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Original Article

TRAF2 gene-silencing inhibits NF-kappa B pathway activation and promotes angiogenesis by endothelial progenitor cells from mice with craniocerebral injury

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Abstract: Objective: To investigate the effects of TRAF2 gene-silencing mediatinig NF-kappa B pathway on the function of endothelial progenitor cells and angiogenesis of craniocerebral injury mice. Methods: A total of 35 male Kunming mice were randomly assigned to the Model group (n=20, craniocerebral injury group) and to the Sham group (n=15, Sham operation group). HE staining was used to observe cerebral histopathological changes and immunohistochemistry was used to detect TRAF2 protein expression changes. The endothelial cells in the brain tissues of the Model group were divided into 5 groups, including the Blank group, NC group, TRAF2-siRNA group, TNF-α group and TNF-α+TRAF2-siRNA group. The qRT-PCR and western blot (WB) were performed to check the mRNA and protein expression of TRAF2, NF-B, VEGF, Bcl-2, Bax gene in cells. MTT assay was used to detect cell proliferation and cell migration distance was detected by scratch test. Angiogenesis was detected by angiogenesis kits. Results: The HE staining result showed the brain tissues of the Model group were flaky and necrotic, while their structures were relatively complete in the Sham group. The immunohistochemistry showed that the positive expression of TRAF2 protein in the Model group was significantly higher than that in the Sham group. Compared with the Sham group, the mRNA and protein expression of TRAF2, NF-kB and Bax in cells increased significantly ($P<0.05$) in the Blank group, NC group, TRAF2-siRNA group, TNF-α group and TNF-α+TRAF2-siRNA group, while the mRNA and protein expression of VEGF and Bcl-2 in those groups decreased significantly (all $P<0.05$). And there was no significant difference ($P>0.05$) between the Blank and NC group. Compared with the Blank group, there was no significant difference in the expression of the TRAF2 in the TNF-α group. The mRNA and protein expression of NF-Kb and Bax increased significantly ($P<0.05$). The trends of the expression of mRNA and protein of VEGF and BCl-2 in the TNF-α group were opposite to that in the TRAF2-siRNA group. The mRNA and protein expression of TRAF2, NF-Kb, Bax decreased significantly in the TRAF2-siRNA group, compared with the Blank group. Compared with Blank group, the mRNA and protein expression of TRAF2 in the TNF-α+TRAF2-siRNA group decreased significantly ($P<0.05$) and there was no significant difference in the mRNA and protein expression of NF-kB, VEGF, Bcl-2 and Bax between the two groups ($P>0.05$). Conclusion: TRAF2 gene silencing could inhibit the activation of NF-kB pathway and promote proliferation, migration and angiogenesis of endothelial progenitor cells which might have a protective effect on mice with craniocerebral injury.

Keywords: TRAF2, NF-kB pathway, ischemic stroke, endothelial progenitor cells, angiogenesis

Introduction

Craniocerebral injury is one of the most important causes of disability and death in clinical practice, especially the severe craniocerebral injury. Therefore, how to effectively promote the repair of neurons and improve neurological function has always been a hot topic [1]. Angiogenesis can improve neurovascular microenvironment of patients with craniocerebral injury. It can also accelerate neuronal regeneration and recovery, so it has always been a research focus [2]. Endothelial cells (EPCs) can differentiate into mature endothelial cells and help repair of inflammatory lesions and promotion of angiogenesis and have been proved to be of great importance in improving the function of nerve recovery [3, 4].

Tumor necrosis factor receptor-associated factor (TRAF) is an important cytoplasmic adaptor protein in human body, which played an impor-

tant role in mediation of biological characteristic of tumor cells [5, 6]. TRAF also regulates human cell immune function and is expressed in both normal and sick human tissues, indicating that it can regulate pathology and normal physiology activities [7, 8]. Some craniocerebral injury-related studies found that inhibiting the TRAF2 expression can significantly improve acute ischemic craniocerebral injury, which suggested TRAF2 can be regarded as a target for the treatment of ischemic craniocerebral injury [9, 10]. Nuclear factor-kappa B (NF-kB) protein is a pleiotropic transcription factor in the human body and its activation can regulate transcription, adhesion yin deficiency and inflammatory factor [11]. Patients with ischemic craniocerebral injury are accompanied with activation of NF-kB that can serve as an important target for craniocerebral injury treatment [12, 13]. However, the specific mechanism between craniocerebral injury and NF-kB signal pathway is not yet clear.

In this study, we investigated the effects that TRAF2 gene silencing mediates NF-kB pathway on the endothelial cell function and angiogenesis in craniocerebral injury mice from the perspective of molecular mechanisms of genes and pathways.

Materials and methods

Animals

Thirty-five male Kunming mice (Shanghai southern model biological research center) were selected, aged from 8 to 12 weeks with body weight of 20-25 g. They were purchased one week before the experiment and housed under standard laboratory conditions with a 12 h day/night circle and food and water ad libitum. This study was approved by the Animal Welfare Ethic Committee of Renmin Hospital of Wuhan University.

Mouse craniocerebral injury model

After weighted, 20 mice were randomly selected as the Model group and the remaining 15 mice were regarded as the Sham group. The mice in the Model group were injected with the 0.6% pentobarbital sodium (P3761, Sigma, Santa Clara, CA, USA) for anesthesia. After fixed on an operation table, the joint sagittal suture and the lambdoidal sutures of brian were cleaned, disinfected and then an incision was made. The scalp and periosteum were peeled off with a pair of medical forceps and a hole was drilled beside the middle of the herringbone on the right side of the brain and coronal sutures. With the help of the PinPoint™ Precision Cortical Impactor (PCI3000, Wuhan Yihong Technology Co., Ltd.), the brains of mice were struck and then the bone and scalp were sutured. For the mice in the Sham group, the bone windows were exposed after anaesthetia and no impact was performed. The mice were placed on a warming to recover to body temperature and subsequent experiments were performed after all the physical signs had returned to a stable state. The cerebral blood flow after middle cerebral artery occlusion in mice was measured by the laser Doppler cerebral hemodynamic analyzer (PF5001, Perimed, Sweden). According to the monitoring of blood flow blocked by the middle cerebral thrombus, whether the model was successful was judged. Once the model was established successfully, the front and rear limbs of the mice were difficult to fully extend and shake to the opposite side. Besides, the mice had unclear consciouses and were unable to act normally. In terms of histology, mice brain tissues were fused with proliferation of glial cells or necrotosis of neurons [14].

HE staining

In the Model group and the Sham group, 5 mice were randomly selected from each group respectively. After that, 50 mg of brain tissue was taken and placed in 4% paraformaldehyde. After 24 hours, conventional dehydration for 1 min and xylene transparent twice (5 min per time), the brain tissue immersed in wax was placed in a paraffin mold at a right position. The slice thickness was 5 μm. The sections were then dried at 60°C for 5 h and dewaxed, with help of hematoxylin (PT001, Shanghai Bogu Biotechnology Co., Ltd. Shanghai, China). After stained at normal temperature for 8 min, rinsed by double water for 60 s, differentiated in 2% alcohol for 30 s and flushed with water for 5 min, the brain tissues were stained with 1% eosin (M027, Shanghai Gefan Biotechnology Co., Ltd. Shanghai, China) in hydrochloric acid at room temperature for 1 min and then washed with water for 5 min. These tissues were dehydrated by gradient alcohol (70%, 80%, 90%,

100%) and the time for each gradient dehydration was 1 min. And then they were clarified twice (1 min per time). Brain tissue sections were mounted using neutral gum and the pathology changes were observed with a light microscope (DSX100, Olympus, Japan).

TRAF2 protein expression detected by immunohistochemistry

In the Model group and the Sham group, 5 mice of each group were randomly selected and brain tissues were taken. The tissues were embedded with 10% formalin in paraffin at a thickness of 5 μm and immunohistochemistry was operated using a SP-9001 kit (Beijing NuoBolaide Technology Co., Ltd., Beijing, China). The brain tissues were placed at room temperature for 20 min and fixed with acetone at 4°C for 12 min. After that, the brain tissues were dewaxed, washed with PBS 3 times (3 min per times) and washed with 5% H_2O_2 for 5 to 10 min to inhibit peroxidase activity. After rinsed for 2 min with PBS, they were placed in 0.01% citric acid buffer, boiled at 90°C for 30 min, cooled to room temperature, rinsed with PBS, and incubated at 37°C for 10 min. After that, rabbit anti-mouse antibody TRAF2 (1:200, ab15191, Abcam, Cambridge, MA, USA) was added, and after being incubated at 4°C overnight, the tissues were rinsed with PBS. The samples were incubated with goat anti-rabbit secondary antibody (DF7852, Shanghai Yao Yun Biotechnology Co., Ltd.) at room temperature for 30 min, and then after coloration with DAB (ab64238, Abcam, Cambridge, MA, USA), they were counterstained with hematoxylin and mounted. The positive cell membrane or cytoplasm was brownish yellow. Five high-power fields (200X) were randomly selected from each slice. A total of 100 cells were selected in each field to calculate the proportion of positive cells. The proportion of positive cells more than 10% was counted as positive (+) and the proportion of negative cells less than 10% counted as negative (-). The experiment was repeated 3 times.

EPCs harvest, culture and identification

In the Model group and the Sham group, 5 mice of each group were randomly selected respectively and 50 mg of brain tissues were taken. After cut to 1 mm^3 tissue blocks, 3 mL of digestive enzymes (containing 0.1% type I collagenase and 0.01% trypsin) were added and the tissues were digested in the 36°C water bath for 70 min. After that, 3 mL of digestive enzymes were added and digested in a 35°C water bath for 20 min and then 5 mL of EBM-2 endothelial cell culture fluid (CC-3156, Lonza, USA) containing 10% fetal calf serum (16000-044, Gibco, Carlsbad, CA, USA) was added. After centrifuged at 1200 r/min for 10 min, the supernatant of solution was discarded and the cells were suspended with added 3 mL EBM-2 culture fluid. The cells were incubated in the incubator at the 37°C, 5% CO_2 condition and the culture fluid was replaced every two days. When the cell fusion reached 80%, the culture solution was passaged. After the cell extracted on the 7th day, the following experiments were performed. The identification of EPCs was performed with the Di-AcLDL and BS-Lectin double staining [15].

Cell grouping and transfection

The endothelial progenitor cells from the Model group were divided into 5 groups: Blank group (transfected blank vector plasmid), NC group (transfected negative control vector plasmid), TRAF2-siRNA group (transfected TRAF2-siRNA plasmid), TNF-α (Tumor Necrosis Factor-α) (AF-315-01A, PeproTech, USA) group (TNF-α as the activator of NF-kB pathway), TNF-α+TRAF2-siRNA group (TNF-α as the activator of transfection of TRAF2-siRNA plasmid+NF-kB pathway). The endothelial progenitor cells in the Sham group were not processed. The cells were inoculated in 96-well plates and transfected at approximately 70% growth density with reference to the Lipofectamine 2000 instructions (11668-019, Invitrogen, New York, California, USA). The cells in the Blank group, NC group, TRAF2-siRNA group, TNF-α group and TNF-α+TRAF2-siRNA group were diluted to 100 pmol with 250 μL serum-free EBM-2 medium (51985042, Gibco, Gaithersburg, MD, USA), and then they were incubated at room temperature for 10 min. Lipofectamine 2000 solution (5 μL) was diluted with serum-free EBM-2 medium (250 μL) and mixed with above transfection respectively. After incubated at room temperature for 30 min, each group was transferred to the cell culture wells. After cultured for 12 h at 37°C and in a 5% CO_2 environment, the culture medium was replaced with complete medium and the cells continued to culture for 24 h and subsequent experiments were performed.

Table 1. Primer sequences

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
β-actin	GGGCACAGTGTGTGTGAC	CTGGCACCACACCTTCTAC
TRAF2	ACACACTATATCACTGGCACC	TTCAGGGAGAAGCGTGTGC
NF-kB	CCTCTGGCGAATGGCTTTAC	GCTATGGATACTGCGGTCTGG
Bcl-2	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTACTCAGTCATCC
Bax	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
VEGF	GGAGATCCTTCTAGGAGCACTT	GGCGATTTAGCAGCAGATATAAGAA

The detection of gene expression level using qRT-PCR

Qiagen miRNeasy MiNi Kit was used to extract total RNA in cells. The primers of TRAF2, NF-kB, VEGF, Bcl-2, Bax, and β-actin were synthesized by Takara (**Table 1**). The RNA was reversely transcribed into cDNA using the PrimeScript RT Kit (RR036A, Takara, Japan) and the reverse transcription system was 10 μL. The reverse transcriptase reaction condition was 37°C and 15 min for 3 times and the condition of reverse transcriptase inactivation was 85°C for 5 s. The reaction solution was subjected to fluorescence quantitative PCR which was performed according to the instructions of SYBR® Premix Ex TaqTM II Kit (RR820A, TaKaRa). The 50 μL reaction system included 25 μL of SYBR® Premix Ex TaqTM II (2×), 2 μL of upstream PCR primer, 2 μL downstream PCR primer, 50 μL of ROX Reference Dye, 4 μL DNA template and 16 μL ddH_2O. ABI7500 quantitative PCR instrument (7500, ABI, USA) was used for real-time quantitative PCR detection. The reaction procedure was pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and cycle 40 times. β-actin was regarded as an internal reference to measure the relative expression levels of TRAF2, NF-kB, VEGF, Bcl-2, and Bax. The relative transcription levels of the target gene mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated 3 times.

Western blot analysis of the expression levels of related proteins in cells after transfection

The protein concentration of each group was measured using the BCA kits (20201ES76, Shanghai Biosen Biotech Co., Ltd.), and 30 μg protein was loaded with deionized water. Samples were mixed with buffer and boiled at 100°C for 5 min. After electrophoresis, the protein was transferred to the NC membrane using the BIO-RAD transfer instrument (Beijing Saibo Technology Co., Ltd.), and blocked with the 5% BSA at room temperature for 1 h. Diluted primary rabbit anti-mouse polyclonal antibody TRAF2 (1:600, ab230795), NF-kB (diluted to 0.5 μg/mL, ab19285), VEGF (diluted to 0.2 μg/mL, ab10972), bcl-2 (diluted to 4 μg/mL, ab27795), Bax (1:1000, ab32503) and β-actin (diluted to 1 μg/mL, ab5694) were added and incubated overnight at 4°C. The membrane was washed in TBST 3 times (5 min per time) and the HRP-labeled goat anti-rabbit IgG (1:1000, Abcam, USA) dilution solution was added. Then it was incubated at the room temperature for 1 h. Finally, the membrane was washed by TBST 3 times (5 min per time) and then the membrane was developed. The ImageJ 1.48u software (National Institutes of Health) was used to calculate the relative amount of the target protein (target protein relative expression amount = target protein band gray value/internal reference band gray value * 100%). Each experiment was repeated three times.

MTT assay for cell proliferation

After 48 h of cell transfection, cells from each group were collected for cell counting and inoculated in 96-well plates from $3*10^3$ to $6*10^3$ cells per well. The cells were incubated for 24 h, 48 h, and 72 h and then the subsequent experiments were performed. The 5 mg/mL MTT solution (Shanghai Boao Biotechnology Co., Ltd.) of 20 μL was added to each well and it continued to be incubated at 37°C for 2 h. After that the culture was terminated. The culture supernatant from the wells was discarded and 150 μL DMSO was added to each well. The OD value of each well was read at 570 nm using the enzyme-linked immunosorbent assay (NYW-96M, Beijing Nuoyawei Instrument Co., Ltd.). Survival rate equals to (OD mean of this group-mean OD of zero group)/(average of OD of untreated group-mean of OD of zero group) * 100%. The experiment was repeated 3 times [16].

The detection of cell migration in each group by scratch test

After 48 h of transfection in each group, they were vaccinated on a 96-well plate. After the

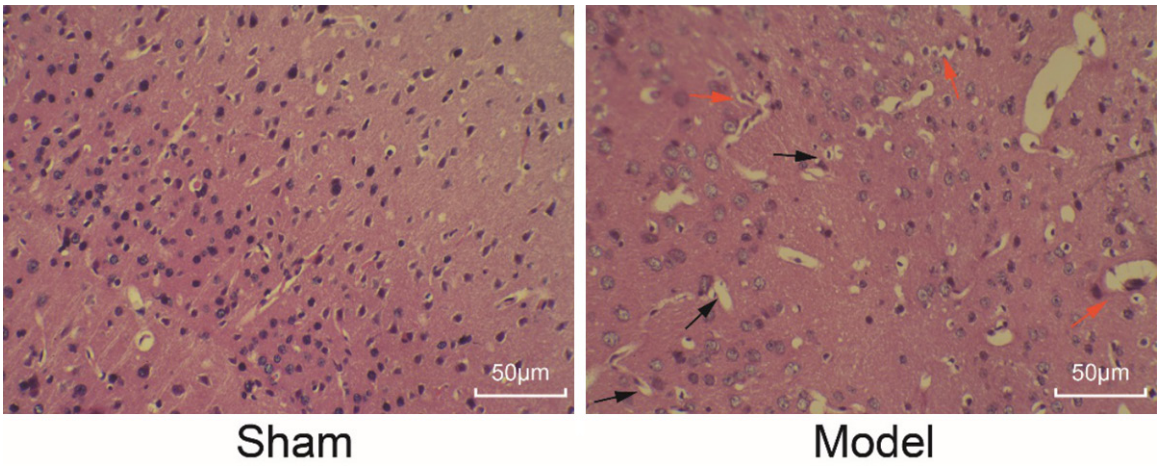

Figure 1. HE staining result of mice in the two groups. The red arrow represents the necrotic lesion. The black arrow represents the glial cell.

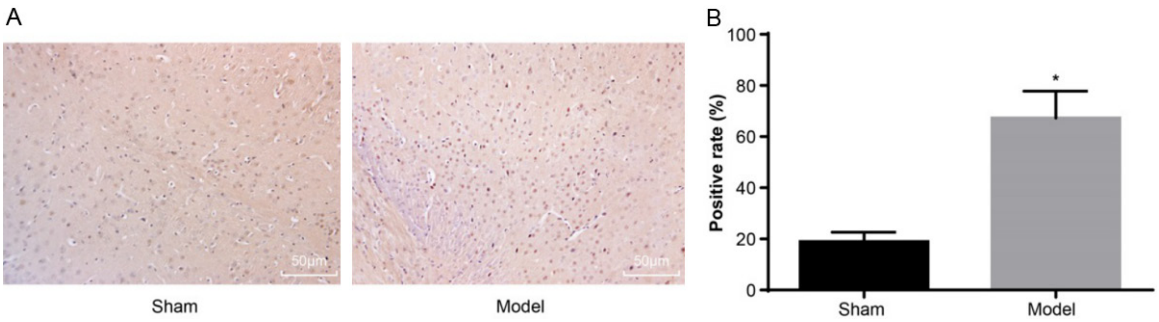

Figure 2. Immunohistochemistry of TRAF2 protein expression. A. Positive expression of TRAF2 protein of the brain tissues of mice in the two groups. B. The histogram of positive expression of TRAF2 protein in brain tissues of mice in the two groups. *P<0.05 vs. Sham group.

cells were adherent, the culture fluid was replaced with serum-free EBM-2 medium. When the cell fusions grew to 90%, 10 μL tips were used to scratch. Per hole was scratched 4 or 5 times and the width of scratches should be same. After being rinsed 3 times with PBS, the remaining cells were continued to be incubated. A inverted microscope (IX53, Olympus, Japan) was used to observe the migration distance of the cells in the scratched area and multiple fields were randomly selected and photographed. Each group was repeated three times [17].

EPCs angiogenesis test

After stored at 4°C overnight, the Matrigel gel was spread in a 24-well plate with dilution of 1:2 and placed in an incubator at 37°C for 1 h. After 48 hours of transfection in each group, trypsin was added and the cells were placed in an incubator at 37°C for 24 hours to form a tube defined as a tubular structure with a length of 4 times the width of the tube. Six visual fields were randomly selected to be observed under the Inverted microscope (100×). The number of tubular structures in the total tube field of view was analyzed by IPP 6.0 image analysis software and the means were calculated. The experiment was repeated three times [18].

Statistical analysis

All data were dealt with using SPSS 21.0 statistical software (SPSS, Chicago, Illinois, USA). Measured data is shown as mean ± standard deviation. T-test was used to process data of the two groups with normal distribution and the one-way ANOVA was used to compare the multiple groups. P<0.05 indicates that the difference was significant.

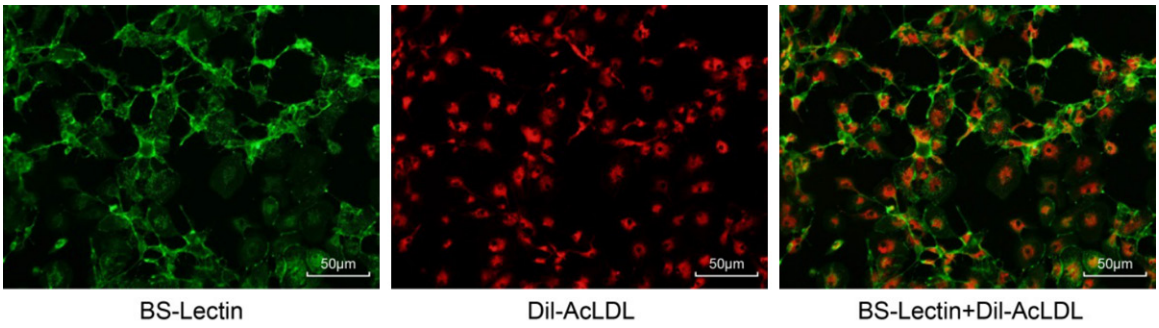

Figure 3. The identification of EPCs by double staining with DiI-AcLDL and BS-Lectin.

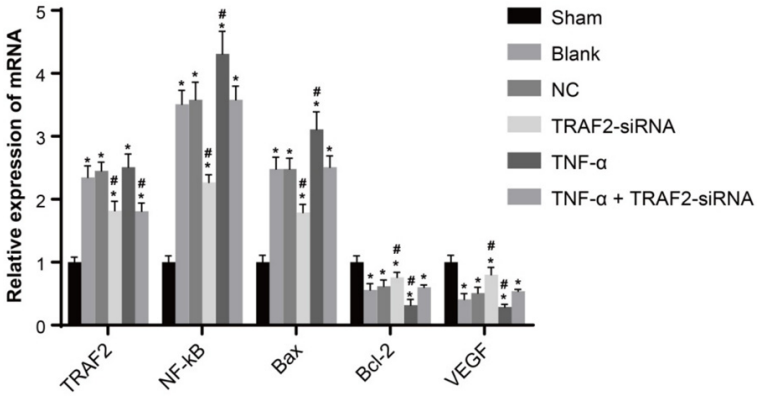

Figure 4. mRNA expression of genes in cells after transfection. *P<0.05 vs. Sham group; #P<0.05 vs. Blank group.

Results

Animal model identification

In the Model group, 3 mice died during the modeling process, and the model formation rate was 85% (17/20), 15 mice were randomly selected for the experiment. After traumatic brain injury to mice, they all suffered from unclear consciousness, hair erection, and arched back and recovered after 1 h. After recovery, the left limb of the mice showed spasm, difficult walking, and leftward deviation.

Pathological observation of brain tissues

HE staining result showed that the brain tissues in the Model group were flaky and partial fusion with increased necrotic lesions and glial cell proliferation. However, in the Sham group, the brain cell structure was complete and arranged neatly (**Figure 1**).

TRAF2 protein expression

The immunohistochemistry detection result showed that the TRAF2 protein appeared tan. The positive rate of TRAF2 protein in the Model group showed significant increase (P<0.05), compared with that in the Sham group (**Figure 2**).

EPCs identification

On the 7th day of culture for EPCs, the collected adherent cells were identified by the double staining of DiI-AcLDL and BS-Lectin. The result showed that EPCs were labelled with green fluorescence by BS-Lectin and labelled with red fluorescence by DiI-AcLDL, which can prove the successful isolation of EPCs (**Figure 3**).

mRNA expression of TRAF2, NF-kB, Bax, VEGF and Bcl-2 after cell transfection in each group

The results of qRT-PCR showed that the mRNA expression of TRAF2, NF-kB, and Bax in the other groups increased significantly, compared with the Sham group (P<0.05) and the mRNA expression of VEGF and Bcl-2 reduced significantly (P<0.05), compared with the Sham group. Compared with NC group (P>0.05), the genes expression in the Blank group didn't show significant difference. The mRNA expression of NF-kB and Bax increased significantly (P<0.05) in the TNF-α group and the mRNA expression of VEGF and BCl-2 reduced significantly (P<0.05), with no significant change for TRAF2, compared with the Blank group.

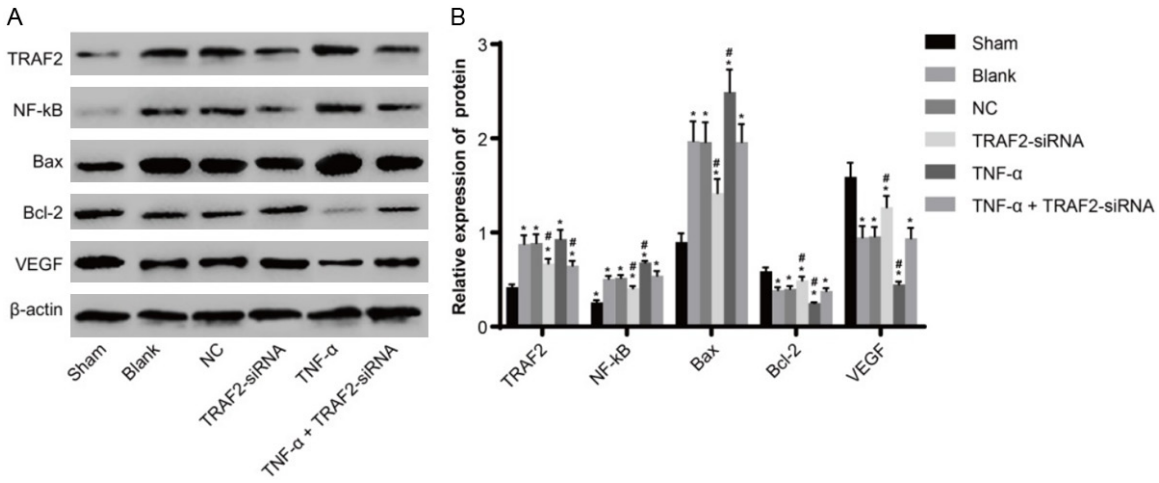

Figure 5. The western blot result of each group after cell transfection. A. The result of gray scale of cells in each group. B. The histogram of protein expression level after cell transfection of each group. *P<0.05 vs. Sham group; #P<0.05 vs. Blank group.

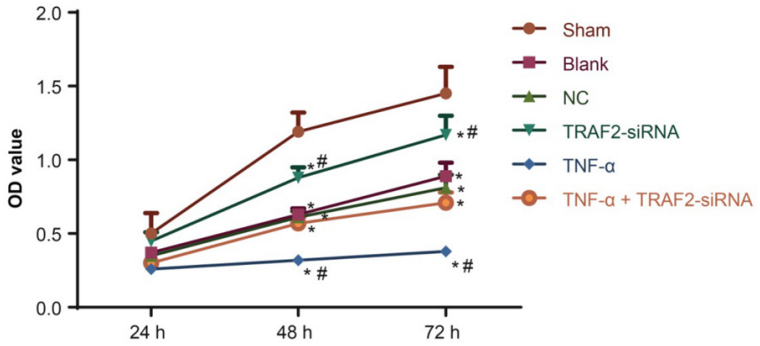

Figure 6. Comparison of cell proliferation in different group after transfection. *P<0.05 vs. Sham group; #P<0.05 vs. Blank group.

Compared with the Blank group, the mRNA expression of VEGF and Bcl-2 in TRAF2-siRNA group significantly increased (P<0.05), and the mRNA expression of TRAF2, NF-kB, and Bax decreased significantly (P<0.05). And the mRNA expression of TRAF2 in the TNF-α+TRAF2-siRNA group decreased significantly (P<0.05) and there was no significant difference for the mRNA expression of NF-kB, VEGF, Bcl-2 and Bax (P>0.05), compared with the Blank group (**Figure 4**).

Protein expression of TRAF2, NF-kB, Bax, VEGF and Bcl-2 genes of cells in each group after transfection

The western blot showed that compared with the Sham group, the protein expression of TRAF2, NF-kB and Bax genes in other groups increased significantly (P<0.05) and the protein expression of VEGF and Bcl-2 reduced significantly (P<0.05). There was no significant difference in protein expression between the Blank and NC groups. Protein expression of NF-kB, Bax gene in the TNF-α group increased significantly (P<0.05) and the protein expression of VEGF and Bcl-2 reduced significantly (P<0.05), with no obvious change of TRAF2, compared with the Blank group. Compared with the Blank group, the protein expression of VEGF, Bcl-2 in the TRAF2-siRNA group increased significantly (P<0.05) and the protein expression of TRAF2, NF-kB and Bax reduced significantly (P<0.05). And the protein expression of TRAF2 in the TNF-α+TRAF2-siRNA group decreased significantly (P<0.05), whereas there was no obvious change in the protein expression of NF-kB, VEGF, Bcl-2 and Bax (P>0.05), compared with the Blank group (**Figure 5**).

Detection of proliferation of cells in each group

Compared with the Sham group, the OD value of the cells decreased (P<0.05) in the Blank, NC, TRAF2-siRNA, TNF-α and TNF-α+TRAF2-siRNA groups after 48 h and 72 h of cell transfection. After 48 h and 72 h of cell transfection, the OD value of cells in the TRAF2-siRNA group increased significantly (P<0.05), compared

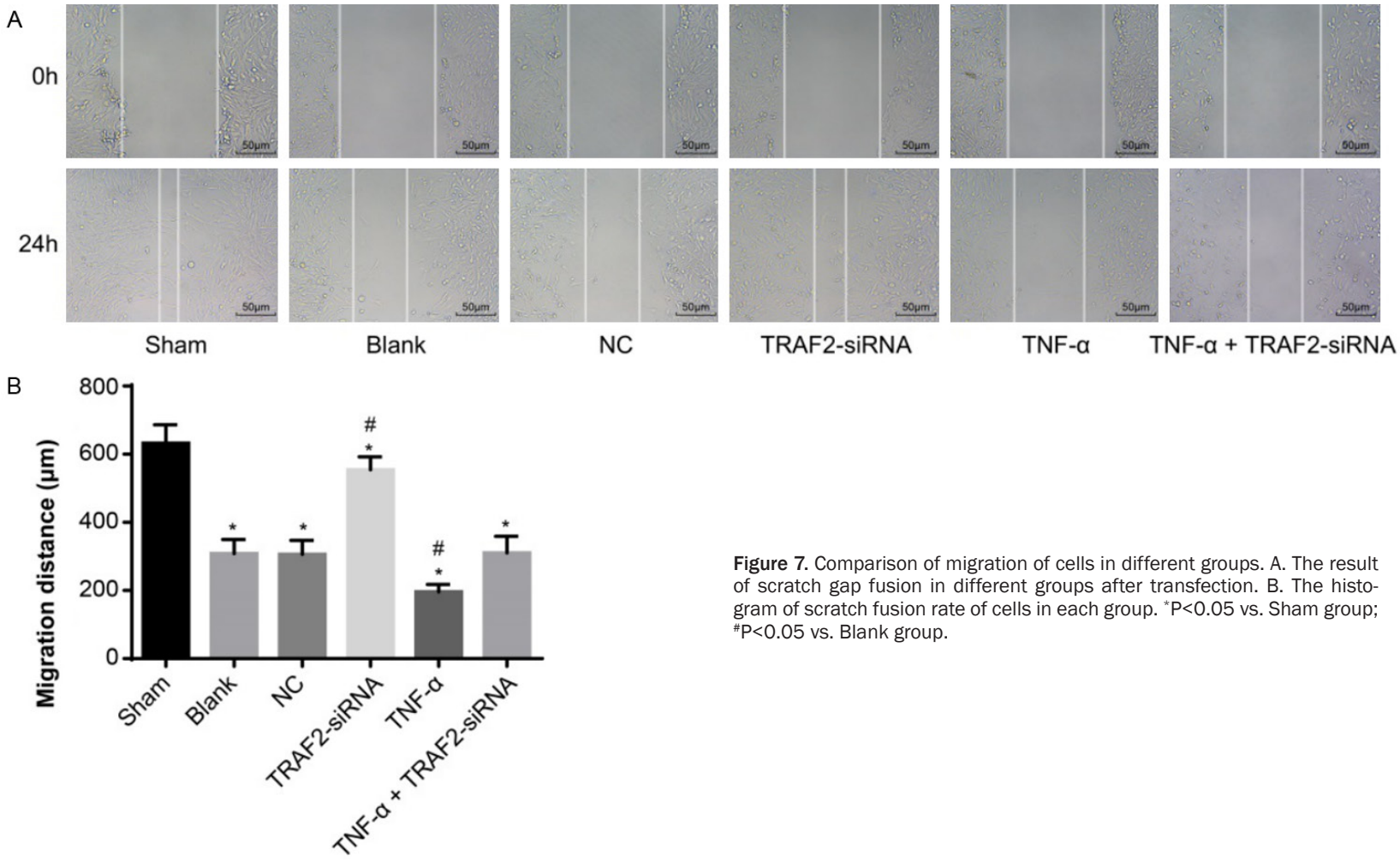

Figure 7. Comparison of migration of cells in different groups. A. The result of scratch gap fusion in different groups after transfection. B. The histogram of scratch fusion rate of cells in each group. *P<0.05 vs. Sham group; #P<0.05 vs. Blank group.

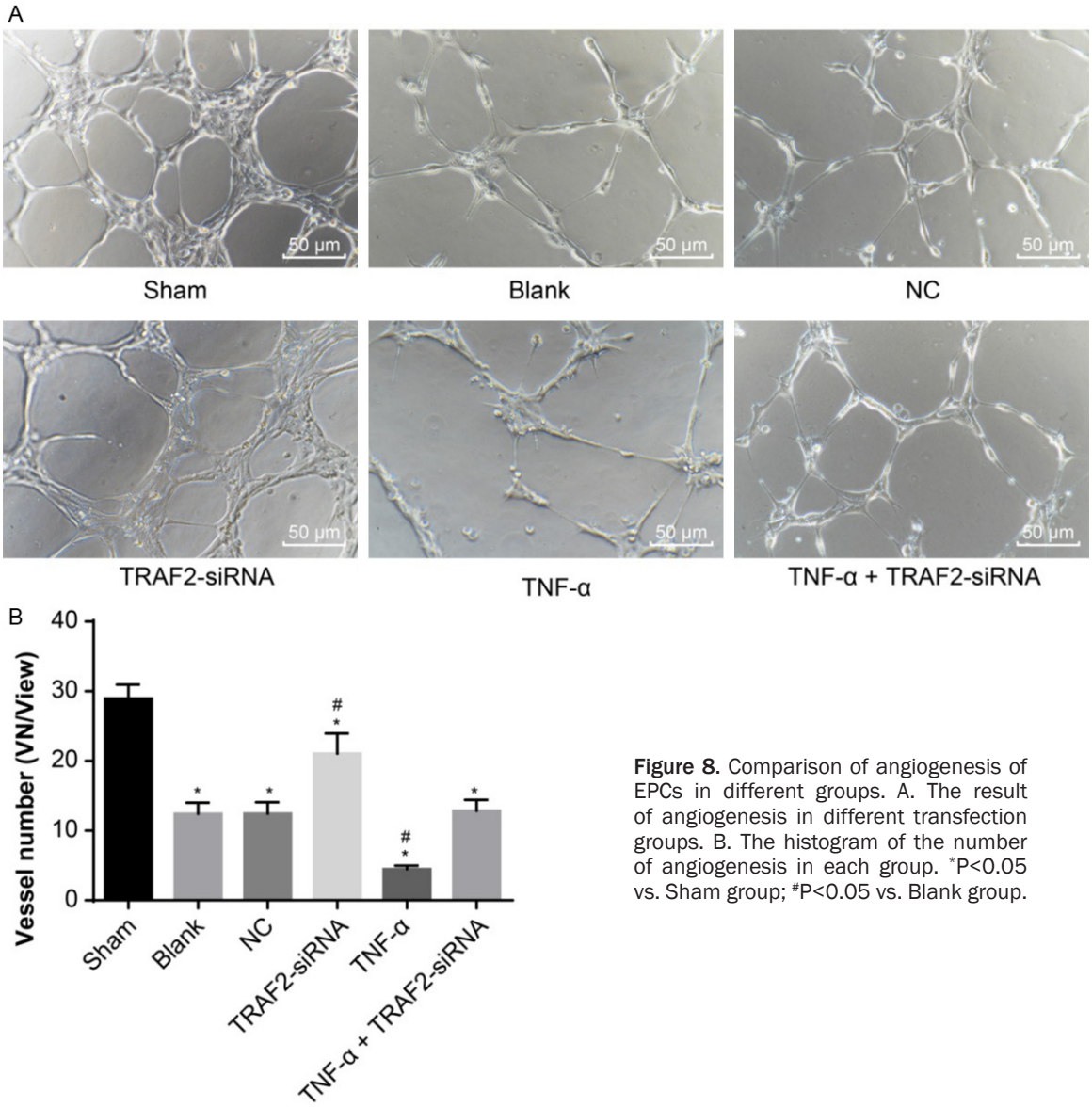

Figure 8. Comparison of angiogenesis of EPCs in different groups. A. The result of angiogenesis in different transfection groups. B. The histogram of the number of angiogenesis in each group. *P<0.05 vs. Sham group; #P<0.05 vs. Blank group.

with the Blank and NC group, while the OD value of cells in the TNF-α group decreased significantly (P<0.05) and there was no significant change in the TNF-α+TRAF2-siRNA group (P>0.05) (**Figure 6**).

Detection of cell migration of each group after transfection with scratch test

There was no significant difference (P>0.05) in the migration ability between the Blank group and the NC group. Compared with the Blank group, the migration ability in the TNF-α group deceased significantly (P<0.05) and the migration ability in the TRAF2-siRNA group increased significantly (P<0.05), while the change of migration in the TNF-α+TRAF2-siRNA group was not significant (P>0.05) (**Figure 7**).

Comparison of EPCs angiogenesis in different groups

Compared with the Blank and NC groups, the angiogenic ability of the TNF-α group was weakened (P<0.05), while the angiogenic ability of the TRAF2-siRNA group was enhanced (P<0.05), with no significant change in the TNF-α+TRAF2-siRNA group (P>0.05) (**Figure 8**).

Discussion

Craniocerebral injury is one of the most common critical illnesses in emergency surgery,

which may even cause systemic inflammatory response or multiple organ failure [19]. EPCs play an important role in the inflammatory response. It can participate in not only the entire inflammatory reaction process, but also the process of inflammatory reaction [20]. The damaged endothelium causes microvascular damage and circular dysfunction, which can aggravate the disease further. Previous studies have confirmed that EPCs transplantation has a good effect on the craniocerebral injury [21, 22]. Therefore, we used TRAF2-siRNA plasmid transfection to silence the TRAF2 gene and explored the possible mechanism of TRAF2-siRNA plasmid transfection and NF-kB signaling pathway in the craniocerebral injury.

The positive expression rate of TRAF2 protein of brain tissues in the Model and Sham groups was detected by the immunohistochemistry and the result showed that the positive expression of TRAF2 in the Model group was significantly higher than that in the Sham group. The positive expression of TRAF2 increased significantly after craniocerebral injury and it was confirmed by previous studies that TRAF2 was highly expressed in a variety of primary tumors [23, 24]. It was also highly expressed in the ischemic craniocerebral injury accompanying the inflammatory cell infiltration [25]. NF-kB is a very important transcription activating factor in the inflammatory response process, which can regulate cytokines and cytokines receptor and then influence apoptosis of cells [26]. Some animal studies have found that NF-kB in the brain tissues increased significantly after craniocerebral injury of mice and the expression level increased with the aggravation of the degree of injury [27].

The result of mRNA and protein expression of cells in each group showed that compared with the Sham group, the mRNA and protein expressions of TRAF2, NF-kB and Bax increased significantly in other groups, while the mRNA and protein expression of VEGF and Bcl-2 decreased significantly. It indicated that the activation of NF-kB signaling pathway in other groups increased apoptosis. The mRNA and protein expression of NF-kB and Bax in the TNF-α group increased significantly, while the mRNA and protein expression of VEGF and Bcl-2 reduced significantly, compared with the Blank group. And there was no significant difference in mRNA and protein expression of VEGF, Bcl-2 and Bax in the TNF-α+TRAF2-siRNA group. VEGF is an important factor affecting capillary permeability and clinical studied have confirmed that the decrease of VEGF was significantly associated with the severity of the disease [28]. Bax is an important promoting-apoptotic protein in the human body and its increase can promote procedural cell death [29]. However, Bcl-2 acts as an anti-apoptotic protein and its decreased expre-ssion can further enhance cell apoptosis [30]. Therefore, we can speculate that TRAF2 gene silencing can affect the NF-kB signaling pathway and inhibit its activation to reduce the apoptosis of EPCs. The proliferation and migration of cells in each group were further examined. The proliferation and migration ability of the TRAF2-siRNA group enhanced significantly, while that decreased in the TNF-α group, compared with the Blank group. In the angiogenic ability experiment, the angiogenic ability of the TRAF2-siRNA group increased significantly, compared with other groups, which indicated that TRAF2 gene silencing plays an important role in promoting angiogenesis in brain-damaged mice. However, the specific mechanism that EPCs participated in the occurrence and development of craniocerebral injury needs to be further studied.

In summary, TRAF2 gene silencing can inhibit the activation of NF-kB pathway and promote proliferation, migration and angiogenesis of endothelial progenitor cells, which have a protective effect on the mice.

Disclosure of conflict of interest

None.

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References

[1] Dagain A, Aoun O, Bordes J, Roqueplo C, Joubert C, Esnault P, Sellier A, Delmas JM, Desse N, Fouet M, Pernot P and Dulou R. Management of war-related ballistic cranioce-

rebral injuries in a french role 3 hospital during the afghan campaign. World Neurosurg 2017; 102: 6-12.
[2] Lu H, Wang L, Zhong W, Qi R, Li N, You W, Su X, Zhuang Z, Cheng H and Shi J. Establishment of swine-penetrating craniocerebral gunshot wound model. J Surg Res 2015; 199: 698-706.
[3] Jiang Z, Xu H, Wang M, Li Z, Su X, Li X, Li Z, Han X. Effect of infusion speed of 7.5% hypertonic saline on brain edema in patients with craniocerebral injury: An experimental study. Gene 2018; 665: 201-207.
[4] He C, Tan X, Lin X, Wang Y, Li Y. Clinical prevention and treatment of acute craniocerebral injury complicated with brain heart syndrome. Clinical Medicine 2017.
[5] Chen T, Gao F, Feng S, Yang T and Chen M. MicroRNA-370 inhibits the progression of non-small cell lung cancer by downregulating oncogene TRAF4. Oncol Rep 2015; 34: 461-468.
[6] Shen RR, Zhou AY, Kim E, O'Connell JT, Hagerstrand D, Beroukhim R and Hahn WC. TRAF2 is an NF-kappaB-activating oncogene in epithelial cancers. Oncogene 2015; 34: 209-216.
[7] Wei B, Ruan J, Mi Y, Hu J, Zhang J, Wang Z, Hu Q, Jiang H and Ding Q. Knockdown of TNF receptor-associated factor 2 (TRAF2) modulates in vitro growth of TRAIL-treated prostate cancer cells. Biomed Pharmacother 2017; 93: 462-469.
[8] Gong H, Fang L, Li Y, Du J, Zhou B, Wang X, Zhou H, Gao L, Wang K and Zhang J. miR873 inhibits colorectal cancer cell proliferation by targeting TRAF5 and TAB1. Oncol Rep 2018; 39: 1090-1098.
[9] Khan MB, Kumar R, Irfan FB, Irfan AB, Bari ME. Civilian craniocerebral gunshot injuries in a developing country: presentation, injury characteristics, prognostic indicators, and complications. World Neurosurg 2014; 82: 14-19.
[10] Dou Y, Shen H, Feng D, Li H, Tian X, Zhang J, Wang Z and Chen G. Tumor necrosis factor receptor-associated factor 6 participates in early brain injury after subarachnoid hemorrhage in rats through inhibiting autophagy and promoting oxidative stress. J Neurochem 2017; 142: 478-492.
[11] Barakat W, Safwet N, El-Maraghy NN and Zakaria MN. Candesartan and glycyrrhizin ameliorate ischemic brain damage through downregulation of the TLR signaling cascade. Eur J Pharmacol 2014; 724: 43-50.
[12] Feng Y, Cui Y, Gao JL, Li MH, Li R, Jiang XH, Tian YX, Wang KJ, Cui CM and Cui JZ. Resveratrol attenuates neuronal autophagy and inflammatory injury by inhibiting the TLR4/NF-kappaB signaling pathway in experimental traumatic brain injury. Int J Mol Med 2016; 37: 921-930.
[13] Tu XK, Yang WZ, Chen JP, Chen Y, Ouyang LQ, Xu YC and Shi SS. Curcumin inhibits TLR2/4-NF-kappaB signaling pathway and attenuates brain damage in permanent focal cerebral ischemia in rats. Inflammation 2014; 37: 1544-1551.
[14] Petraglia AL, Plog BA, Dayawansa S, Chen M, Dashnaw ML, Czerniecka K, Walker CT, Viterise T, Hyrien O, Iliff JJ, Deane R, Nedergaard M and Huang JH. The spectrum of neurobehavioral sequelae after repetitive mild traumatic brain injury: a novel mouse model of chronic traumatic encephalopathy. J Neurotrauma 2014; 31: 1211-1224.
[15] Hu RY, Chen Y, Li H, Zhang L, Jing H, Wu HW. Isolation, culture and characterization of bone marrow derived endothelial progenitor cells from mice. Journal of Southeast University 2014.
[16] Boyarinov GA, Deryugina AV, Zaytsev RR, Boyarinova LV, Yakovleva EI, Soloveva OD, Nikolskiy VO, Galkina MV, Martusevich AA. Morphological changes in myocardial blood microvessels in experimental. Craniocerebral Injury 2016.
[17] Dias HK, Brown CL, Polidori MC, Lip GY and Griffiths HR. LDL-lipids from patients with hypercholesterolaemia and Alzheimer's disease are inflammatory to microvascular endothelial cells: mitigation by statin intervention. Clin Sci (Lond) 2015; 129: 1195-1206.
[18] Kim KA, Shin YJ, Akram M, Kim ES, Choi KW, Suh H, Lee CH and Bae ON. High glucose condition induces autophagy in endothelial progenitor cells contributing to angiogenic impairment. Biol Pharm Bull 2014; 37: 1248-1252.
[19] Zweckberger K, Hackenberg K, Jung CS, Hertle DN, Kiening KL, Unterberg AW and Sakowitz OW. Glibenclamide reduces secondary brain damage after experimental traumatic brain injury. Neuroscience 2014; 272: 199-206.
[20] Tian R, Hou Z, Hao S, Wu W, Mao X, Tao X, Lu T and Liu B. Hydrogen-rich water attenuates brain damage and inflammation after traumatic brain injury in rats. Brain Res 2016; 1637: 1-13.
[21] Riccio A, Pichiorri F, Schettini F, Toppi J, Risetti M, Formisano R, Molinari M, Astolfi L, Cincotti F and Mattia D. Interfacing brain with computer to improve communication and rehabilitation after brain damage. Prog Brain Res 2016; 228: 357-387.
[22] Hsueh YY, Chang YJ, Huang CW, Handayani F, Chiang YL, Fan SC, Ho CJ, Kuo YM, Yang SH, Chen YL, Lin SC, Huang CC and Wu CC. Synergy of endothelial and neural progenitor cells from adipose-derived stem cells to preserve neuro-

vascular structures in rat hypoxic-ischemic brain injury. Sci Rep 2015; 5: 14985.
[23] Benetatos CA, Mitsuuchi Y, Burns JM, Neiman EM, Condon SM, Yu G, Seipel ME, Kapoor GS, Laporte MG, Rippin SR, Deng Y, Hendi MS, Tirunahari PK, Lee YH, Haimowitz T, Alexander MD, Graham MA, Weng D, Shi Y, McKinlay MA and Chunduru SK. Birinapant (TL32711), a bivalent SMAC mimetic, targets TRAF2-associated cIAPs, abrogates TNF-induced NF-kappaB activation, and is active in patient-derived xenograft models. Mol Cancer Ther 2014; 13: 867-879.
[24] Kim J, Moon SH, Kim BT, Chae CH, Lee JY and Kim SH. A novel aminothiazole KY-05009 with potential to inhibit Traf2- and Nck-interacting kinase (TNIK) attenuates TGF-beta1-mediated epithelial-to-mesenchymal transition in human lung adenocarcinoma A549 cells. PLoS One 2014; 9: e110180.
[25] Tesio M, Tang Y, Mudder K, Saini M, von Paleske L, Macintyre E, Pasparakis M, Waisman A and Trumpp A. Hematopoietic stem cell quiescence and function are controlled by the CYLD-TRAF2-p38MAPK pathway. J Exp Med 2015; 212: 525-538.
[26] Sun BZ, Chen L, Wu Q, Wang HL, Wei XB, Xiang YX, Zhang XM. Suppression of inflammatory response by flurbiprofen following focal cerebral ischemia involves the NF-kB signaling pathway. International Journal of Clinical & Experimental Medicine 2014; 7: 3087-3095.
[27] Wu JS, Tsai HD, Cheung WM, Hsu CY and Lin TN. PPAR-gamma ameliorates neuronal apoptosis and ischemic brain injury via suppressing NF-kappaB-driven p22phox transcription. Mol Neurobiol 2016; 53: 3626-3645.
[28] Jin X, Liang B, Chen Z, Liu X and Zhang Z. The dynamic changes of capillary permeability and upregulation of VEGF in rats following radiation-induced brain injury. Microcirculation 2014; 21: 171-177.
[29] Mao W, Yi X, Qin J, Tian M, Jin G. CXCL12 inhibits cortical neuron apoptosis by increasing the ratio of Bcl-2/Bax after traumatic brain injury. Int J Neurosci 2014; 124: 281-290.
[30] Dong P, Zhao J, Zhang Y, Dong J, Zhang L, Li D, Li L, Zhang X, Yang B, Lei W. Aging causes exacerbated ischemic brain injury and failure of sevoflurane post-conditioning: role of B-cell lymphoma-2. Neuroscience 2014; 275: 2-11.