Effect of acupuncture on the TLR2/4-NF-κB signalling pathway in a rat model of traumatic brain injury

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ABSTRACT

Objective To study the effect of acupuncture on the TLR2/4-NF-κB signalling pathway in the cortex of Sprague-Dawley rats following traumatic brain injury (TBI), and investigate the possible mechanism underlying the effects of acupuncture on scar repair.

Methods TBI was established using Feeney's free-falling epidural percussion model. In total, 108 rats were randomly divided into a normal group (n=18), untreated TBI model group (TBI group, n=36) and manual acupuncture-treated TBI group (TBI+MA, n=36). Each group of rats was subdivided into three time groups: 3-day (3d), 7-day (7d) and 14-day (14d). No treatment was given to rats in the normal and TBI groups. The TBI+MA group received manual acupuncture at GV20, GV26, GV16 through GV15, and bilateral LI4. mRNA expression of TLR2, TLR4, NF-κB and protein in the rat cortices was quantified using real-time fluorescence quantitative polymerase chain reaction (qPCR) and Western blot analyses.

Results The modified neurological severity score (mNSS) scores of the TBI+MA group were improved compared with baseline scores 12 hours after modelling, and improved at 7d and 14d compared with the TBI group ($P<0.05$), while the score of the TBI group did not improve until 14d compared to baseline. mRNA and protein expression of TLR2, TLR4 and NF-κB in the TBI group were higher than the normal group at 3d ($P<0.05$), reached a peak at 7d, then began to decrease at 14d. mRNA and protein expression of TLR2, TLR4 and NF-κB were higher in the TBI+MA group compared with the TBI group at 3d ($P<0.05$), were significantly down-regulated at 7d ($P<0.01$), and decreased to normal levels at 14d.

Conclusions Acupuncture has a bidirectional regulatory effect on the TLR2/4-NF-κB signalling pathway-related genes TLR2, TLR4 and NF-κB in the TBI rat cortex, promoting their expression in the early stage and inhibiting it in the later stage.

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INTRODUCTION

Traumatic brain injury (TBI) is a condition caused by blunt or sharp external forces on the brain. TBI may lead to temporary or permanent brain dysfunction. Studies have shown that the prevalence of TBI in the population is skewed towards younger individuals, in whom it has become a leading cause of death and disability. Due to a relative lack of attention, TBI is also known as a 'silent epidemic', and has become a major public health and socio-economic problem worldwide.[1 2]

After TBI, the brain injury produces a severe inflammatory response, leading to microglial activation, macrophage infiltration and astrocyte reactive hyperplasia. Microglia and astrocytes are not only the main immune cells of the central nervous system, but are also closely related to the formation of scar tissue after nerve injury.[3] Glial scarring begins to form after TBI and forms basically at 3 days, and becomes more obvious at 7 days.[4 5] The scar formation has two effects on brain injury: (1) the formation of scar tissue around the lesions separates the inflammatory necrotic tissue from normal tissue in the early injury stage, preventing the inflammation from spreading; and (2) sustained scarring interferes with the growth of neuron axons in the later stage, which curbs the neuronal repair.[6] Our previous studies have found that acupuncture can regulate scar repair and promote the regeneration of neurons.[7–10] A large number of studies[11–13] suggest that there is a close relationship between the TLR2/4-NF-κB signalling pathway and TBI scar repair. In view of the close relationship between nerve repair and glial scar after TBI, and the observation that acupuncture has a positive effect on scar repair after TBI, the aim of this study was to explore the possible mechanism underlying the effects of acupuncture on scar repair after TBI by measuring components of the TLR2/4-NF-κB signalling pathway.

METHODS

Animals and grouping

In total, 108 specific-pathogen-free (SPF) male Sprague-Dawley rats (each weighing 280±20 g) were purchased from Guangdong Medical Experimental Animal Centre (no. 44007200017130). The rats were housed in the Experimental Animal Centre of Jinan University. The temperature of the feeding environment was 20–24°C with a relative humidity of 50%. The rats were fed a normal diet. The experiments were performed under the supervision and assessment of the Laboratory Animal Ethics Committee of Jinan University (permit no. 20160629001). All procedures were in line with the Statute on the Administration of Laboratory Animals approved by China's Council 1988 and the ethical guidelines of the International Association for the Study of Pain. The rats were randomly divided into three groups in accordance with a random number table: normal group (Normal, n=18), TBI model group (TBI, n=36) and manual acupuncture-treated TBI group (TBI+MA, n=36). Eighteen rats were allocated to the Normal group in view of the smaller degree of variance anticipated (based on our previous studies) in keeping with the 3Rs principle of reduction. Then, each group was subdivided into 3-day (3d), 7-day (7d) and 14-day (14d) groups.

Model establishment

We used Feeney's freefall epidural impact method[14] to establish the animal model. The rats were weighed and injected with 3% pentobarbital sodium (1.5 mL/kg, Sigma, USA) intraperitoneally. We incised the scalp along the midline of the rat skull after anaesthesia. We drilled an approximately 5 mm diameter hole with a ZH-RXZ flexible skull drill (Zhenghua, China) 1 mm from the coronal suture and 2 mm to the left of the sagittal suture. Next, we used the injury intensity of a freefall 20 g weight from a height of 30 cm to hit the rivet (diameter 4 mm, length 5 mm) that was on the hole. The impact force was approximately 600 g×cm and resulted in local contusion of the left parietal cortex in the rats that maintained the integrity of the dura. Then, the animals' scalps were sutured. The entire modelling procedure was performed in a sterile operating room, and the rats were kept warm.

Acupuncture treatment

The rats in the acupuncture group began to receive MA treatment 12 hours after the completion of the model with stainless steel needles (13 mm×0.18 mm, HWATO, China). Based on 'Experimental Acupuncture',[15] we selected acupuncture points GV20 (*Baihui*), GV25 (*Renzhong*), GV16 (*Fengfu*) through GV15 (*Yamen*), and bilateral LI4 (*Hegu*). The acupuncture needles were inserted at these points to a depth of approximately 2 mm. The needles remained in place for 15 mins and were twisted once every 5 mins. Each time, intense manual stimulation was applied at every point for 1 min, with a twisting amplitude of 360° and frequency of approximately 160–180 per minute. The 3d, 7d and 14d acupuncture subgroups were treated with acupuncture for 15 min once a day for 3 days, 7 days and 14 days, respectively, at the same time every day until euthanasia. The Normal and model groups did not receive acupuncture treatment; however, the model group rats were fixed for 15 min during the same period. The treatments were performed by the same investigator who was blinded during the treatment.

Neurological behavioural scoring

The rats in each group were trained for ambulation, coordination and balance for 7 days before the modelling. The basal score was measured 1 day before the modelling according to the modified neurological severity score (mNSS).[16] Rats with a score of 4–8 were selected as the experimental subjects and were scored at 12 hours after modelling, while the unmodelled rats were excluded. Eighteen rats whose scores exceeded 8 or were calculated to be under 4, or who died within 12 hours of modelling were excluded. Each group was scored again at 3d, 7d and 14d after the end of treatment.

Tissue sampling

At the end of the treatment period, the rats were euthanased by injecting them with an overdose of pentobarbital sodium intraperitoneally. The thoracic cavity was quickly incised to expose the heart. A puncture needle was inserted from the left apex and extended to the aorta with haemostatic forceps. The right auricle was incised and washed with 100 mL of iced normal saline for approximately 5 min. When the right atrial appendage yielded clear fluid, the cortical tissue around the lesion of the brain tissue was removed and stored in a freezer at −80°C.

Quantitative reverse transcription PCR

mRNA expression of TLR2, TLR4 and NF-κB was measured by real-time fluorescence quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the lesioned cortical tissue using Trizol (Takara, Japan). Then, the total RNA concentration and purity were measured using NanoDrop 2000 (Thermo Scientific, America). Reverse transcription was performed according to the instructions of the Prime Script RT reagent kits (Takara, Japan), and the obtained cDNA was subjected to PCR amplification using the primers detailed in table 1.

Western blotting

Total protein was extracted according to the protein extraction kit instructions. Protein quantification was performed by the BCA (bicinchoninic acid) method. Resuscitated sample protein solution and

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Table 1 Gene sequences for primer synthesis

Gene	Primers	Sequence 5'>3'
TLR2	Sense	GGAATCAACACAATAGAGGGAG
	Anti-sense	CTGAACCAGGAGGAAGATAAAC
TLR4	Sense	CCTCCCTGGTGTTGGATTTTAC
	Anti-sense	AGATGCTTTCTCCTCTGCTGTA
NFκ B	Sense	ATCTATGACAGCAAAGCCCCCAA
	Anti-sense	CCAAATCCTTCCCAAACTCCACC
β-actin	Sense	CTGAACCCTAAGGCCAACCG
	Anti-sense	GACCAGAGGCATACAGGGACAA

PCR conditions were as follows: pre-denaturation at 95°C for 1 min, denaturation at 94°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s for 39 cycles, 55–95°C for every 0.3° and 0.5 s for dissolution curve reading. The experiments were repeated three times. The relative quantification results of the genes were performed using the formula 2-ΔΔCt.

5×loading buffer (4:1) were mixed and boiled for 5 min. After electrophoresis, the protein was transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Germany), blocked with 5% skimmed milk powder, incubated with TBST (tris-buffered saline and Polysorbate 20) for 2 hours at room temperature, washed with TBST. Then the washed PVDF membranes were immersed in a light-emitting liquid for 5 min using a gel automatic imaging system for exposure. Western blotting was performed using Quantity One v4.6.2 for grey value analysis.

Statistical analysis

Statistical analysis of the experimental data was performed using SPSS 13.0 software (SPSS Inc, Chicago, IL, USA). The data are presented as mean±SD. Groups were compared using one-way analysis of variance (ANOVA) followed by post-hoc Student-Newman-Keuls test. A value of P<0.05 indicates a statistically significant difference.

RESULTS

Neurological behavioural score

Before the establishment of the model, the rats were scored according to the mNSS scoring system. The basal score was 0. Twelve hours after model establishment, there were no significant differences between the TBI+MA and TBI groups (P=0.08). Compared with the Normal group, modelled group rats had significantly decreased food intake with reduced activity, were stress unresponsive, and had limb hemiplegia and signs of other neurological deficits, which demonstrated that the modelling had been successful. Compared with the scores 12 hours after modelling, the 7d and 14d scores of the TBI+MA group were significantly improved (P=0.001 and P<0.001, respectively). The neurological score decreased, and the neurological deficit signs gradually improved with extended treatment time. Meanwhile, there was no significant difference in the TBI group until 14d (P<0.001). Compared with the TBI group, the TBI+MA group showed no significant difference on the third day (P=0.22); however, the TBI+MA group scores on 7d and 14d began to improve, and differed significantly from the untreated TBI group (P<0.05), as shown in table 2.

mRNA expression of TLR2, TLR4 and NF-κB in injured cortical tissue

Figure 1 shows that the mRNA expression of TLR2 and TLR4 was significantly up-regulated in the acupuncture group compared with those in the model group at 3d (P<0.05). mRNA expression of TLR2, TLR4 and NF-κB in the acupuncture group decreased significantly at 7d, while that in the model group peaked. mRNA expression in the acupuncture group was down-regulated almost to normal levels at 14d. In contrast, the expression of TLR2, TLR4 (P<0.01) and NF-κB (P<0.05) mRNA in the model group was still higher than normal.

Protein expression of TLR2, TLR4 and NFκB in injured cortical tissue

Figure 2 shows that, compared with the normal group, protein expression of TLR2, TLR4 and NF-κB was significantly up-regulated in the acupuncture group at 3d (P<0.01). Protein expression of TLR2, TLR4 and NF-κB in the acupuncture group decreased significantly compared with the model group at 7d (P<0.01), while protein expression of TLR4 had already decreased to a normal level. Meanwhile, protein expression of all genes in the model group peaked compared with the acupuncture group (P<0.01). At 14d, protein expression of TLR2, TLR4 and NF-κB had almost decreased to normal levels in both acupuncture and untreated model groups.

Table 2 Comparison of neurological behavioural scores in TBI rats ($\bar{x} \pm S$)

	3d Group		7d Group		14d Group	
Group	After 12 hours	3d	After 12 hours	7d	After 12 hours	14d
Normal	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
TBI	6.33±1.30	5.33±1.56	6.50±1.00	5.17±0.94	6.50±1.00	2.33±1.30**
TBI+MA	6.17±1.12	4.50±1.31	5.50±1.45	3.83±0.94*,**	5.83±1.27	1.00±0.85*,**

*P<0.05 compared with the TBI group.
**P<0.01 compared with the corresponding TBI group after 12 hours.
MA, manual acupuncture; TBI, traumatic brain injury. 3d, 3-day; 7d, 7-day; 14d, 14-day (after modelling).

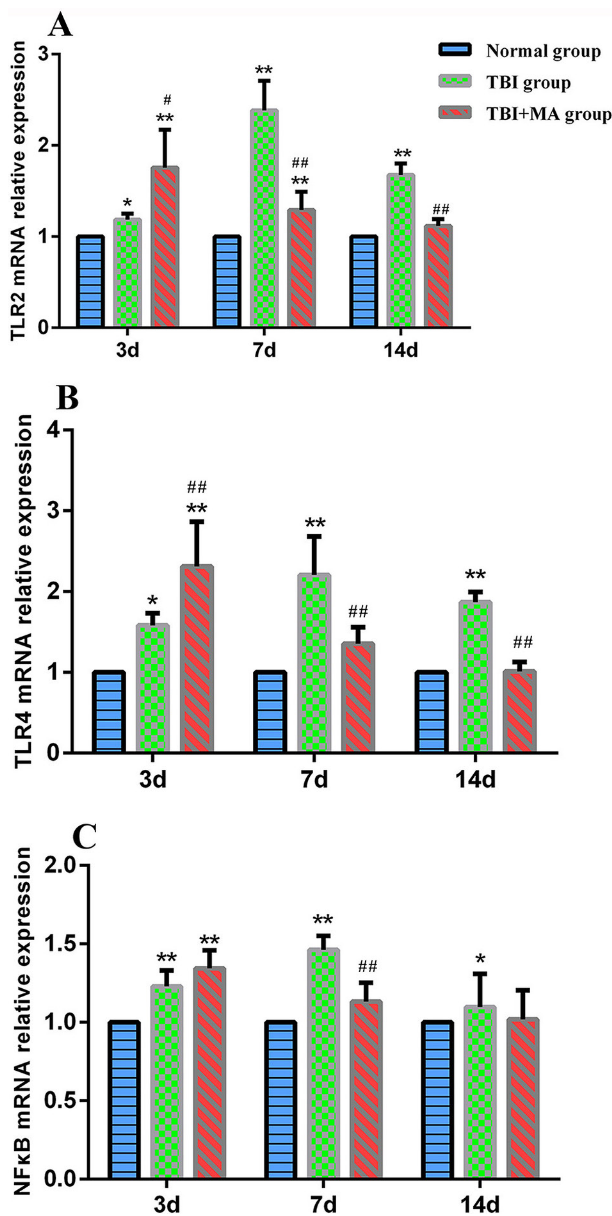

Figure 1 Relative mRNA expression of TLR2 (A), TLR4 (B) and NF-κB (C) at three time points (3, 7 and 14 days) in the cerebral cortex of healthy rats (Normal group, n=6 each) or rats with traumatic brain injury that remained untreated (TBI group, n=12 each) or received manual acupuncture (TBI+MA group, n=12 each). *P<0.05 versus Normal group. **P<0.01 versus Normal group. #P<0.05 versus TBI group. ##P<0.01 versus TBI group.

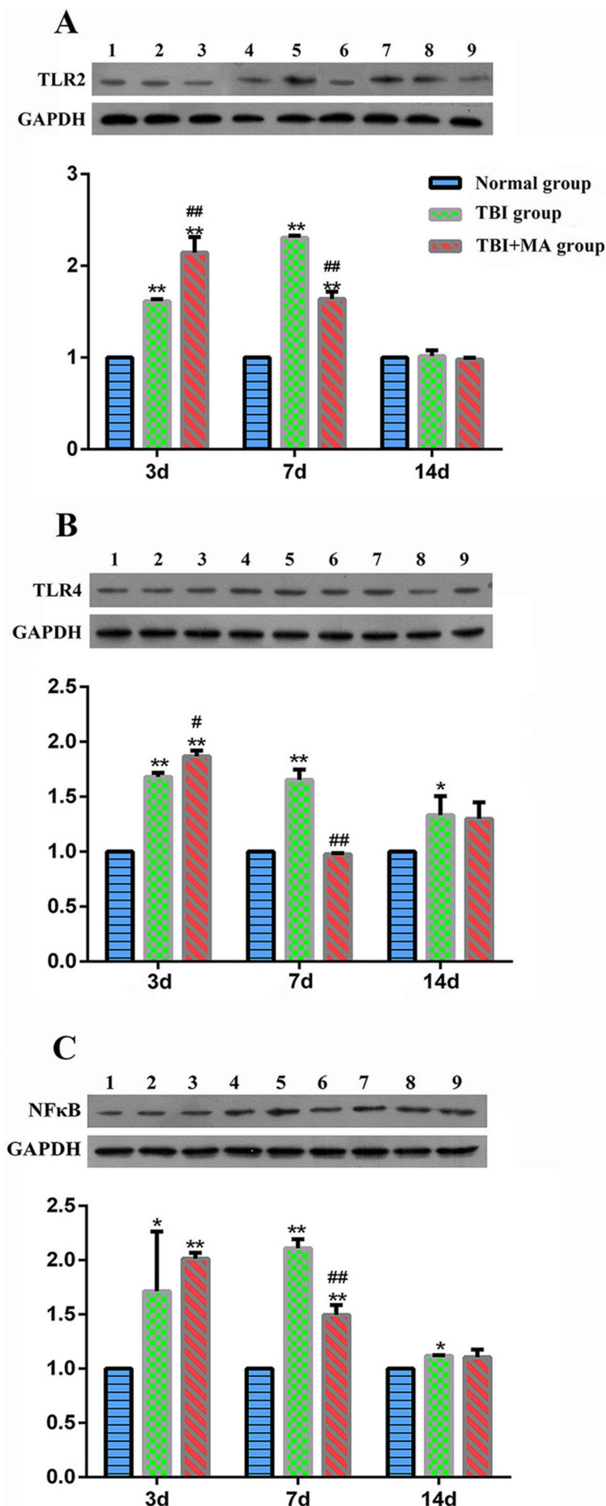

Figure 2 Relative protein expression of TLR2 (A), TLR4 (B) and NF-κB (C) in the cerebral cortex of healthy rats (Normal group, n=6 each) or rats with traumatic brain injury that remained untreated (TBI group, n=12 each) or received manual acupuncture (TBI+MA group, n=12 each). *P<0.05 versus Normal group. **P<0.01 versus Normal group. #P<0.05 versus TBI group. ##P<0.01 versus TBI group.

DISCUSSION

In the present study, we aimed to investigate the effects of acupuncture treatment on the TLR2/4-NF-κB signalling pathway and scar repair after TBI. Acupuncture is considered to be one of the effective treatments for TBI and promotes scar repair following such injury.[17 18] Based on previous preclinical and experimental studies, we selected acupuncture points GV20, GV25, GV16, GV15, and LI4 in this study.[10 19 20] We found that the condition of the TBI rats improved after acupuncture treatment. The mNSS scores in the TBI+MA group were higher than the untreated TBI groups at all three time points and continued to change over time. Accordingly, we would infer that the recovery rate of TBI rats after acupuncture treatment

is faster. In addition, acupuncture treatment appears to affect scar repair after TBI through the TLR2/4-NF-κB signalling pathway.

Toll-like receptors (TLRs) recognise and activate the innate immunity of the human body through pathogen-associated molecular patterns (PAMPs), and constitute the first line of defence of human natural immunity.[21] There are a variety of TLRs on the surface of microglia and astrocytes in the brain. TLR2 and TLR4 are found in different central nervous system injury models.[22] They are found to be more important in the pathogenesis of ischaemia–reperfusion than other TLRs.[23 24] TLR2 and TLR4 are involved in the regulation of scarring after TBI through the TLR2/4-NF-κB signalling pathway.[25] Moreover, TLRs are not only the 'gateways' for identifying and initiating innate immunity, but are also the key proteins that regulate scar repair.[26] TLR2 and TLR4 are expressed in the microglia and astrocytes of the brain.[22] NF-κB plays a key role in the regulation of the TLR2/4-NF-κB signalling pathway. It is considered to be a key gene and its main function is to encode the transcription of cytokine-related genes. It widely participates in the regulation of cell apoptosis and proliferation of glial cells. NF-κB is present in neurons, microglia and astrocytes, and inducible NF-κB is also present in the soma, nucleus and synapses of neurons.[27 28] In the resting state, NF-κB binds to the corresponding inhibitory protein IκB family members in the cytoplasm, whereupon it loses transcriptional activity. When cells are stimulated by cytokines, oxidative stress and transcriptional factors, a second messenger system initiates phosphorylation and ubiquitination; subsequently, the NF-κB and IκB compounds are activated and dissociate. NF-κB is transferred from the cytoplasm into the nucleus, initiating a series of transcriptional processes related to immunity by binding to the target gene locus. NF-κB acts as an important transcription factor compound, which stimulates the production of cytokines and their activation, resulting in cascade amplification of cytokines, which in turn regulates glial cell proliferation.

Traumatic inflammation occurs after TBI, leading to autologous tissue injury or death, followed by the release of heat shock protein (HSP), cellular fibronectin (cFn), hyaluronic acid, high mobility group box 1 protein (HMGB1), and other endogenous ligands.[29 30] These endogenous ligands are recognised and bound by TLR2 and TLR4 in the cell membrane, which activate the TLR2/4-NF-κB signalling pathway, promote downstream NF-κB transcription factor translocation into the nucleus, and mediate the expression of cytokines.[25] Experimental studies have shown that endogenous danger signals following cell damage may be recognised by TLR2 and TLR4 receptors, which activate NF-κB to induce the production of cytokines, which then promote glial cell expression and the formation of glial scars.[31 32] Tu *et al*[33 34] found that inhibition of the TLR2/4-NF-κB signalling pathway can reduce brain injury and protect nerves. Sanz *et al*[35] showed that NF-κB expression levels in the cortex significantly increase after 1 hour in a TBI rat model and peak at 24 hours. TLR2 and TLR4, the important upstream recognition receptors, are essential for NF-κB activation and regulation. Toll-like receptors are upstream switches that recognise danger signals and trigger the secretion of corresponding cytokines after recognising different ligands. This has important potential therapeutic value for regulating scar repair by inhibiting the expression of TLRs and then reducing the activity of NF-κB and down-regulating the expression of cytokines.[36] Kigerl *et al*[11] found that TLRs play an important role in the regulation of glial scar formation after injury. TBI-induced danger signals activate microglia and astrocytes that promote phagoctosis of dead and damaged cells, while glial cell proliferation wraps the lesions and forms glial scars.[37] The activation of astrocytes after differentiation plays a positive role in the repair of nerve injury. But after maturation of the glial scar, it secretes harmful cytokines and forms a chemical glial barrier, seriously affecting nerve regeneration and axonal extension.[38] Therefore, the regulation of glial scar formation significantly impacts the prognosis of TBI.

Our experimental results showed that, compared with the untreated TBI model group, mRNA and protein expression of TLR2, TLR4 and NF-κB were significantly up-regulated after acupuncture treatment for 3d, which indicates that acupuncture can promote the expression of these genes in the early stages of recovery. At 7d, mRNA and protein expression of TLR2, TLR4 and NF-κB in the TBI group were at their highest level, while being down-regulated significantly in the TBI+EA group, which indicates that acupuncture plays an inhibitory role. After 14d, mRNA and protein expression in the TBI+MA group were decreased back to normal level. These results suggest that acupuncture can regulate TLR2, TLR4 and NF-κB bidirectionally—that is, it can promote the expression of TLR2/4-NF-κB signalling pathway-related genes in the early stage, and exert an inhibitory effect in the later stage. Zheng *et al*[39] and Song *et al*[40] found that, following acupuncture treatment of cerebral ischaemia in rats, compared with an utreated model group, the expression of glial fibrillary acidic protein (GFAP) was up-regulated during the first 3 days, whereas it was inhibited 7 days later, and that the proliferation of star glial cells was activated in the early phase and inhibited in the later phase. The trend in expression of tumour necrosis factor (TNF)-α and GFAP is almost consistent, both of them increasing in the early stage after TBI and decreasing in the later stage.[10 41 42] The results of these studies are similar to those of our study. In our previous studies,[8–10 43] we found that the expression of astrocytes in the injured brain of the model group increased significantly at

3d, decreased gradually at 7d, and remained at a high level at 14d. Compared with the TBI model group, expression levels in the TBI+MA group increased significantly at 3d, decreased significantly at 7d (being lower than that in the model group), and reached normal levels at 14d. The results suggest that acupuncture can promote the proliferation of glial cells in the early stage of TBI, which is beneficial to the control of nerve necrosis, and inhibits the excessive proliferation of glial scars in the later stage, which is beneficial to the regeneration of neurons. The trend of glial cell proliferation is consistent with our present experimental study. Liang *et al*[44] and Yang *et al*[45] also suggest that acupuncture can regulate glial cell activation and differentiation. Thus, we infer that acupuncture has a bidirectional modulatory effect on TBI.

A strength of our study is that fact we were able to demonstrate a bidirectional effect of acupuncture treatment for TBI, while others have only studied its promotive or inhibitory effects. We have also further characterised the relationships between acupuncture treatment, scar repair and the TLR2/4-NF-κB signalling pathway in the injured cortex of TBI rats , which may represent a promising therapeutic target in the future. Ultimately, however, the mechanisms of action underlying the beneficial effects of acupuncture on scar repair after TBI are complex. The study also has some limitations including the fact that we only examined the effects of acupuncture at three time points and we did not employ blockade of the signalling pathway to prove the proposed mechanism of action. Our next study will involve inhibition of the TLR2/4-NF-κB signalling pathway by knocking out the TLR2 and TLR4 genes of the rats to further explore the relationship between this signalling pathway and scar repair, and set more time points to study this in greater depth. Furthermore, we will continue to study the bidirectional modulatory effect of acupuncture.

CONCLUSION

Acupuncture has a bidirectional modulatory effect on TLR2/4-NF-κB signalling pathway-related genes TLR2, TLR4 and NF-κB in the injured cortex of rats with TBI, promoting their expression in the early stage and promoting glial scar repair; acupuncture also has an inhibitory effect in the later stage, reducing glial scar hyperplasia, which is conducive to the regeneration of neurons. This may be one of the mechanisms by which acupuncture regulates scar repair in TBI.

Contributors Experiments were designed by S-JL and Y-MZ, and performed by S-JL and Q-FD. Y-MZ and S-BC provided guidance. L-XC, Q-FD and J-HL conducted the data analysis. LP, W-HC, Y-JZ and S-LC contributed materials and reagents. Y-MZ and L-XC wrote the manuscript and all authors approved the final version accepted for publication.

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