

Qingnaopian: A Chinese herbal formula in reducing glial fibrillary acidic protein and inflammation in concussion mice

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Abstract: Qingnaopian has been used in traditional Chinese medicine for treating central nervous system (CNS) injury and inflammatory diseases. The aim of this study was to investigate the effects of Qingnaopian in concussion mice. C57BL/6 mice were used to establish the mild Traumatic Brain Injury (mTBI)/ concussion using the weight-drop techniques. Animal behavioral experiments righting reflex response and locomotor activity were assessed. The expression of pro inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), IL-1 and Glial fibrillary acidic protein (GFAP) were assessed by enzyme-linked immunosorbent assay (ELISA) and Western blot method, respectively. SPSS 19.0 software was used for statistical analysis. The results showed that righting reflex time and locomotor activity were higher in model group compared with those in control group. Qingnaopian treated mice had lower pro inflammatory cytokines, such as IL-1, IL-6 and TNF- α with alleviated GFAP. In short, Qingnaopian treatment improved GFAP induced by blow to head and inflammatory cytokines in concussion mice.

Keywords: Qingnaopian, inflammatory cytokines, GFAP.

INTRODUCTION

Traumatic brain injury (TBI) typically results from a blow to the head. The blow to the head could cause physiological and functional alteration and these changes could induce neurological, cognitive and behavioral symptoms (McCrorry and Meeuwisse, 2013). The severity of brain injury usually ranges from mild to severe and fatal. TBI is one of the common health problems and is the leading cause of mortality and disability in the world. The majority of sports-related TBI, has one or more of the features including headache, dizziness, sleep disturbance, cognitive impairment and loss of consciousness (Arachechige *et al.*, 2012; Simpson, 2011; Halstead and Walter, 2010). It has been estimated that around 1.6 to 3.8 million mTBI new cases occur every year and about 40% of patients who have experienced a concussion, don't try to seek medical cure (Levin *et al.*, 2010; Jeter *et al.*, 2012). Patients with concussion have a high incidence of temporal lobe functional deficits and results in memory disorder. As concussion diagnosis usually based on self-reported neurological symptoms without the positive neuroimaging findings. Therefore, appropriate hall makers of concussion will benefit from objective indicators of injury.

In this study, the concussion model was induced in anesthetized mice via the weight-drop technique. Marmarou *et al.*, technique is widely used and has been modified in mice in this experiment (Viano *et al.*, 2011). The behavioral assessments were used to measure the

deficits in the hippocampal ability of spatial learning and memory. GFAP is a brain-specific protein, which is released in post-concussion stage. Inflammation and pro inflammatory cytokines are involved in the TBI. These biomarkers have been evaluated in mice model and might be used in human study.

Qingnaopian, is a traditional Chinese medicine and is comprised of seven Chinese herbs, including *Radix angelicae*, *Angelica sinensis*, *Ligusticum wallichii*, *Gamdir Vine*, *Asarum sieboldi*, *Os-Draconis* and *Mentha*. Many of these herbs were used to promote blood circulation and are anti-inflammatory in China. The present study aims to evaluate the effects of Qingnaopian in protection of TBI in mice.

MATERIALS AND METHOD

General data

Male C57BL/6 mice, weighting 30-40g, were housed, five per cage under a constant 12-hour light-dark cycle, humidity (50-60%) with free access to food and water. Before experiments, the animals were allowed to habituate to the housing facilities for a week. The present study was conducted with approval from the Animal Ethics Committee of the 152th Center Hospital of PLA. In this study, mice were randomly divided into three groups: (a) Sham group, (b) Model group and (c) Chinese medicine Qingnaopian treated group. Qingnaopian tablet were prepared following Chinese pharmacopoeia standards using HPLC method. The ferulic acid was considered as the major active component, which was higher than 0.01mg/tablet in this study as shown in fig. 1.

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Mice in Qingnaopian treatment group were orally administrated with 1.0g/kg freshly prepared Qingnaopian solution while the mice in control group received vehicle saline daily for 7 days before head impact and for another 30 days after injury. Each mouse was used once for the behavioral test.

Weight-drop (Concussion) model

Experimental concussion model was induced using the weight-drop apparatus as previously described (Milman *et al.*, 2005) Briefly, the device consisted of a 21g metal tube, placed vertically over the mice head. The tube freely fall from the height of 35cm down to the skull of mice. Mice were anesthetized under 5% isoflurane (in air at 2.0L/min). The animals were placed in the holder and the impactor was carefully positioned on the head. The mice of the Sham control group were also placed in the holder with no impact.

Righting reflex response

After the impact, the mice were immediately removed from the holder, placed on the supine position in a clear cage, and measured the time it took to the right posture. The latency period was a method to quantify the neurologic restoration. The time was recorded by three different observers blinded to the treatment.

Locomotor activity testing

Twenty-four hours later to the weight-drop impact session, each mouse was placed in a plastic cage for 30min to evaluate the general activity with a high-resolution 16 photo beam arrays in the horizontal and vertical axis. Locomotor activity was recorded on a computer and analyzed by software fusion.

Western blot assay

Mice were sacrificed by decapitation and the hippocampus were dissected and stored at -80°C. The total proteins in the tissues were homogenized in RIPA buffer containing protease and phosphatase inhibitor cocktail according to the kit instruction. The protein samples concentration was detected by BCA protein quantification kit. Samples of each group were added up

to 40µg and subjected to SDS-PAGE electrophoresis separation, and then transferred to the PVDF membrane. The samples were closed with 5% fat-free milk for 1 hour and added to 50g/L, diluted GFAP antibody (1:500). The membrane was washed by TBST (1ml/L Tween-20) three times (5min/times) after incubating at 4°C overnight, and then added to HRP (Santa Cruz, USA) labeled secondary antibody (1:5000). The membrane was incubated at room temperature for 2h and was washed by TBST three times (10min/times). ECL chemiluminescent darkroom development was used. The protein expression level was normalized by β-actin, and the gray scale was scanned and quantified by software.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β, TNF-α and IL-6 in cerebral spinal fluid (CSF) were measured by ELISA according to ELISA kit instructions. The plates were determined at 450nm with micro plate reader.

STATISTICAL ANALYSIS

Statistical differences were evaluated by software SPSS 19.0. Statistical analysis was performed using one-way analysis of variance, and p-values of less than 0.05 were considered as statistically significant.

Ethical approval

This study was approved by the institutional ethical committee of Chinese PLA General Hospital, Beijing, China. All the experiments were conducted as per NIH guidelines for Lab animals. The reference is 543/IEC-GH/2017.

RESULTS

Quantitation of ferulic acid in qingnaopian

The standard solution of ferulic acid and Qingnaopian solution were injected into high-performance liquid chromatography (HPLC) following Chinese pharmacopoeia's method. A typical HPLC chromatogram of ferulic acid is shown in fig. 1. Contents of ferulic acid used in this study was higher than 0.01mg/tablet.

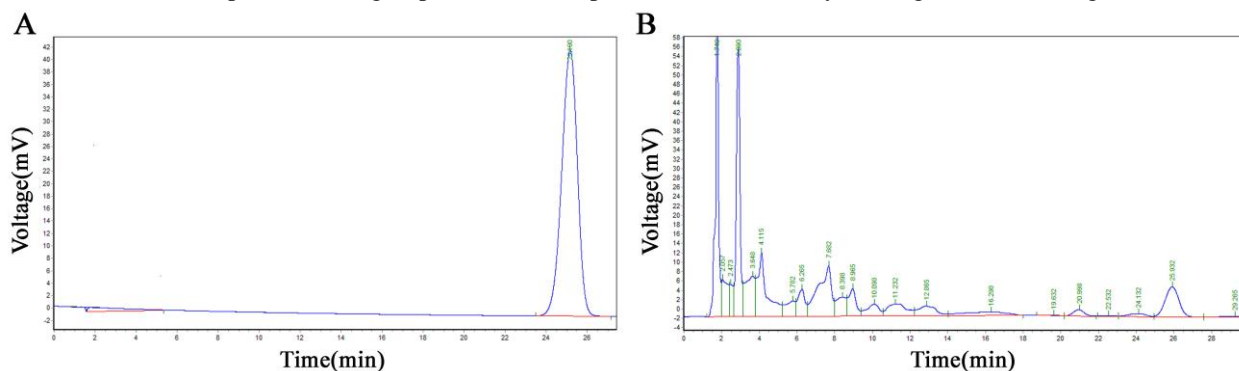


Fig. 1: HPLC Chromatograms of Ferulic Acid (A) Standard solution of ferulic acid (B) Qingnaopian solution

Qingnaopian vs righting time reduction in mice

The righting time of all mice is presented in fig. 2. Recovery time in model group was significantly higher than that in control group ($P < 0.01$), while the recovery time of Qingnaopian treatment group was markedly reduced in the present study ($P < 0.01$). The results demonstrated that the TBI model was well established and the Qingnaopian reduced the righting time in mice.

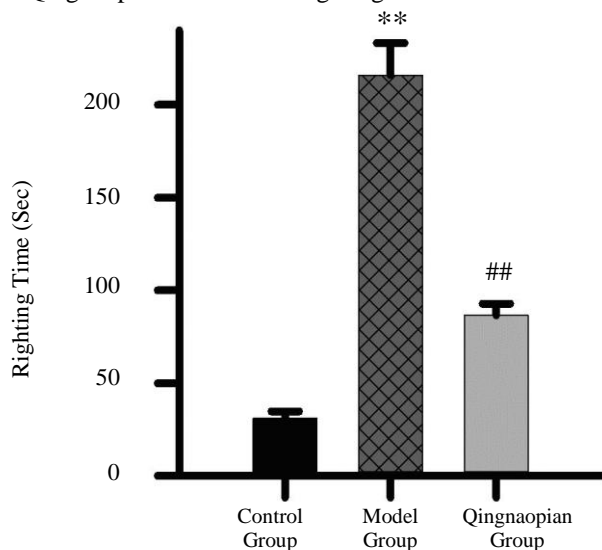


Fig. 2: Recovery of Righting Reflex after TBI. ** $P < 0.01$, compared with control group; ## $P < 0.01$, compared with model group

Qingnaopian relieving brain impairment in mice

Fig. 3 presents the locomotor activity performance on day 7 after head injury. The head impact produced markedly increase compared to that of control group ($P < 0.01$), while the mice treated with Qingnaopian for 15 days significantly decreased the locomotor activity ($P < 0.05$), which indicated that Qingnaopian had relieved the brain impairment in mice in the present study.

Qingnaopian reduction of GFAP expression in mice brain

GFAP is extensively studied TBI biomarker, which is brain-specific protein. Many studies showed that GFAP could be detected in the brain after head injury. As illustrated in fig. 4, the GFAP levels in hippocampus were significantly increased on day 7 after impact ($P < 0.01$) compared to control group. Again, when mice treated with Qingnaopian solution, the levels of brain injury induced GFAP were reduced, although the differences were not significant.

Qingnaopian relieving Pro-inflammatory cytokines in mice

Some studies demonstrated the pro-inflammatory cytokines are involved in the concussion. Therefore, the present study investigated the changes on pro-inflammatory cytokines in CSF. As shown in fig. 5, there

were significant increase in the levels of TNF- α , IL-1 β , and IL-6 in CSF of model mice, as compared with the control group. However, Qingnaopian solution reversed the brain impact and increased the levels of TNF- α , IL-1 β and IL-6 (fig. 4, $P < 0.05$ or $P < 0.01$). Taken together, these results suggested that Qingnaopian has attenuated the expression of TNF- α , IL-1 β and IL-6 in CSF of TBI mice.

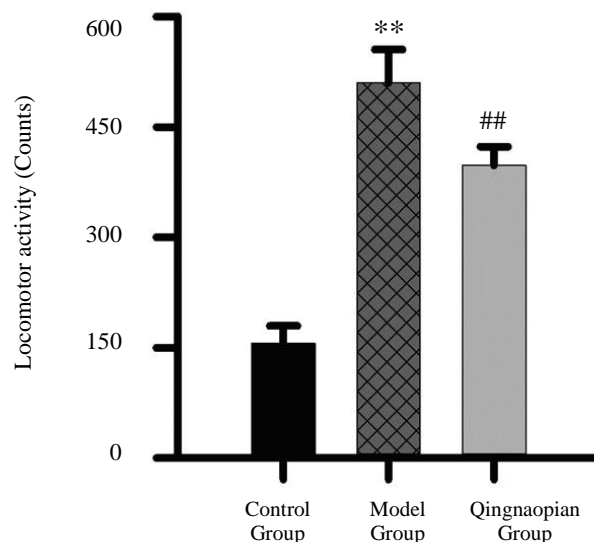


Fig. 3: Effect of TBI on Locomotor Activity. ** $P < 0.01$, compared with control group; # $P < 0.05$, compared with model group

DISCUSSION

Concussion or mild TBI is an increasing major public health problem. However, most of the patients don't seek professional medical care and appeared to recover over time (Wilde and McCauley, 2008; Prins *et al.*, 2010). In fact, lots of patients complained of persisting post concussive symptoms after the recovery. The weight-drop model in mice were used here to mimic the human concussion (Huang and Theilmann, 2009). The present study was conducted to evaluate the effects of Qingnaopian for protecting TBI in mice. Righting time was assessed as indicator of the successful TBI model. The results showed that the blow to the head significantly increased recovery time over control. These results were consistent with Kane *et al* study (Kane *et al.*, 2012). The mice treatment with Qingnaopian remarkably reduced the time. The effects of Qingnaopian in TBI demonstrated that this Chinese herb is an ideal drug to relieve the impairment of head injury.

Mice in concussion model showed coordination deficits and it was consistent with the results from human patients who have experienced concussive brain injury (Guskiewicz, 2011). These injuries were not serious enough to interfere with daily behavior. All mice showed

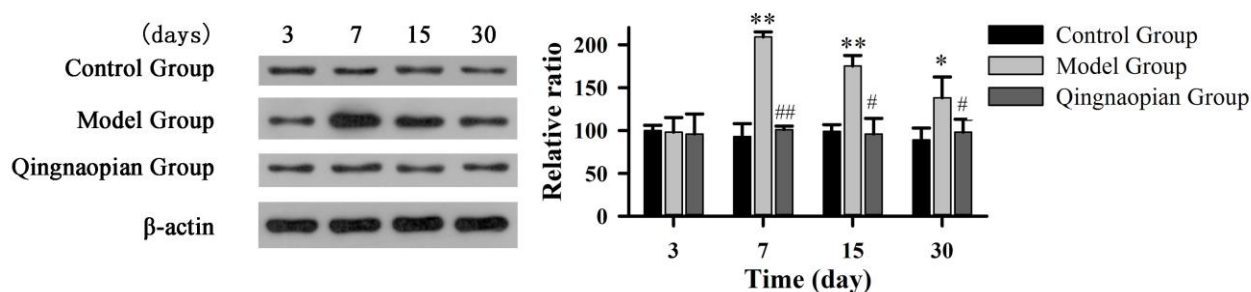


Fig. 4: Effect of TBI on GFAP Expression. **P<0.01, compared with control group; #P<0.05, ###P<0.01, compared with model group

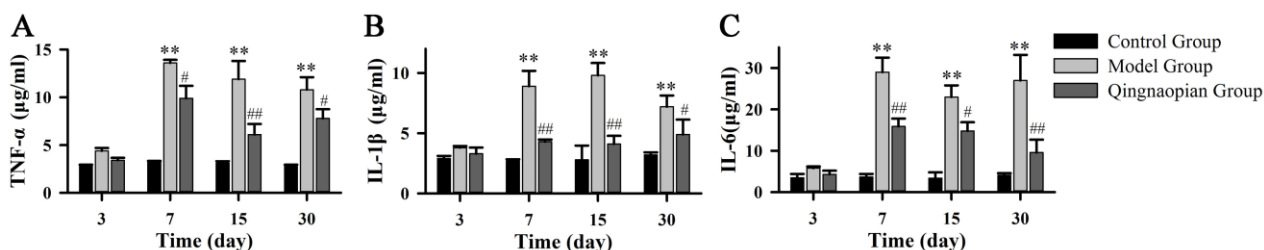


Fig. 5: Qingnaopian Reducing the Expression of TNF- α , IL-1 β and IL-6 in CSF following Head Impact. **P<0.01, compared with control group; #P<0.05, ###P<0.01, compared with model group

the normal grooming and eating food in this study. The outcome related with the impact on head was a transient increase in locomotor activity (Homsí *et al.*, 2010; Bales *et al.*, 2009; Pulella *et al.*, 2006). Mild TBI in mice was resulted in hyperactivity, which was similar to the mTBI human patient, especially in children. As shown in fig. 3, the locomotor activity was significantly increased after brain injury compared to control group. The effects of Qingnaopian could be seen in this behavioral study, which remarkably reduced the locomotor activity.

Previous studies revealed that Glial fibrillary acidic protein (GFAP) was increased after brain injury (McKee *et al.*, 2009; McKee *et al.*, 2010; Meaney and Smith, 2011). Seven days after impact on head, GFAP showed increased expression level of GFAP in hippocampus (fig. 4). GFAP could be used as a biomarker of neuropathological indices that was also found in human patients with brain injuries. Qingnaopian could reverse the impairment induced by weight-drop impact. There is increasing evidence of the inflammatory response and pro-inflammatory cytokines involving brain injury (Lucas *et al.*, 2006). IL-1 is one of the most evaluated cytokines in neurodegenerative conditions and is widely considered as neurotoxic cytokine. TNF- α is another central mediator of brain inflammation and involved in the pathogenesis of several neurological diseases. IL-6 is an endogenous pyrogen which has many effects that are both beneficial and destructive to brain neurons. The cytokines are often related; TNF- α could induce expression of IL-1 and IL-6, and IL-1 could stimulate IL-6 and TNF- α (Gao *et al.*, 2003) As consistent with previous study (Hallenbeck, 2002), the present work

showed cytokines IL-1, IL-6 and TNF- α as significantly increased after the blow to the head. Qingnaopian again reversed these pro inflammatory cytokines in CSF of mice.

CONCLUSION

As a conclusion, Qingnaopian treatment improved GFAP induced by blow to head and inflammatory cytokines in concussion mice model.

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