Acupuncture stimulation of Yamen (GV 15), Fengfu (GV 16), Baihui (GV 20), Shuigou (GV 26) and Hegu (LI 4) reduces brain microglia activation in a traumatic brain injury rat model

Lin Shujun, Zhu Mingmin, Chen Weihao, Zhang Yujuan, Lin Jihuan, Pu Liu, Chen Shulian, Zhang Yimin, Liu Xin

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Correspondence to: Dr. Zhu Mingmin, Prof. Zhang Yimin,
College of Chinese Medicine, Jinan University, 601 Huangpu West Avenue, Guangzhou, Guangdong 510632, China.
Rock3590@163.com, zhangymjnu@163.com
Telephone: +86-20-85226289
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Abstract

OBJECTIVE: To evaluate the effect of acupuncture on neuroinflammation in traumatic brain injury (TBI) rats by stimulating Yamen (GV 15), Fengfu (GV 16), Baihui (GV 20), Shuigou (GV 26) and Hegu (LI 4) acupoints and to investigate the mechanism underpinning this effect.

METHODS: A TBI model was induced in Sprague-Dawley rats using Feeney’s freefall impact method. Acupuncture to stimulate the Yamen (GV 15), Fengfu (GV 16), Baihui (GV 20), Shuigou (GV 26) and Hegu (LI 4) acupoints was performed on the TBI rats. After 3 consecutive days of acupuncture treatment, we investigated signal molecules, receptors and microglia related to neuroinflammation in brain tissue of the TBI rats and analyzed the possible mechanism underlying the effect of acupuncture on neuroinflammation.

RESULTS: After the acupuncture treatment, ionized calcium binding adaptor molecule 1 (Iba1), a protein specific to microglia, was investigated. In the cortical layer of damaged brain tissue in TBI rats, the Iba1-positive area was 3.3% ± 0.9% in the rats that received acupuncture compared with 5.2% ± 1.4% in the TBI rats that did not receive acupuncture, and the microglia were smaller with more slender protrusions in the acupuncture-treated rats. This result indicates that acupuncture can significantly reduce microglia activation in TBI rats. A possible mechanism for this effect is that acupuncture reduces the expression of autotaxin and lysophosphatidic acid. Together, these constitute the autotaxin-lysophosphatidic acid axis, which induces microglial activation in the brains of TBI rats. Acupuncture treatment may downregulate the expression of Lysophosphatidic acid (LPA) receptor (LPAR) 1 and LPAR2 on the microglial cytomembrane, which affects the microglia activation process.

CONCLUSION: Acupuncture stimulating the Yamen (GV 15), Fengfu (GV 16), Baihui (GV 20), Shuigou (GV 26) and Hegu (LI 4) acupoints can effectively inhibit the development of neuroinflammation after TBI. One possible mechanism for this effect is that acupuncture downregulates LPA synthesis and affects the LPA-LPAR pathway by inhibiting LPAR1 and LPAR2, thereby inhibiting microglial activation and reducing neuroinflammation.
Keywords: Acupuncture; Brain injuries, traumatic; Microglia; Lysophospholipids; Receptors, lysophosphatidic acid; Neuroinflammation

INTRODUCTION

After traumatic brain injury (TBI), high levels of neuroinflammation occur in the area surrounding the damaged nerve tissue, which results in secondary damage. This neuroinflammation also seriously affects the prognosis of patients and can result in long-term coma and functional motor and speech impairments. Reducing or eliminating neuroinflammation post-TBI has therefore become a primary aim of TBI treatment. Lysophosphatidic acid (LPA) mediates the process of neuroinflammation, and microglia are the main promoters of neuroinflammation; the two are closely related. LPA is a biologically active small phospholipid that affects many biological processes in pathology. LPA is mainly synthesized extracellularly from lysophospholipase through the mediation of autotaxin (ATX, also known as Enpp2). ATX and LPA together constitute the ATX-LPA axis and exert a range of biological effects. After TBI, the acute levels of both LPA and ATX are significantly increased; this change in the ATX-LPA axis leads to the overactivation of related target cells such as microglia and astrocytes; the activation of related downstream signals; and the secretion of effector factors, which play an important role in forming the toxic internal environment surrounding the damaged area in the brain.

Microglia are a type of macrophage and are the main players in the immune response of the central nervous system (CNS). Microglia maintain the CNS internal environment during normal physiological processes and promote nerve repair upon injury. LPA can directly regulate the activity of microglia through the LPA-LPA receptor (LPAR) pathway. To regulate microglia activity, LPA primarily binds to three receptors on microglial cytomembranes: LPAR1, LPAR2 and LPAR3 [also known as endothelial differentiation gene (EDG) 2, EDG4 and EDG7, respectively]. Studies have shown that acupuncture has a positive effect on TBI. Performing acupuncture after acute TBI can significantly improve the recovery of mind, motor and speech functions in patients. In a randomized controlled trial, the arousal time of TBI patients treated with acupuncture was shorter than patients treated with routine Western medicine over 7 d of treatment. Additionally, acupuncture can promote recovery from brain damage and the regaining of consciousness, and can also improve prognosis. A case report of a TBI patient who received 3 weeks of acupuncture treatment reported that the patient regained consciousness and could tolerate rehabilitation programs, despite starting from a poor condition caused by serious complications after surgery. Additionally, basic research has also demonstrated that electroacupuncture may partially regulate inflammatory processes by modulating interleukin 6 (IL-6) gene expression in cerebral ischemic injury, and that acupuncture can reduce LPA serum levels in patients with acute cerebral infarction, and that acupuncture can inhibit the activation of microglia in ischemic brain damage and reduce neuroinflammation. However, there are no similar studies on the mechanism by which acupuncture acts on the LPA-microglia pathway in TBI.

In rats, neuroinflammation after TBI can activate astrocytes at an early stage, and the activated astrocytes aggregate to form glial scars that clear the damaged or dead brain tissue. However, the excessive formation of glial scars negatively affects nerve repair. We have previously reported that acupuncture can inhibit the formation of excessive glial scarring after TBI, thereby inhibiting neuroinflammation. Microglia are a component of glial scarring, but they are also the main immune cells in the CNS. After TBI, damaged neurons are cleared by activating immune functions in microglia and the damage is thus repaired. Similar to what occurs in astrocytes, microglial overactivation also causes severe neuroinflammation, which causes secondary damage in the brain. In our previous studies, we did not investigate whether acupuncture also regulates neuroinflammation by inhibiting microglial hyperactivity. In this study, we therefore aimed to evaluate the effect of acupuncture stimulation of Yamen (GV 15), Fengfu (GV 16), Baihui (GV 20), Shuigou (GV 26) and Hegu (LI 4) acupoints on neuroinflammation in TBI rats, and to investigate the mechanism underpinning this effect.

MATERIALS AND METHODS

Ethical approval

The experiment was performed in strict accordance with the Regulations for the Administration of Experimental Animals issued by the State Council of China in 1988 and was approved by the Experimental Animal Ethics Committee of Jinan University (batch No. 20150114205420). All rats underwent anesthesia during the construction of the TBI rat model via an intraperitoneal injection of 10 g/L pentobarbital sodium (30 mg/kg). All measures were taken to minimize the pain of the experimental animals.

Developing the TBI rat model

Thirty adult male Sprague-Dawley rats weighing (200 ± 20) g were purchased from the Experimental Animal Center of Southern Medical University [license number: SCXK (Guangdong) 2016-0041, Guangzhou, China]. The experimental animal certificate number was 44002100011064. All animal experiments were conducted at the Experimental Animal Management Center of Jinan University [License No. SCXK (Guangdong) 2012-0117].
After 1 week of adaptive housing, the rats were randomly divided using the random number method into three groups: the normal group, the TBI group and the acupuncture + TBI group. There were eight rats in the normal group, 11 in the TBI group and 11 in the acupuncture + TBI group. Because the study required a model of moderate to severe craniocerebral injury, some experimental rats were killed during the modeling process. Dead TBI rats were excluded, and after modeling, both the TBI group and the acupuncture + TBI group contained just eight rats per group.

The Feeny’s free-fall epidural impact method was used to construct a rat model of severe TBI for the TBI group and the acupuncture + TBI group.10 Rats were weighed before being anesthetized via an intraperitoneal injection of 10 g/L pentobarbital sodium (30 mg/kg) (Sigma, St. Louis, USA). After anesthesia, the scalp was cut along a line 2 mm to the right of the skull median line and separated from the skull. The skull was then drilled with a flexible skull drill to open a round window with a diameter of 5 mm, at a point that was 2 mm to the right of the sagittal suture and 1 mm posterior to the coronal suture; the dura was kept intact (Figure 1A, 1B). A 20-g hammer was allowed to fall freely from a height of 30 cm to hit a flat nail placed on the window of the skull, which results in a local cerebral contusion of the right parietal lobe (Figure 1C, 1D). The wound was rinsed postoperatively using 105 U/L penicillin (Macklin, Shanghai, China) and 1 × 10^5 mg/kg) of 30% alcohol. The normal group, acupuncture + TBI group and the TBI and acupuncture + TBI groups each had eight surviving rats. The rats in the acupuncture + TBI group received immediate treatment. The rats that died were excluded in the fixation frame (Figure 2A), and the TBI group contained just eight rats per group.

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At 1 h after the last treatment, the rats in each group were then anesthetized with 10% chloral hydrate (Sigma, St. Louis, MO, USA) and decapitated. The brains were perfused with ice-cold normal saline before the intact brains were removed, flash frozen, and stored in liquid nitrogen.

**Immunohistochemical labeling of microglia in brain tissue**

Frozen sections of the cortical tissue from around the lesion were made at a thickness of approximately 4-8 μm. After 30 min at room temperature, the sections were fixed in acetone at 4 °C for 10 min. After being allowed to dry slightly, the sections were immersed in a dark box containing 3% H2O2 for 8-20 min to eliminate endogenous peroxidase activity. After three 2-min washes in phosphate buffer saline (PBS), the slides were placed in a humidified box and slightly dried and hydrophobic barrier circles were created around the tissue using an ImmEdge pen. Slides were blocked at 37 °C for 30 min in freshly made working solution (5% bovine serum albumin (Cell Signaling Technolo-
The expression of ATX and LPA was detected by ELISA. Whole protein was extracted from cortical tissue from around the lesion, and 80 μL of sample and 20 μL of 5 x sample loading buffer were mixed and then incubated for 10 min at 100 °C. The samples underwent sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO, USA)-polyacrylamide gel electrophoresis (PAGE) in a 10% separation gel and were then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Danvers, MA USA). The membrane was blocked in 5% fat-free milk for 30 min and then incubated with primary antibodies (rabbit EDG2 antibody, 1: 1000, Abcam, Cambridge, UK; rabbit EDG4 antibody, 1: 1000, Bio-swamp, Wuhan, China; rabbit EDG7 antibody, 1: 200, Abcam, Cambridge, UK;) at 4 °C overnight, followed by incubation in blocking solution at 37 °C for 30 min and three washes in phosphate buffered solution (PBST). Secondary antibodies (goat anti-rabbit IgG-HRP, 1: 100 00, Bio-swamp, Wuhan, China) were then added followed by a PBST wash at room temperature for 1 h. β-Actin was used as the internal reference, and after adding the ECL chemiluminescent agent, the membrane was exposed and imaged in a Sage Creation gel imager.

RESULTS

Effect of acupuncture on the activation of microglia in TBI rats

In our previous study, we used neurobehavioral observations and hematoxylin-eosin (HE) staining to determine whether manual acupuncture (MA) improved brain injury in TBI rats. In the TBI + MA group, neurobehavioral scores were improved on days 7 and 14,
and differed significantly from those of the untreated TBI group. HE results also revealed that the TBI rats without treatment had more significant pathological changes and a slower recovery compared with the TBI + MA rats on days 7 and 14. However, there were no significant differences between the two groups in neurobehavioral scores or HE staining on day 3.

Although the neurobehavioral results and brain tissue changed in the later stages after TBI in our previous study, neuroinflammation is usually severe in the early stages. In this study, we therefore aimed to observe the acute process of neuroinflammation in TBI rats that underwent acupuncture treatment. We used immunohistochemical detection of Iba1 to observe the effects of acupuncture on microglial activation in TBI rats; the activation level of microglia directly reflects the level of neuroinflammation. Immunohistochemistry results were observed under a light microscope at ×400 magnification (Figure 3A). In the normal group, Iba1 expression and density was lower, and Iba1-positive cells were mostly small and thin. The TBI group had more and denser Iba1-positive expression, and Iba1-positive cells were larger and extended into the damaged surrounding area. The acupuncture + TBI group had less Iba1-positive staining and lower density than the TBI group and, similar to the normal group, the Iba1-positive cells were also small and slender. We used ImageJ to measure the ratio of areas with positive staining (Figure 3B). The positive area of Iba1 staining in the normal group was 2.8% ± 0.5% of the total area, whereas the positive area in the TBI group was 5.2% ± 1.4%, which was significantly higher than that of the normal group (P < 0.001). The positive area in the acupuncture + TBI group was 3.3% ± 0.9%, which was significantly lower than that of the TBI group (P < 0.001).

**Effect of acupuncture on the ATX-LPA axis in TBI rats**

The expression of ATX and LPA was detected by ELISA, and the results might reflect the effects of acupuncture on the LPA upstream signal (i.e., LPA synthesis).

As shown in Table 1, ATX expression in brain tissue was significantly higher in the TBI group than in the normal group (P < 0.001) on day 3 of the experiment. After acupuncture treatment, ATX expression in the acupuncture + TBI group was significantly reduced on day 3 (P < 0.001) compared with TBI group. LPA expression followed the same trend as that of ATX for each group; that is, LPA expression was significantly higher in the TBI group than in the normal group (P < 0.001) and LPA expression was significantly lower in the acupuncture + TBI group than in the TBI group (P < 0.001).

**Effects of acupuncture on LPARs in TBI rats**

The expression levels of three LPARs (LPAR1, LPAR2 and LPAR3) were detected using western blotting. The levels of LPARs might reflect the effects of acupuncture on the midstream and downstream signaling of LPA (i.e., the LPA-LPAR pathway, which is the main pathway leading to microglial activation). As shown in Figure 4A, the positions and grayscale values of the internal reference β-actin bands were consistent among the three groups. The TBI group had high LPAR1 expression and the expression of LPAR1 was significantly higher in the TBI group than in the normal group (P < 0.001), whereas LPAR1 expression was significantly lower in the acupuncture + TBI group than in the TBI group (P = 0.033 < 0.05). The trends of LPAR2 expression in the three groups were similar to those of LPAR1 (Figure 4B). LPAR2 expression was significantly higher in the TBI group than in the normal group (P = 0.004 < 0.01), whereas LPAR2 expression was significantly lower in the acupuncture + TBI group than in the TBI group (P = 0.042 < 0.05). Additionally, the pattern of changes in LPAR3 expression in each group was similar to that of LPAR1 and LPAR2: expression of LPAR3 was significantly higher in the TBI group than in the normal group (P = 0.005 < 0.01; Figure 4C). However, although the expression of LPAR3 in the acupuncture + TBI group appeared lower than that in the TBI group, the difference was not significant (P = 0.317 > 0.05).

**Figure 3** Effects of acupuncture on Iba1 expression in cortical tissue around the lesion in the TBI rat

A-C: the staining was observed under a light microscope at a magnification of ×400. Iba1-positive staining (brown) indicates microglia. A: the normal group had relatively low levels of positive staining, and Iba1-positive cells were relatively thin, with slender protrusions (arrows); B: the TBI group had relatively high levels of positive staining, and Iba1-positive cells were relatively large, with protrusions that extended outwards; C: the acupuncture + TBI group had reduced levels of positive staining, and Iba1-positive cells were thin and small, with more slender, shorter protrusions; D: comparison of the percentage of Iba1-positive areas in each group. Normal and TBI groups were only fixed in the fixation frame for 15 min per day. Acupuncture + TBI group received acupuncture treatment for 15 min per day. All groups were treated for 3 consecutive days. TBI: traumatic brain injury. The data are expressed as the mean ± standard deviation. *P < 0.001, compared with normal group; **P < 0.001, compared with TBI group.
Each group of samples was measured three times and the relative grayscale data are expressed as the mean ± standard deviation.

Acupuncture + TBI group received acupuncture treatment for 15 min per day. Acupuncture + TBI group compared with the TBI group, *P < 0.001, compared with normal group; †P < 0.001, compared with TBI group.

### DISCUSSION

Some studies have reported that acupuncture therapy is effective for improving the prognosis of TBI patients. The mechanism underlying this effect is considered to mostly rely on the ability of acupuncture to inhibit neuroinflammation and slow down or eliminate secondary damage caused by neuroinflammation in the CNS after TBI. As mentioned in the introduction, one of the major contributing factors to neuroinflammation is microglia. The activation state and activation level of microglia in the vicinity of the damaged brain area can affect the progression of neuroinflammation. LPA is one of the signaling molecules that can induce microglial activation and affect the process of neuroinflammation. Previous studies have demonstrated that acupuncture therapy can affect LPA expression levels in patients with acute cerebral infarction. However, it remains inconclusive whether acupuncture acts by regulating the LPA signal, thus affecting the activation of microglia and improving post-TBI neuroinflammation.

In the present study, the Yamen (GV 15), Fengfu (GV 16), Baihui (GV 20), and Shuigou (GV 26) acupoints were all located in the governor vessel. In traditional Chinese medicine theory, the governor vessel reaches the head, enters the skull, and connects directly with the brain. Stimulating the relevant acupoints in the governor vessel of the head can therefore treat brain-related diseases. This is the basis of the meridian theory of TBI acupuncture treatment. The literature also shows that governor vessel acupoints have a unique effect on the treatment of brain diseases. Moreover, Hegu (LI 4) belongs to the large intestine vessel, which

![Figure 4](image-url)

Figure 4 Effects of acupuncture on LPAR1 (EDG2), LPAR2 (EDG4) and LPAR3 (EDG7) expression in the cortex surrounding the lesions in TBI rats

A, D: *P < 0.001 compared with the normal group; †P = 0.033 < 0.05 compared with the TBI group; B, E: *P = 0.004 < 0.01 compared with the normal group; †P = 0.042 < 0.05 compared with the TBI group; C, F: *P = 0.005 < 0.01 compared with the normal group; acupuncture + TBI group compared with the TBI group, P = 0.317 > 0.05. Normal and TBI groups were only fixed in the fixation frame for 15 min per day. Acupuncture + TBI group received acupuncture treatment for 15 min per day. All groups were treated for 3 consecutive days. ATX: autotaxin; LPA: lysophosphatidic acid; TBI: traumatic brain injury. Each group of samples was measured three times and the relative grayscale data are expressed as the mean ± standard deviation.
reaches the head from the hands. Thus, stimulation of Hegu (LI 4) can also be used to treat head-related diseases; for example, a previous study has reported that stimulating Hegu (LI 4) can increase the amount and volume of blood flow in the brain.24

Neuroinflammation is usually severe in the early stages after TBI and gradually becomes more stable in the later stages.25 Therefore, in the current study we used a 3-day experimental period so that we could observe the effects of early intervention with acupuncture on the activation of microglia, which is the main influencer of neuroinflammation. There are many pathways for the activation of microglia, but the LPA-LPAR pathway is one of the most important pathways of induction. The results of the present investigation demonstrated that brain Iba1 expression levels were significantly higher in the TBI group than in the normal group, and observations of cell morphology also revealed that Iba1-expressing cells in the TBI group had full soma and extended into the damaged surrounding areas. Iba1 is a protein specific to the microglial cell membrane that is expressed in both the resting and activated states. However, the increased Iba1 protein expression and the full and expanded morphology of Iba1-positive cells in the TBI group indicated that the microglia had an active expression and were in an activated state. Additionally, the expression levels of the ATX-LPA axis and LPARs (LPAR1, LPAR2 and LPAR3) were significantly higher in the TBI group than in the normal group. After TBI in the rats, brain ATX expression was sharply increased and directly caused the synthesis of large quantities of LPA. LPARs were also actively expressed on the surface of microglia, and the binding of LPA and LPARs activated downstream signaling to cause microglia activation, which is consistent with previous studies.26-27 The results of the acupuncture + TBI group revealed that Iba1 expression was lower than in the TBI group and most of the cells expressing Iba1 were thin and small, which suggests that microglial expression levels decreased in the TBI rats after the intervention of acupuncture, and that microglia switched from the activated state to the resting state. The detection of upstream molecules revealed that the ATX-LPA axis and LPAR1 and LPAR2 were also significantly lower in the acupuncture + TBI group compared with the TBI group, which indicates that acupuncture may affect ATX-LPA expression, reduce the expression of LPA and LPARs (mainly LPAR1 and LPAR2), and diminish LPA signal transduction, thereby reducing microglial cell activation and prompting microglia to switch from the activated state to the resting state. The LPAR results demonstrated that LPAR3 expression levels were not significantly different between the acupuncture + TBI group and the TBI group. There are two possible explanations for this: (a) the acupuncture-mediated reduction of microglial activation may only affect the expression of LPA, LPAR1 and LPAR2, but not of LPAR3; or (b) acupuncture also has an effect on LPAR3, but there was no significant effect in the current study because of limitations in the sample size and experimental time period. The LPAR pathway is the main pathway for LPA-mediated induction of microglial activation and neuroinflammation, and it take place in the middle and upper streams of the neuroinflammation mechanism. Currently, the experimental results reveal the molecular mechanism of acupuncture in suppressing TBI neuroinflammation only at the midstream and upper levels of the neuroinflammation mechanism. In future studies, we will continue to explore the downstream components of this mechanism and investigate the influence of acupuncture on LPARs more thoroughly by increasing the sample size. The inhibition of neuroinflammation via acupuncture has been previously investigated. Liu et al.28 used acupuncture to treat patients with acute ischemic cerebrovascular disease and found that acupuncture promoted neurological recovery and may have been associated with decreased plasma LPA levels in patients. In a different study, Liu et al.29 also observed that electroacupuncture inhibited microglia-mediated neuroinflammation in rats with cerebral ischemic injury, thereby improving their movement disorders. These previous findings are consistent with the results of the present study. Moreover, a previous study by our group revealed that acupuncture can effectively improve modified neurological severity scores (mNSS) in TBI rats;20 this score is an evaluation index of neurological function recovery. In addition, acupuncture reduces the aggregation of astrocytes and the formation of glial scarring, thereby inhibiting neuroinflammation.30 Microglia are involved in the formation of glial scars. Therefore, the current study verified the results of our previous study, but from a different perspective. Acupuncture may intervene in the LPA synthesis phase of the LPA-LPAR pathway, inhibit the excessive activation of microglia, and reduce the excessive formation of glial scarring, which together can reduce neuroinflammation, promote damaged nerve repair, and improve neurological function scores. Furthermore, we can apply the acupuncture treatment to TBI patients in the clinic in an evidence-based manner, according to the results from the present study. Our previous clinical trials have shown that acupuncture with an acupoint prescription can effectively promote the recovery of TBI patients.30 This study supports the previously reported clinical effects based on the molecular mechanism of the effects. In conclusion, acupuncture to stimulate Yamen (GV 15), Fengfu (GV 16), Baihui (GV 20), Shuigou (GV 26) and Hegu (LI 4) acupoints can effectively reduce post-TBI neuroinflammation, likely by reducing post-TBI microglial activation and expression levels. These reductions are closely related to the role of acupuncture in inhibiting the ATX-LPA axis, which reduces LPA and LPAR expression and decreases LPA signal transduction.
REFERENCES


