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Research Article

Attenuation of Brain Nitrosative and Oxidative Damage by Brain Cooling during Experimental Traumatic Brain Injury

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The aim of the present study was to ascertain whether brain cooling causes attenuation of traumatic brain injury by reducing brain nitrostative and oxidative damage. Brain cooling was accomplished by infusion of 5 mL of 4°C saline over 5 minutes via the external jugular vein. Immediately after the onset of traumatic brain injury, rats were randomized into two groups and given 37°C or 4°C normal saline. Another group of rats were used as sham operated controls. Behavioral and biochemical assessments were conducted on 72 hours after brain injury or sham operation. As compared to those of the sham-operated controls, the 37°C saline-treated brain injured animals displayed motor deficits, higher cerebral contusion volume and incidence, higher oxidative damage (e.g., lower values of cerebral superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, but higher values of cerebral malondialdehyde), and higher nitrostative damage (e.g., higher values of neuronal nitric oxide synthase and 3-nitrotyrosine). All the motor deficits and brain nitrostative and oxidative damage were significantly reduced by retrograde perfusion of 4°C saline via the jugular vein. Our data suggest that brain cooling may improve the outcomes of traumatic brain injury in rats by reducing brain nitrostative and oxidative damage.

1. Introduction

Evidence has suggested that whole-body cooling prevents oxidative damage after traumatic brain injury (TBI) [1], hemorrhage shock [2], and transient focal cerebral ischemia [3]. Oxidative damage is caused by nitric oxide (NO), hydroxyl radical (OH), and peroxynitrite ($ONOO^-$). Nitric oxide is produced in a reaction that converts arginine to citrulline under control of inducible nitric oxide synthase (iNOS) whereas hydroxyl radicals are cleared by superoxide dismutase (SOD). Superoxide reacts with NO to produce peroxynitrite which reacts with tyrosine to produce 3-nitrotyrosine (3-NT).

Whole body cooling cools the body and the blood and then cools the brain. We have demonstrated that hypothermic retrograde jugular vein flush (HRJVF) without cardiopulmonary bypass reduces both oxidative damage and cerebrovascular dysfunction during heat stroke in rats [4, 5]. Although whole body cooling is effective in reducing oxidative damage after TBI in rats [1], it is interesting to note whether the oxidative damage that occurred during TBI can be affected by selective brain cooling caused by HRJVF in the rat.

The aim of the present study was to investigate the effect of HRJVF caused by infusion of 4°C cold saline via the external jugular vein on oxidative damage that occurred

during TBI. Brain levels of malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione reductase (GR), SOD, catalase, iNOS, and 3-NT were measured as indicators of oxidative stress.

2. Materials and Methods

Adult male Sprague Dawley rats weighing 299–351 g were used in these experiments. Animals were kept under a 12-h/12-h light/dark cycle and allowed free access to food and water. All experimental procedures conformed to National Institute of Health guidelines and were approved by the Chi Mei Medical Center Animal Care and Use Committee to minimize discomfort to the animals during surgery and in the recovery period. Animals were randomly assigned to sham group ($n = 8$), untreated TBI normothermic group ($n = 8$), or TBI hypothermic group ($n = 8$). All tests were run blinded, and the animal codes were revealed only at the end of the behavioral and histological analyses. In TBI normothermic or hypothermic groups, animals were treated with 37°C or 4°C saline (1.7 mL/100 g of body weight over 5 minutes) via the right external jugular vein (cranial direction), respectively, and immediately after injury. Animals used for histological or behavioral studies were provided food and water *ad libitum* throughout the study.

Animals were anesthetized with sodium pentobarbital (25 mg/kg, i.p.; Sigma Chemical Co. St Louis, MO) and a mixture containing ketamine (44 mg/kg, i.m.; Nan Kuang Pharmaceutical, Tainan, Taiwan), atropine (0.02633 mg/kg, i.m.; Sintong Chemical Industrial Co. Ltd. Taoyuan, Taiwan), and xylazine (6.77 mg/kg, i.m.; Bayer, Leverkusen, Germany). The external jugular vein on the right side was cannulated with polyethylene tubing. After cannulation, the wound was sutured, and the animals were turned to the prone position. The animals were placed in a stereotoxic frame, and the scalp was incised sagittally. Animals were subjected to a lateral TBI [6]. After an incision in the scalp was made, a 4.8-mm circular craniotomy was performed midway between lambda and bregma 3.0 mm to the right of the central suture. A modified Luer-Lock connector (trauma cannula), 2.6 mm inner diameter, was secured into the craniotomy with cyanoacrylic adhesive and dental acrylic. A moderate TBI (2.2 atm) was produced by rapidly injecting a small volume of saline into the closed cranial cavity with a fluid percussion device (VCU Biomedical Engineering, Richmond, VA, USA). The animal was removed from the device, the acrylic removed, and the incision sutured. Each injured and sham-injured animals for fluid percussion model was closely evaluated immediately after FPI for behavioral recovery.

Experiment 1, brain cooling was randomly performed immediately after TBI, and their effects on both motor function and infarction volume were assessed 3 days after TBI.

In experiment 2, brain cooling was randomly performed immediately after TBI, and their effects on the concentrations of MDA, SOD, GR, GPx, and catalase in the ischemic cortex were assessed 3 days after TBI.

In experiment 3, brain cooling was randomly performed immediately after TBI, and their effects on the amounts of both iNOS-positive cells and 3-NT-positive cells in the ischemic cortex were assessed 3 days after TBI.

The inclined plane was used to measure limb strength. Animals were placed, facing right and then left, perpendicular to the slope of a 20 × 20 cm ruffer ribbed surface of an inclined plane starting at an angle of 55°C [7]. The angle was increased or decreased in 5° increments to determine the maximal angle an animal could hold to the plane. Data for each day were the means of left and right side maximal angles.

The triphenyltetrazolium chloride (TTC) staining procedures followed those described elsewhere [8]. All animals were sacrificed 3 days after fluid percussion injury. Under deep anesthesia (Sodium pentobarbital, 100 mg/kg, ip), animals were perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 minutes, and sliced into 1.0-mm sections. The brain slices were incubated in 2% TTC dissolved in Ringer's solution for 10 minutes at 37°C, and then transferred to 10% formaldehyde solution for fixation. The volume of contusion, as revealed by negative TTC stains indicating dehydrogenase-deficient tissue, was measured in each slice and summed using computerized planimetry (PC-based image tools software). The volume of infarction was calculated as 1 mm (thickness of the slice) × [sum of the contusion area in all brain slices (mm^2)].

All animals were sacrificed at the third day after fluid percussion injury. Under deep anesthesia (Sodium pentobarbital, 100 mg/kg, i.p.), animals were perfused intracardially with saline. The injured brain tissue proper (core region) was then removed and dissected out the cortex for determination of MDA, GPx, GR, SOD, and catalase contents. As demonstrated in Figure 1, the marked contusion focus was dissected out of the brain for the above-mentioned laboratory tests for free radical compounds.

Lipid peroxidation was assessed by measuring the levels of MDA with 2-thiobarbituric acid (TBA) to form a chromophore absorbing at 532 nm [9]. About 0.1 g of brain tissue was homogenized with 1.5 mL of 0.1 M phosphate buffer at pH 3.5. The reaction mixture (0.2 mL of sample, 1.5 mL of 20% acetic acid, 0.2 mL of 8.1% sodium dodecylsulfate, and 1.5 mL of aqueous solution of 0.8 TBA, up to 4 mL with distilled water) was heated to 95°C for 1 h and then 5 mL of N-butanol and pyridine (15 : 1 v/v) was added. The mixture was vortexed vigorously, centrifuged at 1500 × g for 10 minutes, and the absorbance of the organic phase measured at 532 nm. The values are expressed as nanomoles of TBA-reactive substance (MDA equivalent) per milligram of protein [10].

To measure cytosolic GPx and GR activities, tissues were homogenized in buffer solutions containing 50 mM Tris-HCL (pH7.5), 5 mM EDTA, and 1 mM dithiothreitol. The homogenates were centrifuged at 8500 × g for 15 minutes at 4°C, and supernatants were used for GPx and GR activity assay. The GPx and GR activities were performed with a commercial glutathione peroxidase cellular activity assay kit (Oxis Research, Portland, USA) and a glutathione reductase

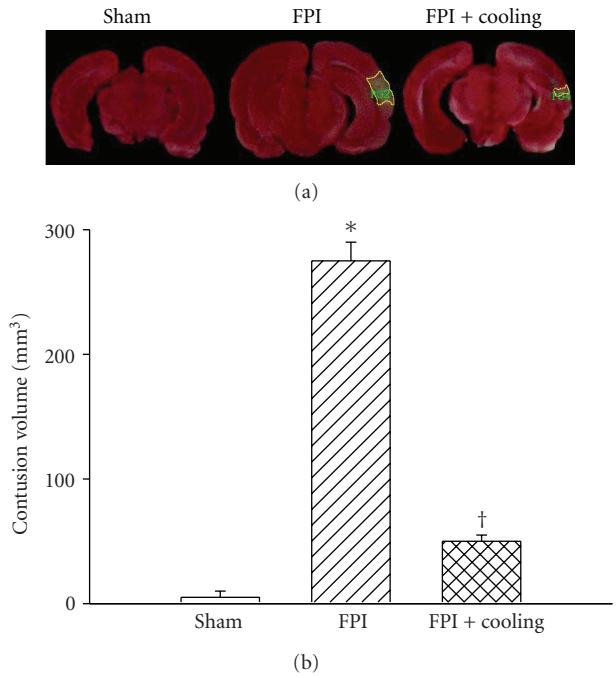

Figure 1: Values of infarction volume for sham-operated rats (first column), 37°C saline-treated FPI rats (second column), and 4°C saline-treated FPI rats (third column). The saline solutions were administered via the jugular vein 3 minutes after the onset of FPI. The data were obtained on day 3 after surgery. Values are mean ± SEM; $n = 8$ per group. $^{*}P < .05$ versus sham-operated controls; $^{\dagger}P < .05$ versus 37°C saline-treated FPI rats. Top photomicrographs depicted the representative picture for a sham-operated rat (Sham), an FPI rat (FPI), and an FPI rat treated with brain cooling (FPI + cooling).

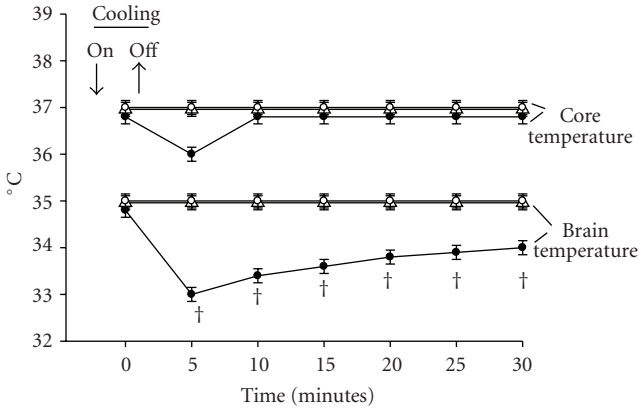

Figure 2: Values of both core and brain temperatures for the sham-operated rats (∘), 37°C saline-treated FPI rats (Δ), and 4°C saline-treated FPI rats (•). The isotonic sodium chloride solutions were administered via the jugular vein 3 minutes after the onset of FPI. Values are mean ± SEM; $n = 8$ per group. $^{\dagger}P < .05$ versus 37°C saline-treated FPI-injured group.

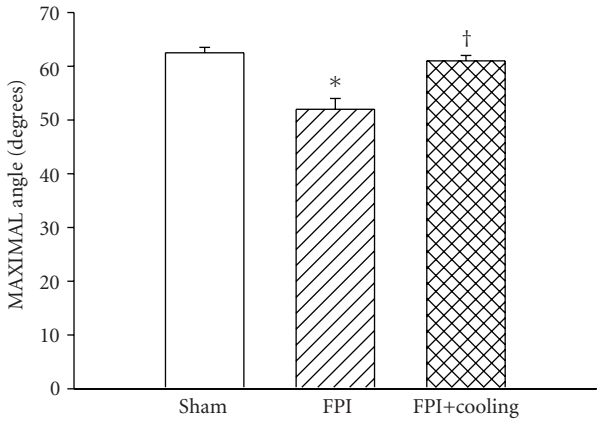

Figure 3: Values of maximal angle animals could cling to an inclined plane for sham-operated rats ((b) first column), 37°C saline-treated FPI rats ((b) second column), and 4°C saline-treated FPI rats ((b) third column). The saline solutions were administered via the jugular vein 3 minutes after the onset of FPI. The data were obtained on day 3 after surgery. Values are mean ± SEM; $n = 8$ per group. $^{*}P < .05$ versus sham-operated controls; $^{\dagger}P < .05$ versus 37°C saline-treated FPI rats.

assay kit (Oxis Research, Portland, USA), respectively. One unit of GPx or GR activity was defined as the amount of sample required to oxidize 1 μmol of NADPH per minute based on the molecular absorbance of 6.22×10^6 for NADPH.

To measure cytosolic SOD and catalase activities, tissues were homogenized in 200 mM phosphate buffer, pH7.0. The homogenates were centrifuged at 3000 × g for 10 minutes at 4°C, and supernatants were used for SOD and catalase activity assay. The SOD and catalase activity were performed with a SOD activity commercial kit (Oxis Research, Portland, USA) and a catalase activity commercial kit (Oxis Research, Portland, USA), respectively. The SOD activity is determined from the ratio of the autoxidation rates in the presence (Vs) and in the absence (Vc) of SOD. One SOD activity unit is defined as the activity that doubles the autoxidation rate of the control bland (Vx/Vc = 2). The catalase activity is determined from the catalase standard curve (Oxis Research, Portland, USA). Protein concentration was determined by the method of Lowry et al. [11].

Autofluorescence was first quenched using the method of Vendrame et al. [12]. Coronal brain sections (7-μm-thick) were cut on a cryostat and were thaw-mounted on gelatin-coated slides. Brain sections were fixed with 4% paraformaldehyde and rehydrated using PBS. Endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS for 10 min. Tissue sections were permeabilized, and free protein binding sites were subsequently blocked for a 60-minute-incubation with 10% normal goat serum/0.4% Triton X-100 in PBS. Tissue sections were then incubated at 4°C with single or a mixture of two primary antibodies (iNOS 1 : 100 BD Biosciences, 3-nitro-L-tyrosine 1 : 100 Upstate) diluted in PBS/1% normal goat serum and 0.25% Triton X-100. The immunoreaction product was visualized using the avidin-biotin peroxidase with secondary antibodies (1 : 200 Sigma) and the ABC substrate kit (Biogenes) diluted at 1 : 100 and diaminobenzidine as chromogen. Alternatively, immunosignals were visualized using fluorescently tagged secondary antibodies (Alexa 568 goat antimouse IgG 1 : 400 Molecular Probe, FITC goat antirabbit IgG 1 : 50 Molecular

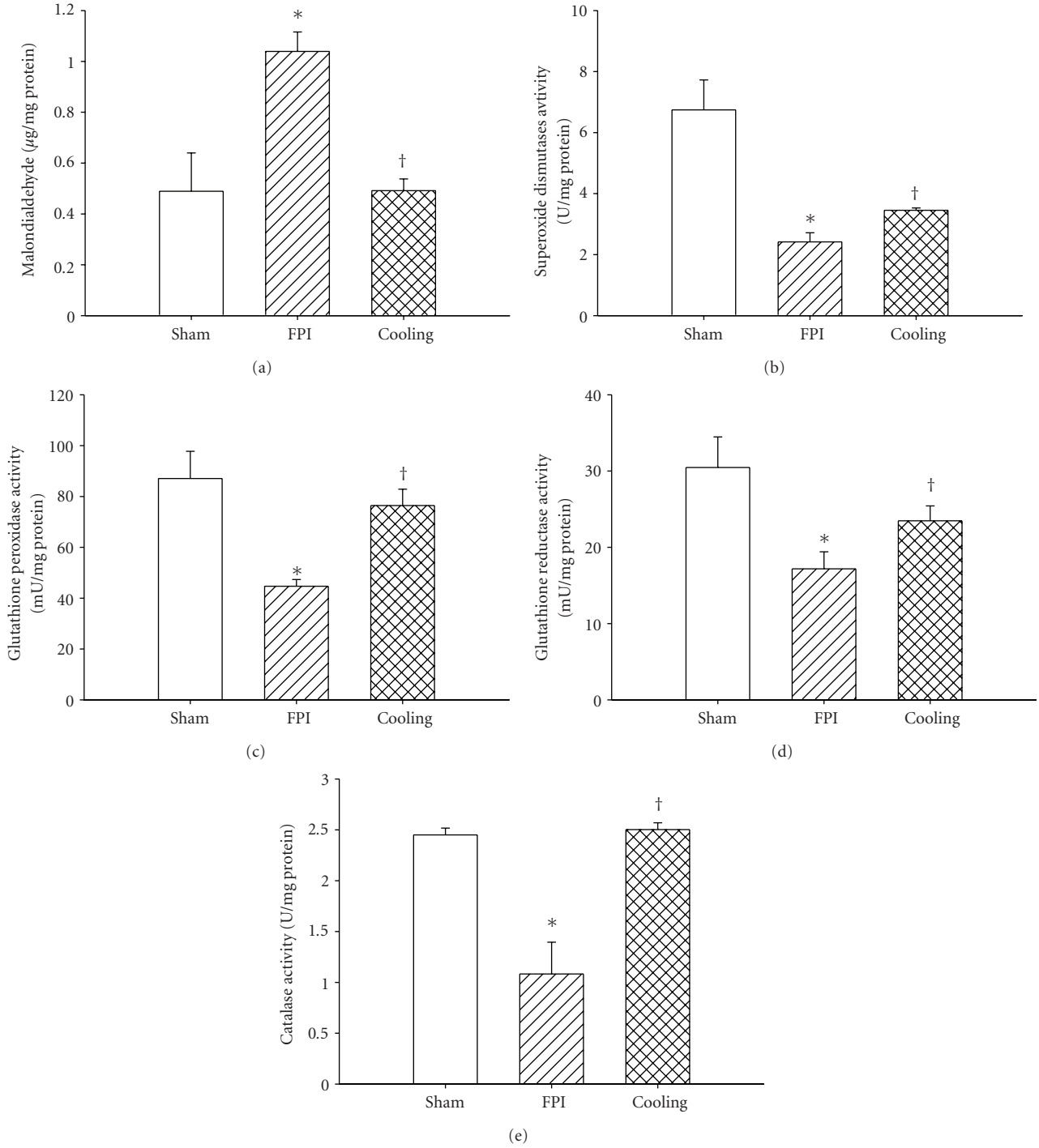

Figure 4: Values of malondialdehyde, superoxide dismutase glutathione peroxidase, glutathione reductase, and catalase in the homogenates of the ischemic for the sham-operated ((b) first column), 37°C saline-treated FPI rats ((b) second column), and 4°C saline-treated FPI rats ((b) third column) rats. The isotonic sodium chloride solutions were administered via the jugular vein 3 minutes after the onset of FPI. The FPI-injured rats were killed on day 3 after surgery. Values are mean ± SEM; $n = 8$ per group. $^{*}P < .05$ versus sham-operated controls; $^{\dagger}P < .05$ versus 37°C saline-treated FPI-injured group.

Probe). Tissue sections were observed under epifluorescence an microscope (Olympus BX51, Tokyo, Japan).

Data are presented as the mean ± SEM. The behavioral data were analyzed using ANOVA with repeated measures and the Kruskal-Wallis H test was used for post hoc comparisons. Infarct size and biochemical data were analyzed with ANOVA and the Newman-Keuls post hoc test. $P < .05$ was considered evidence of statistical significance.

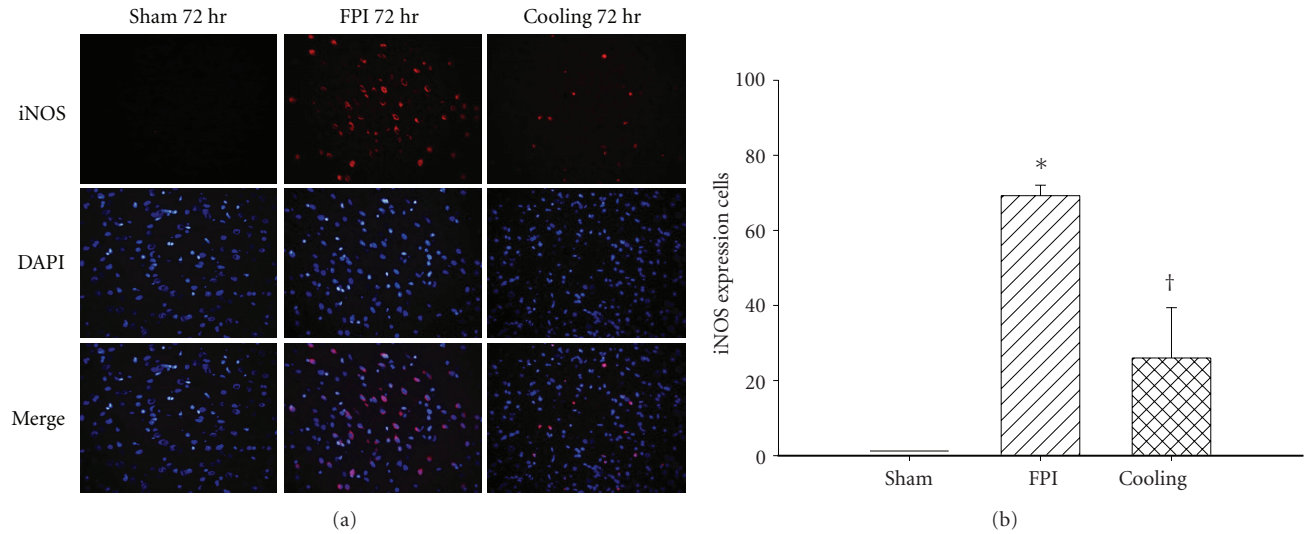

Figure 5: Values of the amounts of iNOS-positive cells in the ischemic cortex (CT) for the sham-operated rats (first column), 37°C saline-treated FPI-treated rats (second column), and 4°C saline-treated-FPI-treated rats (third column). The isotonic sodium chloride solutions were administered via the jugular vein 3 minutes after the onset of FPI. The FPI-injured rats were killed on day 3 after surgery. Values are mean ± SEM; $n = 8$ per group. $^{*}P < .05$ versus sham-operated controls; $^{\dagger}P < .05$ versus 37°C saline-treated FPI group.

3. Results

Figure 2 shows that the core and brain temperature values, respectively, are 37°C and 35°C for the sham-operated rats within a period of 30 minutes. Infusion of 37°C saline via the jugular vein for 5 minutes in the FPI rats had an insignificant effect on both the core and brain temperature. However, brain temperature, but not core temperature, of the FPI rats were significantly (~2°C) decreased by an intravenous infusion of 4°C saline for 5 minutes. As shown in our previous findings [4], during the acute stage (e.g., 2 h) of FPI, both an increase in intracranial pressure (ICP) and a decrease in cerebral perfusion pressure (CPP) were observed in the rat. The decrease in CPP (MAP-ICP) was due to an increase in ICP because an appropriate level of mean arterial pressure (MAP) was maintained within a 2-hour post-FPI period. Additionally, as revealed 3 days after FPI, body-weight loss was noted in rats with FPI. All the decrease in CPP, the increase in ICP, and the body-weight loss that occurred after FPI were significantly reduced following HRJVF.

As compared with those of the sham-operated rats, the maximal angle FPI animals treated with 37°C saline could cling to an inclined plane significantly decreased 72 h after FPI (as shown in Figure 3). However, the FPI-induced reduction in maximal grip angle measured 72 h after FPI was significantly reversed by HRJVF ($P < .05$).

Triphenyltetrazolium chloride staining revealed the marked increase in cerebral contusion in FPI rats treated with 37°C saline compared with sham-operated, controls (Figure 1). Again, the FPI-induced cerebral contusion was significantly reduced by treating with HRJVF (or 4°C saline) ($P < .05$).

As compared with those of the sham-operated rats, the FPI rats treated with 37°C saline had higher values of MDA but lower values of SOD, GP, GR, and catalase in contusion brain (Figure 4). The FPI-induced increased MDA and decreased SOD, GP, and catalase in contusion brain were all significantly ameliorated by HRJVF (or 4°C saline infusion) ($P < .05$; Figure 4).

As shown in Figure 5, the 37°C saline-treated FPI rats had higher numbers of iNOS-positive cells in the contusion brain than those of the sham-operated rats. In addition, the FPI-induced increased numbers of iNOS-positive cells in the contusion brain were significantly reduced by HRJVF (or 4°C saline infusion) (Figure 5).

As demonstrated in Figure 6, the FPI-treated rats that received 37°C saline infusion had higher numbers of 3-NT-positive cells in the contusion brain than those of the sham-operated rats. Furthermore, the FPI-induced increased numbers of 3NT-positive cells in the contusion brain were significantly reduced by HRJVF (or 4°C saline infusion) (Figure 6).

4. Discussion

The mechanisms that have been proposed to lead to secondary brain injury during TBI include excitotoxicity diffuse axonal injury, oxidative stress [12], mitochondrial dysfunction, and apoptosis [13]. Herein we have demonstrated that Nitrostative damage (evidenced by increased iNOS and 3-NT), oxidative damage (evidenced by decreased levels of GP, GR, SOD, and catalase as well as increased levels of malondialdehyde), cerebral contusion and apoptosis, and behavioral deficits all noted 72 h after TBI in a rat model. It is likely that TBI causes cerebral contusion, induces Nitrostative and oxidative damage, and results in cerebral apoptosis, which leads to behavior deficits.

Our current results are in part supported by many investigators. For example, oxidative stress contributes to

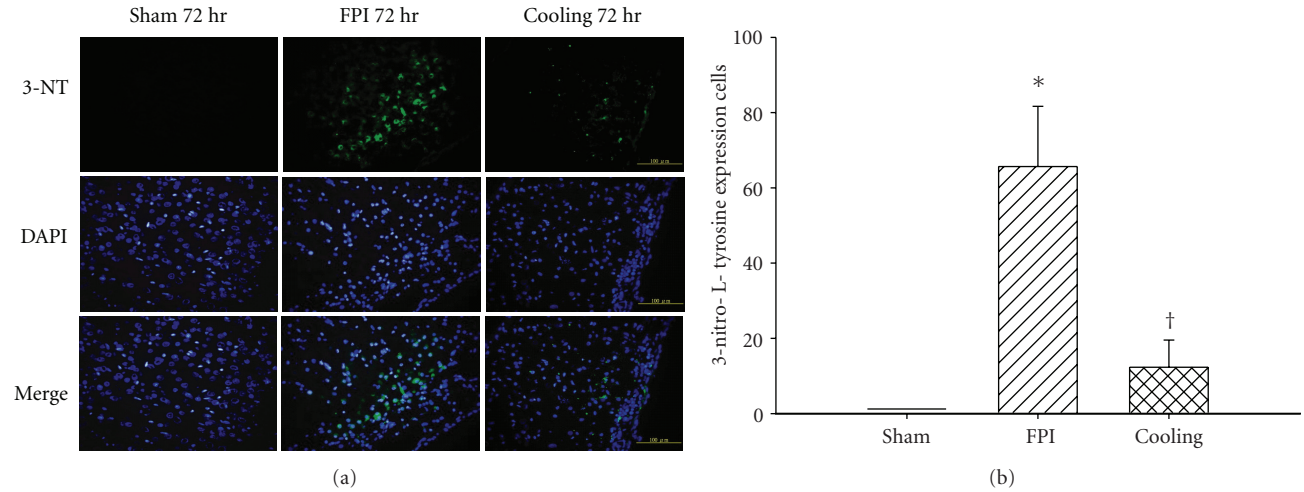

Figure 6: Values of the amounts of 3-nitro-L-tyrosine of glutathione peroxidase positive cells in the ischemic cortex for the sham-operated rats (first column), 37°C saline-treated FPI-treated rats (second column), and 4°C saline-treated-FPI-treated (third column) rats. The isotonic sodium chloride solutions were administered via the jugular vein 3 minutes after the onset of FPI. The PFI-injured rats were killed on day 3 after surgery. Values are mean ± SEM; $n = 8$ per group. $^*P < .05$ versus sham-operated controls; $^\dagger P < .05$ versus 37°C saline-treated FPI-treated group.

secondary brain injury in patients with TBI [14]. Poor neurologic outcome is associated with increased levels of nitrotyrosine in the cerebrospinal fluid (CSF). Superoxide and peroxynitrite play a significant role in secondary brain injury [15]. Pelluffo and coworkers have demonstrated that incubation of motor neurons cultured with nitrotyrosine induced apoptosis [16]. 3-Nitrotyrosine has been reported as a causative in induction of mitochondrial dysfunction after TBI in a mouse model [17].

Although the aforementioned statements tend to suggest that NO derived from the iNOS is an inflammatory product implicated both in secondary damage and in recovery from TBI, several findings promote that iNOS is an endogenous neuroprotectant after TBI in rats and mice [18, 19]. In a model of controlled cortical impact, knockout mice lacking the iNOS gene ($iNOS^{-/-}$) were compared with wild-type mice ($iNOS^{+/+}$). Functional outcome during the first 20 days after injury, and histopathology at 21 days, were assessed in both studies. Treatment of rats with either of the iNOS inhibitors after TBI significantly exacerbated deficits in cognitive performance and increased loss in vulnerable regions of hippocampus. Uninjured $iNOS^{+/+}$ and $iNOS^{-/-}$ mice performed equally well in both motor and cognitive tasks. However, after TBI, $iNOS^{-/-}$mice showed markedly worse performance in the cognitive task than $iNOS^{+/+}$, supporting a beneficial role for iNOS in TBI [18]. Additionally, Bayir and colleagues [19] provided evidence to support a role for iNOS-derived NO as an endogenous antioxidant after TBI.

Although many pharmacologic interventions have been suggested to provide neuroprotection, almost all phase II and III clinical trials in neuroprotection have failed to show consistent improvement in outcome for patients after TBI [20]. In contrast, whole-body cooling (32–34°C) has been considered an effective method for reducing neurological injury in animal and human models of TBI [21, 22]. Although body cooling protects against contusion brain, several problems are associated with whole-body cooling [23, 24]. For example, a faster cooling method is warranted for application of whole body cooling to TBI patients. Whole-body cooling is associated with severe shivering and anesthesia requirement and increases incidents of arterial hypotension, cardiac arrhythmia, hemorrhage, and pneumonia.

Jugular venipuncture is an easy procedure frequently used in the emergency department. In addition, retrograde cold saline infusion via the jugular vein is able to induce rapid brain cooling while maintaining systemic normothermia and normal hemodynamic function in the rat [4, 25]. We have previously demonstrated that brain cooling improves outcomes of TBI by reducing intracranial hypertension, cerebral ischemia and infarction, motor, and proprioception deficits, and body-weight loss in the rat [4]. The present results further show that brain cooling may improve outcomes of TBI in rats by compromising oxidative stress. In particular, Nitrostative damage (evidenced by increased iNOS and 3-NT), oxidative damage (evidenced by decreased GP, GR, SOD, and catalase, but increased malondialdehyde), cerebral apoptosis and contusion, and behavioral deficits that occurring during TBI were all reduced by brain cooling.

The current results are consistent with several investigations. After TBI in rats, enhanced generation of hydroxyl radicals during TBI was attenuated by moderate hypothermia in rats [26]. Treatment of rats with whole-body cooling after TBI increased antioxidant enzyme like SOD activity [27]. Furthermore, therapeutic hypothermia attenuated oxidative stress after sever TBI in infants and children [28]. Cerebral oxidative stress and cerebrovascular dysfunction that occurred during heat stress could be attenuated by brain

cooling in the rat [5]. Local saline infusion into ischemic territory induced regional brain cooling and neuroprotection in rats with transient middle cerebral artery occlusion [29].

Probably, the volume of physiological saline (5 mL of cold saline) infused with this procedure was an important point that may affect the feasibility and safety in clinical settings. This volume (5 mL) was 10–15% of total blood volume in rats with a body weight of approximately 325 g (as used in this study) [30]. In rats, blood flow was approximately 0.87 mL/minutes for the entire brain (brain weight, 2.2 g for 325 g body weight). In humans, the blood flow through the brain tissue averaged 870 mL/minutes in normal adults brain weight, 1500 g [31]. These figures indicate that an infusion volume approximately 870 mL could be applicable in adult patients with an average blood volume of approximately 5000 mL [32]. It is feasible that 70 mL/minutes of infusion volume via the jugular vein can be safely administered to patients.

Finally, it should be pointed out that the inclined plane was used to measure limb strength [6, 7]. Whether it is a suitable index of neuroprotection for traumatic brain injury ([6]; present results) or experimental ischemic stroke [8] in rats needs further explanation.

In summary, our data showed that TBI caused brain contusion, induced nitrostatic and oxidative damage, and resulted in brain apoptosis, which led to behavior deficits in rats. These reactions caused by TBI could be significantly ameliorated by selective brain cooling (caused by retrograde cold saline infusion via the jugular vein). The results suggested that selective brain cooling might improve the outcomes in TBI via reducing brain Nitrostative and oxidative damage in rats without prominent side effects.

Acknowledgment

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