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Original Article

Brain edema and protein expression of c-Fos and c-Jun in the brain after diffused brain injury

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Abstract: Objective: To investigate brain edema and protein expression of c-Fos and c-Jun in brain after diffuse brain injury, and to investigate the pathological change after brain injury, which may provide evidence for the clinical treatment of diffused brain injury. Methods: Marmarou method was used to establish the diffuse brain injury in rats. Results: After diffused brain injury, brain water content increased at 1 h, reached the peak at 1 d and remained at a high level at 7 d when compared with control group. One day after injury, diffuse subarachnoid hemorrhage was observed in the brain. HE staining showed vascular swelling and bleeding at the cortex and corpus callosum at 1 d. β-APP expression was found at the brainstem, hippocampus, thalamus, corpus callosum and periventricular regions. Pathological examination of ultrathin sections showed evidence edema and fracture of axons at 3 d after brain injury. The brain injury caused severe cerebral ischemia. The c-Fos and c-Jun expression increased at 1 h. The c-Fos expression peaked at 3 h ($P < 0.05$), then reduced, reached a maximal level again at 3 d ($P < 0.05$), and reduced significantly at 7 d but remained at a higher level when compared with control group ($P < 0.05$). The number of c-Jun positive cells peaked at 6 h ($P < 0.05$), then reduced, reached a maximal level again at 3 d and reduced markedly but still remained at a higher level when compared with control group ($P < 0.05$). Conclusion: After diffuse brain injury, brain water content and c-Fos/c-Jun expression change over time.

Keywords: Diffused brain injury, brain edema, c-Fos, c-Jun

Introduction

Diffuse brain injury (DBI) is one of common acute disease in the Department of Neurosurgery and has high disability and mortality [1-3]. DBI can be classified as diffuse axonal injury, diffuse brain swelling, diffuse microvascular spasm and hypoxic brain damage. Clinically, to differentiate these subtypes is difficult, and 4 types of injury may be observed in the same patient. Recently, more patients are diagnosed with DBI with the progression of diagnostic techniques. Surgery is unsuitable for the treatment of DBI. Thus, to investigate the pathological change in the brain after DBI is important for the clinical treatment of DBI. In the present study, DBI was introduced in rats by the freefall of an iron hammer, and the brain water content and protein expression of c-Fos and c-Jun were detected in the brain, aiming to investigate the pathological change in the brain after DBI.

Materials and methods

Animals and grouping

Healthy adults SD rats (n = 100) weighing 250 ± 20 g were purchased from the Experimental Animal Center of Shandong University. These animals were randomly assigned into control group (n = 10) and DBI groups. Rats in DBI groups were divided into subgroups according to the time points at which animals were sacrificed (1 h, 3 h, 6 h, 12 h, 1 d, 3 d and 7 d; n = 10 per subgroup). Animals were housed at room temperature and given *ad libitum* access to water and food. This study was approved by the Animal Care Committee of Shandong University and the pain of animal was minimized in the study.

Establishment of animal model

The modified Marmarou method was used to establish the DBI animal model in rats [4]. In

DBI groups, rats were intraperitoneally treated with sodium pentobarbital at 30 mg/kg for anesthesia. Then, animals were placed on a sponge bed with known thickness and coefficient of elasticity, and a special iron helmet was placed on the head of rats with the center of the helmet toward the outlet of the tube. When the animals began to weak and limb movement was initially observed, an iron hammer weighing 450 g freely fell to the helmet from a height of 1.5 m in a tube, and the bed was removed immediately to avoid secondary injury. The breath, limb movement and neurological signs were observed before and after insults.

Detection of brain water content

At different time points, rats were intraperitoneally anesthetized with 1% pentobarbital sodium, and sacrificed by cervical dislocation. Then, about 100 g of cortex was harvested and weighed as the wet weight. Then, the cortex was dried in an oven at 110°C for 24 until the weight was stable. The brain water content was calculated with Elliot formula: brain water content = (wet weight - dry weight)/wet weight × 100%.

Immunohistochemistry

At corresponding time points, animals were intraperitoneally anesthetized with 1% sodium pentobarbital at 30 mg/kg. The chest cavity was opened, and perfusion was transcardially done with normal saline for 10 min and then slowly with 4% paraformaldehyde for 30 min. Subsequently, the animals were sacrificed by cervical dislocation and the brain was harvested. Coronary sections of the brain (5 mm in thickness) were obtained and fixed in 4% paraformaldehyde for 24 h, followed by being embedded in paraffin, sectioned (5 μm in thickness) and HE staining or Bielschowsky staining. In addition, the brain was also harvested for immunohistochemistry for c-Fos and c-Jun.

The paraffin embedded sections were routinely de-paraffinized, hydrated and treated with 3% H_2O_2 for 10 min at room temperature, followed by antigen retrieval for 20 min in a microwave oven. Then, these sections were blocked in goat serum for 20 min, treated with primary antibody (1:100) at 4°C, biotin-conjugated secondary antibody for 30 min at 37°C and then with horseradish peroxidase conjugated streptavidin avidin for 20 min at 37°C. Washing was performed with PBS (pH = 7.4) thrice (5 min at each washing) between any two adjacent procedures except for blocking in serum. Then, visualization was done with DAB, followed by dehydration, transparentization and mounting. In the negative control, the primary antibody was replaced with PBS.

RT-PCR

Animals were sterilized with 75% ethanol and the brain was rapidly collected and weighed. Then, the brain was cut into blocks (30-50 mg for each block). Total RNA was extracted according to manufacturer's instructions. Total RNA was used for reverse transcription into cDNA according to manufacturer's instructions, followed by electrophoresis.

Preparation of ultrathin sections of the brain

Animals were intraperitoneally anesthetized with pentobarbital at 30 mg/kg, and sacrificed by cervical dislocation. The brain was rapidly collected and cut into blocks (1 mm × 1 mm × 1 mm), followed by fixation in 2.5% glutaraldehyde at 4°C over night. Following washing in PBS, tissues were fixed in 1% osmium tetroxide, treated with acetone, embedded, sectioned and stained.

Image analysis and statistical analysis

Image-Pro Plus 5.0 system was used for the image analysis. Images were captured at a high magnification (× 40). Five fields were randomly selected from the cortex and the number of cells positive for c-Fos or c-Jun was determined. Data were expressed as $\bar{x} \pm s$. Statistical analysis was done with SPSS version 10.0, and comparisons were performed with one way analysis of variance, followed by LSD-t test. A value of $P < 0.05$ was considered statistically significant.

Results

Changes in behaviors of animals after DBI

After brain injury, most rats developed coma immediately (absence of auditory reflex, corneal reflex and pain reflex), and apnea within several seconds, and the spontaneous breath was rapid and then became slow, but the magnitude of breath was larger than the normal. Most animals also presented with pilo-erection and

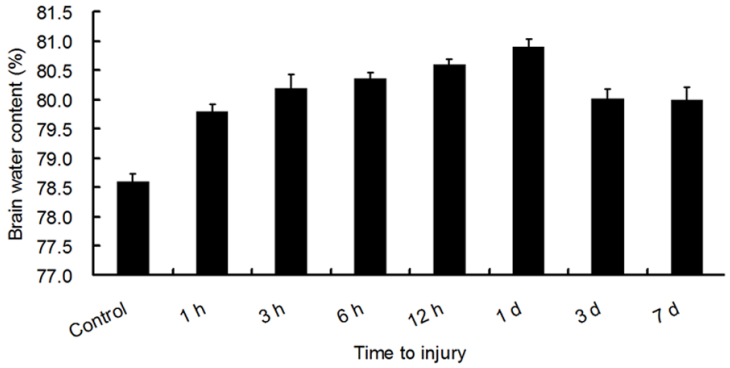

Figure 1. Brain water content at different time points after DBI.

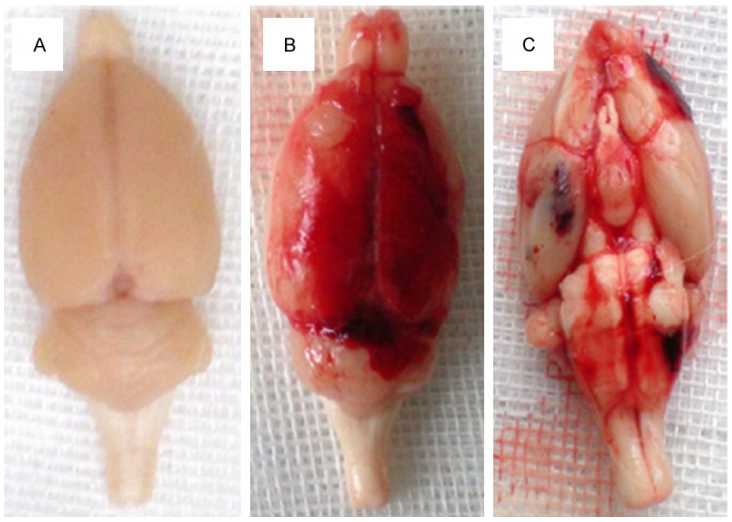

Figure 2. Pathological changes in brains of DBI rats. A: Negative control: brain was normal; B: Diffuse subarachnoid hemorrhage at 1 d after DBI; C: Brain contusion was observed in the rat brain.

systemic convulsion for seconds or longer. After completely waking, above symptoms improved to a certain extent, but slow response and reduction in activity and food intake were common in these animals.

Brain water content at different time points after DBI

After DBI, the brain water content began to increase at 1 h, and increased gradually. Significant elevation of brain water content was observed at 3 h, a high brain water content was noted at 12 h and it peaked at 24 h and then began to reduce. Although the brain water content reduced since 24 h, it was still higher than that in control group. One way analysis of variance showed significant difference in the brain water content among different time points ($P < 0.05$; **Figure 1**).

At 1 h after DBI, the brain water content began to increase and reached a high level at 3 h. It peaked at 1 d and remained at a higher level at 7 d when compared with control group ($P < 0.05$; **Figure 1**).

Pathological changes

When compared with negative control group (sham group; **Figure 2A**), the brain of DBI rats was extensively red, and diffuse subarachnoid hemorrhages were observed in these rats at 1 d (**Figure 2B** and **2C**). In several rats, focal brain contusion was observed (**Figure 2C**). These findings suggest the successful establishment of DBI animal model.

HE staining showed vascular edema at 1 d (**Figure 3A**, **3B**), and heavy hemorrhage (**Figure 3A**) and vascular edema (**Figure 3C**) were also observed at the cortex and corpus callosum. In sham group (**Figure 3D**), the nucleus of cortical neurons was clear, and hemorrhage and vascular edema were not noted.

β-APP is a rapid response protein after brain injury and shows increased expression after brain injury. At 1 d after brain injury (**Figure 4A**), β-APP expression was observed at the hippocampus (**Figure 4B**), thalamus (**Figure 4C**), corpus callosum (**Figure 4D**) and periventricular region (**Figure 4E**). However, in sham group (**Figure 4F**), β-APP expression was undetectable (**Figure 4**).

Ultrastructure of the brain after brain injury

At 3 h, axon fracture and reduction in the myelinated axons of the corpus callosum were observed. At 12 h, the axon fracture was more obvious, accompanied by mitochondrial swelling and intracellular edema. In some regions, myelin swelling and structural change were evident. In sham group, the axons and myelin were intact, showed even distribution, and had normal thickness and dark staining.

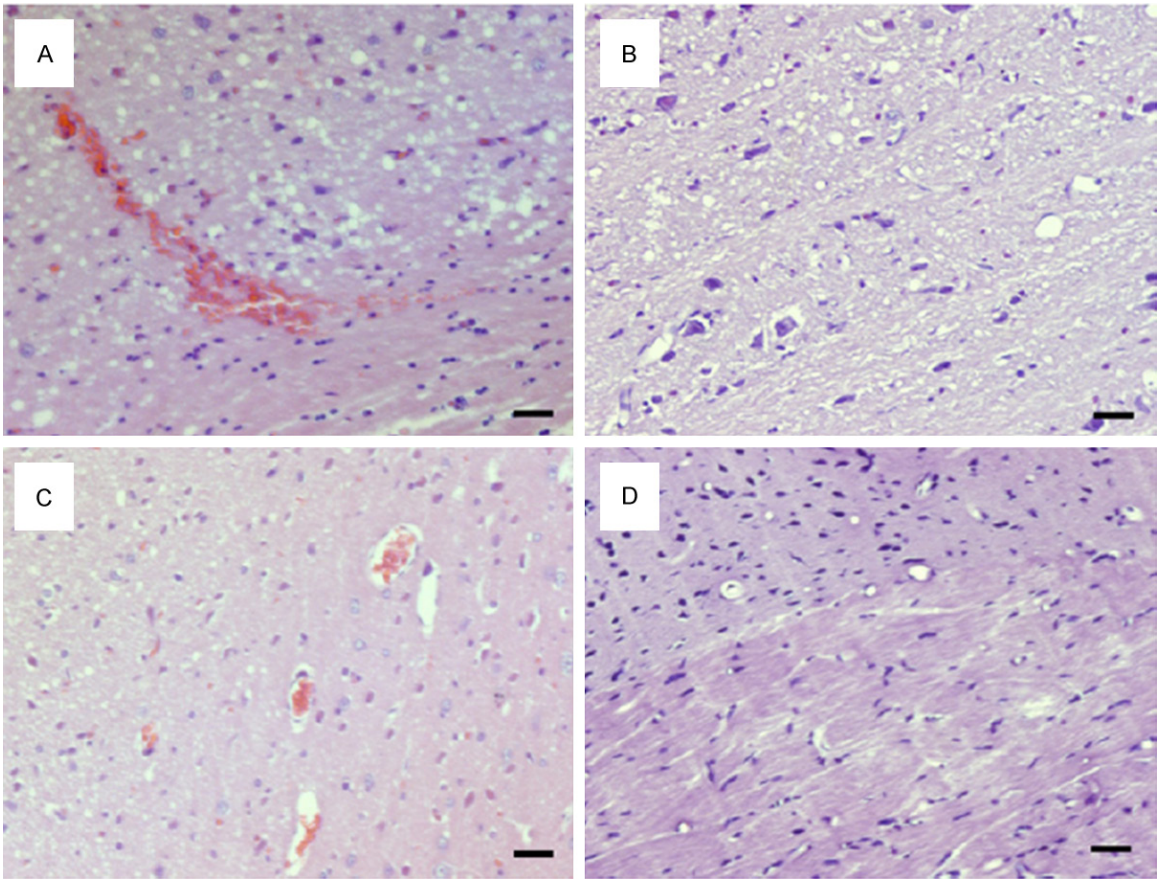

Figure 3. HE staining of the brain at 1 d after DBI. 1 d after DBI, the vascular edema was evident (A, B), hemorrhage was observed at the cortex (A, C). (D) Normal control.

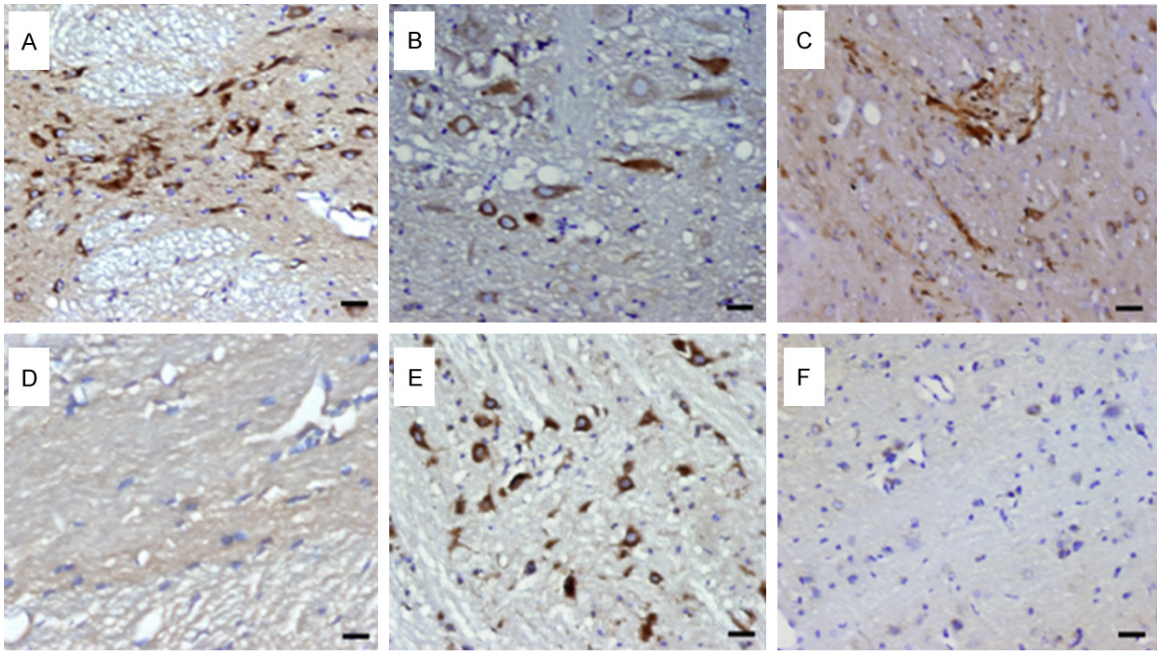

Figure 4. Immunohistochemistry for β-APP at 1 d after brain injury. At 1 d, β-APP positive cells (dark or light brown) were found at the brain stem (A), hippocampus (B), thalamus (C), corpus callosum (D) and periventricular region (E). In control group, β-APP positive cells were not observed (F).

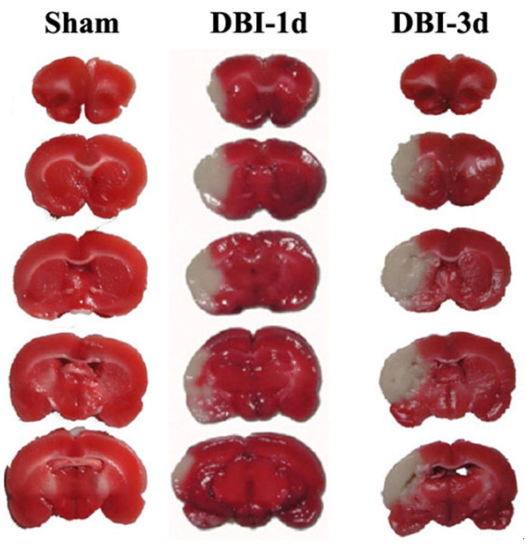

Figure 5. TTC staining of the brain at different time points after injury.

Cerebral hemorrhage after DBI

TTC staining of the brain showed brain infarction after DBI in rats, and the infarct ratio at 3 d and 1 d after brain injury was significantly higher than that in sham group (**Figure 5**).

c-Fos and c-Jun expression after brain injury

In control group, c-Fos or c-Jun positive cells were scattered. At 1 h after brain injury, the number of c-Fos or c-Jun positive cells began to increase. The number of c-Fos positive cells was the highest at 3 h (**Figure 6A**), then reduced and peaked again at 3 d (**Figure 6B**). The number of c-Jun positive cells was the highest at 6 h after injury (**Figure 6C**), then decreased and peaked again at 3 d (**Figure 6D**). The number of c-Fos and c-Jun positive cells at 7 d was significantly higher than that in control group although it reduced since 3 d ($P < 0.05$). In addition, the number of c-Jun positive cells was larger than that of c-Fos positive cells, and the staining intensity of c-Jun was also higher than that of c-Fos. At different time points, the number of c-Fos or c-Jun positive was markedly different from that in control group ($P < 0.05$), and significant difference was noted at any adjacent two time points ($P < 0.05$) (**Table 1**).

Changes in c-Fos and c-Jun expression

After brain injury, c-Fos expression began to increase at 1 h, peaked at 3 h, reduced significantly at 6 h, then began to increase, peaked again at 3 d and decreased again at 7 d, suggesting the double-peak. In addition, c-Jun expression also presented with "double-peak" characteristics with slight difference: c-Jun expression peaked at 6 h, then decreased and peaked again at 3 d (**Figure 7**).

Discussion

Of the pro-oncogene family, oncogenes that can be induced by secondary messengers are also known as immediately early genes (IEGs) or rapid responsive genes [5, 6]. IEGs can encode proteins that have short half-life (20-90 min) and can bind to the specific sequence of DNA [7-9]. IEGs family includes c-fos, c-jun, myc and erg families. To date, c-Fos and c-Jun have been extensively studied.

IEGs are widely distributed in the CNS. Generally, IEGs are highly conservative, and have a low expression. Thus, under normal conditions, IEGs are difficult to detect. After exogenous stimulation, cells in CNS present excitation and secret some neurotransmitters (or hormones) which may serve as primary messengers and act on the target cells. These transmitters may activate the transmembrane transduction and activate the secondary messengers (such as Ca^{2+} and cAMP), inducing specific responses [10-12]. During the responding to stimulation, IEGs in cells (such as c-Fos and c-Jun) are transcribed, and their mRNA translocate into the cytoplasm and are translated into proteins (Fos and Jun proteins) which then enter the nucleus again. In the CNS, IEGs are expression and act on target genes to regulate their transcription, which is involved in the growth and differentiation of neurons and the modulation of repair after injury, acting as a response to exogenous stimulation [13]. Thus, the activation and expression of c-Fos and c-Jun may serve as a functional marker of nerve and gene activity [14]. Under most physiological conditions, c-Fos expression is transient, and c-Fos expression may be a cellular response to injury, but does not mean the irreversible injury and exhaustion of ATP [15, 16]. Thus, c-Fos expression may predict the protection and repair after injury. Under pathological conditions, some stimulators (such as pain, water deprivation, epilepsy or brain injury) may activate IEGs expression [17].

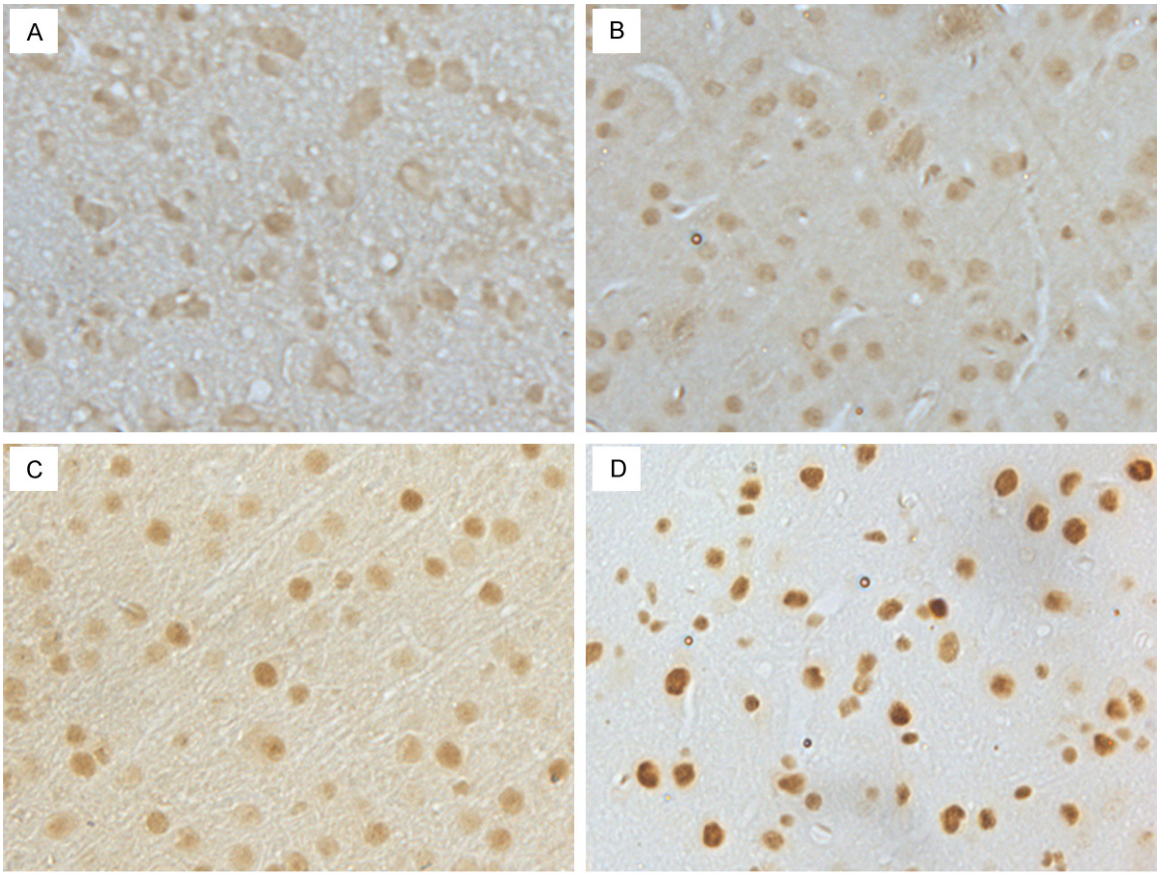

Figure 6. c-Fos and c-Jun expression after brain injury. A: c-Fos expression at 3 h after brain injury; B: c-Fos expression at 3 d after brain injury; C: c-Jun expression at 6 h after brain injury; D: c-Jun expression at 3 d after brain injury.

Table 1. Number of c-Fos and c-Jun positive cells after DBI in rats

Protein	Groups							
	Control	1 h	3 h	6 h	12 h	1 d	3 d	7 d
c-Fos	2.67 ± 2.52	48.67 ± 4.16$^{*,\#}$	72.33 ± 3.05$^{*,\#}$	54.00 ± 45.29$^{*,\#}$	44.00 ± 4.58$^{*,\#}$	59.67 ± 4.93$^{*,\#}$	89.00 ± 4.58$^{*,\#}$	60.33 ± 9.50$^{*,\#}$
c-Jun	12.67 ± 2.38	57.33 ± 3.06$^{*,\#}$	69.33 ± 4.51$^{*,\#}$	97.67 ± 7.51$^{*,\#}$	61.33 ± 9.87$^{*,\#}$	71.00 ± 5.20$^{*,\#}$	92.67 ± 3.79$^{*,\#}$	54.33 ± 3.79$^{*,\#}$

Footnotes: $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. adjacent group.

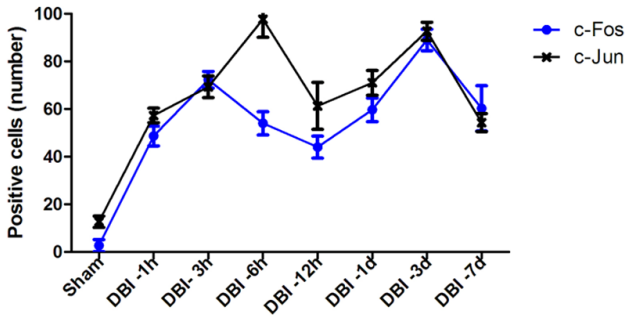

Figure 7. c-Fos and c-Jun expression in the brain of DBI rats at different time points.

DBI may cause dual injury to the brain. One is primary brain injury which is also known as the injury of the brain and blood vessels caused by mechanical factors and characterized by fracture of nerve fibers, disorder of efferent functions and functional disturbance or even death of different neuronal cells [18, 19]. The other is secondary brain injury which refers to the brain injury caused by brain ischemia and edema after primary brain injury. If the primary brain injury is not timely diagnosed and treated, it may cause progressive increase in intracranial pressure and deteriorate the pathology of primary brain injury, resulting in hernia, or even central failure and death.

Our results showed, after brain injury, obstruction of some capillaries and adherence of

inflammatory cells to the vascular wall were observed. In addition, nerve fibers were fractured and showed wave-like or bead-like change, which are features of primary brain injury. Moreover, features of secondary brain injury were also observed. At 1 h after brain injury, the brain water content began to increase, reached a high level at 3 h, and peaked at 1 d, and was still higher at 7 d than that in control group. This indicates that the brain edema is dynamic.

c-Fos and c-Jun are oncogenes and belong to IEGs. Studies have shown that, after exogenous stimulation (such as brain trauma, brain ischemia and epilepsy) of the central nervous system, c-Fos and c-Jun expression responds rapidly, and increases markedly in the neurons at the injured sites. After brain injury, c-Fos and c-Jun may form heterodimer AP1 which is involved in the subsequent expression of survival related genes [20]. There is evidence showing that c-Fos gene and c-Fos protein increase during the neuronal activities and may protect the neurons against injury [21]. In addition, some studies also reveal that c-Fos expression may induce or promote the delayed apoptosis of neurons and thus c-Fos gene should be a pro-apoptotic gene and related to the secondary injury after nerve injury. Studies have indicated that the increased bFGF after brain injury may increase the c-Jun protein expression which may inhibit the glutamate synthase to suppress the apoptosis and exert neuroprotection. In addition, c-Jun is also involved in the JNK mediated apoptosis. On the above findings, c-Fos and c-Jun play important roles in the pathophysiology of the brain [22].

In the present study, closed brain injury was established in rats to mimic brain injury in humans. Results showed the brain water content began to increase at 1 h and peaked at 1 d. One week after brain injury, the brain water content was still higher than that in negative control group. At 1 d after brain injury, diffuse subarachnoid hemorrhage was observed in the brain. HE staining showed vascular edema and heavy hemorrhage in the cortex and corpus callosum. After brain injury, β-APP expression was detected in the brain stem, hippocampus, thalamus, corpus callosum and periventricular region. β-APP is a rapidly responsive protein after brain injury. The increased β-APP expression suggests the successful establishment of animal model. Ultrathin sections showed evident brain edema and obvious axon fracture at 3 d. The brain injury causes severe cerebral ischemia.

RT-PCR and immunohistochemistry showed c-Fos and c-Jun began to express at 1 h after DBI. The c-Fos expression peaked at 3 h, then reduced, and peaked at 3 d, showing the dual-peak phenomenon. The change in c-Jun expression was similar to that in c-Fos expression with slight difference. C-Jun expression peaked at 6 h, which was later than that of c-Fos. This might be ascribed to the larger amount of c-Jun in the brain and compensation of AP-1 complex shortly after injury. In addition, c-Fos and c-Jun may form heterodimer AP-1 (a transcription factor) [23] which binds to the TGACGTCA of c-Fos gene and induces c-Fos expression. This might be also related to the early c-Fos expression. Our results showed c-Fos and c-Jun expression reached a high level at 1 h after brain injury, which is related to the involvement of both in the repair and survival of neurons after injury. At 3 d, the c-Fos and c-Jun expression peaked again, which is associated with the secondary brain injury. c-Jun and c-Fos expression plays important roles in the repair, regeneration and remodeling of neurons after injury. Thus, the degrees of c-Jun and c-Fos expression may provide information for the clinical diagnosis of the severity of brain injury.

Our results showed the brain water content began to increase at 1 h after DBI, and peaked at 1 d. The brain water content at 7 d was still significantly higher than that in control group. At the same time, c-Fos and c-Jun expression increased markedly at 1 h and showed “dual-peak” phenomenon at different time points. The c-Fos and c-Jun expression at 7 d after brain injury was still higher than that in control group. This suggests the dynamic changes in brain water content and c-Fos and c-Jun expression, and c-Fos and c-Jun expression is at a high level in the presence of brain edema. c-Fos and c-Jun are protective on neurons soon after brain injury. Thus, to increase the c-Fos and c-Jun expression may be helpful to control the brain edema. At late stage of brain injury, it is possible to inhibit the c-Fos and c-Jun expression to modulate the outcome of brain edema.

Although the mechanisms underlying the c-Fos and c-Jun expression after DBI have been stud-

ied extensively, these mechanisms are not completely understood. In clinical practice, osmotic diuretics (such as mannitol), opioid receptor antagonists (naloxone, etc.), inhibitors of excitatory amino acids (such as phenobarbital), inhibitors of inflammatory mediator release (such as glucocorticoids) are often used to control brain edema and protect the brain. In future studies, it is necessary to investigate how to control the c-Fos expression and c-Jun expression and how to control the brain edema after brain injury via modulating the c-Fos and c-Jun expression.

Disclosure of conflict of interest

None.

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