Int J Clin Exp Med 2016;9(4):7431-7437
www.ijcem.com /ISSN:1940-5901/IJCEM0016426

Original Article

Protective effects of tamoxifen on traumatic brain injury in rats

Xiaodong Qing[1], Peilong Jiang[1], Li Tang[2]

[1]Department of Neurosurgery, Ningbo Yinzhou Second Hospital, Ningbo, P. R. China; [2]Department of Renal Medicine, Ningbo Yinzhou Second Hospital, Ningbo, P. R. China

Received September 18, 2015; Accepted March 29, 2016; Epub April 15, 2016; Published April 30, 2016

Abstract: Objective: To investigate the neuroprotective effects of tamoxifen on traumatic brain injury in rats and potential mechanisms. Methods: The modified Feeney freefall method was used to establish a rat model of traumatic brain injury. Male Sprague-Dawley (SD) rats were randomly dividedinto four groups: sham surgery group, traumatic brain injury group, traumatic brain injury + solvent treatment group, and traumatic brain injury + tamoxifen treatment group. The experiment included two parts. During the first part, drugs were provided via intraperitoneal injection at 1 and 8 hours after traumatic brain injury, a histopathological examination of the brain tissue was performed at 24 hours after traumatic brain injury in rats, and the relevant mechanisms were explored. During the second part of the experiment, tamoxifen was provided at 1, 8, 24 and 72 hours after injury, and neurological function was assessed after the rats survived for seven days. Results: Tamoxifen treatment significantly inhibited nuclear transfer of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 in the brain tissue surrounding the traumatic brain injury site ($P < 0.05$) and significantly reduced the expression levels of tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1) β and cleaved-caspas-3 ($P < 0.05$). In addition, tamoxifen significantly reduced neuronal apoptosis after traumatic brain injury, the extent of neuronal damage, and neurological defects ($P < 0.05$). Conclusion: Tamoxifen exerts a neuroprotective effect on traumatic brain injury, which may be related to inhibiting the NF-kB-mediated inflammatory response.

Keywords: Brain injury, tamoxifen, inflammatory response

Introduction

Traumatic brain injury has high prevalence, morbidity and mortality worldwide [1]. With the rapid development of the economy and society in China, the prevalence of traumatic brain injury has risen over the years, which has heavily burdened society and patients' families. At present, no drugs effectively treat traumatic brain injury [2]. Recent studies showed that tamoxifen features good neuroprotective effects [3-6], but few studies have investigated its protective effects in traumatic brain injury and potential mechanisms. In this study, we investigated the neuroprotective effects of tamoxifen in traumatic brain injury and the potential mechanisms to provide new ideas and methods for clinically treating patients with traumatic brain injury.

Materials and methods

Experimental animals and groups

For experiment 1, 45 adult healthy male Sprague-Dawley (SD) rats were randomly assigned to one of four groups: the sham surgery group, traumatic brain injury group, traumatic brain injury + solvent treatment group, and traumatic brain injury + tamoxifen treatment group. Five rats died following traumatic brain injury, and additional rats were randomly assigned to replenish the number of rats. None of the rats in the sham surgery group died. The Feeney method was modified to establish a rat model of traumatic brain injury as follows: under anesthesia with chloralhydrate (4 mg/kg, intraperitoneal injection), the rats were positioned on the stereotactic frame. After shaving and

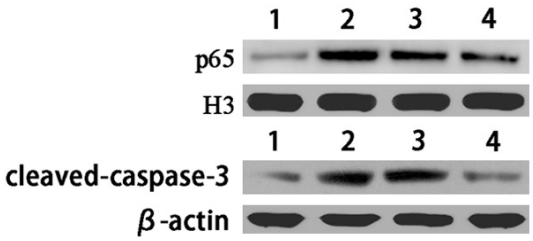

Figure 1. Detection of the p65 and cleaved-caspase-3 protein expression levels in each group (representative images, 1. The sham surgery group; 2. The traumatic brain injury group; 3. The traumatic brain injury + solvent treatment group; and 4. The traumatic brain injury + tamoxifen treatment group).

ethanol disinfection, a 2 cm-long incision was made along the scalp midline. Next, an orthopedic drill was used to make a bone opening 5 mm in diameter at 3 mm right of the coronal suture midline and 3 mm posterior to the sagittal suture. The dura remained intact. A 40 g weight was dropped from 15 cm above and allowed to freefall vertically to strike the right dura pad, which caused an injury 3 mm deep and 4 mm in diameter. The rats in the sham surgery group were only subject to bone opening. A tamoxifen treatment regimen and dose were provided in reference to previous literature [7]. Intraperitoneal injection of tamoxifen was provided 1 and 8 hours after successful establishment of the rat model of traumatic brain injury. Rats in the solvent group only received an intraperitoneal injection of solvent. All rats were sacrificed under anesthesia 24 hours after traumatic injury, and their necks were broken to obtain brain tissue. For experiment 2, 87 rats were randomly assigned to the sham surgery group, the traumatic brain injury group, the traumatic brain injury + solvent treatment group, and the traumatic brain injury + tamoxifen treatment group. All the rats received an intraperitoneal injection of tamoxifen 1, 8, 24 and 72 hours after traumatic brain injury, and their neurological function was assessed 1, 3, 5 and 7 days after traumatic brain injury. Seven rats died following traumatic brain injury, and additional rats were randomly assigned to replenish the number of rats. No rats died in the sham surgery group.

Neurological function assessment in rats after traumatic brain injury

After traumatic brain injury, the neurological function of the rats was assessed using the neurological severity scores (NSS) [8], which mainly included a motor function test, a sensory function test, a balance test, physiological reflex defects, and abnormal movements. The highest score was 18 points. The rats were given 1 point if they could not perform the task or lacked the appropriate response. Moreover, 13-18 points indicated a severe injury, 7-12 points indicated a moderate injury, and 1-6 points indicated a mild injury.

Western blot analysis

Twenty-four hours after traumatic brain injury, the rats were anesthetized with an intraperitoneal injection of chloralhydrate; the chest was then opened, and 100 ml of saline was injected into the left apex. When the liver turned white, the neck was quickly broken, and the brain was removed; the brain tissue surrounding the injury site was then maintained in a freezer at -80°C for later use. The nuclear and cytoplasmic proteins were extracted in strict accordance with the extract kit instructions (Biyuntian Biotech Co., Ltd.), and the Bradford assay was used to measure the protein extract concentration. Next, the protein was mixed with 5 × loading buffer at a 1:4 ratio and boiled in water for 10 minutes; 35 micrograms of the denatured protein was then added to each well for electrophoresis and transferred to a membrane. Next, the membrane was blocked for 1 hour; diluted primary anti-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65, anti-H3, anti-cleaved-caspase-3, and anti-β-actin antibodies (1:1,000, purchased from Cell Signaling Technology, Inc., USA) were then added, and the membrane was placed on a shaker at 4°C overnight. Next, the secondary antibody was added, and the sample was incubated followed by washing the membrane, adding enhanced chemiluminescence (ECL) agents, and gray-scale analysis with the Image J software.

Immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Twenty-four hours after the injury, 10 rats in each group were perfused with paraformaldehyde; their necks were broken, and their brains were removed, embedded in paraffin and prepared as 4 μm sections. For immunohistochemistry, the brain tissue sections were deparaffinized and dehydrated, and antigens were

Table 1. Analysis of the assay results for each group in this study

Group	N	P65	Cleavage caspase-3	TNF-α (pg/mg)	IL-1β (pg/mg)	The number of TUNEL-positive cells	Nissl staining (cells)	The number of cleaved-caspase-3-positive cells
Sham surgery group	10	0.12 ± 0.01	0.09 ± 0.01	42.1 ± 6.3	17.6 ± 3.6	2.3 ± 0.1	87.3 ± 9.6	9.2 ± 0.9
Traumatic brain injury group	10	0.67 ± 0.12*	0.82 ± 0.19*	98.6 ± 10.2*	39.8 ± 7.1*	42.3 ± 8.9*	41.6 ± 7.8*	79.6 ± 6.9*
Traumatic brain injury + solvent treatment group	10	0.62 ± 0.09#	0.85 ± 0.21#	98.1 ± 10.9#	40.1 ± 6.8#	40.5 ± 8.3#	42.9 ± 7.5#	76.1 ± 6.4#
Traumatic brain injury + tamoxifen treatment group	10	0.28 ± 0.06#	0.17 ± 0.07#	62.5 ± 7.8#	23.5 ± 3.8#	22.3 ± 5.1#	69.8 ± 8.2#	38.9 ± 4.6#

*$P < 0.05$, compared with the sham surgery group, #$P < 0.05$, compared with the solvent treatment group.

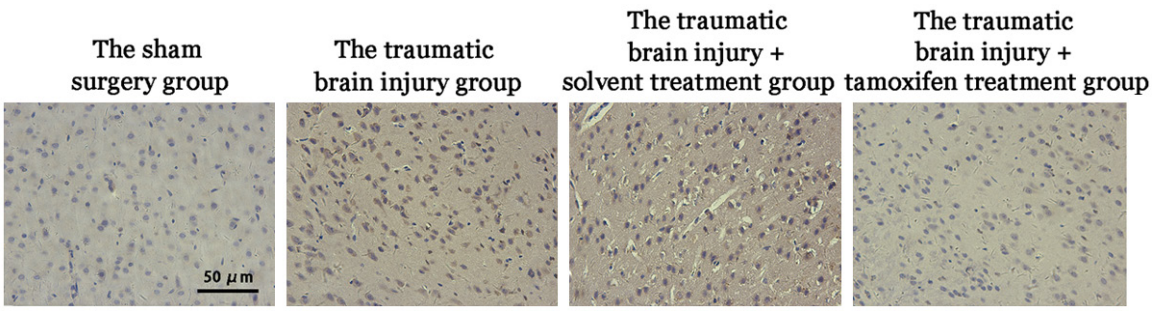

Figure 2. The effects of tamoxifen treatment on cleaved-caspase-3 expression after injury.

repaired in citrate buffer. Next, the slides were blocked with normal goat serum, and the anti-cleaved-caspase-3 antibody (1:50) was added. The slides were placed into a wet pot and incubated for 24 h at 4°C overnight followed by a PBS wash 3 times 5 minutes each. Next, horseradish peroxidase-labeled goat anti-rabbit IgG and horseradish peroxidase-labeled streptavidin-biotin were added; 3,3'-diaminobenzidine was used as the luminescence agent. The slides were mounted and observed under a light microscope; images were collected. Ten fields were counted on each slide, and the mean value was used as the final result for the slide. Four slides were prepared for each rat. A TUNEL staining kit was purchased from Roche (USA), and the assay was performed in strict accordance with the instructions.

Enzyme-linked immunosorbent assay of inflammatory cytokine expression

Tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) β enzyme-linked immunosorbent assay kits were purchased from UNOCI Biotechnology Co., Ltd. For each rat, 50 mg of brain tissue surrounding the injury site was collected, added into 500 ml of PBS, placed into a tissue homogenizer for thorough homogenization, and then stored in a refrigerator at 4°C for later use. During the experiment, the sample was centrifuged at 14,000 rpm for 10 minutes, the supernatant was collected, and the protein concentration was measured using the Bradford assay in strict accordance with the instructions.

Statistical analysis

SPSS 15.0 software was used to process the data, which were expressed as the mean ± standard deviation (x ± S). The neurological function assessment scores were analyzed using a Kruskal-Wallis test, and one-way analysis of variance was performed for the remaining multi-group comparisons.

Results

Effects of tamoxifen on the NF-κB signaling pathway

Compared with the sham surgery group (**Figure 1**; **Table 1**), the NF-κB p65 protein expression level was significantly higher in the brain tissue nuclei surrounding the injury site in the traumatic brain injury group ($P < 0.05$); however, tamoxifen significantly inhibited p65 protein expression in the nucleus ($P < 0.05$).

Effects of tamoxifen on nerve cell apoptosis after traumatic brain injury

The Western blot analyses (**Figure 1**) showed that the expression level of cleaved-caspase-3, which is an apoptosis execution protein, was significantly higher in brain tissue surrounding the injury site after traumatic brain injury; however, tamoxifen significantly reduced the cleaved-caspas-3 protein expression level ($P < 0.05$). Immunohistochemical results show that compared with the sham surgery group, traumatic brain injury in rats yielded significantly more cleaved-caspase-3 positive cells in brain tissue surrounding the injury site. However, tamoxifen significantly reduced the number of cleaved-caspase-3 positive cells (**Figure 2**; **Table 1**, $P < 0.05$), which is consistent with the Western blot analysis. TUNEL-positive cells showed positive results in the nucleus. This study (**Figure 3**; **Table 1**) showed significantly more TUNEL-positive cells in brain tissue surrounding the injury site after traumatic brain injury ($P < 0.05$); however, tamoxifen significantly reduced the number of apoptotic neurons ($P < 0.05$).

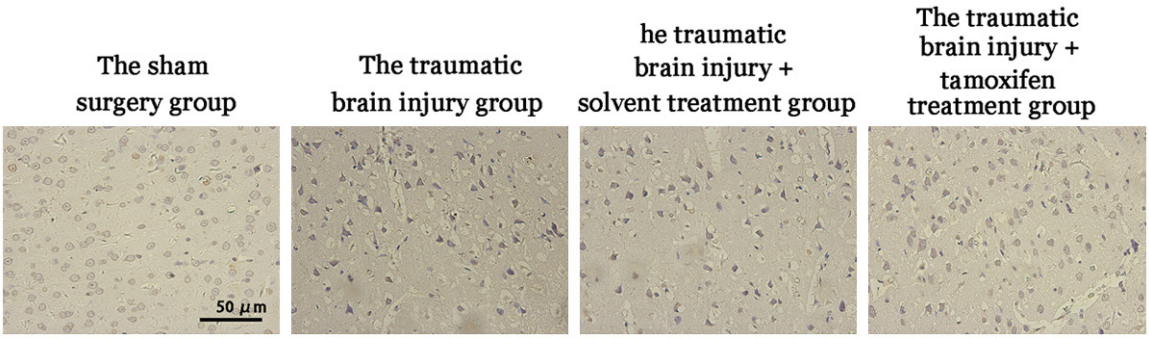

Figure 3. The effects of tamoxifen treatment on apoptosis after injury.

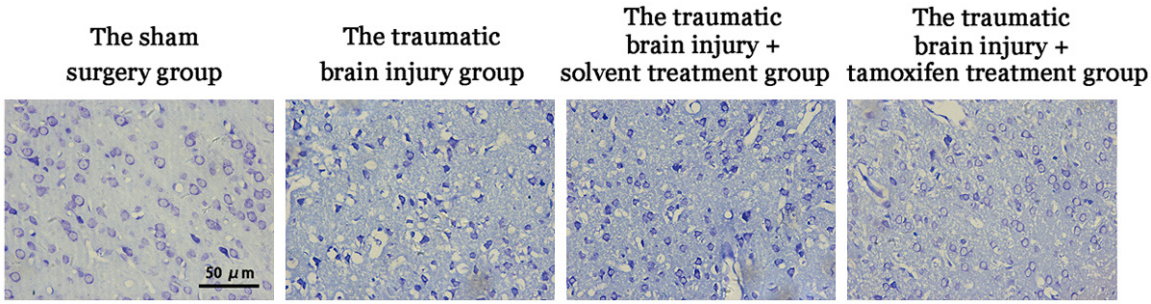

Figure 4. Nissl staining results.

Table 2. The effects of tamoxifen on the neurological function assessment score after traumatic brain injury in rats

Group	N	1 d	3 d	5 d	7 d
Sham surgery group	20	1.21 ± 0.01	1.1 ± 0.01	0.9 ± 0.02	0.6 ± 0.05
Traumatic brain injury group	20	16.1 ± 3.36*	15.39 ± 2.12*	14.64 ± 1.96*	13.51 ± 2.01*
Traumatic brain injury + solvent treatment group	20	16.2 ± 2.13*	15.49 ± 1.96*	14.75 ± 1.92*	13.69 ± 2.19*
Traumatic brain injury + tamoxifen treatment group	20	13.8 ± 2.05#	12.3 ± 1.53#	10.5 ± 1.49#	8.2 ± 1.97#

*P < 0.05, compared with the sham surgery group, #P < 0.05, compared with the traumatic brain injury group.

Effects of tamoxifen on inflammatory cytokine expression after traumatic brain injury

Compared with the control group (**Table 1**), the expression levels of inflammatory cytokines TNF-α and IL-1β significantly increased in brain tissue surrounding the injury site after traumatic brain injury ($P < 0.05$); furthermore, compared with the solvent treatment group, tamoxifen significantly reduced the TNF-α and IL-1β expression levels ($P < 0.05$).

Nissl staining results

Nissl staining showed that normal neurons were round with no staining, but the damaged neurons exhibited an irregular shape or shrank with staining. Compared with the sham surgery group (**Figure 4**; **Table 1**), the traumatic brain injury and solvent treatment groups ($P < 0.05$) exhibited fewer undamaged neurons; however, tamoxifen significantly increased the number of undamaged neurons ($P < 0.05$).

Effects of tamoxifen treatment on the neurological function assessment score after traumatic brain injury in rats

Compared with the sham surgery group, the neurological defect score was significantly higher 1, 3, 5 and 7 days after traumatic brain injury in the traumatic brain injury and solvent treat-

ment groups (**Table 2**; $P < 0.05$); however, tamoxifen treatment significantly reduced the neurological defect score (**Table 2**; $P < 0.05$).

Discussion

Traumatic brain injury can be categorized as primary or secondary; primary brain injury refers to brain damage directly caused by an external force, which is beyond the scope of clinical interventions, and secondary brain injury occurs a few hours to a few days after injury, which is the focus of traumatic brain injury clinical treatments [9]. Previous studies have shown that a variety of pathological factors, such as oxidative stress, the inflammatory response and apoptosis, are involved in secondary brain injury after traumatic brain injury. Furthermore, early interventions to reduce the level of oxidative stress and the extent of the inflammatory response can significantly reduce the extent of traumatic brain injury [10]. Tamoxifen is a synthetic partial agonist of the estrogen receptor with certain estrogen-like effects and is now widely used to clinically treat breast cancer. Tamoxifen is convenient to administer and is quickly absorbed into the body after oral administration. Its serum concentration peaks as soon as 4-7 hours after oral administration and its elimination half-life is approximately 7 days [11]. Recent studies have shown that tamoxifen exerts good neuroprotective effects, which are related to its anti-inflammatory and antioxidant role [5, 12]. This study showed that tamoxifen significantly reduced neuronal apoptosis and the number of damaged neurons after traumatic brain injury. In addition, tamoxifen significantly improved neurological defects after traumatic brain injury, which suggests that tamoxifen exerts good neuroprotective effects after traumatic brain injury.

Tamoxifen not only reduces lipopolysaccharide-induced activation of microglias, thus exerting an anti-inflammatory effect on in vitro experiments, but also reduces the extent of the inflammatory response and pathological damage to brain tissue. Thus, it exerts significant neuroprotective effects in animal models of spinal cord injury and subarachnoid hemorrhage [5, 6, 13]. After the data confirmed that tamoxifen exerts good neuroprotective effects after traumatic brain injury, we further explored the potential mechanisms with a focus on the effects of tamoxifen on NF-κB-mediated inflammatory responses. Previous studies have shown that NF-κB plays an important role in secondary brain injury after traumatic brain injury; NF-κB activity was significantly increased in brain tissue surrounding the injury site after traumatic brain injury, and inhibiting NF-κB activity significantly reduced the extent of brain injury [14]. Once transferred into the nucleus, NF-κB p65 binds a specific target gene sequence to regulate downstream inflammatory cytokine expression (TNF-α and IL-1β). The TNF-α and IL-1β expression levels significantly increased in brain tissue, cerebrospinal fluid and blood after traumatic brain injury, which are closely related to a patient's prognosis. Inhibiting TNF-α and IL-1β expression significantly reduced the extent of pathological damage after traumatic brain injury [15]. Caspase-3 is a critical apoptosis execution protein after traumatic brain injury and is expressed in animal models of traumatic brain injury and human brain tissue. Inhibiting cleaved-caspase-3 (mature caspase-3 protein) expression significantly reduced nerve injury [16]. This study showed that the NF-κB p65, cleaved-caspas-3, TNF-α and IL-1β expression levels significantly increased in brain tissue surrounding the injury site. However, tamoxifen significantly reduced the nuclear transfer of NF-κB p65 and the expression levels of TNF-α and IL-1β in brain tissue surrounding the injury site, which is consistent with previous studies and suggests that inhibition of NF-κB-mediated inflammatory signaling pathways may be one mechanism underlying the neuroprotective effects of tamoxifen.

In summary, tamoxifen plays a significant neuroprotective role in the animal model of traumatic brain injury, which may be related to inhibition of NF-κB-mediated inflammatory pathways. Based on its safety profile in human and the results of this study, tamoxifen may be used to treat patients with traumatic brain injury in clinical practice. Future studies should include a more detailed and comprehensive investigation of the mechanisms underlying tamoxifen's neuroprotective effects to lay a solid theoretical foundation for its clinical application.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Li Tang, Department of Renal Medicine, Ningbo Yinzhou Second Hospital,

1 Qianhe Road, Yinzhou District, Ningbo 315100, Zhejiang, P. R. China. Tel: +86 574-55662011; 137-32119148; Fax: +86 574-55662011; 1373211-9148; E-mail: tangli508@126.com

References

[1] Chantsoulis M, Mirski A, Rasmus A, Kropotov JD, Pachalska M. Neuropsychological rehabilitation for traumatic brain injury patients. Ann Agric Environ Med 2015; 22: 368-79.

[2] Bergold PJ. Treatment of traumatic brain injury with anti-inflammatory drugs. Exp Neurol 2016; 275 Pt 3: 367-80.

[3] Wei HY, Ma X. Tamoxifen reduces infiltration of inflammatory cells, apoptosis and inhibits IKK/NF-kB pathway after spinal cord injury in rats. Neurol Sci 2014; 35: 1763-8.

[4] Franco Rodríguez NE, Dueñas Jiménez JM, De la Torre Valdovinos B, López Ruiz JR, Hernández Hernández L, Dueñas Jiménez SH. Tamoxifen favoured the rat sensorial cortex regeneration after a penetrating brain injury. Brain Res Bull 2013; 98: 64-75.

[5] Salgado IK, Torrado AI, Santiago JM, Miranda JD. Tamoxifen and Src kinase inhibitors as neuroprotective/neuroregenerative drugs after spinal cord injury. Neural Regen Res 2015; 10: 385-90.

[6] Sun X, Ji C, Hu T, Wang Z, Chen G. Tamoxifen as an effective neuroprotectant against early brain injury and learning deficits induced by subarachnoid hemorrhage: possible involvement of inflammatory signaling. J Neuroinflammation 2013; 10: 157.

[7] Wang GH, Zhang XG, Jiang ZL, Li X, Peng LL, Li YC, Wang Y. Neuroprotective effects of hyperbaric oxygen treatment on traumatic brain injury in the rat. J Neurotrauma 2010; 27: 1733-43.

[8] Si D, Li J, Liu J, Wang X, Wei Z, Tian Q, Wang H, Liu G. Progesterone protects blood-brain barrier function and improves neurological outcome following traumatic brain injury in rats. Exp Ther Med 2014; 8: 1010-1014.

[9] Paterniti I, Cordaro M, Navarra M, Esposito E, Cuzzocrea S. Emerging pharmacotherapy for treatment of traumatic brain injury: targeting hypopituitarism and inflammation. Expert Opin Emerg Drugs 2015; 20: 583-96.

[10] Gyoneva S, Ransohoff RM. Inflammatory reaction after traumatic brain injury: therapeutic potential of targeting cell-cell communication by chemokines. Trends Pharmacol Sci 2015; 36: 471-80.

[11] Morad SA, Cabot MC. Tamoxifen regulation of sphingolipid metabolism-Therapeutic implications. Biochim Biophys Acta 2015; 1851: 1134-1145.

[12] Boulos AS, Deshaies EM, Dalfino JC, Feustel PJ, Popp AJ, Drazin D. Tamoxifen as an effective neuroprotectant in an endovascular canine model of stroke. J Neurosurg 2011; 114: 1117-26.

[13] Sun XB and Ma C. Neuroprotective effect of tamoxifen on early brain injury in rat model with subarachnoid hemorrhage. Jiangsu Medical Journal 2013; 39: 1888-1890.

[14] Yang L, Tao LY, Chen XP. Roles of NF-kappaB in central nervous system damage and repair. Neurosci Bull 2007; 23: 307-13.

[15] Corps KN, Roth TL, McGavern DB. Inflammation and neuroprotection in traumatic brain injury. JAMA Neurol 2015; 72: 355-62.

[16] Keane RW, Kraydieh S, Lotocki G, Alonso OF, Aldana P, Dietrich WD. Apoptotic and antiapoptotic mechanisms after traumatic brain injury. J Cereb Blood Flow Metab 2001; 21: 1189-98.