Hypothermic Neuroprotection
A Global Ischemia Study Using 18- to 20-Month-Old Gerbils

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Background and Purpose Previous studies from this laboratory have shown that mild ischemic or prolonged (i.e., 12 to 24 hours) postischemic hypothermia conveys long-lasting (1 to 6 months) protection against CA1 injury. However, these studies have used young animals (aged 3 to 5 months). Stroke incidence rises sharply in late middle age at a time when changes in brain chemistry could alter the response to neuroprotective treatments. Therefore, we evaluated the efficacy of hypothermia in an older population (aged 18 to 20 months) of gerbils.

Methods Three groups of gerbils were exposed to a 5-minute episode of global ischemia or sham occlusion. One group was cooled during ischemia (mean brain temperature of 32°C). A second group was maintained at normothermia (36.4°C) during occlusion and the first hour of reperfusion. Beginning 1.0 hour after occlusion, these gerbils were gradually cooled to 32°C and maintained at this level before gradual rewarming to 37°C at 25 hours after ischemia. The third ischemic group was kept at normothermia during surgery and the first hour of reperfusion. After surgery, all animals were tested for acute (i.e., within 30 hours of ischemia) changes in locomotor activity as well as for chronic (i.e., 5, 10, and 30 days after ischemia) habituation deficits in an open field test.

Results Both ischemic and postischemic hypothermia provided robust protection (P<.0001) of hippocampal CA1 neurons when assessed 30 days after ischemia. However, ischemic hypothermia was more effective than postischemic hypothermia in providing behavioral protection.

Conclusions This study demonstrates that both ischemic and prolonged postischemic hypothermia provide robust and lasting (30-day survival) histological protection against a severe ischemic insult. The extent of behavioral protection with postischemic hypothermia was less than that previously observed in younger animals. This suggests that neuroprotective treatments in young animals may lose efficacy as a result of aging. (Stroke. 1997;28:2238-2243.)

Key Words • aging • cerebral ischemia • hypothermia • neuroprotection • gerbils

Almost 75.0% of all strokes affect people aged 65 years or older.12 Despite these statistics, it is standard practice to use young animals in models of cerebral ischemia.2 This may be problematic for several reasons. First, elderly individuals may simply not tolerate aggressive stroke interventions. Second, the aging brain undergoes numerous biochemical,2-6 morphological,7-10 and electrophysiological11,12 changes that could alter the pattern of vulnerability to cerebral ischemia. Third, in older animals there is evidence for depletion of ATP, increased accumulation of intracellular calcium, poorer recovery of ion homeostasis, mitochondrial impairment, and increased formation of free radicals.5,6,12,14 All of these events might be expected to worsen ischemic outcome in the older animal. However, in other respects the aged brain may be less vulnerable to ischemia since NMDA receptor populations and NMDA-responsivity are decreased.9,13

In the few studies that compared susceptibility to ischemia between young and old animals it was found that older animals are generally more vulnerable, but there is some controversy.16 In one global ischemia study, 18- to 22-month-old rats exhibited greater CA1 and striatal neuronal injury than 5- to 6-month-old animals.17 However, a more recent study reported region specific changes in sensitivity to forebrain ischemia, with the striatum and cortex being more vulnerable but CA1 less vulnerable in older rats.16 In focal ischemia, infarct volumes appear larger in older animals.16,18 Given the above results, it is possible that treatments found effective in young animals would not necessarily be as effective in older animals. Since clinical trials are largely based on demonstrated efficacy in animal models, it is important to know whether results derived from young animals can be extrapolated to an older population. Thus, we examined the neuroprotective efficacy of intraschismic and postischemic hypothermia in a gerbil model of global ischemia that used 18- to 20-month-old animals. Hypothermia was selected because it has repeatedly been shown to convey lasting functional and histological protection16,24 in young animals to a degree unsurpassed by current pharmacological treatments.24 Neuroprotection was assessed with a combined histological and behavioral approach that has been described previously.20,22,25,26

Materials and Methods

Subjects

Thirty female Mongolian gerbils were used in this study after approval by the Memorial University Animal Care Committee.
in accordance with guidelines established by the Canadian Council of Animal Care. Gerbils were obtained from High Oak Ranch (Baden, Ontario, Canada) at approximately 12 weeks of age. Animals were housed in groups of up to 4 per cage until approximately 18 months of age, when they were used in the present experiment and subsequently housed individually. All gerbils had free access to food and water throughout this experiment.

One group of gerbil was subjected to 5 minutes of normothermic ischemia (36°C) without any treatment (I; n=5). Other groups were sham operated (S; n=8); subjected to ischemia under mild (32°C) brain hyperthermia (IH; n=8); and subjected to normothermic ischemia followed 1 hour later by 24 hours of mild (32°C) postischemic hyperthermia (PH; n=9).

Temperature Measurement and Control

All gerbils were implanted with a 5.0-mm guide cannula as described previously.14 Implant surgery was performed under 1.5% to 2.0% halothane (70% N₂O/30% O₂) followed by a 35 mg/kg IP postoperative dose of sodium pentobarbital.

Two days after cannula implantation, gerbils were briefly anesthetized with halothane while the telemetry brain probe (model XM-FH, Mini-Mitter Co, Inc) was inserted. The probe sampled the temperature of the anterior dorsal striatum, and the data were recorded three times per minute. Baseline temperature was collected over a 3-hour period (early afternoon) in freely moving animals followed by probe removal.

Two days after baseline temperature measurement, gerbils were anesthetized with 1.5% to 2.0% halothane (70% N₂O/30% O₂). Animals were then wrapped in a homoeothermic body blanket (Harvard Apparatus), and a water blanket (Med-T-Pads, model TP-3E, Gaymar Industries Inc) was wrapped around the dorsal and lateral surfaces of the head. The brain temperature probe was reinserted, and a rectal probe was inserted to sample core temperature. After a midline neck incision, the common carotid arteries were carefully isolated. Once brain temperature stabilized at normothermic levels (ie, 36.4°C), bilateral carotid artery occlusion (5-minute duration) was induced in I, IH, and PH groups. Sham-operated gerbils were treated similarly but did not undergo occlusion. In the IH group, brain temperature was selectively lowered to 30°C (achieving a mean brain temperature occlusion temperature of 32°C) by perfusing cold water through the water blanket surrounding the head immediately after placement of the microaneurysm clips. Rewarming began just after clip removal. In all animals subjected to ischemia, the carotid arteries were visually inspected after clip removal to verify reflow. The wound was sutured, and each animal was returned to its cage for further temperature measurement/control. A 100-W lamp was used to maintain brain temperature if it fell below 37°C during the first hour after surgery. This usually was not necessary since bilateral carotid occlusion in gerbils produces a slight (~0.7°C) rise in postischemic brain temperature.27,28

Beginning 1 hour after ischemia, animals in the PH group were slowly cooled at a rate of 1°C/10 min to 32°C. They were then maintained at 32°C (±0.2°C) for 24 hours until gradually rewarmed to 37°C at 25 hours after ischemia. This 24-hour whole-body cooling period was manually produced in the awake, freely moving animal by a combination of an overhead fan, water spray, and a 100-W lamp, as described elsewhere.19,20 Such extended hypothermic periods have been repeatedly found to be safe in the unanesthetized rodent.19,20,24,25

At approximately 30 hours after ischemia, all gerbils were briefly anesthetized with 2.0% halothane while the brain probes were removed. Animals were then returned to the vivarium where they were housed except for days on which behavioral testing occurred.

Behavioral Testing

Since the Mini-Mitter brain probes also provide a measure of gross activity levels, which are useful in predicting the severity of an ischemic insult, this acute measure of activity was recorded simultaneously with the core body temperature for a 30-hour period after ischemia/sham surgery. To test for chronic habituation impairments, gerbils were placed in an open field on days 5, 10, and 30 after surgery, as previously described.19,20,26 Each test session lasted 10 minutes, during which the activity scores were collected by an automated image analysis system. This test has been previously shown to accurately predict the degree of ischemia-induced hippocampal damage.20,22,26,21 Elevated open field scores reflect impaired habituation as opposed to simple motor hyperactivity,28 which normally subsides by 2 days after ischemia.29

Histology

Gerbils were killed 30 days after ischemia with an overdose of sodium pentobarbital. They were then transcardially perfused with 15 mL of heparinized saline followed by 50 mL of 10% formalin. The brains were left in fixative for 1 day before removal from the skulls. After further fixation, paraffin-embedded brains were then sectioned at 6 μm and stained with hematoxylin and eosin. As previously reported,29,30 the number of viable CA1 neurons in medial, middle, and lateral sectors (sector length=0.2 mm) was determined at −1.7 (level A) and −2.2 (level B) mm to bregma and in a single sector (medial CA1) at −2.8 (level C) mm.31 The summed number of histologically viable neurons from levels A, B, and C was analyzed by ANOVA, as previously described.19,20

Results

Baseline temperature data were similar in all groups with an overall average of 36.7°C, which is similar to previous results in younger animals.19,20,22,29,32 There were no significant group differences (F1,26 <1). Brain temperature during and for the first hour after surgery (Fig 1, Table) was manipulated close to the desired values outlined in "Materials and Methods." Note that the PH group had a slight (≈0.2°C) but not significantly (t1,12.54, P=.24) higher temperature during ischemia compared with the I group. In addition, during the first postischemic hour the average temperature of the IH group was ≈0.3°C to 0.4°C below that of the I and PH groups. This difference was due to the extra time required to rewarmed the IH group to normothermic levels after ischemia. Although the average temperature of the PH group was significantly above the IH group average temperature during the first postischemic hour (F1,26 =5.33, P=.03), it is unlikely that these negligible differences (ie, 0.4°C) account for any of our experimental findings.

Acute activity levels increased dramatically in the I group ≈2 hours after occlusion and remained high for nearly the entire 30-hour monitoring period (Fig 1). Both intraischemic and postischemic hyperthermia blunted the magnitude and duration of this acute ischemia-induced behavioral activation.

Open field testing (Fig 2) revealed significant group differences (F1,26 =3.62, P=.026) (main effect). Specific contrasts showed that the I group was significantly impaired (higher activity scores) compared with the S
Fig 1. Brain temperature and acute activity profiles during the 30-hour postischemia monitoring period. Note the similar brain temperature profiles between the sham (S), intraischemic hypothermia (IH), and 5-minute ischemic (I) groups. Acute activity levels increased dramatically in the I group ~2 hours after occlusion and remained high for nearly the entire 30-hour period. Both intraischemic and postischemic hypothermia blunted the magnitude and duration of this ischemia-induced activation.

Group (F_{1,28}=7.39, P = 0.012). Intraischemic hypothermia significantly reduced this habituation impairment (F_{1,28}=7.86, P = 0.009). Postischemic hypothermia did attenuate this impairment, but this was not significant (F_{1,28}=1.70, P = 0.204). Repeated exposure to the open field resulted in reduced activity scores (F_{2,28}=49.67, P < 0.001) compared with the first test session 5 days after ischemia/surgery (Fig 2). There was, however, a significant group by day interaction (F_{5,62}=2.52, P = 0.032). Notably, the IH group had a different trend over days than the I (F_{2,22}=3.54, P = 0.008) and PH (F_{2,22}=3.92, P = 0.026) groups. This was due to the activity levels of the IH group dropping dramatically on day 10 to the level of S animals (Fig 2).

Normothermic ischemia in these aged gerbils consistently resulted in extensive CA1 necrosis at all three levels assessed (Fig 3). This is similar to previous findings in young animals,\textsuperscript{19,20,22} except that injury was slightly greater in the most posterior level (level C, ~86% necrosis in old animals versus 71% necrosis in young animals). However, this difference was not significant (t_{1}=1.94, P = 0.069). Intraischemic hypothermia markedly reduced CA1 injury (versus the I group) at all

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Values are mean±SD, expressed in degrees Celsius.
levels of the hippocampus ($P<.001$). In fact, cell counts were not statistically different from S animals ($P>.13$). Similarly, 24 hours of posts ischemic, mild hypothermia provided robust and significant neuroprotection at all CA1 levels ($P<.001$). Cell counts in the PH group were statistically different from the S group at 1.7 (level A) mm posterior to bregma ($t_{24}=2.65, P<.018$). The degree of neuroprotection was quite similar in both the IH and PH groups with no significant differences at any anterior-posterior level of CA1 ($P>.16$).

It was noted that all animals gradually lost weight over the course of the experiment (30-day survival), partly as a result of being segregated into individual cages. However, in the PH group animals lost an additional 5 g by the first posts ischemic day, as previously noted,20 with young gerbils subjected to extended posts ischemic cooling. As before, the weight of the PH gerbils recovered to that of the other animals in the study by the fifth posts ischemic day. Other than this transient effect, no deleterious effects of prolonged posts ischemic hypothermia were observed.

**Discussion**

The highly significant histological protection of CA1 neurons across three anterior-posterior levels of the hippocampus (86.8%, 89.9%, and 98.9% of age-matched S controls) 30 days after intras ischemic hypothermia in these older gerbils is similar to the degree of protection we observed in young animals.22 Posts ischemic hypothermia also provided substantial protection of CA1 neurons. However, protection across all three hippocampal levels was on average $\approx 12\%$ less effective (71.6%, 82.6%, and 87.6%, respectively, for levels A, B, and C) than our previous results with posts ischemic hypothermia in young gerbils.19 None of these differences were significant ($t_{24}=P>.05$). The posterior CA1 region of the hippocampus is considerably more resistant to ischemia than anterior CA1.34 Thus, it is notable that the extent of CA1 necrosis (≈86%) observed at the posterior boundary of the hippocampus in old gerbils (group I), although less than more anterior regions, was somewhat, although not significantly, greater than that (≈71%) observed in our previous studies utilizing young gerbils.20,22 This observation, coupled with the modest reduction in efficacy seen with posts ischemic hypothermia, suggests that CA1 neurons may be more vulnerable in older gerbils, but this speculation awaits confirmation by directly comparing larger groups of young and old animals in a single study. Although systematic cell counts were not performed in brain regions other than CA1, there was no indication of enhanced neuronal loss in the caudate and cortex. With the occlusion durations used in the present study, these brain regions are rarely affected in 3- to 5-month-old gerbils.

Our histological results are consistent with those described initially by Yao et al.,17 who noted increased damage to CA1 neurons after 20 minutes of forebrain ischemia in 18- to 22-month-old female rats. A recent study16 reporting reduced CA1 injury in aged male rats is difficult to reconcile with our results. The discrepancy could be due to many factors, including posts ischemic survival time (ie, 7 versus 30 days), methods of histological analysis, and sex differences in vulnerability to ischemic damage.25 Obviously more studies are required to identify the factors that most markedly affect ischemic outcome in aged animals.

The dramatic and protracted increase in nonspecific motor activity that begins 2 to 3 hours after ischemia36-38 was almost completely negated by intras ischemic hypothermia and to a lesser extent by posts ischemic hypothermia. Prolonged cooling produces some sedation in normal animals; however, activity levels return to normal within 1 day after reestablishment of normothermia (F.C., unpublished data, 1996). As we have previously noted in young gerbils,30 acute activity patterns on the first day after ischemia can be used to gauge the efficacy of ischemic treatments.

Intras ischemic hypothermia also prevented chronic habituation deficits in the open field, confirming our findings in young animals,22 as well as similar functional protection reported by others.13,31 In contrast to our previous findings in young gerbils,20 posts ischemic hypothermia did not provide significant behavioral protection in the open field test in aged gerbils, although there was a trend toward better performance. This may reflect a real difference between young and old animals. It is possible that CA1 neurons are more vulnerable to ischemia in older animals and that additional or more potent therapies will be required to protect cells from damage. For example, increasing the duration of posts ischemic hypothermia from 12 to 24 hours has been shown to greatly enhance long-term survival of CA1 neurons.19,20,24 Perhaps increasing the duration of posts ischemic hypothermia in the present study from 24 to 36 or even 48 hours would have increased the degree of CA1 protection.

It should be noted that the sample size in the present study was small (n=5 to 9 per group), and the variability in open field behavior among older animals is substantially greater than that observed in young animals. Thus, larger group sizes and the use of additional, more complex behavioral tests (eg, T maze) that have greater selectivity for hippocampal function might have revealed greater functional savings after posts ischemic hypothermia. In addition, it is possible that the behavioral deficits arose from a combined loss of several neuronal groups in addition to CA1. For example, somatostatin-positive hilar neurons and CA2 neurons are as sensitive, if not more sensitive, to ischemic injury than CA1 neurons.39,40 In older animals these neurons may have been protected by intras ischemic hypothermia but might have been irreversibly injured before effective posts ischemic hypothermic levels (ie, 32°C) were achieved. Finally, it is conceivable that ischemia induces subtle but functionally important forms of neuronal injury (eg, loss of dendritic spines) not revealed by Nissl stains, and posts ischemic hypothermia may not have prevented this injury. If so, such possibilities underscore the need to employ behavioral and electrophysiological end points when assessing neuroprotection.22,41

Intras ischemic hypothermia has been termed the “gold standard” of neuroprotection,42 and the present data extend this claim to include older animals. Posts ischemic hypothermia, although less effective than intras ischemic hypothermia, is nonetheless a very effective treatment. The present results suggest that treatments found effective in young animals may not convey the same degree of protection in an older population because of a variety of age-related changes that could alter sensitivity to ische-
mia. Therefore, it would be wise to confirm the efficacy of a particular therapy in young and old animals, preferably in several models, before it is advanced to clinical trials.

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The study by Corbett and colleagues assessed the effects of intrasichemic and postschismic hypothermia on aged (18- to 20-month-old) gerbils. The question of whether or not hypothermia is protective in other animals is an important one in regard to the human condition of stroke. Thus, the finding that intrasichemic and postschismic hypothermia both provide significant histopathological protection in aged gerbils is a contribution to the literature. In addition, behavioral correlations suggesting some lessening of hypothermic protection in old animals underscore the importance of age in experimental ischemia studies.

Published studies have shown that the gerbil CA1 hippocampus can be protected by a wide range of therapeutic interventions after cerebral ischemia. Thus, the authors correctly point out that additional studies are required in other animal species before clinical trials are initiated based on these findings. Indeed, global ischemia studies in rats have shown that restricted periods of postschismic hypothermia (ie, 3 to 7 hours) can delay neuronal damage without providing chronic protection of the CA1 hippocampus. In those cases, postschismic hypothermia combined with pharmacotherapy was required to provide long-lasting histopathological protection.

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