The Influence of Hypothermia on Outcome After Intracerebral Hemorrhage in Rats

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Background and Purpose—Late hypothermia (HYPO) reduces injury after collagenase-induced intracerebral hemorrhage (ICH), whereas early HYPO does not because it exacerbates the protracted bleeding that occurs in this model. We hypothesized that early HYPO would not increase bleeding after whole blood infusion and thus expected early HYPO to improve outcome through reducing secondary consequences of ICH (eg, inflammation).

Methods—Autologous blood (100 μL) was infused into the striatum. Rats were maintained at normothermia or subjected to mild (33°C to 35°C) HYPO for 2 days starting 1 (HYPO-1) or 4 hours (HYPO-4) after ICH. Hematoma volume was measured at 12 hours to determine whether HYPO-1 aggravated bleeding. We measured blood–brain barrier (BBB) disruption and edema 2 days after ICH in all groups. At 4 days, we counted degenerating neurons, neutrophils, and iron-positive cells (eg, macrophages) in the lesioned hemisphere. Recovery was assessed using several behavioral tests (ie, staircase reaching task, ladder walking task, limb use cylinder test) over 7 or 30 days, at which time we quantified lesion volume.

Results—HYPO did not increase bleeding. Both HYPO treatments reduced BBB disruption and infiltration of inflammatory cells. HYPO-1 treatment modestly reduced edema and provided limited to no functional benefit in the behavioral tests. HYPO did not affect lesion volume.

Conclusions—HYPO reduced edema, BBB disruption, and inflammation. Although encouraging, HYPO treatment must be improved so that histological and functional benefit are obtained before clinical investigation. Otherwise clinical failure is anticipated. (Stroke. 2006;37:1266-1270.)

Key Words: neuroprotection stroke temperature

Spontaneous intracerebral hemorrhage (ICH) causes high mortality and poor recovery in survivors. Recombinant activating factor VII (rFVIIa) is currently the only drug that improves outcome for ICH patients.1 However, it is limited to those who promptly receive attention and who undergo hematoma expansion. Thus, cytoprotective treatments are needed. In ICH, significant tissue damage occurs quickly because of space-occupying effects and toxicity of the degrading hematoma. Secondary consequences of ICH and ischemia include inflammation,2 edema,3 and oxidative damage,4 which all contribute to cell death.5,6

Mild, prolonged hypothermia (HYPO) improves outcome in rodent models of global7 and focal ischemia.8,9 Furthermore, HYPO can be safely applied to stroke victims and significantly benefits cardiac arrest patients.10 Given the overlap in mechanisms contributing to injury after ischemia and ICH and the fact that HYPO favorably affects deleterious processes common to both, it makes sense to test HYPO after ICH. Early work shows that HYPO reduces edema after intrastrial thrombin injections11 and after ICH12,13 in rats. Also, local HYPO reduces edema in a pig ICH model.14 However, contrary to ischemia, in which earlier cooling is more efficacious,7,9 HYPO initiated soon after collagenase-induced ICH, during active bleeding, increases hematoma size.15 This effect is apparently attributable to side effects of HYPO (eg, elevated blood pressure, coagulopathy), which would counteract beneficial effects of HYPO during the early post-ICH period. Not surprisingly then, HYPO delayed 12 hours after ICH improves recovery and lessens tissue loss because bleeding would not be aggravated at that time.15 Accordingly, we hypothesized that early HYPO should improve outcome in the whole blood model of ICH because bleeding is expected to end at or soon after infusion. Therefore, we assessed whether post-ICH HYPO affects bleeding, blood–brain barrier (BBB) permeability, edema, inflammation, neuronal degeneration, lesion size, and behavioral recovery.

Materials and Methods

Animals

We used 257 male Sprague-Dawley rats obtained locally (~16 weeks old; ~375 g). All procedures were approved by the University of Alberta and followed the Canadian Council on Animal Care guidelines.
**General Procedures**

Surgical procedures were performed aseptically under isoflurane (4% induction; 2% maintenance in 70% N₂O and 30% O₂).

**Temperature Probe Implantation**

A telemetry probe (TA10TA-F40; Transoma Medical) was implanted into the peritoneum 3 days before ICH. Core temperature was sampled every 30 seconds and the day before ICH served as a baseline. Brain temperature was not measured because of technical difficulty in securing a head cap while permitting striatal blood infusion.

**Intracerebral Hemorrhage**

Anesthetized animals were placed in a stereotaxic frame. A midline scalp incision was made and a burr hole was drilled 3.5 mm right of and at the anteroposterior level of Bregma. To create an ICH, 100 μL of autologous blood withdrawn from the tail was injected into the striatum (depth of 6.5 mm) over 10 minutes. After another 10 minutes, the needle was slowly removed. A metal screw sealed the hole, and the scalp was closed, followed by application of Marcaine (Sanofi). During surgery (~45 minutes), core normothermia (NORMO) was maintained (ie, 36.5°C to 37.5°C). The abdomen and back were shaved in all animals to facilitate cooling in HYPO rats and to prevent our knowledge of group identity in NORMO rats. Rats were weighed daily after ICH up to 7 days. In experiment 2, which was taken to represent all studies, we measured mean arterial blood pressure (via tail artery) throughout surgery and arterial physiological measurements (pH, pCO₂, pO₂, hemoglobin, glucose) before and after ICH. A small amount of heparinized saline was used to prevent clotting in the tail artery and catheter. This was not done in other studies.

**Post-ICH Temperature**

Rats were maintained near NORMO for 1 hour after ICH. The NORMO group was regulated >36.5°C for 48 hours. Others were slowly cooled at a rate of 2°C per hour to 33°C starting 1 (HYPO-1) or 4 hours (HYPO-4) after ICH and maintained at this level for 24 hours. Rats were warmed (2°C per hour) to 35°C for an additional 24 hours after rewarming to NORMO (Figure 1) as done previously. Temperature was precisely servoregulated using infrared lamps, fans, and water misters.

**Experiment 1: Hematoma Volume**

Hematoma volume was measured in NORMO (n=7 included) and HYPO-1 (n=7) rats 12 hours after ICH using a spectrophotometric hemoglobin assay described previously.15,16

**Experiment 2: BBB Disruption**

We assessed Evan’s blue extravasation (n=20 per group) 48 hours after ICH. Evan’s blue dye (Sigma; 2% in saline; 4 mL/kg) was injected intravenously. Two hours later, rats were perfused with saline, and each hemisphere was weighed, homogenized in saline, and centrifuged. The supernatant was incubated with 50% trichloroacetic acid, centrifuged, and absorbance was read on a spectrophotometer at 610 nm. Four unoperated rats served as a nonhematoma control. Thirteen additional rats were used to generate a standard curve using known amounts of dye (0.1 to 1.0 μL) added to unoperated control hemispheres. The amount of extravasated Evan’s blue dye was calculated from this curve.

**Experiment 3: Brain Water Content**

Brain water content was measured in 6 unoperated rats and at 2 days after ICH (n=10 in each of 3 groups). After decapitation (under anesthesia), the cerebellum and 4-mm-thick sections of striatum and cortex of each hemisphere were weighed (wet weight), baked at 100°C for 24 hours, and reweighed (dry weight). Water content was determined by ([wet weight−dry weight]/wet weight)×100.

**Experiment 4: Assessment of Iron-Positive Cells, Neutrophils, and Degenerating Neurons**

Four days after ICH, all groups (n=10 per group) were euthanized, and brains were cut into 10-μm coronal frozen sections. Iron-positive cells and neutrophils were stained using Perl’s Prussian blue for ferric iron6,17 and Leder’s stain18 for chloracetate esterase activity (Sigma), respectively. Degenerating neurons were stained with Fluoro-Jade B (BioChemika).19 The total number of iron-positive cells, neutrophils, and degenerating neurons was counted in the lesioned hemisphere at the level of maximum hematoma diameter.

**Experiment 5: Long-Term Outcome**

Rats (n=21 per group) were trained on cylinder, horizontal ladder, and staircase tests.20 These tests measure spontaneous forelimb usage, walking and skilled reaching ability, respectively, and are sensitive to striatal ICH.23-24 Baseline performance and training was done before core probe implantation.15 Rats were evaluated on the ladder (% successful steps) and cylinder (asymmetry score; ipsilateral−contralateral touches) tests 7 and 30 days after ICH, and on the staircase from days 24 to 28 days.

Thirty days after ICH, rats were euthanized with pentobarbital (80 mg/kg) and perfused with saline then 10% formalin. Then 40-μm coronal brain sections taken every 400 μm were stained with cresyl violet. Lesion volume (cellular debris, ventriculomegaly, and cavity) was manually determined using Scion Image J.15 Volume of tissue lost=remaining volume of normal hemisphere−remaining volume of lesioned hemisphere. Volume of a hemisphere=average (area of hemispheric coronal section−area of ventricle−area of damage)×section interval×number of sections.

**Experiment 6: Short-Term Outcome**

NORMO and HYPO-1–treated rats (n=8 each) survived for 7 days after ICH. Functional outcome was assessed using the ladder and cylinder at 7 days, and lesion volume was assessed.

**Statistics**

All procedures were done by experimenters blind to group identity. Using SPSS (version 12), data were analyzed with ANOVA and least significant difference post hoc tests if needed. Data are presented as the mean±SEM. A P value of <0.05 was considered to be statistically significant.

**Results**

In addition to the number of rats stated in each experiment, an additional 21 rats were excluded, of which 20 were excluded because of technical problems (eg, computer crash during temperature regulation), and the other rat was euthanized 8 days after ICH for failure to maintain NORMO.

**Physiological Variables**

Blood gases, hemoglobin, pH, and glucose (experiment 2) were in normal ranges and did not differ significantly among groups (Table). During surgery, mean arterial blood pressure was slightly but significantly higher in the HYPO-4 group than the NORMO and HYPO-1 groups. Baseline core tem-
per temperature collected before ICH was similar among groups (36.76±0.04°C; data not shown), and post-ICH temperature was regulated as desired. For instance, in experiment 5 (Figure 1) temperatures were on average 33.0±0.04°C; data not shown), and post-ICH temperature was regulated as desired. For instance, in experiment 5 (Figure 1) temperatures were on average 33.0±0.01°C and 34.9±0.02°C during the designated periods of 33°C and 35°C cooling, respectively.

Experiment 1: Hematoma Volume
The hematoma volume was not significantly different in NORMO (79.2±6.1 μL) and HYPO-1 groups (82.1±8.9 μL).

Experiment 2: Evans Blue Extravasation
We detected a small amount of Evan’s blue dye in the nonhematoma control brains (0.7±0.2 μg dye/g tissue). Evans blue extravasation, corrected for this baseline reading, in the ipsilateral hemisphere was significantly reduced by HYPO-1 and HYPO-4 treatments (Figure 2). Little extravasation occurred in the contralateral hemisphere, which was not significantly different among groups.

Experiment 3: Brain Water Content
Compared with unoperated controls (78.3±0.1%), the NORMO rats had significantly increased brain water content in the damaged striatum (80.0±0.2%), which was significantly reduced by HYPO-1 (79.4±0.2%) but not HYPO-4 treatment (79.8±0.3%).

Experiment 4: Iron-Positive Cells, Neutrophils, and Degenerating Neurons
Neutrophils infiltrated the hematoma and surrounding tissue by 4 days (Figure 3A), whereas iron-positive cells (likely activated microglia or macrophages) were in the surrounding tissue (Figure 3B). Both HYPO treatments significantly reduced neutrophil and iron-positive cell infiltration. The number of degenerating neurons did not differ among groups.

Experiment 5: Long-Term Outcome
Fifteen rats (5 per group) failed to retrieve ≥9 pellets per side on the last 3 days of training in the staircase,23 and were excluded from just this analysis. Analysis of contralateral forelimb success revealed a significant GROUP×TRIAL interaction. On further analysis, the HYPO-1 group obtained significantly more pellets on the first day (versus NORMO; Figure 4A), but all groups performed similarly afterward (ie, full recovery). In the ladder-walking test, the percentage of successful steps made with the contralateral forelimb on days 7 or 30 was similar among groups (data not shown). The HYPO-1 group made more successful steps with the contralateral hindlimb on day 7 (versus NORMO and HYPO-4 groups; Figure 4B) but not day 30. Limb use (asymmetry score) in the cylinder was equivalent in all groups on days 7

| Physiological Variables Measured Before (top) and After ICH (bottom) |
|--------------------------|-----------------|-----------------|
|                          | NORMO           | HYPO-1          | HYPO-4          |
| pH                       | 7.42±0.01       | 7.43±0.01       | 7.42±0.01       |
| pCO₂, mm Hg              | 42.9±1.1        | 40.7±0.7        | 42.9±0.9        |
| pO₂, mm Hg              | 132.4±2.3       | 132.1±2.6       | 128.4±2.6       |
| Hemoglobin, g/dL         | 13.7±0.1        | 13.5±0.5        | 14.1±0.2        |
| Glucose, mmol/L          | 14.6±0.4        | 13.5±0.5        | 14.3±0.3        |
| MABP, mm Hg             | 96.7±1.2        | 97.0±1.4        | 102.2±0.9       |

Except for mean arterial blood pressure (MABP; averaged throughout surgery), which was slightly higher in the HYPO-4 group, the values were within normal ranges and similar among groups both before and after ICH.

Figure 2. Evan’s blue extravasation (μg dye/g tissue±SEM) in the ipsilateral and contralateral hemispheres 48 hours after ICH (experiment 2; n=20 per group). Ipsilateral extravasation was significantly reduced by HYPO-1 and HYPO-4 treatments.

Figure 3. Number of neutrophils (A), iron-positive cells (B), and degenerating neurons (C) in the lesioned hemisphere 4 days after ICH (mean±SEM; experiment 4; n=10 per group). Neutrophils were detected in the hematoma and surrounding tissue, whereas iron-positive cells surrounded the hematoma. Infiltration of neutrophils and macrophages was significantly reduced in the HYPO-1 and HYPO-4 groups. The number of degenerating neurons did not differ among groups.

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and 30 (Figure 4C). After ICH, damage occurred primarily to the striatum and corpus callosum (Figure 5B). HYPO treatments did not affect the volume of tissue lost (Figure 5A).

Experiment 6: Short-Term Outcome
behavioral impairments in the cylinder (19.5±15.2 versus 15.2±7.0) and ladder (forelimb success 84.8±5.5% versus 85.4±3.9%; hindlimb success 84.1±6.4% versus 85.3±6.1%) tests as well as lesion size (7-day survival; 30.0±2.3 mm³ versus 34.8±3.8 mm³) were not significantly different between NORMO and HYPO-1 groups, respectively.

Discussion
The present findings do not support the use of HYPO soon after ICH because our short- and long-term outcome studies largely found no benefit with either HYPO treatment. Nonetheless, HYPO significantly but modestly reduced edema and substantially reduced BBB disruption and inflammation. Accordingly, reductions in edema, inflammation, or BBB disruption may not necessarily translate into functional and histological improvements. Therefore, any putative therapy should be comprehensively assessed (eg, recovery, injury, edema, etc.) before clinical investigation.

As discussed, HYPO provides significant protection in models of global and focal cerebral ischemia. Conversely, hyperthermia aggravates ischemia. Our present findings with HYPO and our recent findings with induced hyperthermia suggest that ICH is less temperature sensitive than ischemia. In our hyperthermia study, forced elevations in temperature did not significantly worsen outcome after ICH. Interestingly, the clinical findings on ICH and hyperthermia are contentious.

There are several study limitations that warrant consideration. First, we did not determine whether HYPO affects edema, BBB disruption, and inflammation at other times. Thus, HYPO may have postponed these processes as found in focal ischemia, in which HYPO delayed inflammation. If so, longer cooling may be more efficacious. Second, it appears that HYPO provides better protection in the collagenase model than in the whole blood model. However, further study is needed to confirm this because we did not directly compare treatments (eg, onset delay) in models matched for insult severity. For instance, our collagenase study found benefit with cooling delayed for 12 hours, which was not presently tested. Thus, it is possible that later cooling would provide benefit in the whole blood model. However, aside from HYPO aggravating bleeding in the collagenase model, there is no obvious mechanism as to why later cooling would provide better protection, and this was not seen in comparing HYPO-1 and HYPO-4 groups. Third, we used heparinized saline to prevent clotting in the tail artery in experiment 2, which was accessed for blood pressure and blood gas analysis. Although the rest of the studies did not use heparin, it is possible that the results of experiment 2 are somehow affected compared with the other experiments. Fourth, the statistically significant reduction in edema must be considered modest at best. Therefore, the apparent lack of benefit of this edema reduction must be interpreted with caution. Indeed, we expect that greater reductions in edema would provide some benefit, although this should always be tested. Fifth, the HYPO-1 treatment improved hindlimb success in the ladder at day 7 after ICH in experiment 5 but not 6. Because there is no obvious explanation for this, it is possible that these hindlimb findings in experiment 5 are attributable to chance, especially considering that forelimb success was not different in either experiment, and generally, other tests showed no benefit. Finally, assessing functional outcome in the whole blood model was problematic because...
animals showed good recovery with each test. Thus, without substantial behavioral impairments, one cannot easily assess protective effects (ie, ceiling effect). We attempted to overcome this by repeatedly using several tests sensitive to striatal injury.

In summary, early and prolonged HYPO reduced several consequences of ICH but provided very little functional benefit and no discernible histological protection. Accordingly, further study is needed to improve HYPO before it is applied to ICH patients. Importantly, side effects of HYPO must be identified and countered. Our previous collagenase study showed that early HYPO aggravated bleeding, which did not occur presently in the whole blood model but may occur in patients experiencing rebleeding or in patients undergoing hemorrhagic transformation after ischemia. Safer treatment may be achieved through using alternate cooling methods (eg, local cooling) as well as drug cotreatment (eg, rFVIIa to treat coagulopathy). Interestingly, the activity of rFVIIa is only slightly affected by cooling to 33°C, and it reduces bleeding in NORMO and HYPO pigs with liver injury. Thus, rFVIIa and HYPO may be an especially effective approach for treating ICH.

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