Cerebral vascular responsiveness after experimental traumatic brain injury: the beneficial effects of delayed hypothermia combined with superoxide dismutase administration

Laboratory investigation

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Object. Traumatic brain injury (TBI) induces cerebral vascular dysfunction reflected in altered responses to vasodilators such as acetylcholine and hypercapnia. It has been demonstrated that the use of either posttraumatic hypothermia or free radical scavengers offered vascular protection when those treatments were delivered early after the injury, losing efficacy when the initiation of either treatment was delayed. Because immediate posttraumatic treatment is not realistic in the clinical setting, the authors undertook this study to investigate whether the combination of delayed hypothermia and the delayed administration of the free radical scavenger superoxide dismutase (SOD) could result in improved vascular protection.

Methods. Male Sprague–Dawley rats were anesthetized and subjected to either an impact-acceleration or sham injury. Animals were treated either with hypothermia (32°C) initiated 60 minutes after TBI, delayed SOD (60 U/ml) applied 90 minutes after TBI, or a combination of delayed hypothermia (32°C) and delayed SOD (60 U/ml) applied 15 minutes prior to the cessation of hypothermia. In this investigation, the diameter of cerebral pial arterioles was measured at rest and then challenged with vasodilator acetylcholine and hypercapnia. Four vessels were assessed per animal prior to injury and then again up to 6 hours after injury.

Results. Delayed SOD treatment did not enhance vascular function, while delayed hypothermia treatment only partially preserved pial vascular function. However, the combination of delayed hypothermia and delayed SOD significantly preserved vascular function after the injury.

Conclusions. The results of these studies demonstrate that delayed hypothermia partially preserves vascular function after TBI, while expanding the therapeutic window over which agents such as SOD can now provide enhanced protection. (DOI: 10.3171/JNS/2008/109/9/0502)

KEY WORDS • brain injury • hypothermia • pial vasculature • rat • vasoreactivity

Abbreviations used in this paper: ACh = acetylcholine; BP = blood pressure; CSF = cerebrospinal fluid; ICP = intracranial pressure; SEM = standard error of the mean; SOD = superoxide dismutase; TBI = traumatic brain injury.

Traumatic brain injury remains a serious health care problem, affecting the physical, social, and economic status of the nation. To date, in the experimental setting, several new protective interventions have been shown to reduce the damaging effects of brain injury; nevertheless, these benefits have not been confirmed in clinical studies. In this vein, the use of posttraumatic hypothermia has yielded promising results, yet it was found ineffective in a national multicenter clinical trial. The reasons for this initial failure were multiple; however, based upon promising results in a subset analysis of the patient population studied in this trial, Clifton and colleagues have initiated a new trial of hypothermia in TBI with more rigorous inclusion and treatment criteria (Clifton, personal communication, 2005).

Traumatic brain injury is well known to trigger multiple brain parenchymal and vascular responses. Important among these are sustained cerebral vascular alterations associated with impaired vascular responses to vasodilators. Such vascular impairment is assumed to play a role in the brain’s increased vulnerability to secondary insults after TBI as the impaired vessels cannot adequately respond to secondary challenges that include hypoxia or transient ischemia. To date, it is known that hypothermia initiated early after injury reverses the
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injury-induced impaired vascular reactivity to the vasodilator ACh and hypercapnia.21,22 In the clinical setting, early posttraumatic treatment is not feasible because of the time lapses that typically occur between the traumatic episode and hospital admission. In view of this, the initiation of delayed hypothermic intervention was pursued in the laboratory with initial research suggesting that the hypothermic treatment was only partially successful when the initiation of treatment was delayed for 1 hour after the experimental TBI. Additional studies, however, indicated that even this delayed hypothermic treatment could prove efficacious in reducing TBI-induced vascular abnormalities if the duration of the hypothermic period was prolonged and if it was followed by slow rewarming.22 These findings parallel similar reports of the use of hypothermia in the stroke literature, where it has been demonstrated that delayed but prolonged hypothermia can be neuroprotective.4,5

In addition to the posttraumatic vascular protective effects of hypothermia, benefit may also be obtained through the application of free radical scavengers, which are also known to protect against injury-induced brain damage and vascular abnormalities.15,17,24 Like hypothermia, free radical scavengers have been most effective when used early in the posttraumatic period.17 The lack of prolonged protection was consistent with the observation that the surge in posttraumatic perivascular, oxygen radical production, triggered by the accelerated metabolism of arachidonic acid, was relatively brief (<30 min).17 Given that the use of both hypothermia and oxygen radical scavengers proved efficacious only when applied early postinjury, the question arose as to whether a combined therapeutic approach would prove more efficacious and/or extend the therapeutic window. As hypothermia is known to decrease metabolism while blunting oxygen radical production,15,19 it appeared rational that the use of delayed hypothermia could provide initial partial vascular protection while elongating the therapeutic window over which free radical scavengers could exert delayed and perhaps enhanced protection. To this end, we examined the combined effects of delayed administration of a free radical scavenger (SOD) and delayed hypothermia on the injury-induced pial vascular reactivity. Following TBI, the initiation of hypothermia was delayed for 60 minutes, with the animals then receiving a delayed administration of SOD followed by slow rewarming to normothermia. To validate the effects of such a combined treatment approach, single and double cranial windows were installed. The 2-window approach allowed us to apply SOD in only 1 of 2 windows, enabling comparison of pial vascular reactivity to vasodilators between hemispheres in the same animal, while also controlling for the injury severity and the extent of a hypothermic intervention.

Methods

Surgical Preparation

All experiments were carried out in a fashion consistent with the policies of the animal care and use committee of Virginia Commonwealth University. Male Sprague–Dawley rats, weighing ~300–380 g were anesthetized with sodium pentobarbital (60 mg/kg administered intraperitoneally). The femoral artery and vein were cannulated with a PE50 catheter for the continuous arterial BP monitoring and the periodic administration of pharmacological agents and blood gas evaluations. Blood gases and pH were determined prior to each measurement of the pial vessel diameter described below. A tracheotomy was performed and each animal was placed on a respirator on room air. Pancuronium bromide (3 mg/kg) was then administered intravenously to produce skeletal muscle paralysis. A PCO2 of 35–40 mm Hg was maintained throughout the experiment by modifying the rate or volume of the respirator. Body temperature was measured by a rectal probe, and brain temperature was monitored by a thermistor placed in the temporalis muscle.

Single Cranial Window Placement

Animals underwent placement of a single cranial window using protocols described in detail elsewhere.18,19 In brief, a midline sagittal incision was performed to expose the skull, which was cleaned and dried. A 2 × 4–mm craniotomy was then completed over the left parietal bone, and the dura mater was cut. A cranial window, consisting of a glass plate inside a metal ring, was placed over the craniotomy site, and fixed in place by bone wax and dental acrylic, allowing for the visualization and measurement of the underlying pial arterioles. The cranial window, which was filled with artificial CSF, contained 3 outlet/inlet ports. One port allowed for the delivery of ACh and other drugs, another served as a disposal outlet, and the third was connected to the Statham pressure transducer for the ICP measurement. The ICP was preset at 5 mm Hg, and the CSF pH was adjusted to 7.35. The pial resting diameter was measured in at least 4 different arterioles using a Vickers image-splitting device, and the resting vessel diameter was recorded preinjury and at 4, 5, and 6 hours postinjury for each pial arteriole.

Dual Cranial Window Placement

In the current study, the single cranial window placement procedure was modified to also allow the use of 2 separate cranial windows. These 2 separate windows were used to compare the vessel reactivity between windows in the same animal under different conditions. To this end, 2 craniotomies were performed, one on each side of the central suture. Using bone wax, a dividing barrier was built between the craniotomies. Modified glass plates were cut to fit over each craniotomy site and were affixed to the skull bone with additional bone wax and dental acrylic. The isolation of the windows, with respect to their CSF environment, was tested using a topical dye (Evans Blue) at the end of the experiment. The retention of the dye in one window indicated no leakage into the contralateral window, which was assumed to reflect the isolation of the window’s CSF and microvascular compartments.

Vasodilator Challenge

Vasoreactivity to hypercapnia was evaluated in half
of the animals while they were receiving mechanical ventilation with room air. Both 3% and 5% CO₂-containing gases were used for at least 7 minutes to induce PCO₂ change in the animals. Following the completion of those studies focusing on the different levels of hypercapnia, the same pial arterioles were assessed for their vasoreactivity to ACh. These responses were measured using 2 concentrations of ACh (0.1 µM and 10.0 µM) dissolved in artificial CSF. Using the inlet ports described above, different concentrations of ACh were applied to the pial surface for 2–4 minutes before the vessel diameter measurements were recorded. For both the hypercapnia and ACh studies, any observed change in vascular diameter was expressed as a percent change from the resting diameter at that time interval.

**Traumatic Brain Injury**

After the baseline/resting vessel diameter and ACh-induced or hypercapnia-induced vessel diameter were measured, the rats were subjected to impact acceleration injury, described in detail elsewhere. In brief, after the completion of the pial vessel measurements, the cranial window(s) were gently removed. Exposed brain tissue was covered with a CSF-rinsed cotton cloth and a stainless steel helmet was secured with the dental acrylic over the craniotomy site. The rat was placed on a foam pad under the 2.5-m Plexiglas tube in such a way that the steel disk was centered directly under the 450 g brass weight, which was released to impact the helmet from 2 m. The rat was returned to the respirator immediately after the injury and reconnected to the BP-monitoring device. Five minutes after the injury, the steel helmet was removed and the cranial window(s) were reinstalled over the craniotomy sites. Such injuries were considered of moderate severity as they triggered vascular dysfunction without contusional change or any overt brain tissue damage. Histopathological analyses typically revealed the presence of traumatic axonal injury. Despite these axonal and vascular abnormalities, however, these injuries were not associated with any deaths.

**Experimental Design**

In this investigation, 2 independent protocols were used in the animals subjected to TBI. These animals were treated with combinations of hypothermia and/or SOD (60 U/ml). In Study 1, independent groups of animals, equipped with a single cranial window, were analyzed, with 1 group containing animals that were injured and received a delayed hypothermic intervention without SOD administration and the other containing animals injured and treated with delayed hypothermia as well as delayed topical application of SOD. In Study 2, animals receiving hypothermic interventions were equipped with dual cranial windows, allowing for the assessment, in the same animal, of any potential additive protective effects of SOD. In this approach, the effects of topically applied SOD were assessed in one window and compared with results in the opposite untreated window. The use of these 2 different study groups allowed us to critically assess the effects of hypothermia and SOD, not only in different groups of animals but also in the same animal population.

Throughout all experiments, normothermic temperatures were maintained at 37°C, while hypothermia was maintained at 32°C. Core temperatures were measured with a rectal thermometer, and brain temperature was monitored with the temporalis muscle thermometer. The resting diameter of the vessels was measured before the injury and then again before each test with ACh or hypercapnia at 4, 5, and 6 hours after TBI. The animals were randomly assigned to one of the following groups.

**Study 1**

**Sham Group.** Animals in this group (6 rats) were not subjected to impact acceleration injury, but they did undergo surgery as well the same surgical preparation procedures as the animals in the other groups. Anesthesia was induced, mechanical ventilation was initiated, and then a craniotomy was performed and the cranial window was placed. The rats’ body temperature was maintained at normothermic levels at 37°C during the vessel measurements in response to ACh and hypercapnia throughout the time course.

**Injured, Untreated Group.** Animals in this group were prepared for surgery in the manner described above (as were all animals in this study) and a craniotomy was performed for pial measurements. The animals were then subjected to an impact acceleration injury and maintained at normothermic levels during the pial measurements. The vasodilator responses to ACh (6 rats) and hypercapnia (6 rats) were measured preinjury and at 4, 5, and 6 hours postinjury. No treatment was administered to the animals in this group.

**Injured, SOD-Treated Group.** Animals in this group (6 rats) were subjected to an impact acceleration injury and maintained at normothermic levels during the pial measurements. Superoxide dismutase (60 U/ml; 3000 U/mg protein from bovine blood; Sigma-Aldrich Co.) dissolved in artificial CSF was administered into the CSF-containing space underlying cranial window through the inlet 90 minutes after the injury. In this fashion, the topically applied SOD bathed the cerebral arterioles that were evaluated. The vascular response to ACh and hypercapnia was measured preinjury and postinjury at 4, 5, and 6 hours postinjury.

**Injured, Delayed Hypothermia Group.** Animals in this group were subjected to an impact acceleration injury and 1 hour postinjury were cooled to 32°C with ice packs and maintained at hypothermic levels for 1 hour. Animals were slowly rewarmed with a heat lamp increasing the body and brain temperatures by 1°C per 20 minutes. The vascular responses to ACh (6 rats) and hypercapnia (6 rats) were measured preinjury and at 4, 5, and 6 hours postinjury.

**Injured, Delayed Hypothermia, and SOD Group.** Animals in this group were subjected to an impact acceleration injury and 60 minutes postinjury were cooled to 32°C with ice packs. Hypothermia was maintained for 60
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minutes, then the animals were slowly rewarmed with a heat lamp, with body temperature being increased by 1°C per 20 minutes. In addition to this hypothermic treatment, animals received topically administered SOD through one of the inlets on the cranial window 15 minutes before the end of the hypothermic treatment. Vessel measurements in response to ACh (6 rats) and hypercapnia (6 rats) were taken preinjury and 4, 5, and 6 hours after TBI.

**Study 2**

**Injured, Delayed Hypothermia, and SOD/Cranial Windows Group.** For this study, the animals (8 rats) were anesthetized and prepared for the double window installation. A cranial window was built with 2 independent chambers for the infusion of CSF, ACh, or SOD. Each animal was then injured and at 1 hour after the injury cooled to 32°C with ice packs. The hypothermia was maintained for 1 hour with slow rewarming back to normothermic temperature levels as described above. Baseline/resting diameters were measured in at least 4 different cerebral arteries in each of the 2 windows in every animal. Responses to ACh were tested in both windows to ascertain that no side differences in responsiveness existed. The use of the dual windows allowed for the measurement of the vessel diameter in 2 different windows. Infusion of SOD 105 minutes after TBI (15 minutes prior to cessation of the hypothermic treatment) occurred in only 1 of the 2 windows, permitting the assessment of the vessels with SOD treatment in 1 window and without the SOD treatment in the other window. This dual window preparation served as an internal control since the animal was injured and then kept hypothermic at 32°C for an hour but was treated with SOD in only 1 of the 2 windows, allowing a comparison of the vessel reactivity between the 2 different windows within the same animal.

**Statistical Analysis**

All values were expressed as means ± SEM. To compare the baseline measurement to measurements obtained at each of the successive time points, a paired t-test was used. To compare differences between treatments, the data were tested with an analysis of variance followed by a Duncan post hoc test for multiple comparisons between the treatment groups.

Differences were considered significant at probability values < 0.05.

**Results**

The physiological parameters of mean arterial BP and blood gases did not differ significantly between groups (data not shown). Animals maintained PaCO₂ at ~35 mm Hg, with elevations to 50 mm Hg when inhaling 3% CO₂ and up to 60 mm Hg with 5% CO₂ inhalation.

**Study 1**

The use of 2 concentrations of ACh (0.1 μM and 10.0 μM) in the sham-injured animals resulted, respectively, in 10 and 20% increases in the vessel diameter (Fig. 1). In the injured, untreated group, the vasodilatory response was significantly diminished in comparison with baseline measurements, and this reduced response persisted at 4, 5, and 6 hours after TBI for both 0.1 μM ACh (p < 0.005) and 10.0 μM ACh (p < 0.05). Rats treated with SOD 90 minutes after the injury also showed a significant reduction in vasodilator response (compared with baseline values) for both concentrations of ACh at all time points (p < 0.005). Animals in the injured hypothermia-treated group (Fig. 1) demonstrated a significantly reduced vasodilator response at 5 and 6 hours at the lower concentration of ACh (p < 0.005) and at all time points at the high ACh concentration (p < 0.001). However, the vasodilator response in the injured hypothermia and SOD-treated animals was not significantly different from the baseline measurement at all time points for both concentrations of ACh.

Using analysis of variance for between-group comparison, animals treated with a 60-minute long hypothermic intervention at 32°C, initiated 60 minutes after TBI and those treated with SOD only, revealed no significant enhancement of the microvessel function in response to ACh in comparison with the animals in the sham group. A slight trend for vascular protection was demonstrated in comparison with the injured untreated animals, yet this response was still significantly diminished in comparison with the sham group data (p < 0.05). Animals treated with hypothermia in combination with a topical application of SOD maintained a vasodilator response that was not significantly different from the animals in the sham group (Fig. 1).

Comparable to the observations obtained with the use of ACh, the vascular responses to hypercapnia (Fig. 2) were decreased in injured untreated animals in comparison with the baseline measurements (p < 0.005). The injured animals treated with delayed hypothermia and slow rewarming revealed only partial vascular protection, yet it was still significantly decreased when compared with baseline data (p < 0.05). However, this protection became virtually complete with the combined treatment of delayed hypothermia and SOD. In addition, between-group analysis indicated that injured animals treated with both hypothermia and SOD demonstrated significantly improved response (p < 0.05) to hypercapnia in comparison with injured untreated and injured hypothermia-treated groups.

**Study 2**

The effectiveness of the combination treatment was verified in the group of animals with the double cranial windows, with each one placed over a hemisphere and SOD infused into only 1 of the 2 windows (Fig. 3). In the animals treated with hypothermia after TBI, the vascular response to ACh in the window through which SOD was applied paralleled baseline data. In contrast, vascular measurements taken in the second cranial window, containing CSF alone, revealed a decreased vascular response to ACh (p < 0.05), despite the use of the hypothermic intervention.

**Discussion**

It is well established that TBI causes cerebral vascular
abnormalities and tissue damage. We previously investigated the efficacy of posttraumatic treatment of delayed hypothermia on cerebral microcirculation in brain-injured rats and reported that moderate hypothermia at 32°C for 1 hour partially protected the reactivity of cerebral arterioles, provided that the animals were rewarmed gradually.21 In the present study, we demonstrated in the same experimental setting (60 minutes of hypothermia initiated 60 minutes after injury) that the administration of SOD 15 minutes prior to the termination of the cooling period (~105 minutes after injury) provided added protection in terms of cerebral vascular function; whereas delayed application of SOD alone at 90 minutes postinjury was not associated with any therapeutic effect. Although evidence in the stroke literature suggests that hypothermia extends the time window for gene therapy and provides enhanced neuroprotection,12 to our knowledge, this is the first report of a comparable phenomenon in the context of vascular protection following TBI.

We previously reported that the time window for effective postinjury treatment in this rat model of TBI was ~1 hour after impact acceleration brain injury. Beyond this time, neither brief (1 hour) hypothermia nor drug treatment proved efficacious.22 While the exact mechanism through which the combination of delayed SOD and hypothermia treatment provides enhanced vascular protection is not known, it is clear that the use of hypothermia, possibly through reduced metabolism,1,9 extends the therapeutic window, allowing SOD to exert its protective effects via the scavenging of the oxygen radicals known to be released following TBI.11,15,24 The beneficial effects of delayed SOD application in this study may also imply that other pharmacological agents that have previously failed to provide significant posttraumatic therapeutic benefit, may now provide additional benefit to brain-injured animals and possibly patients, when given in combination with hypothermia treatment, an issue that mandates continued investigation.
We believe that our demonstration of the beneficial effects of hypothermia in extending the therapeutic window constitutes an important proof of concept. While immediate cooling following brain injury would be ideal and is easily initiated in the laboratory, we acknowledge that its application in patient care is unrealistic. However, because of an extended therapeutic window, the combination of hypothermia and other pharmacological agents merits further investigation so that more aggressive approaches in treating brain-injured patients can be considered. We recognize that prolonged hypothermic intervention, as reported by authors from other laboratories as well as our own, is more protective than short-term hypothermia. The use of prolonged hypothermia, however, poses its own problems as it is associated with potentially serious side effects, such as hypothermia-induced diuresis and electrolyte loss. Accordingly, an overall reduction in the duration of hypothermic treatment seems desirable, if it does not compromise effectiveness.

There is substantial evidence that the vascular injury in TBI is due to the generation of free radicals. The origin of superoxide anions in TBI is related to the accelerated metabolism of arachidonate via cyclooxygenase. The injured vessels dilate and display abnormal reactivity to both vasoconstrictor and vasodilator stimuli. The dilated vessels and the breakdown of blood–brain barrier favor the formation of edema, potentially triggering a possible secondary injury. There is evidence that hypothermia reduces the production of free radicals. However, it is unclear whether the production of free radicals continues to affect the cerebral vasculature following cooling, or whether the scavenging action of SOD is impeded in the presence of hypothermia. Our findings on the combined effects of hypothermia and SOD indicate that the release of free radicals most likely continues beyond 2 hours postinjury, and that the action of SOD is not impeded by the production of hypothermia. This premise was further confirmed in those experiments where double-window technique was used, wherein SOD was applied under one window in the same animal undergoing systemic hypothermic treatment following TBI. In response to known vasodilators, cerebral arterioles on the SOD-treated side consistently outperformed those on the nontreated side in every animal for up to 6 hours postinjury.

**Conclusions**

In conclusion, this study demonstrated that hypothermia and SOD after TBI in rats...
thermic treatment at 32°C initiated 60 minutes after TBI allowed SOD, applied 45 minutes after the initiation of hypothermia, to provide a virtually complete vascular protection. This finding was particularly striking in that, when administered alone at 90 minutes post-TBI, SOD did not improve dilator function in the pial vasculature. Similarly, hypothermic treatment alone, initiated 60 minutes after TBI, demonstrated only partial restoration of the vascular function, which was still significantly diminished from the sham animals. Collectively, these findings argue for the enhanced beneficial effects of combined therapy. The vascular response in the window with SOD was significantly enhanced (indicated by §, p < 0.05) compared with the response in the window without the SOD throughout the experiment. Data are expressed as means ± SEMs.

Fig. 3. Graph illustrating the vasodilator response to ACh in an injured group of animals surgically prepared with 2 cranial windows and demonstrating the importance of the synergistic effect of a hypothermic treatment together with an SOD application. Postinjury responses that were significantly different from the baseline responses are indicated by the an asterisk (p < 0.05). The bars with black vertical lines represent cerebral vascular response in the windows without SOD, and the checkered bars show the response in the windows with SOD. Since these data points were derived from the same animals, one can clearly see the added advantage of the SOD application when administered with the delayed hypothermic treatment. The vascular response in the window with SOD was significantly enhanced (indicated by §, p < 0.05) compared with the response in the window without the SOD throughout the experiment. Data are expressed as means ± SEMs.

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References
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