Neuroprotective effects of bloodletting at Jing points combined with mild induced hypothermia in acute severe traumatic brain injury

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Graphical Abstract

Bloodletting at Jing points combined with mild induced hypothermia for acute severe traumatic brain injury

Abstract

Bloodletting at Jing points has been used to treat coma in traditional Chinese medicine. Mild induced hypothermia has also been shown to have neuroprotective effects. However, the therapeutic effects of bloodletting at Jing points and mild induced hypothermia alone are limited. Therefore, we investigated whether combined treatment might have clinical effectiveness for the treatment of acute severe traumatic brain injury. Using a rat model of traumatic brain injury, combined treatment substantially alleviated cerebral edema and blood-brain barrier dysfunction. Furthermore, neurological function was ameliorated, and cellular necrosis and the inflammatory response were lessened. These findings suggest that the combined effects of bloodletting at Jing points (20 μL, twice a day, for 2 days) and mild induced hypothermia (6 hours) are better than their individual effects alone. Their combined application may have marked neuroprotective effects in the clinical treatment of acute severe traumatic brain injury.

Key Words: nerve regeneration; Jing points; bloodletting; mild induced hypothermia; acute severe traumatic brain injury; brain edema; brain water content; blood-brain barrier; neural regeneration

Introduction

Traumatic brain injury (TBI) results from strong mechanical forces on the head. TBI includes primary craniocerebral injury as well as secondary lesions that disrupt the blood-brain barrier (BBB) and cause brain edema, eventually resulting in neuronal cell death (Schmidt et al., 2005). TBI may bring about coma, paralysis, mental disorder, and even death. Consequently, effective treatments for TBI are urgently needed.

Bloodletting therapy is an ancient traditional medical approach that has therapeutic efficacy. In particular, bloodletting at Jing points (one of the five Shu points located at the tips of the fingers and toes; Figure 1) has been used to treat coma since ancient times. The Jing points in the extremities, known as the source of water, allow for Qi to flow out of these sites.
Furthermore, therapy at Jing points may improve cerebral edema (Jiang et al., 2013). Mild induced hypothermia (MIH), a treatment strategy in which the body is maintained at a controlled temperature of 32–35°C, is a non-pharmacological approach that has long been used to treat TBI. Several studies have shown that MIH can decrease intracranial pressure, reduce cerebral edema, diminish adverse reactions, and play a protective role in the brain after TBI (Jia et al., 2009; Cheng et al., 2013; Li et al., 2015). However, the effectiveness of bloodletting therapy at Jing points combined with hypothermia for TBI remains unclear. Therefore, in this study, we examined the effect of bloodletting at Jing points, the effect of hypothermia, as well as the effect of their combined application in a rat model of TBI, with the aim of developing a new method for the clinical treatment of TBI.

Materials and Methods

Ethics statement

The animal studies were approved by the Institutional Animal Care and Use Committee of Logistics University of China Armed Police Force (license No. 201404831) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment.

Animals

A total of 100 adult male Sprague-Dawley rats, weighing 280–300 g and 7–8 months of age, were provided by the Experimental Animal Center of Military Medical Sciences (Beijing, China; Approval No. SCXK (Army) 2012-0004). All animals were housed under a 12/12-hour light/dark cycle at 22–25°C in cages, and allowed free access to food and water. The rats were randomly assigned into the following five groups (n = 20 rats per group): sham-operated (sham), TBI, bloodletting (BL), MIH, and bloodletting plus MIH (B + M).

Establishment of an animal model of acute severe TBI

The rats were intraperitoneally anesthetized with 5% chloral hydrate (0.6 mL/100 g), and the head was placed on a stereotactic frame in the prone position. After shaving and sterilizing, a midline longitudinal incision was performed. A right parietal craniotomy (4 mm in diameter, 2 mm from the sagittal suture and 2 mm from the coronal suture) was performed using a dental drill, with the cerebral dura kept intact (Cheng et al., 2013). A cortical contusion injury (Custom & Design Company, Richmond, VA, USA) model of TBI in the rat was employed for this study. Injury was induced by a pneumatic piston containing a 3-mm-diameter tip at a rate of 4 m/s and a depth of 3 mm. Sham-operated rats underwent the same craniotomy without cortical impact (Cheng et al., 2013).

Application of bloodletting at Jing points and hypothermia

In the BL group, disposable syringe needles (Tianjin Hanaco Medical Co., Ltd., Tianjin, China) were perpendicularly inserted into the skin to a depth of 1 mm in the distal ends of the fingers bilaterally for bloodletting (Figure 1). Blood (20 μL) was removed at each point, twice a day, for 2 days. Comparative anatomy was used for point selection, with reference to human anatomical acupoints (Jiang et al., 2013). Rats in the MIH group were subjected to MIH immediately after TBI using a Blanketrol Hypo/Hyperthermia System (Cincinnati Sub-Zero Products, Cincinnati, OH, USA). Temperature probes were used to measure rectal temperature, maintaining a body temperature of 32 ± 0.5°C. Then, 6 hours later, rats in the MIH group were warmed to 37 ± 0.3°C, slowly over a 1-hour period, with a heat lamp. Rats in the B + M group underwent the two treatments simultaneously. MIH was administered only once.

Measurement with magnetic resonance imaging (MRI)

MRI was performed using a standard 3T clinical dedicated wrist MR scanner (Siemens Trio Tim, Berlin, Germany) after TBI for 48 hours. After anesthesia, the head was fixed in the prone position and coronally scanned, anterior to posterior, starting from the olfactory groove. T2-weighted images were acquired with the following parameters: repetition time = 4,000 ms, echo time = 75 ms, image matrix = 320 × 320. Sequences were gathered for 8 slices, each 2 mm thick.

Neurological function assessment

Neurological function assessment was performed using the modified neurological severity score (mNSS). For each rat, function tests were performed 48 hours after TBI. The mNSS is a composite of motor, sensory, reflex and balance tests (Lu et al., 2003). The mNSS is graded on a scale of 0–18, where a total score of 18 points indicates severe neurological deficit and a score of 0 indicates normal performance; 13–18 points indicate severe injury, 7–12 indicates moderate injury, and 1–6 indicates mild injury. All functional tests were performed by an investigator adequately trained in functional measurements and blinded to the experimental groupings.

Brain water content measurement

Rats were anesthetized with 5% chloral hydrate (0.6 mL/kg) and decapitated 48 hours after TBI. The brains were quickly removed. The olfactory lobe and cerebellum were removed, and excess water was removed from the surface of the brain by absorbing with filter paper. The samples were immediately weighed to obtain the wet weight, then heated at 100°C for 24 hours (Lee et al., 2008) and weighed again to obtain the dry weight. Brain water content was calculated as a percentage using the following formula: (wet weight − dry weight)/wet weight × 100%.

BBB integrity evaluation

The integrity of the BBB was evaluated by measuring the extravasation of Evans Blue dye 48 hours after TBI. The rats were anesthetized, and Evans Blue (2% in saline; 3 mL/kg; Sigma, Tianjin, China) was injected intravenously via the
femoral vein 2 hours before sacrifice. The rats were perfused transcardially with saline to remove the intravascular dye, and decapitated immediately thereafter. A piece of the cerebral cortex containing the region of damage was dissected out, homogenized in 5 mL formamide, and incubated at 37°C for 24 hours. Samples were then centrifuged at 12,000 r/min for 20 minutes, and the supernatant was transferred to a 96-well plate. The absorbance values were measured at 620 nm using a microplate reader (Thermo, Waltham, MA, USA). The Evans Blue content was calculated and expressed as μg/g of brain tissue.

Observation of pathology
Rats were anesthetized and perfused transcardially with saline, followed by 4% paraformaldehyde 48 hours after TBI. Brains were postfixed in 4% paraformaldehyde overnight, and then transferred to 30% sucrose solution and stored at 4°C. Coronal sections of frozen brains were cut using a cryostat microtome, with a thickness of 20 μm for Fluoro-Jade C staining, and a thickness of 5 μm for hematoxylin and eosin staining. Fluoro-Jade C staining (FJC, Millipore, MA, USA) was performed to evaluate degenerating neurons (Schmued et al., 1997). First, brain sections were immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 minutes, and rinsed with 70% ethanol and distilled water for 2 minutes. Then, sections were treated with 0.06% potassium permanganate for 20 minutes, and washed with distilled water for 2 minutes. Subsequently, the tissue was stained in 0.0004% Fluoro-Jade C for 20 minutes and washed three times with distilled water for 5 minutes per wash. Finally, the tissue was incubated in 0.01% 4',6-diamidino-2-phenylindole for 10 minutes and washed as above.

For hematoxylin-eosin staining (Gao et al., 2014), 5-μm sections were prepared and stained. First, the slides were dipped in a jar containing hematoxylin for 3 minutes and then rinsed in water for 20 seconds. The slides were rinsed in differentiation media for 30 seconds and then in water for 10 minutes. Then, the slides were stained with eosin for 20 seconds and then rinsed with water for 20 seconds. Thereafter, the sections were dehydrated through a graded alcohol series for 2 minutes in each solution. Subsequently, one or two drops of neutral balsam were added, and the sections were covered with a coverslip after drying. Sections were observed by light microscopy (Leica Microsystems, Wetzlar, Germany).

Statistical analysis
Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA), and the values were expressed as the mean ± SD. Factor analysis was conducted to compare data among multiple groups. A value of $P < 0.05$ was considered statistically significant.

Results
MRI images of rats with acute severe TBI treated with bloodletting at Jing points combined with MIH
MRI T2-weighted images (T2WI) showed no abnormal changes in rats in the sham group, with the midline structures remaining centered. Brain edema, hematoma and midline shifting were more severe in the TBI group than in the BL, MIH and B + M groups (Figure 2).

Bloodletting at Jing points combined with MIH improved neurological function in rats with acute severe TBI
At 48 hours after TBI, compared with the TBI group, mNSS was higher in the BL, MIH and B + M groups ($P < 0.01$). The mNSS was significantly lower in the B + M group than in the BL and MIH groups ($P < 0.01$). There was no significant difference in the mNSS between the B + M group and the sham group ($P > 0.05$; Figure 3).

Bloodletting at Jing points combined with MIH reduced brain water content in rats with acute severe TBI
Evans Blue assay showed that brain edema occurred after TBI in each group. Bloodletting at Jing points, MIH and their combined application all attenuated brain edema compared with the TBI group ($P < 0.05$), especially in the MIH and B + M groups ($P < 0.01$). Rats in the B + M group had a brain water content similar to that in the sham group ($P > 0.05$; Figure 4).

Bloodletting at Jing points combined with MIH lessened injury to the BBB of rats with acute severe TBI
Compared with the TBI group, BBB permeability was significantly improved in the BL, MIH and B + M groups ($P < 0.01$). Furthermore, BBB integrity was better in the MIH and B + M groups compared with the BL group ($P < 0.05$ and $P < 0.01$, respectively). There was no statistically significant difference between the B + M and sham groups ($P > 0.05$; Figure 5).

Bloodletting at Jing points combined with MIH alleviated pathological changes in rats with acute severe TBI
Hematoxylin-eosin staining revealed heavy bleeding at the injury site in the TBI group. A large number of inflammatory cells were visible in the TBI group. Compared with the sham and B + M groups, there was a reduction in the number of neurons as well as in the total number of cells. Cell shrinkage and morphological abnormalities were visible in the TBI group (Figure 6). Fluoro-Jade C staining was carried out to evaluate degenerating neurons. Degenerating neurons were significantly increased in the TBI group compared with the sham and B + M groups (data not shown; Figure 7).

Discussion
TBI is a major cause of disability and mortality (Garling et al., 2014). Cerebral edema is a major complication. At present, the primary treatments for cerebral edema are diuretics, hyperosmotic agents, hormones, drugs and surgery. However, all of these treatments have severe side effects. In comparison, bloodletting and MIH, which are non-pharmacological measures, do not have adverse effects. In this study, we investigated whether bloodletting combined with MIH could improve neurological function and cerebral edema, stabilize BBB permeability, reduce apoptosis, and exert a
Several studies have demonstrated that bloodletting at Jing points can increase O₂ partial pressure, inhibit H⁺ accumulation in ischemic areas, regulate the concentration of K⁺ and Na⁺, prevent Ca²⁺ influx, maintain ionic homeostasis, improve brain blood flow, and lessen cytotoxic edema in ischemic models (He et al., 2002; Guo et al., 2003; Gao et al., 2012). Brain edema after trauma has pathogenetic similarities to edema after ischemia. This suggests that the effect of bloodletting on ischemic brain edema may be similar to that in traumatic brain edema.

MIH exerts a significant neuroprotective effect that may reduce secondary cerebral damage after TBI. The neuroprotective effect of MIH may be mediated by its ability to reduce cerebral oxygen metabolism (Liu and Yenari, 2007). MIH has been used as a prophylactic neuroprotectant in the acute phase and to control brain edema in the sub-acute phase. The neuroprotective effects of MIH include inhibiting apoptotic/necrotic processes, lessening neuronal/cellular damage, inhibiting the early stress response, reducing cerebral glucose demand and cerebral thermo-pooling, and suppressing excitotoxicity in the acute phase (Soukup et al., 2002; Colbourne et al., 2003; Polderman, 2004; Liu and Yenari, 2007; Oddo et al., 2009; Dietrich and Bramlett, 2010; Truettner et al., 2011). Subsequently, during the sub-acute phase, MIH reduces BBB disruption and limits brain swelling, inhibits inflammation, and diminishes seizure activity (Deng et al., 2003; Sahuquillo and Vilalta, 2007; Schreckinger and Marion, 2009; Atkins et al., 2010; Dietrich and Bramlett, 2010). Sun et al. (2013) demonstrated that MIH substantially decreases intracranial hypertension by reducing blood volume and inhibiting inflammatory after TBI, thereby lessening BBB leakage.

TBI is characterized by high incidence, disability and death rates, and is a serious threat to human well-being (Kabadi and Faden, 2014; Shin et al., 2015). A series of pathological
Figure 5 Effects of bloodletting at Jing points and MIH on blood-brain barrier integrity in rats with TBI.

(A) Images of Evans Blue (EB) leakage 48 hours after TBI. (B) EB content in the region of damage 48 hours after injury. Data are expressed as the mean ± SD. Factor analysis was conducted to compare data among multiple groups. **P < 0.01, vs. sham group; ###P < 0.01, vs. TBI group; ††P < 0.01, vs. B + M group; &P < 0.05, vs. MIH group. TBI: Traumatic brain injury; BL: bloodletting; MIH: mild induced hypothermia; B + M: bloodletting plus mild induced hypothermia.

Figure 6 Effects of bloodletting at Jing points combined with MIH on pathological changes in rats with TBI (hematoxylin-eosin staining).

Heavy bleeding, a reduced number of neurons and many inflammatory cells were seen in the TBI group. Compared with the sham group and the B + M group, the number of neuronal cells and the total number of cells appeared reduced, and cell shrinkage and irregular morphology were observed in the TBI group. TBI: Traumatic brain injury; BL: bloodletting; MIH: mild induced hypothermia; B + M: bloodletting plus mild induced hypothermia.

Figure 7 Effects of bloodletting at Jing points combined with MIH on neuronal degeneration in the brain of rats with TBI (FJC staining).

Green fluorescence represents necrotic neurons. In the TBI group, there was an increase in the number of degenerating neurons, compared with the sham and B + M groups. TBI: Traumatic brain injury; BL: bloodletting; MIH: mild induced hypothermia; B + M: bloodletting plus mild induced hypothermia; DAPI: 4',6-diamidino-2-phenylindole; FJC: Fluoro-Jade C.
changes occur after TBI, including cellular leakage, secondary neuronal necrosis, inflammation, fluid and electrolyte imbalance, and increased brain water content, causing cerebral edema and increasing intracranial pressure (Wilson and Montgomery, 2007). Single treatment methods have limited efficacy. Thus, a combination of various methods and strategies is necessary to obtain a satisfactory outcome. Our current findings suggest that bloodletting combined with MIH can improve neurological function and lessen cerebral edema, stabilize the BBB, reduce apoptosis, and exert a neuroprotective effect after TBI.

Author contributions: YT and XMM conducted the experiment. TLY performed MRI. XYC bought the dye. HTS analyzed the data. SXC was responsible for data analysis. SZ proofread the paper. All authors approved the final version of the paper.

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