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Early hyperbaric oxygen therapy inhibits aquaporin 4 and adrenocorticotrophic hormone expression in the pituitary gland of rabbits with blast-induced craniocerebral injury★

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Abstract

In the present study, rabbits were treated with hyperbaric oxygen for 1 hour after detonator-blast-induced craniocerebral injury. Immunohistochemistry showed significantly reduced aquaporin 4 expression and adrenocorticotrophic hormone expression in the pituitary gland of rabbits with craniocerebral injury. Aquaporin 4 expression was positively correlated with adrenocorticotrophic hormone expression. These findings indicate that early hyperbaric oxygen therapy may suppress adrenocorticotrophic hormone secretion by inhibiting aquaporin 4 expression.

Key Words

hyperbaric oxygen; blast-induced injury; craniocerebral injury; aquaporin 4; pituitary gland; adrenocorticotrophic hormone

Research Highlights

Early hyperbaric oxygen suppresses adrenocorticotrophic hormone secretion in the pituitary gland by inhibiting aquaporin 4 expression

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INTRODUCTION

Stress reactions occurring after craniocerebral injury can cause neuroendocrine disorders, destabilization of the internal environment, and even death^[1]. Stress reactions occurring after injury are characterized by hypothalamic-pituitary-adrenal axis activation, as well as changes in related hormones, neurotransmitters and cytokines^[2]. Damage to the hypothalamic-pituitary-adrenal axis occurs within several hours after craniocerebral injury, resulting in excess adrenocorticotrophic hormone secretion in the acute stage of injury^[3]. In patients with mild or moderate craniocerebral injury, adrenocorticotrophic hormone levels were associated with trauma severity and prog-

nosis, while no correlation was found in patients with severe injury^[4]. Another study revealed that aquaporin 4 in pituitary gland cells may be involved in hormone secretion following cerebral edema^[5], but evidence to support this hypothesis is currently lacking. Blast-induced traumatic brain injury can influence neuroendocrine function^[6-7]. In the present study, we, for the first time, used a rabbit model of blast-induced craniocerebral injury to detect aquaporin and adrenocorticotrophic hormone expression in the pituitary gland following 1 hour of hyperbaric oxygen treatment. Our objective was to investigate the correlations between these factors and the curative effects of early hyperbaric oxygen on hypothalamic-pituitary-adrenal axis dysfunction caused by

blast injury.

RESULTS

Quantitative analysis of experimental animals

A total of 150 New Zealand rabbits were randomly assigned to the hyperbaric oxygen ($n = 70$), untreated blast injury ($n = 70$) and untreated control ($n = 10$) groups. Blast-induced craniocerebral injury was induced in the hyperbaric oxygen and untreated blast injury groups. The hyperbaric oxygen group received hyperbaric oxygen for 1 hour after blast injury, while the other groups received air at normal air pressure. Rabbits with blast-induced apnea received respiratory tract nursing. Their breath became deep, slow, and gradually accelerated. They regained consciousness 3–5 hours later, but they were depressed with a poor appetite. Nine rabbits developed epilepsy and three suffered limb palsy (not excluded from the study); five of these rabbits died because of starvation at 3 days after blast injury. These rabbits were replaced. The rabbits with blast injury survived for more than 7 days, and 150 were included in the final analysis. Ten rabbits from each the hyperbaric oxygen and untreated blast injury groups were assessed at 1, 6, 12, 24 and 72 hours, and at 7 and 14 days after blast injury.

Early hyperbaric oxygen inhibited aquaporin expression in the pituitary gland after blast-induced craniocerebral injury

Immunohistochemistry was used to determine aquaporin expression in the adenohypophysis, intermediate lobe and neurohypophyseal cells. Aquaporin was highly expressed in the region surrounding the sinusoidal capillary. Aquaporin expression in the pituitary gland increased over time after blast injury compared with that in the

control group, reaching a peak level at 72 hours after injury ($P < 0.05$). By contrast, aquaporin expression in the pituitary gland decreased after early hyperbaric oxygen treatment, with statistically significant differences from 6 hours after injury compared with the control group ($P < 0.05$), but then gradually increased to the normal level by 14 days ($P > 0.05$; Figure 1, Table 1, supplementary Figure 1 online).

Early hyperbaric oxygen inhibited adrenocorticotrophic hormone expression in the pituitary gland after blast-induced craniocerebral injury

Immunohistochemistry revealed that adrenocorticotrophic hormone was mainly expressed in the cytoplasm, and some positive particles were found in the cytoplasm around the nuclei. Adrenocorticotrophic hormone expression in the pituitary gland increased with time after injury compared with the control group, reaching a peak at 72 hours after injury ($P < 0.05$), while hyperbaric oxygen treatment reduced adrenocorticotrophic hormone expression in the pituitary gland ($P < 0.05$; Figure 2, Table 2, supplementary Figure 2 online). Pearson correlation analysis showed that aquaporin expression was positively correlated with adrenocorticotrophic hormone expression ($r = 0.959$, $P < 0.001$; Figure 3).

Aquaporin and adrenocorticotrophic hormone were co-expressed in pituitary cells after early hyperbaric oxygen therapy

Immunohistochemical double-labeling studies showed that aquaporin was expressed in the membrane and cytoplasm of acidophilic, basophilic, chromophobe and follicular cells in the adenohypophysis, while adrenocorticotrophic hormone was expressed in the cytoplasm of basophilic cells.

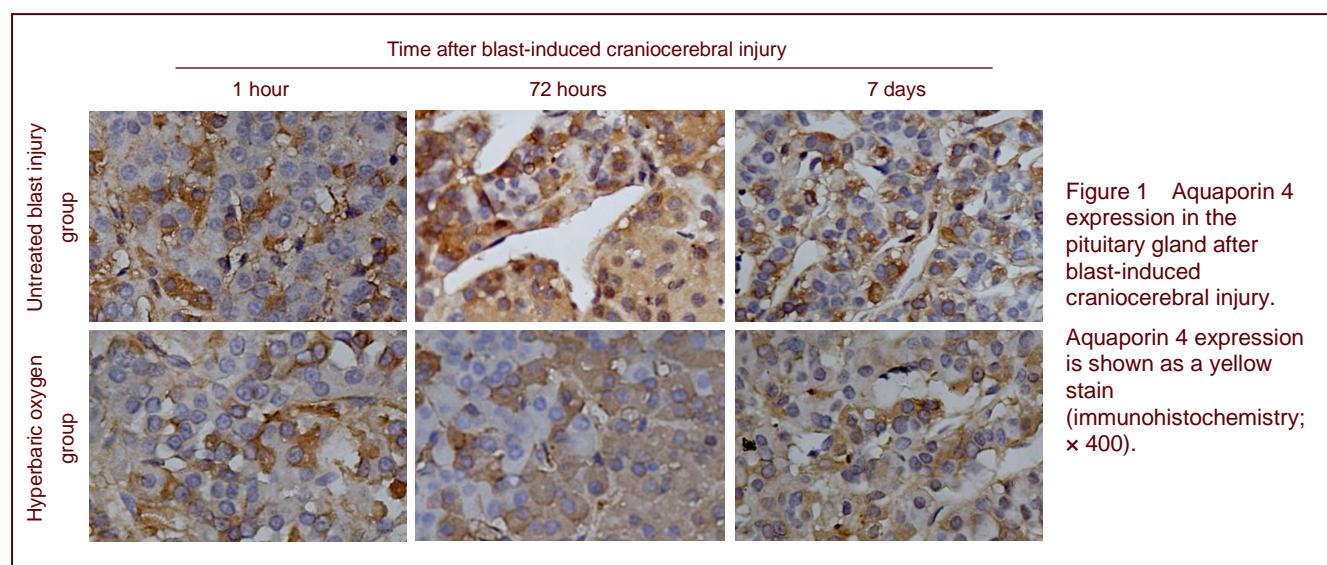


Table 1 Relative aquaporin 4 expression in the pituitary gland after blast-induced craniocerebral injury (mean absorbance; immunohistochemistry)

Group	Time after injury						
	1 hour	6 hours	12 hours	24 hours	72 hours	7 days	14 days
Untreated blast injury	0.412±0.031 ^a	0.461±0.018 ^a	0.511±0.028 ^a	0.573±0.030 ^a	0.799±0.129 ^a	0.611±0.057 ^a	0.388±0.025 ^a
Hyperbaric oxygen	0.388±0.021 ^a	0.421±0.019 ^{ab}	0.452±0.022 ^{ab}	0.490±0.030 ^{ab}	0.648±0.046 ^{ab}	0.557±0.051 ^{ab}	0.328±0.012 ^b
Control	0.333±0.012	0.333±0.012	0.333±0.012	0.333±0.012	0.333±0.012	0.333±0.012	0.333±0.012

Higher absorbance values represent greater aquaporin 4 expression. Results are expressed as mean ± SD of 10 rabbits per group at each time. Differences between multiple groups were compared using one-way analysis of variance, and differences between two groups were compared using the Student-Newman-Keuls method. ^a*P* < 0.05 vs. the control group; ^b*P* < 0.05, vs. the untreated blast injury group.

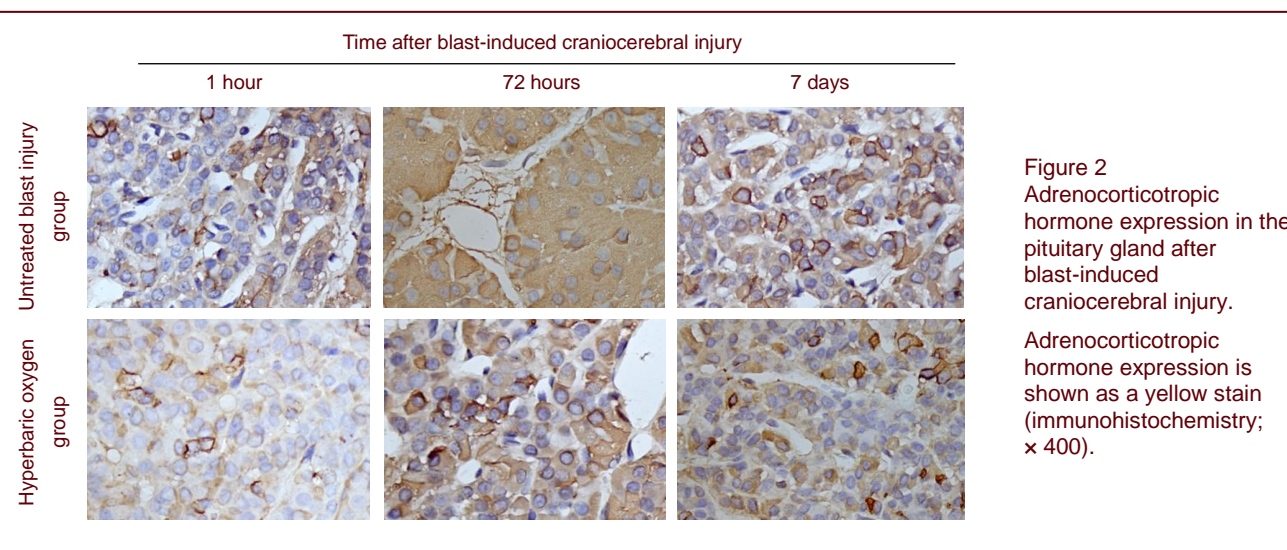


Figure 2 Adrenocorticotrophic hormone expression in the pituitary gland after blast-induced craniocerebral injury. Adrenocorticotrophic hormone expression is shown as a yellow stain (immunohistochemistry; × 400).

Table 2 Relative adrenocorticotrophic hormone expression in the pituitary gland after blast-induced craniocerebral injury (mean absorbance value; immunohistochemistry)

Group	Time after blast-induced craniocerebral injury						
	1 hour	6 hours	12 hours	24 hours	72 hours	7 days	14 days
Untreated blast injury	0.413±0.036 ^a	0.454±0.036 ^a	0.592±0.040 ^a	0.662±0.046 ^a	0.854±0.055 ^a	0.616±0.037 ^a	0.294±0.032
Hyperbaric oxygen	0.374±0.023 ^{ab}	0.413±0.023 ^{ab}	0.475±0.035 ^{ab}	0.530±0.040 ^{ab}	0.577±0.040 ^{ab}	0.479±0.028 ^{ab}	0.323±0.021 ^{ab}
Control	0.310±0.023	0.310±0.023	0.310±0.023	0.310±0.023	0.310±0.023	0.310±0.023	0.310±0.023

Higher absorbance values represent greater adrenocorticotrophic hormone expression. Results are expressed as mean ± SD of 10 rabbits per group at each time. Differences between multiple groups were compared using one-way analysis of variance, and differences between two groups were compared using the Student-Newman-Keuls method. ^a*P* < 0.05, vs. the control group; ^b*P* < 0.05, vs. the untreated blast injury group.

A number of cells co-expressing aquaporin and adrenocorticotrophic hormone were observed in the adenohypophysis of the untreated blast injury group, mixed with aquaporin-positive cells and adrenocorticotrophic hormone-positive cells. The number of double-labeled cells decreased significantly after hyperbaric oxygen therapy compared with that in the untreated blast injury group at 72 hours after injury (Figure 4).

DISCUSSION

Aquaporins are cell membrane transport proteins re-

sponsible for water transport. Aquaporin is extensively expressed in the brain, where it plays an important role in water transport and balance^[8]. In the present study, aquaporin was expressed on the membrane of adrenal pituitocytes, consistent with previous results^[5]. The pituitary gland lacks a blood-brain barrier, and its endothelial cells on the venous side of the capillaries are window-shaped, with abundant perivascular space around the vessels^[9]. In the present study, aquaporin was extensively expressed in the region surrounding the sinusoidal capillary, indicating that aquaporin may play a physiologically important metabolic role within the pituitary gland and other brain tissues. Aquaporin

protein expression changed dynamically in the pituitary gland of rabbits after blast injury; its expression gradually increased over time, reaching a peak at 72 hours after injury, consistent with previous results^[10].

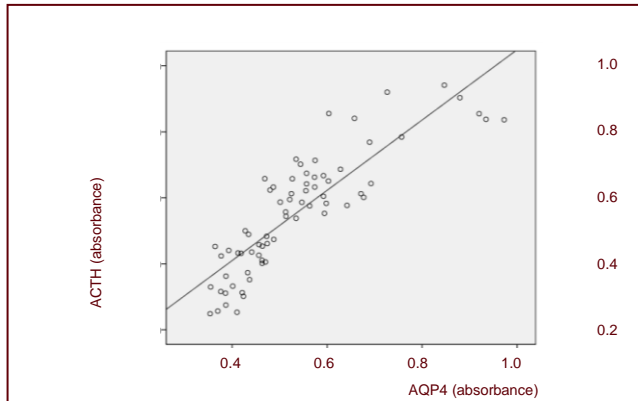


Figure 3 Correlation between aquaporin 4 (AQP4) and adrenocorticotrophic hormone (ACTH) expression in the pituitary gland after blast-induced craniocerebral injury. Pearson correlation analysis showed that AQP4 expression was positively correlated with ACTH expression ($r = 0.959$, $P < 0.001$).

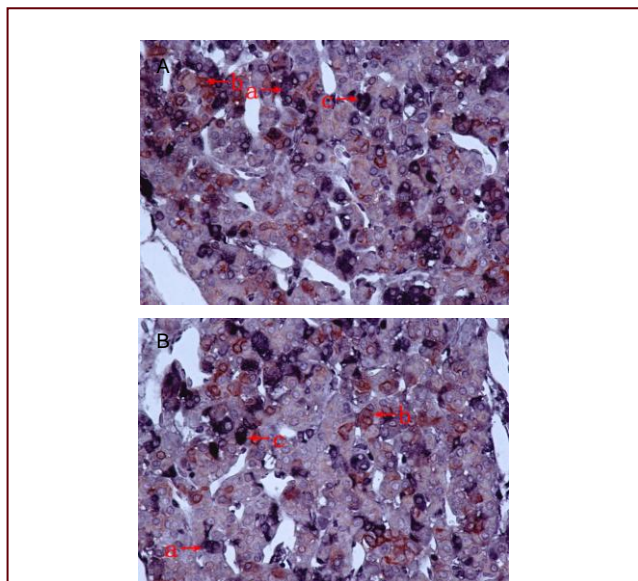


Figure 4 Co-expression of aquaporin 4 (AQP4) and adrenocorticotrophic hormone (ACTH) in pituitary cells at 6 hours after blast-induced craniocerebral injury (immunohistochemical double-labeling; $\times 400$). Arrow a shows AQP4 expression. AQP4 was mainly expressed in the membrane and cytoplasm of some cells. Arrow b shows ACTH expression. ACTH was expressed in the cytoplasm of corticotrophs. Arrow c shows AQP4 and ACTH co-expression.

(A) Untreated blast injury group.
(B) Hyperbaric oxygen group.

Studies have shown that aquaporin expression is positively correlated with the severity of cerebral edema in mice with water intoxication^[12-15]. Manley *et al*^[16] proposed that aquaporin acts as an osmoreceptor or receptor to generate rapid and sensitive increases in cell capacity by enhancing osmotic pressure. Therefore, there is a dynamic interaction between blood-brain barrier injury-induced osmotic pressure changes, aquaporin and cerebral edema^[17-20]. Nielsen *et al*^[21-22] reported that aquaporin participates in pituitary vasopressin secretion and regulation. Studies have also shown that aquaporin in endocrine cells may be involved in the synthesis, secretion, transport and regulation of hormones^[5, 23]. In the present study, the changes in adrenocorticotrophic hormone expression were similar to those of aquaporin in the untreated blast injury group, and correlation analysis showed a positive correlation between adrenocorticotrophic hormone and aquaporin. These results indicate that aquaporin participates in adrenocorticotrophic hormone secretion and regulation. Blast-induced craniocerebral injury in rabbits affects blood-brain barrier permeability, which enhances aquaporin expression in the pituitary gland, resulting in regional osmotic pressure changes and adrenocorticotrophic hormone release. However, the precise molecular mechanism requires investigation. Hyperbaric oxygen can balance cerebral blood flow, improve injury-induced brain tissue hypoxia, restore cell membrane permeability, accelerate clearance of accumulated toxic substance and facilitate the repair of injured tissue^[24-26]. Results of the present study showed that hyperbaric oxygen therapy did not significantly alter aquaporin expression in the pituitary gland of rabbits at 1 hour after injury, but did reduce adrenocorticotrophic hormone expression. It is likely that hyperbaric oxygen therapy suppresses adrenocorticotrophic hormone release induced by pituitary gland ischemia/hypoxia. At 72 hours after blast-induced craniocerebral injury, the co-expression of aquaporin and adrenocorticotrophic hormone was significantly reduced in the hyperbaric oxygen-treated group compared with the untreated blast injury group. We believe that hyperbaric oxygen improved the partial pressure of oxygen within the pituitary gland, relieved tissue hypoxia and the abnormal distribution of intracellular and extracellular ions, inhibited aquaporin expression, and maintained regional osmotic pressure surrounding adrenocorticotrophic hormone cells, thereby reducing adrenocorticotrophic hormone secretion. In summary, early hyperbaric oxygen therapy can inhibit aquaporin expression in anterior pituitary cells, thereby reducing adrenocorticotrophic hormone secretion.

MATERIALS AND METHODS

Aquaporin expression is highly correlated with the structure and function of the blood-brain barrier^[11]. Stu-

Design

A randomized, controlled animal study.

Time and setting

The animal experiments were performed at the Animal Experimental Center, Daping Hospital of Third Military Medical University, China between February and October 2009. Immunohistochemical studies were performed in the Laboratory of Immunohistochemistry, Anhui Medical University, China, between February and July 2010.

Materials

A total of 150 healthy, adult New Zealand rabbits, males and females, weighing 2.0–2.5 kg, were provided by the Animal Experimental Center, Daping Hospital (license no. SYXK (army) 2007-017). Rabbits were housed at $26 \pm 1.5^\circ\text{C}$ with a humidity of 60%. All animal procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China^[27].

Methods

Establishment of blast-induced craniocerebral injury

Rabbits were anesthetized by an intravenous injection of 3% pentobarbital sodium (1 mg/kg) *via* the ear, and placed under a self-made explosion frame. A thick cotton blanket was prepared and used to protect the neck and chest against blast or burn injury. A paper detonator (Chongqing 845 Factory, Chongqing, China), equivalent to 600 mg trinitrotoluene, was fixed 6.5 cm above the rabbit's brain, 1.5 cm to the median, 2 cm anterior to the line of both ears, to induce an explosion. The scalp was sutured after the blast. The patency of the respiratory tract of animals was maintained and cardiopulmonary resuscitation was used if necessary^[28-29] (supplementary Figure 3 online).

Early hyperbaric oxygen therapy

A transparent oxygen animal experimental chamber was purchased from Shanghai Decompressor Factory (Shanghai, China). Rabbits received hyperbaric oxygen therapy for 1 hour after blast injury, and were exposed to pure oxygen at 2 atmospheres (equivalent to 2.03 MPa). The rabbits received pure oxygen for 5 minutes, followed by a period of increasing pressure for 30 minutes, stable pressure for 1 hour and a period of reducing pressure for 30 minutes. This treatment was performed twice daily, once in the morning and once in the afternoon, with an interval of 8 hours between treatments. Rabbits in the other groups were exposed to air at normal pressure.

Preparation of pituitary gland paraffin sections

Ten rabbits from each group were selected at 1, 6, 12,

and 24 hours, and at 7 and 14 days after injury. Rabbits were anesthetized by an intravenous injection of 3% pentobarbital sodium (1 mg/kg). The heart was perfused with 4% paraformaldehyde, and the rabbits were sacrificed. Pituitary gland tissues were harvested (supplementary Figure 4 online)^[30], fixed in 4% paraformaldehyde, dehydrated, immersed in paraffin, embedded, and sectioned into three serial sections (5 μm thick). Hyperbaric oxygen therapy was performed 1 hour after inducing blast injury, and sections were prepared immediately after completing hyperbaric oxygen therapy.

Immunohistochemistry for aquaporin and adrenocorticotrophic hormone expression in the pituitary gland

The sections were dewaxed, hydrated, washed with PBS three times for 3 minutes each, immersed in 3% H_2O_2 and left at room temperature for 10 minutes. The sections were then washed three times with PBS for 3 minutes each, followed by antigen retrieval in a microwave. The sections were washed with PBS, mixed with goat serum blocking solution at room temperature for 30 minutes, and incubated with 100 μL of rabbit anti-aquaporin or rabbit anti-adrenocorticotrophic hormone polyclonal antibodies (1:200; Boster, Wuhan, China) overnight at 4°C . The sections were warmed to room temperature for 10 minutes, washed with PBS three times for 5 minutes each, and then incubated with 100 μL of goat anti-rabbit IgG (1:100; Boster) per section at 37°C for 30 minutes. After washing the sections with PBS three times for 5 minutes each, they were treated with 100 μL of streptavidin-biotin complex solution (Boster) per section, washed with PBS three times for 5 minutes each, and colorized with diaminobenzidine for 5–10 minutes. Sections were then washed with tap water, counterstained with hematoxylin for 1 minute, differentiated with hydrochloric acid and ethanol for about 10 seconds, washed with tap water for 10–15 minutes, dehydrated, cleared, mounted, dried, and observed under a light microscope (Nikon80i; Nikon, Tokyo, Japan). The mean absorbance value in randomly selected fields of view (magnification, $\times 400$) was calculated using the JEDA 801D morphologic image analysis system (Molecular Devices, Sunnyvale, CA, USA) and MetaMorph software (Molecular Devices)^[31].

Immunohistochemical double-labeling of aquaporin and adrenocorticotrophic hormone

The sections were dewaxed, hydrated, mixed with 3% H_2O_2 and left at room temperature for 10 minutes, followed by antigen retrieval under a microwave (twice at high temperature for 5 minutes, with 5 minutes between each heating). The sections were blocked with goat serum at room temperature for 30 minutes and incubated

with 100 μ L of rabbit anti-aquaporin polyclonal antibody (1:50) overnight at 4°C. The sections were warmed to room temperature for 10 minutes and incubated with 100 μ L of peroxidase-labeled goat anti-rabbit IgG (1:100; Boster) per section at 37°C for 30 minutes. The sections were then washed with PBS three times for 5 minutes each, treated with 100 μ L of streptavidin-biotin complex solution (Boster) per section, colorized with blue diaminobenzidine for 10 minutes, washed with distilled water, blocked with goat serum for 30 minutes, and treated with 100 μ L of rabbit anti-adrenocorticotrophic hormone polyclonal antibody (1:50) overnight at 4°C. The sections were warmed to room temperature for 10 minutes and incubated with 100 μ L of peroxidase-labeled goat anti-rabbit IgG (1:100; Boster) per section at 37°C for 30 minutes. The sections were then washed with PBS three times for 5 minutes each, treated with 100 μ L of streptavidin-biotin complex solution (Boster) per section, colorized with red diaminobenzidine for 10 minutes, washed with distilled water three times for 5 minutes each, dehydrated, cleared, glycerol-mounted, and observed under a light microscope (magnification, \times 400).

Statistical analysis

Data were analyzed using SPSS software version 17.0 (SPSS, Chicago, IL, USA) and are expressed as mean \pm SD. Differences between each group at different times were compared using one-way analysis of variance, and paired comparisons were performed using the Student-Newman-Keuls method. Values of $P < 0.05$ were considered statistically significant.

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Author contributions: Jian Huo, Yanyan Yang, Shaonian Xu and Wenjiang Sun collected and interpreted the experimental data. Jian Huo, Jiachuan Liu, Chunlin Wang, Yongming Zhang and Jinbiao Wang conceived and designed the study. Jian Huo conducted data analysis, wrote the manuscript, and provided technical and statistical support. Jiachuan Liu was in charge of the funds, revised the manuscript and guided the study.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of the Third Military Medical University,

China.

Supplementary information: Supplementary data associated with this article can be found in the online version, by visiting www.nrronline.org.

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