Research report

Failure of delayed and prolonged hypothermia to favorably affect hemorrhagic stroke in rats

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Abstract

Prolonged hypothermia reduces global and focal cerebral ischemic injury in rodents even when delayed for hours. However, it is not known whether hypothermia can reduce injury following intracerebral hemorrhage (ICH). Accordingly, we studied striatal injury and concomitant motor deficits after 2 days of hypothermia, induced 1 h after creation of an ICH by infusion of bacterial collagenase. Rats were first trained to retrieve food pellets in the Montoya staircase task. They were then implanted with core temperature telemetry probes and later subjected to normothermic ICH or sham operation (vehicle injection). Half self-regulated temperature after surgery; others were cooled to 33°C (24 h) and then 35°C (24 h). Hypothermia did not affect behavioral scores of sham animals (89.8% of baseline in 3rd staircase test) or histology. Untreated (normothermic) ICH rats lost 23.1 mm of tissue at a 1-month survival, which significantly impaired food pellet retrieval (66.0% retrieval) with the contralateral limb (tested on days 21–25). Contrary to our hypothesis, hypothermia failed to lessen either the reaching impairment (62.8%) or the lesion (22.2 mm\textsuperscript{3}). While other hemorrhagic insults or complications may be improved with hypothermia, our data suggest that it will not salvage tissue that is quickly lost after ICH. We also assessed walking across a horizontal ladder and spontaneous paw usage in a cylinder test at 1–4 weeks after ICH, but neither test was sufficiently sensitive to this mild insult. This indicates that skilled reaching is more severely disrupted than spontaneous paw usage or walking after a striatal hemorrhage.

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1. Introduction

Intracerebral hemorrhage (ICH), which commonly occurs in the striatum, thalamus, cerebellum and pons, accounts for approximately 10–15% of strokes in Western populations [27] and is one of the most devastating types of stroke. Indeed, the 30-day mortality rate is ~50%, and neurological recovery in survivors is often poor [5]. Neurological deficits result from direct tissue destruction, space-occupying effects of the hematoma, a reduction of cerebral blood flow in surrounding tissue [28], and cerebral edema [41]. A variety of treatment strategies have been tested such as the surgical removal of the hematoma [2,3,22], and treatment of raised intracranial pressure (ICP; [18]). Experimental treatments aim to reduce either tissue damage or the secondary consequences of an ICH, such as inflammation [16,33] or raised ICP. Unfortunately, ICH remains a clinically difficult problem to treat [6] and experimental studies have so far failed to show exceptional benefit from any therapy.

Delayed hypothermia reduces ischemic injury in rodents and promotes functional recovery [10,20]. For example, mild postischemic hypothermia, when prolonged (e.g. 24 h), provides effective and long-lasting protection of hippocampal CA1 neurons in both gerbil [7,8] and rat [11] models of global ischemia. Delayed hypothermia also reduces infarct size and functional impairments following middle cerebral artery occlusion (MCAO) in rats...
have acquired the task when they retrieved at least nine Canada) to produce intracerebral hemorrhage (ICH) or 

Rats were considered to bacterial collagenase (Type IV -S, Sigma, Oakville, ONT, Quebec, Canada) were entered into this study. Rats were anesthetized with isoflurane (1.5–2% maintenance in 70% \( \text{N}_2\text{O} \), 30% \( \text{O}_2 \)) and telemetry core temperature probes (model TA10TA-F40; Data Sciences, St. Paul, MN, USA) were implanted into the peritoneal cavity. The abdominal wound was treated with a local anesthetic (Marcaine; Sanofi Canada, Markham, ONT, Canada) and sutured closed. Rats were then housed individually upon receivers (RPC-1; Data Sciences) interfaced to a computer running DataSciences telemetry software which sampled temperature every 30 s. Data from the complete day prior to ICH or sham surgery served as a baseline. Temperature data were analyzed with ANOVA followed by a Scheffé correction for individual comparisons (SPSS, v 11.0).

Core temperature, when measured with telemetry probes, correlates well with brain temperature in normal awake or anesthetized rats. For instance, we (DeBow and Colbourne, unpublished data) compared core and brain temperatures in awake rats, both measured with telemetry probes, and found \( r=0.97 \) (\( P<0.0001 \)). Similar correlations are found in anesthetized rats as long as cerebral blood flow is not impaired. Notably, however, brain temperature is \(-0.7^\circ\text{C} \) lower than core temperature. Brain temperature was not measured in this study because of the desire to maintain a closed cranium, and therefore to allow ICP to rise after the hemorrhagic stroke, and due to technical problems with securing a probe on the head while allowing access to the striatum for collagenase infusion (see Section 2.4).

2.3. Core temperature telemetry probe implantation

Six days after the end of staircase training, rats were briefly anesthetized with isoflurane anesthesia (4% induction; 1.5–2% maintenance in 70% \( \text{N}_2\text{O} \), 30% \( \text{O}_2 \)) and telemetry core temperature probes (model TA10TA-F40; Data Sciences, St. Paul, MN, USA) were implanted into the peritoneal cavity. The abdominal wound was treated with a local anesthetic (Marcaine; Sanofi Canada, Markham, ONT, Canada) and sutured closed. Rats were then housed individually upon receivers (RPC-1; Data Sciences) interfaced to a computer running DataSciences telemetry software which sampled temperature every 30 s. Data from the complete day prior to ICH or sham surgery served as a baseline. Temperature data were analyzed with ANOVA followed by a Scheffé correction for individual comparisons (SPSS, v 11.0).

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2.4. Intracerebral hemorrhage/sham surgery

Rats were anesthetized with isoflurane (1.5–2% maintenance) and placed in a stereotaxic frame. During anesthesia (~30 min) core temperature was maintained near \( 37^\circ\text{C} \) (normothermia) with a heating pad. Following a midline scalp incision (earlier treated with Betadine), a small burr hole was made 3 mm contralateral to preferred paw (as determined by performance in the staircase test; random if no clear preference) and 0.2 mm posterior to bregma. A 28-gauge needle (Hamilton syringe, Hamilton, Reno, NV, USA) was then lowered 6 mm below the surface of skull and we infused 0.7 \( \mu\text{l} \) of sterile saline containing 0.14 U bacterial collagenase (Type IV-S, Sigma, Oakville, ONT, Canada) to produce intracerebral hemorrhage (ICH) or
sterile saline alone (SHAM procedure) into the striatum over a period of 5 min. The needle remained in place for an additional 5 min following infusion. A metal screw (model MX-080-2, Small Parts, Miami Lakes, FL, USA) was inserted to the thickness of the skull bone thus sealing the burr hole and allowing for subsequent ICP elevations due to the hemorrhage. The scalp wound was then infiltrated with Marcaine and closed with staples. All rats were shaved (part of abdomen and back) to facilitate subsequent hypothermia treatment or to prevent knowledge of group identity in the normothermic groups. Importantly, prior to surgery all rats were randomly assigned to SHAM or ICH groups and then randomly assigned to either normothermic or hypothermic conditions.

The dose of collagenase was chosen to produce a mild–moderate sized lesion and was based upon studies by Peeling et al. [32]. A mild lesion was presumed to be more amenable to treatment with hypothermia than a severe insult, thus maximizing our chances of detecting beneficial effects.

2.5. Post-surgery temperature control

The ICH (n=8 included) and SHAM (n=5) animals were allowed to freely regulate their own temperature. ICH+HYPO (n=8) and SHAM+HYPO (n=6) groups were slowly cooled, starting at 1 h after ICH induction, by a rate of 1 °C per 30 min to a core temperature of 33 °C and maintained at this level for 24 h. Rats were then slowly warmed (1 °C/30 min) to 35 °C and kept at that temperature for an additional 24 h. Following this, core temperature was maintained between 36 °C and 37 °C (low normothermia range) for 24 h. Temperature was precisely (±0.3 °C) regulated using a servo-controlled system that employed fans and fine water misters for cooling and infrared lamps for warming [9]. Temperature was monitored in all animals for 7 days after surgery before telemetry probes were quickly removed under isoflurane anesthesia.

Three additional rats were excluded due to technical problems (e.g. significant bleed upon insertion of needle). Another three rats were later excluded due to aberrant lesion location. No mortality occurred during this experiment.

2.6. Behavioral testing

2.6.1. Limb-use asymmetry test

Rats were put in a transparent cylinder (45 cm in height and 33 cm in diameter) for 5 min and allowed to spontaneously explore the cylinder on days 7, 14, 21, and 28 following ICH/SHAM surgery. A video camera set up below the cylinder recorded all forelimb placements, which were recorded and analyzed according to Tillerson et al. [40]. Briefly, forelimb use was noted for initiating a rearing movement, exploration of cylinder walls, and landing after a rearing movement. A push-off was considered the independent use of either forelimb (or simultaneous use) when a rearing movement began. Wall exploration included the initial contact of a forelimb with the wall and subsequent single-limb contacts during lateral movements across the wall while maintaining a vertical posture. The use of the opposite limb while maintaining an initial wall contact was scored as a simultaneous use. Landings included the use of either forelimb (or both) to land after a rearing movement. Push-off, wall exploration, and landing forelimb placements were scored independently and expressed in terms of: (1) the percentage of impaired limb use in relation to the total number of limb-use movements, (2) the percentage of non-impaired limb use in relation to the total number of limb-use movements, and (3) the percentage of simultaneous use of forelimbs or ‘co-use’ relative to the total number of limb placements. This data were analyzed with ANOVA with a Scheffé correction for individual comparisons.

2.6.2. Horizontal ladder walking test

On days 7, 14, 21, and 28 post-surgery, rats were tested on the horizontal ladder walking test [29]. Each session consisted of three consecutive trials in which the rat walked across a horizontal ladder with randomly spaced ‘rungs’ or bars (spacing between bars ranged from 1 to 3 cm). For each limb, the total number of steps and the slips (limb falls through bars) made to traverse a 0.5-m segment was analyzed with ANOVA with a Scheffé correction for individual comparisons.

2.6.3. Staircase test

Rats were food-deprived to 90% of their free-feeding weight prior to staircase testing which consisted of 10 trials (two trials per day) on days 21–25 after ICH/SHAM surgery. The total number of pellets retrieved on each side, which was expressed as a percent of baseline (average of the last 10 training trials), was recorded for each trial and analyzed with ANOVA with a Scheffé correction for individual comparisons.

2.7. Histology

Rats were allowed to survive for 30 days following ICH or sham operation. They were euthanized with an overdose of Somnotol (80 mg/kg) and were transcardially perfused with 0.9% saline and then 10% formalin. Forty-μm coronal sections were taken with a cryostat. Sections were taken every 200 μm starting at +1.7 mm to bregma and extending back to −4.8 mm to bregma. Sections were stained with cresyl violet and using Scion Image J 4.0, the volume of the lesion plus atrophy (e.g. ventricular enlargement) was quantified and expressed as follows:
Volume of tissue lost  
= remaining volume of normal hemisphere  
− remaining volume of injured hemisphere

Volume of a hemisphere = average (area of the complete  
coronal section of the hemisphere  
− area of ventricle − area of damage)  
× interval between sections × number of sections

Volume of tissue lost (i.e. a combination of the hemorrhagic infarct, cavity and ventricular enlargement) was analyzed with a one-way ANOVA.

In addition to calculating the volume of tissue lost, we estimated tissue destruction by the methods of Altumbabic et al. [1]. Briefly, the area of the contralateral and ipsilateral striatum as well as the hematoma was measured. Striatal loss was considered to be the difference between the contralateral striatum and the remaining ipsilateral striatum (ipsilateral striatum − hematoma).

3. Results

3.1. Temperature

Baseline core temperature, measured with the telemetry probes, was collected the day before ICH/SHAM surgery and was similar among groups (37.0°C ± 0.1 S.D.) and similar to previous studies. Temperature during and after surgery (Fig. 1) was regulated as desired (see Section 2). For 12 h after surgery, ICH rats were slightly warmer than SHAM rats (37.5 vs. 37.2°C, respectively; ANOVA on mean temperature; P = 0.003).

3.2. Body weight

The core probe implantation procedure caused a small drop (~4 g) in body weight, which was similar in all groups (F3, 26 < 1). The ICH and SHAM groups had similar postoperative (days 4, 7, 14, 21 and 28) weights (% surgery day) after the infusion procedure. However, hypothermia treatment in the SHAM+HYPO (vs. SHAM; P = 0.045) and ICH+HYPO (vs. ICH; P < 0.001) groups did result in statistically significant weight loss on days 4 and 7, but this never exceeded 10% of their pre-surgery body weight. All groups had regained any lost weight and were not significantly different on days 14, 21 and 28 (P = 0.345). Induced weight loss (~9% of baseline overall) during staircase testing was not significantly different among groups (F3, 23 < 1).

3.3. Staircase testing

All groups performed similarly on the training phase of the staircase test (data not shown). As expected, there was no significant difference (P = 0.726) in the food pellet retrieval (% baseline which was defined as the average performance on the last 10 trials of training) of the SHAM and SHAM+HYPO groups with either the ipsilateral or contralateral forelimb (Fig. 2). Thus, these groups were combined for subsequent analysis. Neither the ICH nor the ICH+HYPO groups had statistically significant ipsilateral reaching impairments (P = 0.083), whereas both obtained significantly less pellets than SHAMs (combined groups) with the contralateral forelimb (P = 0.010). Hypothermia did not lessen this deficit (P = 0.967).

3.4. Horizontal ladder walking test

The total number of steps taken to cross the 0.5-m length of horizontal ladder was very similar among groups (data not shown). There were no significant group effects for any limb (P = 0.091) although there was a trend towards an impairment (higher % of falls) with the contralateral forelimb in the ICH and ICH+HYPO groups (vs. SHAMs; Fig. 3). These group comparisons did not reach statistical significance with the Scheffé test (P = 0.153) or even with simple contrasts (P = 0.056), which did not correct for multiple comparisons (i.e. less likely to make a type II error).

3.5. Limb-use asymmetry test

There were no significant differences between SHAM and SHAM+HYPO groups on measures of paw placement on the wall, on push off and on landing (ANOVA; P = 0.385); thus these groups were combined for subsequent analyses. While there were some trends towards diminished usage (vs. SHAMs) of the contralateral forelimb in ICH groups (e.g. placement on wall; Fig. 4), there were no significant group main effects for usage of the contralateral paw (P = 0.133), ipsilateral paw (P = 0.127) or co-usage (P = 0.555) on paw placement on the
Fig. 2. Performance (% baseline) in the Montoya staircase test given on days 21–25 post-ICH or SHAM surgery for the ipsilateral (A) and contralateral (B) forelimb. Both ICH and ICH+HYPO groups were significantly impaired (vs. SHAMs) with the contralateral limb, but were not significantly different from each other.

wall, and on landing. For paw placement on push off we found no significant group effect for the contralateral limb ($P = 0.112$) or co-usage ($P = 0.071$), but did find a significant group effect for the ipsilateral limb ($P = 0.048$), which was due to the ICH+HYPO group using their ipsilateral limb more frequently than SHAMs on weeks 1 ($P = 0.042$) and 4 ($P = 0.009$) only. There were no significant differences between these groups on weeks 2 and 3; nor were any differences found between the ICH and ICH+HYPO groups. Accordingly, this test was not very useful in distinguishing among groups.

3.6. Volume of lesion

Injection of collagenase in ICH rats resulted in a mean total tissue loss of $23.1 \text{ mm}^3 \pm 12.4$ S.D. This largely included striatum, more medially (Fig. 5) but often lateral as well. Sometimes the lesion affected thalamus, globus pallidus and the internal capsule. Notably, prominent ventricular dilation was seen ipsilateral to the lesion and somewhat anterior and posterior to the obvious hemorrhagic stroke focus. Post-ICH hypothermia did not significantly affect the volume of tissue lost ($22.2 \text{ mm}^3 \pm 9.5$ S.D.; $P = 0.982$ vs. ICH). Sham groups did not sustain any

Fig. 3. Performance (failure rate −%) with the contralateral forelimb in traversing the horizontal ladder on days 7, 14, 21 and 28 post-surgery. This test was not sufficiently sensitive to the ICH lesion although there was a trend towards impairment with the contralateral forelimb.

Fig. 4. Percentage of paw placements on the wall in the cylinder test, which was given at 7, 14, 21 and 28 days post-surgery. There were no significant differences among the groups. Trends for push-off and landing were similar (data not shown).
damage aside from the needle tract except for one SHAM rat (noted in Section 2), which had a significant cortical lesion. Since this animal was noted to have a large bleed upon needle insertion it was excluded.

Using the methods of Altumbabici et al. [1], tissue loss (in mm$^3$) was estimated using the section with the maximum hematoma diameter. There was a strong correlation ($r=0.84$; $P<0.001$) between the area of striatal loss calculated from that single section and the total volume of injury calculated from sections encompassing the entire lesion (36 sections per rat from ICH and ICH+HYPO groups). In order to determine whether it is necessary to take sections every 200 μm, the volume of lesion was also estimated using sections 400 and 600 μm apart. Lesion volume (all ICH and ICH+HYPO rats) was similar when sections were taken at 200 μm (22.7 mm$^3$), 400 μm (21.7 mm$^3$) and 600 μm (21.9 mm$^3$) intervals ($F_{2, 45}<1$).

4. Discussion

Prolonged mild hypothermia failed to reduce either the volume of tissue lost following ICH or the behavioral deficits (skilled reaching) that accompany unilateral striatal injury. This is in stark contrast to the beneficial effects of similar hypothermia treatments induced hours after global [8,11] and focal cerebral ischemia [12,13] and the use of hypothermia after thrombin injection to reduce edema [24]. The infusion of bacterial collagenase resulted in a mild to moderate-sized ICH affecting largely the striatum, but extending to include thalamus, globus pallidus and internal capsule. Most tissue destruction, previously characterized by Del Bigio et al. [15], occurs over several hours. Thus, our hypothermia treatment, which was initiated 1 h after infusion of collagenase, should be considered an early intervention. We intentionally used a mild to moderate insult and early induction of hypothermia to maximize the likelihood of finding a beneficial effect while maintaining clinical relevance (i.e. delayed induction of therapy). Contrary to our expectations, hypothermia had no discernable effects on the volume of tissue lost or the skilled reaching deficit. There are several likely explanations: (1) this type of hemorrhagic insult does not have a substantial ischemic component and thus hypothermia, an anti-ischemic therapy, would be ineffective, (2) most injury occurs too rapidly to be effectively treated by delayed treatments or the insult was too severe, (3) the therapy was sub-optimal (e.g. too brief), and (4) benefit was only transient.

The severity of an insult dramatically affects the amount of neuroprotection observed with any treatment. For example, a 12-h duration of hypothermia barely reduces CA1 cell death after 5 min of normothermic forebrain ischemia in gerbils, whereas near-total protection is observed after a 3-min insult [7]. Thus, one could argue that a milder hemorrhagic insult than that presently used might be amenable to hypothermia therapy. However, the ICH insult used was relatively mild since it did not result in significant (or at least persistent) impairments on two of our behavioral tests (i.e. the horizontal ladder and the cylinder task) or in any mortality. Furthermore, the impairment observed in the staircase test (Fig. 2) was similar to that observed after cortical infarction after distal MCAO, an insult that is amenable to delayed hypothermia treatment [12]. Accordingly, our insult was not likely too severe. Indeed, it is possible that our insult was not sufficiently severe to properly assess hypothermia. For example, prolonged cooling might attenuate those deleterious events (e.g. blood–brain barrier breakdown, edema, raised ICP) that accompany severe ICH as it has been shown to do after thrombin injection [24]. While such changes may occur following the present ICH insult, they were not sufficiently severe to result in mortality and may not have contributed substantially to outcome. Consequently, it is possible that hypothermia therapy may not provide benefit after mild hemorrhagic insults, but may be indicated for severe insults (e.g. those with delayed edema and ICP elevations), which we plan to assess.

The duration of hypothermia markedly affects neuroprotection. For example, 12 h of hypothermia provides little persistent protection in CA1 after global ischemia while a 24-h duration provides near-total protection [7]. As we used over 2 days of mild hypothermia, which in models of global and focal ischemia provide substantial functional and histological protection, it is not likely that much would be gained by extending hypothermia therapy further. Nonetheless, more protracted cooling may be beneficial especially in models of ICH that result in a significant amount of delayed edema and raised ICP.

The striatum is a difficult structure to protect after either global or focal ischemia, likely due, in part, to vascular
differences and intrinsic differences in cell types (e.g., cortex or CA1). In fact, several studies of hypothermia find better protection in cortex than striatum [13,21,23,25]. For instance, hypothermia delayed for 1 h after a focal ischemic insult, produced by temporary suture occlusion of the middle cerebral artery, resulted in a substantial reduction in cortical injury but only a trivial amount of striatum was protected [13]. Thus, it is possible that hemorrhagic insults in other structures, such as cortex, might be more amenable to hypothermia therapy.

A final consideration is that hypothermia (and other therapies) sometimes provides only transient benefit after cerebral insults. For example, after global ischemia brief hypothermia (e.g., 3 h) will reduce CA1 sector cell death for several days only [7,17]. Whereas prolonged hypothermia, as presently used, results in persistent benefit after global and focal ischemia [7,8,11–13] it may only transiently (e.g., by day 7) attenuate histological damage and behavioral impairments after ICH.

The failure to detect deficits in the horizontal ladder task is in contrast to ICH-induced beam-walking deficits observed in other studies (e.g., Ref. [1,15,31]). Several explanations seem plausible. First, the tests are not identical and walking a beam could more difficult than traversing perpendicularly placed bars. Second, most studies utilizing the beam-walking test used it as a part of a series of neurological tests and report only the total score. Thus, the beam-walking task may not yield large deficits in those studies. Third, many studies of focal and hemorrhagic stroke use neurological test batteries during the first week after the insult. Subsequent to this time deficits on these tests largely resolve [1,14,31]. We did not perform neurological testing during the first week after ICH because half of our animals were being cooled up to and including the third day and we allowed several more days to recover (e.g., weight) after this therapy. Finally, there may be significant differences among these hemorrhage studies in location and size of lesion, which would certainly affect the magnitude and persistence of functional deficits. Thus, a larger lesion or a more laterally placed striatal lesion [34] may have resulted in greater deficits and may account for beam-walking deficits in other studies. Indeed, a more recent hemorrhagic stroke study in our laboratory (DeBow et al., in preparation), which used a larger and more lateral striatal hemorrhagic lesion, did find significant and persistent deficits on the horizontal ladder task.

The cylinder task has not been used previously for striatal ICH. However, it is sensitive to focal ischemic injury [4,36] and 6-hydroxydopamine lesions [40]. The likely reason we did not observe a significant (there were trends) deficit is that our insult was relatively mild, the lesion too medial, or that there was sufficient recovery (e.g., by resolution of edema) by the time the first test was administered. For example, untreated ICH rats were observed to have their contralateral forelimb in a retracted posture in the days following ICH induction and they often did not use this paw when making contact with the wall of their home cage.

There are a variety of histopathological assessment methods and little consensus (e.g., number of sections to take, staining methods, etc.) on appropriate ways to determine the volume of brain injury. We found that the simple area assessment of the coronal section with the largest lesion was representative of the volume of tissue lost when many sections were obtained and a volume was determined. However, the collagenase injection did result in prominent ventricular enlargement in sections anterior, through, and posterior to the hemorrhagic lesion; an effect that is likely due to dendritic and axonal injury. Accordingly, it is important to consider these sections in any measure of lesion volume especially in cases of cytoprotection, which might differentially affect ventriculomegaly. Our findings suggest that a dozen equally spaced sections can be sufficient. Prolonged hypothermia did not affect ventriculomegaly, which is in line with the behavioral data showing absence of a beneficial effect. Nonetheless, it is possible that some beneficial (or detrimental) effects went unnoticed (e.g., axonal injury). Further study is needed with more sensitive functional tests and histological methods, especially given the positive finding of Kawai et al. [24].

In summary, delayed and prolonged hypothermia lessened neither subcortical lesion volume nor skilled reaching deficits in a collagenase model of ICH in the rat. Although unexpected, these results are consistent with the lack of substantial structural protection or functional benefit (in the Montoya staircase test) with other therapies (e.g., surgical aspiration, anti-inflammatory drugs, or free-radical scavengers) using this ICH model. While we do not exclude other possible beneficial effects of hypothermia (e.g., ICP reduction), tissue that is quickly lost following ICH will not likely be salvaged.

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