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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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A B S T R A C T

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 FeCl₂ (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.

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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 H₂O₂ 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 FeCl₂ (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 FeCl₃ (Willmore et al., 1980) and FeCl₂ 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...
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 FeCl₂ 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 FeCl₂ 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 FeCl₂ 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% N₂O, balance O₂). 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 FeCl₂ (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 FeCl₂ 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 (\(\frac{\text{Wet Weight} – \text{Dry Weight}}{\text{Wet Weight}}\)) × 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 FeCl₂ 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: \(\frac{\text{ipsilateral forelimb contact} + 1/2 \text{both}}{\text{contralateral forelimb contact} + \text{ipsilateral forelimb contact} + \text{both}}\) × 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:

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

Hemisphere volume

= average (area of the complete coronal section of the hemisphere – area of ventricle – area of damage if any) × interval between sections × number of sections.
Golgi–Cox staining (Experiment 3)

We first conducted a pilot study with a low dose of FeCl₂ (0.95 μg, n = 3) where animals were euthanized (pentobarbital) at 7 days for assessing lesion volume (as in Experiments 2 and 4). We deliberately used a low dose to avoid causing extensive striatal damage that would prevent us from evaluating peri-lesional dendritic atrophy (i.e., enough striatum remained to obtain sufficient numbers of surviving striatal neurons). For this study, rats were euthanized at 7 days after iron (0.95 μg, n = 11) or a saline infusion control (n = 9). Rats were transcerebrally perfused with saline and the brains were removed and immersed in Golgi–Cox solution for 14 days. Vibratome sections were cut at 200 μm (Leica VT 1200 S) and stained according to established procedures (Gibb and Kolb, 1998; MacLellan et al., 2011). Medium spiny neurons in the perinsult and contralateral striatum were drawn (20 × camera lucida). Neurons (n = 5–6 per region/animal) had to be fully impregnated and unobstructed (e.g., by blood vessels) to be drawn. These were quantified using Sholl analysis, which estimates the dendritic length, and branch order analysis to evaluate complexity (Kolb et al., 1998; MacLellan et al., 2011). These data were averaged per region per animal. Note that it is not possible to accurately determine lesion volume from Golgi–Cox stained tissue due to the nature of the stain and the use of thick sections necessary for determining dendritic arborization.

Fluoro-Jade stain (Experiment 4)

The Fluoro-Jade B stain marks degenerating neurons (Schmued and Hopkins, 2000). Slides were rinsed in water and then dehydrated using alcohols and incubated with 0.06% potassium permanganate for 15 min. Slides were then washed with water, incubated in 0.001% Fluoro-Jade (Chemicon, Temecula, CA) for 30 min in the dark, and cover slipped. Sections at the level of significant injury were qualitatively evaluated to determine whether there was recent neuronal death or not.

Perls’ stain (Experiment 4)

Perls’ stain (Wu et al., 2003) was used to identify iron positive cells (e.g., macrophages). 4% potassium ferrocyanide was mixed with 4% HCl into which sections were incubated for 60 min. The sections were then washed with water for 10 min. 0.4% DAB solution was prepared and slides were incubated for 45–60 min. Then the slides were quickly dehydrated with alcohol and kept in Citrosolv (Fisher Scientific, USA) before cover slipping.

Statistical analysis (all experiments)

Data are presented as mean ± standard error of mean (SEM) except NDS, which is reported as median. Data were analyzed by analysis of variance (ANOVA) with Tukey HSD post-hoc testing when appropriate. For NDS we used Kruskal–Wallis or Mann–Whitney tests (SPSS v.17.0, SPSS Inc., Chicago, IL).

Results

Exclusions

One rat from Experiment 1 died during surgery. No other unplanned mortality occurred. Also, whereas other studies have noted the occurrence of seizures following cortical injections of iron, we observed none in our striatal infusion paradigm. Finally, two rats in Experiment 4 were excluded because of technical problems with surgery.

Experiment 1

Significant edema (24 h survival), comparable to that commonly reported in ICH models, occurred in ipsilateral striatum and cortex for all groups (p ≤ 0.004 vs. contralateral side, Fig. 1). Edema in the injured cortex and striatum was lowest in the 3.8 μg group (p ≤ 0.005) compared to 5 and 10 mmol/L groups, which were similar (p ≥ 0.841).

Experiment 2

Iron infusion caused noticeable striatal damage with additional injury to the corpus callosum and cortex at higher doses (Fig. 2B). While there was a dose-dependent increase in tissue loss (p < 0.001), it was not linear (Fig. 2C). Specifically, the 3.8 μg dose caused less damage than the higher doses (p ≤ 0.002), but the two highest doses were not statistically different from each other (p = 0.594). The two saline controls, which were added after the iron experiments were completed, sustained only minimal injury from the needle insertion and infusion procedure (Figs. 2A and C). These groups did not differ significantly from each other (p = 0.235), and they had considerably less damage than the iron groups (e.g., the 19.0 μg FeCl₂ group had many times more damage than the acidity matched saline control).

Behavioral data were collected for the 3.8 to 38.0 μg groups for the NDS (Fig. 3A), cylinder (Fig. 3B) and corner turn tests (Fig. 3C). All animals had normal baseline NDS scores. Groups were not significantly different on days 1 (p = 0.358) and 7 (p = 0.090), but on day 4 the 38.0 μg group was slightly more impaired than the other two (p ≤ 0.005). Repeated-measures ANOVA on the cylinder task revealed a time effect (p < 0.001), but no interaction (p = 0.068) or group effect (p = 0.400). Thus, all groups showed a bias for using the ipsilateral (non-impaired) forelimb after the lesion, but there were no differences among groups at baseline or day 7. A repeated-measures ANOVA on the corner turn test revealed a significant time effect (p < 0.001), but no interaction (p = 0.509) or group effect (p = 0.707). Thus, post-infusion scores were higher than baseline (i.e., turn bias), but there were no differences among the FeCl₂ groups. The additional saline controls, which were analyzed separately (added after the 3.8–38 μg groups were completed), also showed normal behavioral scores before and after infusion (e.g., NDS scores of 0, and no bias in the corner turn test) that did not differ when comparing baseline with post-infusion scores (data not shown).

Experiment 3

The pilot study showed that the 0.95 μg FeCl₂ infusion caused only a very small amount of tissue loss at 7 days as expected (8.55 ± 3.07 mm³). Nonetheless, for the Golgi–Cox study, the striatal neurons (Fig. 4A) near the lesion were markedly atrophied compared to the normal side (Fig. 4B). Sholl analysis (Fig. 4C), which estimates overall dendritic length, showed that surviving peri-lesion neurons.
had considerably shorter dendrites ($p < 0.001$). Conversely, the contralateral side was normal (vs. saline, $p = 0.599$). The comparison of ipsi- and contralateral neurons within the iron group was significant ($p < 0.001$). Thus, iron caused remarkable dendritic atrophy ($\sim 50\%$) of peri-lesion striatal neurons. For the branch order analysis (Fig. 4D), which reflects dendritic complexity, we found a significant interaction ($p < 0.001$) in the 2-factor design for the peri-lesion neurons. Thus, further analysis showed that the iron group had fewer lower-order branches (1 through 5, $p \leq 0.002$ vs. saline). The 6 and 7 orders were near zero normally and thus not expected to be different ($p \geq 0.232$). There was a branch order main effect ($p < 0.001$) for the contralateral neurons (i.e., fewer distal bifurcations), but no group effect ($p = 0.991$) nor interaction ($p = 0.827$, data not shown). Thus, iron infusion significantly reduced dendritic length and number of branches indiscriminately only in the peri-lesion zone. The saline control infusion did not have a noticeable effect on dendritic structure and there was minimal injury (e.g., needle tract) as seen in Experiment 2.

**Experiment 4**

A 3.8-μg infusion of iron caused considerable tissue loss at 7 (Fig. 5A) and 60 days (Fig. 5B). Damage was $\sim 50\%$ greater at 60 days (Fig. 5C, $p = 0.017$). Fluoro-Jade staining in the ipsilateral hemisphere showed that at least some of this continuing tissue loss resulted from ongoing neuronal death observed at 7 (Fig. 6A) and 60 days (Fig. 6B), but not in the contralateral side. Cell counts were not performed, but examining several sections per animal revealed that there were considerable (hundreds per coronal section) numbers of Fluoro-Jade positive cells at day 7 whereas at day 60 there were mild to moderate number of cells in every animal as shown in representative photomicrographs. The Perls' stain was used to qualitatively show the number and spatial distribution of iron-positive cells (macrophages, microglia). Sample photomicrographs bordering the lesion cavity are shown at 7 (Fig. 6C) and 60-day survival times (Fig. 6D). Intense staining was observed at both times in all animals. There was no labeling further away from the lesion (e.g., ventral cortex) or in the contralateral side (not shown). Thus, the spatial localization of iron overlaps with atrophied and degenerating cells.

**Fig. 2.** Representative sections illustrating damage caused by saline infusion (A) and the 3.8-μg iron infusion (B). Control groups had damage limited to the injection tract whereas extensive destruction affecting striatum, corpus callosum, and cortex occurred with iron infusion. Tissue lost at 7 days is shown in C ($p < 0.05$ vs. 3.8 μg group, $p < 0.05$ vs. combined saline controls, which were not different).

**Discussion**

An intracerebral infusion of iron is well known to cause edema and rapid cell death (Huang et al., 2002; Nakamura et al., 2006; Willmore et al., 1980; Willmore and Rubin, 1982), as the present findings confirm and extend upon by documenting the relationship between increasing iron dose and greater cell death/edema. Beyond this, our study primarily sought to determine whether ferrous iron causes protracted cell death and tissue loss in striatum, which did occur. Thus, our data supports the belief that iron released from degrading erythrocytes accounts for much of the delayed, secondary damage observed in animal models of ICH (Del Bigio et al., 1996; Felberg et al., 2002; MacLellan et al., 2008; Nguyen et al., 2008), which typically targets the striatum and rarely the cortex (MacLellan et al., 2012). As well, we sought to determine if surviving striatal neurons would show dendritic atrophy as others have shown in cortex after iron infusion in rat (Willmore et al., 1980) and cat (Reid et al., 1979) and we have seen the following collagenase-induced ICH in striatum of rat (Nguyen et al., 2008). Indeed, we found that the surviving striatal neurons in the peri-lesion zone were strikingly atrophied after a low-dose FeCl$_2$ infusion, which caused a relatively minor lesion at the low end of the range commonly produced in ICH studies. Accordingly, this supports the hypothesis that iron released into brain parenchyma after an ICH contributes to neuronal atrophy.

Our conclusions about the importance of iron in mediating secondary (chronic) degeneration are in line with numerous studies showing that free radical scavengers (Nakamura et al., 2008; Peeling et al., 2001) and iron chelators (Gu et al., 2009; Hua et al., 2006;
Huang et al., 2002; Song et al., 2008; Wan et al., 2006; Wu et al., 2012) limit injury, edema and behavioral dysfunction after ICH. In most cases chelators were given acutely, but our findings suggest that delayed chelation therapy might provide additional protection against protracted cell death and atrophy, perhaps because not all iron is sufficiently liganded (Kell, 2010). However, some studies find that iron chelators administered soon after ICH do not improve outcome. For example, we (Warkentin et al., 2010) reported that deferoxamine failed to affect cerebral edema, tissue loss or behavioral outlook in the collagenase model despite using protocols found effective in the whole blood model. More recently we reported that early treatment with deferoxamine lowers total iron levels (measured by rapid scanning X-ray fluorescence imaging—RS-XRF) after collagenase-induced ICH while failing to improve neurological recovery (Auriat et al., 2012). Differences among studies, including model and hematoma size, which influence inflammation, the timing and amount of iron released, changes in ferritin levels, etc., may contribute to study discrepancies. As well, chelators or free radical scavengers alone may not be sufficient in some cases, but require combination treatment to maximally reduce oxidative stress (Kell, 2010) and to noticeably attenuate lesion size.

The surprising amount of protracted injury occurring many weeks after FeCl₂ infusion does not explain differences between the whole blood and collagenase models with regard to the extent of delayed injury. Specifically, infusing collagenase can cause considerable delayed tissue loss over weeks (MacLellan et al., 2008; Nguyen et al., 2008), as we find with FeCl₂, and much more than we have observed in the whole blood model. Differences among ICH models, such as the extent of inflammation, the size of the initial insult, and the extent of parenchymal ultrafiltration by blood (and damage it causes), must be considered (Frantzias et al., 2011; MacLellan et al., 2012). For instance, little secondary damage appears to occur in very small collagenase-induced insults (Wasserman and Schlichter, 2007). As well, there appears to be differences between the whole blood and collagenase models with regard to dendritic atrophy (Auriat et al., 2010; MacLellan et al., 2011; Nguyen et al., 2008), but again this might be influenced by initial lesion size. Similar model differences have been reported in the ischemia literature (Gonzalez and Kolb, 2003). Regardless, there are undoubtedly other factors important in delayed injury that are not modeled with FeCl₂ infusion.

Several additional observations deserve consideration. First, there was a non-linear relationship at the higher iron doses. This is potentially due to limited diffusion distances at the higher doses (e.g., barriers such as the corpus callosum), or alternatively by differences in how inflammatory cells respond to limit extreme injury. Linear relationships might also
be found had we evaluated other survival times (e.g., edema at 3 days or histology at 4 weeks). Second, the behavioral tests show similar levels of dysfunction despite a considerable range in injury, which also overlaps with that produced in ICH models. Some behavioral tests are effective “lesion detectors” but are sometimes insensitive to variations in lesion size (MacLellan et al., 2006) as the present findings also indicate. This is a concern as some treatments may significantly reduce injury without being behaviorally noticeable on many routine tests. However, it is possible that later testing would reveal dose-dependent deficits. We did not conduct behavioral testing in the Golgi–Cox and long-term survival studies in order to avoid the potential impact that this could have on neuronal death, dendritic atrophy and tissue destruction (MacLellan et al., 2012). Third, because dendritic arborization is strongly tied to functional performance (Kleim and Jones, 2008; Kolb et al., 1998; Murphy and Corbett, 2009) and clearly affected by ICH (Nguyen et al., 2008) and iron (present findings), one ought to consider dendritic damage in ICH studies — not only rehabilitation studies (Auriat et al., 2010; Takamatsu et al., 2010) but also when testing neuroprotectants. For instance, iron chelators and/or free radical scavengers may partly work to improve behavioral recovery by attenuating cellular atrophy and not just by reducing cell death.

There are several methodological limitations that warrant consideration. First, while the infusion of FeCl₂ is clearly toxic, the release of iron from degrading erythrocytes occurs slowly after ICH allowing for protective measures to be initiated, such as increasing ferritin production to limit iron toxicity (Wu et al., 2003). We opted to use the simplified commonly used method to determine whether iron alone (vs. mechanical trauma, etc.) causes striatal neuronal atrophy and progressive tissue loss including from neuronal death. However, future studies might consider more protracted release (e.g., cannula and mini-pump) to better mimic ICH.

Second, we did not count dead neurons because this data cannot be used to calculate its contribution to tissue loss (mm³), which is our primary endpoint. Cell counts in the peri-lesion zone are also biased by tissue changes (loss of neuropil and lesion expansion) making it difficult to compare survival times. Thus, our findings only show that prominent and lasting neuronal death occurred. Likely, dendritic atrophy and delayed neuronal death both contribute to the increasing volume of tissue lost with time, but one cannot determine the exact contribution of each to tissue loss or functional impairment.

Third, with the Golgi–Cox method we could not determine whether the dendritic atrophy caused by FeCl₂ infusion would have recovered or eventually led to cell death. We suspect that many neurons would have eventually succumbed based upon prominent Fluoro-Jade staining in this zone and the remarkable atrophy found. Our previous collagenase study showed neuronal atrophy at 7 but not 60 days after ICH (Nguyen et al., 2008). This implies that atrophied cells recover, but changes in lesion size over time and the use of a between subjects design must be considered. Notably, atrophied neurons observed soon after an ICH may eventually die off (contributing to lesion expansion) leaving only more peripheral, healthy-looking neurons at later survival times (i.e., sampling location bias). While the Golgi method is well suited to striatal insults, an alternative would be to evaluate atrophy after cortical hemorrhage with 2-photon microscopy. Ischemia studies using this method show dendritic atrophy near the infarct, which is balanced by growth of these cells’ dendrites that are located away from the infarct (Brown et al., 2010). Presently, all branches of striatal neurons appeared markedly atrophied after FeCl₂ infusions, but it is possible that there is a distance-to-lesion effect in this model and after ICH.

Fourth, total iron levels cannot be quantified with Perls’ staining, which was the histochemical method presently used. An alternative is to use RS-XRF, which we recently used to show that total iron levels are significantly increased in the hematoma and immediate peri-hematoma region, but not in areas farther away from the ICH (Auriat et al., 2012). This, of course, fits with the sequestering of iron by microglia and macrophages (Perls’ positive cells) that migrate to

![Fig. 6. Representative photomicrographs showing the many degenerating neurons (FluoroJade positive cells, arrows) that were found around the iron infusion (3.8 μg) site at 7 (A) and 60 days (B). Representative photomicrographs also show heavy Perls’ staining at 7 (C) and 60 days (D) after iron infusion (iron positive cells, arrows). The contralateral side was always normal.](image-url)
the hematoma and peri-hematoma areas after ICH (Wang, 2010), and similarly, injured areas caused by FeCl2 infusion. Presently, we can only visually relate the extent of injury to the localization of iron in the damaged and immediate surrounding zone. Even with RS-XRF to quantify total iron, we would still need to know the type (Fe2+ vs. Fe3+) and molecular localization of iron to draw stronger conclusions. For instance, one would have to determine how much of the total iron load is bound to ferritin and other proteins that render it harmless. Thus, further mechanistic studies are needed to relate iron-mediated oxidative stress and other potential mechanisms (e.g. abnormal electrical activity, deafferentation and inflammation) to atrophy and cell death. Unfortunately, the RS-XRF method is not compatible with Golgi-Cox staining and Perls’ histochemistry cannot be used to accurately quantify iron levels. Thus, directly relating iron load to the distribution to neuronal death and dendritic atrophy is difficult to do.

In summary, our study proves that iron overload leads to edema, substantial dendritic damage, and a remarkable amount of delayed neuronal death and tissue loss in the striatum of rat. These findings strongly support the assertion that iron toxicity contributes to delayed, secondary degeneration occurring after striatal ICH. Therefore, treatments targeting iron overload and its consequences are logical therapeutic targets, especially considering that the progression of cell death with iron toxicity is quite long and beyond the time frame commonly targeted in neuroprotection trials. Future mechanistic work is warranted with the FeCl3 model, and studies must also evaluate other ICH specific factors (e.g., thrombin) contributing to very delayed injury and neuronal atrophy.

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