lack of DFX clinical trials that investigate any form of haemorrhage besides ICH. Given the preclinical effects of DFX and similarities in the role of iron in SAH and IVH to that ICH, advancement of DFX into large-scale clinical trials for IVH and SAH should be considered.

Minocycline

Effect on brain injury in animal models

Minocycline is another iron chelator. A tetracycline derivative, it has high lipophilicity allowing it to cross the BBB.¹⁹³ It was originally investigated for its therapeutic potential as a macrophage/microglial inhibitor.^{194–196} It decreases post-ICrH damage mediated by thrombin-activated microglia¹⁹⁷ by inhibiting matrix metalloproteinase and PARP-1 activation.^{198 199} However, more recently, minocycline has been investigated for its potential as an iron chelator. In vitro, it reduces iron-induced injury in cortical neuron with higher activity than DFX.²⁰⁰ In vivo, it reduces increases in serum iron levels, reduces iron overload and neuronal death and attenuates iron-induced brain oedema and BBB disruption following ICH.²⁰¹ Coinjection of minocycline +FeCl₂ compared with FeCl₂ alone decreased Ft, CP, HO-1, Tf and TfR upregulation.²⁰² Owing to its iron-chelating abilities, as well as its ability to inhibit harmful microglial activation, minocycline is a promising therapeutic option for ICrH.

Clinical trials

Minocycline currently used clinically as an antibiotic. There have, however, been few human pilot studies

conducted that investigate the efficacy of minocycline treatment following haemorrhage or TBI. A study conducted with 152 patients saw a significant improvement in patient outcome with minocycline treatment compared with placebo.²⁰³ In the ongoing MINOS (Minocycline to Improve Neurologic Outcome in Stroke) trial, minocycline treatment has been shown to be safe in 10 mg/kg intravenous dosages.²⁰⁴ That trial has shown that minocycline decreases the expression of the potentially harmful matrix metalloproteinase-9 and the inflammatory cytokine IL-6 after stroke.^{205 206} However, it has yet to release results on the overall efficacy of minocycline. However, a pilot study conducted in 2013 involving 95 participants investigated the effects of minocycline in ischaemic and haemorrhagic stroke. It found that minocycline is safe, but not particularly efficacious.²⁰⁷ There is therefore significant controversy about the efficacy of minocycline in human trials. Given the concordance on the safety of the treatment, however, it seems sensible to move forward into a larger clinical trial.

Other iron chelators

Deferiprone

In addition to DFX and minocycline, there are several other iron-chelating drugs that have therapeutic potential with respect to ICrH and TBI. Deferiprone (DFP) is a bidentate chelator, requiring three molecules of DFP per iron cation chelated²⁰⁸ as opposed to the hexadentate DFX (1:1 ratio). DFP is effective at attenuating neurodegeneration induced by brain iron overload in Friedreich's ataxia.²⁰⁹ However, it has not been investigated as thoroughly as a treatment for ICrH damage. The few studies that have performed so have had mixed results; DFP administration at 8 hours post-SAH resulted in significantly decreased cerebral vasospasm in a rabbit model.²¹⁰ However, in a rat ICH model, DFP failed to decrease ROS generation, oedema formation and morbidity, despite successfully reducing iron content.⁹⁶ This latter study used a high dosage of DFP (125 mg/kg, which is above the 75–100 mg/kg typical dosage usually used to treat thalassaemia), and found that a dosage of 200 mg/kg was highly toxic to the rats in the study. It may therefore be of interest to reassess DFP's abilities as a treatment if used at a lower dose and possibly in conjunction with DFX administration (as is performed commonly in thalassaemia and other iron overload conditions).

Deferasirox

Deferasirox (DFS) is bidentate, requiring two equivalents of chelator for every one iron equivalent.²⁰⁸ It is delivered orally and is often used to treat anaemias or thalassaemia when DFX treatment is insufficient. It has only been cursorily investigated with respect to TBI. Systemic administration of DFS allows for mild improvements in hindlimb function following traumatic spinal cord injury at the T8 vertebra.²¹¹ The link between this

improvement and any decrease in iron overload has not been investigated, however. Moreover, there have been no studies to the best of our knowledge investigating DFS following TBI or ICrH within the brain itself.

Clioquinol

The iron chelator Clioquinol (CQ) has attracted interest in the treatment of Alzheimer's and Parkinson's disease due to its ability to chelate a variety of metals, including copper, zinc and iron.^{212 213} Its hydrophobic nature allows it to pass through the BBB. In a rat model of ICH, CQ administration ameliorated motor dysfunction due to its ability to reduce ROS production in oligodendrocytes.²¹⁴ In addition, CQ improved neurological outcome, reduced brain oedema and improved mortality rates in a different rat model while enhancing expression of the iron exporting protein FP1.⁹⁶ These findings suggest CQ as a promising upcoming treatment option for ICH. However, its ability to treat other forms of ICrH has yet to be investigated, nor has it been clearly demonstrated that its effects are due to iron chelation.

2,2'-Dipyridyl

Findings on 2,2'-dipyridyl (DP) have been mixed. A lipid-soluble ferrous chelator that acts intracellularly and can bypass the BBB, it showed promise in treating SAH-induced cerebral vasospasm, as it was shown to be effective in a primate model.²¹⁵ In addition, DP has been shown to have greater cell membrane permeation and intracellular iron sequestering ability than DFX.⁶¹ However, to the best of our knowledge, there has been no subsequent research on its effectiveness in SAH since the turn of the century. In ICH, its effectiveness is controversial. Wu *et al*²¹⁶ found that DP pretreatment decreased iron deposition and neurodegeneration following ICH, while additionally attenuating ROS production and microglial activation. Furthermore, this study found that DP post-treatment ameliorated neurobehavioral deficits and brain oedema formation while not affecting mortality rates. However, more recently, Caliaperumal *et al*²¹⁷ performed a series of experiments assessing DP's effect following ICH and found that it failed to chelate non-haem iron levels following ICH, failed to reduce oedema in the ipsilateral cortex and failed to lessen tissue loss or decrease neurodegeneration. These somewhat contradictory findings could be partly explained by the difference in experimental setup (the first was more focused on a pretreatment vs post-treatment administration while the second was simply assessing post-treatment in a variety of circumstances), but they still warrant further investigation and clarification.

FUTURE DIRECTIONS

Over the past few decades, there has been an absence of improvements to the therapeutic approaches available for ICrH. Mortality and incident rates have been

remained largely unchanged. This review stresses the extent and severity of the roles that iron may play in all types of ICrH. It is therefore imperative that more investigation into all types of iron-related brain damage be conducted. Current upcoming treatment options such as DFX and minocycline are promising in animal models and preliminary human studies. Despite this, advancement into clinical trials for these drugs has been slow.

Additional therapeutic targets should be investigated as well. It may not be sufficient to simply chelate extracellular iron, as iron can act via the plethora of signalling pathways as discussed in this review. Therefore, investigation into additional iron-related pathways would be prudent. For example, there has been an absence of investigations finding ways to target specific iron-related proteins after injury. Targets such as hepcidin, IRP-1 and IRP-2, FPI and Ft are well understood in terms of normal function, but methods to treat dysfunction following ICrH are lacking. Ferroptosis, the iron-dependent form of cell death, has yet to be investigated with respect to ICrH.

Additionally, the interplay between ICrH and neurodegenerative diseases merits continued investigation. While amyloid angiopathy is a major cause of ICH, proteins such as APP and phenomena such as neurofibrillary tangles suggest that there are further links between neurodegenerative diseases and haemorrhage/iron.

The recent identification of neuronal CD163 is a promising area of research. It provides a pathway by which Hb-bound iron may significantly enter neurons and thereby cause neuronal iron overload and death. Investigation into neuronal CD163 as a therapeutic target is currently being undertaken in this and other laboratories. Identification of ways to increase Hb reuptake by non-neuronal cells, decrease iron uptake by neurons and/or increase neuronal ability to sequester excess iron influx are important areas of study.

As with all neurological conditions, the delivery of therapeutics is a concern. Most therapeutics developed by the pharmaceutical industry do not cross the BBB. For example, DFX has limited BBB permeability. Strides are being made in delivery systems designed to deliver therapeutics to the brain and alternative routes of administration (eg, intranasal) are being investigated. Hopefully, those strides can be used to develop therapeutics for ICrH.

CONCLUSION

Iron has beneficial and detrimental actions. While it is essential to countless critical cellular functions, it can be harmful if concentrations overwhelm the defence mechanisms of the brain. Following haemorrhage, the brain is exposed to iron-rich Hb which can be disastrous should the existing homeostatic mechanisms be insufficient. Iron is a popular topic in a variety of neurodegenerative diseases, and indeed, it is gaining attention as a therapeutic target in ICrH and TBI, yet there are many

iron-related therapies and experiments that have not yet been investigated. This review stresses the importance of iron in the secondary damage following ICrH. It is clear that the oxidative damage is a key factor in the poor prognosis associated with ICrH and TBI. Current treatment options have not seen a decrease in morbidity or mortality, and so it is of critical importance that we continue to seek out new therapies that are centred on the role of iron following ICH, IVH and SAH.

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