Original Article
Research on the effects and mechanisms of IL-17 on traumatic brain injury in rats

Fuyu Gao, Zhilin Cao, Benjun Bi

Department of Orthopedic Surgery, Yantaishan Hospital, Yantai, Shandong, China

Abstract: Objective: To investigate the effect and possible mechanism of IL-17 on traumatic brain injury (TBI) in rats. Methods: Seventy two healthy adult male Sprague-Dawley (SD) rats were randomly divided into three groups: sham operation group, TBI group, secukinumab (IL-17 inhibitor) and TBI group (S+T group). And each group was further divided into four subgroups (6 h, 12 h, 24 h and 48 h) of 6 rats on the basis of different observation times. TBI model was made by using the brain hydraulic impact method. In S+T group, secukinumab was intraperitoneally injected for 3 days in advance and TBI model was made at the specified observation time on the fourth day. In sham group, only craniotomy was performed. Neurological severity scores were applied in functional assessment of brain in each group; enzyme linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (PT-PCR) method were applied to detect the contents of rat peripheral blood cytokines such as IL-6, TNF-α and IL-17 in each group; western blot (WB) was also applied to detect the level of IκB and p-P65 in brain tissues in each group. Results: Compared with shame group, ethology scores at each observation time, contents of cytokines (IL-6, TNF-α and IL-17) in peripheral blood, expressions of IL-6, TNF-α, IL-17 mRNA in brain tissues, degradation amount of IκB and expression of p-P65 were significantly increased in TBI group. In S+T group, however, the above indexes were between the sham group and TBI group (P<0.05). Conclusion: Traumatic brain injury may be caused by IL-17 mediated activation of the downstream NF-κB pathway.

Keywords: Traumatic brain injury (TBI), interleukin-17 (IL-17), interleukin-6 (IL-6), NF-κB pathway

Introduction

The incidence rate of traumatic brain injury (TBI) rises year by year in China, which brings great physical and mental anguish to patients and their families and causes great losses to the economic development of China [1, 2]. Therefore, further researches about pathogenesis of brain injury and how to take effective measures to mitigate injury and improve prognosis are always the research hotspots in the medical field [3, 4]. In recent years, studies have shown that IL-17 secreted by Th17 cells plays a key role in brain injury [5].

Previous studies have shown that in experimental autoimmunity cerebrospinal meningitis model, the reinfusion of IL-17 secreted by Th17 cells to normal mice can induce the more severe experimental autoimmunity cerebrospinal meningitis in these normal mice. Besides, its severity is closely related to the content of IL-17 in reinfusion [6-8]. However, in TBI inflammatory responses, whether IL-17 is involved and how to influence the progression of TBI have not been fully elucidated. This paper aims to further elucidate the specific occurrence mechanism of IL-17 and the signaling pathway that may be involved in the TBI inflammatory responses, and then tries to seek therapeutic target and provide the clinical evidence for the treatment of TBI.

Materials and methods

Grouping of experimental animals

Seventy two healthy adult male SD rats weighting 250±20 g were provided by the experimental animal center of 2nd Army Medical College, China. Then, these rats were randomly divided into sham, TBI and S+T groups. The sham, TBI

and S+T groups consisted of 24 rats respectively. Each group was further divided into four subgroups (6 h, 12 h, 24 h and 48 h) of 6 rats on the basis of different observation times [23]. There was no statistical significance in the difference of nutritional status and physical growth in all rats.

Establishment of TBI model

The rats' heads were fixed and their hair overhead was shaved after anesthesia via intraperitoneal injection of 5% pentobarbital (0.1 ml/100 g). Their skins were then sterilized by iodophor and draped. After that, their scalps were scissored a long the median sagittal suture and periosteum of the bones of the cranium was isolated to expose the bones of the cranium. Under the operating microscope of neurosurgery, the drill was used to grind 2 mm away from the right sagittal suture, and holes were drilled at 2.5 mm after the coronal suture and a round bone window with a diameter of 4 mm was then grinded. The dura mater was confirmed to be intact during the process [23]. In sham group, only craniotomy was performed according to the way described above while TBI model was not made and none reagent was injected. In TBI group, TBI model was made at the specified time point using the brain hydraulic impact method but none reagent was injected. In S+T group, rats were injected intraperitoneally with secukinumab (IL-17 inhibitor) for three consecutive days before craniotomy (a total of three times). And on the fourth day, the brain hydraulic impact method was applied to establish TBI model at the specified time point. Success criteria for establishing TBI model: pupillary light reflex, corneal reflex and plantar reflex disappeared immediately after injury, but recovered within 30 minutes.

Collection of blood from rats

The rats in each group were immediately fixed on the operating table and placed in the supine position after successful anesthesia via intraperitoneal injection of 5% pentobarbital (0.1 ml/100 g). After that, their abdominal cavities were opened with the abdominal aortas being exposed. Then, after the abdominal aortas were punctured by the puncture needles, vacutainers were used to store the blood. Blood was collected as much as possible.

ELISA detection of IL-6, TNF-α and IL-17 contents in peripheral blood

Blood of all rats in each group were collected in chronological order and these rats were then sacrificed. Appropriate amount of EDTA was added to the blood samples for anticoagulation and then stood for 1 h. After that, the blood samples were centrifuged at 12,000 r/min for 15 min and the blood serums were then taken for cryopreservation at -80°C for later detection. The ELISA kits for IL-6, TNF-α and IL-17 which were purchased from the Xitang Biology were applied, and all steps were operated in strict accordance with the procedures specified by the kit. The OD values of plasma IL-6, TNF-α and IL-17 levels in rats among each group were measured by the microplate reader at 562 nm wavelength to analyze the changes of inflammatory factors.

Collection of total RNA from brain tissues and RT-PCR analysis of IL-6, TNF-α and IL-17 mRNA expressions

The rats were sacrificed after vein sampling in accordance with the method described previously. Then the brain tissues of rats were taken out and cleaned in the sterile PBS and then placed in the 1 ml Trizol EP tube. The total RNA was extracted from the brain tissues in accordance with the introduction of Trizol kit. Reverse transcription was operated in accordance with the instruction of TAKARA reverse transcriptase kit to get the cDNA. Subsequently, cDNA was used as a template to retrieve the Genebank to get the mRNA sequences of IL-6, TNF-α and IL-17A (synthesized by the Shanghai Biotechnology Co., Ltd.). And the expression levels of genes were detected by the SYBR Ex Taq kit from the TAKRA Company.

WB detection of protein expressions of IκB and p-P65

The rats were sacrificed and their brain tissues were taken out after vein sampling in accordance with the method described previously. Then, about 100 mg clean area of brain tissues at the same site were cleaned in the sterile PBS and then put on the ice with 100 μl protein lysis buffer being added. After being lysed for 30 min and centrifuged at 12,000 r/min for 20 min at 4°C, the supernatants were then collected. The protein levels of samples were

Table 1. The number of successful cases of rat brain injury model

Group	Successful cases (number)	
	TBI group	S+T group
6 h	6	6
12 h	6	6
24 h	6	6
48 h	6	6

detected by the BCA kit from the Beyotime Institute of Biotechnology. Then protein lysis buffer was evenly distributed with the equal protein level, and 5× protein buffer was added and then mixed. After metal bath at 95°C for 15 min and aliquot, these samples were stored in the fridge at -80°C for the later detection of protein semiquantitative.

After SDS-PAGE electrophoresis and protein isolation, the separated bands were transferred to the PVDF membrane. 5% nonfat milk was blocked for 1.5 hours at room temperature and rinsed in TBST for 15 min for a total of 3 times. After that, the antibody IκB (diluted in 1:1000) and antibody p-P65 (diluted in 1:2000) were added and stored overnight at 4°C and then rinsed in TBST for 15 min for a total of 3 times. The two antibodies diluted by TBST were incubated in greenhouse for 1.5 h, and rinsed in TBST for 15 min for a total of 3 times. The membrane was put on a membrane in the dark room, with appropriate amount of ECL working solution evenly covered on it, and then exposed and visualized by X-ray. The expression scores for protein β-actin were taken as the internal parameter, the software Image Lab was applied for image analysis.

Ethology observation

Ethology observation after brain injury in rats

In TBI group and S+T group, the changes in general appearances including breathing, heart rate, pupil, hair and so on after the brain hydraulic impact were observed: the pupils of rats were irradiated with the flashlights instantly after the impact in the two groups to observe whether there was pupillary light reflex; the corneas of rats were swabbed with cotton swabs to observe whether there was corneal reflex; the cotton swabs were similarly used to stimulate the pelma of rats to observe whether there

was pain reflex. The time of occurrence and disappearance were recorded respectively. If the recovery happened within 30 min, the model would be regarded as a success. The number of successful cases can be seen in **Table 1**.

Neurological severity scores

The neurological severity of rats in TBI group and S+T group were scored according to the rats neurological severity scores improved by Shibani et al. [10], and 10 standards were as follows: success was recorded as 0 while failure as 1; and the highest score was 10 which showed the severe neurological score (**Figure 1**).

Statistical analysis

In this experiment, the measurement data were presented as mean ± standard deviation and analyzed using the SPSS16.0. The comparisons of differences between groups were detected using the t test. The above indexes of rats in these three groups were collected and the software of Graphpad Prism 6.0 was applied to draw the corresponding histograms, conduct the statistical analysis, and calculate the *P* values. P<0.05 was considered to indicate a statistically significant difference.

Results

Results of the model of brain injury in rats

In this experiment, 30 SD rats were used to establish the model of brain injury in TBI group. Among them, 3 rats died for accidental anesthesia and 3 rats died for the apnea and cardiac arrest after the impact. All other 24 survived rats appeared the symptoms of subdural hematoma after the brain hydraulic impact. Once they got injured, pupillary light reflex, corneal reflex and plantar reflex were instantly disappeared but recovered in 30 min. All these appearances supported that the TBI model was established successfully.

In S+T group, 30 SD rats were used in the model establishment. Among them, 4 rats died for accident anesthesia and 2 rats died for apnea and cardiac arrest after impact. The model established by the other 24 survived rats were successful.
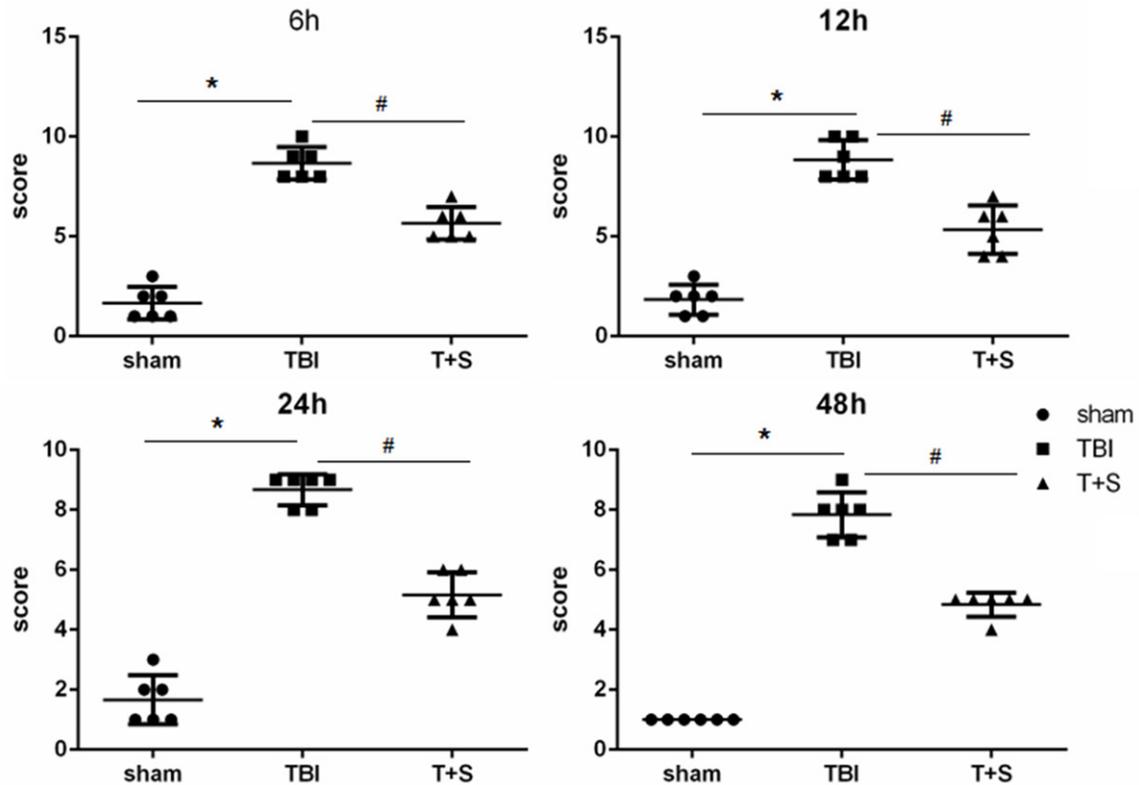
Figure 1. The neurological scores at different time points in each group. Note: *P<0.05, compared with sham group; #P<0.05, compared with TBI group.
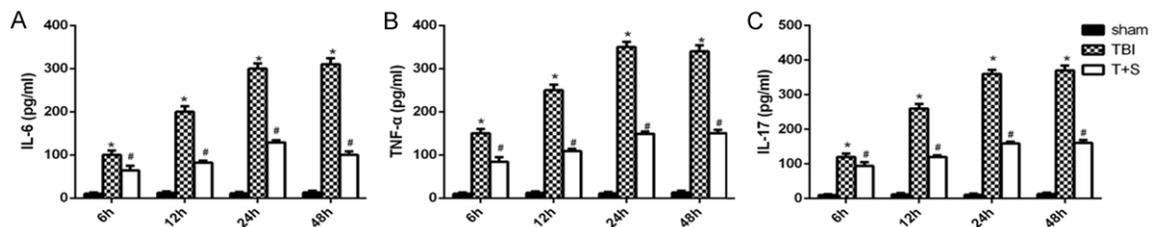

Figure 2. The levels of IL-6, TNF-α and IL-17 in peripheral blood at different time points in each group. Note: A: The levels of cytokines IL-6 in peripheral blood at different time points in each group; B: The levels of TNF-α cytokines in peripheral blood in each group; C: The levels of IL-17 cytokines in peripheral blood in each group. **P<0.01, compared with sham group; ##P<0.05, compared with TBI group.

Neurological severity scores

The neurological functions of rats in each group were evaluated by using the neurological severity score scale at 6 h, 12 h, 24 h and 48 h after brain injury. Compared with sham group, the average neurologic scores in TBI group were increased, and the neurologic scores in S+T group were lower than TBI group but higher than sham group (P<0.05) (**Figure 1**).

ELISA detection results of IL-6, TNF-α and IL-17 in peripheral blood

Compared with sham group, the levels of IL-6, TNF-α and IL-17 in peripheral blood detected by ELISA in TBI group were obviously increased at 6 h, 12 h, 24 h and 48 h after TBI and peaked at 48 h after TBI (P<0.01). Compared with TBI group, the above levels in S+T group were remarkably decreased at 6 h, 12 h, 24 h and
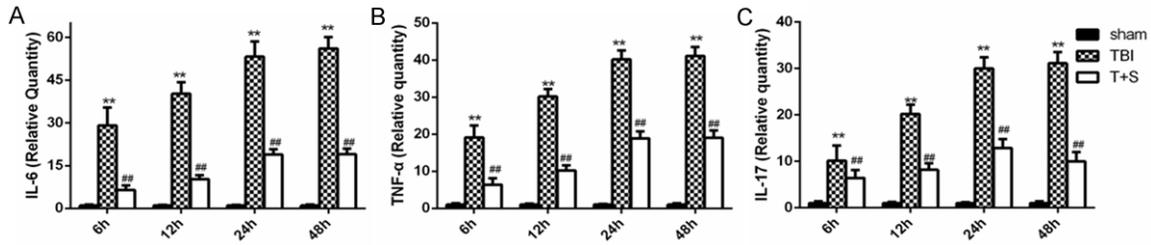
Figure 3. The levels of IL-6, TNF-α and IL-17 mRNA in brain tissues at different time points in each group. Note: A: The levels of IL-6 in peripheral brain tissues in each group; B: The levels of TNF-α in brain tissues in each group; C: The levels of IL-17 mRNA in brain tissues at different time points in each group. **P<0.01, compared with sham group; ##P<0.01, compared with TBI group.
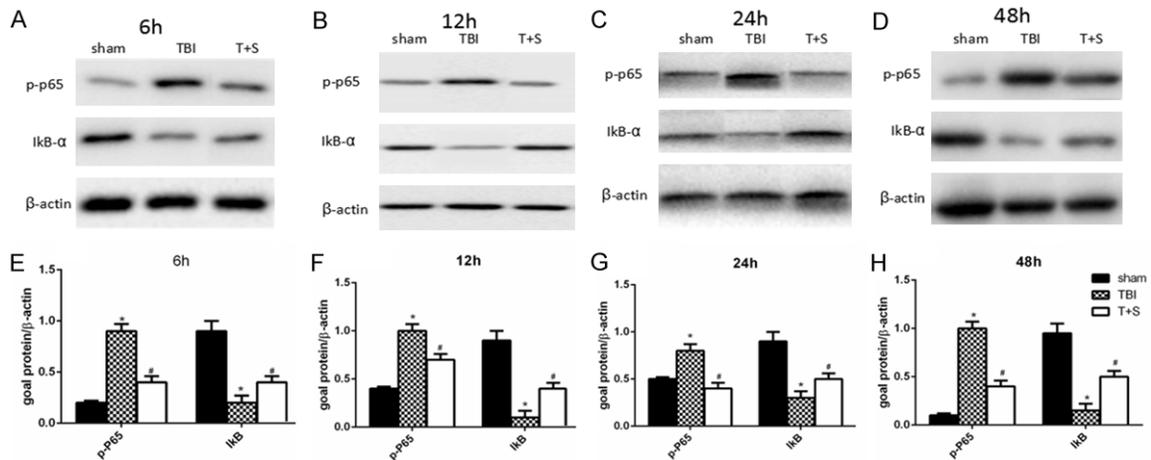

Figure 4. The expressions of IκB and p-P65 protein in brain tissues at different time points. Note: A, E: The expression levels of IκB, p-P65 protein and analytic statistics of gray levels in brain tissues at 6 h; B, F: The expression levels of IκB, p-P65 protein and analytic statistics of gray levels in brain tissues at 12 h; C, G: The expression levels of IκB, p-P65 protein and analytic statistics of gray levels in brain tissues at 24 h; D, H: The expression levels of IκB, p-P65 protein and analytic statistics of gray levels in brain tissues at 48 h. *P<0.05, compared with sham group; #P<0.05, compared with TBI group.

48 h after TBI. And all the levels of IL-6, TNF-α and IL-17 in peripheral blood detected by ELISA peaked at 48 h after TBI (P<0.01), which was consistent with TBI group (**Figure 2**).

RT-PCR detection results of IL-6, TNF-α and IL-17 mRNA in brain tissues

Compared with sham group, the expressions of IL-6, TNF-α and IL-17 mRNA in brain tissues in TBI group were obviously increased at 6 h, 12 h, 24 h and 48 h after TBI and peaked at 48 h after TBI (P<0.01). Compared with the TBI group, the above levels in S+T group were remarkably decreased at 6 h, 12 h, 24 h and 48 h after TBI. And all the expressions of IL-6, TNF-α and IL-17 mRNA in brain tissues peaked

at 48 h after TBI (P<0.01), which was consistent with TBI group (**Figure 3**).

Detection results of IκB and p-P65 protein in brain tissues

Compared with sham group, IκB in brain tissues in TBI group were degraded gradually at 6 h, 12 h, 24 h and 48 h after TBI and the content of degradation peaked at 48 h after TBI. The levels of p-P65 phosphorylation were gradually increased at 6 h, 12 h, 24 h, 48 h after TBI and peaked at 48 h after TBI (P<0.01). In S+T group, the degradation of IκB and increase of p-P65 phosphorylation levels at 6 h, 12 h, 24 h, 48 h after TBI were less obvious than TBI group. But the degradation amount of IκB and p-P65 phos-

phorylation levels peaked at 48 h after TBI (P<0.01), which were consistent with TBI group (**Figure 4**).

Discussion

TBI, as a quite common clinical disease, often accumulates to central nervous system and has high morbidity and mortality [11, 12]. With the development of diagnosis and treatment technology of neurosurgery, patients with brain injury can be considered almost recovered. However, the treatment of TBI hasn't reached the ideal therapeutic effect yet [13]. It has been reported that TBI can cause substantial inflammatory factors released by parts of brain tissues and the whole body, production and release of free radical, the occurrence of lipid peroxidation and so on [14, 15]. Therefore, the inflammatory response is an important part of pathophysiology in TBI. And the inflammatory response can cause serious secondary brain insults (SBI), such as cerebral edema, turbulence of brain blood flow, abnormal metabolism of brain and the death of neuron, etc.

In this study, the TBI models were made by using the brain hydraulic impact method; and the inflammatory factors (IL-6, TNF-α and IL-17) in peripheral blood, the mRNA expressions of above inflammatory factors in brain tissues, IκB degradation amount and p-P65 phosphorylation expression amount in rats after injury were analyzed. Moreover, IL-17 inhibitor was also applied to intervene and then to confirm whether the signaling pathway of NF-κB cells was involved in the process of the occurrence and development of TBI which was mediated by IL-17. The results showed that the inflammatory factors (IL-6, TNF-α and IL-17) in peripheral blood and parts of brain tissues were released progressively after TBI while these inflammatory factors would decreased significantly if the IL-17 inhibitor was given in advance. Meanwhile, it was also found that the degradation of nuclear factor NF-κB was obvious and the levels of phosphorylation were increased distinctly while they would decreased significantly after the intervention of IL-17 inhibitor. All the results above indicated that inflammatory response was stronger after TBI, and IL-17 was involved in the process of inflammatory response and played a role in activating the NF-κB signaling pathway.

IL-17 is a kind of cytokine secreted by Th17 cells to promote the inflammatory response [16]. Up to now, 6 family members of IL-17 have been discovered: IL-17A (IL-17), IL-17B, IL-17C, IL-17D, IL17-E (also known as IL-25) and IL-17F. Among them, IL-17 is the prototype of IL-17 family [6]. It can strengthen the inflammatory response through promoting the release of inflammatory factors. Th17 cells can not only secrete IL-17A and IL-17F but also IL-6, TNF-α and so on. These cytokines can mobilize collectively, recruit and activate neutropenia and mediate the process of neutropenia mobilization [17, 18]. After combining with IL-17R, IL-17 plays its biological role through the MAP kinase pathway and nuclear transcription factor κB (NF-κB) pathway, so as to effectively mediate the inflammatory response of tissues [19, 20]. This study showed that the expression of NF-κB (p-P65) increased significantly in the occurrence of TBI and decreased significantly after the treatment of IL-17 inhibitors.

In this study, after using the IL-17 inhibitor (Secukinumab) for rats in advance, the inflammatory factors and the involved NF-κB signaling in peripheral blood and brain tissues were significantly improved when compared with the pure TBI. Secukinumab is a kind of inhibitor of IL-17 which can selectively combine with IL-17A to neutralize IL-17A's role in promoting inflammation [9]. At present, it's used to treat the autoimmunity inflammatory diseases in the clinical stage, including psoriasis, rheumatoid arthritis, ankylosing spondylitis, etc., and has got a good therapeutic effect [9]. It also has been reported that the expressions of IL-17 and its related cytokines are out of balance after acute spinal cord injury in rats [21], and the use of IL-17 inhibitor (Secukinumab) has got a good effect in the treatment of secondary spinal cord injury after the spinal cord injury in rats [22]. This also provides us with a new idea for the application of Secukinumab in the treatment of acute brain injury.

The inflammatory response accompanied by TBI involves a variety of inflammatory signaling pathways. This study paid more attention to the inflammatory response mediated by the NF-κB (p65) pathway. NF-κB is a multifunctional nuclear transcription factor protein family, which plays a central role in mediating the transcriptional control of cell signal. After being

activated, NF-κB can mediate the gene transcription of cytokines that related to the inflammation. The subunits of NF-κB can form dimer to combine with the specific sequences on the target gene and then modulate the gene transcription. Among them, the most common one is the heterodimer formed by P50 and P65. When stimulated by external stimuli, I-κB kinase (IKK) can be activated, which further results in the phosphorylation and polyubiquitylation of I-κB and finally the degradation of I-κB protein. The released NF-κB can be further activated, quickly transferred to the nucleus, and then bound with the target gene in the nucleus, thereby promoting the transcription of the target gene. The experimental results in this study showed that the activated NF-κB (p-P65) were increased constantly in TBI group and peaked at 48 h. However, the activation of NF-κB was significantly reduced after using IL-17 inhibitor in advance.

In conclusion, the application of IL-17 inhibitor in advance can attenuate the inflammatory response in parts of brain tissues and the whole body in TBI as well as decrease the NF-κB activation, thereby interdicting the occurrence of secondary brain injury and providing a new target for the clinical treatment of TBI.

It's quite complicated that NF-κB signaling pathway is involved in the functional mechanism of TBI mediated by IL-17. In this study, we only investigated the pathway activation and the expression changes of inflammatory factors after TBI. Therefore, further experiments are still needed to verify the specific mechanism, and find the specific signaling molecules on the pathway, so as to provide a new target for clinical treatment.

At the same time, the establishment of TBI models made by using the brain hydraulic impact method might fail. And the number of rats in experimental group and control group might be deficient at each time point. So, the phenomena and mechanism of this experiment need to be further verified with a larger sample of rats.

Disclosure of conflict of interest

None.

Address correspondence to: Benjun Bi, Department of Orthopedic Surgery, Yantaishan Hospital, No. 91 Jiefang Road, Yantai 246000, Shandong, China. Tel: +86-0535-6602001; E-mail: benjunbi246@126.com

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