The neuronal protective effects of local brain cooling at the craniectomy site after lateral fluid percussion injury in a rat model

Che-Chuan Wang, MD,a,b,c,d Yuan-Shen Chen, MD,e Bor-Shyh Lin, PhD,f Chung-Ching Chio, MD,a Chiao-Ya Hu, BD,b and Jinn-Rung Kuo, MD, PhDa,b,g,*

a Department of Neurosurgery, Chi-Mei Medical Center, Tainan, Taiwan
b Department of Medical Research, Chi-Mei Medical Center, Tainan, Taiwan
c Institute of Photonic system, National Chiao-Tung University, Tainan, Taiwan
d Department of Child Care, Southern Taiwan University of Science and Technology, Tainan, Taiwan
e Department of Neurosurgery, National Taiwan University Hospital Yun-Lin Branch, Yunlin, Taiwan
f Institute of Imaging and Biomedical Photonics, National Chiao-Tung University, Tainan, Taiwan
g Department of Biotechnology, Southern Taiwan University of Science and Technology, Tainan, Taiwan

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Abstract
Background: The aim of the present study is to investigate whether local brain cooling at the craniectomy site causes attenuation of traumatic brain injury (TBI) induced by fluid percussion injury (FPI).

Methods: Anesthetized male Sprague–Dawley rats were divided into two major treatment groups. Immediately after the onset of fluid percussion TBI, a craniectomy window of 6 × 8 mm was made at the right parietal, and a cold water bag (0°C–1°C or 5°C–6°C) was applied locally for 30 min. Additional groups of rats were used as craniectomy and craniectomy + FPI controls. Physiological parameters, such as brain and colonic temperature, mean arterial pressure, and heart rate, were monitored during FPI. Functional motor outcomes were evaluated using the inclined plane test (maximal grasp angle). Cellular infarction volume was calculated using triphenyltetrazolium chloride staining. Apoptosis and neuronal marker–positive cells in the cortex were measured by immunofluorescence staining. All functional and morphologic parameters were assessed 72 h after injury.

Results: Compared with the craniectomy + FPI control groups, the groups treated with 5°C–6°C local cold water therapy showed significant attenuation of the FPI-induced motor deficits, weight loss, and cerebral infarction but no effect on colonic temperature. The FPI-induced apoptosis and neuronal loss were also significantly reduced by local cooling.

Conclusions: Our results suggest that local cooling with 5°C–6°C cold water therapy may ameliorate TBI in rats by reducing infarction volume, neuronal cell loss, and apoptosis, resulting in improved functional outcome. We propose that the use of local cooling at the craniectomy site after FPI might have clinical benefits in the future.

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*Corresponding author. Department of Neurosurgery, Chi-Mei Medical Center, 901 Chung Hwa Road, Yung Kang City, Tainan, Taiwan. Tel.: +886 6 281 2811; fax: +886 6 282 8928. E-mail address: kuojinnrung@gmail.com (J.-R. Kuo).

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1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability and remains a critical public health challenge [1]. TBI affects up to 2% of the population per year [2]. Despite aggressive treatment, an unfavorable outcome rate ranging from 30%–90% is reported [3,4]. Thus, developing a new and effective therapeutic strategy for head-injured patients remains an important issue.

Induced therapeutic whole-body hypothermia for TBI was first studied in 1943 [5]. Since this time, hypothermia has been shown to be protective after TBI. However, whole-body cooling has serious complications, such as severe shivering, arterial hypotension, cardiac arrhythmia, coagulopathy, and infection [6,7]. Therefore, selective cooling of the damaged brain and preventing complications related to whole-body cooling could be a promising strategy for TBI treatment [8–12].

Recently, we successfully demonstrated that selective brain cooling using retrograde jugular vein flush with 4°C saline immediately after TBI has neuronal protective effects without interfering with body temperature [13,14]. Although this technique is efficient for cooling the entire brain, its clinical application is limited because of its difficulty and the possible complications of cannulation. We, therefore, considered whether hypothermia could be performed non-invasively without inducing systemic complications but still effectively reduce intracranial temperature. This study aimed to collect data that would be useful for future innovation in hypothermic therapy.

Decompressive craniectomy (DC) is a common clinical practice and is considered to be a life-saving procedure in treating severe TBI patients [15]. Brain hypothermia induced by local ice-bag cooling is effective for the control of intracranial pressure (ICP) in patients who had previously undergone DC [16]. However, there is little knowledge regarding local brain cooling at the craniectomy site (LBCCS) therapy after TBI at this time.

To address this question, we conducted this investigation on the therapeutic effects of LBCCS of animals subjected to TBI. In addition, this study attempted to compare the temporal profiles of physiological change, neuronal loss, apoptosis, cerebral infarction, body weight loss, and motor deficits during TBI in rats with or without local cooling.

2. Materials and methods

2.1. Experimental design

Table summarizes the overall experimental procedures. The end point for our study was 72 h after TBI as most investigations indicate that severe lateral fluid percussion causes motor and cognitive dysfunction, which persists from 72 h–1 y after TBI [17].

2.2. Animals

Adult male Sprague–Dawley rats weighing 280–300 g were used in these experiments. The animals were kept under...
a 12-h light-to-dark cycle and allowed free access to food and water. All experimental procedures were approved by the Animal Research Committee of the Chi-Mei Medical Center. These procedures were performed in compliance with the regulations of the National Institutes of Health for the use of animals in research to minimize discomfort in the animals during surgery and recovery. At the end of the experiments, the control and other rats that survived TBI were sacrificed with an overdose of urethane.

2.3. TBI

The animals were anesthetized with an intraperitoneal administration of sodium pentobarbital (50 mg/kg), ketamine (intramuscularly [IM], 44 mg/kg), Xylazine hydrochloride (Rompun) (IM, 6.77 mg/kg), and atropine (IM, 0.026 mg/kg). A craniectomy 2 mm in radius, 4 mm from bregma, and 3 mm from sagittal sutures over the right parietal cortex was performed via stereotaxic frame. After the craniectomy and implantation of injury cannula, the fluid percussion device was connected to the animal via the Luer-lock fitting, and the brain was injured with a 25-ms percussion of 2.0–2.2 atm. This produces a moderate-severity brain trauma as originally described by McIntosh et al. [18]. A transient hypertensive response, apnea, and seizure were observed immediately after fluid percussion injury (FPI), and this combination was used as the inclusion criterion for successful FPI. The animals were then separated into FPI + craniectomy or FPI + craniectomy + LBCCS groups.

2.4. Surgery and physiological parameter monitoring

The rats’ right femoral artery was cannulated with polyethylene tubing under sodium pentobarbital anesthesia for blood pressure monitoring. All recordings were made on a four-channel Gould polygraph. The rats’ core temperatures were monitored continuously using a thermocouple, and the mean arterial pressure (MAP) and heart rate (HR) were continuously monitored using a pressure transducer. Frontal cortex brain temperature was monitored using a digital electric thermometer (model DP80; Omega Engineering, Inc, Stanford, CT) with a 0.15-mm-diameter temperature probe (model YP-033-1-T-G-60-SMP-M; Omega Engineering, Inc, Stanford, CT) inserted 4.0 mm ventral to the surface of the skull. The probe was removed before FPI and replaced immediately after injury. Colon temperatures were measured with an analog electronic thermometer (model 43 TE; YSI, Inc, Yellow Springs, OH) and temperature probe (series 400; YSI, Inc).

2.5. Experimental groups

The animals were randomly assigned to craniectomy, FPI plus brain craniectomy, FPI plus brain craniectomy and local cooling with 5°C–6°C of cold water, and the FPI plus brain craniectomy and local cooling with 0°C–1°C of cold water groups. All tests were blinded, and the animal codes were revealed only at the end of the analyses. The animals were treated with local cooling after the craniectomy procedure was completed (15 min after FPI), and they then received local cooling for 30 min. The craniectomy window was of 1-mm size and located 7 mm from bregma and 3 mm and 8 mm from sagittal sutures in the right parietal cortex; the craniectomy was performed via stereotaxic frame. The cold water temperature was monitored using an analog electronic thermometer (model 43 TE; YSI, Inc) and temperature probe (series 400; YSI, Inc). For testing the safety of local cooling therapy, two groups, including craniectomy + local cooling with 0°C–1°C and 5°C–6°C of cold water, were assigned.

2.6. Cerebral infarction assay

Infarct volume was assessed using 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St Louis, MO) staining as demonstrated previously [19]. All animals were sacrificed 3 d after injury. The brain slices, in 2-mm sections, were incubated in 2% TTC dissolved in phosphate-buffered saline (PBS) for 30 min at 37°C and then transferred to a 5% formaldehyde solution for fixation. The volume of infarction, as revealed by negative TTC stains (pale color), was measured in each slice and summed using computerized planimetry (personal computer–based Image Tools software; Media Cybernetics, Inc). The infarction volume was calculated as 2 mm (slice thickness) × (sum of the infarction area in all brain slices [mm²]).

2.6.1. Cell apoptotic assay

Three days after injury, apoptotic cells were identified by staining with terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL) [20] using previously described procedure [21]. The number of TUNEL-positive cells was calculated in five coronal sections from each rat in the samples and summed using computerized planimetry (personal computer–based Image Tools software). The following antibodies were used in this study: monoclonal mouse anti–neuronal-specific nuclear protein (NeuN, MAB377; Chemicon Millipore Corporation, Billerica, MA) and Alexa Fluor 568 anti-mouse IgG (A11031; Life Technologies Co, Grand Island, NY).

2.6.2. Neuronal loss in cortex

Adjacent 50-μm sections corresponding to coronal coordinates 0.20 –0.70 mm anterior to bregma were incubated in 2 mol/L of HCl for 30 min, rinsed in 0.1 mol/L of boric acid (pH 8.5) for 3 min at room temperature, and then incubated with primary antibodies in PBS containing 0.5% normal bovine serum at 4°C overnight. After being washed in PBS, the sections were incubated with secondary antibodies for 1 h at room temperature. The following antibodies were used in this study: mouse anti-NeuN at 1:200 dilution and Alexa Fluor 568 goat anti-mouse IgG. The sections were then washed in PBS with 0.6% Triton X-100, incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG in 1% bovine serum albumin/PBS/0.1% Triton X-100 for 60 min, and mounted with anti-fade mounting media. The number of labeled cells was calculated in five coronal sections from each rat and expressed as the mean number of cells per section. For negative control sections, all procedures were performed in the same manner without the primary antibodies.
2.7. Functional outcome

An inclined plane was used to measure limb strength. The animals were placed facing right and then left, perpendicular to the slope of a 20 × 20 cm rubber ribbed surface of the inclined plane starting at an angle of 55° [22]. The angle was increased or decreased in five increments to determine the maximal angle at which an animal could hold onto the plane. The measurement for each day was the mean of the left- and right-side maximal angles.

2.8. Statistical analysis

Immunoreactive cell count and lesion volumes were evaluated for Gaussian (normal) distribution and expressed as means ± standard errors of the mean. The data were analyzed with one-way analysis of variance and if \( P < 0.05 \), analyzed using a Newman–Keuls post hoc test. Differences were considered to be statistically significant when \( P < 0.05 \). All data were analyzed with SigmaPlot, version 11.0, for Windows (Systat Software, San Jose, CA).

3. Results

3.1. Acute effects of FPI

The average impact intensity of the fluid pulse delivered to animals in the injured group was 2.09 ± 0.02 atm (mean ± standard error of the mean), and the impact time was 0.0246 ± 0.0004 s. Immediately after this impact, all rats experienced a transient of apnea (38.8 ± 9.0 s), hypertension (up to approximately 140 mm Hg), and tachycardia (approximately 400 beats/min). Craniectomy control animals showed no apnea, hypertension, or tachycardia. There was no difference between the two treatment groups.

3.2. LBCCS affected neither MAP nor HR after FPI

The craniectomy + local cooling (0°C–1°C and 5°C–6°C) with cold water groups showed decreased MAP, HR, and colon temperature within an 80-min timecourse, but these effects were not significant (Fig. 1).

3.3. LBCCS significantly decreased brain temperature but not rectal temperature

The craniectomy + FPI group maintained their rectal temperature and insignificantly decreased their brain...
temperature, to 36.5°C and 34.5°C, respectively, over an 80-min timecourse. Both the craniectomy + local cooling groups showed significant decreases in brain temperature over an 80-min timecourse, with temperatures of 32.5°C to 33.5°C and 31.5°C to 33.0°C for the 5°C to 6°C and 0°C to 1°C of cold water-treated groups, respectively (Fig. 2).

3.4. LBCCS with 5°C–6°C of cold water significantly reduced the FPI-induced cerebral infarction volume

The TTC-stained sections, 3 d after FPI, showed a significant increase in the infarcted area in the craniectomy + FPI group compared with that in the craniectomy controls. The FPI-induced infarction volume was significantly decreased by LBCCS with 5°C–6°C of cold water treatment but not by the 0°C–1°C ice-water treatment ($P < 0.05; n = 6$) (Fig. 3).

Fig. 3 – The effects of local cooling on the FPI-induced infarction volume in the ischemic cortex 3 d after FPI. *$P < 0.05$, craniectomy group compared with the FPI + craniectomy group; +$P < 0.05$, FPI + craniectomy group compared with the FPI + craniectomy + local cooling (5°C–6°C) group. LC = local cooling.

To examine the safety of local cooling, we tested the craniectomy control groups receiving LBCCS treatment with 0°C–1°C or 5°C–6°C of cold water without FPI. These results showed no significant difference in infarction volume between the 0°C–1°C and 5°C–6°C groups (Fig. 3).

3.5. LBCCS with 5°C–6°C of cold water significantly decreased the FPI-induced apoptosis

Based on the results of the infarction volume, we next evaluated the effect of LBCCS with 5°C–6°C of cold water on FPI-induced apoptosis, neuronal loss, body weight loss, and motor deficiency. The craniectomy + FPI group exhibited a significant increase in the number of TUNEL-positive cells in the ischemic cortex 3 d after FPI compared with the craniectomy control group (96.8 ± 11.7 versus 0; $P < 0.01, n = 6$). The craniectomy + FPI + local cooling (with 5°C–6°C of cold water) group had significantly fewer TUNEL-positive cells compared with the craniectomy + FPI group (56.7 ± 1.5 versus 96.8 ± 11.7; $P < 0.05, n = 6$) (Fig. 4).

3.6. LBCCS with 5°C–6°C of cold water significantly decreased the FPI-induced neuronal loss

The craniectomy + FPI group exhibited significantly fewer NeuN-positive cells in the ischemic cortex 3 d after FPI than the craniectomy control group (67.3 ± 8.1 versus 180.7 ± 11.6; $P < 0.01, n = 6$). The craniectomy plus FPI and local cooling
(5°C–6°C of cold water-treated) group had significantly more NeuN-positive cells than the craniectomy plus FPI group (121.0 ± 8.7 versus 67.3 ± 8.1; *P < 0.05, n = 6) (Fig. 5).

3.7. **LBCCS with 5°C–6°C of cold water significantly decreased the FPI-induced body weight loss**

The body weight loss 3 d after FPI was significantly greater than that in the craniectomy controls (31.5 ± 4.2 g versus 52.1 ± 2.1 g; *P < 0.05; n = 6). The FPI-induced body weight loss was significantly decreased by the treatment of LBCCS with 5°C–6°C of cold water after FPI (52.1 ± 2.1 g versus 39.5 ± 3.5 g; *P < 0.05; n = 6) (Fig. 6).

3.8. **LBCCS with 5°C–6°C of cold water significantly attenuates FPI-induced motor deficits**

Three days after the FPI, behavioral tests revealed that rats in the craniectomy + FPI group performed significantly worse on a motor function test than the craniectomy controls (54.5 ± 1.5 versus 60.0 ± 1.4; *P < 0.05; n = 6). The TBI-induced motor dysfunction was significantly reduced by LBCCS with 5°C–6°C of cold water in an ice bag (54.5 ± 1.5 versus 59.5 ± 0.9; *P < 0.05; n = 6) (Fig. 7).

**4. Discussion**

4.1. **Novelty of the present study**

In the present study, LBCCS with 5°C–6°C of cold water counteracted the deficits induced by TBI in rat, including infarction volume, apoptosis, neuronal survival in the ischemic cortex, and neurologic deficits, without affecting the systemic temperature or inducing systemic complications. To the best of our knowledge, this is the first study to report brain temperature profiles using local brain cooling methods in a traumatic central nervous system injury experimental model.
4.2. Safety of the LBCCS

As the clinical implications could be very serious, the safety of selective LBCCS with 5°C–6°C of cold water should be evaluated. First, in the present study, we cooled only the damaged brain without affecting the systemic temperature. The complications of whole-body cooling, such as severe shivering, increased incidence of arterial hypotension, cardiac arrhythmia, hemorrhage, and infection [6,8], did not occur in the present study. Second, when the control animals (no FPI) were exposed to local brain cooling (5°C–6°C), although the infarction volume was small (7 ± 2 mm³), the overall LBCCS with 5°C-6°C of cold water significantly reduced FPI-induced cerebral infarction volume 3 d after FPI. We consider that LBCCS is likely to be safe at least in the short term; nevertheless, the long-term complications must be evaluated.

4.3. Rationale for LBCCS

Besides the use of DC, the management guidelines for serious TBI recommended by the Asia-Oceania Neurotrauma Society include the management of ICP and cerebral perfusion pressure, as well as the use of sedatives [23]. However, after TBI, most patients do not require a craniectomy. If selective brain cooling is considered, whole-brain cooling, such as retrograde jugular vein flush with 4°C saline [8,13,24] and intracarotid artery with 5°C cold solution perfusion [25], do not require this kind of surgical procedure. Cooling on the craniectomy site may have clinical limitations. However, DC has been considered to be a life-saving procedure after moderate to severe TBI [15,26]. After DC was performed, ICP has been shown to decrease and the cerebral perfusion pressure to increase, with these effects being maintained at levels that were ideal for patients [27,28]. Furthermore, the removal of skull bone and duraplasty at the brain injury site facilitates the heat transfer by conduction,
convention, and radiation from the brain parenchyma, allowing for more effective neuronal cooling [16]. Thus, DC in certain cases may be beneficial if it is clinically indicated.

Consistent with the study by Prandini et al. [29], using a middle cerebral artery occlusion animal model, in the present study, LBCCS reduced the infarction volume in an experimental rat TBI model. This result also showed that local cooling on the previously performed craniectomy site may prevent secondary brain damage when craniectomy is clinically indicated.

Compared with whole-brain cooling by retrograde jugular vein flush with 4°C saline [8,13,24], intracarotid artery with 5°C cold solution perfusion [25], and cooling cuff containing cold circulating water wrapped around the bilateral common carotid arteries [30], our LBCCS method is noninvasive, concerns less complications, and is easily performed and effective. We believe that the least invasive and most selective methods of hypothermia hold the greatest promise of becoming practical measures for providing neuroprotection after TBI.

Similar to the previous results using invasive retrograde jugular vein flush brain cooling in a rat model [8,22,31] and epidural cooling in a monkey model [32], in the present results, LBCCS with 5°C–6°C of cold water for 30 min after FPI significantly induces rapid brain cooling (5 min to 32°C and 20 min to 33.5°C for approximately 80 min) and improves outcome of severe TBI in the rat. At the same time, the systemic temperature was insignificantly changed (maintained at approximately 36°C). Thus, it appears that LBCCS specifically induces brain cooling without interfering with body temperature and would thus be safer and not require intensive care. Because local cooling is a simple procedure that is frequently used in the intensive care unit for fever control, we propose that LBCCS may be useful in clinical applications in TBI patients who have undergone DC for neuroprotection.

4.4. Local brain cooling effects on neuroprotection

In the present study, consistent with previous whole-brain cooling studies (mean brain temperature of 33°C) [8,13], LBCCS with 5°C–6°C of cold water (maintaining the brain temperature at 32.5°C–33.5°C) was neuroprotective. This result was demonstrated by the preservation of neurons and the reduction of apoptosis and infarction volume after TBI. Other mechanisms of neuroprotection provided by whole-brain cooling after brain injury were reported to be by reducing brain lactate accumulation [8]; reducing brain nitrosative and oxidative damage [14]; modulating reactive astrogliosis, microgliosis, caspase 3 activation, neuronal apoptosis, and neurogenesis [13]; and attenuating systemic inflammation, activated coagulation, and tissue ischemia and injury [33]. It is likely that LBCCS with 5°C–6°C of cold water may act through similar mechanisms to improve the outcome of TBI. Based on our results, we propose that maintaining brain temperature at 32.5°C–33.5°C may be the threshold temperature required to obtain a therapeutic effect. However, this hypothesis requires verification.

Why was the LBCCS with 0°C–1°C of cold water ineffective at decreasing infarction volume and functional outcome? In the present study, the brain temperature stayed in the range of 31.5°C–33.0°C on LBCCS with 0°C–1°C of cold water. Whether cold injury using 0°C–1°C of cold water at the craniectomy site induced brain injury, such as reduction in regional cerebral blood flow, increased lactate-to-pyruvate ratio and local tissue hypoxia [34], or severe impairment of
the microcirculation and brain–blood barrier breakdown [35,36], was involved in our TBI model remains to be evaluated.

4.5. Limitations of the present study

Several drawbacks of LBCCS should be considered. First, the uneven distribution of cooling to various brain regions would make LBCCS ineffective for injuries to deeper structures [37]. Second, severe TBI is often complicated by intracranial hypertension and usually involves damage to both sides of the brain rather than just the unilateral DC site. Clinically, patients who require craniectomy have extensive brain injury and edema. The injury is usually not localized to one part of the brain [4]. How effective local cooling will be in the setting of extensive systemic injury must be evaluated in the future. Therefore, further research is clearly indicated to delineate the risks and benefits associated with LBCCS therapies. Third, application of local cooling to a craniectomy site that was created immediately after the injury may have no clinical correlation. Although we have successfully demonstrated the beneficial effects of LBCCS on a TBI animal model, the therapeutic window of LBCCS must be evaluated, comparing animals craniectomized immediately after TBI or with a delay of 1, 3, or 8 h as recommended by Plesnila [38]. These concerns would suggest that the clinical applicability and effectiveness of LBCCS are premature.

5. Conclusions

Our results suggest that local cooling therapy with 5°C–6°C of cold water in an ice bag may ameliorate TBI in rats by reducing infarction volume, neuronal loss, and neuronal apoptosis, resulting in improvements in functional outcome and body weight loss. We also suggest that local cooling shortly after craniectomy may have clinical benefits.

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