Treatments (12 and 48 h) with systemic and brain-selective hypothermia techniques after permanent focal cerebral ischemia in rat

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Abstract
Mild hypothermia lessens brain injury when initiated after the onset of global or focal ischemia. The present study sought to determine whether cooling to \(33^\circ C\) provides enduring benefit when initiated 1 h after permanent middle cerebral artery occlusion (pMCAO, via electrocautery) in adult rats and whether protection depends upon treatment duration and cooling technique. In the first experiment, systemic cooling was induced in non-anesthetized rats through a whole-body exposure technique that used fans and water mist. In comparison to normothermic controls, 12- and 48-h bouts of hypothermia significantly lessened functional impairment, such as skilled reaching ability, and lesion volume out to a 1-month survival. In the second experiment, brain-selective cooling was induced in awake rats via a water-cooled metal strip implanted underneath the temporalis muscle overlying the ischemic territory. Use of a 48-h cooling treatment significantly mitigated injury and behavioral impairment whereas a 12-h treatment did not. These findings show that while systemic and focal techniques are effective when initiated after the onset of pMCAO, they differ in efficacy depending upon the treatment duration. A direct and uncomplicated comparison between methods is problematic, however, due to unknown gradients in brain temperature and the use of two separate experiments. In summary, prolonged cooling, even when delayed after onset of pMCAO, provides enduring behavioral and histological protection sufficient to suggest that it will be clinically effective. Nonetheless, further pre-clinical work is needed to improve treatment protocols, such as identifying the optimal depth of cooling, and how these factors interact with cooling method.

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Introduction
Mild hypothermia, in the range of 32 to 35 °C, is the most extensively studied therapy for ischemic brain injury. Based upon many animal studies (reviewed in MacLellan et al., 2009; Polderman, 2008; van der Worp et al., 2007), hypothermia was successfully translated to the clinic where it has been proven to reduce morbidity and mortality after cardiac arrest (Bernard et al., 2002; The Hypothermia After Cardiac Arrest Study Group, 2002) and to improve outcome in infants suffering from a hypoxic–ischemic event (Gluckman et al., 2005; Shankaran et al., 2005). Despite these successes and encouraging animal data, hypothermia has not yet been clinically proven to lessen the devastation of a focal cerebral ischemic insult (stroke) in adults. Nonetheless, initial clinical findings are encouraging, such as the use of cooling to lessen raised intracranial pressure, and further study is underway to test the ability of hypothermia to improve functional outcome (Polderman, 2008).

In most adult patients, systemic hypothermia is used. This is induced and maintained through surface-cooling blankets and adhesive pads, the use of an endovascular cooling system and sometimes cold intravenous fluids initially (Polderman, 2008; Polderman and Callaghan, 2006). These more advanced systems allow for a rapid induction and steady control of hypothermia, which are improvements over older methods. Nonetheless, inducing systemic hypothermia carries considerable risk, including arrhythmias, dehydration and increased infection rates, which become more troublesome at temperatures below 32 °C and with more prolonged treatment (Polderman, 2008; Schubert, 1995). Thus, several methods, including cooling helmets, epidural cooling pads and intranasal cooling systems, have been devised to selectively cool the brain thereby avoiding systemic side effects altogether. While the safety and effectiveness of such methods have yet to be fully determined, animal studies are promising (Covaci et al., 2008; Wagner and Zuccarello, 2005). Although, not all agree that a significant amount and duration of selective brain cooling is truly possible in humans, at least not without some drop in core temperature.

As in humans, one may induce either systemic or brain-selective hypothermia in rodents. Most methods to systemically cool, such as use of cooling pads, require anesthesia and are appropriate for cooling periods shorter than 6 h. The use of cold rooms or exposure techniques, including our fan and water spray system (Colbourne et al., 1996), are effective for inducing more prolonged cooling in awake...
rodents, but side effects still occur, including altered blood pressure and weight loss. Several methods have been devised to induce brain-selective hypothermia, such as by cooling the head directly (Nurse and Corbett, 1994; Taniguchi et al., 2005) or via the carotid arteries (Wei et al., 2008), but they are limited to anesthetized animals and a brief cooling treatment. Nonetheless, they are effective after focal ischemia, at least with early intervention, as are systemic cooling techniques (MacLellan et al., 2009; Polderman, 2008; van der Worp et al., 2007). Given the expected need for more prolonged cooling, we recently devised a simple method to induce long-term, brain-selective hypothermia in conscious rodents (Clark and Colbourne, 2007). The technique involves surgically implanting a metal coil or strip between the temporals muscle and the skull. This device is then attached to an overhead swivel and a cold water source. With this method, mild hypothermia can be safely induced in one hemisphere without the cardiovascular effects and loss of body weight seen with exposure cooling. As in humans, the efficacy of prolonged brain-selective cooling for stroke in rats is unknown. However, several studies have used 12 h to 6 days of focal brain cooling to treat intracerebral hemorrhage with limited success (Fingas et al., 2007, 2009; Wagner et al., 2006).

Aside from avoiding systemic complications, focal cooling offers the possibility of using deeper and more prolonged hypothermia protocols. Treatment duration is especially important. For instance, in global ischemia, brief cooling provides little or only transient benefit whereas prolonged cooling is needed to permanently rescue brain tissue (Colbourne and Corbett, 1994; Dietrich et al., 1993; MacLellan et al., 2009). Similar findings have been reported in animal models of focal ischemia (Clark et al., 2006; Maier et al., 1998; Yanamoto et al., 1996). However, not all studies have found delayed hypothermia to be effective (Campbell et al., 2008; Doerfler et al., 2001; Moyer et al., 1992; Ridenour et al., 1992; Yanamoto et al., 1996), and a recent meta-analysis concluded that better protection was observed in studies that used shorter cooling (van der Worp et al., 2007). Accordingly, further study is needed to identify the factors that determine when hypothermia is effective, including the insult severity, intervention delay, treatment duration, and cooling method.

Presently, we investigated the effects of 12 and 48 h of systemic and brain-selective hypothermia induced starting 1 h after the onset of a permanent middle cerebral artery occlusion (pMCAO) in adult rats. A model of permanent ischemia was chosen because it reflects a common clinical presentation (Kassem-Moussa and Graffagnino, 2002; Rha and Saver, 2007). Additionally, the use of short, transient occlusion models, mimicking the ideal but rare clinical situation of rapid reperfusion, leads one to generally overestimate treatment effects achievable in the stroke population (Rha and Saver, 2007). We compared 12- and 48-h bouts of hypothermia because we had shown these to differ in efficacy out to a 7-day survival after pMCAO (Clark et al., 2008). As well, these are within the range used clinically for brain injury (Bernard et al., 2002; Clifton et al., 2001; Gluckman et al., 2005; Schwab et al., 1998; Shankaran et al., 2005; The Hypothermia After Cardiac Arrest Study Group, 2002). Outcome was assessed with several functional tests out to a 1-month survival, at which time lesion volume was determined. The use of a long-term survival time with multiple behavioral tests is needed to thoroughly evaluate treatment efficacy (Corbett and Nurse, 1998, Stroke Therapy Academic Industry Roundtable (STAIR), 1999) because neuroprotective treatments, including hypothermia, may simply postpone ischemic cell death (Colbourne and Corbett, 1994; Colbourne et al., 1999; Dietrich et al., 1993; Doerfler et al., 2001; Vatlysson et al., 1994).

Methods

Subjects

One hundred and forty-six male, young-adult, Sprague-Dawley rats were used. They were housed individually on a diurnal light cycle (on time: 07:00–19:00 h) with free access to food, except as described below, and water. These experiments were approved by the University of Alberta Biosciences Animal Care and Use Committee and were in accordance with the guidelines of the Canadian Council on Animal Care. Two experiments were conducted to evaluate the efficacy of prolonged hypothermia initiated 1 h after pMCAO. The first used whole-body hypothermia whereas the second used a brain-selective cooling technique. Both experiments compared 12- and 48-h hypothermia treatments to a normothermic control group. Lesion volume and several behavioral endpoints were evaluated as illustrated in Fig. 1.

Experiment 1 used 84 rats of which 8 were excluded before treatment randomization because of technical problems (e.g., surgical error). This left 76 rats in the 12-h systemic hypothermia group (SH-12), 25 in the 48-h systemic hypothermia group (SH-48) and 24 in the normothermic control group (NOR (SH)). Experiment 2 used 62 rats of which 9 were excluded for similar technical reasons prior to randomization. First, a small pilot study (N = 6), which measured core (T_c) and brain temperature (T_br) via telemetry, was done to establish and illustrate a focal hypothermia cooling protocol. Of these 6 rats, 3 were cooled for 48 h while 3 were kept normothermic. This information guided our main efficacy experiment, where 13 rats were subjected to 12 h of focal hypothermia (FH-12), 16 were subjected to 48 h of focal cooling (FH-48) and 18 rats served as a normothermic control group (NOR (FH)).

Anesthesia/surgery

All surgeries were performed under isoflurane anesthesia (4% induction; 2% maintenance; 60% N_2O, balance O_2). Aseptic techniques were used and wounds were treated with local anesthetic (Marcaine; Sanofi Canada, Markham, Ontario, Canada) to reduce post-operative pain. Rats also received 5 mL of saline S.C. to aid in recovery after surgery.

Core probe implantation

Sterilized telemetry probes (model TAT10TA-F40, Transoma Medical, St. Paul, MN) were surgically implanted into the peritoneal cavity 3 days prior to the stroke surgery. These probes, which measure T_c, were previously calibrated against a laboratory standard—a calibration-grade...
glass thermometer. In order to record temperature, the rats were housed individually in cages that rested upon receivers (RPCL-1, Transoma Medical) interfaced to a computer running A.R.T. software (v.2.2, Transoma Medical). Temperature was sampled twice per minute and we used the data from the days before stroke to confirm that the rats’ temperature were normal, which was always the case. In conscious rats, $T_c$ normally correlates well with $T_b$ and these readings are usually within 1°C of each other (DeBow and Colbourne, 2003). Core temperature probes were used in Experiment 1 and in the pilot experiment. We did not use core probes in the main part of Experiment 2 because the pilot study showed that $T_c$ does not change in rats subjected to this focal brain cooling protocol, which confirms earlier findings (Clark and Colbourne, 2007; Fingas et al., 2007, 2009).

**Permanent middle cerebral artery occlusion**

Rats were food deprived for ~15 h prior to surgery to help keep glucose within a narrow range. After anesthetization, skull temperature ($T_s$) was measured with a thermocouple probe (model HYPO-33–1-T-G–60-SMC-M, Omega, Stanford, CT) and maintained near a target temperature of 37.2°C using a heating pad and overhead infrared lamp (175 W). Next, the tail artery was catheterized to measure mean arterial blood pressure (MABP) and to collect blood (100 μL) for determination of blood gases and glucose levels (blood gas analyzer model ABL 810, Radiometer, Copenhagen, Denmark). Finally, rats were subjected to pMCAO via electrocauterization of the distal MCA (Clark et al., 1996; DeBow and Colbourne, 2003). Finally, rats were immersed in the main part of Experiment 2 because the pilot study showed that $T_c$ does not change in rats subjected to this focal brain cooling protocol, which confirms earlier findings (Clark and Colbourne, 2007; Fingas et al., 2007, 2009).

**Hypothermia protocols**

**Systemic hypothermia**

Body temperature was regulated after pMCAO surgery with a servo-regulated system that used fans and fine water misters to cool and infrared lamps to heat (Colbourne et al., 1996; DeBow and Colbourne, 2003). All rats were maintained at normothermia for 1 h after stroke onset. At that time, rats that were randomized to receive hypothermia treatment began to be cooled at a clinically-feasible rate of 2°C/h to 33°C, which was maintained (often within 0.3°C) for 12 or 48 h prior to slow re-warming at a rate of 1°C/h. Temperature was then kept from falling below 36°C until 96 h after stroke onset. The NOR (FH) group had their temperature regulated such that they were not permitted to fall below 36°C for first 96 h after pMCAO onset.

**Focal brain hypothermia**

In Experiment 2, all rats were surgically implanted with a cooling strip immediately following pMCAO. This cooling implant was modified from our published method (Clark and Colbourne, 2007). Briefly, the temporalis muscle was retracted to allow for the placement of a 7-mm long × 3-mm wide hollow stainless steel strip against the skull. The strip was secured with dental cement and stainless steel screws. This was the end of surgery in the main experiment; however, additional procedures were completed in the pilot study. In these 6 rats, 3 burr holes were made in the dorsal surface of the skull. This was for placement of metal screws needed to secure a plastic cylinder that encased a calibrated, telemetry probe (model VM-FH-BP, Mini-Mitter Co. Inc, Sun River, OR). The shaft of this probe was inserted into the cortex to measure $T_b$.3 mm below the skull surface at 0.5 mm anterior and 3.5 mm lateral to bregma on the side of the stroke. This site was presumed to border the ischemic core. Following anesthesia, $T_b$ was measured every 30 s whereas $T_s$ was sampled once per day (noon) for 3 days. Simultaneous measurement of $T_b$ and $T_c$ with these telemetry probes is not possible owing to signal interference (Clark and Colbourne, 2007; DeBow and Colbourne, 2003).

Note that animals in this pilot study were only used for their temperature data because the use of a brain probe in some animals would have confounded the study (e.g., longer surgery time, needle tract injury from the telemetry probe). Furthermore, $T_b$ was not measured in all animals in order to avoid the additional injury caused by the brain probe, which might also be aggravated by stroke-induced cerebral edema.

**Behavioral testing**

**Neurological deficit scale**

In both experiments, a neurological deficit scale (NDS) score was measured prior to and at 7 days after stroke onset. However, in the first experiment only a subset of rats were evaluated (SH-12: N = 10; SH-48: N = 8; NOR (SH): N = 8). The score was determined from 5 behaviors that assess motor and sensory function including: hind limb retraction, contralateral forelimb flexion, bilateral forepaw grasp, ability to traverse a narrow beam and forelimb placing. The total score ranged from 0, indicating the absence of impairment, to 13, which denotes maximum impairment (Clark et al., 2008; MacLellan et al., 2006).

**Horizontal Ladder**

Rats were evaluated on a horizontal ladder task (Metz and Whishaw, 2002) prior to and at 7 and 28 days after stroke onset. This test is used to determine the rat’s ability to traverse a series of parallel bars variably spaced 1–3 cm apart. Error rate was determined as the percentage of slips (foot falls below the level of the bars) made while traversing the middle 0.5 m segment of the apparatus. This test is sensitive to pMCAO-induced injury (Clark et al., 2008).

**Montoya staircase reaching task**

Skilled reaching was evaluated with the Montoya staircase test (Montoya et al., 1991), which is sensitive to MCAO-induced cortical injury (Colbourne et al., 2000). Body weight was measured and rats were then food deprived to 90% of their free-feeding weight and trained in the staircase over 40 trials (2 trials/day, 5days/week) prior to pMCAO. Rats had to obtain at least 9 pellets (45 mg each; Bio-Serv, Frenchtown, NJ) per side out of a possible 21 by the last 5 consecutive days of training or they were excluded from the analysis. Rats were returned to ad lib feeding following training which ended 3 days before pMCAO. Skilled reaching, under food deprivation, was assessed over 10 trials on days 28 – 32 following pMCAO onset.

**Histology**

Rats were euthanized at 32 days after pMCAO onset by an intraperitoneal injection of sodium pentobarbital (~100 mg/kg) and transcardially perfused with 0.9% saline followed by 10% formalin. Brains were frozen and 50 μm coronal sections were obtained and later stained with cresyl violet. The Scion Image J program (Scion...
Corporation, Frederick, MD) was used to measure the area of normal tissue at 400-μm intervals extending through the entire brain. The volume of tissue lost was calculated as:

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\text{Volume of tissue lost} = \text{remaining volume of normal hemisphere} - \text{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.

Statistics

A p value of < 0.05 was considered statistically significant. The NDS scores were analyzed with Kruskal–Wallis tests, and, if significant, follow up comparisons were done with the Mann–Whitney U test. The Wilcoxon sign ranks test was used for comparisons across time for the NDS data (SPSS, v. 12). All other data were analyzed with the analysis of variance (ANOVA) with LSD post-hoc tests if needed. Mortality was evaluated with a Chi-square test.

Results

Experiment 1: systemic hypothermia treatment

Exclusions and protocol violations

Several premature deaths occurred after group assignment: 5 in the NOR (SH) group (21%), 3 in the SH-12 group (11%) and 5 in the SH-48 group (20%). The cause of death is not known, but perhaps due to extensive brain injury and cerebral edema. Mortality rates did not differ significantly among groups (p = 0.587). Five rats were excluded from staircase test analysis because they failed to reach criterion-level performance during baseline training (NOR (SH): N = 2; SH-12: N = 2; SH-48: N = 1). The rest of their data were used. A computer hard drive failure resulted in loss of temperature data leaving files for 40 rats. It should be noted that all rats were servo-regulated in a similar fashion and their data were frequently monitored during collection to ensure proper temperature control.

Physiological variables

Physiological measurements (pH, etc.) taken during surgery are presented in Table 1. There were no significant Group effects (p > 0.221) except for Tc (p = 0.024). However, differences between groups were ≤0.3 °C, which is expected to be of little biological importance. Indeed, a correlation between Tc and infarct size yielded r² = 0.104 (p = 0.010), which indicates that very little (10%) of the variability in infarct size can be accounted for by Tc. A repeated-measures ANOVA on body weight data (day of pMCAO surgery, and 7 and 32 days later; Table 1) revealed a significant Time effect (p < 0.001) with a non-significant interaction (p = 0.060) and Group main effect (p = 0.671). Thus, while cooled rats generally had lower body weights by day 7, it was not statistically significant. Core temperature (Fig. 2A) was regulated as desired.

Behavioral tests

The NDS scores were not significantly (p = 0.096) different among groups at baseline (data not shown) as scores indicated that there was no deficits. Post-stroke scores were significantly higher than baseline (p < 0.001) and there was a significant difference among groups at 7 days post-stroke onset (p = 0.019, Fig. 3A). Here the SH-48 group was significantly better than the NOR (SH) group (p = 0.009) whereas the other comparisons were non-significant (p > 0.064). Thus, only

![Fig. 2.](image-url)

(A) Body temperature, measured via telemetry, is shown for the 72 h after stroke onset in NOR (SH), SH-12 and SH-48 groups. (B) Brain temperature in NOR (FH) and FH-48 groups for 3 days after stroke onset (pilot study). Rates of cooling and re-warming were matched in both experiments.
the SH-48 group significantly lessened neurological impairment on the NDS.

A repeated-measures ANOVA on the stepping error rate with the contralateral-to-stroke forelimb in the ladder test (Fig. 4A) showed a significant Day effect ($p < 0.001$) with error rates that were significantly higher on both test days compared to baseline ($p < 0.001$). However, neither the Group main effect ($p = 0.236$) nor the interaction ($p = 0.116$) was significant. Furthermore, baseline scores were not different among groups ($p = 0.137$) and a repeated-measures ANOVA on only the post-stroke data showed a non-significant Group effect ($p = 0.168$) and a non-significant interaction ($p = 0.379$). The Day effect was significant ($p < 0.001$). Thus, stepping error rates significantly increased after stroke, but this was not significantly attenuated by systemic hypothermia treatment.

The staircase data (number of pellets consumed) were analyzed for the ipsi (Fig. 5A)- and contralateral-to-stroke forelimb (Fig. 5B). Initially a repeated-measures ANOVA with within-subjects contrasts was used to analyze all of the data, which included the baseline average, in order to determine whether scores after stroke onset were significantly lower than baseline performance. For both limbs, this was true over all test days ($p \leq 0.007$). As expected, baseline scores for each limb were not different among groups ($p \geq 0.292$). Post-stroke scores for each limb were then analyzed with repeated-measures ANOVAs, which revealed significant Day effects ($p < 0.001$) due to improved scores over test days, non-significant interactions ($p \geq 0.202$) and significant Group main effects ($p \leq 0.022$). Post-hoc tests showed that both cooled groups retrieved significantly more pellets than the NOR (SH) control group ($p \leq 0.027$). The SH-12 and SH-48 groups were not different ($p \geq 0.598$).

### Histology

Lesion volume (Fig. 6A) was significantly different among groups ($p = 0.006$). Furthermore, the NOR (SH) control group (Fig. 6C) had a significantly larger lesion than the SH-12 ($p = 0.005$) and SH-48 ($p = 0.004$; Fig. 6D) groups, which were not different ($p = 0.897$). Thus, both hypothermia treatments equally lessened tissue loss.

### Experiment 2: brain-selective hypothermia treatment

#### Pilot experiment

Brain temperature remained normothermic in control rats tethered to the cooling apparatus, whereas $T_b$ declined to between $\sim 31$ and $32 \, ^\circ C$ in the cooled rats (Fig. 2B). Re-warming was at the desired rate of $\sim 1 \, ^\circ C/h$, matching that found in Experiment 1. Core temperature averaged $37.1$ and $37.5 \, ^\circ C$ in the control and cooled groups, respectively.

#### Main study

### Exclusions and protocol violations

In Experiment 2, premature death after randomization occurred in the NOR (FH) ($N = 3$, 17% mortality), SH-12 ($N = 2$, 15%) and SH-48 ($N = 1$, 6%) groups, but this mortality was not significantly different among groups ($p = 0.353$). One SH-48 rat was excluded from the staircase analysis because it failed to obtain the criterion level of performance. The remainder of its data were used in the study.

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**Fig. 4.** Stepping error rate in the horizontal ladder test on the day of baseline assessment and at 7 and 28 days after pmCAO surgery in Experiments 1 (A) and 2 (B). Error rates significantly increased after stroke. Despite trends, the systemic hypothermia treatments did not significantly lessen error rate. The FH-48 treatment significantly improved outcome, whereas the trend towards benefit with FH-12 was not statistically significant.
**Fig. 5.** Mean number of pellets consumed in the staircase test with the ipsilateral (A and C) and contralateral-to-stroke (B and D) forelimbs for Experiments 1 (A and B) and 2 (C and D). The SH-12, SH-48 and FH-48 treatments significantly improved skilled reaching success. The FH-12 treatment did not (BL = baseline average of the last 10 training trials).

**Fig. 6.** Average lesion volume at 32 days after pMCAO surgery in Experiments 1 (A) and 2 (B). The SH-12 and SH-48 treatments significantly and equally reduced tissue loss whereas only the FH-48 treatment significantly reduced tissue loss. The typical extent of injury after untreated stroke in Experiment 1 is illustrated in C whereas D illustrates the typical animal protected by the SH-48 treatment. The black region denotes necrotic tissue and lesion cavity.
Physiological variables

Physiological measurements taken during pMCAO surgery are presented in Table 1. There were no Group effects ($p ≥ 0.059$) except for $T_1$ ($p = 0.048$). However, differences between groups were $<0.4^\circ$C, which is expected to be of little biological importance. Furthermore, $T_2$ over this small range was not significantly related to infarct size ($r^2 = 0.008$, $p = 0.582$). Body weight data was analyzed with a repeated-measures ANOVA. It showed a significant Time effect ($p < 0.001$), but the interaction ($p = 0.848$) and Group main effect ($p = 0.470$) were non-significant.

Behavioral tests

The NDS scores were not significantly different among groups at baseline (data not shown) as scores indicated that there were no impairments. Scores on day 7 after stroke onset were significantly higher than baseline ($p < 0.001$) and significantly different among groups ($p = 0.033$, Fig. 3B). Specifically, the FH-48 group was significantly better than the NOR (FH) group ($p = 0.009$) whereas the other comparisons were non-significant ($p ≥ 0.148$). Thus, only the FH-48 group significantly lessened neurological impairment on the NDS.

A repeated-measures ANOVA on the stepping error rate with the contralateral-to-stroke forelimb in the ladder test (Fig. 4B) showed a significant Day effect ($p < 0.001$) with error rates being significantly higher on both test days compared to baseline ($p < 0.001$). The Group main effect ($p = 0.002$) and the interaction ($p = 0.010$) were significant. Baseline scores were not different among groups ($p = 0.739$). A repeated-measures ANOVA on the post-stroke data also showed a significant Group effect ($p = 0.002$) and a non-significant interaction ($p = 0.548$) with the Day effect being significant ($p < 0.001$). Here the FH-48 treatment significantly reduced stepping error rate ($p < 0.001$), while the FH-12 treatment did not ($p = 0.081$).

The staircase data (number of pellets consumed) were analyzed for the ipsi (Fig. 5C)- and contralateral-to-stroke forelimb (Fig. 5D). Initially, a repeated-measures ANOVA using within-subjects contrasts was used to analyze all of the data for each forelimb separately, which included the baseline average. These ANOVAs determined whether scores after stroke were significantly lower than baseline performance, which was true over all test days and for both limbs ($p < 0.001$). As expected, ANOVAs on baseline scores were non-significant for each limb ($p > 0.108$). Post-stroke scores for each limb were then analyzed with repeated-measures ANOVAs, which revealed significant Day effects ($p < 0.001$), due to improved scores over test days, along with significant Group main effects ($p = 0.002$) and non-significant interactions ($p ≥ 0.203$). Furthermore, the FH-48 group retrieved significantly more pellets with each limb than the NOR (FH) and FH-12 groups ($p ≤ 0.007$), which were not significantly different ($p ≥ 0.269$). Thus, only the FH-48 treatment significantly improved skilled reaching ability.

Histology

Lesion volume (Fig. 6B) was significantly different among groups ($p = 0.031$). Specifically, the FH-48 group had a smaller lesion than the NOR (FH) group ($p = 0.014$) and the FH-12 ($p = 0.042$) group. The latter groups were not different ($p = 0.787$). Thus, only the FH-48 treatment significantly lessened tissue loss.

Discussion

In this study, we used a model of severe pMCAO in which to test 12- and 48-h durations of therapeutic hypothermia initiated slowly at 1 h after onset of stroke. We used a permanent stroke model in order to increase the translational potential of this research because, in humans, many occlusions are permanent (Kassem-Moussa and Graffagnino, 2002; Rha and Saver, 2007), and rtPA is still administered to only a small minority of patients (Allen et al., 2009). The 12- and 48-h durations of systemic hypothermia were both persistently neuroprotective (32-day survival). In contrast, the 48-h period of focal hypothermia was neuroprotective whereas the 12-h duration was not. Reductions in lesion volume were associated with improvement in functional recovery seen in protected groups. Mortality rates, however, were not significantly different among groups in either experiment. Regardless, this study shows that delayed cooling can provide enduring behavioral and histological protection after pMCAO. While hypothermic neuroprotection is duration dependent, our findings suggest that this relationship may vary with the method of cooling.

Although our data do not explain this discrepancy between focal and systemic cooling, there are several possible explanations. First, while we matched the onset delay, the rates of cooling and rewarming and the durations of systemic and focal cooling, it was impossible to perfectly match the depth of hypothermia. Systemic cooling is expected to cause a more uniform pattern of brain hypothermia than focal cooling, which is known to induce temperature gradients (Clark and Colbourne, 2007). Thus, salvageable tissue within the penumbra, especially that located further away from the cooling strip, would not have been as cold as tissue residing directly underneath the cooling device. Accordingly, the longer focal cooling treatment may have been needed because a milder level of hypothermia was induced in some of the penumbral tissue. In contrast, the penumbra may have been cooler during systemic hypothermia treatment, thereby providing sufficient protection when applied for just 12 h. However, some reports do not support this hypothesis as they found that milder cooling can be more efficacious (Hu et al., 2000; Kollmar et al., 2007). Unfortunately, it is not possible to spatially map out temperature gradients with available telemetry probes, which would require recording temperature simultaneously from multiple sites in the core and penumbra. A second possible explanation is that one or more of the systemic side effects of whole-body cooling may be neuroprotective. For instance, inducing systemic hypothermia in conscious rodents can modestly and transiently elevate blood pressure (MacLellan et al., 2004), which might improve collateral blood flow and limit injury (Shin et al., 2008). Conversely, our focal cooling technique does not appear to appreciably affect blood pressure (Clark and Colbourne, 2007). Other physiological effects of systemic cooling that might be important include changes in corticosterone levels, glucose levels, blood oxygenation, etc. Finally, it appears that the second experiment produced a 20% greater lesion volume than the first experiment for unknown reasons (e.g., physiological variables were similar). Insult severity is known to require a longer hypothermia treatment to be efficacious (Colbourne and Corbett, 1994; MacLellan et al., 2008), but the small difference in lesion volume between these studies likely does not fully account for the results. Further study comparing cooling methods is needed, and it should not be assumed that focal cooling will provide equal benefit to systemic hypothermic protocols, even when brain temperature is matched.

Our present findings with systemic cooling do not perfectly mirror our earlier work in the pMCAO model (Clark et al., 2008). In that study, we observed significantly better histological protection at a 7-day survival with 48 h of cooling than with a 12-h treatment. Importantly, we used the same insult done by the same surgeon, and we used the same cooling protocols. Furthermore, the lesion volume in normothermic rats was quite similar between studies, arguing against insult severity as a factor. While we did use different survival times, if anything, one would expect greater histological protection at the earlier survival time (Clark et al., 2008) that then disappeared by the longer survival time used in the present study. This was not the case. If there had been significantly less protection in that SH-12 group, then the data would fit with our previous study and with the findings in Experiment 2 of this study.

Numerous reviews and the STAIR reports have noted the importance of behavioral assessment in pre-clinical, stroke...
neuroprotection studies. Thus, we evaluated recovery with three tests: the NDS, the horizontal ladder and the staircase test. As previously seen, each test was sensitive to the brain injury caused by pMCAO, and overall these behavioral data mirrored the histology. Indeed, our primary functional endpoint, a priori, was the staircase test owing to its sensitivity to lesion size (Colbourne et al., 2000; MacLellan et al., 2006). Indeed, the present results with this test reflected the infarct size data. The data in Experiment 1 were more variable than that in the second experiment for unknown reasons and we may not have had the statistical power to show an effect on the ladder test, despite large Ns. Regardless, it is not uncommon to find partial functional benefit and our overall findings support the use of these hypothermic protocols.

The heterogeneity of stroke (e.g., location and duration of occlusion) in humans along with numerous other variables (e.g., age, gender, and treatment delay) and species differences makes it impossible to perfectly define an optimal treatment protocol from animal studies. Indeed, it is unlikely that an ideal treatment protocol could ever be identified and applied to all strokes. Animal studies, such as the present one, can only suggest a reasonable protocol that should be effective in humans. For instance, initial findings in the gerbil model of global ischemia (Colbourne and Corbett, 1994, 1995) correctly identified cooling protocols that were later shown to improve outcome after cardiac arrest (Bernard et al., 2002; The Hypothermia After Cardiac Arrest Study Group, 2002). Thus, the present findings, along with other studies, strongly suggest that prolonged cooling is needed to effectively improve outcome after focal ischemia, especially after an intervention delay. Importantly, the current findings also demonstrate long-term functional and histological benefit after a permanent focal ischemic insult, similar to that previously demonstrated in transient focal ischemia (Colbourne et al., 2000; Corbett et al., 2000; Yamamoto et al., 2001). Thus, we expect that mild hypothermia will improve outcome in many ischemic stroke patients, if cooling is instituted early and for long enough.

While the use of more prolonged treatments appears to be more efficacious overall, extended cooling increases the risk of systemic complications as well as the possibility that innate repair mechanisms (neuroplasticity) will be retarded. The latter effect was observed with the delayed use of a matrix metalloproteinase inhibitor after focal ischemia (Zhao et al., 2006). Our findings with hypothermia show that this effect either did not happen or was insufficient to negatively affect behavioral recovery, at least for the 12- and 48-h periods of mild cooling used. For instance, 12 and 48 h of systemic cooling provided for an equivalent reduction in infarct size, yet the additional cooling in the 48-h group did not hamper recovery on any of the tests used. Had cooling impeded stroke-induced repair mechanisms (e.g., synaptogenesis), then the SH-48 group should have had a poorer behavioral recovery than the SH-12 group. This did not occur. Similar findings occurred in our previous pMCAO study that assessed outcome to 7 days (Clark et al., 2008) and in our intracerebral hemorrhage studies where we have cooled for up to 6 days (Fingas et al., 2009). Nonetheless, cooling for much longer or to deeper levels may be harmful to brain recovery. Extended cooling has been used to treat persistently elevated intracranial pressure (Polderman, 2008), or it might occur unintentionally (Ford and Reardon, 2006; Merchant et al., 2006).

As noted, prolonged cooling has been repeatedly used after brain injury to treat cerebral edema and raised intracranial pressure in an effort to lessen morbidity and mortality (MacLellan et al., 2009; Polderman, 2008). In this study, mortality rates did not significantly differ among groups. While this allows us to clearly interpret the behavioral and histological effects of hypothermia without a mortality rate confound, it also suggests that cooling will not lessen post-stroke mortality. This latter conclusion, however, is premature. First, these experiments were not sufficiently powered to confidently accept the null hypothesis (no effect on mortality) because mortality rates were relatively low in this model (~20% in untreated rats). Second, we did not identify the cause of premature death or its relationship to hypothermia treatment and its side effects. Third, a cardiectomy model of pMCAO is not well suited, on its own, to test the effects of cooling on mortality because the cardiectomy itself can be beneficial by lowering intracranial pressure (Walberer et al., 2008). Given the clinical interest in craniotomy, further work is needed to evaluate the impact of hypothermia with and without craniotomy. In this setting, it seems especially important to also evaluate the effects of re-warming rate as elevated intracranial pressure may reoccur upon warming from systemic and perhaps focal brain cooling. We warmed slowly at 1 °C/h in both experiments, but it is possible that even slower rates would have resulted in greater benefit.

In summary, prolonged mild hypothermia, induced systemically or with a brain-selective cooling method, reduced infarct size and behavioral impairment after pMCAO out to a 1-month survival. These findings build upon earlier studies using systemic and focal hypothermia protocols. Despite significant protection, however, considerable tissue loss still occurred, which is not surprising given that we modeled severe, permanent, focal ischemia. Accordingly, further improvements to hypothermia therapy and the use of combination treatments, including tPA (Tang et al., 2009), are urgently needed. Our findings also suggest that efficacy may depend somewhat upon the method of inducing hypothermia. Thus, further animal and clinical study is needed to develop alternative cooling methods, such as brain-selective and drug-induced hypothermia protocols (e.g., H2S; Florian et al., 2008) and to ensure that such methods are persistently effective in reducing brain injury.

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