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Intra-parenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage

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ABSTRACT

Intracerebral hemorrhage (ICH) is a devastating stroke causing considerable tissue destruction from mechanical trauma and secondary degeneration. Free iron, released over days from degrading erythrocytes, causes free radicals that likely contribute to delayed injury. Indeed, an intracerebral injection of iron rapidly kills cells and causes cerebral edema. We expanded upon these observations by: determining a dose–response relationship of iron infusion, examining the structural appearance of surviving striatal neurons, and evaluating injury over months. First, we measured 24-h edema in rats given 3.8, 19.0 or 38.0 μg infusions of $FeCl_2$ (i.e., 30 μL of a 1, 5 or 10 mmol/L solution). Second, rats were given these infusions (vs. saline controls) followed by behavioral assessment and histology at 7 days. Third, dendritic structure was measured in Golgi–Cox stained neurons at 7 days after a 0.95-μg dose (30 μL of a 0.25 mmol/L solution). Last, rats survived 7 or 60 days post-injection (19.0 μg) for histological assessment. Larger doses of iron caused greater injury, but this was generally not reflected in behavior that indicated similar deficits among the 3.8–38.0 μg groups. Similarly, edema occurred but was not linearly related to dose. Even after a low iron dose the surviving neurons in the peri-injury zone were considerably atrophied (vs. contralateral side and controls). Finally, continuing tissue loss occurred over weeks with prominent neuronal death and iron-positive cells (e.g., macrophages) at 60 days. Iron alone may account for the chronic degeneration found after ICH in rodent models.

Introduction

Intracerebral hemorrhage (ICH) is a particularly devastating stroke causing higher death rates than ischemia, and in survivors, long-term neurological problems (Sacco et al., 2009). A better understanding of disease pathophysiology will improve treatment options. Since the initial damage results from mechanical trauma as blood dissects through tissue, investigators have been targeting secondary (delayed) damage, which is thought to result from factors such as thrombin, inflammation, and especially iron (Frantzias et al., 2011; Wang, 2010; Xi et al., 2006).

After an ICH ferrous iron is released from lysed erythrocytes after hemoglobin breakdown (Wu et al., 2003). Parenchymal levels of non-heme and total iron increase over a few days and persist for months in animal models (Auriat et al., 2012; Hua et al., 2006; Wu et al., 2003) and patients (Wu et al., 2010). Ferrous iron reacts with H_2O_2 to generate reactive hydroxyl radicals (OH·), and this oxidative stress damages proteins, lipids, DNA, etc. (Nakamura et al., 2005; Triggs and Willmore, 1984). Apart from inducing cell death, iron also causes blood brain barrier dysfunction and cerebral edema (Huang et al., 2002). Indeed, direct proof of iron-mediated toxicity was shown by injecting $FeCl_2$ (ferrous iron) into rat brain and observing DNA damage (Nakamura et al., 2005). Cell death and neuronal atrophy of cortical neurons have also been clearly demonstrated following $FeCl_2$ (Willmore et al., 1980) and $FeCl_3$ infusions (Reid et al., 1979). Additional evidence for iron mediated secondary damage after ICH comes from studies that limited toxicity with free radical scavengers such as NXY-059 (Peeling et al., 2001), and iron chelators, such as deferoxamine (Gu et al., 2009; Hua et al., 2006; Huang et al., 2002; Song et al., 2008; Wan et al., 2006) and 2,2′-dipyridyl (Nakamura et al., 2006; Wu et al., 2012). These promising data have led to a clinical trial with deferoxamine, which is currently underway (Selim et al., 2011). It should be noted, however, that not all animal studies find that deferoxamine improves outcome after ICH (Auriat et al., 2012; Warkentin et al., 2010; Wu et al., 2011).

In animal models, secondary damage occurs over the first few days but sometimes for much longer. The extent and timing of injury have been studied in the whole blood and collagenase models. For instance, significant tissue loss occurs over weeks after collagenase infusion (MacLellan et al., 2008; Nguyen et al., 2008). Noting the persistence of iron in the brain after ICH, we hypothesized that such delayed tissue loss is mediated by iron. As well, striatal neurons in the peri-hematoma region have atrophied dendrites after ICH (Nguyen et al., 2008), which we hypothesize is partly caused by iron toxicity as

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shown, for example, to occur after cortical infusion of ferric iron in cat (Reid et al., 1979). One must distinguish this dendritic atrophy from the common usage of 'atrophy' to describe a general loss of tissue over time, as evidence by ventriculomegaly. The latter can be due to dendritic atrophy, cell death and other reasons (e.g., increased pressure within the ventricles). Dendritic arborization is a key measure of neuroplasticity and clearly an important factor in behavioral recovery (Kleim and Jones, 2008; Kolb et al., 1998; Murphy and Corbett, 2009). Thus, assessing dendritic atrophy as well as total atrophy is important.

Presently we used the intra-striatal infusion of $FeCl_2$ as a simplified model (Nakamura et al., 2006; Willmore et al., 1980; Willmore and Rubin, 1982) to test the role of iron in edema, early and late tissue losses, and dendritic atrophy. We measured edema 24 h after 3.8–38.0 μg infusions (in 30 μL of saline) of $FeCl_2$ were infused into the striatum of rats (Experiment 1). A range in doses was used to mimic the range in hematoma volume, edema and brain damage commonly encountered in ICH research, and as done by others (Nakamura et al., 2006). Next, we evaluated behavior and lesion size at 7 days in groups given various doses of $FeCl_2$ or saline controls (Experiment 2). A 0.95 μg dose was subsequently used to produce a small lesion and using this dose we evaluated dendritic structure (Experiment 3). We used the Golgi–Cox stain to examine dendritic arborization. Finally, we compared histological outcome at 7 and 60 days after a 19.0 μg infusion of iron (Experiment 4). We hypothesized that protracted neuronal death would occur leading to significant enlargement of tissue lost over time associated with iron deposition.

Material and methods

Animals

All procedures followed the guidelines of the Canadian Council of Animal Care and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta. We used 84 male Sprague–Dawley rats (250–350 g, ~3 months old) randomly assigned to 4 experiments (n = 18–23 each) with blinded assessment. Rats were grouped 4/cage in standard polycarbonate cages (wood chip bedding), except for single housing used in Experiment 2. There we wished to avoid any influence of social housing on dendritic shape (Kolb et al., 1998). Food and water were provided ad lib and rats were kept in a temperature and humidity controlled room (lights on from 7 am–7 pm).

Surgery (all experiments)

Surgical procedures were performed aseptically. Rats were anesthetized with isoflurane (4% induction, 1.5–2.5% maintenance in 60% N_2O, balance O_2). Body temperature was maintained at 37 °C during anesthesia with a heated water blanket and a rectal temperature probe. After placing the animals in a stereotaxic frame a hole was drilled 3.5 mm right and 0.2 mm anterior to Bregma. A 26 gauge needle was inserted 6.5 mm into the striatum to infuse $FeCl_2$ (0.95, 3.8, 19.0 or 38.0 μg contained in 30 μL of 0.25, 1, 5 and 10 mmol/L solutions of $FeCl_2$ in saline, respectively) or saline alone (at pH of 4 or 5.2) over 10 min (Nakamura et al., 2006). The needle was removed following an additional 10 min. A small metal screw was inserted in the hole, bupivacaine was applied and clips were used to close the wound.

Brain water content (BWC) (Experiment 1)

The BWC was measured 24 h after infusing 3.8, 19.0 or 38.0 μg of iron (n = 5, 5, 8 each). Briefly, animals were anesthetized with isoflurane and quickly decapitated. The brain was blocked 2 mm anterior to 2 mm posterior to the injection and separated into cortex and striatum. The cerebellum served as a control. The wet–dry weight method was used. Basically, sample wet weight was taken before and after 24 h at 100 °C. We calculated BWC as ((Wet Weight–Dry Weight)/Wet Weight) × 100 (Wu et al., 2003).

Behavioral evaluation (Experiment 2)

Three groups (n = 6, 6 and 5 each) were initially done (3.8, 19.0 or 38.0 μg infusions). Subsequently, we added two control groups (saline at pH of 4 and 5.2; n = 4 each) to approximate the pH range of $FeCl_2$ groups. This allowed us to test whether the injury was due to acidity, which previous studies indicate is not the case (Willmore et al., 1980; Willmore and Rubin, 1982). All of these groups were subjected to behavioral evaluation followed by lesion volume determination at a 7-day survival.

Neurological deficit scale

A neurological deficit scale (NDS) that is sensitive to striatal damage (Del Bigio et al., 1996; Hua et al., 2002; MacLellan et al., 2006; Peeling et al., 2001) was used at 1, 4 and 7 days post injection (vs. day before injection). Briefly, the rats were evaluated on: spontaneous circling, hind limb retraction, bilateral forepaw grasp, contralateral forelimb flexion, and beam walking. A maximum score of 14 denotes greatest impairment.

Forelimb asymmetry

Rats were placed in the vertical cylinder (45 cm in height and 20 cm in diameter) and video recorded for ~10 min on the day prior to iron injection and 7 days afterward. This 'cylinder test' is used to evaluate forelimb contact during wall exploration, and is sensitive to striatal injury. The % ipsilateral forelimb use was: (ipsilateral forelimb contact + 1/2 both)/(contralateral forelimb contact + ipsilateral forelimb contact + both) × 100 (Hua et al., 2002; MacLellan et al., 2006).

Corner turn test

The corner turn test measures turning bias when rats turn away after entering a 30° corner (Hua et al., 2002; Warkentin et al., 2010). Rats are placed in front of two angled Plexiglas walls (41 cm in height; 30.5 cm in length) and those with striatal injury turn ipsilateral when exiting. Testing was done on the day prior to iron injection and 7 days post-injection.

Lesion volume (Experiments 2 and 4)

Based upon Experiment 2, we selected the 3.8-μg dose for the last experiment to allow for injury progression to occur and to avoid possible ceiling effects with severe insults or floor effects with very mild insults. Rats were euthanized with pentobarbital (100 mg/kg IP) and transcardially perfused with 0.9% saline, then formalin. Coronal frozen sections (40 μm) were stained with cresyl violet. Lesion volume was determined with Scion Image J (4.0; Scion Corporation, Frederick, MD) as routinely done on digitized images of coronal brain sections taken so that they extended from anterior, through and beyond the sections with obvious tissue damage (Auriat et al., 2012; MacLellan et al., 2006). The volume of tissue lost was calculated:

$$\text{Tissue lost } (\text{mm}^3) = \text{volume of normal hemisphere} - \text{volume of injured hemisphere.}$$

$$\text{Hemisphere volume} = \text{average}\,(\text{area of the complete coronal section of the hemisphere} - \text{area of ventricle} - \text{area of damage if any}) \times \text{interval between sections} \times \text{number of sections.}$$