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A Rapid Fluorescent Method to Quantify Neuronal Loss after Experimental Intracerebral Hemorrhage

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Abstract

Neuronal loss in tissue surrounding an intracerebral hemorrhage (ICH) is usually quantified by labor-intensive histological methods that are subject to bias. Fluorescent protein expression has been successfully used as a marker of cell viability in vitro and in retinal studies in vivo, but not in any ICH model to date. The potential of this approach was investigated using transgenic mice that constitutively express the red fluorescent protein variant dTomato in central neurons under the control of the Thy1 promoter. Breeding and growth of these mice were similar to their wild-type counterparts; behavioral phenotyping by digital analysis of home cage video recordings detected no differences. Bright fluorescence was evident in fresh brain samples with minimal background fluorescence, and was reduced in tissue surrounding the hematoma. In order to assess fluorescence loss as an injury marker in a planned study, these mice were crossed with heme oxygenase (HO)-2 knockouts and wild-type controls; striatal hemorrhage was induced by stereotactic injection of collagenase. Fluorescence in hemorrhagic striata was reduced to $86.4 \pm 3.9\%$, $62.2 \pm 5.1\%$, and $58.3 \pm 3.0\%$ of contralateral on days 1, 4 and 8, respectively, and correlated closely with reduction in striatal cell viability as quantified by MTT assay. HO-2 knockout and wild-type values did not differ significantly. Similar results were observed with stereological cell counts of striatal neurons identified by NeuN immunoreactivity. These results suggest that loss of constitutive dTomato fluorescence is an accurate and efficient marker of neuronal loss in tissue surrounding a striatal hematoma.

Keywords

Heme oxygenase; Intracerebral hemorrhage; Stroke models

1. Introduction

Intracerebral hemorrhage (ICH) is modeled in rodents by stereotactic injection of bacterial collagenase or autologous blood, most often into the striatum, a common site of clinical ICH. Neuronal loss in tissue surrounding the hematoma is then quantified by histological analysis, using various stains to identify normal and degenerating cells (MacLellan et al., 2012). Specific methods vary considerably from laboratory to laboratory, but do share common features of being laborious, operator-dependent, and quite expensive. These

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characteristics may account in part for the limited scope of many studies, which typically test only one hemorrhage volume and one or two drug doses. Since the efficacy of any therapeutic intervention will likely be a function of both clot size and drug dose, a more efficient and reproducible method to quantify neuronal injury is urgently needed.

A rapid alternative to cell counting is direct quantification of cell viability in fresh tissue samples. Since ICH does not produce a discrete infarction with defined boundaries (Hua et al., 2007), this method is most accurately accomplished by excising tissue of interest, placing it into cell culture medium, and quantifying its viability by MTT or similar colorimetric assay (Qu et al., 2007; Qu et al., 2005). Two characteristics of the mouse striatum make it particularly well-suited to this ex-vivo approach. First, it is architecturally-distinct in fresh tissue preparations and easily dissected free of surrounding tissue. Second, its small size (mean volume $23.6 \pm 0.6 \text{ mm}^3$, (Rosen and Williams, 2001) allows complete analysis of the entire structure, thereby eliminating sampling bias. However, MTT and other tetrazolium salts have key limitations. Since they are also converted to formazan dyes by non-neuronal cells, they do not give information specific to neurons, and can be confounded by gliosis and inflammatory infiltrates. In addition, they must be delivered homogeneously to cells, requiring cell dissociation methods that are a potential source of variability.

Loss of fluorescent protein expression has been validated as an injury marker in cell culture neurotoxicity models (Anegawa et al., 2000; Hansen et al., 2010). However, in vivo application of this method has been sparse, and to date has been used only for noninvasive monitoring of retinal ganglion cell injury (Hayworth et al., 2008; Leung and Weinreb, 2009). No study has characterized fluorescent protein expression as an index of cell viability after hemorrhagic stroke. Transgenic mice that constitutively express the red fluorescent protein variant dTomato in neurons are commercially available (Livet et al., 2007). In the present study, we hypothesized that dTomato fluorescence in striatal tissue lysates correlates with perihematomal cell viability, and can be used to rapidly quantify cell injury after ICH. In order to evaluate this method in parallel with other established approaches in a study that had been planned in our laboratory, we incorporated the dTomato transgene into heme oxygenase (HO)-2 knockout mice and wild-type controls. The effect of this knockout on collagenase-induced ICH was then assessed.

2. Materials and Methods

2.1. Experimental Animals

Mouse breeding and housing were conducted at our animal care facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal care and treatments complied with the standards described in *Guide for Care and Use of Laboratory Animals* and were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Animals were provided with food and water ad libitum and a 12-hour light/dark cycle. Mice that constitutively express the red fluorescent protein (RFP) variant dTomato in neurons were purchased from The Jackson Laboratory ("Brainbow" mice (Livet et al., 2007), Stock #007910, C57BL6 background) and a breeding colony was established. They were then crossed with HO-2 knockout mice (Poss et al., 1995) from a colony maintained in our animal facility for several years. Offspring heterozygous at the HO-2 locus and expressing dTomato were then selected as breeding pairs for this study. HO-2 genotype was determined at 2–4 weeks by PCR using genomic DNA isolated from tail clippings and previously published primers (Rogers et al., 2003). The Brainbow construct incorporating the dTomato gene was detected by PCR using the following primers:

Brainbow forward: 5'-GGCACGCTGATCTACAAGGT-3'; Brainbow reverse: 5'-GGGAGGTGTGGGAGGTTTTT-3'; Brainbow band ~450bp.

Control Forward: 5'-CAAATGTTGCTTGTCTGGTG-3'; Control Reverse: 5'-gTCAGTCgAgTgCACAgTTT-3'; Control band ~200bp.

dTomato expression could be detected at three weeks by skull transillumination using a Nikon TRITC filter. HO-2 protein expression was determined by Western blotting using a previously-described method (Chen and Regan, 2004). All mice used in experiments were the first or second-generation offspring of breeders that were heterozygous at the HO-2 locus.

2.2. Striatal Collagenase Injection

All experiments were conducted on 4–6 month old mice (n = 132, 75 males and 57 females). Homozygous HO-2 knockout and wild-type mice expressing neuronal dTomato were anesthetized with 1.5% isoflurane in oxygen, and were secured into a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Collagenase was purchased as a lyophilized product from Sigma Aldrich, St Louis, MO (C9572-7.5KU, 1917 units/mg solid). Careful handling of collagenase was found to be necessary to produce a consistent hemorrhagic effect. After weighing (0.1–0.4 mg), collagenase was dissolved in sterile cell culture grade distilled water (Gibco, Farmingdale, NY, 0.1 mg/33µl), aliquoted (2 µl), and stored at –70°C until used. When frozen at this dilution, it was stable for approximately one month. On the day of injection, an aliquot was rapidly thawed, placed on ice, and diluted in 4 µl ice-cold sterile artificial CSF (NaCl 148 mmol/L, KCl 3 mmol/L, CaCl₂ 1 mmol/L, MgCl₂ 0.8 mmol/L, Na₂HPO₄ 0.8 mmol/L, NaH₂PO₄ 0.2 mmol/L) to make the working dilution. Injecting solution was then made by adding either 1 µl (for 0.014 unit injection) or 2 µl (0.028 unit injection) of working dilution to 68 µl artificial CSF, and mixing well. In order to minimize loss of collagenase by binding to the plastic tube or glass syringe, both were precoated with injecting solution for five minutes prior to preparation of fresh injecting solution in the same tube. Collagenase (1 µl/min) was injected through a small burr hole at the following coordinates relative to bregma: 2.5 mm lateral, 0.5 mm anterior, and 3.0 mm below the surface of the skull. After 9 minutes, the needle was slowly withdrawn 1 mm, followed by the remaining 2 mm four minutes later. The skin was then sutured, and the mouse was allowed to recover in a warm environment. Surgical control mice were treated in the same manner, but were injected with an equal volume of artificial CSF only.

2.3. Striatal Hemoglobin Assay

At 24 hours after collagenase injection, mice were deeply anesthetized with isoflurane and were quickly euthanized by cervical dislocation. Brains were rapidly removed and placed into 60-mm culture dishes. Striata were then excised under a dissecting microscope and placed into separate 2-ml tubes with 0.5 ml phosphate-buffered saline. After three freeze-thaw cycles, samples were sonicated for 15 seconds on ice, and debris was then removed by centrifugation (16,200×g, 5 minutes). The hemoglobin content of the supernatant was then assayed using the method of Winterbourne (Winterbourn, 1990).

2.4. MTT and dTomato Fluorescence Assays

At 1, 4, or 8 days after collagenase injection, mice were euthanized as described above. Brains were immediately removed and placed into 60 mm culture dishes containing dissecting medium (Hanks Balanced Salt Solution supplemented with 27.8 mM glucose, 20.5 mM sucrose, and 4.2 mM sodium bicarbonate (Rose et al., 1993). Injected and contralateral striata were excised under a dissecting microscope, minced with forceps, and placed into separate centrifuge tubes in 1 ml dissecting medium. Tissue was then dissociated by trituration through a Pasteur pipette, followed by passage through another pipette with

narrowed (flame-polished) tip. MTT and striatal dTomato fluorescence assays were conducted on the same samples. The former procedure has previously been described in detail (Qu et al., 2005). Briefly, after addition of 1 ml of 0.25 mg/mL MTT, tubes were incubated in a water bath at 37°C for four minutes. The cell suspension was then collected (1380×g, 2 minute), supernatant was removed, and the purple formazan was extracted in 2 ml isopropanol. The formazan absorbance in isopropanol was determined at 562 nm with a reference wavelength of 650 nm, and was normalized to that in the contralateral striatum. Red fluorescence was not detected in the isopropanol supernatant, but was released from the cell pellet by sonication in 500 µl PBS. After removal of debris by centrifugation (16,200×g, 5 minutes), lysate dTomato fluorescence was determined using a Perkin Elmer fluorescence spectrometer (ex 557 nm, em 585 nm) and was also normalized to that in the contralateral striatum. At the dilution used, the relationship between fluorescence intensity and dTomato concentration was linear.

2.5. Tissue Processing

For preparation of fresh tissue slices, mice were deeply anesthetized with isoflurane and were quickly euthanized by cervical dislocation. Brains were rapidly removed and embedded in 1.5% high gel strength low melting point agarose type I-B (Sigma A0576). Serial sections (300 µm) were cut coronally through the striatum using a VF-300 microtome (Precisionary Instruments, Greenville, NC).

For histological analysis, mice were deeply anesthetized with isoflurane and then were perfused via the left ventricle with 5 ml of 0.9% saline for 1 minute, followed by 60 ml of cold 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 15 minutes at room temperature. Brains were then carefully removed from the skull, postfixed for 7 hours at 4°C, and cryopreserved in 0.1 M phosphate buffer (pH 7.4) containing 20% sucrose at 4°C; the sucrose solution was replaced after 12 hours of immersion. Sectioning and immunostaining were carried out by FD Neurotechnologies, Inc., Columbia, MD, USA. Serial frozen sections (50 µm) were cut coronally through the cerebrum containing the striatum, from approximately bregma +2.68 mm to bregma -2.30 mm (Paxinos and Franklin, 2001). Every sixth section was immunostained with anti-NeuN (Millipore, Billerica, MA; Cat. MAB377B, 1:400 dilution), using 3–3' diaminobenzidine chromagen. Nonfluorescent staining of neurons was chosen for these experiments to avoid variability caused by photobleaching, which often occurs during the long light exposures needed to count cells using designed-based stereology. Sections were mounted on Superfrost Plus slides.

2.6. Stereology Methods

NeuN positive cells in collagenase-injected and contralateral striata were quantified using the optical fractionator method (West, 1993) and the *Stereologer* system (Long et al., 1998) (Stereology Resource Center, Chester, MD). Each hemi-section was first imaged under low magnification (2x); the striatum was outlined according to coordinates of Paxinos and Franklin (Paxinos and Franklin, 2001). NeuN positive cells were counted with a 100x oil immersion lens, maintaining a guard volume of 1 µm to avoid cutting artifacts on the section surface. Counting criteria for neurons were nuclear NeuN immunoreactivity and a defined nuclear membrane. All counts were conducted by an observer blind to the mouse genotype and experimental condition. These laborintensive experiments were conducted only at 4 days after collagenase injection, since preliminary studies had indicated that most cell loss was complete by that time point.

2.7. Behavioral Testing

At 1, 3, 5 and 7 days after collagenase injection, behavioral parameters were quantified using previously described methods (Chen et al., 2011), according to the following schedule: 1) Quantification of cage locomotor activity, 10 AM – 1 PM; 2) Corner test, 2PM to 3PM; 3) Adhesive removal test, 3PM to 4PM; Elevated body swing test, 4PM to 5PM.

Quantification of Cage Activity—Each mouse was placed into a separate cage that was identical to its home cage, with fresh bedding, food and water. A video camera (Sony DCR-HC62) was positioned perpendicular to the long axis of the cage so that the field of view included the entire length of the cage. Mouse activity was recorded for 3 hours, and was analyzed using HomeCageScan (Version 3.0, Clever Systems Inc., Reston, VA USA), quantifying the following behaviors: walking, feeding, vertical hanging, rearing, and jumping. The time spent in each behavior was summed to yield an index of total activity.

Corner test—Two Styrofoam boards (30x20x1cm) were attached at one end at a 30 degree angle. As the mouse entered the corner to explore, both sides of the vibrissae were stimulated, resulting in rearing upward and turning towards the open end. The percentage of right turns was recorded for six trials. Turning that was not associated with rearing was not scored. A mouse with a right striatal lesion will preferentially turn to the right (Zhang et al., 2002)

Elevated body swing test—The mouse was held by the examiner 3 cm from the base of its tail, and lifted so that its head was 3 cm above the base of the same empty cage that was used for the adhesive removal test. Left and right swings were recorded over 45 seconds. A right striatal lesion produces a leftward swinging bias [13]

Adhesive removal test—After habituation in an empty cage for 5 minutes, adhesive dots were attached to the medial aspect of the left or right forepaw, with randomization of placement order. The mouse was then returned to its empty cage, and the interval until dot removal was recorded in two trials, with a maximum time of 120 seconds. Mean removal time from the right (ipsilateral to ICH) forepaw was subtracted from that of the left to calculate an asymmetry score (MacLellan et al., 2006). Four training sessions were conducted prior to formal testing.

2.8. Statistical analysis

Data were analyzed with one-way ANOVA in experiments containing three or more groups, followed by the Bonferroni multiple comparisons test. Mortality data were analyzed with Fisher's exact test. Significance was assigned to a p-value < 0.05.

3. Results

Brainbow mice that constitutively express dTomato in neurons (Livet et al., 2007) were bred without any difficulty and were grossly indistinguishable from wild-type mice. Behavioral phenotyping by digital analysis of cage video recordings did not demonstrate any differences in activity (Fig. 1A). Detection of the Brainbow construct containing the dTomato gene was easily accomplished by PCR-based genotyping and was confirmed by skull transillumination after age 3 weeks using a TRITC filter (Fig 1B, C). Bright fluorescence was apparent in fresh brain specimens viewed under a stereo microscope (Figs. 1C, 2). At the brief exposures needed, nonspecific fluorescence was not detected in wild-type brains. Reduced striatal fluorescence was observed in mice sustaining an ICH due to collagenase injection (Fig 2A–D).

3.1. Correlation of dTomato Fluorescence and MTT assay

Striatal cell viability 1 day after injection of 0.014 units collagenase, as assessed by MTT assay, was $85.2 \pm 8.8\%$ of that in the contralateral striatum in HO-2 wild-type mice. It was reduced to $55.1 \pm 4.3\%$ and $61.2 \pm 3.1\%$ of contralateral at 4 days and 8 days, respectively. Mean striatal dTomato fluorescence values at these three time points, also normalized to those in contralateral striata, were $86.4 \pm 3.9\%$, $62.2 \pm 5.1\%$, and $58.3 \pm 3.0\%$. These values were not significantly altered in mice lacking the HO-2 gene (Fig. 3A–C).

Increasing the collagenase dose to 0.028 units increased mortality in both wild-type and knockout mice (Table 1). The mortality rate was not affected by absence of the HO-2 gene. Striatal cell viability in surviving mice as measured by MTT assay was similar to that observed after injecting 0.014 units collagenase, and was not altered by HO-2 knockout. MTT results were also in close agreement with dTomato fluorescence values at this higher collagenase dose (Figs. 3D, 4). Comparison of normalized results of the MTT and dTomato assays for each mouse in this series of experiments (both tests conducted on same tissue samples) indicated a high degree of correlation (Fig. 5).

Quantification of striatal hemoglobin at 24 hours after collagenase injection demonstrated that HO-2 knockout did not alter hemorrhage volume at either collagenase dose (Fig. 6).

3.2. Histological quantification of neuronal loss

Using standard methods of unbiased stereology (Long et al., 1998; West, 1993), the mean number of neurons (defined by NeuN immunoreactivity) in striata contralateral to the stereotactic collagenase injection was $1.92 \pm 0.15 \times 10^6$ for wild-type mice and $1.90 \pm 0.08 \times 10^6$ for HO-2 knockout mice at four days (Fig. 7). These values were decreased by approximately 35% in the ipsilateral striata of both knockout and wild-type mice at this time point. No significant loss of NeuN immunoreactivity was observed in striata injected with artificial CSF instead of collagenase.

3.3. Behavioral Outcome

Mouse cage activity, defined as the total time spent walking, feeding, vertical hanging, rearing, and jumping during the three hour recording interval, was markedly reduced at one day after collagenase injection (Fig. 8). Mean activity levels increased over the subsequent 6 days, but remained below baseline, in agreement with prior observations (Chen et al., 2011). Deletion of the HO-2 gene had no effect on mouse cage activity.

Focal motor deficits were assessed using the adhesive removal, corner, and elevated body swing tests (Chen et al., 2011). All three tests detected deficits that persisted throughout the one week observation period. A significant difference between HO-2 knockout and HO-2 WT mice was observed only on day one and only with the adhesive removal test. Results of all other tests indicated that absence of the HO-2 gene had no effect at any time point (Fig. 8B–D).

4. Discussion

This study accomplishes two ends. First, we present data indicating that neuronal loss in tissue surrounding an experimental ICH can be accurately estimated in mice expressing the dTomato red fluorescent protein variant by simply measuring the fluorescence of a striatal lysate. This procedure, which can easily be completed within 30 minutes of brain harvesting, correlated very well with results of the MTT cell viability assay conducted on the same samples, and was consistent with stereological cell counts. Second, we used these methods to test the effect of HO-2 gene knockout on striatal injury produced by collagenase-induced

ICH. In contrast to our prior observations using the autologous blood injection ICH model (Qu et al., 2007), no neuroprotection was detected. The effect of HO-2 after ICH therefore appears to be model-specific.

Quantifying neuronal loss after a striatal hemorrhage in mouse models is quite challenging, since a well-defined infarct as observed after ischemic stroke is not present. In the days after the hemorrhage, tissue surrounding the hematoma is edematous with areas of incomplete cell loss that may not be accurately detected by measuring lesion volumes after TTC, MTT or other staining (Hua et al., 2007). No standard method of histological analysis is currently in use to assess neuronal loss in this injured tissue. Table 2 lists the approaches that have been employed in recent publications. In addition to the labor-intensive nature of these methods and the potential to introduce distortions during tissue processing, three limitations are apparent. First, counts of the percentage of degenerating neurons/field may not reflect cumulative neuron loss over time. A degenerating neuronal cell body is detectable by histological stains for only 2–4 days, following which it is presumably cleared from tissue and would not be counted (Franssen, 2007). Second, since it is impossible to count the 1.4–2.5 million neurons in the mouse striatum (Rosen and Williams, 2001), all studies count only a small fraction of cells. Protocols for selecting sections and fields vary widely and may be subject to bias, particularly when cell profiles are counted without application of unbiased stereology. Third, the criteria used to identify a degenerating cell are often not precisely defined. Results may therefore be confounded by investigator-specific or laboratory-specific factors.

Methods that quantify cell viability by retention of fluorescent proteins have been used for several years in vitro. Most relevant to the current study, loss of green fluorescent protein was validated as an accurate injury marker in models of microglial neurotoxicity and excitotoxicity, correlating very well with propidium iodide, trypan blue, and Cell-Tracker viability assays (Anegawa et al., 2000; Hansen et al., 2010). The availability of transgenic mice that express fluorescent proteins in central neurons provides an opportunity for high throughput analysis of neuronal loss in experimental models of acute CNS injury. However, this opportunity has been exploited to date only in retinal studies. Transgenic mice expressing cyan fluorescent protein driven by the Thy1 promoter have been used to noninvasively monitor retinal ganglion cell loss in models of rotenone toxicity (Hayworth et al., 2008), glaucoma (Tosi et al., 2010), and optic nerve transection (Chauhan et al., 2012). The present results suggest that attenuation of striatal fluorescence in neuronal dTomato-expressing mice accurately reflects cell loss over time, in agreement with these retinal studies. In this deep ICH model, striatal fluorescence could not be detected with sufficient precision to be monitored noninvasively, so it was quantified ex vivo after microdissection of the striatum. Since brain fluorescence was easily detected by skull transillumination, longitudinal in vivo monitoring of neuronal loss may be feasible when injury is primarily cortical.

In a recent study, Jin et al. (Jin et al., 2013) reported that perihematomal neuronal loss correlated with reduced expression of dopamine- and cAMP-regulated phosphoprotein Mr 32 kDa (DARPP-32), a cytosolic protein that is highly enriched in striatal neurons. On tissue sections, the DARPP-32-negative area correlated with necrosis as measured by propidium iodide staining, with a matching time course on immunoblots. These results suggest that loss of DARPP-32 expression is also a marker of neuronal death after striatal ICH, and may complement dTomato fluorescence measurements using the same tissue lysates.

In order to evaluate this fluorescent method in a planned study, dTomato transgenic mice were crossed with mice lacking the gene for HO-2, the predominant neuronal HO isoform. We had previously reported that these knockouts sustained less striatal injury when injected

with autologous blood or hemoglobin (Qu et al., 2007; Qu et al., 2005). However, in our collagenase model, HO-2 knockout did not increase neuronal viability in the hemorrhagic striatum at any time point, and had no effect on mortality. The only difference detected between HO-2 knockout and wild-type mice was an increased response time in the adhesive removal test in the former. This behavioral deficit was observed only at 24 h and was not consistent with results of other neurological outcome measures, suggesting an effect that is weak and transient. A modest increase in early neurological deficits after collagenase-induced ICH in HO-2 knockouts was previously reported by Wang et al. (Wang et al., 2006), who also noted a reduction in lesion volume but no change in brain edema. The current results are therefore in part consistent with these prior observations.

Since a selective inhibitor of HO-2 is not available, its effect on acute neural cell injury has been most often investigated using HO-2 knockout mice. Results reported to date have been quite variable and highly dependent on both cell type and the injury model. In primary cultures, HO-2 knockout neurons were less susceptible than wild-type cells to hemin or hemoglobin, but were more susceptible to inorganic iron (Regan et al., 2004; Rogers et al., 2003). In contrast, astrocytes lacking HO-2 were more vulnerable to hemin than their wild-type counterparts (Chen and Regan, 2004), while cerebral microvascular endothelial cells were more vulnerable to glutamate and TNF- α (Basuroy et al., 2006; Parfenova et al., 2006). The deleterious effect of HO-2 on neurons exposed to hemoglobin or hemin may be due to iron released as a consequence of breakdown of hemin, as it was not seen when apotransferrin is present in the culture medium (Wang et al., 2006). In vivo, HO-2 knockout increases brain injury due to ischemia, trauma, or injection of excitatory amino acids (Chang et al., 2003; Dore et al., 1999). The minimal effect of HO-2 knockout in our collagenase model may indicate that any attenuation of hemoglobin or hemin neurotoxicity is largely balanced by exacerbation of other injury mechanisms. The only difference was observed 24 hours after collagenase injection using the very sensitive adhesive removal test. This early time point precedes erythrocyte lysis in rodent ICH models (Wagner et al., 2003), suggesting that HO-2 has a beneficial effect on an injury cascade that is unrelated to hemoglobin or hemin toxicity.

The autologous blood injection and collagenase ICH models can be calibrated to produce hematomas that have a grossly similar appearance. However, since hematomas are produced by markedly different mechanisms in these models, divergence of injury cascades in adjacent tissue should not be surprising. The major criticism of the autologous blood model is that it fails to simulate clinical ICH because the hematoma is not produced by hemorrhage in situ (James et al., 2008). The collagenase model addresses that concern, but raises others. Since a bacterial protein is injected into the brain, it may initiate a local inflammatory response, enhancing that produced by the hematoma per se. Another concern is that breakdown of multiple vessels near the injection site by collagenase may reduce local blood flow sufficiently to produce ischemia, which is not a feature of clinical ICH or the blood injection model (Qureshi et al., 1999; Zazulia et al., 2001). When initial hematoma volumes were equal, MacLellan et al. (MacLellan et al., 2008) reported that the collagenase model produced more rapid blood-brain barrier breakdown, greater injury to adjacent tissue, and more severe and persistent neurological deficits than the blood injection model. These differences may account for the observation that the iron chelator deferoxamine, which is currently in clinical ICH trials, improved outcome in studies using the blood injection model (Nakamura et al., 2004; Qing et al., 2009) but had a variable effect in the collagenase model (Auriat et al., 2012; Warkentin et al., 2010; Wu et al., 2011). Our current and prior results (Qu et al., 2007) suggest that the effect of HO-2 on injury to perihematomal neurons depends at least in part on the method used to create the hematoma. The model that best recreates the cellular microenvironment in tissue surrounding a clinical ICH remains undefined and seems a worthy topic of further investigation.

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Abbreviations

HO	heme oxygenase
ICH	intracerebral hemorrhage
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RFP	red fluorescent protein

References

- Anegawa NJ, Guttman RP, Grant ER, Anand R, Lindstrom J, Lynch DR. N-Methyl-D-aspartate receptor mediated toxicity in nonneuronal cell lines: characterization using fluorescent measures of cell viability and reactive oxygen species production. *Brain Res Mol Brain Res*. 2000; 77:163–175. [PubMed: 10837912]
- Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, Colbourne F. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. *Exp Neurol*. 2012; 234:136–143. [PubMed: 22226595]
- Basuroy S, Bhattacharya S, Tcheranova D, Qu Y, Regan RF, Leffler CW, Parfenova H. HO-2 provides endogenous protection against oxidative stress and apoptosis caused by TNF-alpha in cerebral vascular endothelial cells. *Am J Physiol Cell Physiol*. 2006; 291:C897–C908. [PubMed: 16822952]
- Chang EF, Wong RJ, Vreman HJ, Igarashi T, Galo E, Sharp FR, Stevenson DK, Noble-Haesslein LJ. Heme oxygenase-2 protects against lipid peroxidation-mediated cell loss and impaired motor recovery after traumatic brain injury. *J. Neurosci*. 2003; 23:3689–3696. [PubMed: 12736340]
- Chauhan BC, Stevens KT, Levesque JM, Nuschke AC, Sharpe GP, O'Leary N, Archibald ML, Wang X. Longitudinal in vivo imaging of retinal ganglion cells and retinal thickness changes following optic nerve injury in mice. *PLoS One*. 2012; 7:e40352. [PubMed: 22768284]
- Chen J, Regan RF. Heme oxygenase-2 gene deletion increases astrocyte vulnerability to hemin. *Biochem. Biophys. Res. Commun*. 2004; 318:88–94. [PubMed: 15110757]
- Chen L, Zhang X, Chen-Roetling J, Regan RF. Increased striatal injury and behavioral deficits after intracerebral hemorrhage in hemopexin knockout mice. *J Neurosurg*. 2011; 114:1159–1167. [PubMed: 21128737]
- Dore S, Sampei K, Goto S, Alkayed NJ, Guastella D, Blackshaw S, Gallagher M, Traystman RJ, Hurn PD, Koehler RC, Snyder SH. Heme oxygenase-2 is neuroprotective in cerebral ischemia. *Mol Med*. 1999; 5:656–663. [PubMed: 10602774]
- Franssen, C.; Switzer, RC., IV; Switzer, RC. 2007 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience; 2007. Neurotoxicity study design: the essential element of sacrifice times. Program No. 25.14. Online., 2007.
- Hansen E, Krautwald M, Maczurek AE, Stuchbury G, Fromm P, Steele M, Schulz O, Garcia OB, Castillo J, Korner H, Munch G. A versatile high throughput screening system for the simultaneous identification of anti-inflammatory and neuroprotective compounds. *J Alzheimers Dis*. 2010; 19:451–464. [PubMed: 20110593]
- Hayworth CR, Rojas JC, Gonzalez-Lima F. Transgenic mice expressing cyan fluorescent protein as a reporter strain to detect the effects of rotenone toxicity on retinal ganglion cells. *J Toxicol Environ Health A*. 2008; 71:1582–1592. [PubMed: 18850458]
- Hua Y, Keep RF, Hoff JT, Xi G. Brain injury after intracerebral hemorrhage: the role of thrombin and iron. *Stroke*. 2007; 38:759–762. [PubMed: 17261733]
- James ML, Warner DS, Laskowitz DT. Preclinical models of intracerebral hemorrhage: a translational perspective. *Neurocrit Care*. 2008; 9:139–152. [PubMed: 18058257]
- Jin H, Xi G, Keep RF, Wu J, Hua Y. DARPP-32 to quantify intracerebral hemorrhage-induced neuronal death in basal ganglia. *Transl Stroke Res*. 2013; 4:130–134. [PubMed: 23543809]

- Leung CK, Weinreb RN. Experimental detection of retinal ganglion cell damage in vivo. *Exp Eye Res.* 2009; 88:831–836. [PubMed: 18926816]
- Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, Sanes JR, Lichtman JW. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature.* 2007; 450:56–62. [PubMed: 17972876]
- Long JM, Kalebica AN, Muth NJ, Hengemihle JM, Jucker M, Calhoun ME, Ingram DK, Mouton PR. Stereological estimation of total microglia number in mouse hippocampus. *J Neurosci Methods.* 1998; 84:101–108. [PubMed: 9821640]
- MacLellan CL, Auriat AM, McGie SC, Yan RH, Huynh HD, De Butte MF, Colbourne F. Gauging recovery after hemorrhagic stroke in rats: implications for cytoprotection studies. *J Cereb Blood Flow Metab.* 2006; 26:1031–1042. [PubMed: 16395282]
- MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. *J Cereb Blood Flow Metab.* 2012; 32:612–627. [PubMed: 22293989]
- MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, Colbourne F. Intracerebral hemorrhage models in rat: comparing collagenase to blood infusion. *J Cereb Blood Flow Metab.* 2008; 28:516–525. [PubMed: 17726491]
- Nakamura T, Keep RF, Hua Y, Schallert T, Hoff JT, Xi G. Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *J Neurosurg.* 2004; 100:672–678. [PubMed: 15070122]
- Parfenova H, Basuroy S, Bhattacharya S, Tcheranova D, Qu Y, Regan RF, Leffler CW. Glutamate induces oxidative stress and apoptosis in cerebral vascular endothelial cells: contributions of HO-1 and HO-2 to cytoprotection. *Am J Physiol Cell Physiol.* 2006; 290:C1399–C1410. [PubMed: 16371440]
- Paxinos, G.; Franklin, KBJ. *The Mouse Brain in Stereotaxic Coordinates.* Second Edition ed. San Diego: Academic Press; 2001.
- Poss KD, Thomas MJ, Ebralidze AK, TJ OD, Tonegawa S. Hippocampal long-term potentiation is normal in heme oxygenase-2 mutant mice. *Neuron.* 1995; 15:867–873. [PubMed: 7576635]
- Qing WG, Dong YQ, Ping TQ, Lai LG, Fang LD, Min HW, Xia L, Heng PY. Brain edema after intracerebral hemorrhage in rats: the role of iron overload and aquaporin 4. *J Neurosurg.* 2009; 110:462–468. [PubMed: 19025353]
- Qu Y, Chen-Roetling J, Benvenisti-Zarom L, Regan RF. Attenuation of oxidative injury after induction of experimental intracerebral hemorrhage in heme oxygenase-2 knockout mice. *J. Neurosurg.* 2007; 106:428–435. [PubMed: 17367065]
- Qu Y, Chen J, Benvenisti-Zarom L, Ma X, Regan RF. Effect of targeted deletion of the heme oxygenase-2 gene on hemoglobin toxicity in the striatum. *J. Cereb. Blood Flow Metab.* 2005; 25:1466–1475. [PubMed: 15902196]
- Qureshi AI, Wilson DA, Hanley DF, Traystman RJ. No evidence for an ischemic penumbra in massive experimental intracerebral hemorrhage. *Neurology.* 1999; 52:266–272. [PubMed: 9932942]
- Regan RF, Chen J, Benvenisti-Zarom L. Heme oxygenase-2 gene deletion attenuates oxidative stress in neurons exposed to extracellular hemin. *BMC Neurosci.* 2004; 5:34. [PubMed: 15377391]
- Rogers B, Yakopson V, Teng ZP, Guo Y, Regan RF. Heme oxygenase-2 knockout neurons are less vulnerable to hemoglobin toxicity. *Free Rad. Biol. Med.* 2003; 35:872–881. [PubMed: 14556851]
- Rose, K.; Goldberg, MP.; Choi, DW. Cytotoxicity in murine neocortical cell culture. In: Tyson, CA.; Frazier, JM., editors. *Methods in Toxicology; Part A: In Vitro Biological Systems.* San Diego: Academic Press; 1993. p. 46–60.
- Rosen GD, Williams RW. Complex trait analysis of the mouse striatum: independent QTLs modulate volume and neuron number. *BMC Neurosci.* 2001; 2:5. [PubMed: 11319941]
- Tosi J, Wang NK, Zhao J, Chou CL, Kasanuki JM, Tsang SH, Nagasaki T. Rapid and noninvasive imaging of retinal ganglion cells in live mouse models of glaucoma. *Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging.* 2010; 12:386–393. [PubMed: 19937134]
- Wagner KR, Sharp FR, Ardizzone TD, Lu A, Clark JF. Heme and iron metabolism: role in cerebral hemorrhage. *J Cereb Blood Flow Metab.* 2003; 23:629–652. [PubMed: 12796711]

- Wang J, Zhuang H, Doré S. Heme oxygenase 2 is neuroprotective against intracerebral hemorrhage. *Neurobiol Dis.* 2006; 22:473–476. [PubMed: 16459095]
- Warkentin LM, Auriat AM, Wowk S, Colbourne F. Failure of deferoxamine, an iron chelator, to improve outcome after collagenase-induced intracerebral hemorrhage in rats. *Brain Res.* 2010; 1309:95–103. [PubMed: 19879860]
- West MJ. New stereological methods for counting neurons. *Neurobiology of aging.* 1993; 14:275–285. [PubMed: 8367009]
- Winterbourn CC. Oxidative reactions of hemoglobin. *Meth. Enzymol.* 1990; 186:265–272. [PubMed: 2172706]
- Wu H, Wu T, Xu X, Wang J. Iron toxicity in mice with collagenase-induced intracerebral hemorrhage. *J Cereb Blood Flow Metab.* 2011; 31:1243–1250. [PubMed: 21102602]
- Zazulia AR, Diringner MN, Videen TO, Adams RE, Yundt K, Aiyagari V, Grubb RL Jr, Powers WJ. Hypoperfusion without ischemia surrounding acute intracerebral hemorrhage. *J Cereb Blood Flow Metab.* 2001; 21:804–810. [PubMed: 11435792]
- Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniago P, Li Q, Lu M, Chopp M. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J Neurosci Methods.* 2002; 117:207–214. [PubMed: 12100987]

Highlights

- Red fluorescent protein (RFP) expression was evaluated as a cell viability marker in an intracerebral hemorrhage model.
- Fluorescence loss correlated with cell death as measured by MTT assay.
- The RFP transgene was incorporated into HO-2 knockout and wild-type mice.
- HO-2 had no effect on fluorescence loss, in agreement with other outcome measures.

Figure 1.

A) Behavioral phenotyping by digital analysis of video recordings demonstrated no difference in cage activity of mice lacking the dTomato gene and wild-type at the HO-2 locus (WT), transgenic dTomato mice with the wild-type HO-2 gene (RFP-WT), and dTomato transgenic HO-2 knockout mice (RFP-KO). Total minutes spent walking, rearing, feeding, vertical hanging and jumping were summed to provide index of total activity; B) Representative gels identifying: top panel, HO-2 protein expression by western blotting; middle panel, HO-2 wild-type gene and knockout insert by PCR, control is from HO-2 heterozygous knockout mouse; bottom panel, Brainbow construct containing dTomato gene by PCR, control is from wild-type mouse without dTomato gene; C) Identification of dTomato expression in living mouse by skull transillumination after hair clipping (left 4 photos), and in freshly harvested brain (right 4 photos), using a TRITC filter. Scale bar = 3 mm.

Figure 2.

Brightfield (top panel) and corresponding fluorescent (bottom panel) images of dTomato-expressing brain (wild-type at HO-2 locus), 4 days after injection of 0.028 units collagenase into the right striatum. A) whole brain before dissection; B) left striatum (contralateral to collagenase injection) is exposed by removal of overlying cortex, demonstrating bright fluorescence; C) injected right striatum is exposed, demonstrating reduced fluorescence in superior aspect of striatum; D) left and right striata have been removed; reduced fluorescence of hemorrhagic right striatum is apparent. Scale bar = 3mm.

Figure 3.

Loss of dTomato fluorescence after striatal ICH correlates with attenuation of MTT reduction to formazan. Bars represent mean striatal cell formazan or striatal dTomato fluorescence 1, 4 or 8 days after stereotactic injection of 0.014 or 0.028 units collagenase (high dose). Controls were injected with an equal volume of artificial CSF instead of collagenase. All mice used expressed dTomato; WT and KO indicate gene at the HO-2 locus. Values are normalized to those in the contralateral striatum (= 100), n = 5–12 mice/condition.

Figure 4.

dTomato fluorescence is reduced in the right striata of HO-2 WT and KO mice after collagenase-induced ICH. Serial sections (300 μ m) were cut coronally through the striatum, 4 days after striatal injection of 0.028 units collagenase. Top row (WT) and third row (KO) are brightfield images; second row (WT) and last row (KO) are images under fluorescence microscope.

Figure 5.
Correlation of dTomato fluorescence and MTT reduction to formazan in mice (n=52) receiving striatal injection of collagenase or artificial CSF.

Figure 6.
HO-2 gene knockout does not alter striatal hemorrhage after collagenase-induced ICH.
Mean striatal hemoglobin in HO-2 knockout and wild-type striata 24 hours after injection of
0.014 or 0.028 units collagenase (n=5/condition).

Figure 7.

Bars represent mean number of striatal NeuN immunoreactive cells in HO-2 wild-type (WT) and knockout (KO) mice 4 days after injection of collagenase (0.028 units) or artificial CSF, and in contralateral striata. Total cell numbers are based on sampling using design-based stereology. Photographs are of representative WT and KO sections stained with anti-NeuN; C,G are from contralateral striata, D,H are from collagenase-injected striata. Note that stereological cell counting requires thick sections, so some neuron cell bodies are out of focus. Scale bar = 10 μ m.

Figure 8.

Effect of HO-2 knockout on behavioral outcome after ICH. Mouse cage activity and performance on adhesive removal, corner, and elevated body swing tests at indicated days after 0.014 unit collagenase injection (* $P < 0.05$ v. corresponding WT condition, Bonferroni multiple comparisons test, $n = 6-22$ /condition, with higher value for pre-injection testing).

Table 1

Mortality in WT and HO-2 KO mice receiving 0.014 units or 0.028 units collagenase. All deaths occurred within 24 hours of collagenase injection. $P > 0.05$, WT v. KO at both collagenase doses., $P = 0.001$, high dose v. low dose.

Collagenase	0.014U		0.028U	
Genotype	WT	KO	WT	KO
Living	23	22	25	28
Dead	2	3	16	13
Total	<u>25</u>	<u>25</u>	<u>41</u>	<u>41</u>

Table 2

Histological methods used to quantify peri-hematoma neuronal loss in recently published papers, demonstrating variable methods for section and field selection. FJB, FJC: Fluoro Jade B or C. NS: not specified.

Study	Stain	Section and Field Selection	Fields Counted
Xue, Stroke 40:2199–204	FJB	3 levels, 200 μ m apart	12
Karki, Stroke 40: 3384–9	H+E, NeuN	0.5 mm intervals through hematoma	NS
Gu, Stroke 40: 2241–43	FJC	Adjacent 0.14 mm ² fields	4
Grasso, Neurosurgery 65:763–9	H+E, NeuN	10 random sections	NS
Zhao, Brain Inj 23:353–7	TUNEL	3 consecutive sections	27
Savard, Comp.Med 59:444–8	Hematoxylin + Saffron	Every 100 μ m through hematoma	NS
Lekic, J Neurotrauma 27:627–37	Cresyl Violet	Site of maximum hematoma volume	5
Wu, Neurocrit Care 14:109–17	Cresyl Violet	3 sections per mouse	12
Sun, J. Neurotrauma 28:2513–2521	FJC	3 sections adjacent to hematoma	12
Khatibi, Anesth Analg 113:343–8	TUNEL	3 perihematoma regions	NS
Masuda, Brain Res 1355:228–39	NeuN, FJC	3 sections at bregma per mouse	12
Chang, Am J Path 178:1749–1761	FJB, TUNEL	700x900 μ m ² adjacent to hematoma	12
Wu, J Neurosurg 114:1805–1811	FJC	1 mm anterior and posterior to injection site	6
Matsushita, JCBFM 31:222–34.	NeuN	4 sections every 120 μ m from injection site	NS
Ohnishi, Neuropharm 61:975–980	NeuN	Peripheral region of hematoma	NS
Hijioka, Neuroscience 222 :10–19	TUNEL, NeuN	4 sections every 120 μ m from injection site	NS
Wu, Neurobiol Dis 45:388–94	FJC	3 sections, lateral edge of hematoma	12