Treatment of intracerebral hemorrhage in rats with 12 h, 3 days and 6 days of selective brain hypothermia

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ABSTRACT

Intracerebral hemorrhage (ICH) is a devastating stroke with no proven treatment to reduce brain injury. In this study we modeled ICH by injecting 100 μL of autologous blood into the striatum of rats. We then tested whether hypothermia would reduce brain injury and improve recovery as has been repeatedly observed for ischemic and traumatic brain damage. Aside from reducing blood-brain barrier disruption, inflammation and edema, hypothermia has not consistently improved behavioral or histological outcome after ICH in animal studies. As this might relate to the choice of cooling method and the duration of hypothermia, we used a system that selectively cooled the injured hemisphere to ~32 °C (striatum) while the body remained normothermic. Cooling (vs. normothermia) started 1 h after ICH and lasted for 12 h, 3 days or 6 days followed by slow re-warming (~1 °C/h). Functional impairment was evaluated from 2 to 3 weeks post-ICH at which time brain injury was determined. The ICH caused significant impairment on a neurological deficit scale and in tests of walking (horizontal ladder), skilled reaching (tray task) and spontaneous limb usage (cylinder test). Only the limb use asymmetry deficit was significantly mitigated by hypothermia, and then only by the longest treatment. Lesion volume, which averaged 16.9 mm³, was not affected. These results, in conjunction with earlier studies, suggest that prolonged mild hypothermia will not be a profound neuroprotectant for patients with striatal ICH, but it may nonetheless improve functional recovery in addition to its use for treating cerebral edema.

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Introduction

Intracerebral hemorrhage (ICH) accounts for 10–20% of all strokes, and it results in high mortality and morbidity (Broderick, 1994; SACCO et al., 2008). The rapid release of blood into the parenchyma causes considerable mechanical injury that might only be attenuated by limiting hematoma volume; although rFVIIa, which limits hematoma growth, does not improve outcome (Mayer et al., 2008). Nonetheless, secondary injury occurs from the toxic effects of blood components, such as thrombin, as well as erythrocyte rupture (e.g., iron-catalyzed free radical reactions). These events along with blood-brain barrier disruption, the development of edema and inflammation are leading therapeutic targets especially because they persist for days to weeks following ICH (Xi et al., 2006).

Many therapies have been directed at these targets (James et al., 2008), including hypothermia. Mild to moderate hypothermia lessens injury and improves recovery in models of global and focal ischemia (Dietrich et al., 2009; MacLellan et al., 2009; Nagel et al., 2008; van der Worpe et al., 2007). Furthermore, clinical findings show that cooling improves outcome in adults suffering cardiac arrest (Bernard et al., 2002; The Hypothermia After Cardiac Arrest Study Group, 2002) and infants suffering hypoxia/ischemia (Shankaran et al., 2005). Thus, one would expect hypothermia to improve outcome after ICH, which shares mechanisms of injury with ischemia (Xi et al., 2006). Although there is some clinical work (Feng et al., 2002), it is not known whether hypothermia will improve survival and lessen disability after ICH. Furthermore, animal studies are inconclusive, and compared to ischemia, there is a paucity of neuroprotection studies for this stroke type that still results in over 50% mortality (Sacco et al., 2008).

It is apparent, however, that hypothermia attenuates cerebral edema after injections of thrombin (Kawai et al., 2001) and whole blood (Fingas et al., 2007; Kawanishi et al., 2008; MacLellan et al., 2006a, 2006b; Wagner et al., 2006). Inflammation and blood-brain barrier disruption is also markedly attenuated (Kawanishi et al., 2008; MacLellan et al., 2006a, 2006b; Wagner et al., 2006). Thus, one would expect cooling to limit cell death thereby improving recovery. Nonetheless, this has not been clearly demonstrated. For instance, 2 days of systemic hypothermia (33 °C) failed to improve outcome in the whole blood model of striatal ICH in rats (MacLellan et al., 2006a, 2006b). Similarly, 4 days of focal (brain-selective) hypothermia (~32 °C) lessened edema, but did not improve recovery or reduce...
The horizontal ladder test measured skilled recovery after ICH (MacLellan et al., 2006a, 2006b). An explanation for the discrepancy between the whole blood and collagenase models is not clear, but probably stems from differences in the extent and time course of injury (MacLellan et al., 2008; Xue and Del Bigio, 2003) as well as the use of different treatment protocols among studies.

Presently, we investigated the effects of three durations (12 h, 3 days and 6 days) of focal hypothermia (32 °C) induced starting 1 h after whole blood infusion into the striatum of rats. The duration of cooling is usually of paramount importance with more prolonged treatment (days) providing greater protection than with briefier treatment after global ischemia (Colbourne and Corbett, 1994), focal ischemia (Clark et al., 2008) and traumatic brain injury (Jiang et al., 2006). Thus, duration-dependent protection would be expected on the basis of those findings and the fact that inflammation, edema and cell death continue for days after the ICH event. However, we also tested a brief treatment as more protracted cooling might impede post-stroke plasticity (e.g., synaptogenesis) thereby counteracting hypothermia’s beneficial effects. For example, delayed treatment with a matrix metalloproteinase inhibitor suppressed remodeling and impaired recovery after stroke (Zhao et al., 2006). Focal cooling was used to avoid potentially deleterious effects associated with systemic hypothermia (e.g., weight loss, alterations in blood pressure), especially likely with prolonged treatment (MacLellan et al., 2004). Furthermore, we slowly re-warmed after hypothermia to avoid potentially harmful effects associated with rapid re-warming (Berger et al., 2007; Nakamura et al., 1999; Ueda et al., 2004). Indeed, our recent failure to improve outcome after ICH with focal hypothermia may have stemmed from the use of near-instantaneous re-warming after 4 days of cooling (Fings et al., 2007). Behavioral recovery was evaluated with a neurological deficit scale (NDS) (MacLellan et al., 2006a, 2006b), the tray task that assessed skilled reaching (Whishaw, 2000), the horizontal ladder test that measured walking ability (Metz and Whishaw, 2002), and the cylinder test of spontaneous forelimb usage (Schallert, 2006). Lesion size was determined at 3 weeks post-ICH.

**Methods**

**Animals**

We used 60 young-adult, male, Sprague–Dawley rats (∼200 g) obtained from the Bioscience breeding colony at the University of Alberta. All procedures were approved by the Biosciences Animal Care and Use Committee at the University of Alberta and were in accordance with the Canadian Council on Animal Care guidelines. Animals were randomly assigned to one of 4 groups or used in a small pilot study.

**Intracerebral hemorrhage (ICH)**

Rats were anesthetized with isoflurane (4% induction, 2% maintenance in 60% N2O and 40% O2) for aseptic ICH surgery as previously described (Fings et al., 2007; MacLellan et al., 2006a, 2006b). After shaving the skull, ∼0.2 mL of Marcaine (Sano-Flam Co. Inc, Sun River, OR, USA) intermittently during cooling and re-warming after ICH in a non-anesthetized rat. Briefly, a core temperature probe was surgically (isoflurane anesthesia) implanted into the peritoneal space 4 days before ICH surgery, whereas a brain temperature probe was secured to the skull while the temperature sensing tip resided within the dorsal striatum (Debow and Colbourne, 2003). Brain temperature was not measured in the main study in order to avoid compounding the ICH-induced injury.

**Behavioral assessment**

Baseline and post-stroke testing was conducted by an experimenter unaware of treatment assignment. Rats were trained prior to ICH and tested out to 21 days after ICH. Testing was not done in the first week after ICH owing to the fact that the H144 group was cooled for 6 days.

A composite NDS scale, ranging from 0 (not impaired) to 11 (severely impaired), was determined from 5 tasks including: hindlimb retraction, bilateral forepaw grasp, contralateral forelimb flexion, beam-walking and vibrissae-elicited forelimb placing. A baseline assessment was conducted 3 days prior to ICH and post-stroke
assessments were done at 13 and 20 days after ICH. A similar scale was previously shown to be sensitive to striatal ICH (MacLellan et al., 2006a, 2006b, 2008).

Walking ability was evaluated by the horizontal ladder test (Metz and Whishaw, 2002), which is sensitive to motor system damage, including striatal ICH (MacLellan et al., 2006a, 2006b). Briefly, this test measures a rat’s ability to traverse a 1 m long parallel series of horizontal bars (2 mm thick) randomly spaced 3–5 cm apart and elevated above a countertop. The total number of steps and slips (i.e., limb falls below the level of the bars) made with each limb are averaged from 4 walks across the ladder per test day. A baseline assessment was conducted 3 days before ICH and post-stroke testing occurred on day 13 and 20.

Spontaneous forelimb usage was determined in a cylinder test (Schallert, 2006), which is sensitive to the damage caused by striatal ICH (Fingas et al., 2007; Hua et al., 2002). Rats were individually placed in a transparent vertical cylinder (20 cm diameter, 45 cm high) for 10 min and videotaped from below. From the videos we determined the rats’ spontaneous limb usage with each forelimb by counting the initial placements of a forelimb on the wall and during subsequent movements along the wall. The data were excluded if the rat failed to make at least 10 independent touches as this was deemed too low to accurately judge asymmetry. An asymmetry score (e.g., 50% = no asymmetry, whereas <50% indicates diminished use of the contralateral-to-ICH limb) was calculated according to the formula:

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\text{Asymmetry score} = \frac{(\text{number of contacts with contralateral forelimb} + 1)}{2 \times (\text{number of contacts with both forelimbs})}
\]

Skilled reaching ability was evaluated with the tray task (Whishaw, 2000), which is sensitive to striatal injury caused by ICH (Nguyen et al., 2008a,b). Rats were food deprived to 90% of their normal feeding weight prior to and during training days (5 days/week for 2 weeks). Each daily session lasted for 30 min during which they were placed in a Plexiglas box (19 cm wide × 25 cm high × 27 cm long) and encouraged to reach for food pellets (17% Layer Pro-stock feed; Masterfeeds, Edmonton, AB, Canada) placed in a shallow tray (4 cm wide × 0.5 cm deep) just outside a parallel array of vertically spaced bars (9 mm apart). With this design rats could not obtain food with their tongue or by dragging it into the cage because it would drop through a wire grid floor. Thus, rats had to reach and grasp the food to be successful. The last training session (3 days prior to ICH) was videotaped to determine paw preference and reaching success (obtaining food) with that paw (number of successful reaches/number of reaches ≥ 40%) for their reaching data to be included in the study. Following unilateral motor system injury, rats sometimes switch to using their unimpaired limb during reaching tasks. Therefore, in those few rats we prevented this behavior by temporarily wrapping an adhesive bandage around the non-impaired forelimb, which prevented reaches through the bars with that limb. This manipulation does not impede the rat’s ability to use the limb for walking, support or food handling. Rats were then returned to ad lib feeding until 2 days prior to testing on days 14 and 21 post-ICH.

Lesion volume determination

The rats were euthanized under 100 mg/kg pentobarbital at 21 days post-ICH. They were perfusion fixed with 4% paraformaldehyde after being flushed with 0.9% phosphate-buffered saline. Coronal brain sections extending beyond and throughout the lesion were cut at 14 μm with a cryostat and stained with cresyl violet. The lesion volume was determined using Scion image J. (v4.0, Scion Corporation, Frederick, MD, USA), as routinely done (Fingas et al., 2007; MacLellan et al., 2004):

Volume of tissue lost = remaining volume of normal hemisphere − remaining volume of injured hemisphere.

Volume of a hemisphere = area of remaining tissue × distance between sections × number of sections analyzed.

Statistics

Data are presented as mean ± S.E.M., except NDS scores which are presented as medians. Data were analyzed by SPSS (v. 15) using ANOVA (1 and 2 way designs) for interval or ratio data (e.g., body weight), and non-parametric statistics for any such data that either violated the assumption of homogeneity of variance or was ordinal in nature (e.g., NDS scores). Specifically, we used the Kruskal–Wallis H and Wilcoxon Sign Ranks non-parametric tests for between group and repeated measures comparisons, respectively.

Results

Temperature measurement in the pilot study

Brain temperature was normothermic (~36.4 °C) prior to the onset of hypothermia. Cooling was induced rapidly (~5 min) whereas re-warming was controlled over the course of 5 h (Fig. 1). Core temperature was sampled once per day during hypothermia, and remained normothermic at each sampling time point (37.1–37.6 °C). The same cooling protocol (flow rate through the cooling coil) was used in the rest of the study.

Animals and physiological variables

Nine rats were excluded from this study because of complications arising during hypothermia treatment (e.g. broken or detached PE50 tubing causing early re-warming). The remaining group sizes were: NOR (N = 13); H12 (N = 11), H72 (N = 11) and H144 (N = 12). Of these, glucose, pH, hemoglobin, blood gases and surgical weight were all in the expected range and did not differ significantly among groups (p ≥ 0.083, one-way ANOVAs, Table 1). Kruskal–Wallis H statistics were also computed owing to significant heterogeneity (Levene statistic) among groups in some of these comparisons. These analyses yielded the same conclusions; that is, there were no group differences (p ≥ 0.129).

Behavioral testing

The NDS scores were similar among groups during baseline assessment with 42 of 47 rats attaining a NDS score of zero, or no impairment (p = 0.130, Kruskal–Wallis H test). Significant impairment (p < 0.001 vs. baseline, Wilcoxon Sign Ranks test) was found on
days 13 (Fig. 2A) and 20 (Fig. 2B). However, there were no Group effects on either test day \((p \geq 0.143; \text{Kruskal–Wallis H tests})\). Thus, ICH resulted in lasting neurological deficits that were not affected by any of the hypothermia treatments.

A repeated measures ANOVA on the error rate with the contralateral-to-stroke forelimb in the horizontal ladder task (Fig. 3A) revealed a significant effect of Time \((p < 0.001)\) with a non-significant Time × Group interaction \((p = 0.347)\), and a non-significant Group main effect \((p = 0.065)\). Repeated measures contrasts showed that the error rate was significantly higher on days 13 and 20 compared to the baseline period \((p < 0.001)\), indicating that ICH significantly and persistently impaired stepping accuracy. Owing to heterogeneity these data were also analyzed with non-parametric statistics which yielded the same findings (data not shown). Similarly, an ANOVA on the hind limb stepping accuracy (Fig. 3B) showed a Time effect \((p < 0.001)\), but no Group main effect \((p = 0.422)\) or interaction \((p = 0.189)\). Again the post-ICH error rates were significantly higher than the baseline scores \((p < 0.001)\). Thus, while ICH persistently impaired stepping accuracy with the contralateral fore and hind limbs, these impairments were not influenced by hypothermia treatment.

The data from 8 rats were excluded from the tray-reaching task analysis because of a failure to pass the baseline inclusion criterion. Although these rats were excluded from this analysis, they were included in the rest of the study. A repeated measures ANOVA on the remaining data yielded a significant Time effect \((p < 0.001)\), a non-significant Group effect \((p = 0.848)\), and a non-significant interaction \((p = 0.371)\). Repeated measures contrasts showed that post-ICH reaching success (days 14 and 21) was significantly lower than baseline performance \((p < 0.001)\). Thus, ICH significantly and persistently impaired reaching, which was not influenced by any of the hypothermia treatments (Fig. 4).
A small number of data points (7/144 = ∼5%) were lost due to rats not exceeding our criterion for minimum activity in the cylinder test (Fig. 5) and this data loss was distributed among the 4 groups and test times. Owing to a significant interaction (p = 0.011) in a repeated measures ANOVA (baseline, days 13 and 20), we analyzed only the post-ICH data, which showed the Time main effect (p = 0.788) and Time × Group (p = 0.949) interaction were non-significant while the Group main effect was significant (p = 0.006). Post hoc testing (LSD) showed that the H144 group had less asymmetry than the NOR (p = 0.003) and H72 groups (p = 0.002), whereas all other comparisons were non-significant (p ≥ 0.096). Importantly, differences did not exist at baseline (Group main effect: p = 0.596) where scores approached 50% (no asymmetry). Furthermore, significant asymmetry was found overall in comparing day 13 (p = 0.001) and day 20 post-ICH (p = 0.011) scores to baseline performance. Thus, the test was sensitive to the ICH.

Lesion volume assessment

The data for 4 animals (2 NOR, 1 H12, and 1 H144) were lost due to a histological processing problem. In the remainder, it was clear that injury occurred in the lateral and mid-striatum, with ventricular enlargement in the lesioned hemisphere (Fig. 6A). Tissue loss averaged 16.9 ± 8.6 mm³ at 21 days post-ICH, which did not differ among groups (p = 0.369, ANOVA, Fig. 6B).

Discussion

The use of a 6-day hypothermia treatment eliminated the spontaneous limb use asymmetry (cylinder task) that developed after striatal ICH. In contrast, general neurological impairment, skilled reaching deficits, stepping errors and lesion volume were not significantly affected by the H144 treatment. Furthermore, neither the brief (12 h) nor moderate length (3 days) hypothermia treatment improved outcome on any measure. Thus, these data are largely in agreement with our previous study that used a 4-day cooling regimen that reduced edema but did not improve functional recovery or a reduction in cell death (Wagner et al., 2006). The hypothermia findings in rodent models of ICH suggest that reductions in edema do not necessarily translate into improved outcome; although, some benefit would be expected clinically, but probably only in cases of severe edema (e.g., to reduce mortality).

The efficacy of delayed hypothermia is markedly influenced by insult severity with less benefit occurring after more severe or prolonged ischemia (MacLellan et al., 2009). While the final lesion volume was modest in the present study (vs. % injury sustained in humans), one might still argue that delayed cooling would be more efficacious when applied after a smaller ICH (e.g., 50 μl blood injection) than that presently used. Although this remains to be tested, we predict that no additional benefit would occur as there would be fewer peri-hematoma neurons at risk, and it would be even more difficult to detect the behavioral effects of saving those few mild hypothermia, induced either systemically or with brain-selective protocols. Notably, these protocols significantly lessen edema, blood-brain barrier disruption and inflammation after ICH (Fingas et al., 2007; Kawamishi et al., 2008; MacLellan et al., 2006a, 2006b) as well as being neuroprotective after ischemia. Thus, although there is some ongoing cell death in this model (Felberg et al., 2002; Wasserman and Schlichter, 2007), it appears to be either insensitive to hypothermia treatment, or of insufficient amount to noticeably influence lesion size. Similarly, most drug treatments have failed to influence lesion size in this model. Conversely, the collagenase model causes greater and more prolonged injury than the whole blood model when both have similar initial hematoma volumes (MacLellan et al., 2008). This might explain why delayed cooling can reduce lesion volume and improve outcome in the collagenase model (MacLellan et al., 2004). Given the limitations of the collagenase and whole blood models in rodents (James et al., 2008), such as the limited white matter injury (vs. humans), it seems appropriate that hypothermia also be assessed in other models. Notably, one study induced ICH in pigs and found that profound local cooling reduced edema, but they did not assess whether that translated into improved functional recovery or a reduction in cell death (Wagner et al., 2006). The hypothermia findings in rodent models of ICH suggest that reductions in edema do not necessarily translate into improved outcome; although, some benefit would be expected clinically, but probably only in cases of severe edema (e.g., to reduce mortality).

Fig. 5. Asymmetry score (% contralateral forelimb use; mean ± S.E.M.) prior to and at 13 and 20 days after ICH. There were no group differences prior to stroke. However, the H144 group was significantly better than the NOR group after ICH.

Fig. 6. (A) Injury occurred in the lateral and mid-striatum, with ventricular enlargement in the hemisphere with the ICH (modified from Paxinos and Watson, 1998). (B) The volume of tissue lost (mm³; mean ± S.E.M.) at 21 days after ICH was unaffected by hypothermia treatment.
neurons. Finally, similar durations of systemic (MacLellan et al., 2009) and brain-selective hypothermia (D. Clark, M. Penner, S. Wowk, I. Orellana-Jordan and F. Colbourne, unpublished data) reduce injury after severe brain ischemia (permanent middle cerebral artery occlusion) in rats. Lack of benefit, therefore, has more to do with the nature of ICH (mechanisms of injury, rate of cell death), including model-dependent characteristics, than with the lesion size or treatment protocol. Likewise, the failure of many other experimental treatments to markedly reduce lesion volume after ICH, including milder insults, suggests that it is not particularly amenable to treatment. Thus far, clinical findings, including surgical removal of the hematoma (Mendelow et al., 2005) and the use of rFVIIa (Mayer et al., 2008), support this assertion.

A comparison of our current findings, especially the H72 treatment, to our earlier work that used 4 days of focal hypothermia with near-instantaneous re-warming, suggests that slowing the rate of re-warming after hypothermia had no impact. This indicates that re-warming rate is not important after focal cooling as it appears to be after systemic hypothermia. However, further study is warranted in other models of brain injury, with other treatment protocols (depth and duration of cooling) and in situations where neuroprotection clearly occurs. Until then, it is advisable to use slow re-warming rates in animal studies and clinically.

In rats, focal brain hypothermia offers several advantages over systemic cooling protocols including reduced body weight loss and fewer cardiovascular effects (Clark and Colbourne, 2007). While prolonged brain-selective hypothermia can be administered without mortality, it remains possible that extended or profound hypothermia will interfere with post-stroke neuroplasticity. This might be especially problematic with the current cooling system wherein the cerebral cortex underlying the cooling coil experiences a greater drop in temperature than the target, which was the striatum. Indeed, we estimate that region of cortex to be at ∼27 °C during the current cooling protocol (Clark and Colbourne, 2007). Unfortunately, the present findings cannot be used to determine whether this overcooling effect is helpful or harmful to recovery. However, the findings that even 6 days of cooling did not worsen outcome suggests that there will be no net harm from prolonged brain-selective cooling. Similarly, we have cooled the motor cortex (∼32 °C) of normal rats for 3 weeks without obvious signs of post-cooling behavioral abnormality or cellular death (A. Auriat, G. Silasi, M. Penner, D. Clark and F. Colbourne, unpublished data). Further work is needed to compare focal and systemic cooling protocols after ischemic and hemorrhagic stroke in order to characterize differences in efficacy and side effects. Selective behavioral protection, as presently found, might be due to spatially-limited neuroprotection (e.g., protecting sensory and not motor systems) and/or the timing and nature of testing (e.g., task difficulty). Given many such issues, reviewers have argued that neuroprotection studies should use several tests of functional outcome in order to avoid missing or overestimating treatment effects (Corbett and Nurse, 1998; MacLellan et al., 2006a, 2006b). In this study, the H144 treatment prevented the ICH-induced decline in the use of the contralateral-to-stroke limb during vertical exploration of a cylinder, whereas performance was not improved on the ladder or reaching tasks. Similarly, others have reported selective behavioral protection in the absence of obvious histological benefit with hypothermia after whole blood-induced ICH (Kawanishi et al., 2008). Such selectivity presumably stems from the nature of these tests and the treatment. However, one must also wonder whether the benefit on one test, among many, simply occurred by chance, which goes up with the number of tests used. Thus, it seems warranted that additional experiments determine whether these findings can be replicated. If true, one might speculate that prolonged hypothermia acted to either promote neuroplasticity or to block abnormal plasticity. For instance, prolonged hypothermia might mitigate the dendritic injury that occurs in the peri-hematoma region after ICH (Nguyen et al., 2008a,b), an effect which would likely be missed with standard measures of lesion volume. Such effects could explain why an ICH study in rat found that delayed rehabilitation worked better if it was preceded with systemic hypothermia treatment (2 day duration), which on its own did not reduce lesion volume (MacLellan et al., 2005).

In summary, the use of brief (12 h) to prolonged (6 days) treatments with focal hypothermia did not reduce lesion volume and, for the most part, did not influence functional outcome after whole blood induced ICH in rats. These findings suggest that mild hypothermia will be of limited neuroprotective potential for ICH despite its clear ability to reduce inflammation, blood-brain barrier disruption and edema. Not only do these findings set limits to what might be expected of hypothermia in the clinic (e.g., limited to treating edema), but they suggest that ICH may not be particularly amenable to any treatment. Nonetheless, further testing of hypothermia after ICH with alternative treatment regimens (e.g., profound focal cooling) and especially in alternate experimental models is certainly warranted before any definitive predictions can be made of hypothermia’s efficacy as a neuroprotectant for ICH. Similarly, the interactive effects of hypothermia and drug therapies (e.g., rFVIIa, etc.) must be considered. Such studies are also important in order to understand the effects of cooling in the setting of hemorrhagic transformation after focal ischemic stroke.

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