Delayed induction and long-term effects of mild hypothermia in a focal model of transient cerebral ischemia: neurological outcome and infarct size

CAROLINA M. MAIER, PH.D., GUO HUA SUN, M.D., PH.D., DAVID KUNIS, B.S., MIDORI A. YENARI, M.D., AND GARY K. STEINBERG, M.D., PH.D.

Departments of Neurosurgery and Neurology and the Stanford Stroke Center, Stanford University Medical Center, Stanford, California

Object. The goals of this study were to determine the effects of delaying induction of mild hypothermia (33°C) after transient focal cerebral ischemia and to ascertain whether the neuroprotective effects of mild hypothermia induced during the ischemic period are sustained over time.

Methods. In the first study, rats underwent 2 hours of middle cerebral artery (MCA) occlusion. Animals in one group were maintained under normothermic conditions (N group, 23 rats) throughout the period of ischemia and reperfusion. Rats in four additional groups were exposed to 2 hours of hypothermia, which commenced at ischemia onset (H0 group, 11 rats) or with delays of 90 (H90 group, 10 rats), 120 (H120 group, 10 rats), or 180 (H180 group, five rats) minutes, and allowed to survive for 3 days. In the second study, animals underwent 1.5 hours of MCA occlusion and were maintained under normothermic (48 rats) or hypothermic (44 rats) conditions during the ischemia period, after which they survived for 3 days, 1 week, or 2 months. All animals were evaluated for neurological findings at 24 hours and 48 hours postischemia and before they were killed. Regions of infarct were determined by examining hematoxylin and eosinstained brain slices obtained at six coronal levels.

Conclusions. Mild hypothermia conferred significant degrees of neuroprotection in terms of survival, behavioral deficits, and histopathological changes, even when its induction was delayed by 120 minutes after onset of MCA occlusion (p < 0.05) compared with normothermic conditions. Furthermore, the neuroprotective effect of mild hypothermia (2-hour duration) that was induced during the ischemia period was sustained over 2 months. These studies lend further support to the use of mild hypothermia in the treatment of stroke.

Key Words • hypothermia • cerebral infarction • focal cerebral ischemia • therapeutic window • neuroprotection • rat

A critical factor in developing a therapeutic strategy against stroke is the time window available. Results of studies in which animal models of focal cerebral ischemia have been used indicate that there is penumbral viability for a few hours after ischemia but that the magnitude of infarct reduction achieved by most stroke therapies is probably greatest when treatment is commenced during the ischemia period. A more clinically relevant issue that still needs to be addressed is whether mild hypothermia, instituted during the postischemia period, is also cerebroprotective. Investigators have demonstrated significant reductions in neuronal damage and functional deficits in response to delayed but prolonged hypothermia following global cerebral ischemia. In focal cerebral ischemia, delaying the onset of mild hypothermia (1–3 hours duration) up to 1.5 hours (but not beyond) has shown benefits. Recently, Huh and colleagues showed that, following a 2-hour period of MCA occlusion, a 3-hour period of cranial cooling to 32°C initiated 2 hours after ischemia onset, followed by an additional 2-hour period at 35°C, resulted in a 69% reduction in infarct volume at 72 hours. However, the use of postischemic mild hypothermia with delays up to 3 hours have not been studied in a focal ischemia model. This is important to determine how long mild hypothermia’s induction can be delayed without loss of benefit, which in turn would provide clues about the neuroprotective mechanisms involved. The first study was undertaken to determine the effects of delaying induction of mild hypothermia up to 3 hours following transient MCA occlusion by the intraluminal suture method.

Another issue that is unclear is whether hypothermia may simply delay the onset of cell death. In global models of cerebral ischemia, postischemic induction of mild hypothermia has been shown to be only transiently effective unless the hypothermic period was extended to 24 hours. With prolonged mild hypothermia, sparing of neurons could be observed even at 6 months postinjury. The use of extended periods of hypothermia is controversial, however, because some investigators have documented a hypothermia-related increase in the incidence of infections, mostly pulmonary in nature, with cooling periods that last longer than 24 hours.
Mild hypothermia to treat experimental stroke

Following focal ischemia, one group of investigators has shown that moderate hypothermia (30°C) delivered during the ischemia period provided protection up to 7 days postinjury, but longer time points have not yet been examined. The aim of the second study was to examine the long-term effects of inducing mild hypothermia during the ischemia period following transient MCA occlusion both in terms of neurological outcome and histopathological findings.

Materials and Methods

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

Stroke Model

Anesthesia was induced in male Sprague–Dawley rats (Charles Rivers, Wilmington, DE) weighing 285 to 305 g each by administering a 3% halothane mixture, which was delivered by mask. The anesthesia was maintained at a surgical level by administering 1% halothane in oxygen (200 ml/minute) and air (800 ml/minute) without the use of paralytic agents. The depth of anesthesia was assessed every 15 minutes by applying a hindlimb pinch. Rectal temperature was maintained at 36.5 to 37.5˚C before ischemia was induced. To allow for better postoperative recovery and long-term survival, we chose not to use laser Doppler flowmetry or a brain temperature probe, because both of these procedures require a craniotomy. Furthermore, we have previously shown a high correlation (r = 0.91, p < 0.0001) between brain and rectal temperatures in this model, with brain temperature being higher than rectal temperature by 0.2 to 0.7°C. Electrocardiographic leads were placed on the animals to monitor heart rates and respirations. A midline incision was made in the neck to expose the common carotid artery, ECA, ICA, and pterygopalatine artery. The common carotid artery, ECA, and pterygopalatine artery were ligated using a No. 6-0 silk suture. The stroke was produced by inserting a No. 3.0 monofilament suture with a flamed tip 18 to 23 mm from the ICA–ECA bifurcation, thus occluding the ostium of the MCA. In the first study the suture was kept in place for 2 hours. Animals were randomly assigned to one of five different experimental groups: 1) normothermic control rats (N group: 37°C, 23 animals); 2) rats that were exposed to 2 hours of hypothermia therapy, which was initiated at the onset of ischemia (H0 group: 33°C, 11 animals); 3) rats that underwent 2 hours of hypothermia therapy, which was initiated 90 minutes into the ischemia period (H90 group: 33°C, 10 animals); 4) rats that were subjected to 2 hours of hypothermia therapy, which was initiated at reperfusion onset (H120 group: 33°C, 10 animals); and 5) rats that underwent 2 hours of hypothermia therapy, which was initiated 1 hour after reperfusion onset (H180 group: 33°C, 5 animals). The H180 group was added before completion of the original study, and thus the numbers of animals used in the other groups had to be increased to randomize the animals into five experimental groups.

In the second study the occlusion period was shortened to 90 minutes to reduce the high mortality rate observed in normothermic animals as we reached longer survival end points. Animals were kept either normothermic (37°C, 48 animals) or hypothermic (33°C, 44 animals) during the 90-minute ischemia period. Total body cooling was achieved by spraying alcohol onto each animal and cooling it to the desired temperature using a fan. Rewarming was achieved using a heating pad placed under the animal and a lamp positioned over the animal's body. Both cooling and rewarming were achieved within 10 minutes. Postanesthesia recovery time, defined as the time from discontinuation of anesthesia until the animal regained its righting reflex, was recorded. The animal was then transported to the intensive care unit at the Veterinary Services Center, where it was closely monitored throughout the recovery period and evaluated for neurological deficits. Rectal temperature was monitored hourly for the first 3 hours following recovery from anesthesia. Fluids (1–2 ml normal saline/100 g body weight) were given as needed, and the analgesic agent butorphanol tartrate (0.015–2 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. During Study I, animals in all five groups were maintained in a halothane-induced state of anesthesia for the same amount of time. At the end of the experiment all animals were returned to normothermic condition, and their blood was allowed to reperfuse for 3 days (Studies I and II) or 1 week or 2 months (Study II).

Behavioral Analysis

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

Infarct Analysis

Only animals surviving for the entire study (desired end point of 3 days, 1 week, or 2 months) were used for histological and histochemical analysis. The rats were killed by administration of a halothane overdose, and their brains were quickly removed and sliced into 3-mm-thick coronal sections. The brain slices were incubated in 2% TTC at 37°C for 15 minutes and fixed in 10% buffered formalin (pH 7.4) for 1 week. Following paraffin embedding, 6-μm-thick sections were stained with hematoxylin and eosin. The infarcts in both the cortex and striatum were evaluated in a blinded fashion by using light microscopy. Histological criteria for confirming the presence of an infarct included areas of pan necrosis with shrunken dark neurons and glial pallor. Areas of infarct on hematoxylin and eosin–stained sections (six coronal levels) were traced and measured using an image analysis system (Microcomputer Imaging Device; Imaging Research, Inc., St. Catherines, ON, Canada).

Statistical Analysis

Statistical analyses were performed using analysis of variance for continuous data and nonparametric tests for noncontinuous data. All data are expressed as means ± SEM, and a probability value less than 0.05 was considered significant.

Results

Study I

Forty-five percent of the normothermic animals died during this study, compared with no animals in any of the...
hypothermic animal groups (p < 0.05). As seen in Fig. 1A and B, normothermic animals had an average neurological deficit score of 35 at 24 and 72 hours, respectively. Animals exposed to mild hypothermia exhibited significant improvement at both 24 and 72 hours when hypothermia was induced during the ischemia period or after delays of up to 120 minutes following onset of ischemia (p < 0.05, Kruskal–Wallis test and Mann–Whitney U-test), but not when induction of hypothermia was delayed by 180 minutes.

Histopathological analysis showed that mild hypothermia induced at the time of ischemia onset significantly reduced the size of the infarct at all levels (55% in the hemisphere, 54% in the cortex, and 46% in the striatum). Delaying induction of hypothermia by 90 minutes resulted in similar reductions in infarct size (61% in the hemisphere, 66% in the cortex, and 61% in the striatum). There was also a significant reduction in infarct size in the cortex (47% decrease) when induction of mild hypothermia was delayed by 120 minutes, but no protection was observed in the striatum (Fig. 2). Delaying induction of hypothermia by 180 minutes after ischemia onset was not protective in any region examined.

Study II

As was the case in Study I, mild hypothermia significantly reduced the mortality rate compared with normothermia. In normothermic animals, death occurred in 23% of the rats in the 3-day and 1-week groups and 39% of the rats in the 2-month group; in hypothermic animals, death occurred in 7% of the rats in the 3-day survival group, 0% in the 1-week survival group, and 6% in the 2-month survival group (p < 0.005). An examination of average postanesthesia recovery times showed that animals in the hypothermia group recovered at a significantly faster rate than those in the normothermia group (26 ± 9 minutes compared with 32 ± 10 minutes, respectively; p < 0.05). Neurological deficit scores at both 3 and 7 days postischemia were significantly better for animals exposed to hypothermia (p < 0.05; Fig. 3A and B, respectively). In particular, these animals were more alert and more responsive to handling; exhibited normal eating, drinking, and grooming behaviors; and displayed increased exploratory behavior; whereas normothermic animals were often very lethargic and showed no interest in their surroundings. At 2 months postinjury, there was a trend toward improved neurological outcomes in hypothermic animals compared with normothermic controls, but this difference was not statistically significant (Fig. 3C). Normothermic animals demonstrated an average weight loss of approximately 18 g by Day 1 posts ischemia, whereas the average weight loss of hypothermic animals was 12 g. By Day 5 posts ischemia, hypothermic animals had gained significantly more weight than normothermic control animals (312 ± 13 g compared with 270 ± 21 g, respectively; p < 0.05).
As seen in Fig. 4, histopathological examination revealed that mild hypothermia decreased the infarct area by 37% at 3 days (p = 0.038), 48% at 7 days (p = 0.038), and 56% at 2 months (p = 0.045) postischemia. Animals in the 2-month normothermic group tended to have smaller infarcts than animals in the 3-day or 1-week groups, although this difference was not statistically significant. This is most likely due to the increased number of deaths before the 2-month evaluation in animals with very large infarcts, thus lowering the average size of infarct in that group. It is worth mentioning that TTC was not useful in delineating the infarct area at the 7-day time point. We have previously shown a good correlation between TTC and hematoxylin and eosin staining up to 3 days post-injury.30 At 7 days postischemia, the border of the infarct could not be easily discerned using TTC because the infarct exhibited pink coloring instead of the usual pale appearance, presumably due to the high concentrations of monocytes and foamy macrophages observed at that time point (Fig. 5).

Discussion

These studies are the first to show the following: 1) mild hypothermia lasting only 2 hours confers a significant degree of neuroprotection in terms of survival, neurobehavioral deficits, and histopathological changes, even when its induction is delayed by 120 minutes following ischemia onset; and 2) the neuroprotective effects of mild hypothermia instituted during ischemia and continued for 2 hours are sustained over time. Our findings are particularly relevant to the field of neurosurgery in which temperature reductions of short duration can be achieved in a controlled surgical setting. Mild hypothermia may be used as a preventive intraoperative measure for neurosurgical patients at high risk for stroke, as well as in the acute phase of an ischemic event such as vasospasm following aneurysmal subarachnoid hemorrhage.
From a scientific point of view, these findings are important because they provide clues about the cerebroprotective mechanisms that are potentially involved. Explanations for hypothermic neuroprotection include decreases in cerebral metabolic rate, restoration of cerebral blood flow, preservation of the blood–brain barrier, and reduction of cytotoxic edema. Another potential neuroprotective mechanism that has been extensively studied involves alterations in neurotransmitter release. Excitatory amino acids are known to play a significant role in mediating ischemic injury, and it has been demonstrated that hypothermia reduces the biosynthesis, release, and uptake of certain neurotransmitters. Following global ischemia, glutamate levels increase within 10 to 20 minutes after onset of ischemia and then decrease by 30 to 50 minutes. Following focal cerebral ischemia, glutamate levels typically peak within 60 minutes of onset of ischemia and return to baseline or decrease substantially by 90 to 120 minutes. Mild hypothermia appears to blunt this peak and, in some instances, delays it by 20 minutes. A few studies, including this one, have shown that mild hypothermia is still effective even when applied after glutamate peaks (hypothermia delayed by 60–120 minutes). Therefore, there is reason to believe that mild hypothermia also exerts its protective effects by altering processes downstream of excitatory amino acid release.

The reduction in the extent of neuronal damage that results from inducing hypothermia postischemia may also be due to alterations in the activity of protein kinases, resynthesis of cellular repair proteins, decreases in lipid peroxidation, and alterations in hydroxyl radical production. The possible neuroprotective effect of extending halothane-induced anesthesia into the postischemia period is also worth mentioning. Using a 5-minute bilateral carotid artery occlusion model, Kuroiwa and colleagues have shown that anesthesia induced postischemia by the administration of 1% halothane prevented the spontaneous and detrimental 1.5°C increase in cranial and rectal temperatures observed in gerbils and significantly protected CA1 neurons against ischemic damage. In Study I, this was controlled by keeping the duration of halothane-induced anesthesia constant for all animals and carefully maintaining temperature throughout the experiment.

Study II provided the first demonstration that the protective effect against infarction and death conveyed by mild hypothermia induced during ischemia is sustained over time in a model of transient focal ischemia. Our results indicate that there is also significant improvement in neurological outcome up to 7 days postischemia and a trend toward improvement at 2 months postischemia. It is important to note, however, that the lack of significant differences between the two temperature groups at 2 months was due not to worsening deficits in the hypothermic animals, but to an improvement in neurological deficit scores of the normothermic control animals. At 2 months postinjury, no animals exhibited the characteristic circling beha-
Mild hypothermia to treat experimental stroke

behavior that initially was associated with this stroke model, and there were no other gross sensorimotor deficits. Both normothermic control and hypothermic animals were alert and responsive; exhibited normal eating, drinking, and grooming behaviors; and displayed increased exploratory behavior 2 months after the stroke. This may represent a recovery of function by cerebral plasticity; however, the neurological grading scale that we used may not be sensitive enough to detect fine sensorimotor or behavioral deficits. Several normothermic control animals that had very large infarcts did not reach the 2-month end point; therefore, that group tended to have a smaller average infarct size. However, death was also included as a measure of neurological outcome and thus the smaller infarct size in these animals should not have altered their overall neurological deficit scores. Our results differ from those of Yanamoto and colleagues who have shown that, in response to mild hypothermia conditions maintained during ischemia (2-hour duration) and postischemia (3-hour duration), the reduction in infarct volume observed at 2 days post-MCA occlusion is lost at 30 days postocclusion. In their study, however, maintaining hypothermia for an additional 21 hours after surgery had long-lasting benefits. The same group had previously shown a significant reduction in infarct volume in cases in which mild hypothermia was induced immediately on reperfusion and maintained for 21 hours, whereas an immediate but brief (1-hour) period of hypothermia was ineffective.

Because TTC is a mitochondrial enzyme stain, the pink color observed in the infarcted area at 7 days postischemia, particularly in normothermic control animals, indicated infiltration of some cell type into the affected tissue. Examination of that tissue aided by light microscopy revealed a large number of inflammatory cells in the ischemic area. Although we had previously shown that mild hypothermia inhibits neutrophil infiltration at 3 days following MCA occlusion, monocytes and macrophages may also be decreased in number. Blood-borne monocytes and macrophages infiltrate infarcted tissue after the initial invasion of neutrophils and persist for a long time (> 60 days) after maturation of the infarct. As seen in Fig. 5, at 7 days postinjury, monocytes and foamy macrophages are present within ischemic regions under normothermic conditions. After arriving in the ischemic area, these cells can contribute to the exacerbation of reperfusion damage by producing cytotoxic factors such as glutamate, tumor necrosis factor-α, nitric oxide, and free radicals. Qualitative observations from this study indicate that levels of monocytes and macrophages are reduced in regions of tissue injury under conditions of mild hypothermia. Thus, hypothermia may work by attenuating the inflammatory response that accompanies ischemia–reperfusion injury. On the other hand, because hypothermia was only induced during the ischemia period, it is possible that the reduction in inflammatory cell infiltration is merely an epiphenomenon. It could, for example, be simply the result of decreased capillary permeability or reduced blood–brain barrier breakdown, both of which have been shown to occur with hypothermia.

The results of the studies presented here conclusively show that mild hypothermia, whether induced during ischemia or delayed, can substantially improve functional and histological outcome with long survival times. Induction of whole-body hypothermia may not be the most desirable way to achieve the targeted temperature in humans; however, methods for selective brain cooling might provide a viable alternative. At first glance, the therapeutic effectiveness of hypothermia (2 hours after ischemia onset) may appear to be relatively narrow, offering potential benefit during the controlled intraoperative surgical setting, but being less applicable for treating patients suffering from spontaneous stroke. However, combination therapy using mild hypothermia and pharmacological intervention may be efficacious. For example, the beneficial effects of tissue plasminogen activator in thrombolytic stroke rely on early intervention; however, the addition of induced hypothermia may prolong the therapeutic window of tissue plasminogen factor. The same may be applied to N-methyl-D-aspartate antagonists. Ginsberg, et al., showed that neither moderate hypothermia (30°C) nor the N-methyl-D-aspartate antagonist dextromethorphan alone protected against global ischemic damage in rats, but the combination was protective. Similar results were obtained by Dietrich and associates who showed that neither delayed MK-801 (dizocilpine) nor postischemic hypothermia alone was effective in attenuating ischemia-related behavioral deficits, whereas combination therapy provided long-lasting protection against transient global ischemia. Thus, the main role of mild hypothermia against stroke may be to extend the therapeutic window of other treatment modalities.

To date, hypothermia is by far the most potent neuroprotector available against experimental cerebral ischemia. Although further work must be accomplished to optimize its use in humans, clinical studies have already been started to test its efficacy in the treatment and prevention of stroke.

References


C. M. Maier, et al.