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The Journal of Trauma, Injury, Infection and Critical Care
Brain Cooling-Stimulated Angiogenesis and Neurogenesis Attenuated Traumatic Brain Injury in Rats

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**Background:** Although brain cooling has been reported as effective in improving the outcome after traumatic brain injury (TBI) in rats, the mechanisms of brain cooling-induced neuroprotective actions remain unclear. This study was to test whether angiogenesis and neurogenesis attenuating TBI could be brain cooling-stimulated.

**Methods:** Anesthetized rats, immediately after the onset of TBI, were divided into two groups and given the brain cooling (infusing 5 mL of 4°C saline via the external jugular vein) or no brain cooling (infusion 5 mL of 37°C saline via the external jugular vein).

**Results:** Brain cooling without interference with the core temperature in rats significantly attenuated TBI-induced cerebral infarction (90 mm³ vs 250 mm³) and motor (61° vs 57° maximal angle) and proprioceptive (14% MPE vs 42% MPE) function deficits, significantly reduced TBI-induced neuronal (24 NeuN-TUNEL double positive cells vs 62 NeuN-TUNEL double positive cells) and glial (5 GFAP-TUNEL double positive cells vs 35 GFAP-TUNEL double positive cells) apoptosis (increased TUNEL-positive and caspase-3-positive cells), neuronal loss (102 NeuN-positive cells vs 66 NeuN-positive cells), and gliosis (40 GFAP-positive cells vs 66 GFAP-positive cells; 66 Iba1-positive cells vs 89 Iba1-positive cells), and significantly promoted angiogenesis (5 BrdU/endothelial cells vs 1 BrdU/endothelial cell; 58 VEGF-positive cells vs 31 VEGF-positive cells) and neurogenesis (33 BrdU/NeuN positive cells vs 14 BrdU/NeuN positive cells).

**Conclusions:** Resultantly, brain cooling-stimulated angiogenesis and neurogenesis attenuated TBI in rats hereby.

**Key Words:** Traumatic brain injury, Brain cooling, Angiogenesis, Neurogenesis,
Gliosis
INTRODUCTION

Traumatic brain injury (TBI) results in significant long-term disability (a significant socioeconomic burden)\(^1\). Besides the primary insult-caused local neuronal damage, TBI secondarily causes a progressive cascade of related events that contribute to neuronal damage including ischemia, brain edema, excitotoxicity, oxidative stress, and dysregulation of calcium homeostasis.\(^2,3\) Additionally, neuronal loss after TBI is typically observed at the frontal and temporal poles, and in the orbital frontal lobes and the cortex above the sylvian fissure.\(^4\) The hippocampus is known to be damaged frequently in human beings, with neuronal loss in greater than 80% of fatal TBI.\(^5,6\) Continual neurogenesis in the adult human hippocampus\(^7\) and subventricular zone (SVE)\(^8,9\) is proposed. Angiogenesis may be neurogenesis-associated in vitro and in vivo.\(^10,11\) Neuronal loss in TBI may be functionally counterbalanced by generating new neurons.

Ensuantly, brain cooling (infusing 4\(^\circ\)C normal saline via the external jugular vein) improved TBI in rats.\(^12\) However, it is unknown whether brain cooling-stimulated angiogenesis and neurogenesis attenuates TBI.

To deal with the problem, in the present study, brain cooling (infusing cold saline via the jugular vein) was random immediately after TBI in rats, and the effects on cerebral infarction (evaluated by the triphenyltetrazolium chloride, TTC, staining), neuronal loss (evaluated by the numbers of neuronal-specific nuclear (Neu-N) protein-positive cells), gliosis (evaluated by the numbers of the astrocytes and microglia), apoptosis (evaluated by terminal deoxynucleotidyl-transferase-mediated and duDP-biotin
nick end-labeling, TUNEL, staining), angiogenesis (evaluated by the numbers of both 
BrdU-positive endothelial and VEGF-positive cells), neurogenesis (evaluated by the 
numbers of NeuN-positive and BrdU-plus NeuN double-positive cells), and motor and 
proprioceptive dysfunction were assessed 3 days after TBI.
MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 298 to 355 g were used in these experiments. Animals were kept under 12-h/12-h cycle and allowed free access to food and water. All experimental procedures conformed to the instructions of National Science Council of Republic of China 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. The animals were randomly assigned to the sham group, TBI brain normothermia group, or TBI brain cooling group. All the tests were blinded, and the animal codes were revealed only at the end of the behavioral and histological analyses.

In the TBI normothermia or brain cooling group, 37°C or 4°C saline (1.7 mL/100 g body weight) via the external jugular vein in a (cranial direction) was offered immediately after surgery. In the histological or behavioral studies, food and water throughout the study were provided spontaneously.

Fluid Percussion Injury (FPI)

The animals were anesthetized with sodium pentobarbital (25 mg/kg, i.p.; Sigma Chemical Co., St. Louis, MO, USA) 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), and in a stereotaxic frame were with the scalp incised sagittally and subjected to a lateral FPI. After an incision in the scalp, 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 leur-lock connector (trauma cannula), 2.6 mm inner diameter, was secured into the cranial cavity with cyanoacrylic adhesives and dental acrylic. A moderate FPI (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). An animal was removed from the device; the acrylic, removed; the incision, sutured. Each injured and sham-injured animal for behavioral recovery was closely evaluated immediately after FPI. Core and brain temperatures were thermocouples-monitored continuously after FPI and brain cooling.

**Experimental Procedures**

In Experiment 1, effects of intravenously infusing 37°C (n=8) or 4°C (n=8) saline were on both core and brain temperature in TBI-treated rats.

In Experiment 2, intravenously infusing 37°C (n=8) or 4°C (n=8) saline was random immediately after FPI, and the effects on infarction volume, motor function, and proprioception were assessed 3 days after FPI. Another 8 rats were sham controls.

In Experiment 3, intravenously infusing 37°C (n=8) or 4°C (n=8) saline was random immediately after FPI, and the effects on amounts of the NeuN plus TUNEL positive cells, the GFAP plus TUNEL positive cells, and active caspase-3-positive cells in the ischemic cortex were assessed 3 days after FPI. Another 8 rats were sham controls.

In Experiment 4, intravenously infusing 37°C (n=8) or 4°C (n=8) saline was random, and the effects on amounts of Neu N-positive and Brdu/NeuN double-positive cells in the ischemic cortex were assessed 3 days after FPI. Another 8 rats were sham controls.
controls.

In Experiment 5, intravenously infusing 37°C (n=8) or 4°C (n=8) saline was random, and the effects on amounts of both GFAP- and 1ba1-positive cells were assessed in the ischemic cortex 3 days after FPI. Another 8 rats were sham-operated controls.

In Experiment 6, intravenously infusing 37°C (n=8) or 4°C (n=8) saline was random, and the effects on amounts of both Brdu endothelial double positive and VEGF-positive cells in the ischemia cortex were assessed 3 days after FPI.

**Inclined Plane**

The inclined plane was to measure limb strength. The 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 55°, which was increased or decreased by 5° to determine the maximal angle an animal could hold to the plane. Data for each day were the means of the maximal angles on the left and right side.

**Proprioception**

Proprioception appraising was tactile placing and hopping (resting; posture; postural reactions). The functional deficit was graded as: [a normal or 0% maximal possible effect (MPE)]; 2 (slightly impaired); 1 (severely impaired); 0 [a completely or 100% MPE]. Here lifting the frontal half of the animal and one hind limb off the ground was tested for it to stand on just one limb and be moved laterally, which normally evoked a prompt hopping with the weight-hearing limb in the direction of movement to prevent the animal from falling. For the prevention a
predominant proprioceptive block delayed hopping followed by greater lateral hops, opposed to a predominant motor block which would cause a prompt but weaker-than-normal response. For the full blockade, the would be no hopping.\textsuperscript{17}

\textbf{Cerebral infarction assay}

The triphenyltetrazolium chloride (TTC) staining followed those described elsewhere.\textsuperscript{18} All the animals euthanized at 3 days after FPI were saline-perfused intracardially under deep anesthesia (100 mg/kg sodium pentobarbital, i.p.). The brain tissue was successively: removed; immersed in cold saline for 5 min; sliced into 2.0-mm sections; incubated in 2\% TTC dissolved in phosphate-buffered saline for 30 min at 37\textdegree C; transferred into 5\% formaldehyde solution for fixation. The infarction volume, 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), and calculated as 2 mm (thickness of the slice) $\times$ (sum of the infarction area in all brain slices [mm$^2$]).\textsuperscript{18}

\textbf{TUNEL Assay}

The TUNEL assay was using the same brain sections histochemically. The color was developed using 3,3-diamino-benzidine tetrachloride (DAB). The sections were sequently: xylene- and ethanol-treated for paraffin removal and for dehydration; PBS-washed and 3\% H$_2$O$_2$ solution-incubated for 20 min; 5 $\mu$g/ml proteinase k-treated at room temperature for 2 min; PBS-rewashed (0.1 M, pH7.4); treated with a TUNEL reaction mixture (nucleotide Rochel Mannheim, Germany) at 37\textdegree C for 1 h; distilled water
(D/W)-washed; re-incubated in horse-radish peroxidase-conjugated fluorescent antibodies at room temperature for 30 min; rewashed; visualized using the avidin-biotin-peroxidase complex (ABC) technique and 0.05% DAB (Sigma) as a chromogen. The TUNEL-positive cells were pathologists-counted in 30 fields/sections (×200 magnification). The blinding was for pathology grading of the results.

**Bromodeoxyuridine Labeling**

To evaluate the proliferation of cells, 5-bromodeoxyuridine (BrdU) (Roche Diagnostics, Indianapolis, USA; 50 mg/kg) dissolved in PBS were administered intraperitoneally once daily for 3 days after FPI. The rats were sacrificed 3 days later.

**Immunohistochemistry**

Adjacent 50-μm sections corresponding to coronal coordinates 0.20 mm to 0.70 mm anterior to the bregma were consecutively: 2 mol/L HCl-incubated for 30 min; 0.1 mol/L boric acid-rinsed (pH 8.5) at room temperature for 3 min: primary antibodies-incubated in PBS containing 0.5% normal bovine serum at 4°C overnight; secondary antibodies-incubated at room temperature for 1 h. The antibodies therein were sequentially: rat monoclonal anti-BrdU antibody (1:10; Oxford Biotechnology, Oxfordshire, UK), mouse anti-vascular endothelial growth factor (VEGF), GFAP, Iba1, or anti-neuronal-specific nuclear protein (NeuN, antibody in 1:200 dilution); Alexa-Fluor ®, 568 goat anti-mouse (IgG) antibody for detecting the sections. For endothelial cells, biotinylated Lycopersicon esculentum (Tomato) lectin (1:200, Vector Laboratories, Burlingame, CA, USA) was used in the same manner instead of the other primary
antibodies and visualized using fluorescein-labeled streptavidin (1:200, Vector Laboratories, Burlingame, CA, USA). For caspase-3 activity, cortical sections were serially: incubated with the cleaved caspase-3 (ASP/75) antibody (Cell Signaling Technology, Inc., Beverly, MA, USA), which only recognized the large fragment of activated caspase-3 (17-20 kDa), in 3% BSA/PBS/0.1% TX-100 at 4°C overnight; washed in PBS/0.6% TX-100 and FITC-conjugated anti-rabbit IgG incubated in 1% BSA/PBS/0.1% TX-100 for 60 min; antifade mounting media-mounted. The labeled cells were calculated in 5 coronal sections from each rat and expressed as the mean number of cells per section. For negative control sections, all the procedures were without the primary antibodies.

**Statistical Analysis**

The data were presented: mean ± s.d. The repeated measures analysis of variance was to test the treatment-by-time interactions and the effects of treatment over time on each score. The Duncan’s multiple-range test was for post hoc multiple comparisons among means. Mann-Whitney U-test was for Table 1. P<0.05 was considered significant.
RESULTS

Brain Cooling Reduced Brain Temperature but not Core Temperature

Brain temperature in brain cooling-treated rats was significantly lower at 5-30 min after the start of FPI than in the 37°C saline-treated FPI controls. As in Fig. 1, resuscitation by brain cooling (4°C saline infusion-induced) immediately after FPI decreased the brain temperature from 35°C to 33°C within 30 min. However, brain cooling insignificantly changed core temperature.

Brain Cooling Improved Motor and Proprioceptive Function after FPI

At 3 days after FPI in behavioral tests, the 37°C saline-treated FPI rats had significantly lower performance in both motor and proprioceptive function than the sham-operated controls (Table 1). The FPI-induced motor and proprioceptive dysfunction could be significantly brain cooling (4°C saline infusion-induced)-reduced significantly.

Brain Cooling Decreased Infarct Area after FPI

In the TTC-stained sections at 3 days after FPI, the infarcted area of the brain cooling (4°C saline infusion-induced)-treated FPI group was significantly decreased (Table 1; Fig. 2). The cortical and hippocampal infarction areas were distinctly smaller in the brain cooling-treated rats than in the 37°C saline-treated ones.

Brain Cooling Decreased both Neuronal and Glial Apoptosis after FPI

The TUNEL plus NeuN-stained, TUNEL plus GFAP-stained and caspase-3-stained sections at 3 days after FPI, the positive cells in the ischemic cortex compared with those
of sham controls were significantly increased (Table 1). The FPI-induced increased numbers of these cells in the ischemic cortex were **brain cooling (4°C saline infusion-induced)**-reduced significantly (Table 1).

**Brain Cooling Promoted Neurogenesis after FPI**

In the perischemic area, NeuN-positive (Fig. 3) and BrdU plus NeuN double-positive cells were increased in the brain cooling (4°C saline infusion-induced) treated group (P<0.05) compared with the 37°C saline-treated group at 3 days after FPI (Table 1).

**Brain Cooling Promoted Angiogenesis after FPI**

In the perischemic area, BrdU-positive endothelial and VEGF-positive cells increased in the brain cooling (4°C saline infusion-induced) treated group (P<0.05) compared with the 37°C saline-treated group at 3 days after FPI (Table 1).

**Brain Cooling Attenuated Brain Gliosis after FPI**

The GFAP- (Fig. 4-a) and Iba1-positive cells (Fig. 4-b) in the ischemic brains of the 37°C saline-treated FPI group were significantly increased compared with the sham controls at 3 days after FPI (Table 1). Again, the increase in GFAP- and iba1-positive cells in the ischemic cortex of 37°C saline-treated FPI rats was significantly brain cooling-decreased (Table 1).
DISCUSSION

The current results, brain cooling without interference with the core temperature in rats attenuated TBI-induced cerebral infarction and motor and proprioceptive dysfunction, reduced TBI-induced neuronal apoptosis and gliosis, and significantly promoted angiogenesis and neurogenesis in response to injury in the ischemic cortex. In our data, brain cooling-stimulated angiogenesis and neurogenesis improved the recovery from TBI in the rats.

With the continual neurogenesis confirmed in the adult human hippocampus and subventricular zone (SVE), there was also evidence for the latent endogenous neural progenitor cells (NPCs) activated at the cortical injury sites in the mammalian brain. In this study, the BrdU-labeled cells that expressed NeuN, a postmitotic neurons-expressed nuclear protein, were approximately two and half fold at 3 days after TBI in the brain cooling-treated rats; thence brain cooling could significantly enhance the proliferation in the ischemic cortex, attenuated the injury-induced neuronal apoptosis and cell loss, and after reducing apoptosis in the ischemic cortex 3 days after TBI may have improved neuronal loss. However, it is mysterious whether the brain cooling preventing neuronal loss is due to the proliferated cells generated from the hippocampus and SVE and migrated and differentiated in the ischemic cortex and/or activation of NPCs activated at sites of cortical injury sites. In fact, it has been shown that juvenile mammals recover to a greater extent than adults after TBI. Thus, brain cooling-related differences hypothesized herein in cortical neurogenesis after TBI may have helped partly for greater motor and proprioceptive recovery.
Although the adult rat brain generates new neurons in response to TBI, glial formation could be TBI-induced in our data. To overcome the inhibitory environment of the glial scar, combination treatments proposed for a growth-related pathway across lesion cavities while enhancing the neurons ability to elongate by manipulating growth inhibitors in the glial scar environment may allow long-distance functional regeneration after TBI. As shown in the current results, the injury-induced gliosis of astrocytes and microglia brain cooling-reduced significantly. Thus, it was likely that brain cooling inhibiting glial scar formation enhanced actual functional neuronal regeneration.

Moreover, in the present results, brain cooling stimulated VEGF production and increased the amounts of BrdU-positive endothelial cells in the ischemic cortex. These findings supported the hypothesis that brain cooling may have promoted an environment assisting to revasculize ischemic brain to proceed neuronal regeneration. A rich vascular environment, along with VEGF generation, may have enhanced subsequent angiogenesis and neurogenesis. In a more recent report, systemically administrating Premarin, a conjugated estrogen, stimulated VEGF production, increased the amounts of BrdU-positive endothelial cells in the injured brain, attenuated cerebral infarction and apoptosis, and restored normal behavioral function in TBI rats. Like Premarin therapy, brain cooling enhancing neovessel formation and accelerating endogenous neurogenesis may have improved behavioral outcome and cerebral infarction after FPI.

After TBI, oxidative stress is important in early as well as in secondary neuronal and vascular damage after TBI; generation of lipid peroxidation and consumption of antioxidants in rats is enhanced. Evidence has accumulated to
indicate that TBI-related oxidative stress can be reduced by whole body cooling therapy. For example, the increased levels of free fatty acids in cerebrospinal fluids from patients with TBI could be reduced by whole body cooling.\textsuperscript{35} Therapeutic hypothermia preserved antioxidant defenses after severe TBI in rats,\textsuperscript{36} infants and children.\textsuperscript{37} Indeed, in our unpublished data (Kuo, Chang, Chio, Lin, and Lin), brain cooling, like whole body cooling, significantly attenuated the TBI-induced cerebral oxidative damage in rats.
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29. Wei EP, Kontos HA, Beckman JS. Mechanisms of cerebral vasodilation by


Table 1. Effects of brain cooling on behavioral and histopathologic characteristics in the brain cooling and normothermic groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Animal groups</th>
<th>Sham controls</th>
<th>FPI+37°C saline</th>
<th>FPI+4°C saline</th>
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<tbody>
<tr>
<td>1. Maximal angle (degree)</td>
<td>62±0 (8)</td>
<td>57.5±0.7 (8)*</td>
<td>61.2±0.7 (8)†</td>
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<td>2. % MPE</td>
<td>0±0 (8)</td>
<td>41.5±5.6 (8)*</td>
<td>14.1±6.7 (8)†</td>
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<td>3. Infarction volume (mm³)</td>
<td>0±0 (8)</td>
<td>250±20 (8)*</td>
<td>90±17 (8)†</td>
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<td>4. Number of NeuN-TUNEL double positive cells (per section)</td>
<td>0±0 (8)</td>
<td>62±3 (8)*</td>
<td>24±13 (8)†</td>
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<tr>
<td>5. Number of GFAP-TUNEL double positive cells (per section)</td>
<td>0±0 (8)</td>
<td>35±7 (8)*</td>
<td>5±3 (8)†</td>
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<tr>
<td>6. Number of active caspase-positive cells (per section)</td>
<td>0±0 (8)</td>
<td>34.7±0.7 (8)*</td>
<td>17.7±5.7 (8)†</td>
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<tr>
<td>7. Number of NeuN-positive cells (per section)</td>
<td>132±15 (8)</td>
<td>66±2 (8)*</td>
<td>102±5 (8)†</td>
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<tr>
<td>8. Number of BrdU/NeuN double positive cells</td>
<td>0±0 (8)</td>
<td>14±3 (8)*</td>
<td>33±7 (8)†</td>
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<td>9. Number of BrdU/endothelial cells</td>
<td>0±0 (8)</td>
<td>1.3±1.3 (8)*</td>
<td>5.3±0.9 (8)†</td>
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<td>10. Number of VEGF positive cells</td>
<td>0±0 (8)</td>
<td>31±3 (8)*</td>
<td>58±2 (8)†</td>
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<tr>
<td>11. Number of GFAP-positive cells</td>
<td>32±5 (8)</td>
<td>66±9 (8)*</td>
<td>40±5 (8)†</td>
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<tr>
<td>12. Number of Iba1-positive cells</td>
<td>53±4 (8)</td>
<td>89±3 (8)*</td>
<td>66±3 (8)†</td>
<td></td>
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</table>

Data as mean±s.d. followed by the numbers of animals in parentheses. *P<0.05 in comparison with sham controls; †P<0.05 in comparison with (FPI+37°C saline) group (Mann-Whitney U-test). FPI, fluid percussion injury; MPE, maximal possible effect.
The legends of the figures

Fig. 1 Brain cooling significantly decreased brain temperature but not rectal temperatures. The 37°C saline-treated FPI group maintained their rectal temperature (△) and brain temperature (○) at 36.5°C and 35°C within a 30 min course. The FPI plus cooling (4°C saline infusion-induced)-treated group showed the significantly decreased brain temperatures (33°C-34.5°C) (●) and the insignificantly decreased rectal temperature (▲) within a 30 min course. Each point manifested mean ± s.d. of 8 animals per group.

Fig. 2 Brain cooling decreased the FPI-induced infarction volume. The 37°C saline-treated FPI group showed the significantly increased infarction volume at 3 days after FPI compared with the sham controls (*P<0.01). The cooling (4°C saline) plus FPI group had the significantly decreased infarction volume compared with the 37°C saline-treated FPI group (†P<0.01). Each column and bar manifested mean ± s.d. of 8 animals per group. Top panels depicted representative TTC staining at 3 days after FPI for a sham control (A), an FPI rat (B), and an FPI + cooling-treated rat (C).

Figure 3. Brain cooling decreased the FPI-induced neuronal loss. As compared with the sham controls, the 37°C saline-treated FPI rats had the decreased NeuN-positive cells in the ischemic cortex at 3 days after FPI. The brain cooling (4°C saline infusion-induced) plus FPI group had the significantly increased NeuN-positive cells. Each column and bar manifested mean±s.d. of 8 animals per group.

Figure 4. Brain cooling decreased the FPI-induced gliosis. The 37°C saline-treated FPI
group had the significantly increased GFAP-positive (a) and Iba1-positive (b) cells in the ischemic cortex at 3 days after FPI compared with the sham controls (†P<0.01). The brain cooling (4°C saline infusion-induced) plus FPI group had the significantly decreased GFAP-positive and Iba1-positive cells (†P<0.01) compared with the 37°C saline-treated FPI group. Each column and bar manifested mean±s.d. of 8 animals per group.
Figure 1.
Figure 2.
Figure 3
Figure 4