NEURAL REGENERATION RESEARCH
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Heme as an inducer of cerebral damage in hemorrhagic stroke: potential therapeutic implications

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Intracerebral hemorrhage (ICH) consists of the rupture of a cerebral artery leading to bleeding into the surrounding parenchyma. This event has a primary phase of brain injury consisting of mechanical tissue damage due to the mass effect, followed by a secondary phase of brain injury triggered by the presence of blood components released at the site of bleeding (Bulters et al., 2018). Despite the high rates of mortality and morbidity from ICH, no effective treatment is available so far.

The contribution of the secondary phase of brain injury to ICH outcomes has been intensively studied as a promising area for therapeutic research. Our recent work focused on the standardization of a mouse model of intracerebral heme injection comparing it to a broadly used model of ICH (autologous blood injection). We showed that intracerebral heme injection is a feasible model to evaluate the mechanisms of heme/iron involvement in neurologic disorders. Moreover, we demonstrated that heme injection induces oxidative damage, neuroinflammation and long-term astrogliosis, and provided evidence that the induction of neurologic deficits in this model is partially dependent on NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3)-inflammasome sensing (Vasconcellos et al., 2021).

Adaptive protective mechanisms against heme toxicity and their therapeutic implications for ICH: In bleeding events, extravasated red blood cells release into the tissue huge amounts of hemoglobin, a molecule with a fundamental role in transporting O_2 to the body tissues (**Figure 1**). In fact, this O_2 binding capacity of hemoglobin is mediated by heme, which is the prosthetic group of proteins involved in numerous important biological processes. Nevertheless, in pathological events such as ICH, heme has a harmful role when released extracellularly, in part due to its redox potential. Adaptive strategies were developed by mammals to deal with free heme, contributing to its buffering and clearance. Among them are the heme-scavenger action mediated by hemopexin, which induces the internalization of heme via receptor-mediated endocytosis, and the intracellular degradation of heme by heme-oxygenase (HO), breaking heme into iron, carbon monoxide and bilirubin (Bulters et al., 2018). Iron excess is deleterious and can trigger a unique modality of cell death termed ferroptosis, which is also triggered in neurons treated with heme. To prevent the detrimental consequences of an excess of free iron, ferritin can store in the cytosol huge amounts of iron in its inert form. In line, heme detoxifying mechanisms, including those mediated by hemopexin, HO and ferritin, are extremely important for the neurological outcome in experimental models of ICH, though the efficacy of HO-1 regulators in ICH is still under debate (Bulters et al., 2018; Li et al., 2018) (**Figure 1**). We demonstrated that HO-1 and ferritin levels are increased in the brain after heme injection (Vasconcellos et al., 2021). Moreover, others have demonstrated an increased expression of the referred proteins in the mouse brain in standard models of ICH, as well as surrounding the hematoma in patients with ICH (Chu et al., 2018). These findings indicate that understanding the adaptive responses developed by mammals to deal with heme/iron might open new roads for the development of novel therapeutic approaches for ICH (**Figure 1**, boxes #1 and #3). Despite the increasing number of studies regarding the protective roles of hemopexin (Bulters et al., 2018) and HO-1 regulators (Li et al., 2018) in preclinical models of ICH, problems such as the high toxicity, low bioavailability to the brain and poor pharmacologic properties of the compounds tested still need to be overcome for future clinical trials. Iron chelators are already being tested in humans (ClinicalTrials.gov identifiers NCT02175225, NCT01662895, NCT00598572, NCT00777140, NCT02216513, NCT02875262 and NCT03754725), and the results of a recent phase 2 trial demonstrated the lack of efficacy of deferoxamine mesylate in spontaneous ICH, although this drug was shown to be safe (Selim et al, 2019). Nonetheless, little is known about the effects of iron chelators in a combined therapeutic approach, which needs further investigation.

Therapeutic targeting the alarmin effects of heme: In the last decades, the mechanisms involved in the inflammatory processes triggered by heme have been described. Heme acts as a warning signal activating innate immune receptors and triggering inflammation in several biological systems. One of the proposed mechanisms is the activation of the intracellular damage receptor NLRP3, which culminates in NLRP3–apoptosis-associated speck-like protein containing CARD–pro-caspase-1 complex formation that, in turn, induces caspase 1 self-cleavage and interleukin (IL)-1β processing and release (Dutra et al., 2014) (**Figure 1**). In this context, NLRP3 inflammasome activation is associated with increased neurological deficits in experimental models of ICH. We showed that heme induces the production and cleavage of IL-1β to its active form in the brain in an NLRP3-dependent manner (Vasconcellos et al., 2021). Accordingly, others have shown that IL-1β and IL-1 receptor interaction is important for heme-induced brain damage (Chu et al., 2018) and that selective NLRP3 inhibitors have beneficial effects in experimental models of ICH (Ren et al., 2018). However, we have not investigated the source of IL-1β in the brain and we do not discard the possibility that heme could activate others signalling pathways including other inflammasome platforms. The investigation of the source and role of inflammatory mediators in ICH is crucial to grasp the mechanisms involved in neuroinflammation after an ICH event and for the identification of targets for preclinical studies. Taken together, data from studies that have used the heme injection model and the standard models of ICH have

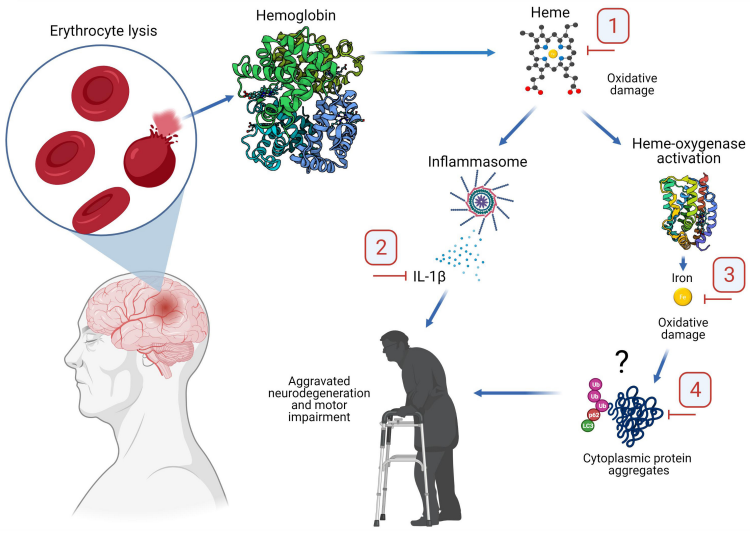

Figure 1 | **The role of heme in the secondary effects of intracerebral hemorrhage (ICH).**
In ICH, the disease onset occurs with a bleeding event and the extravasation of blood components into brain parenchyma. With time, extravasated erythrocytes are lysed, releasing their cytosolic components in the brain, including huge amounts of hemoglobin. Free hemoglobin in the extracellular spaces suffers oxidation and releases heme in its free form, which has an inflammatory and pro-oxidative potential with the ability to activate the inflammasome. Adaptive strategies were developed during evolution to deal with free heme released in the extracellular environment, such as its binding to hemopexin forming a complex that is then internalized via receptor-mediated endocytosis. Once in the intracellular environment, heme can be degraded by heme-oxygenase, generating equimolar amounts of carbon monoxide, biliverdin and iron. Due to the intrinsic oxidative potential of iron, free iron can be harmful to the cells and can induce protein modification and intracytoplasmic protein aggregation. Both inflammasome-dependent interleukin (IL)-1β release and heme-induced protein aggregation might aggravate ICH, exacerbating brain damage, a hypothesis that still needs to be confirmed. The four numbered boxes indicate potential therapeutic targets for ICH. Created with BioRender.com.

pointed out the promising therapeutic potential of IL-1β or NLRP3 signalling inhibition, which includes the use of drugs already approved for clinical use in other indications (**Figure 1**, box #2). An example that is already being evaluated in clinical trials in patients with spontaneous ICH is Anakinra, an IL-1 receptor antagonist already approved for the treatment of inflammatory diseases (ClinicalTrials.gov identifiers NCT03737344 and NCT04834388). Gasdermin D, a protein that acts as the final effector of inflammasome activation and an executor of pyroptosis, forming a pore in the plasma membrane that enables IL-1β secretion, is another molecular target that should be explored in ICH. Promising results were obtained with gasdermin D inhibition in preclinical models of ischemic stroke, but this strategy still needs to be investigated for the treatment of ICH (Wang et al., 2021).

The role of heme/iron in neurodegeneration: Iron overload is observed in post-hemorrhagic brains, and an increasing number of studies have demonstrated its importance in ICH neuropathology as well as its occurrence in other neurological disorders, including neurodegenerative diseases such as Parkinson's disease. Iron accumulation is also the main characteristic of a group of rare genetic disorders known as neurodegeneration with brain iron accumulation, whereas heme metabolism defects play a causal role in several rare neurodegenerative disorders (Chiabrando et al., 2018; Ndayisaba et al., 2019). Another aspect that should be considered is that, similarly to the prevalence of neurodegenerative diseases, the prevalence of cerebral microbleeds and ICH (and consequently the presence of heme and iron in the brain parenchyma) increases with ageing, and there is evidence that cerebral microbleeds are more prevalent in patients with Alzheimer's disease than in the general population (Puy et al., 2021).

Neurodegenerative diseases are frequently associated with protein modification, misfolding and aggregation, leading to the formation of intracellular and extracellular aggregates. Intracellular protein aggregates have two main routes for degradation, the proteasome and autophagy, the former mainly degrading misfolded proteins and the latter degrading huge protein aggregates. Therefore, the accumulation of intracellular protein aggregates can be associated with a dysfunction in the degradation pathways.

The mechanisms linking iron or heme dyshomeostasis to neurodegeneration were reviewed in detail elsewhere (Chiabrando et al., 2018; Ndayisaba et al., 2019). In this regard, our group has demonstrated that heme induces transient protein aggregation mediated by oxidative stress through a nuclear factor erythroid 2-related factor 2-dependent anti-stress homeostatic response. HO-1 activity was found to be essential for protein aggregation and iron was the minimal requirement for this phenomenon. Autophagy deficiency increased the cytoplasmic accumulation of aggregates, highlighting the importance of autophagy-mediated clearing processes (Vasconcellos et al., 2016). Our data using the model of intracerebral heme injection are in line with these findings. We observed lipid peroxidation and increased p62 expression (a nuclear factor erythroid 2-related factor 2-regulated protein that loads cargo for autophagic degradation) in the brain of heme-injected mice, supporting the possibility of heme-induced protein aggregation via oxidative stress (Vasconcellos et al., 2021), a hypothesis that still needs to be tested (**Figure 1**). In this sense, we propose that heme ability to induce the accumulation of intracellular protein aggregates should be taken into consideration for further investigation on how hemorrhagic events and/or heme/iron dyshomeostasis can affect the progression of neurodegenerative processes. If this hypothesis is confirmed in models of heme injection and ICH, it will open the possibility of new therapeutic strategies involving iron chelators combined with drugs that promote the clearance of protein aggregates (such as autophagy inducers) immediately after the hemorrhagic event (**Figure 1**, box #4).

Potential applications of the heme injection model of ICH: The major advantage of the intracerebral heme injection model is the possibility to investigate in vivo the consequences of the exposure of brain cells to heme and the mechanisms behind the toxic and pro-inflammatory effects of this blood component. It is notorious that certain differences exist in comparison with the autologous blood injection model, in which the presence and interplay of other blood components might play a role in the effects observed. Two important aspects to highlight are differences in the mortality rates and the severity of neurological impairments, indicating a more severe injury in the autologous blood model (Vasconcellos et al, 2021). In our view, the model of intracerebral heme injection can be used to reveal new aspects of ICH pathophysiology related to heme and for the screening of therapeutic targets, whereas the standard ICH models (autologous blood and collagenase injection) are more suitable for preclinical testing of novel therapies, as they mimic multiple aspects of the disease.

Concluding remarks: The initial management after ICH is crucial for the patient's outcome and to prevent recurrent hemorrhagic events. The period when secondary brain damage is developing represents an important therapeutic window for possible interventions that could avoid the detrimental effects of blood components in the brain parenchyma. In our opinion, the investigation of the mechanisms involved in neurological damage after hemorrhagic events and the understanding of the role of heme/iron on ICH prognosis are essential steps in the hunt for new therapeutic opportunities (**Figure 1**). Further research is therefore needed to address how heme can cause neural dysfunction through mechanisms that go beyond its pro-oxidant effects, and the murine model of intracerebral heme injection provides a useful tool for these studies. Strategies focused on this suitable therapeutical window should be investigated deeper in order to protect neural cells and improve the long-term prognosis of ICH.

LRCV received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement (No. 845908).

PMPC received funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Instituto Nacional de Ciência e Tecnologia em Medicina Regenerativa.

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Date of submission: June 15, 2021
Date of decision: August 31, 2021
Date of acceptance: September 18, 2021
Date of web publication: February 8, 2022

https://doi.org/10.4103/1673-5374.335148
How to cite this article: *Vasconcellos LRC, Pimentel-Coelho PM (2022) Heme as an inducer of cerebral damage in hemorrhagic stroke: potential therapeutic implications. Neural Regen Res 17(9):1961-1962.*

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P-Reviewer: Zhang L; C-Editors: Zhao M, Liu WJ, Qiu Y; T-Editor: Jia Y