Comparative neuroprotective efficacy of prolonged moderate intraischemic and postischemic hypothermia in focal cerebral ischemia

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Object. The purpose of this study was to compare the effects of prolonged hypothermia on ischemic injury in a highly reproducible model of middle cerebral artery (MCA) occlusion in rats.

Methods. Male Sprague-Dawley rats were anesthetized with halothane and subjected to 120 minutes of temporary MCA occlusion by retrograde insertion of an intraluminal nylon suture coated with poly-L-lysine through the external carotid artery into the internal carotid artery and the MCA. Two levels of prolonged postischemic cranial hypothermia (32°C and 27°C), and one level of intraischemic cranial hypothermia (32°C) were compared with the ischemic normothermia (37°C) condition. Target cranial temperatures were maintained for 3 hours and then gradually restored to 35°C over an additional 2-hour period. The animals were evaluated using a quantitative neurobehavioral battery of tests before inducing MCA occlusion, during occlusion (at 60 minutes postonset in all rats except those in the intraischemic hypothermia group), and at 24, 48, and 72 hours after reperfusion. The rat brains were perfusion fixed at 72 hours after ischemia and infarct volumes and brain edema were determined. Both intraischemic and postischemic cooling to 32°C led to similar significant reductions in cortical infarct volume (by 89 and 88%, respectively) and total infarct volume (by 54 and 69%, respectively), whereas postischemic cooling to 27°C produced lesser reductions (64 and 49%, respectively), which were not statistically significant. All three hypothermic regimens significantly lessened hemispheric swelling and improved the neurological score at 24 hours. Our data confirm that a high degree of histological neuroprotection is conferred by postischemic cooling to 32°C, which is virtually equivalent to that observed with intraischemic cooling to the same level.

Conclusions. These results may be relevant to the design of future clinical trials of therapeutic hypothermia for acute ischemic stroke.

Key Words * brain temperature * hypothermia * middle cerebral artery occlusion * vascular occlusion * neuroprotection * stroke * rat

Abbreviations used in this paper: ANOVA = analysis of variance; MCA = middle cerebral artery; SEM = standard error of the mean.
A large number of studies conducted over the past decade have established, in principle, the efficacy of using moderate degrees of cranial hypothermia to confer neuroprotection in the settings of both focal and global cerebral ischemia.[10] Although moderate cranial hypothermia initiated during focal vascular occlusion is capable of reducing infarct size by 90% or more,[4,43] the therapeutic window for neuroprotection achieved with postischemic hypothermia has not been extensively investigated, nor has the optimal depth of cooling been carefully defined.

In the present study, we have used a highly reproducible model of temporary MCA occlusion to compare the neuroprotective efficacy of three potentially clinically relevant hypothermic regimens: prolonged periods of cranial cooling to a target temperature of 32°C, begun either at the onset of ischemia or at the onset of reperfusion 2 hours later, and cooling to a target temperature of 27°C begun at the time of reperfusion. Using selective cranial cooling, we avoided any adverse events that might be associated with systemic hypothermia.

MATERIALS AND METHODS

Animal Preparation

Twenty-five adult male Sprague-Dawley rats weighing 274 to 340 g each were denied food overnight but allowed free access to water. Animal protocols for these studies were approved by the University of Miami Animal Care and Use Committee. After an intraperitoneal injection of atropine sulfate (0.5 mg/kg), anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and a balance of oxygen. The rats were orally intubated, immobilized with intravenous pancuronium bromide (0.6 mg/kg), and mechanically ventilated. Temperature probes were inserted into each animal's rectum and left temporal muscle for temperature monitoring. Cranial temperature was maintained (at levels specified in Experimental Groups) by means of a warming lamp and a cooling fan (delivering liquid nitrogen vapor) placed above the rat's head. Rectal temperature was separately regulated by placing a warming blanket beneath the animal. The right femoral artery and vein were catheterized for continuous blood pressure monitoring and periodic blood sampling for arterial gas levels, pH, and plasma glucose (15 minutes before MCA occlusion, 15 minutes after onset of MCA occlusion, and hourly for 5 hours after reperfusion). Mean arterial blood pressure was measured through an indwelling femoral arterial catheter connected to a precalibrated Statham pressure transducer and was recorded continuously. Serial measurements of arterial blood gas levels, pH, and plasma glucose were made.

Middle Cerebral Artery Occlusion

The right MCA was occluded for 2 hours by using the intraluminal-filament method. [45] which we had modified.[6] In brief, the right common carotid artery was exposed through a midline neck incision and dissected free of surrounding nerves; the occipital branches of the external carotid artery were coagulated; and the pterygopalatine artery was ligated. A 4-cm length of No. 3-0 monofilament nylon suture was inserted through the proximal external carotid artery into the internal carotid artery and the MCA, a distance of 19 to 21 mm from the common carotid artery bifurcation according to the animal's weight. Before using the suture, its tip was blunted by heat and a 20-mm distal segment of it was coated with poly-L-lysine solution (0.1% [wt/vol]) and dried at 60°C for 1 hour. We have shown that this coating procedure enhances the reproducibility of the resulting infarct.[6] Animals were allowed to awaken from anesthesia and were assessed using a standardized neurobehavioral scale (see Behavioral Testing). Rats that did not demonstrate a left upper-extremity paresis were excluded from further study. After 2 hours of MCA occlusion, the rats were reanesthetized with the same combination of anesthetic
agents. Temperature probes were reinserted, and the intraluminal suture was carefully removed. The neck incision was closed with silk sutures and the animals were allowed to survive for 3 days with free access to food and water. Rectal temperature and body weight were measured before MCA occlusion and periodically throughout the 3-day survival period.

**Experimental Groups**

**Group A--Normothermic.** Cranial and rectal temperatures were held at 37°C during 2 hours of MCA occlusion and maintained at that level for 5 hours after onset of reperfusion.

**Group B--Intraischemic Hypothermia of 32°C.** The brain temperature was reduced to 32°C (body temperature was kept at 37°C) and maintained at that level for 2 hours during MCA occlusion and for 1 hour of reperfusion, after which cranial temperature was gradually restored to 35°C over an additional 2-hour period.

**Group C--Postischemic Hypothermia of 32°C.** Cranial and rectal temperatures were held at 37°C during the 2-hour MCA occlusion period. Cranial temperature was then reduced to 32°C (and rectal temperature to 35°C) and maintained at that level for the first 3 hours of recirculation. Cranial temperature was gradually restored to 35°C over an additional 2-hour period.

**Group D--Postischemic Hypothermia of 27°C.** Cranial and rectal temperatures were kept at 37°C during the 2-hour MCA occlusion period. Cranial temperature was then reduced to 27°C (and rectal temperature to 35°C) and maintained at that level for the first 3 hours of recirculation. Cranial temperature was gradually restored to 35°C over an additional 2-hour period.

The four temperature protocols of this study are illustrated diagrammatically in Fig. 1.
Fig. 1. Diagram showing the four cranial temperature groups in this study. Group A: normothermia (37°C). Group B: intraischemic hypothermia, 32°C. Group C: postischemic hypothermia, 32°C. Group D: postischemic hypothermia, 27°C.

Behavioral Testing

A battery of behavioral tests was performed in rats before MCA occlusion, during occlusion (at 60 minutes after onset in all groups except Group B), and at 24, 48, and 72 hours after reperfusion. The battery consisted of two tests that have been used previously to evaluate various aspects of neurological function: 1) the postural reflex test, to examine upper body posture while the animal is suspended by the tail;[5] and 2) the forelimb-placing test, to examine sensorimotor integration in forelimb-placing responses to visual, tactile, and proprioceptive stimuli.[17] Neurological function was graded on a scale of 0 to 12 (normal score 0, maximum score 12) as previously described.[6] The investigator who rated neurobehavioral was blinded to the experimental group.

Quantitation of Infarct Volume and Brain Swelling

Following a 3-day survival time, the rats were reanesthetized with halothane/nitrous oxide and their brains were perfusion-fixed as previously described[35] with a mixture of 40% formaldehyde/glacial acetic acid/methanol (1:1:8 by volume) delivered into the ascending aorta at 110 mm Hg. The head was placed in the same mixture at 4°C overnight. The brains were then removed and brain blocks were embedded in paraffin. Ten-micron-thick sections were cut in the coronal plane and stained with hematoxylin and eosin. To quantitate infarct volume and depict infarct frequency distribution, histological sections were digitized at nine standardized coronal levels by a charge-coupled device-based
camera system, equipped with a macrolens and green filter, that was interfaced to an image-analysis system from which data were exported to a computer workstation for image processing. An investigator blinded to the experimental groups outlined the zones of infarction, which were clearly demarcated, as well as the outlines of the left and right hemispheres on each tissue section. Software developed by us was used to quantitate the outlined areas and compute integrated volumes and infarction frequency distribution.[49,50] The volume of infarction was calculated as the integrated product of the cross-sectional area for all sections and the distance between sections. To compensate for brain swelling in the ischemic hemisphere,[42] total infarct volume in each rat was corrected by first computing the volume of the left and right hemispheres and then applying the following formula: corrected infarct volume = left hemisphere volume - (right hemisphere volume - measured infarct volume). The degree of associated brain edema was determined as the difference in brain volume between the two hemispheres.

**Statistical Analysis**

Infarct volumes, brain swelling, neurological score, and physiological variables were compared among groups by ANOVA. Repeated-measures ANOVA was used for intergroup comparisons of infarct areas at nine coronal levels. Fisher's exact test was used to compare the frequency of infarction between groups. A probability level less than 0.05 was regarded as significant. Values are presented as mean values ± SEM.

**Sources of Supplies and Equipment**

The Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) and mechanically ventilated using a ventilation unit obtained from Stoelting (Wood Dale, IL). Mon-a-therm 7000 temperature probes were purchased from Mallinckrodt, Inc. (St. Louis, MO). Mean arterial blood pressure was measured using a Statham P23XL pressure transducer available from Viggo-Spectramed, Inc. (Oxnard, CA) and recorded using an RS3400 polygraph from Gould, Inc. (Valley View, OH). Serial measurements of arterial blood gas levels and pH were obtained using equipment (model ABL 330) purchased from Radiometer America, Inc. (Westlake, OH) and measurements of plasma glucose from equipment (model 2300 Stat) purchased from Yellow Springs Instrument Co., Inc. (Yellow Springs, OH). The charge-coupled device-based camera system was obtained from Xillix Technologies Corp. (Vancouver, BC, Canada), equipped with a Micro-Nikkor macrolens and green filter available from Nikon Corp. (Tokyo, Japan), and interfaced to an MCID image-analysis system (Imaging Research, Inc., Brock University, St. Catharines, ON, Canada), from which data were exported to a DEC-Alpha workstation purchased from Digital Equipment Corp. (Maynard, MA).

**RESULTS**

**Physiological Variables**

The mean physiological variables obtained in the animals are presented in Table 1. Plasma glucose, blood gas levels, and arterial blood pressure were not significantly different among groups.
Neurological Assessment

High-grade contralateral forelimb-placing deficits (score 11 ± 0) were clearly present at 60 minutes of
MCA occlusion in all rats tested. (Animals in Group B, the intraschemic hypothermia group, could not be tested during MCA occlusion because of the need to initiate prompt cooling.) In all intraschemic and postischemic hypothermia groups (B, C, and D), the neurological score at 24 hours was significantly improved compared with the ischemia normothermia group (Group A; p < 0.001, one-way ANOVA by ranks followed by Dunnett's test; Fig. 2). In the two 32°C hypothermia groups (Groups B and C), but not in the 27°C hypothermia group (Group D), the improvement was also demonstrable at 48 to 72 hours when compared with the 32°C normothermic Group A.

Fig. 2. Total neurological score (normal score 0; maximum score 12). Dashed line denotes the mean neurological score a 60 minutes after onset of MCA occlusion. Bars depict each group's neurological score 24 hours after MCA occlusion (means ± SEM, six to seven animals/group). Asterisks denote a significantly improved neurological score at 24 hours postocclusion compared with the normothermic group (37°C) (p < 0.05, Kruskal-Wallis one-way ANOVA on ranks, followed by Dunnett's test for multiple comparisons). intraisch = intraschemic; MCAo = MCA occlusion; postisch = postischemic.

Infarct Volumes and Brain Swelling

In normothermic (Group A) rats subjected to 2 hours of MCA occlusion, a large cortical and subcortical infarct was evident at multiple coronal levels (Fig. 3).
Fig. 3. Histopathological infarct-frequency maps obtained at eight coronal levels (ranging from bregma ± 5.2 mm to -7.3 mm) in the four groups in this study. Colors depict numbers of rats with histological evidence of infarction at each pixel location. Normothermic (37°C) animals show a large consistent cortical and subcortical infarct at multiple coronal levels. Both intraischemic and postischemic hypothermia to 32°C are associated with marked reductions in the frequency of cortical infarction. intra = intraischemic; post = postischemic.

The mean cortical infarct volume in this group was 130.9 ± 13.1 mm$^3$ and the mean subcortical infarct volume was 74.2 ± 8.6 mm$^3$ (mean ± SEM). Total infarct volume, corrected for brain swelling, averaged 124.2 ± 20.4 mm$^3$, which amounted to 36% of normal (left) hemisphere volume (Fig. 4).
Prolonged cranial hypothermia of 32°C begun either during MCA occlusion (Group B) or at the onset of recirculation (Group C) was highly effective in reducing cortical (p < 0.005) and total (p < 0.028) infarct volumes (Figs. 3 and 4). Mean infarct-volume reductions amounted to 88.8% (cortex) and 54.3% (total) for Group B, and 87.5% and 68.8%, respectively, for Group C. In both 32°C groups, significant reductions in the infarct area were evident at seven coronal levels (Fig. 5). Intraischemic hypothermia to 32°C tended to be slightly more neuroprotective than postischemic cooling to 32°C; however, this difference did not achieve statistical significance (Fig. 6).
Fig. 5. Bar graphs indicating the cortical infarct areas at nine coronal levels in the four...

37°C group

32°C-intraischemic group

32°C-postischemic group

27°C-postischemic group
groups (A-D) in this study. Asterisks denote significant differences from the normothermic (37°C) group (p < 0.05, repeated-measures ANOVA followed by Dunnett's multiple comparison procedure).

Postischemic cooling to 27°C (Group D) also tended to reduce cortical and total infarct volumes (by 64.3% and 48.6%, respectively), but these reductions were not statistically significant. However, repeated-measures ANOVA revealed that cortical infarct areas were significantly reduced by 27°C hypothermia at six coronal levels, compared with the normothermic group (Fig. 5). Striatal infarct volume averaged 61.6 mm³ for the entire series and was not affected by hypothermia in any group (Fig. 3).
Brain edema was significantly reduced in all intraischemic and postischemic hypothermia groups compared with the normothermic group ($p = 0.001$; Fig. 3). However, although brain edema was significantly reduced at multiple coronal levels in both 32°C groups, animals of the 27°C group showed a significant reduction of brain edema at only one coronal level.

There were no animal deaths in any group studied.

**DISCUSSION**

The chief objectives of this study were: 1) to compare the neuroprotective efficacy of prolonged hypothermia of 32°C initiated during temporary MCA occlusion with that of the same hypothermia initiated at the onset of reperfusion; and 2) to compare the effects of two levels of postischemic hypothermia (32°C and 27°C). Our results demonstrate that hypothermia, whether applied immediately after MCA occlusion or 2 hours later, confers neuroprotective effects on cortical infarct volume, brain edema, and neurological score compared with the normothermic state. Two observations are of particular clinical relevance. First, prolonged hypothermia of 32°C was strikingly and virtually equally effective in reducing cortical infarct volume, whether instituted during MCA occlusion (intraischemic) or following recirculation (postischemic). Second, the more profound level of postischemic hypothermia (27°C) failed to confer a statistically significant reduction in infarct volume and tended to be less effective in combating brain swelling. We also observed that the animals appeared to tolerate postischemic cooling to 32°C better than cooling to 27°C and recovered from anesthesia more rapidly. Thus, our study supports the use of prolonged cooling to 32°C as an appropriate level of hypothermic therapy to achieve consistent neuroprotection.

The hypothermia-induced reduction in infarct volume was paralleled by a striking reduction in the extent of brain swelling measured volumetrically. In the case of the 32°C hypothermic regimens, this amounted on average to 73%. Behaviorally, animals displayed consistent high-grade neurological deficits when examined during the MCA occlusion period itself, but hypothermic rats exhibited a greater degree of subsequent neurological recovery. The protective effect of hypothermia in this study could not be explained by differences in arterial blood pressure, plasma glucose, or arterial blood gas levels, because
these variables were carefully controlled and did not differ among groups.

Ischemia Model

The use of a highly consistent model of MCA occlusion was essential to demonstrate the comparative efficacy of three hypothermic regimens in the present study. We have previously shown that the use of a poly-L-lysine-coated intraluminal suture enhances the consistency and reproducibility of this model.[6] In our experience, a 2-hour period of normothermic MCA occlusion gives rise to a large, consistent cortical and subcortical infarct, a consistent high-grade neurological deficit, and virtually no incidence of operative mortality. We have used this model extensively in previous studies to characterize local blood flow and metabolism in the ischemic penumbra,[8] to undertake pixel-based correlations of metabolism and blood flow with local histopathological findings,[48] and to establish the efficacy of neuroprotective interventions.[7,9]

Moderate Hypothermia: Influence of Delay and Duration

We recently analyzed previous reports on the neuroprotective efficacy of therapeutic hypothermia in focal ischemia in the rat as a function of the duration of the ischemic insult, the intensity and duration of cooling, and the delay in its initiation.[19] In many reported studies, cooling was initiated at the onset of MCA occlusion,[4,13,22,33,37] whereas in others cooling was initiated at later time points. The study conducted by Xue, et al.,[43] is particularly instructive. These authors investigated rats that were subjected to 3 hours of focal ischemia and cooled to 32°C in various regimens. Hypothermia lasting 3 hours and begun at the time of MCA occlusion led to a 92% reduction in cortical infarct size. Cooling for 1.5 hours was equally effective if begun at the onset of MCA occlusion or deferred by 1.5 hours (approximately 45-49% cortical rescue), whereas a 3-hour cooling period deferred by 1.5 hours achieved greater protection (73% cortical salvage). In other studies,[3,24,26] delays of up to 2 to 3 hours in initiating cooling were considered, but in none of these studies were longer durations of cooling systematically evaluated.

Studies in experimental global ischemia have shown that, although shorter durations of postischemic moderate hypothermia are not permanently neuroprotective,[18] prolonged reductions in body temperature confer sustained behavioral and histological neuroprotection.[15,16] Prolonged postischemic cooling in focal ischemia has been less extensively investigated.[31,44,46,47] One such study compared 1- and 3-hour periods of cooling to 30°C, which were initiated after a 2-hour MCA occlusion period.[47] Cooling for 3 hours reduced cortical infarct volume by 84%, whereas cooling for 1 hour was not significantly protective. A recent study, in which 30-minute and 2-hour cooling periods to 33°C were compared in a rat MCA occlusion model, similarly led to the conclusion that longer cooling durations enhanced the degree of neuroprotection.[29] In the present study, we maintained target cranial temperatures (32 or 27°C) for 3 hours, followed by gradual rewarming to 35°C over an additional 2-hour period. Previous studies in both focal[3] and global[10] cerebral ischemia have shown that cranial temperature measured in the temporal muscle is closely predictive of intraparenchymal brain temperature. Our results confirm the efficacy of prolonged cooling. We believe that the use of selective cranial hypothermia and gradual posthypothermia rewarming helped to minimize any adverse consequences of cooling.

Clinical Relevance

The results of the present study take on added relevance in light of ongoing and planned clinical trials of
Hypothermic neuroprotection for acute traumatic brain injury and ischemic stroke. Marion, et al.,[30] conducted a randomized controlled trial of patients with severe closed-head injury; hypothermic patients were cooled to 33°C within 10 hours of injury and were maintained at 32 to 33°C for 24 hours. Patients with Glasgow Coma Scale scores of 5 to 7 showed significantly improved outcomes at 3 to 6 months. A larger Phase III study of moderate hypothermia in severe brain injury has subsequently been conducted[14] and the results will soon be announced. In patients with severe ischemic stroke in the MCA territory, Schwab, et al.,[41] demonstrated the feasibility of direct brain-temperature monitoring and achieved sustained hypothermia (33-34°C) by systemic cooling. In 25 patients with severe MCA territory strokes whose bodies were cooled to 33°C for 48 to 72 hours, intracranial pressure was reduced during the cooling period and the mortality rate was 44%. This contrasts with an expected mortality rate of 80% when standard treatment is used.[40]

Well-known adverse effects of hypothermia include cardiac arrhythmia, decreased cardiac function, and disturbances of coagulation; these typically occur at temperatures of 30°C or lower.[34,38] By contrast, recent clinical studies support the comparative safety of prolonged moderate hypothermia (32-33°C). In patients with severe ischemic stroke who were treated with 33°C core body temperature reductions for 48 to 72 hours, the sole frequent complication was pneumonia in 40% of cases; other severe side effects were undetectable.[40] Similarly, Marion, et al.,[30] reported no important cardiac or coagulopathic complications in patients with traumatic brain injury who were subjected to induced hypothermia of 30 to 32°C for 24 hours. Recent clinical trials have incorporated gradual posthypothermic rewarming protocols in order to reduce adverse events.[14,30] In the present study, we also permitted cranial rewarming to occur gradually, over a 2-hour period. By contrast, previous animal studies of hypothermia have had much more rapid rewarming periods,[29,31] and this may have explained the occasional occurrence of adverse cardiorespiratory events.

Deleterious Effects of Hyperthermia in the Injured Brain

The potential clinical importance of moderate hypothermia in ischemic stroke is reinforced by observations that the acutely ischemic or traumatized brain appears to be inordinately susceptible to the damaging influence of even small degrees of brain temperature elevation.[20] A number of clinical studies have shown that elevated temperature is associated with poor outcome in patients with acute stroke.[1,23,36] In a recent report, Castillo, et al.,[11] demonstrated that hyperthermia (defined as an axillary temperature exceeding 37.5°C) occurring within the first 24 hours after stroke onset was strongly correlated with larger infarct volumes observed on computerized tomography scans and with more severe neurological deficits at 3 months. Cerebrospinal fluid concentrations of glutamate and glycine correlated with increased body temperature and were therefore viewed as possibly contributory to the increased brain damage observed in patients with temperature elevations.[12] In experimental studies, we have shown that hyperthermia, even if delayed by 24 hours following an ischemic event, worsens the histopathological outcome of both focal and global cerebral ischemia.[2,28] The fact that brain temperature may exceed core body temperature in brain-injured patients[32,39] makes the need to correct even mild levels of hyperthermia in this context even more compelling.

Mechanisms of Hypothermic Neuroprotection

A large number of basic studies have shown that the mechanisms of neuroprotection conferred by moderate hypothermia are undoubtedly multifactorial (for reviews, see additional articles[10,16,20,21,27]). Mechanisms of injury that were ameliorated by moderate hypothermia (and
exaggerated by moderate hyperthermia) include: release of excitatory neurotransmitters; activation of protein kinases, blood-brain barrier dysfunction; oxygen radical production with peroxidative damage to lipids, proteins, or DNA; altered gene expression; ischemic depolarizations; cytoskeletal breakdown; and microglial activation. Moderate hypothermia also exerts a general effect in reducing cerebral metabolic rate. A recent study[25] in which magnetic resonance spectroscopy was used has shown that moderate hypothermia increases the fraction of glucose metabolism shunted through the pentose phosphate pathway. Upregulation of this pathway may play an important role in maintaining cellular integrity and function during ischemia by maintaining membrane potential, stabilizing mitochondrial permeability, and countering potential oxidative damage.[25]

CONCLUSIONS

We have shown that cooling of the brain to 32°C for 3 hours (followed by a 2-hour gradual rewarming period), initiated at the time of recirculation after a 2-hour period of MCA occlusion, confers high-grade histological and behavioral protection that is virtually equivalent to that seen with intraischemic cooling. By contrast, postischemic cooling to 27°C was less effective and appeared to be less well tolerated. We believe that these results may be of relevance in guiding the design of future clinical trials.

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