

## Original Article

# Electroacupuncture modulated the inflammatory reaction in MCAO rats via inhibiting the TLR4/NF- $\kappa$ B signaling pathway in microglia

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Received June 27, 2015; Accepted July 29, 2015; Epub September 1, 2015; Published September 15, 2015

**Abstract:** In this study, we aim to investigate the effects of electroacupuncture on the TLR4/NF- $\kappa$ B signaling pathway in microglia. Male Wistar rat of SPF grade (weighing 200 $\pm$ 20 g) were randomly divided into (i): sham control group, which was subjected to sham operation (ii) vehicle group, which underwent the occlusion of middle cerebral artery; (iii-v): acupuncture groups, which were subjected to the occlusion of middle cerebral artery and treated with acupuncture on the Neiguan acupoint (P6), Quchi acupoint (LI11), and Diji acupoint (SP8), respectively. HE staining was performed to detect the necrotic rate of neurons. Mediators of inflammation were measured using ELISA. Immunofluorescence was performed to measure the expression of TLR4, HMGB1, TRAF6, IKK $\beta$  and NF- $\kappa$ B p65 in microglia. Severe decrease was noticed in the neurological score, necrotic rates of neuron, expression of IL-1 $\beta$ , IL-6, TLR4, HMGB1, TRAF6, IKK $\beta$  and NF- $\kappa$ B p65 in microglia. Compared with the vehicle group, significant decrease was revealed in the neurological score, necrotic rate, IL-1 $\beta$ , TLR4, TRAF6, IKK $\beta$  and NF- $\kappa$ B p65 in Neiguan group and Quchi group, respectively. In addition, remarkable decrease was observed in the expression of TNF- $\alpha$  and IL-6 in Quchi group. Compared with the Diji group, the necrotic rate of neurons in hippocampus region was significantly decreased in the Quchi group ( $P < 0.05$ ). In Neiguan group, the expression of TLR4 and IKK $\beta$  was significantly attenuated ( $P < 0.05$ ). The expression of TRAF6 was remarkably decreased in the Neiguan group and Quchi group, respectively. Electroacupuncture on Neiguan and Quchi could improve the neurological injury, attenuate the inflammation, and inhibit the activity of TLR4/NF- $\kappa$ B signaling pathway in microglia.

**Keywords:** Electroacupuncture, microglia, TLR4/NF- $\kappa$ B signaling pathway

## Introduction

Stroke is a great threat to the public health worldwide. Ischemic stroke, accounting for nearly 80% of the cerebrovascular disorders, causes severe sequels and financial burdens to the survivals [1]. As an important part of traditional Chinese medicine, acupuncture has been proved to improve the life quality of the patients through attenuating the functional disorders and morbidity, as well as few side effects [2]. Although acupuncture has been commonly used in China for treating stroke, its mechanism is still not well defined.

Recently, extensive studies have been conducted to investigate the potential mechanism of cerebral ischemia [3, 4]. Up to now, most stud-

ies approved that inflammatory reactions played crucial roles in the pathogenesis of cerebral ischemia [5]. To be exact, the inflammatory reactions may contribute to the secondary brain injury during the cerebral ischemia, which is considered as the major cause for the ischemic injuries. Therefore, it is reasonable to speculate that anti-inflammatory therapy may be effective in preventing and treating cerebral ischemia.

Microglia is considered as the main immune effector cells in the brain tissues. Its activation, depending on the interaction between the receptor and ligands, has been acknowledged as the initiating factor for triggering the immune reactions in cerebral tissues [6]. To our best knowledge, the major type of receptor in the immune system is Toll like receptors (TLRs),

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among which TLR4 plays a crucial role in the ischemic injury [7, 8]. It is highly expressed in the microglia, and is closely associated with the activation of NF- $\kappa$ B signaling pathway in the ischemic tissues.

Previous study showed electroacupuncture could attenuate the activation of microglia in rats with ischemic reperfusion injury [9]. Moreover, acupuncture showed inhibitory effects on the inflammatory reactions in the cerebral ischemia through modulating the release of TNF- $\alpha$ , IL-1 and IL-6. Our previous study revealed that acupuncture could attenuate the infiltration of inflammatory cells in animal models of cerebral ischemia [10]. In the early stage of ischemia, massive inflammatory cell infiltration was noticed in the ischemic region in the animal models. On the contrary, less infiltration was revealed in animals treating with acupuncture. On this basis, we hypothesize that electroacupuncture on certain acupoints may play a protective role in the ischemic stroke through modulating the activation of microglia.

In this study, MCAO model was established to investigate the effects of acupuncture on the effects of inflammatory reactions in rats with cerebral ischemia. We determined the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in ischemic tissues. Also, the expression of TLR4, HMGB1, TRAF6, IKK $\beta$  and NF- $\kappa$ B p65 in microglia was determined. Our study revealed acupuncture could modulate the inflammatory reaction in MCAO rats via inhibiting the TLR4/NF- $\kappa$ B signaling pathway in microglia.

### Materials and methods

#### *Animals*

Male Wistar rat of SPF grade (weighing 200 $\pm$ 20 g) were purchased from Vital River Laboratories (Beijing, China). Then the animals were fed for adaptation for 1 week in a chamber at a temperature of 25°C and a humidity of 60%. All the protocols used in this study are approved by the Ethical Committee of Shandong University of Traditional Chinese Medicine.

#### *Experimental design*

The animals in each experiment were randomly divided into (i): sham control group, which was

subjected to sham operation (ii) vehicle group, which underwent the occlusion of middle cerebral artery; (iii-v): acupuncture groups, which were subjected to the occlusion of middle cerebral artery and treated with acupuncture on the Neiguan acupoint (P6), Quchi acupoint (LI11), and Diji acupoint (SP8), respectively. The frequency of the sparse-dense wave was set at 2/15 Hz. The interval of the sparse wave and dense wave was 1.5 seconds. The output current was set as 1 mA. For the electroacupuncture group, the animals underwent acupuncture once per day with a duration of 30 min for consecutive 5 days. The animals were sacrificed 2 h after the acupuncture.

#### *Induction of MCAO model*

The induction of MCAO model was performed according to the previous description [6]. Briefly, the rats were anesthetized with chloral hydrate via intra-peritoneal injection. The right arteria carotis communis (CCA) was exposed through a midline incision in the neck. The CCA was separated until reaching the bifurcation of external carotid artery (ECA) and internal carotid artery (ICA). Subsequently, an incision was made at the position that was 3 mm from the CCA, followed by insertion of ICA (about 20 mm). Finally, the CCA was ligated using a surgical suture, and the incision was sutured gradually. The animals with neurologic deficits progressed from 1 to 3 were used in the subsequent studies.

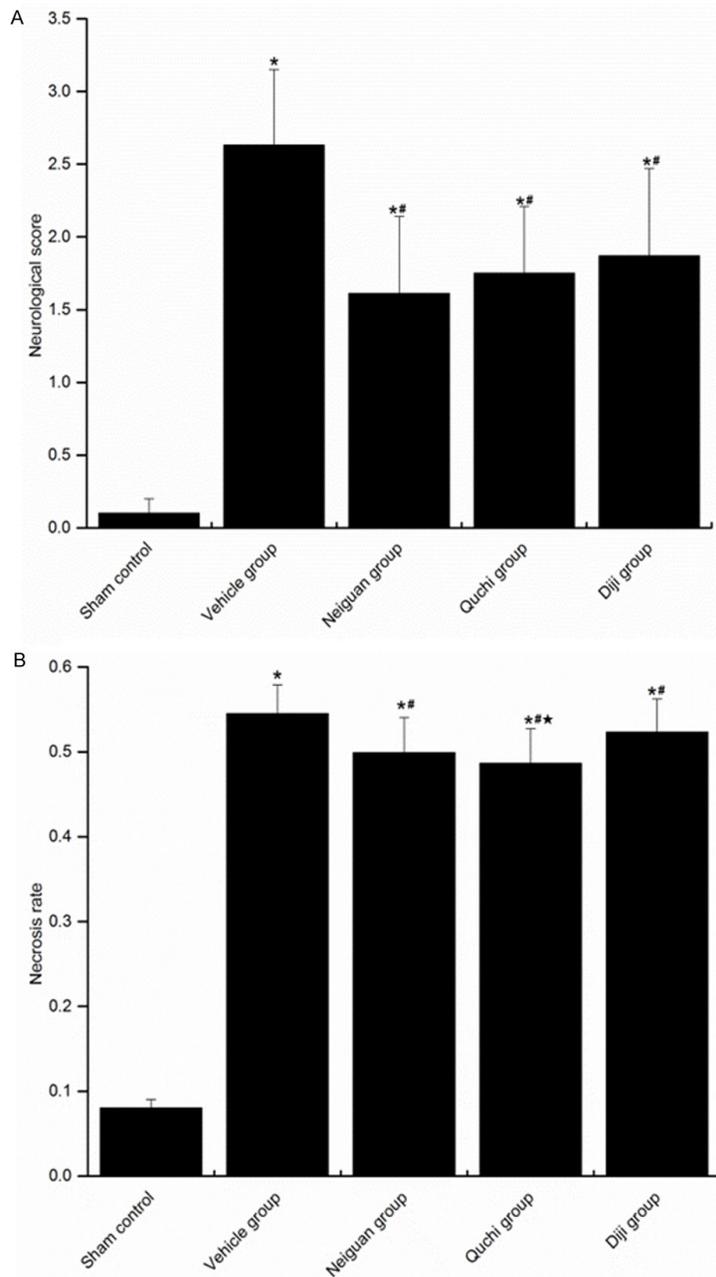
#### *Neurological examination*

Neurological tests were scored on a five-point scale. 0: No neurological deficit; 1: Failure to extend left forepaw fully on lifting whole body by tail; 2: Circling to left; 3: Falling to left; 4: Unable to walk spontaneously and had depressed levels of consciousness.

#### *Hematoxylin and eosin staining*

Hematoxylin and eosin (HE) staining was performed to determine the rate of necrosis of neuron as conventionally described. The frozen sections obtained from the anterior part of the brain (the brain was divided into two anterior and posterior part along the coronal plane) was fixed using 4% paraformaldehyde at 4°C for 4 h, dehydrated through xylenes and alcohols and embedded in paraffin. Sections were routinely

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**Figure 1.** Effects of the acupuncture on the neurological scores (A) and necrosis rates (B) in each group. \*,  $P < 0.01$ , compared with sham control; #,  $P < 0.05$ , compared with the vehicle group; ★,  $P < 0.05$ , compared with Dijji group.

cut at  $5 \mu\text{m}$  and stained with hematoxylin and eosin. The CA3 region of the ischemic side was observed using an OLYMPUS BX53 optical microscope at a magnification of  $400\times$ . For each section, 5 fields were randomly selected. HE staining results were defined as positive in the presence of dense staining, karyopyknosis or karyorrhexis.

### Determination of $\text{TNF-}\alpha$ , $\text{IL-1}\beta$ and $\text{IL-6}$

For the determination of the cytokines, about 150 brain tissues derived from the ischemic tissues were homogenated using cold physiological saline. After centrifugation at  $4000 \text{ r/min}$  at  $4^\circ\text{C}$  for 15 min, the supernatant was collected. The concentration of  $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$  and  $\text{IL-6}$  were determined using commercial ELISA kits purchased from Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China).

### Immunofluorescence

After deparaffinage, the sections were subject to hydration followed by incubation with EDTA antigen retrieval solution for 1 min in a microwave oven. After cooling to room temperature, the mixture was washed with PBS, followed by blocking for 30 min. Then cells were incubated with primary antibody of IBA1 (1:200, Abcam, Cambridge, UK) paired with TLR4 (1:200, Abcam, Cambridge, UK), HMGB1 (1:200, Abcam, Cambridge, UK), TRAF6 (1:200, Abcam, Cambridge, UK),  $\text{IKK}\beta$  (1:200, Abcam, Cambridge, UK), or  $\text{NF-}\kappa\text{B p65}$  (1:200, Abcam, Cambridge, UK) at  $4^\circ\text{C}$  overnight. Subsequently, cells were incubated with Daylight 549 labeled secondary antibody and FITC fluorescent secondary antibody (1:500, Dingguo Biotechnology Co., Ltd. Beijing, China) for 1 h at  $37^\circ\text{C}$ . The images were observed using a confocal laser scanning microscope.

The IBA1 stained cells were in a red color. For the cells with expression of IBA1, TLR4, HMGB1, TRAF6, and  $\text{IKK}\beta$ , the cells were presented in a yellow color. For the cells with expression of IBA1 and  $\text{NF-}\kappa\text{B p65}$ , the cells were presented with red color in cytoplasm and green color in nucleus. Five sections were randomly selected for each group, and five fields were randomly

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**Table 1.** Protein expression levels of TLR4, HMGB1, TRAF6, IKK $\beta$ , NF- $\kappa$ B p65 in microglia of MCAO model rats

Group	TLR4	HMGB1	TRAF6	IKK $\beta$	NF- $\kappa$ B p65
Sham-operation group	67.1 $\pm$ 15.58	61.91 $\pm$ 20.74	77.37 $\pm$ 8.71	75.8 $\pm$ 10.93	3.6 $\pm$ 2.41
Model control group	362.47 $\pm$ 12.16*	209 $\pm$ 30.62*	406.19 $\pm$ 56.08*	400.01 $\pm$ 28.51*	49 $\pm$ 2.74*
Neiguan group	298.04 $\pm$ 16.89* $\cdot$ $\Delta$ $\cdot$ $\#$	164.7 $\pm$ 33.22*	230.94 $\pm$ 49.26* $\cdot$ $\Delta$ $\cdot$ $\#$	306.14 $\pm$ 46.27* $\cdot$ $\Delta$ $\cdot$ $\#$	39.6 $\pm$ 7.54* $\cdot$ $\Delta$
Quchi group	320.04 $\pm$ 38.31* $\cdot$ $\Delta$	178.87 $\pm$ 36.57*	295.17 $\pm$ 33.91* $\cdot$ $\Delta$ $\cdot$ $\#$	349.18 $\pm$ 34.87* $\cdot$ $\Delta$	37 $\pm$ 2.45* $\cdot$ $\Delta$
Diji group	340.94 $\pm$ 28.74*	175.83 $\pm$ 51.47*	350.83 $\pm$ 37.26* $\cdot$ $\Delta$	371.11 $\pm$ 25.74* $\cdot$ $\Delta$	40.2 $\pm$ 8.17* $\cdot$ $\Delta$

\*P < 0.05, vs. sham-operation group;  $\Delta$ P < 0.05, vs. model control group;  $\#$ P < 0.05, vs. DJ group.

selected at a magnification of 200 $\times$ . IPP software was used to evaluate the average optical density of the yellow signals, or the percentage of cells presented with red color in cytoplasm and green color in nucleus.

### Statistical analysis

Data were present as mean  $\pm$  standard deviation. SPSS 13.0 software was used for the data analysis. One-factor analysis of variance was performed for the inter-group comparison.  $P$  < 0.05 demonstrated statistical difference.

### Results

#### *Effects of acupuncture on the neurological scores and necrosis in hippocampal neurons*

Compared with the sham control, the scores in the neurological tests were remarkably increased in the vehicle group ( $P$  < 0.01) and acupuncture groups ( $P$  < 0.01), together with significant increase of necrosis in the hippocampal neurons ( $P$  < 0.01, **Figure 1**). Compared with the vehicle group, the neurological scores were significantly decreased in the acupuncture groups, respectively ( $P$  < 0.05). In addition, remarkable decrease was noted in the necrosis of hippocampal neurons in Neiguan group and Quchi group, compared with the vehicle group ( $P$  < 0.05). Further, compared with the Diji group, significant decrease was noticed in the necrosis in the hippocampal neurons in the Quchi group ( $P$  < 0.05, **Table 1**).

#### *Levels of TNF- $\alpha$ , IL-1 $\beta$ and IL-6 in MCAO rats*

The levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were remarkably increased in the vehicle group compared with the sham control group ( $P$  < 0.01, **Figure 2**). The levels of TNF- $\alpha$  and IL-6 were significantly lower in the Quchi group compared with those of the vehicle group ( $P$  < 0.05). In addition, the

level of IL-1 $\beta$  was remarkably decreased in the Neiguan group and Quchi group compared with the vehicle group ( $P$  < 0.05).

#### *Expression of TLR4, HMGB1, TRAF6, IKK $\beta$ and NF- $\kappa$ B p65 in microglia*

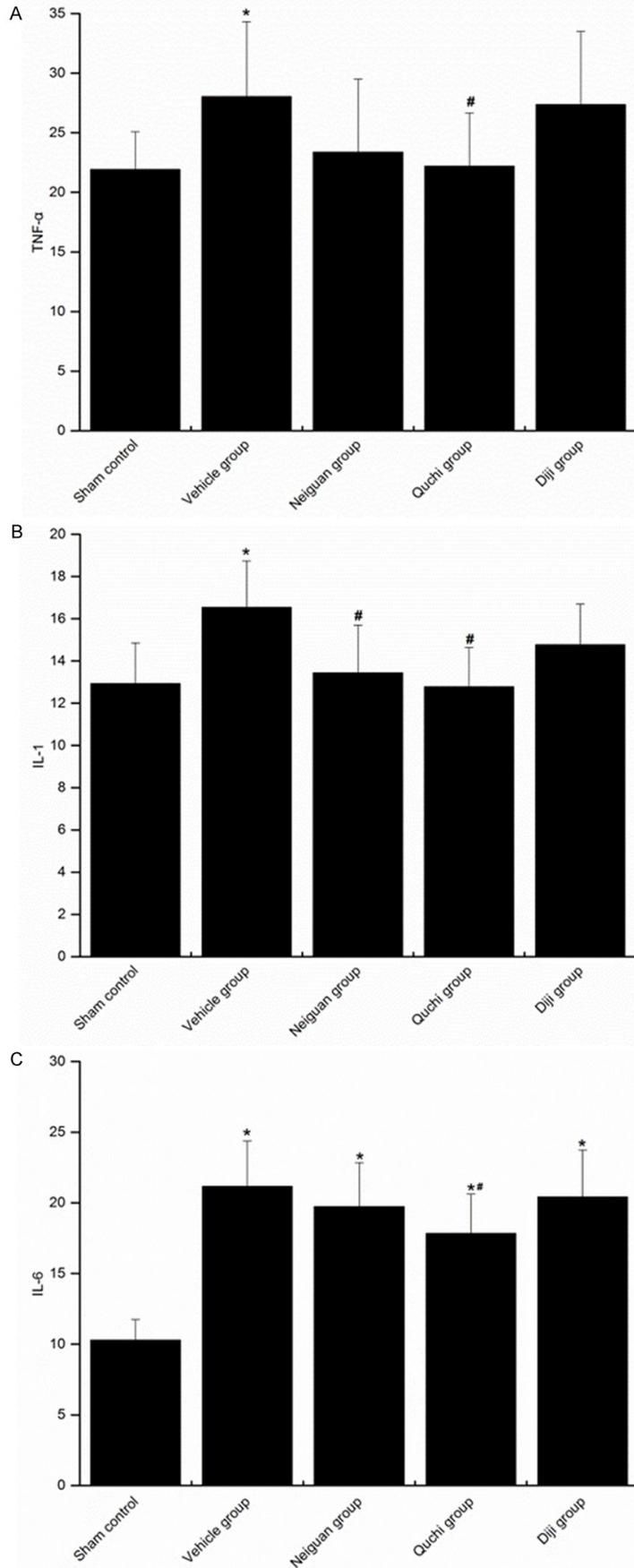
The expression of TLR4, HMGB1, TRAF6, IKK $\beta$  and NF- $\kappa$ B p65 in microglia of the brain tissues was remarkably up-regulated in the vehicle group ( $P$  < 0.05) and acupuncture groups ( $P$  < 0.05), compared with the sham control group (**Figure 3**). Compared with the vehicle group, the expression of TLR4 in the microglia was significantly down-regulated in the Neiguan group ( $P$  < 0.05) and Quchi group ( $P$  < 0.05), respectively. Similarly, the expression of TRAF6, IKK $\beta$  and NF- $\kappa$ B p65 in microglia was down-regulated in the Neiguan group, Quchi group, and Diji group, respectively. The expression of TLR4 and IKK $\beta$  was down-regulated in the microglia in the Neiguan group compared with the Diji group ( $P$  < 0.05).

### Discussion

It has been well acknowledged that inflammatory reactions involve in the cerebral injury after ischemic stroke. Initially, the inflammatory reactions were immediately triggered ever after cerebral ischemia, which may result in deterioration of delayed cerebral injury caused by brain ischemia and poor functional prognosis of nerve cells. In this process, microglia as the major immune effector cell in the central nervous system plays a crucial role in the activation of the immune inflammation [11].

Microglia formed the inherent immune system in brain, and the activated microglia contributed to the phagocytosis of the pathogen, cell debris, as well as the secretion of anti-inflammatory substances and nerve growth factors [12]. Also, it plays neurotrophic roles through en-

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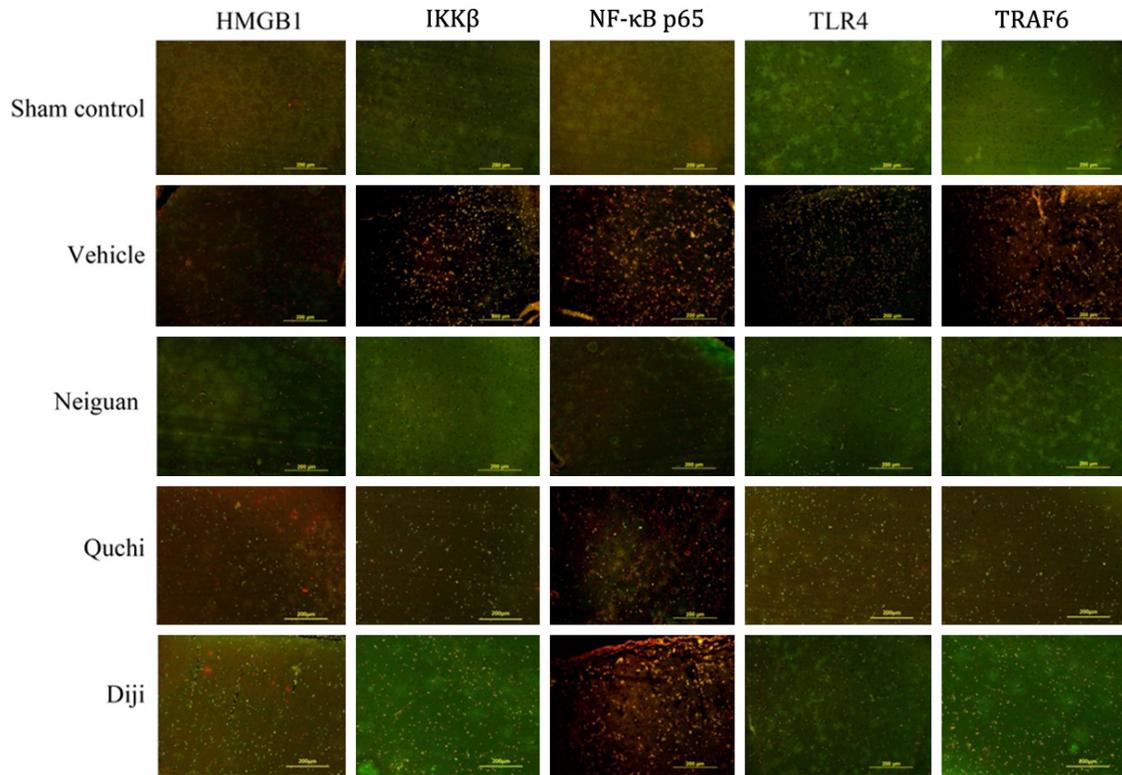


**Figure 2.** Effects of acupuncture on the expression of TNF- $\alpha$  (A), IL-1 $\beta$  (B) and IL-6 (C) in brain tissues. \*, P < 0.01, compared with sham control; #, P < 0.05, compared with the vehicle group.

abling the regeneration of neurons and survival of peripheral neurons in proximity to the neurons underwent necrosis [13]. However, in the presence of out-of-control or aberrant proliferation of immune-transmitter, microglia may lead to secondary cerebral injuries through releasing and/or section of substances with neurotoxicity, inflammatory factor, and certain enzymes [14]. On this basis, it is reasonable to develop new strategies to modulate the inflammatory reactions in cerebral ischemia through inhibiting the excessive activation of microglia.

TLRs play crucial roles in the activation of microglia through interacting with the ligands [15]. Microglia was highly expressed in the cellular surface, while its expression in the central nervous system was less than 10% [16]. A large number of cytokines were expressed in the microglia, among which TLR4 was highly expressed and played vital roles in the ischemic injury. For example, mice with knock-out of *TLR4* gene were not apt to develop cerebral ischemia/reperfusion injury compared with the normal control. In addition, the area of the infarction in the mice with knock-out of *TLR4* gene was smaller than that of the normal control [17]. Lehnardt et al reported that massive neuronal death was induced in the presence of activation of TLR4 in vitro, and this process was depended on the presence of the microglia [18]. In addition, LPS stimulation to the innate immune system could convert the hypoxic-ischemic insult from

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**Figure 3.** Expression of TLR4, HMGB1, TRAF6, IKK $\beta$  and NF- $\kappa$ B p65 in microglia in MCAO rats.

no or mild neuronal injury to severe neuronal loss. However, animals with loss-of-function mutation of the *TLR4* gene were resistant to neuronal injury, and the cultured microglia separated from the mice with mutation of *TLR4* gene was not responded to the LPS stimulation.

HMGB1 is an important endogenous ligand for TLR4 in the non-infectious immune inflammation induced by cerebral ischemia [19]. In rats with cerebral ischemia, the expression of TLR4 and HMGB1 was up-regulated in the activated microglia [20]. The interaction between TLR4 and HMGB1 could trigger several MyD88 depending or none-MyD88 depending signaling pathways. Moreover, in mice with knock-out of TLR4, the signals activated through the NF- $\kappa$ B were attenuated within 24 h after cerebral ischemia, together with decreased cerebral infarction area [21]. This demonstrated modulation of the microglia activation was crucial for the stimulating of immune inflammation after cerebral ischemia, in which the activation of NF- $\kappa$ B signaling pathway induced by TLR4 was of prime importance.

In this study, acupuncture on the Neiguan and Quchi acupoints could significantly decrease the neurological score of MCAO model and the necrosis of hippocampal neuron. In addition, it could inhibit the aberrant expression of certain factors involved in the TLR4/NF- $\kappa$ B signaling pathway after cerebral ischemia. Moreover, acupuncture on these acupoints could inhibit the activation of the microglia, and attenuate the up-regulation of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in brain tissues after ischemia. Taken together, we conclude that the anti-inflammatory effects of acupuncture may be closely related with the activation of microglia.

### Acknowledgements

This study is supported by the National Natural Scientific Foundation (No. 81202765, No. 81303053, and No. 81403492).

### Disclosure of conflict of interest

None.

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