Optimal Depth and Duration of Mild Hypothermia in a Focal Model of Transient Cerebral Ischemia
Effects on Neurologic Outcome, Infarct Size, Apoptosis, and Inflammation

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Background and Purpose—Mild hypothermia is possibly the single most effective method of cerebroprotection developed to date. However, many questions regarding mild hypothermia remain to be addressed before its potential implementation in the treatment of human stroke. Here we report the results of 2 studies designed to determine the optimal depth and duration of mild hypothermia in focal stroke and its effects on infarct size, neurological outcome, programmed cell death, and inflammation.

Methods—Rats underwent a 2-hour occlusion of the left middle cerebral artery. In the first study (I) animals were kept (intraischemically) at either 37°C (n = 8), 33°C (n = 8), or 30°C (n = 8). Study II consisted of 4 groups: (1) controls (37°C, n = 10), (2) 30 minutes of hypothermia started at ischemic onset (33°C, n = 9), (3) 1 hour (33°C, n = 8), and (4) 2 hours (33°C, n = 8). Brain temperature was measured by a thermocouple probe placed in the contralateral cortex. After suture removal, all animals were rewarmed and reperfused for 22 hours (I) or 70 hours (II).

Results—Mild hypothermia to 33°C or 30°C was neuroprotective (17 ± 7% and 27 ± 6%, respectively) relative to controls (53 ± 8%, P < 0.02), but 33°C was better tolerated and recovery from anesthesia was faster. The neurological score of hypothermic animals was significantly better than that of controls (I & II) at both 24 and 72 hours postischemia except for the 30-minute group (II), which showed no improvement. In Study II, 2 hours of hypothermia reduced injury by 59%, 1 hour reduced injury by 84% whereas 30 minutes did not reduce injury. Normalized for infarct size, 2 hours of mild hypothermia decreased neutrophil accumulation by 57% whereas both 1 hour and 30 minutes had no effect. At 72 hours, 1 and 2 hours of mild hypothermia decreased transferase dUTP nick-end labeling (TUNEL) staining by 78% and 99%, respectively, and 30 minutes of hypothermia had no effect.

Conclusions—Intraischemic mild hypothermia must be maintained for 1 to 2 hours to obtain optimal neuroprotection against ischemic cell death due to necrosis and apoptosis. (Stroke. 1998;29:2171-2180.)

Key Words: hypothermia ■ ischemia ■ neuroprotection ■ apoptosis ■ inflammation

Stroke is a major source of disability and thus much research emphasis is being placed on the treatment and prevention of stroke. Neuroprotective strategies include the use of glutamate receptor antagonists, calcium channel blockers, and free-radical scavengers.Thrombolytic agents have already been shown to improve outcome by accelerating recanalization. In recent years, there has also been a resurgence of interest in mild hypothermia as a method of cerebral protection.

Small decreases in brain temperature (2°C to 6°C) are well tolerated and have been shown to confer a significant degree of neuroprotection in some animal models of cerebral ischemia.1-5 Although research in this area has been conducted for more than 30 years, many questions regarding mild hypothermia remain unanswered, including the degree of temperature reduction needed, when it should be instituted, and for how long a period it should be maintained. The mechanism of cerebral protection by mild brain hypothermia is also unclear and still a source of controversy. Its neuroprotective effects have been ascribed to a decrease in cerebral metabolic rate, restoration of cerebral blood flow, and preservation of the blood-brain barrier. Other potential protective mechanisms include alterations in neurotransmitter release, activity of protein kinases, and resynthesis of cellular repair proteins.6 More recently, there have also been reports that hypothermia may lead to specific inhibition of apoptosis7 and the attenuation of the inflammatory response that often follows an ischemic insult.8

Mild or moderate hypothermia has been shown to reduce neurological deficits if started before, during, or after cerebral ischemia, but few studies have examined functional outcome in detail after experimental cerebral ischemia with hypothermia.9-13 Furthermore, much of the work with mild hypothermia has centered around global models of cerebral ischemia.
and less is known about focal ischemia. Here we report the results of two studies designed to determine the optimal depth and duration of mild hypothermia in a model of middle cerebral artery occlusion (MCAO) with special attention directed to the animal’s intraoperative systemic physiological parameters, postanesthesia recovery time, and behavioral assessment over a 24- to 72-hour period. We also examined the effects of mild hypothermia on infarct size, apoptosis using TUNEL staining plus morphologic criteria and analysis of DNA fragmentation, and inflammation by myeloperoxidase (MPO) staining of neutrophils.

Materials and Methods

The following animal protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care. The following animal protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care. The following animal protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care.

Stroke Model

Male Sprague Dawley rats weighing 290 to 350 g (Charles Rivers, Wilmington, Del) were anesthetized with 3% halothane (delivered by mask) and were maintained in surgical plane of anesthesia with 1% halothane in 200 mL/min oxygen and 800 mL/min air without the use of paralytic agents. Depth of anesthesia was assessed every 15 minutes by hind limb pinch. A thermistor probe was inserted 50 mm into the rectum and rectal temperature was maintained at 36.5°C to 37.5°C before ischemia. ECG leads were placed to monitor heart rate and respirations. The animal’s head was immobilized in a stereotactic frame and a small scalp incision was made. A small burr hole was drilled to permit insertion of a 33-gauge thermocouple temperature probe to measure cortical brain temperature in the nonischemic hemisphere. The probe was inserted into the cortex approximately 3 mm deep. The probe site was sealed with dental cement, and the animal was placed in supine position. The right femoral artery was catheterized with PE-50 tubing for monitoring blood pressure, collecting blood samples, and infusion of normal saline and sodium bicarbonate (8.4%), as necessary, to correct arterial base deficit. Blood glucose and hematocrit values were measured before and during the ischemic period. Blood gases were measured (alpha-stat method) with an automatic pH/BlOD Gas Analyzer Model 178 (Ciba Corning Diagnostics Corp, Medfield, Mass). A midline incision was made in the neck to expose the common carotid (CCA), external carotid (ECA), internal carotid (ICA), and pterygopalatine (PPA) arteries. The CCA, ECA, and PPA were ligated with a 6-0 silk suture. The stroke was produced by inserting a 3-0 monofilament suture (with a flamed tip) 18 to 23 mm from the bifurcation of the ICA and ECA, thus occluding the ostium of the middle cerebral artery (MCA). The suture was kept in place for 2 hours. In the hypothermic groups, total body cooling was achieved by spraying alcohol onto the animal and cooling it to the desired temperature with a fan. This method decreased brain temperature from 37°C to 30°C within 10 minutes. In the first study, the brain thermocouple probe was removed just before anasthesia was discontinued until the animal regained its righting reflex, was recorded. The animal was then transported to the intensive care unit (ICU) at the Veterinary Services Center at Stanford University, Stanford, Calif, where it was closely monitored throughout the recovery period and evaluated for neurological findings. Rectal temperature was monitored hourly for the first 3 hours after recovery from anesthesia. Fluids (1 to 2 mL normal saline per 100 grams of body weight) were given as needed, and the analgesic butorphanol tartrate (0.05 to 2.0 mg/kg) was administered if the animal was perceived to be in pain or in undue distress. The animal was allowed free access to food and water after surgery.

Behavioral Analysis

The animals were monitored continuously and assessed for neurological findings at the end of the experiment by an individual blinded to the experimental groups. A neurological grading scale was used (Table 1). The animal’s weight was recorded at 24, 48, and 72 hours posts ischemia, and mortality was also used as an endpoint.

Infarct Analysis

Only animals surviving the entire study were used for histologic and histochemical analysis (24-hour survival for Study I, 72-hour survival for Study II). Animals were euthanized with a halothane overdose, and the brains were quickly removed and sliced into 3-mm thick coronal sections. The brain slices were then incubated in 2% tetrazolium chloride (TTC) at 37°C for 15 minutes and fixed in 10% buffered formalin (pH 7.4) for 1 week. After paraffin embedding, 10-μm thick sections were stained with hematoxylin and eosin (H & E). In Study 2, additional sections were cut for TUNEL and MPO staining. Infarct was evaluated by light microscopy, in a blinded fashion, in both the cortex and striatum. Histologic criteria for infarct included areas of pan-necrosis with shrunken dark neurons and glial pallor.14 The area of infarct (determined from H&E-stained sections) was expressed as a percentage of the total area of left hemisphere, cortex, or striatum (Study I). Infarct volume was also calculated corrected for edema by use of the method of Swanson et al.15 Infarct areas on TTC-stained slices were traced and measured with an image analysis system (MCID, Imaging Research Inc, Ontario, Canada). Infarct volume was calculated as the sum of the infarcted areas.

**TABLE 1. Neurological Grading Scale**

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of consciousness</td>
<td></td>
</tr>
<tr>
<td>Normal (alert)</td>
<td>0</td>
</tr>
<tr>
<td>Stuporous</td>
<td>5</td>
</tr>
<tr>
<td>Comatose</td>
<td>15</td>
</tr>
<tr>
<td>Sensorimotor function</td>
<td></td>
</tr>
<tr>
<td>No withdrawal to pain (front)</td>
<td>2</td>
</tr>
<tr>
<td>(back)</td>
<td>2</td>
</tr>
<tr>
<td>No righting reflex</td>
<td>10</td>
</tr>
<tr>
<td>Gait</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Moderate ataxia</td>
<td>5</td>
</tr>
<tr>
<td>Able to stand</td>
<td>10</td>
</tr>
<tr>
<td>Unable to stand</td>
<td>15</td>
</tr>
<tr>
<td>Behavior</td>
<td></td>
</tr>
<tr>
<td>No grooming</td>
<td>6</td>
</tr>
<tr>
<td>No eating/drinking</td>
<td>10</td>
</tr>
<tr>
<td>No exploratory behavior</td>
<td>10</td>
</tr>
<tr>
<td>Death</td>
<td>70</td>
</tr>
</tbody>
</table>

*Modified from Baker et al.1
multiplied by the distance between brain slices. Brain swelling was calculated as follows: [(volume of ipsilateral hemisphere − volume of contralateral hemisphere)/volume of contralateral hemisphere (×100%)].

Transferase dUTP Nick-End Labeling (TUNEL) Staining
TUNEL staining was performed with the ApopTag Plus in situ Apoptosis Detection Kit (Oncor, Gaithersburg, Md). Tissue sections were deparaffinized in xylene and hydrated in a sequence of ethanol washes, followed by a final wash in phosphate-buffered saline (PBS). Nuclei of tissue sections were stripped of proteins by incubation with 20 μg/mL of proteinase K (42°C) for 15 minutes. Tissue slices were then washed in distilled water and PBS and incubated in 3% hydrogen peroxide to remove endogenous peroxidases. After equilibration, the sections were incubated at 42°C in terminal deoxynucleotidyl transferase (TdT) enzyme and digoxigenin-labeled substrate for 1 hour. Antidigoxigenin was then applied, and visualization was accomplished with a diaminobenzidine (DAB) substrate solution. The sections were then counterstained with methyl green, cleared, and mounted. Sections treated with the DNAse I enzyme were used as positive controls (Amer sham, Cleveland, Ohio), and sections in which TdT treatment was omitted were used as negative controls. TUNEL-labeled cells were counted in the cortex and striatum.

Myeloperoxidase (MPO) Staining
Tissue sections were deparaffinized, hydrated, and washed in PBS. The sections were then incubated with 20 μg/mL of proteinase K for 15 minutes, washed with distilled water and PBS, and incubated with 3% hydrogen peroxide to remove endogenous peroxidases. Blocking serum was applied for 20 minutes at 42°C. The sections were then sequentially incubated in primary rabbit anti-human MPO antibody (1:200) for 30 minutes, biotinylated goat anti-rabbit IgG antibody (1:200) for 30 minutes, and ABC reagent (avidin and biotinylated horseradish peroxidase complex) for 30 minutes (ABC kit, Vector, Burlingame, Calif). Visualization was accomplished with a DAB substrate solution until the desired staining intensity was obtained (Oncogene Research Products, Cambridge, Mass). The sections were then counterstained with H & E, cleared, and mounted.

Animals that died before the desired endpoint were excluded from any histologic outcome determination (ie, infarct size quantification, and TUNEL and MPO staining) to avoid potentially biasing the data. Six coronal TUNEL-stained sections were counted on 2 animals from each group (total of 8 animals) to identify the coronal level(s) with the highest number of stained cells/nuclei. Then this coronal level was assessed for the number of TUNEL-stained nuclei detected in two high power fields at the level of the anterior commissure for each animal that reached the desired 72-hour endpoint in Study II. All data from MPO staining refer to the number of polymorphonuclear leukocytes (PMNLs) detected in an entire left hemisphere section at the level of the AC (for each surviving animal).

Analysis of DNA Fragmentation
Four animals (2 normothermics and 2 hypothermics, 33°C, 2-hour duration) underwent 2 hours of MCA occlusion followed by 72 hours of reperfusion as previously described. At the end of reperfusion period, DNA was harvested from the cortex and striatum of both the ipsilateral and contralateral hemispheres. The tissue samples were immediately frozen on dry ice or immediately digested in 100 mmol/L Tris-HCl (pH 8.0), 200 mmol/L NaCl, 5 mmol/L EDTA, and 0.2% SDS containing 5 mg/mL proteinase K overnight at 55°C. The DNA solution was incubated for 1 hour at room temperature with 5 mg/mL RNase A and then extracted with phenol-chloroform. DNA was separated on 0.8% agarose gels and visualized with ethidium bromide staining. 16,17 A 123 bp DNA ladder (Life Technologies, GibcoBRL, Gaithersburg, Md) was used as a control in all gels.

Results
Figure 1 shows the overall correlation coefficient between brain and rectal temperature (r = 0.91, P < 0.0001), with brain temperature higher than rectal temperature by 0.2°C to 0.7°C. There were no significant differences between groups in systemic physiological parameters or volume of fluid infused with the exception of the desired changes in brain and rectal temperature (Studies 1 and 2), and a lower intraschismic pH value for the 2-hour hypothermic group compared with the 30-minute hypothermic group (Study 2). Of note, however, was the increased difficulty in controlling the systemic parameters of the 30°C group versus the 33°C group because of the former group’s decreased respiratory rate and occasional cardiac arrhythmias (Study 1, Table 2). Average postanesthesia recovery time showed that the animals in the 33°C group recovered at a significantly faster rate (21 ± 4 minutes, P < 0.05) compared with the normothermic animals (37 ± 4 minutes). No significant improvement in recovery time was found in the 30°C group (28 ± 4 minutes). In Study 2, normothermic animals had an average weight loss of approximately 28 g by postischemic day 1, whereas the average weight loss for hypothermic animals was significantly lower (P < 0.05): 25 g (H0.5), 20 g (H1), 18 g (H2). By 3 days postischemia, normothermic animals had lost, on average, 51 g, and hypothermic animals lost 30 g (H0.5), 31 g (H1), and 32 g (H2), but these differences were not statistically significant.

In Study 1, neurological outcome showed that hypothermic animals tended to score better than the normothermic animals, although this difference was not statistically significant (P = 0.08 by Kruskal-Wallis test). In Study 2, neurologic scores at both 24 and 72 hours postischemia were significantly better for animals subjected to 1 or 2 hours of hypothermia. In particular, these animals were more alert and more responsive to handling; showed normal eating, drinking and grooming; and had increased exploratory behavior; whereas normothermic animals...
TABLE 2. Physiological Variables at Baseline and During Ischemic Period

<table>
<thead>
<tr>
<th>Group</th>
<th>MABP, mm Hg</th>
<th>Heart Rate, beats/min</th>
<th>pH</th>
<th>PaCO2, mm Hg</th>
<th>PaO2, mm Hg</th>
<th>Glucose, mg/dL</th>
<th>Hct, %</th>
<th>Rectal Temperature, °C</th>
<th>Brain Temperature, °C</th>
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</tr>
<tr>
<td>b</td>
<td>92±2</td>
<td>298±4</td>
<td>7.32±0.03</td>
<td>36±2</td>
<td>106±4</td>
<td>119±3</td>
<td>36±1</td>
<td>36.7±0.1</td>
<td>36.4±0.1</td>
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<td>i</td>
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<tr>
<td>b</td>
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<td>120±4</td>
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<td>301±4</td>
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<tr>
<td>b</td>
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<tr>
<td>b</td>
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<td>335±5</td>
<td>7.31±0.01</td>
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<td>132±2</td>
<td>116±4</td>
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<tr>
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<td>332±5</td>
<td>7.34±0.02</td>
<td>45±1</td>
<td>136±6</td>
<td>114±5</td>
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<td>7.31±0.01</td>
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<td>122±9</td>
<td>96±4</td>
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<td>32.9±0.1</td>
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<td>H2 (n=8)</td>
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<tr>
<td>b</td>
<td>81±2</td>
<td>329±6</td>
<td>7.32±0.01</td>
<td>44±2</td>
<td>131±6</td>
<td>118±4</td>
<td>40±1</td>
<td>36.8±0.1</td>
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<tr>
<td>i</td>
<td>84±3</td>
<td>315±5</td>
<td>7.30±0.01*</td>
<td>49±2</td>
<td>131±6</td>
<td>119±10</td>
<td>38±1</td>
<td>32.9±0.1</td>
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</tbody>
</table>

Data are mean±SEM. Hct indicates hematocrit; b, baseline; i, ischemic period.
†No significant difference between groups by 1-way ANOVA (excluding brain and rectal temperature, and *). *P<0.05; H2 vs H0.5 (during the ischemic period) by Student t test.

were often very lethargic and showed no interest in their surroundings. Treatment with thirty minutes of hypothermia showed no neurologic improvement when compared with the normothermic controls. Results from the neurological deficit scores are shown in Figure 2a (Study 1), 2b (Study 2 at 24 hours postischemia), and 2c (Study 2 at 72 hours postischemia). A maximum score of 70 indicates that the animal died before the 24 or 72-hour endpoint.

In Study 1, histopathology revealed an 68% and 51% reduction in the area of cortical infarct for the 33°C group (17±7%, P<0.005) and the 30°C group (27±6%, P<0.02), respectively, compared with the normothermic animals (53±8%) as seen in Figure 3. In the second study, 2 hours of mild hypothermia decreased infarct area by 59% (hemispheric), 72% (cortical), and 25% (striatal); 1 hour of mild hypothermia reduced injury by 84% (hemispheric), 94% (cortical), and 60% (striatal). At first these results may appear to show that 1-hour duration of mild hypothermia is more protective than 2 hours, mainly because of a single animal in the 2-hour duration group that had a very large infarct. Therefore, this is a very conservative analysis that does not exclude the outlier in the 2-hour group. Furthermore, there is no statistically significant difference between the 1- and 2-hour duration of hypothermia groups at any level studied. Thirty minutes of mild hypothermia did not protect against neuronal damage. Figure 4 shows the infarct volumes obtained in the various groups as determined from TTC-stained slices. There was a good correlation between TTC- and H & E-stained tissue both at 24 (r=0.843) and 72 hours (r=0.917) postischemia. One hour of hypothermia significantly reduced cerebral edema (1.9±1.3%, P=0.002) compared with normothermic controls (12.6±2.1%). Two hours or 30 minutes of hypothermia did not reduce brain swelling to a significant degree (8.8±3% and 11.8±2.1%, respectively). The mortality results for Study 2 were as follows: 50% for the normothermic group, 44% for the 30 minutes hypothermic group, and 0% for both 1- and 2-hour hypothermic groups.

Data from a representative normothermic and a 2-hour hypothermic animal (Study II) are displayed in Figure 5, which shows that for both MPO and TUNEL staining, the level of the AC (coronal level 3) and coronal level 4 had the highest numbers of stained cells/nuclei. Cells were counted as apoptotic only if they were TUNEL-positive and showed characteristic nuclear morphology typical of apoptosis (ie, cells containing pyknotic nuclei).
plus apoptotic bodies). TUNEL staining was absent in animals that survived 6 hours or less of reperfusion, but was prominent starting at 24 hours postischemia (data not shown). At 72 hours, 1 and 2 hours of mild hypothermia decreased TUNEL staining by 78% and 99%, respectively, compared with normothermic controls. Thirty minutes of mild hypothermia reduced the number of TUNEL-stained nuclei by 17%, however, this was not statistically significant. Although TUNEL-stained nuclei were found scattered throughout the ischemic area, they tended to concentrate in the boundaries of the striatum and were also prominent in the amygdala (Table 3, Figures 5 and 6). Results from DNA extraction at 72 hours postischemia showed evidence of internucleosomal laddering in both ipsilateral cortex and striatum of normothermic and hypothermic (2-hour duration) animals, but no laddering was observed in the contralateral cortex or striatum in either group (Figure 7). Although visualization with ethidium bromide is not quantitative, it is important to note that visualization of DNA laddering in hypothermic animals was difficult, presumably because of the reduced number of apoptotic cells observed in those animals.

After 70 hours of reperfusion, neutrophils could be easily detected in both cortex and striatum and had a similar visualization compared with controls

Table 3. Quantification of Ipsilateral Apoptotic Nuclei and Neutrophils at 72 Hours Reperfusion after 2-Hour MCAO

<table>
<thead>
<tr>
<th>Stain/Region</th>
<th>N (n=5)</th>
<th>H0.5 (n=6)</th>
<th>H1 (n=8)</th>
<th>H2 (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tunel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctx</td>
<td>58±11</td>
<td>48±5</td>
<td>13±5†‡</td>
<td>0±1†‡</td>
</tr>
<tr>
<td>St</td>
<td>93±20</td>
<td>64±11</td>
<td>26±10†‡</td>
<td>5±2‡</td>
</tr>
<tr>
<td>MPO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctx</td>
<td>69±19</td>
<td>42±14</td>
<td>17±7†</td>
<td>19±6‡</td>
</tr>
<tr>
<td>St</td>
<td>189±11</td>
<td>145±21</td>
<td>35±13*‡</td>
<td>43±10‡*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. TUNEL indicates stain for apoptotic nuclei; MPO, Myeloperoxidase stain for neutrophils; Ctx, cortex; and St, striatum. P<0.001 compared with controls (N) by Student t test; †P<0.05 compared with 30 min group (H0.5) by Student t test.
distribution to the TUNEL-stained nuclei; however, a higher concentration of PMNLs was found within the ischemic core compared with TUNEL-stained cells. At 72 hours postischemia, 1 hour of mild hypothermia reduced neutrophil accumulation by 75%, and 2 hours of mild hypothermia decreased it by 72%. Thirty minutes of hypothermia reduced PMNLs infiltration by 39%, but this was not statistically significant (Table 3, Figures 5 and 6). Normalized for infarct size, 2 hours of mild hypothermia decreased neutrophil accumulation by 57% in the hemisphere, 27% in the cortex, and 76% in the striatum. One hour or 30 minutes of hypothermia did not decrease PMNL infiltration after normalization.

Discussion

Mild hypothermia is already being used in the treatment of traumatic brain injury18 and there is renewed interest at the clinical level in developing guidelines for its therapeutic use in stroke patients.19 Two key issues that need to be addressed are the optimal depth and duration of mild hypothermia. Although we find no differences in the degree of neuroprotection afforded by either hypothermia group, in the present studies we show that 33°C is associated with more stable intraoperative hemodynamic and respiratory status as well as with improved recovery from anesthesia compared with 30°C. Furthermore, we show that just 1 hour of intraischemic mild hypothermia is sufficient to obtain very significant neuroprotection. Neurologic outcome and mortality are the two most important endpoints in any clinical study on stroke. Most experimental studies, however, use histologic outcome to determine the efficacy of the therapeutic strategy in question. Here we report the results of two studies that show a strong association between histopathologic findings and neurobehavioral outcome.

Our first study demonstrates that both mild (33°C) and moderate (30°C) intraischemic hypothermia are protective against ischemic neuronal damage in a rat model of transient focal cerebral ischemia. Although we find no significant differences between the two hypothermic groups, Goto et al20 have reported temperature-dependent reductions in cerebral infarction. In that study, infarct volume was reduced by 22.4% in mildly hypothermic animals (33°C) and by 49.5% in moderately hypothermic animals (29°C). This suggests that a more intense ischemic challenge, such as the one used in that study (3 hours MCAO plus bilateral carotid artery occlusion), may require larger temperature reductions to obtain significant neuroprotection. In fact, the 3-hour model of reversible ischemia may be more analogous to permanent focal ischemia, since the size of infarction obtained in that study is not different from what others have found using permanent MCAO.21 In the present study, both temperatures afford a marked and comparable degree of neuroprotection; however, mild (33°C) hypothermia is devoid of the systemic complications, including decreased respiratory rate and cardiac arrhythmias, which were observed in animals treated with moderate (30°C) hypothermia. Furthermore, average post-anesthesia recovery time showed that animals in the 33°C group recovered at a significantly faster rate than normothermic controls, whereas animals in the 30°C group showed no improvement in recovery time. Therefore, mild hypothermia (33°C) may be a safer, more manageable alternative to moderate hypothermia. Based on these results, 33°C was used as the target temperature in the second study that was designed to determine the optimal duration of intraischemic hypothermia.

Results from Study 2 show that both 1 and 2 hours of intraischemic hypothermia, started at the ischemic onset, can reduce the behavioral and histopathological deficits associated with transient focal cerebral ischemia. Thirty minutes of mild hypothermia, however, does not afford any protection against neuronal damage and does not improve neurological outcome. Others who have studied this question have found conflicting results. Kader et al22 found that 33°C or 34.5°C for 1 hour, induced at ischemic onset, reduced infarction due to permanent MCAO with a 24-hour endpoint. Another study,23 however, found that 33°C for 1 hour reduced infarct size at 96 hours after transient but not permanent focal ischemia. Karibe et al24 found a significant reduction in infarction if mild intraischemic hypothermia was introduced within 30 minutes of transient (2 hours) MCAO, but the protection was lost if hypothermic induction was delayed by more than 60 minutes. Thus, although it may not be necessary to maintain intraischemic hypothermia for more than 1 hour if started within 30 minutes of the focal ischemic onset, longer periods of
hypothermia may be needed to achieve neuroprotection following permanent vessel occlusion or if initiated in a delayed fashion after ischemic onset.

The observation that reducing the temperature for 1 hour but not 30 minutes immediately after MCAO results in cerebroprotection in our model, suggests that mild hypothermia affects some of the injury mechanisms that occur early in the ischemic process. During ischemia there is an uncoupling of cerebral blood flow and metabolism that results in cerebral energy failure and a concomitant disruption of ion homeostasis that leads to the accumulation of intracellular calcium.25 This activates several enzymatic systems that, if severe and prolonged, can lead to irreversible cell damage. Mild hypothermia may work early on by reducing the energy demands of the cell (ie, slowing down energy failure), thereby delaying activation of deleterious catabolic processes.26 Hypothermia may also act by preventing adenosine triphosphate depletion27 and intracellular acidosis,28 by reducing the release of excitatory amino acids and catecholamines,29 and by preventing the ischemic inhibition of protein kinases.30

Cytotoxic edema also occurs early in the ischemic process. By lowering the rates of catabolic reactions, mild hypothermia may help maintain tissue integrity and therefore reduce edema formation.31 In Study 2 we show that 1 hour of mild hypothermia reduces cerebral edema by 85%. Based on the behavioral and histological data, the lack of significant protection against brain swelling with 30 minutes of hypothermia is not surprising. However, 2 hours of mild hypothermia does not significantly reduce brain edema. This may be explained by the variability in infarct sizes and edema observed in that group. One animal in the 2-hour group had a large infarct with a correspondingly large edema component (4% higher than the largest value in the normothermic group). That animal had a low (near normal) neurological score at 24 hours postischemia but, unlike the other animals in that group, showed increased behavioral deficits at 72 hours.

In recent years two other delayed injury mechanisms have come into focus: activation of apoptosis, and inflammation. Several investigators have recently shown that apoptosis may be a contributing factor in neuronal death following both global22-24 and focal13,33-39 ischemia. Results from our second study show that at 3 days posts ischemia, 1 and 2 hours of mild hypothermia is sufficient to decrease the number of apoptotic cells, as determined by TUNEL staining and morphology, by 78% and 99%, respectively. Thirty minutes of hypothermia, however, has no effect. This is particularly significant because the only other study that has demonstrated robust effects of hypothermia on the number of cells undergoing

![Figure 6](image6.png)

**Figure 6.** Photomicrographs. A, DNA nick-end labeling of cells in the cortex of a normothermic animal 72 hours after transient focal cerebral ischemia (×100). Yellow arrows indicate apoptotic cells (deep brown), black arrow shows a necrotic cell with diffuse brown staining (counterstaining with methyl green). B, High magnification of a labeled cell displaying the characteristic features of chromatin condensation and apoptotic bodies (arrow). C, TUNEL staining followed by a methyl green counterstain in a respective cortical region of interest in a mildly hypothermic animal (1-hour duration, ×100); note absence of TUNEL-positive cells. D, DNA nick-end labeling of apoptotic cells (arrows) in mammary tissue (positive control) counterstained with methyl green. E, Neutrophils (arrows) stained dark blue in paraffin-embedded tissue of a normothermic animal 72 hours postischemia. F, Respective region of interest in a mildly hypothermic animal (2-hour duration, ×250); note absence of neutrophils.

![Figure 7](image7.png)

**Figure 7.** Representative agarose gels exhibiting DNA fragmentation in the cortex (Ctx) and striatum (St) after 2 hours of MCA occlusion followed by 72 hours of reperfusion. Lane 1, 123 bp standard DNA ladder; lane 2, right (contralateral hemisphere to MCAo) cortex (R Ctx); lane 3 left (ipsilateral) cortex (L Ctx); lane 4, right (contralateral) striatum (R St); lane 5, left (ipsilateral) striatum (L St).
apoptosis (in transient global hypoxia-ischemia) used a 12-hour cooling period commenced postinsult.\(^7\) In that study, mild hypothermia (34.9°C mean tympanic membrane temperature) had no effect on the fraction of cells undergoing necrosis, although the fraction of apoptotic cells was significantly reduced, suggesting that hypothermia specifically inhibited apoptosis. The authors speculate that, although the trigger of programmed cell death may occur during the ischemic insult, cellular commitment to death occurs later, thus allowing hypothermia to interrupt the biochemical cascade leading to apoptosis.

In our study we find that TUNEL staining is absent before 6 hours of recirculation but is clearly visible at 24 hours. By 72 hours postischemia, TUNEL staining is very prominent in both normothermic and hypothermic animals, although to a significantly different degree. Results from the DNA fragmentation analysis confirm the latter findings. After 3 days of reperfusion we see evidence of DNA laddering in the cortex and striatum (ipsilateral to the MCAO) of both normothermic and hypothermic animals. At first this would appear to contradict results from a study by Charriaut-Marlangue et al\(^37\) in which the initial stages of DNA fragmentation were observed as early as 3 hours into the recirculation period following 1 hour MCAO and bilateral CCA occlusion. However, full DNA laddering was not evident until 18 hours postischemia. Li et al\(^40\) also investigated the temporal profile of in situ DNA fragmentation following 2 hours MCAO and found that apoptosis first appears within 30 minutes of reperfusion, peaks at 24 to 48 hours, and persists for 4 weeks after the insult. The differences observed in those studies and ours may be attributed to variations in the ischemic model, as well as in the duration of vessel occlusion. It is possible that apoptosis is triggered by various mechanisms that themselves may have unique temporal profiles and be differentially activated depending on infarct severity. On the other hand, the variability between studies may simply reflect differences in the sensitivity of the assays used in each study. The anatomical distribution of TUNEL-stained cells is another issue that deserves attention. In their studies, Li and colleagues\(^40,41\) showed that apoptotic cells are primarily localized to the inner boundary zone of the ischemic infarct and that they appear earlier in the preoptic area and striatum than in the cortex. This is consistent with the idea that apoptosis is an active process that requires energy, so the pericore regions of the affected hemisphere may provide the necessary conditions for the induction of programmed cell death. In our study, however, we find a somewhat different distribution pattern of TUNEL-stained nuclei. In both normothermic animals and those treated with 30 minutes of mild hypothermia, we find that apoptotic cells, although dispersed throughout the MCA territory, are concentrated primarily in the boundaries of the entire striatum and are also prominent in the amygdala. Although the significance of this spatial distribution is unclear, it is possible that apoptotic cells appear first in the boundaries of the ischemic core and are still detectable 72 hours after the initial insult even though the lesion has already expanded to pericore regions by this time. No distinct distribution pattern of TUNEL-stained cells was found in animals treated with either 1 or 2 hours of mild hypothermia.

Although the significance of the inflammatory response in infarct development has attracted the attention of many researchers over the years, little work has been done to assess the effects of mild hypothermia on leukocyte accumulation in ischemically-injured neuronal tissue. Polymorphonuclear leukocytes are at the center of the inflammatory process and may play an important role in the development/progression of an infarct.\(^42\) PMNLs can adhere to the endothelium of the microvasculature within the ischemic territory, thereby plugging vessels ("no reflow" phenomenon) and worsening ischemic damage.\(^43\) Activated PMNLs may also mediate injury of the vascular endothelium through the generation of free radicals, thus intensifying the ischemic condition.\(^44\) Finally, once leukocytes have infiltrated the neuropil, they can injure neurons and glia directly via proteolytic enzyme release.\(^45\) In 1994, Garcia et al\(^46\) using a rat model of permanent MCAO, showed that PMNLs could be detected in capillaries and venules within 30 minutes of ischemic onset in areas where the microvascular patency was impaired. Others\(^47-50\) have studied the role of leukocytes in inducing neutropenia or blocking adhesion molecules involved in neutrophil migration, particularly ICAM-1, CD11b, and CD18. Those studies show that inhibition of these molecules can reduce infarct volume, and that this reduction in damage is dose dependent and associated with significant functional improvement. More recently, a study by Toyoda et al\(^51\) examined the effects of intraischemic moderate hypothermia (30°C) on neutrophil infiltration following transient (3 hours) MCAO. Results from that study showed that, at 24 hours postischemia, hypothermic animals exhibited significantly less MPO activity in the pericore region compared with normothermic controls, although no differences were observed in the core. In Study 2 we find that 1 hour of mild hypothermia is sufficient to reduce neutrophil accumulation by 75% and 2 hours of hypothermia decreases it by 72%. We observe a decrease in neutrophil accumulation within the core of the infarct. Thirty minutes of hypothermia reduces PMNL infiltration by 39%, although this is not statistically significant. When normalized for infarct size, only 2 hours of mild hypothermia significantly reduces accumulation of PMNLs, whereas 1 hour shows a trend and 30 minutes is not effective.

There are some limitations to the present studies. Although we showed that mild hypothermia is effective at reducing neuronal injury when applied intraischemically, a more clinically relevant issue is whether hypothermia can protect if applied hours after ischemia or once reperfusion has occurred. However, other studies\(^41,50\) on focal ischemia have shown that delaying hypothermic onset up to 1.5 hours can still be beneficial, provided hypothermia is maintained for at least 1 to 3 hours. Longer durations of mild hypothermia with 2 to 3 hours of delay have not yet been studied in this model. Secondly, since we assessed injury at 24 and 72 hours postischemia, we cannot exclude the possibility that mild hypothermia simply delays neuronal damage. Indeed, work by Dietrich et al\(^52\) on global ischemia has shown that mild hypothermia (applied for 3 hours at reperfusion onset) is very effective at reducing neuronal damage 3 days postischemia, but the protective effect is less impressive at 7 days. By 2 months, cytoprotection is lost unless hypothermia is applied.
intraschemically. Finally, we cannot exclude the possibility that the neuroprotective effects observed with hypothermia are due to changes in blood flow. Indeed, 30°C (but not 33°C) has been shown to decrease blood flow in the ischemic brain following permanent MCAO. To date, no other single therapeutic approach has been developed that can reduce ischemic neuronal injury to the extent that is observed with hypothermia. As suggested by the present and other studies, mild hypothermia’s neuroprotective benefit may be secondary to attenuation of several detrimental processes involved in both necrotic and apoptotic cell death. Furthermore, this may be a cost-effective therapy (particularly in the surgical setting) that is easily implemented, and may prove to be of value by itself or in combination with more traditional pharmaceutical approaches. Although further work needs to be done to evaluate its long-term effects, clinical studies of mild hypothermia in the treatment and prevention of stroke should be considered.

Acknowledgments

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The authors suggest that studies should be initiated to evaluate efficacy of hypothermia in the clinical setting. They argue that it may be preferable to use 33°C rather than 30°C, evaluating ultimate efficacy. As this therapy moves toward clinical trials, it is also possible that details related to the rate and mechanism of cooling and rewarming may become important in determining ultimate efficacy.

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