Malva Sylvestris Attenuates Cognitive Deficits in a Repetitive Mild Traumatic Brain Injury Rat Model by Reducing Neuronal Degeneration and Astrocytosis in the Hippocampus

Malva Sylvestris (MS) is a widely used herbal medicine known for its neuroprotective properties. However, the mechanisms by which MS attenuates cognitive deficits in a repetitive mild traumatic brain injury (MTBI) rat model remain unclear. This study aimed to evaluate the effect of MS on cognitive dysfunction in a repetitive MTBI model.

**Background:** The aim of our study was to evaluate the effect of Malva sylvestris (MS) on cognitive dysfunction in a repetitive mild traumatic brain injury (MTBI).

**Material/Methods:** MTBI was induced in all the study animals by hitting a metallic pendulum near the parietal-occipital area of the skull three times a day for ten days. Animals were treated with MS (250 mg/kg and 500 mg/kg) intragastrically per day for seven consecutive days. Cognitive function was estimated by the Morris water maze (MWM) method. Histopathology studies were performed on the hippocampal region by Nissl staining and anti GFAP staining. Concentrations of reactive oxygen species (ROS), and oxidative parameters including superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation (LPO), and inflammatory cytokines in the brain tissues were measured.

**Result:** Treatment with MS significantly improved cognitive function compared to the negative control. Histopathology studies suggested that treatment with MS significantly decreased the count of neurodegenerative cells and induction of astrocytosis in the MTBI treated group compared to the negative control group. However, the concentrations of ROS and LPO, and the activities of SOD and CAT were significantly decreased in the MS treated groups of MTBI rats compared to the negative control group. Inflammatory cytokines, such as IL-1β, IL6, and TNF-α were significantly decreased in the brain tissues of the MTBI treated group compared to the control group of rats.

**Conclusions:** This study concluded that treatment with MS significantly improved cognitive dysfunction by reducing neurodegeneration and astrocytosis in brain tissues via decreasing oxidative stress and inflammation in neuronal cells.

**MeSH Keywords:** Astrocytes • Brain Injuries • Neurodegenerative Diseases

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**Authors’ Contribution:**

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| Hailin Qin | Jie Qin | Junmin Hu | He Huang | Lianting Ma | 

1 Department of Neurorsurgery, Southern Medical University, Guangzhou, Guangdong, P.R. China
2 Department of Neurorsurgery, Wuhan General Hospital of PLA, Wuhan, Hubei, P.R. China

**Corresponding Author:** Lianting Ma, e-mail: liantingma@hotmail.com

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ANIMAL STUDY

Background

Mild traumatic brain injury (MTBI) affects approximately 42 million people worldwide [1], and it is thought that these injuries induce neurobehavioral changes in patients [2]. MTBI may also result in the development of neurodegenerative diseases such as cognitive dysfunction, amyotrophic lateral sclerosis (ALS), Parkinson disease, and Alzheimer disease [3]. Additionally, mechanical injury can initially induce MTBI and subsequently cause secondary injuries as the disease progresses [4]. The literature suggests that neuro-inflammation and increased levels of oxidative stress are responsible for the progression of the neurodegeneration associated with MTBI [5–7].

Neuro-inflammation is the result of immune reactions occurring in the brain that enhance glutamate levels and increase the production of reactive oxygen species (ROS), which, in turn, promote inflammatory reactions [8]. Moreover, glial scars may form due to neuro-inflammation, which may prevent neuronal regeneration and impair cognitive function [9]. Although the limited number of drugs available for the treatment of MTBI can effectively manage its symptoms, this disorder significantly enhances the risk of cognitive dysfunction [10]. Traditional Chinese medicines and compounds derived from them have been used to effectively manage MTBI [11,12]. For example, *Malva sylvestris* L. (MS) of the Malvaceae family, which is traditionally used as medicine in China, reportedly exerts anti-inflammatory, antioxidant, bacteriostatic, anti-nociceptive, and anti-influenza activities [13–15]. Phytochemical investigations have revealed that MS herbs contain several biologically active chemical components that include essential oils, sesquiterpenes, leucoanthocyanidins, anthocyanins, coumarins, and anthoacyaninds [13]. Coumarins and anthocyanins are known to protect against neurodegeneration in cases of cerebral ischemia [16,17]. Thus, the present study evaluated the effects of MS on cognitive deficits induced in a repetitive MTBI rat model.

Material and Methods

Animals

Sprague-Dawley rats of 200 g to 250 g body weight were used in this study. All the rats were housed under a controlled condition specified as per guidelines. All the experiments used in this study are approved by the Animal Ethical Committee of the Southern Medical University, China (IEC/SMU/2016/19); this study followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use.

Preparation of extract

Whole plants of *Malva sylvestris* (MS) were collected and authenticated from the Botanical Department of the Southern Medical University, China. Whole plants were air dried after washing. Dried plants were cut into small pieces and coarsely ground into powder using a grinder. The powder was kept in 95% methanol in a bottle for seven day for maceration. The extract was filtered out and the filtrate was dried using a rotary evaporator. The practical yield of the extract was found to be 16.5% w/w.

Induction of MTBI

MTBI was induced as per previously reported methods by hitting a metallic pendulum near the parietal-occipital area of the skull three times a day for ten days. The following formula was used to assess the severity of injury: record the timing of recovery of physiological parameter instantly after the injury and score it accordingly as apnea (xp1), external auditory meatus reflex (xp2), righting reflex response (xp3), corneal reflex (xp4), or response to painful stimulus (xp5). Then according to the formula Y=0.133xp1+0.264xp2+0.027xp3+0.196xp4−0.006xp5−2.982, if Y <0.319 and injured rat with apnea, then the rat was defined as MTBI. All the animals defined as MTBI were selected for further study.

Treatment protocol

All the MTBI animals were separated into four groups: control group received solvent only, negative control group received no treatment, and MS treated groups received the extract (250 mg/kg or 500 mg/kg) intragastrically per day for the duration of seven consecutive days.

Assessment of cognitive function by Morris water maze

Cognitive function was tested by Morris water maze (MWM) method by having a tank dimension of 180 cm diameter and 70 cm height. The tank was separated into four quadrants with suitable labeling and the stage kept 2 cm below the water level. All rats were trained for five days, two sessions a day, at an interval of two hours after the production of injury. There were four trials in each session, performed at an interval of 30 seconds. In each trial, the rat was placed in a quadrant and permitted to search for the stage. If the rat was not able to locate the stage in two minutes then the rat was placed on the stage for two minutes for consideration of the escape latency. Spatial memory of the trained rats were investigated by removing the stage from the quadrant and estimating the duration of time spent by a rat in a specified quadrant at the end of the protocol. All the MWM trials were performed at a specific time of the day to avoid time of day related variation in performance.
Tissue preparation

At the end of protocol, all the animals were injected with 10% chloral hydrate intraperitoneally to produce anesthesia. The brain was isolated from each animal and fix in 4% paraformaldehyde. Then the brain was stored at 4°C in a 30% sucrose solution. The isolated brain was separated into two sections. One section of the brain was used for anti GFAP staining and Nissl staining. The other section of the brain of each rat was homogenized instantaneously in a solution containing Tris-HCl (50 mM, pH 7.4) and sucrose (300 mM). The homogenate was centrifuged at 10,000 g for the duration of 10 minutes, and the supernatant was separated for biochemical estimation.

Nissl staining

Isolated rat brain sections were placed into 4% paraformaldehyde for one day and dehydrated with alcohol. Dehydrated sections were dipped into paraffin and then using a microtome, sliced into 5 μm tissue sections. The tissue slices were stained with Nissl staining in 1% thionin. Neuronal cells counting was done using a light microscope (400×) focused on the hippocampus areas Ca1, Ca3, and DG.

Anti GFAP staining

The isolated brain was cut into sections using a microtome. Tissue sections were kept under citric acid buffer solution for 45 minutes to estimate antigens, and PBS was used to rehydrate the tissue sections for 10 minutes for the estimation of astrocytosis. Later, permeabilization buffer (PBS containing 0.1% Triton X and 4% goat serum) was used for 60 minutes to permeabilized the tissue block sections. GFAP specific monoclonal primary antibody (1: 400, MAB360) was incubated with the tissue sections at 4°C overnight. Thereafter, on the next day, biotin-conjugated secondary antibody was incubated with tissue sections at room temperature for two hours. Tissues were further treated with streptavidin-peroxidase for 12 minutes after washing with 1% Triton X and 0.01 M PBS solution. Moreover, tissue sections were treated with developing agent diaminobenzidine for 10 minutes; thereafter tissue sections were dehydrated by adding alcohol (95%) twice, and tissues were mounted on the slides using neutral gum solution. The hippocampus area of the brain (Ca1, Ca3, and DG) was photographed by using trinocular microscope at 400×.

Estimation of ROS generation

The estimation of the production of ROS was done as per He et al. Tissue homogenates were kept for the estimation of intensity of fluorescence at wavelength of 488 nm for excitation and 530 nm for emission using flow cytometer. Moreover, swelling of the mitochondria was determined by estimating the optical density at a wavelength of 520 nm as per a previous study. The degree of swelling of the mitochondria was reflected by the turbidity of the reactant product.

Estimation of markers of oxidative stress

Superoxide dismutase (SOD) was estimated in the brain tissue of rats by using the riboflavin sensitized method; the alteration in absorbance was observed for four minutes at 460 nm. The level of lipid peroxidation (LPO) was estimated (using the Ohkawa method) in the tissue homogenate; the amount of malondialdehyde (MDA) was estimated at 532 nm.
The activity of catalase (CAT) in the brain tissue was assessed based on the ability to catalase to oxidize H$_2$O$_2$. The alteration in the level of absorbance was estimated for three minutes at one minute intervals at 240 nm.

**Statistical analysis**

Data is represented as mean ±SD (n=10). All the results were analyzed statistically by one way ANOVA and post hoc study by Dunnett. In this study, values of $p<0.05$ was considered as significant.

The level of cytokines, such as IL1β, IL6, and TNFα, were estimated in the tissue homogenate by using specific enzyme-linked immunosorbent assay (ELISA) kits as per the instruction given in the individual kits.
Results

Effect of MS on cognitive function

The effect of MS on cognitive function in the repetitive MTBI in rats using MWM apparatus is shown in Figure 1. There were significant \( p<0.01 \) increases in the escape latency in the negative control group (no treatment) compared to the control group (solvent-only). However, treatment with MS significantly decreased the escape latency compared to the negative control group in the MTBI rat model (Figure 1A). The time spend in the target quadrant significantly decreased \( p<0.01 \) in MTBI rats compared to the control group, and MS treated group showed significant reduction in the time spend in the target quadrant compared to the negative control group (Figure 1B).

Effect of MS on neurodegeneration

Nissl staining was done to estimate the neurodegeneration on the hippocampus of the repetitive MTBI rats as shown in Figure 2. It was observed that the degeneration of neuron was significantly enhanced in the MTBI rats compared to the control rats \( p<0.01 \). However treatment with MS 250 mg/kg and MS 500 mg/kg significantly decreased the count of neurodegeneration in Ca1 and Ca3 regions of hippocampus of the MTBI rats compared to the negative control rats \( p<0.01 \).
Effect of MS on the astrocytosis induction

Anti GFAP staining was done to estimate the effect of MS on astrocytosis induced by MTBI in the hippocampus of the rats as shown in Figure 3. There was a significant increase in the count of GFAP positive cells in the hippocampal region CA1 and CA3 of the negative control rats. It was observed that treatment with MS at 250 mg/kg and 500 mg/kg significantly decreased the enhanced count of GFAP positive cells in the hippocampal region CA1 and CA3 of MTBI rats compared to the negative control rats.

Effect of MS on the production of ROS and oxidative stress parameters

The effect of MS on the production of ROS and oxidative stress parameters in repetitive MTBI rats are shown in Figure 4 and Table 1. It was observed that the production of ROS in brain tissue of MTBI rats was significantly enhanced compared to control rats. However, the quantity of ROS production significantly decreased in the MS treated groups compared to the negative control group.

Oxidative stress parameters, such as SOD, LPO, and CAT, also get altered in MTBI rats. The activity of SOD significantly decreased while CAT and LPO activity was significantly enhanced in the brain tissue of the MTBI rats compared to the control rats. These altered activities of SOD, CAT, and LPO were found to be significantly attenuated in the MS treated rats compared to the negative control rats.

Effect of MS on inflammatory cytokines

The effect of MS on the concentration of inflammatory cytokines, such as IL1β, IL6, and TNFα, in the brain tissues of the MTBI rats is shown in Figure 5. There were significant increases in the concentration of IL1β, IL6, and TNFα in the brain tissue of the MTBI rats (negative control group) compared to the control rats. Treatment with MS 250 mg/kg and MS 500 mg/kg significantly decreased the concentration of IL1β, IL6, and TNFα in the brain tissues of the MTBI rats compared to the negative control rats in a dose dependent manner.

Discussion

MTBI affected patients suffer from neurodegenerative disorders and there are a limited number of drugs available for the management of these neurodegenerative disorders. In the recent era, scientists have focused on alternative therapies for the management of cognitive dysfunction induced by MTBI. Thus, this report evaluated the effect of MS on cognitive deficits in a MTBI rat model.

The literature suggests that MTBI alters cognitive functions by enhancing neurodegeneration [11]. The results of our study suggested that the time spent in the target quadrant and the escape latency were significantly decreased in MTBI rats compared to the control group. However, treatment with MS significantly attenuated the cognitive function by improving the time spent in the target quadrant and the escape latency in the MTBI rats. Cognitive defects due to neurodegeneration and the drugs that protect neurodegeneration seem to attenuate the cognitive deficit in MTBI rats [18]. Our study results

Table 1. Effect of Malva sylvestris on SOD, LPO & CAT in the brain tissue of repetitive mild traumatic brain injured rat.

| Sr. No. | Group           | SOD (Unit/mg protein) | LPO (nmol MDA/mg protein) | CAT (µmol H₂O₂ consumed/min/mg protein) |
|---------|-----------------|-----------------------|---------------------------|----------------------------------------|
| 1       | Control         | 14.2±1.2              | 7.9±0.5                   | 49.1±2.1                               |
| 2       | Negative control| 3.8±0.4**             | 15.1±1.3**                | 72.5±5.5**                             |
| 3       | MS 250 mg/kg    | 10.2±1.1**            | 10.3±0.6**                | 60.3±3.2**                             |
| 4       | MS 500 mg/kg    | 13.1±1.3**            | 8.3±0.3**                 | 53.9±2.4**                             |

Values are means ±SD; ** p<0.01 than Control group, *** p<0.01 than Negative control group.
suggested that treatment with MS significantly decreased the neurodegeneration count and MTBI-induced astrocytosis in the brain tissue of MTBI rats.

The literature has reported that in MTBI, enhanced oxidative stress results in neurodegeneration, and antioxidant drugs effectively reduce/prevent neurodegeneration by decreasing oxidative stress [19,20]. In a MTBI rat model, by reducing oxidative stress and the generation of free radicals, drugs improve cognitive functions and reduce neuronal death [21]. Our study also found that treatment with MS 250 mg/kg and MS 500 mg/kg significantly decreased the concentration of ROS and the activity of LPO, and increased the activity of SOD and CAT enzymes in the brain tissues of MTBI rats.

MTBI-induced cognitive dysfunction occurs due to neuro-inflammation, and the induction of astrocytosis plays a vital role in neuro-inflammation [22–24]. MS is reported to possess strong anti-inflammatory activity and anti-cholinesterase activity. The result of our study showed that treatment with MS 250 mg/kg and MS 500 mg/kg significantly reduced the concentration of IL1β, IL6, and TNFα in the brain tissue of MTBI rats. Moreover, the histopathology of the MS treated group suggested that induction of astrocytosis was significantly reduced by the decreasing number of GFAP positive cells and thereby improved cognitive function. Thus, MS effectively attenuated the cognitive function in MTBI rats by decreasing neuro-inflammation concentrations in the brain tissues.

**Conclusions**

This study concluded that treatment with MS significantly improved cognitive dysfunction by reducing neurodegeneration and astrocytosis in brain tissues via decreasing oxidative stress and inflammation in neuronal cells.

**Conflict of interest**

None.

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**Figure 5.** Effect of *Malva sylvestris* on the concentration of inflammatory cytokines in repetitive mild traumatic brain injured rats. Values are means ±SD; **p <0.01 compared to the control group, ##p <0.01 compared to the negative control group.
References:

1. Laurer HL, Bareyre FM, Lee VM et al: Mild head injury increasing the brain’s vulnerability to a second concussive impact. J Neurosurg, 2001; 95: 859–70

2. Miller KJ, Ivins BJ, Schwab KA: Self-reported mild TBI and postconcussive symptoms in a peacetime active duty military population: Effect of multiple TBI history versus single mild TBI. J Head Trauma Rehabil, 2013; 28: 31–38

3. Esopenko C, Levine B: Aging, neurodegenerative disease, and traumatic brain injury: The role of neuroimaging. J Neurotrauma, 2015; 32: 209–20

4. Bramlett HM, Dietrich WD: Progressive damage after brain and spinal cord injury: Pathomechanisms and treatment strategies. Prog Brain Res, 2007; 161: 125–41

5. Tsai YD, Liliang PC, Cho CL et al: Delayed neurovascular inflammation after mild traumatic brain injury in rats. Brain Inj, 2013; 27: 361–65

6. Roth TL, Nayak D, Atanasijevic T et al: Trans cranial amelioration of inflammation and cell death after brain injury. Nature, 2014; 505: 223–28

7. Faden AI, Loane DJ: Chronic neurodegeneration after traumatic brain injury: Alzheimer disease, chronic traumatic encephalopathy, or persistent neuroinflammation? Neurotherapeutics, 2015; 12: 143–50

8. Abdul-Muneer PM, Schuetz H, Wang F et al: Induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mild traumatic brain injury induced by primary blast. Free Radic Biol Med, 2013; 60: 282–91

9. Blostein PA, Jones SJ, Buechler CM, Vandongen S: Cognitive screening in mild traumatic brain injuries: systematic review, Br J Psychiatry J Mental Sci, 2013; 203: 31–38

10. Cooper C, Li R, Lyketsos C, Livingston G: Treatment for mild cognitive impairment: A review. J Am Geriatr Soc, 2013; 61: 461

11. Song H, Xu L, Zhang R et al: Rosemary extract improves cognitive deficits in a rat model of traumatic brain injury via an anti-inflammatory pathway. Sci Rep, 2016; 6: 20040

12. Qin H. et al.: Malva sylvestris attenuates cognitive deficits... © Med Sci Monit, 2017; 23: 6099-6106

13. Prudente AS, Loddi AM, Duarte MR et al: Pre-clinical anti-inflammatory aspects of a cuisine and medicinal millennium herb: Malva sylvestris L Food Chem Toxicol, 2013; 51: 324–31

14. Cheng C, Wang ZY: Bacteriostatic activity of anthocyanin of Malva sylvestris. Journal of Forestry Research 2006; 17: 83-85.

15. Patricia FE, SATO A, Esquibel MA, Campos-buzz F, Meira AV, Filho C. Antinociceptive Activity of Malva sylvestris L. Lat Am J Pharm, 2009; 28: 454–56

16. Wang C, Pei A, Chen J, Yu H, Sun ML, Liu CF, Xu X: A natural coumarin derivative esculetin offers neuroprotection on cerebral ischemia/reperfusion injury in mice. J Neurochem, 2011; 121: 1007–13

17. Min J, Yu SW, Baek SH et al: Neuroprotective effect of cyanidin-3-O-glucoside anthocyanin in mice with focal cerebral ischemia. Neurosci Lett, 2011; 500: 157–61

18. Itoh T, Satou T, Nishida S et al: Edaravone protects against apoptotic neuronal cell death and improves cerebrovascular function after traumatic brain injury in rats. Neurochem Res, 2010; 35: 348–55

19. Abdul-Muneer PM, Schuetz H, Wang F et al: Induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mild traumatic brain injury induced by primary blast. Free Radic Biol Med, 2013; 60: 282–91

20. Readnower RD, Chavko M, Aede S et al: Increase in blood-brain barrier permeability, oxidative stress, and activated microglia in a rat model of blast-induced traumatic brain injury J Neurosci Res, 2010; 88: 3530–39

21. Chkhikvishvili I, Sanikidze T, Gogia N et al: Rosmarinic acid-rich extracts of Satureja hortensis (L.) protect Jurkat T cells against oxidative stress. Oxid Med Cell Longevity, 2013; 2013: 456253

22. Rossi D: Astrocyte physiopathology: At the crossroads of intercellular networking inflammation and cell death, Prog Neurobiol, 2015; 130: 86–120

23. Mouzon B, Chaytow H, Crynen G et al: Repetitive mild traumatic brain injury in a mouse model produces learning and memory deficits accompanied by histological changes. J Neurotrauma, 2012; 29: 2761–73

24. Petraglia AL, Plog BA, Dayawansa S et al: The pathophysiology underlying repetitive mild traumatic brain injury in a novel mouse model of chronic traumatic encephalopathy. Surg Neurol Int, 2014; 5: 184