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Original Article

Effect of Mongolian medical brain vibration therapy on inflammation and neuroprotection in rats with concussion

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Abstract: Cerebral concussion, also referred to as mild traumatic brain injury (mTBI), is a complex injury that can lead to cognitive dysfunction and even permanent disability. The purpose of this study was to assess the effects of Mongolian medical brain vibration therapy (MMBVT) and its possible mechanism, on rats with a simple cerebral concussion. An experimental model of mTBI was established by striking the head, while normal Sprague-Dawley (SD) rats were used as controls. At 24 h after mTBI, models of cerebral concussion were treated with MMBVT. Morris water maze (MWM) test was performed to assess spatial learning and memory abilities. When compared with the model group, the escape latencies of rats in the treatment group were significantly shorter and the number of crossings over the target quadrant was larger, especially on the 4th day and 7th day after mTBI ($P<0.05$), indicating that MMBVT may represent an effective treatment strategy. Meanwhile, MMBVT ameliorated the mTBI-induced neuronal apoptosis and the abnormal expression of choline acetyl transferase (ChAT) and tryptophan hydroxylase (TPH) ($P<0.05$), which support the neuroprotective effect of MMBVT. Finally, results demonstrated that MMBVT could lower the levels of inflammatory cytokines (TNF-α, IL-6, and IL-8) in brain tissue of rats modeled with cerebral concussion ($P<0.05$), implying that the neuroprotective effect of MMBVT is mediated, at least in part, by reducing inflammation. To our knowledge, this is the first study to indicate that MMBVT significantly improves neurological and cognitive impairments, and these effects are associated with the prevention of mTBI-induced neuroinflammation and neuronal apoptosis.

Keywords: Mild traumatic brain injury, concussion, Mongolian medical brain vibration therapy, neuroinflammation

Introduction

Cerebral concussion, also known as mild traumatic brain injury (mTBI), is a type of traumatic injury to the brain, which has attracted medical attention due to its high incidence, high disability rate, and high mortality rate [1]. Some patients with cerebral concussions can recover when a week of rest is given, although additional concussions can result in physical, emotional, and cognitive symptoms [2].

To develop effective therapies to prevent or reduce long-term damage following cerebral concussion, it is important to understand the pathophysiological cascade of events and the mechanisms underlying neurological abnormalities. It has been reported that TBI-induced apoptotic neuronal cell death, results in progressive neuronal degeneration [3]. Additionally, recent studies have demonstrated that neuroinflammation was considered an important pathological feature of cerebral concussion [4]. After trauma, a series of inflammatory events occur, such as the activation of macrophages and microglia, and the production of cytokines and chemokines [5]. Persistent neuroinflammation may also participate in neuronal degeneration and cognitive dysfunction [6]. Moreover, it is postulated that concussive head injury-

induced activation of pro-inflammatory cytokines may impact the availability of neurotransmitters in the brain [7]. Kawa et al. found that the expression of the 5-hydroxytryptamine (5-HT) rate-limiting enzyme tryptophan hydroxylase 2 (TPH2) was increased. It is well known that dysfunctions in TPH2 play a role in the pathogenesis of emotional and cognitive disorders through the modulation of the biosynthesis of 5-HT [8]. In contrast, Zhang et al. reported that the levels of acetylcholine (Ach) and the Ach synthetic enzyme choline acetyltransferase (ChAT) were significantly decreased in rats suffering from TBI [9].

At present, the treatment for cerebral concussion generally involves physical and cognitive rest [10]. Several preclinical studies have been performed to assess the efficacy of treatment strategies, and current targets focus on suppressing inflammation, pre-programmed cell death, and associated neurodegenerative-like pathology [11]. However, there is a lack of high-quality evidence for the treatment of cerebral concussion. It is of great significance to find an appropriate, safe and effective way for the treatment of patients with cerebral concussions.

Vibration therapy is already generally used for the treatment and prevention of diverse human diseases, such as Parkinson's disease, chronic stroke, spinal cord injury, and multiple sclerosis [12-15]. Mongolian medicine, one of the outstanding traditional cultures of the Mongolian people, has been developing for thousands of years [16]. Mongolian medical brain vibration therapy (MMBVT) has been used in the treatment of cerebral concussion for a long time. The application of MMBVT as a nonpharmacological approach has the potential to ameliorate symptoms of a concussion. It plays role in regulating blood and strengthening immunity, and preventing and treating diseases. Our previous studies showed that MMBVT has a preferable effect on mTBI, but its mechanism remains unclear [17]. Thus, in the present study, we assess the effect of MMBVT on a rat model of cerebral concussion. Moreover, its underlying mechanisms were also analyzed. Elucidating the beneficial effect and mechanism of MMBVT on cerebral concussion would aid in expanding the knowledge of treatment strategies for disease.

Materials and methods

Animals

Adult male Sprague-Dawley (SD) rats (weighing 220-250 g) were obtained from the Chinese Academy of Military Science (Beijing, China). Animals were housed in individual cages with free access to food and water and were kept in a temperature-controlled room (25°C) under a constant 12/12 h light/dark (lights on at 07:00) cycle. After the 7-day acclimatization period, experimental animals were randomly assigned to either sham or concussion groups as well as to the treatment group. Animal experiments were approved by Baotou Medical College Animal Care and Use Committee (Approval number: 2021037).

Establishment of the rat model of cerebral concussion

Experimental concussion was induced similar to those described previously [17]. Briefly, rats were anesthetized with 3% isoflurane and were fixed on the experimental platform. A concave metal disc was adhered to the skull of the restrained rat to transmit the impact power to the whole brain. The impounder tip of the injury device was then extended to a stroke distance of 44 mm and impacted the metal disc with a velocity of 6.0 m/s. After the injury, the animals were kept warm (37°C) until they regained consciousness. Sham-operated rats underwent the same procedures except for the impact. Treatment groups were treated with MMBVT at 24 h after mTBI. At the end of the study, rats were anesthetized by intraperitoneal injection of a lethal dose of pentobarbitone sodium (250 mg/kg body weight) for brain tissue collection.

Micro-PET/CT imaging

Micro-PET/CT Imaging (SIEMENS Inveon MM, Siemens Ltd., Munich, Germany) of rats was performed 24 h after mTBI, which provides important information, such as the presence of the brain hemorrhage. The model rats were anesthetized with 10% chloral hydrate solution (0.3 mg/kg) and were fixed in a prone position on a scanning bed of micro-PET/CT. Micro-PET/CT scans were then carried out with voltage 80 kV, current 500 μA, slice thickness 0.06 mm, and exposure time 600 ms.

Behavioral assessment

The effects of MMBVT on the spatial learning and memory abilities of the rats were evaluated at days 4, 7, and 10, following treatment with a 48-h interval. Morris water maze (MWM) was conducted in a circular basin (120 cm diameter; 45 cm deep). The maze was filled with water (25-26°C) to a depth of 30 cm. The water was made opaque by adding non-toxic white tempera paint. A Plexiglas platform was submerged 2.5 cm beneath the water surface and placed in the center of one quadrant of the pool. Rats were placed into the water facing the wall of the maze at one of the equally spaced starting positions. During each trial, the rat was allowed to swim freely to find the submerged platform or until 60 s elapsed. Animals were given four trials per day with a 5-min inter-trial interval. The latency to find the hidden escape platform was recorded. Spatial memory was also assessed during a 60 s probe trial in which the platform is removed from the pool as described previously [18]. The number of times the animal crossed the quadrant where the platform was originally located was recorded.

Nissl staining

The specimens were fixed with formaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. Tissue sections were deparaffinized using xylene, rehydrated in an alcohol concentration gradient, and washed in tap water. Nissl staining was performed using Nissl staining Kit (Boster Biotech, Wuhan, China). The images were taken with a light microscope.

Enzyme-linked immunosorbent assay

Inflammatory factor levels in brain tissue were detected with commercially available ELISA kits (Boster Biotech, Wuhan, China) for TNF-α, IL-6, and IL-8 according to the manufacturer's instructions at days 4, 7, and 10, following treatment. The standards were run in duplicate and samples in triplicate. Absorbance was measured at 450 nm using a microplate reader.

Western blot analysis

Total protein was extracted by homogenizing samples of the parietal cortex, hippocampus, and hypothalamus in a lysis buffer. Protein concentrations were assessed using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Each sample (30 μg) was separated by SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The blotted membranes were blocked with blocking solution (5% nonfat dry milk, 0.05% Tween-20 in PBS) at room temperature, and then incubated with the following primary antibodies overnight at 4°C: anti-ChAT (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-TPH (1:1000; Cell Signaling Technology, Danvers, MA, USA), or anti-GAPDH (1:40,000; Sigma-Aldrich, St. Louis, MO, USA). After being washed with PBS containing 0.1% Tween-20, the membranes were incubated with secondary antibodies (1:2000; Cell Signaling Technology, Danvers, MA, USA). The protein bands were visualized by the ECL detection system (GE Healthcare, Pittsburgh, PA, USA).

Statistical analysis

All data are analyzed with IBM SPSS Statistics for Windows (version 20) and expressed as mean ± SE. Differences between means were assessed by one-way measures analysis of variance (ANOVA) followed by Tukey's post hoc analysis. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Animal characteristics in micro-PET/CT imaging

Micro-PET/CT of rats in the model group (n=10) and the treatment group (n=10) was performed. The results confirmed that 8 rats in the model group appeared normal. Only 2 rats had a small amount of subarachnoid hemorrhage (**Figure 1**). Moreover, the rats in the treatment group (n=10) were examined using the micro-PET/CT and all rats appeared normal. There was no death among the test animals, implying that this method to establish the model of cerebral concussion was safe. Therefore, 8 rats in the model group and 10 rats in the treatment group, appeared normal on the micro-PET/CT, which were selected for further analysis.

Mongolian medical brain vibration therapy reversed behavioral dysfunction

Spatial learning was assessed in the MWM test at 4, 7, and 10 days following the control procedure or mTBI (**Figure 2A**). The results demonstrated that an increased latency time was

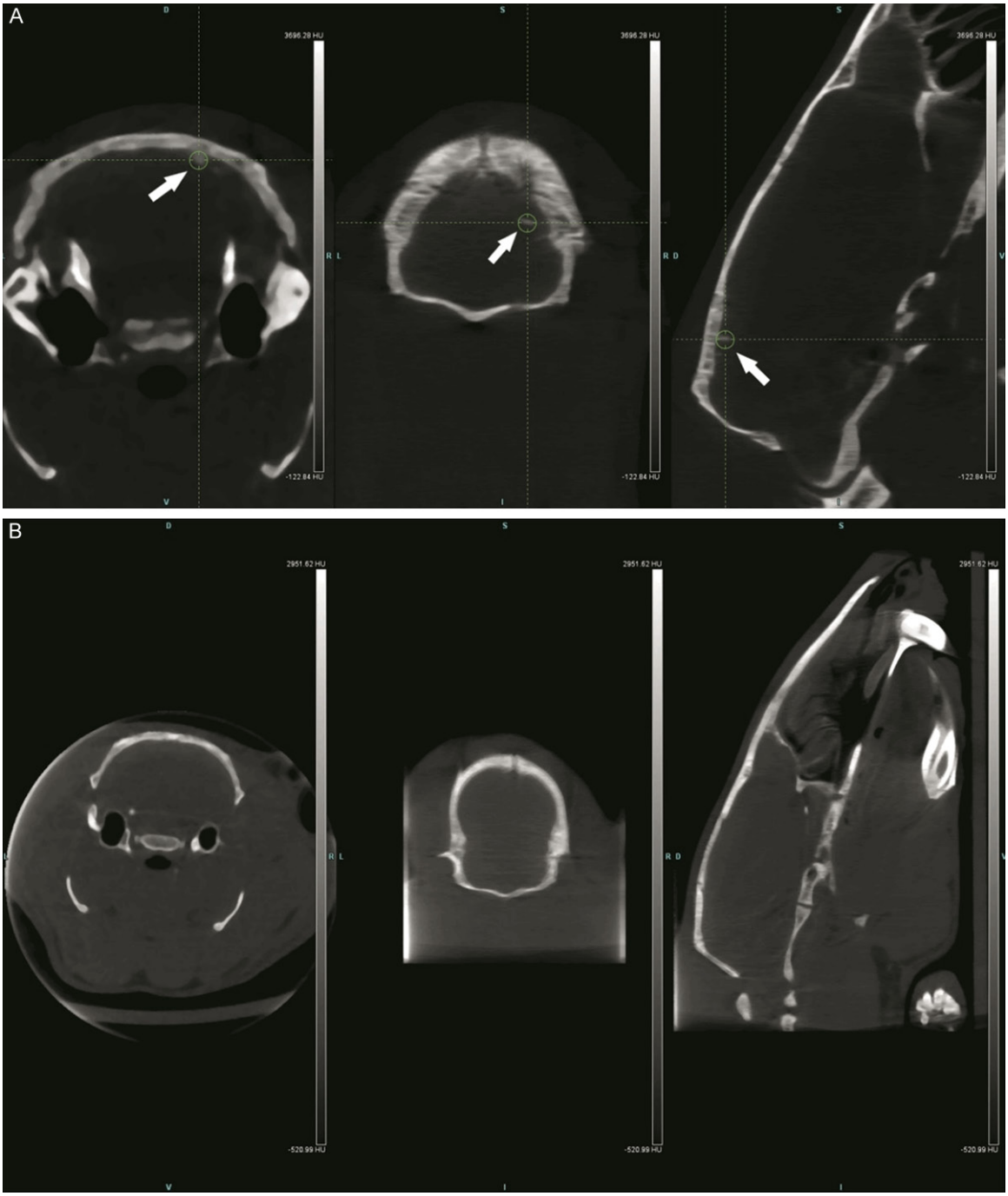

Figure 1. Micro-PET/CT imaging after a mild traumatic brain injury. A. The arrows represent subarachnoid hemorrhage. B. There was no significant cerebral hemorrhage or intracranial hemorrhage.

required for the rats modeled with cerebral concussion to locate the platform compared to the control rats ($P<0.05$). Compared with the model group, the treatment group rats showed a significant reduction in latency time at the 4th and 7th days of post-injury ($P<0.05$). Meanwhile, memory retention ability was assessed in the MWM at 4, 7, and 10 days following the control procedure or mTBI (**Figure 2B**). The number of crossings over the platform zone was significantly reduced in the concussed rats compared to the control rats ($P<0.05$). Moreover, significant reversal effects were observed in the treatment group ($P<0.05$). The overall findings showed that MMBVT rescued spatial learning and memory dysfunction.

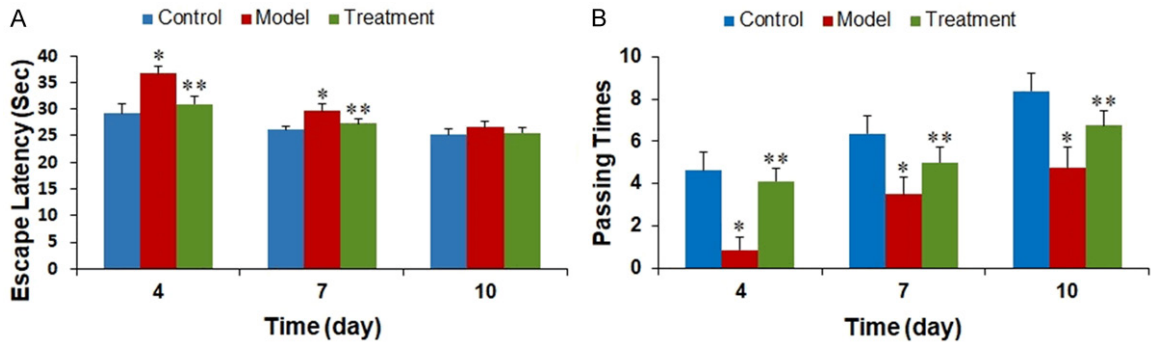

Figure 2. Effect of the Mongolian medical brain vibration therapy on spatial learning and memory abilities in rats. A. In the hidden platform tests, the treatment group showed a shorter latency to escape at the 4th and 7th days, compared to the model group. B. In the probe trial, the treatment group traveled into the platform zone, where the hidden platform was originally located, significantly more times than the model group. Values are expressed as the means ± SE, n=8 rats in the model group and n=10 rats in the control group and the treatment group. *P<0.05, *vs* control group. **P<0.05, *vs* model group.

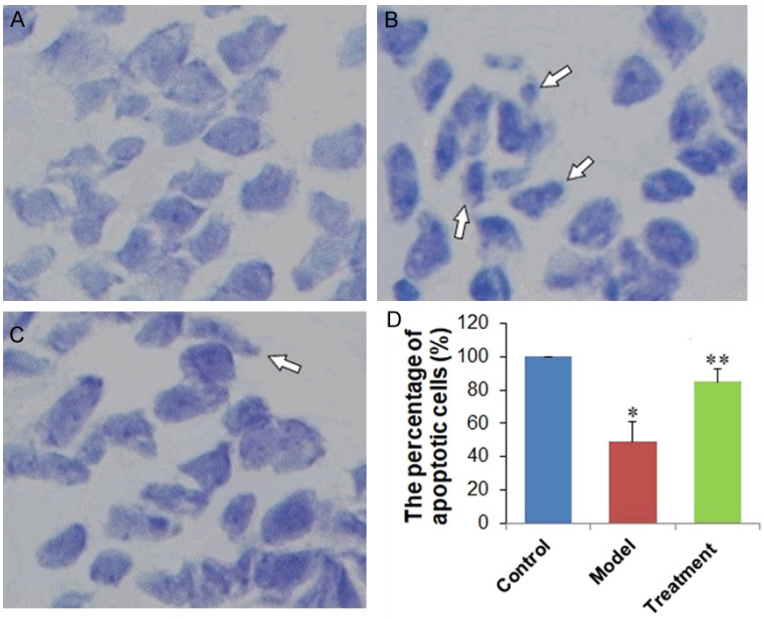

Figure 3. The Mongolian medical brain vibration therapy protected neurons against mild traumatic brain injury-induced neuronal apoptosis 4 days after injury. A. Control group; B. Model group; C. Treatment group; D. The percentage of apoptotic cells. Values are expressed as the means ± SE, n=8 rats in the model group and n=10 rats in the control group and the treatment group. *P<0.05, *vs* control group. **P<0.05, *vs* model group.

Mongolian medical brain vibration therapy reduced neuronal loss

Nissl staining was used to identify the apoptotic neurons at 4 days following the control procedure or mTBI (**Figure 3**). The apoptotic neurons were dark and shrunken with cytoplasmic vacuoles, while the normal neurons have a relatively full soma with round and large nuclei. Nissl staining revealed that the percentage of apoptotic cells was higher in the model group than in the control group (P<0.05). However, the number of degenerated neuronal cells was significantly lower in the treatment group than in the model group (P<0.05).

Mongolian medical brain vibration therapy ameliorated the expression of ChAT and TPH

Total protein extracted from the cortices, hippocampi, and hypothalamus of the rat brains was analyzed for ChAT and TPH. Protein levels in the control, model or treatment groups were compared (**Figure 4**). Compared to the control group, ChAT and TPH levels were significantly increased and decreased in the model group, respectively (P<0.05). Interestingly, the expression levels of these proteins were significantly reversed in the MMBVT-treated group (P<0.05). Original data for the expression of ChAT and TPH showed in **Figure 4** were presented in Supplementary Figures 1, 2.

Mongolian medical brain vibration therapy reduced neuroinflammation

The results showed that the model group had significantly higher expression levels of IL-6, IL-8, and TNF-α than the control group, while

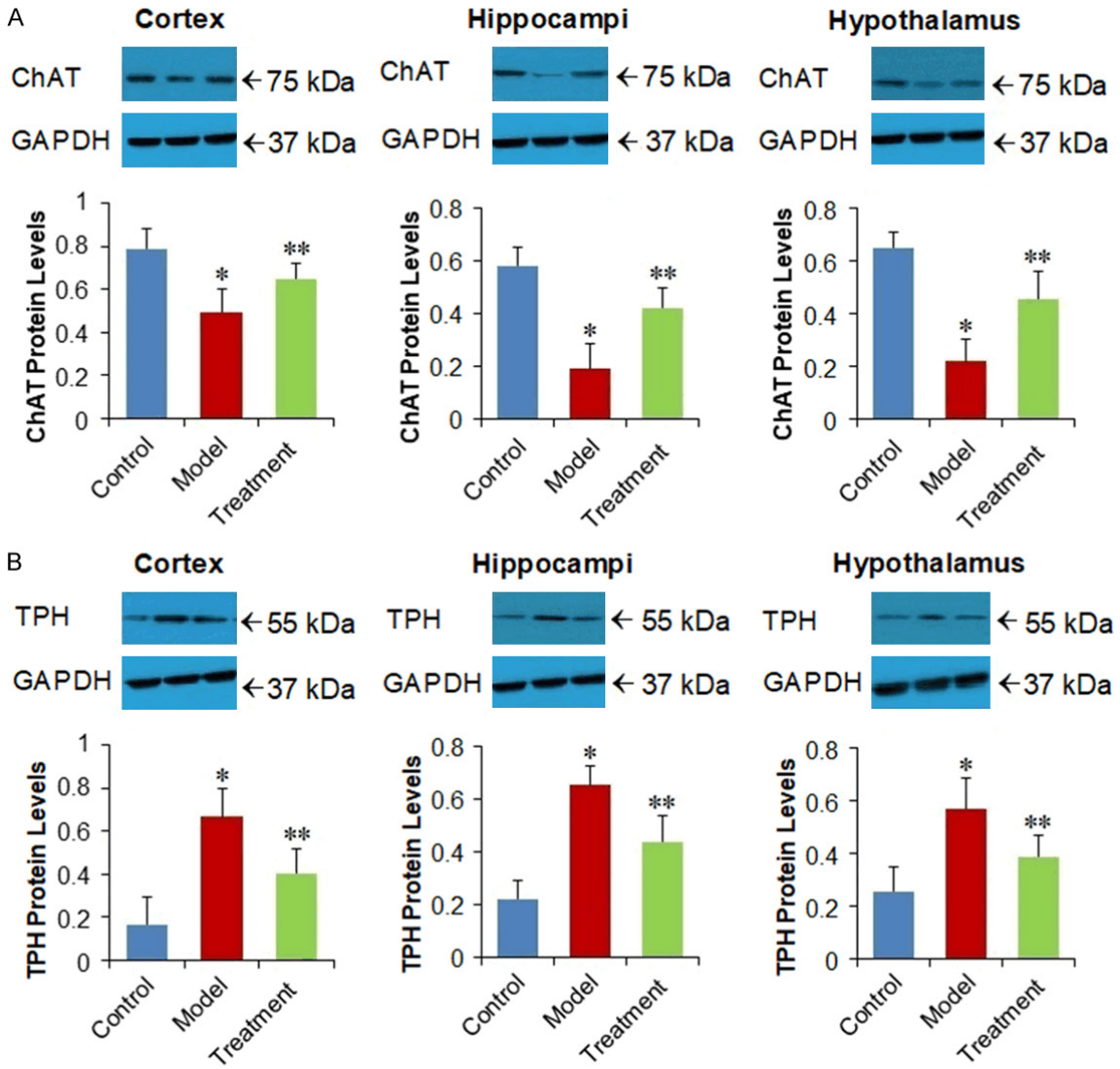

Figure 4. The Mongolian medical brain vibration therapy ameliorated the expression levels of ChAT and TPH. Values are expressed as the means ± SE, n=8 rats in the model group and n=10 rats in the control group and the treatment group. *P<0.05, *vs* control group. **P<0.05, *vs* model group.

the MMBVT treatment decreased the mTBI-induced enhancement of inflammatory factor levels (P<0.05) (**Figure 5**). These findings suggested that the MMBVT treatment alleviated the inflammatory response in the brain mediated by mTBI.

Discussion

Cerebral concussion or mTBI may produce acute as well as long-term functional consequences associated with cognitive disorders and post-concussive syndromes [19]. In the present study, we report for the first time that MMBVT exerted significant neuroprotective effects on rats with cerebral concussions. Importantly, we further confirmed that the neuroprotective effect of MMBVT is mediated, at least in part, by inhibiting neuroinflammation and neuronal apoptosis.

To confirm whether a cerebral concussion had occurred, a behavioral test was conducted in the current study. We found that concussed rats demonstrated cognitive dysfunction in spatial learning at the 4th and 7th days post-injury. Additionally, the rats in the model group performed significantly worse compared to the

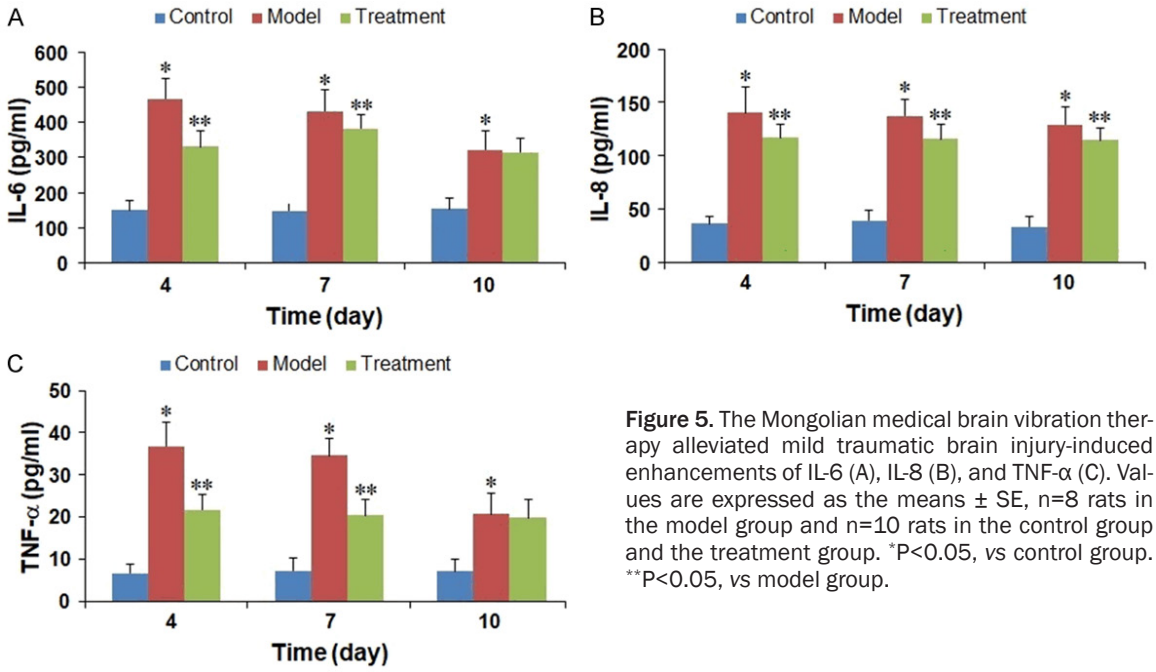

Figure 5. The Mongolian medical brain vibration therapy alleviated mild traumatic brain injury-induced enhancements of IL-6 (A), IL-8 (B), and TNF-α (C). Values are expressed as the means ± SE, n=8 rats in the model group and n=10 rats in the control group and the treatment group. $^{*}P<0.05$, *vs* control group. $^{**}P<0.05$, *vs* model group.

control rats in the probe trial. On the 10th day post-injury, there was no significant difference among these 3 groups in latency time. This might be because mTBI leads to a temporary disturbance in brain function, and the cognitive impairments were ameliorated gradually with the time, which is in agreement with previous reports [20]. Interestingly, we further found that all of these behavioral abnormalities were strongly ameliorated by MMBVT, indicating that MMBVT may represent an effective treatment strategy.

Recent findings have emphasized the potentially significant effects of apoptotic cell death and neurodegeneration on cognition, learning, and behavior [21]. The present study confirmed that the MMBVT protected neurons against mTBI-induced neuronal apoptosis and improved neurological function. It is therefore intriguing to speculate that MMBVT reversed the mTBI-induced apoptosis of neuronal cells and may responsible for its treatment efficacy on cerebral concussion.

The alterations of neurotransmitter utilization in discrete brain areas are the characteristic feature of disturbances after mTBI [21]. Previous studies reported that ChAT and TPH are enzymes that play key roles in the biosynthesis of 5-HT and Ach, respectively [8, 9]. The expression levels of ChAT and TPH can indirectly represent the contents of 5-HT and Ach. Therefore, we further explored the expressed levels of ChAT and TPH in the parietal cortex, hippocampus, and hypothalamus of the rat brains. Our results indicated that mTBI elevated the level of TPH and decreased the level of ChAT in the above-mentioned regions of the rat brains, which is in agreement with other reports [22, 23]. The results further confirmed the dysfunctional state of neurons, which may partly explain the development of concussion brought about by the mechanical trauma. Moreover, the altered expressions of ChAT and TPH were reversed by the MMBVT. We, therefore, speculated that MMBVT regulated the levels of these proteins by preventing mTBI-inducing neuronal loss, resulting in the alteration of 5-HT and Ach, respectively.

The TBI-mediated inflammatory response plays an important role in neural damage and secondary pathological processes [24]. Neuroinflammation can lead to persistent impairment in neuronal structure [25, 26]. Our study showed that the levels of inflammatory cytokines (TNF-α, IL-6, and IL-8) increased after the mTBI. It was interesting to observe that the MMBVT significantly inhibited the inflammatory response following mTBI, implying that MMBVT

has the potential for reducing inflammation and promoting neuronal survival.

In conclusion, the current study firstly demonstrates that the MMBVT inhibited neuroinflammation and exerted neuroprotective effects, providing new insights into the development of a natural therapy for the treatment of cerebral concussion.

Limitations

This study tried to address the effect of the MMBVT on inflammation and neuroprotection in rats with a concussion. However, the study was not without limitations. Further research should be made to understand the role of the upstream related signaling pathways in the regulation of the inflammatory response following the mTBI.

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Disclosure of conflict of interest

None.

Abbreviations

Ach, Acetylcholine; ChAT, Acetyl choline transferase; ELISA, Enzyme-linked immunosorbent assay; MMBVT, Mongolian medical brain vibration therapy; mTBI, Mild traumatic brain injury; MWM, Morris water maze; SD, Sprague-Dawley; TPH, Tryptophan hydroxylase; 5-HT, 5-hydroxytryptamine.

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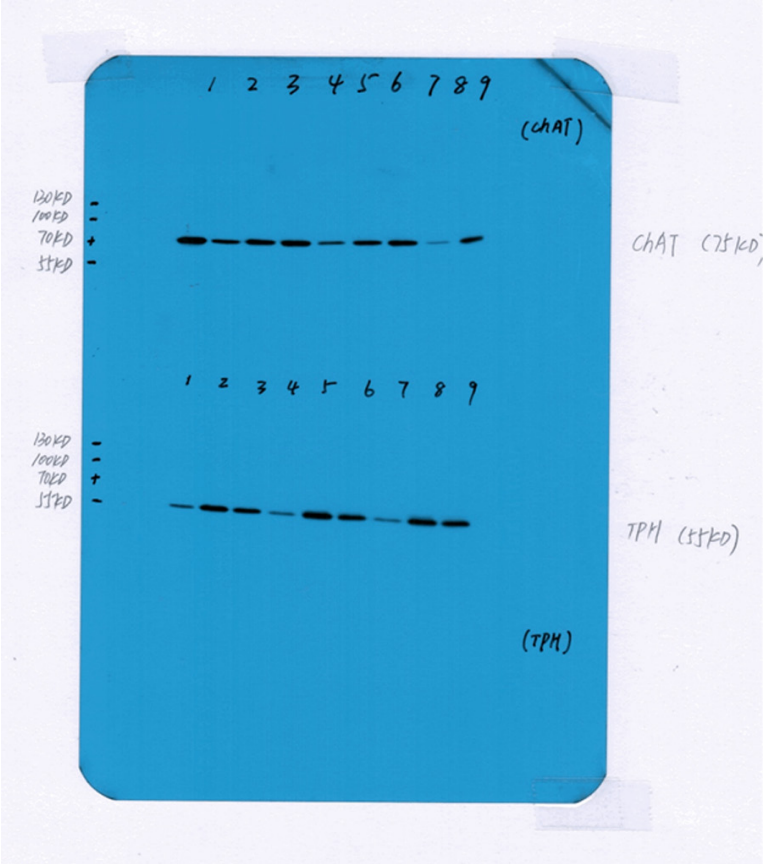

Supplementary Figure 1. Lane 1-3: The expression levels of ChAT and TPH in the cortices of the control group, the model group and the treatment group, respectively. Lane 4-6: The expression levels of ChAT and TPH in the hippocampi of the control group, the model group and the treatment group, respectively. Lane 7-9: The expression levels of ChAT and TPH in the hypothalamus of the control group, the model group and the treatment group, respectively.

Supplementary Figure 2. Lane 1-3: The expression levels of GAPDH in the cortices of the control group, the model group and the treatment group, respectively. Lane 4-6: The expression levels of GAPDH in the hippocampi of the control group, the model group and the treatment group, respectively. Lane 7-9: The expression levels of GAPDH in the hypothalamus of the control group, the model group and the treatment group, respectively.