Temperature-Regulated Model of Focal Ischemia in the Mouse
A Study With Histopathological and Behavioral Outcomes

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Background and Purpose—The importance of mouse stroke models has increased with the development of genetically manipulated animals. We hypothesized that immediate postischemia hypothermia may attenuate ischemic brain injury in the mouse.

Methods—Intraabdominal radio frequency probes were implanted in animals and core temperature monitored. Groups included: MCAO-45-REG (45 minutes middle cerebral artery occlusion [MCAO]) temperature-controlled in the postischemic period /H11022/34°C for 24 hours; MCAO-45 (45 minutes MCAO) were allowed to self-regulate core temperature during recovery; MCAO-30-REG (30 minutes MCAO), with the same temperature protocol as MCAO-45-REG; MCAO-30 (30 minutes MCAO), with temperature protocol the same as MCAO-45. Behavior and histological score was assessed at 7 days. The qualitative histological score assessed for injury in 18 specified regions.

Results—MCAO-45-REG core temperature (mean 34.94°C /H11006/0.8°C) was significantly different than the self-regulating (MCAO-45, mean 33.1°C /H11006/2.3°C) for the first 4 hours after anesthesia (\textit{P} \leq 0.01). There was a trend toward similar differences in temperatures for MCAO-30-REG and MCAO-30 (\textit{P} = 0.08). At 7 days, a greater improvement in behavior score was observed for MCAO-45 and MCAO-30 compared with MCAO-45-REG and MCAO-30-REG (\textit{P}<0.001). The histological score confirmed reduced injury in unregulated temperature groups (MCAO-45-REG mean 38\pm10 and MCAO-45 30\pm5.1, \textit{P}<0.05; MCAO-30-REG 41\pm10 and MCAO-30 30\pm9, \textit{P}<0.05).

Conclusions—Hypothermia is an important confounder of stroke injury in the intraluminal filament mouse model. Future mouse stroke studies must use strict temperature regulation. (\textit{Stroke}. 2004;35:1720-1725.)

Key Words: ischemic attack, transient ■ hypothermia ■ behavior

Despite extensive animal research on the pathophysiology of stroke, very little has been translated into clinically effective therapies. Among the many clinical trials, only a few have been “positive.” There is growing evidence that revascularization therapies are efficacious clinically, reducing neurological disability,\textsuperscript{1} illustrating that these approaches can translate from the laboratory to the bedside. In contrast, cytoprotectants effective in rodents have failed in human clinical trials.\textsuperscript{2} The most commonly cited failings of preclinical studies include poor physiological monitoring, short outcomes, and an absence of behavioral endpoints.\textsuperscript{3,4}

To date, hypothermia is the only stroke treatment that has translated to humans.\textsuperscript{5} Hypothermia used after cardiac arrest improved functional and cognitive recovery. The effect of mild hypothermia in reducing injury after global ischemia, and infarct volume in transient focal ischemia is well-established in the laboratory.\textsuperscript{6,7}

Mouse stroke models are being used increasingly to research complex molecular processes after ischemia. Exciting developments concerning neuroprotective strategies have involved the genetic manipulation of endogenous enzymes that may play a role in the progression of ischemic injury. However, little is known about temperature regulation in mouse strains after focal ischemia. This may be very important because hypothermia or hyperthermia affects ischemic brain injury after middle cerebral artery occlusion (MCAO) in animals.\textsuperscript{8} There are many studies using transgenic or gene-depleted mouse species but none has controlled temperature in the postischemic period.\textsuperscript{9–15} Another limitation of these studies was a failure to assess long-term and functional outcome because substantial cell death may progress over time and histology does not always predict functional outcome.\textsuperscript{16}

Preliminary observations suggested that transient hypothermia occurred postischemia in the transient MCAO in the mouse, and we hypothesized that this postischemia hypothermia profoundly mediates ischemic brain injury. In this study,
we assessed the effect of controlling core temperature during and after focal ischemia in the mouse on both behavior and histological injury at 7 days. We describe the use of telemetry to regulate core temperature in the posts ischemic mouse.

Materials and Methods

Animals
All experiments and procedures were approved by the local animal care committee and were in line with the Canadian Council of Animal Care guidelines. Male C57 Black 6 mice (3 months old, 25 to 35g; Charles River Breeding Laboratories, Ontario, Canada) were prepared for transient MCAO using the intraluminal filament method.17 A total of 54 animals were used: 48 in 4 experimental groups and 6 to determine blood pressure and arterial blood gases. The 4 groups included: MCAO-45-REG (45 minutes MCAO, N = 14) temperature-controlled throughout surgery for 2 hours at 36.5°C, and subsequently regulated at >34°C for 24 hours; MCAO-45 (45 minutes MCAO, N = 14) temperature-controlled during surgery as in MCAO-45-REG, after which animals self-regulated core temperature; MCAO-30-REG (30 minutes MCAO, N = 10), with core temperature regulation as in MCAO-45-REG; MCAO-30 (30 minutes MCAO, N = 10), with temperature protocol the same as MCAO-45. The threshold temperature of 34°C is consistent with the normal minimum daytime diurnal temperature. Animal weight was recorded before surgery and daily thereafter. Core temperature and blood pressure were monitored as described.

Transient Focal Ischemia
Anesthesia was induced with isoflurane (3% initial, 1% to 1.5% maintenance) in O2, and air (80%:20%). Briefly, under the operating microscope, the left common carotid artery (CCA), the left external carotid artery (ECA), and the left internal carotid artery (ICA) were isolated and a 6-0 suture was tied at the origin of the ECA and at the distal end of the ECA. The left CCA and ICA were temporarily occluded. The silicon-coated nylon suture was introduced into the ECA and pushed up the ICA until resistance was felt and the filament was inserted ~9 to 10 mm from the carotid bifurcation, effectively blocking the middle cerebral artery (MCA). The diameter of the tip of coated suture was considered acceptable between 180 and 220 micrometers.18 The suture remained inserted for 30 or 45 minutes, after which it was removed and the ECA was permanently tied. Subcutaneous normal saline (0.9%) was administered daily, adjusting the volume according to the animal’s weight loss. We estimated that the daily requirement for water was 2 mL, and if the weight decreased by 1 gram over 24 hours an additional 1 mL of 0.9% saline was administered. This was continued until the animal’s weight increased to within 5% of baseline.

Measurement of Cerebral Blood Flow
Transcranial measurements of cerebral blood flow (CBF) were made by laser-Doppler flowmetry (LDF). A 0.5-mm diameter microfiber laser-Doppler probe (Probe 418; Perimed) was attached to the skull with cyanoacrylate glue 6 mm lateral and 1 mm posterior of bregma. While under general anesthesia, regional cerebral blood flow (rCBF) was monitored within the infarct core and in the parietal cortex (penumbra). The surgical procedure was considered adequate if ≥70% reduction in rCBF occurred immediately after placement of the intraluminal occluding suture; otherwise, mice were excluded. Blood flow velocity was measured pres ischemia, during MCAO and reperfusion, using data collection software (Perisoft Version 1.3; Perimed Inc.). Data were expressed as a mean percentage of the baseline preischemia value.

Temperature Regulation
Mice were implanted with intraabdominal radiofrequency probes (TA10TA-F20; Transoma Medical) 7 days before MCA occlusion. Core temperature was sampled every 30 seconds using receivers (RLA-1020; Data Sciences Int) interfaced to a computer running ART 2.2. This telemetry system minimizes stress and allows temperature monitoring/control in the freely moving animal.4 Core temperature and activity (counts per 30 seconds) was initially monitored for 7 days (N = 15). The control (LH) was defined as the period of the treatment condition (L.H.). The score used was as follows: 0, normal; 1, consistent forward gait and no weight loss; 2, consistent lateral to lesion side when lifted by the tail; 2, observations of score 1 with consistently reduced resistance to lateral push and gait toward the paretic side; 3, observations of score 2 plus large or weak circling toward the paretic side; 4, score 2 plus small circling toward the paretic side; and 5, score 2 plus tight circling toward the paretic side.

Histology
Animals were euthanized with sodium pentobarbital (70 mg/kg intra-peritoneally) and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde. The brain was then embedded in paraffin; sectioned at 6 μm thickness, and stained with hematoxylin and eosin. In addition, consecutive sections were assessed for degree and extent of cellular injury and pan-necrosis in a total of 18 regions through the striatum (2 sections) and hippocampus (2 sections). The distance between sections was ~1 mm. The specific regions include cortical areas C1-C6; medial striatum (SM) and lateral striatum (SL); and hippocampus (H). In each region, degree of neuronal injury was assessed according to a scale of 0 to 4: 0, normal; 1 denotes ≤10% selective neuronal injury; 2 denotes 10% to 50% neuronal injury; 3 denotes >50% neuronal injury; and 4 indicates confluent areas of pan-necrosis. The minimum total score was 0, indicating a normal brain; a maximum score of 72 indicates pan-necrotic tissue in all 18 areas (Figure 1). The grading system accounts for the degree of injury at each site and the extent of the damage (ie, number of sites affected). The histology was analyzed blinded to animal identity and the interobserver reliability of the score was assessed between each interpreter (P.A.B., L.H.). The histological assessment was also performed for the most anterior striatal slice (bregma 1.54 mm) and a more posterior hippocampal slice (bregma −3.52 mm), therefore including a total of 6 slices. The cerebral infarct volume was calculated by measuring the ipsilateral and contralateral hemispheric volume and subtracting the difference (mm³), assuming that the contralateral hemisphere was unaffected by ischemia. This method was used because at 7 days, the infarct could not be clearly defined.

Statistics
For all experiments with parametric data, a paired t test was used for comparison of continuous data. Mann-Whitney U test was used to compare the means of ordinal data. One-way ANOVA was used for the interobserver correlation coefficient between observers (P.A.B., L.H.). Comparisons of proportions were assessed using 2 × 2 tables and exact methods. Spearman correlation coefficients were used to determine the relationship of animal activity and temperature and of histological injury and behavior. Data are presented as mean ± SD, with a value of P < 0.05 considered to be significant statistically.
Results

The mean weight of animals was 30.6 ± 2.3 grams (MCAO-45-REG: 30.4 ± 2.6; MCAO-45: 30.6 ± 2.6; MCAO-30-REG: 30.2 ± 2.0; MCAO-30: 31.3 ± 1.9; P > 0.5). Cortical blood flow measured using LDF decreased after MCA occlusion by at least 70% in all animals. After insertion of the intraluminal filament, the mean rCBF decreased to 11.5 ± 4.9% of the preocclusion value. There was no difference statistically in the intraischemic relative rCBF between MCAO-45-REG (mean 10.1 ± 4.9) and MCAO-45 (mean 13.9 ± 5.6, P = 0.07), or between MCAO-30-REG (mean 11.4 ± 6.1) and MCAO-30 (mean 10.21 ± 5.1, P > 0.5). Immediately during reperfusion, the LDF reading returned to 88.9% ± 26.7% of the baseline preocclusion value. Similarly, there was no significant difference in the LDF recordings during reperfusion between MCAO-45-REG (mean 81.2 ± 27.3) and MCAO-45 (mean 100.5 ± 32.8, P > 0.1), or MCAO-30-REG (mean 91.6 ± 19.7) and MCAO-30 (mean 80.3 ± 17.1, P > 0.2). LDF measurements were recorded for 60 minutes after reperfusion (Figure 2) and confirmed a restoration of CBF to within 90% of preocclusion values, which decreases dramatically, revealing a relative hypoperfusion (mean values 30%). The blood pressure recordings and blood gas analysis are in the Table.

Temperature was monitored for 7 days in free-moving animals housed in a day/night light cycle (N = 15). The mean temperature during this period was 36.23 °C ± 0.5 °C (range 33 °C to 38 °C). The general activity (counts per 30 seconds, averaged hourly and including 168 data points over 7 days) correlated very highly with the temperature variation (r = 0.72, P < 0.001) shown in Figure 3.

In Figure 4 the temperature recordings are shown for the self-regulating animals (MCAO-45 and MCAO-30) and those controlled at 34 °C (MCAO-45-REG and MCAO-30-REG). MCAO-45-REG core temperature (mean 34.94 °C ± 0.8 °C) was significantly higher than the unregulated temperature (mean 34.39 °C ± 0.8 °C). Additionally, the MCAO-45-REG animals maintained a more constant temperature during the experiment.

Blood Pressure and Arterial Blood Gas Measurements During 30 and 45 Minutes MCAO

<table>
<thead>
<tr>
<th></th>
<th>45-Min MCAO</th>
<th>30-Min MCAO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP (mm Hg)</td>
<td>101 (7.9)</td>
<td>106 (7.6)</td>
<td>0.42</td>
</tr>
<tr>
<td>BP postreperfusion (mm Hg)</td>
<td>102 (14.1)</td>
<td>99.7 (4.5)</td>
<td>0.75</td>
</tr>
<tr>
<td>pH (mm Hg)</td>
<td>7.32 (0.1)</td>
<td>7.31 (0.1)</td>
<td>0.70</td>
</tr>
<tr>
<td>PCO2 (mm Hg)</td>
<td>45.2 (10.2)</td>
<td>43.6 (8.3)</td>
<td>0.83</td>
</tr>
<tr>
<td>PO2 (mm Hg)</td>
<td>93.3 (17.7)</td>
<td>107.3 (2.5)</td>
<td>0.25</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.9 (0.2)</td>
<td>15.1 (0.4)</td>
<td>0.48</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>8.6 (1.7)</td>
<td>8.6 (1.5)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; Min, minute.
groups (MCAO-45, mean 33.1°C±2.3°C) for the first 4 hours after the end of surgical temperature regulation ($P<0.05$). A different mean core temperature was observed between MCAO-30-REG (mean 35.4°C±0.9°C) and MCAO-30 (mean 34.6°C±1.0°C), which was not significant ($P=0.088$). Figure 4 also presents the relative animal activity (counts per minute), and the reduced initial activity corresponds to reduced core temperature in groups MCAO-45 and MCAO-30.

Behavioral deficits were recorded 4 hours after anesthesia and at 7 days. In MCAO-45-REG and MCAO-45, the behavior score was equivalent at 4 hours, but at 7 days an improvement in behavior score was observed, which was greater in the unregulated groups (Figure 5A). Similar results are shown for MCAO-30-REG and MCAO-30. There were 4 animal deaths in MCAO-30-REG and MCAO-30. There were 4 animal deaths in MCAO-45-REG (compared with MCAO-30-REG, $P<0.05$) and 3 for MCAO-45 (compared with MCAO-30, $P=0.06$).

Histological sections were assessed for neuronal injury and frank necrosis in the specified regions. The mean of the scores (34.6±9.6, range 18 to 57) correlated with behavioral outcome ($r=0.46$, $P=0.003$). The histological score and infarct volume measurements confirmed reduced injury in unregulated temperature groups (Figure 5B). The use of the histological score of 6 slices (including bregma 1.54 and −3.52) produced a similar result ($P<0.01$). All animals had hypothalamic damage. The intraclass correlation coefficient between histological raters was 0.88 (95% CI, 0.81 to 0.95) and indicates excellent agreement.

**Discussion**

Mild hypothermia occurs after MCAO in the mouse, and this hypothermia improves behavioral and histological outcome. The fact that hypothermia confounds experimental results is not new because hypothermia has confounded previous pharmacotherapeutic studies. For example, $N$-methyl-$D$-aspartate antagonists (eg, MK-801) provide little or no protection when temperature is maintained. Furthermore, the cytoprotection afforded by NBQX, an AMPA antagonist, is caused by protracted hypothermia.

Insight into why mice become mildly hypothermic after focal ischemia can be obtained by studying their temperature patterns and the relationship to animal activity. The baseline diurnal temperature strongly correlates to the animal’s activity. As their daytime motor activity decreases, there is a decrease in the core temperature. The converse is true at night when motor activity and temperature increases. The daytime/nighttime core temperature differential is up to 4°C, a phenomenon not seen in gerbils or rats ($\approx$1°C). The postischemic mouse is inactive for several hours while thermoregulation is poorly maintained and heat is lost through their relatively large surface area. In addition, we found hypothalamic injury. This is probably caused by transient occlusion of hypothalamic arterial perforators originating off the internal carotid artery by the intraluminal filament.
was considered to partly explain variability in infarct volume. It is also worth mentioning that postischemic hypothermia has also been documented in global cerebral ischemia. One additional alternative explanation is that the rCBF decreases because of reduced metabolic demand by the injured brain tissue.

This study has some limitations. Long-term histological and functional outcomes were not assessed. Most cell death evolves rapidly within days if the insult is moderate to severe, but substantial cell death may occur beyond this time frame if the ischemia is mild or when a cytoprotectant is used. In addition, we used a neurological deficit score, which was initially developed in rats and modified for mice. These types of neurological deficit score are not standardized and vary among laboratories.

In summary, hypothermia confounds mouse studies of stroke and can easily be controlled for with the use of telemetry temperature probes. The study demonstrates that 7-day survival can be achieved with consistent and reproducible histological injury.

References


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