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Apoptosis-related protein expression in rabbits with blast brain injury following early hyperbaric oxygen therapy[★]

Shaonian Xu, Jiachuan Liu, Yongming Zhang, Chunlin Wang, Jinbiao Wang, Yanyan Yang, Jian Huo, Wenjiang Sun

Department of Neurosurgery, the 105 Hospital of Chinese PLA, Hefei 230031, Anhui Province, China

Abstract

We treated detonator-explosion-induced craniocerebral injury in rabbits with hyperbaric oxygen 1–24 hours post-injury. Expression of the apoptosis-regulating protein cytochrome c, the pro-apoptotic protein Bax and the apoptosis marker caspase-3 in the tissues surrounding the area of injury was significantly reduced, while that of the anti-apoptotic protein Bcl-2 was significantly increased. Our findings indicate that the curative effects of early hyperbaric oxygen on cortical cell apoptosis is associated with suppression of cytochrome c release from mitochondria. This mechanism underlies the observed reduction in Bax expression and upregulation of Bcl-2 expression.

Key Words

hyperbaric oxygen; craniocerebral injury; caspase-3; cytochrome c; Bax; Bcl-2; neural regeneration

Abbreviations

HBOT, hyperbaric oxygen therapy; PBS, phosphate buffered saline

Shaonian Xu[★], Master, Department of Neurosurgery, the 105 Hospital of Chinese PLA, Hefei 230031, Anhui Province, China

Corresponding author: Jiachuan Liu, Master, Chief physician, Master's supervisor, Department of Neurosurgery, the 105 Hospital of Chinese PLA, Hefei 230031, Anhui Province, China
ljc571017@sina.com

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INTRODUCTION

The mitochondrion is a eukaryotic cell organelle that plays a critical role in cell apoptosis^[1]. Indeed, the mitochondrion-mediated pathway is one of two pathways of apoptosis, the other being death receptor-mediated^[2-3]. Early signal transduction in apoptosis mainly occurs *via* the mitochondrion pathway, in which, the mitochondrion acts as a receptor and amplifier of death signals in cells^[4-5]. Cytochrome c is distributed in the mitochondrial inner and outer membranes^[6] where it functions as an important cell regulator^[7]. Its release from mitochondria is regarded as an early critical event of apoptosis^[8-11]. In the presence of ischemia or hypoxia injury in brain tissues, changes in mitochondrial morphology, membrane

potential and function result in elevated cytochrome c levels in the cytoplasm and reduced concentrations in the mitochondria^[12-13], which finally triggers the caspase-induced apoptotic pathway^[14-15]. In apoptosis related gene families, bcl-2 and bax may be involved in this process^[16-18]. Previous studies found that hyperbaric oxygen therapy (HBOT) can maintain the mitochondrial membrane potential such that cell apoptosis can be reduced in rats with cortical injury^[19-20]. In addition, early HBOT resulted in inhibitory effects on brain cell autophagy and apoptosis in an animal model of cerebral edema induced by explosive injury^[21-23]. Although clinical HBOT also achieves curative effects after craniocerebral injury, the precise mechanism behind this remains unknown. We therefore carried out HBOT after brain injury and determined changes in caspase-3,

cytochrome c, Bax and Bcl-2 levels in brain tissues of rabbits.

RESULTS

Quantitative analysis of experimental animals

A total of 150 New Zealand rabbits were randomly assigned to control ($n = 10$), model ($n = 70$) and HBOT ($n = 70$) groups. A blast-induced brain injury was established in the model and HBOT groups, and the HBOT group received HBOT treatment 1 hour after injury. Control and model groups received air at normal pressure instead of HBOT. Animals with blast-induced apnea were subjected to respiratory tract nursing or cardio-pulmonary resuscitation if necessary. Vital signs gradually stabilized and the animals regained consciousness 3-4 hours later, but they were depressed

with a low appetite. They were housed in the laboratory animal center, but five died 3 days post-injury. A total of 155 rabbits were analyzed following supplementation.

Early HBOT suppressed cytochrome c expression in brain tissues around the injury site

Immunohistochemical detection revealed little cytochrome c expression in the cerebral cortex of control rabbits. Significant cytochrome c expression was detected in the brain tissues around the injury site 1 hour after the blast injury. This gradually increased with time, peaked at 12 hours, then gradually decreased and had restored to normal levels by day 3 ($P > 0.05$). Cytochrome c expression in the brain tissues around the injury site was significantly reduced in the early stages (1-24 hours post-injury) of HBOT ($P < 0.05$), but remained higher than control group ($P < 0.05$) then restored to normal levels by day 3 ($P > 0.05$; Figure 1).

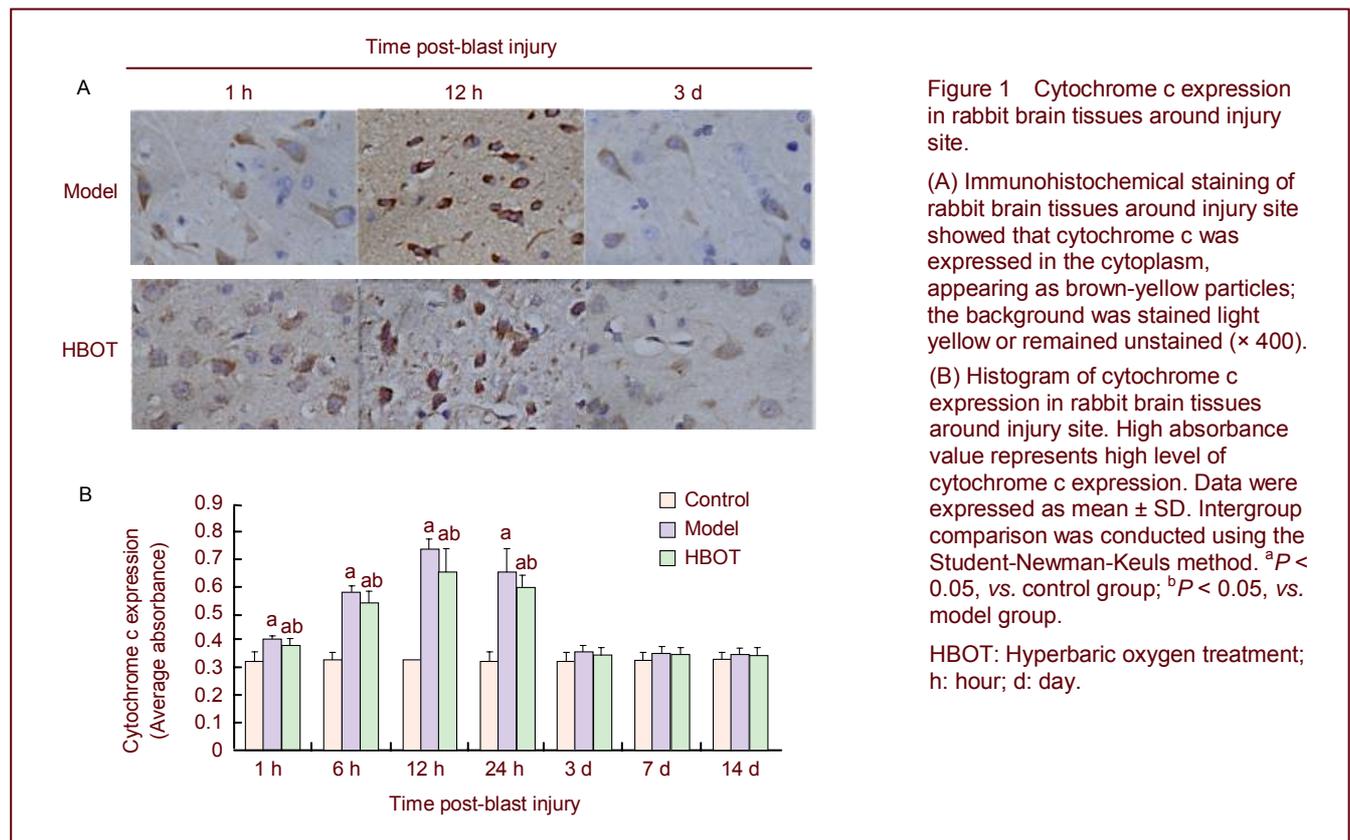


Figure 1 Cytochrome c expression in rabbit brain tissues around injury site.

(A) Immunohistochemical staining of rabbit brain tissues around injury site showed that cytochrome c was expressed in the cytoplasm, appearing as brown-yellow particles; the background was stained light yellow or remained unstained ($\times 400$).

(B) Histogram of cytochrome c expression in rabbit brain tissues around injury site. High absorbance value represents high level of cytochrome c expression. Data were expressed as mean \pm SD. Intergroup comparison was conducted using the Student-Newman-Keuls method. ^a $P < 0.05$, vs. control group; ^b $P < 0.05$, vs. model group.

HBOT: Hyperbaric oxygen treatment; h: hour; d: day.

Early HBOT suppressed Bax expression in rabbit brain tissues around the injury site

Immunohistochemistry showed little Bax expression in the cerebral cortex of control group rabbits. Bax expression was detected in the brain tissues around the injury site 1 hour post-blast injury, gradually increased over time, peaked at 12 hours, gradually decreased and was restored to normal levels by day 14 ($P > 0.05$). Bax expression in the brain tissues around the injury site was reduced in the early stage (1-24 hours post-injury) of

HBOT ($P < 0.05$) but remained higher than the control group ($P < 0.05$), and was restored to normal levels by day 7 ($P > 0.05$; Figure 2).

Early HBOT suppressed Bcl-2 expression in rabbit brain tissues around the injury site

Immunohistochemistry showed little Bcl-2 expression in the cerebral cortex of control group rabbits. By contrast, Bcl-2 expression was detected in the brain tissues around the injury site 1 hour post-blast injury, gradually

increasing over time and peaking at 6 hours. Levels then gradually decreased and were restored to normal by day 7 ($P > 0.05$). Bcl-2 expression in the brain tissues around

the injury site was significantly increased in the early stage (1-24 hours post-blast injury) of HBOT compared with the model group ($P < 0.05$; Figure 3).

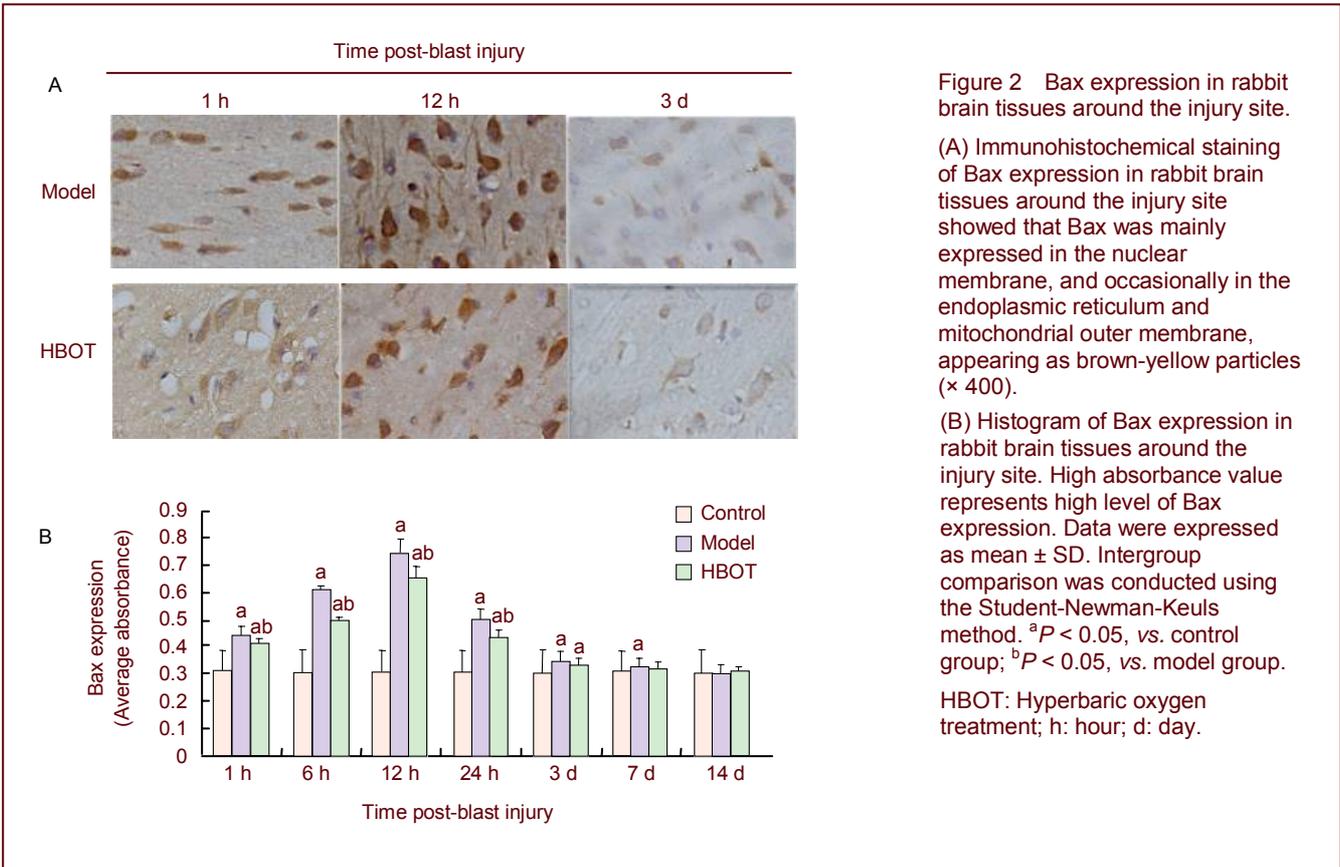


Figure 2 Bax expression in rabbit brain tissues around the injury site.

(A) Immunohistochemical staining of Bax expression in rabbit brain tissues around the injury site showed that Bax was mainly expressed in the nuclear membrane, and occasionally in the endoplasmic reticulum and mitochondrial outer membrane, appearing as brown-yellow particles ($\times 400$).

(B) Histogram of Bax expression in rabbit brain tissues around the injury site. High absorbance value represents high level of Bax expression. Data were expressed as mean \pm SD. Intergroup comparison was conducted using the Student-Newman-Keuls method. ^a $P < 0.05$, vs. control group; ^b $P < 0.05$, vs. model group.

HBOT: Hyperbaric oxygen treatment; h: hour; d: day.

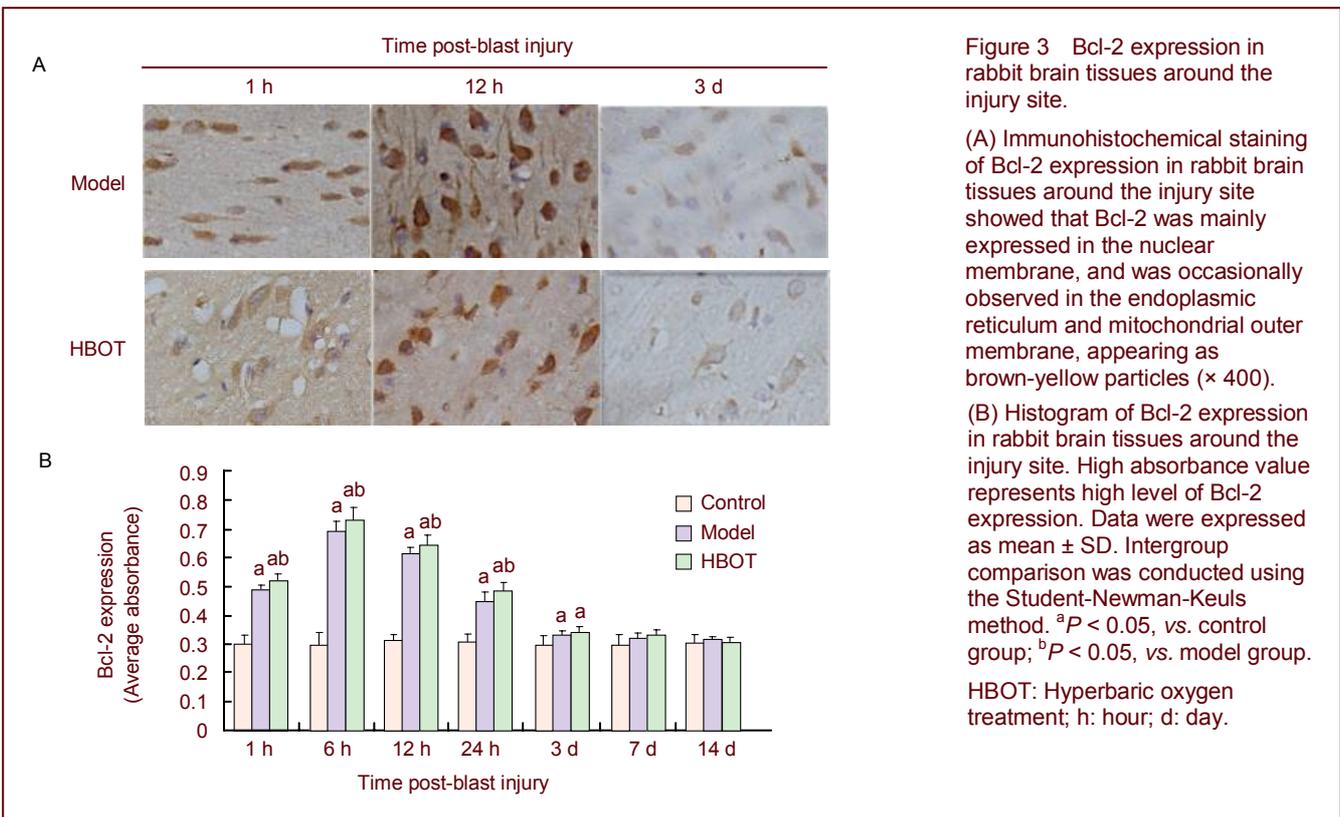


Figure 3 Bcl-2 expression in rabbit brain tissues around the injury site.

(A) Immunohistochemical staining of Bcl-2 expression in rabbit brain tissues around the injury site showed that Bcl-2 was mainly expressed in the nuclear membrane, and was occasionally observed in the endoplasmic reticulum and mitochondrial outer membrane, appearing as brown-yellow particles ($\times 400$).

(B) Histogram of Bcl-2 expression in rabbit brain tissues around the injury site. High absorbance value represents high level of Bcl-2 expression. Data were expressed as mean \pm SD. Intergroup comparison was conducted using the Student-Newman-Keuls method. ^a $P < 0.05$, vs. control group; ^b $P < 0.05$, vs. model group.

HBOT: Hyperbaric oxygen treatment; h: hour; d: day.

Early HBOT suppressed caspase-3 expression in rabbit brain tissues around the injury site

Caspase-3 activation is a marker of apoptosis in its irreversible stage^[24], and, as such, is used as a major marker for apoptosis. Immunohistochemistry showed little caspase-3 expression in the cerebral cortex of control group rabbits. However, caspase-3 expression was detected in the brain tissues around the injury site 1

hour after the blast. Levels then gradually increased, peaked at 24 hours, gradually decreased and were restored to normal by day 14 ($P > 0.05$). Caspase-3 expression in brain tissues around the injury site was decreased in the early stage (1–24 hours post-blast injury) of HBOT ($P < 0.05$) but remained higher than the control group ($P < 0.05$), and was restored to normal levels by day 14 ($P > 0.05$; Figure 4).

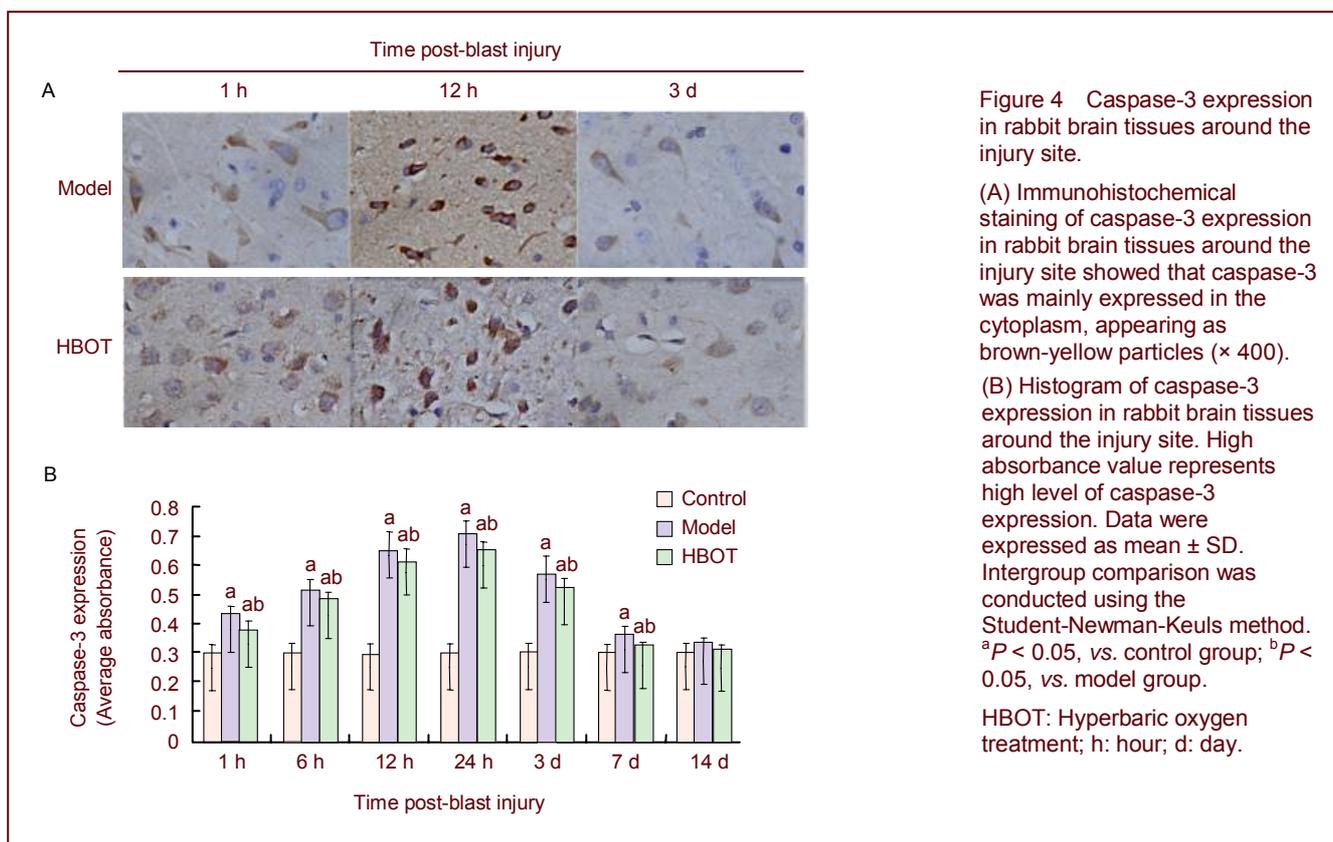


Figure 4 Caspase-3 expression in rabbit brain tissues around the injury site.

(A) Immunohistochemical staining of caspase-3 expression in rabbit brain tissues around the injury site showed that caspase-3 was mainly expressed in the cytoplasm, appearing as brown-yellow particles ($\times 400$).

(B) Histogram of caspase-3 expression in rabbit brain tissues around the injury site. High absorbance value represents high level of caspase-3 expression. Data were expressed as mean \pm SD. Intergroup comparison was conducted using the Student-Newman-Keuls method. ^a $P < 0.05$, vs. control group; ^b $P < 0.05$, vs. model group.

HBOT: Hyperbaric oxygen treatment; h: hour; d: day.

DISCUSSION

In the present study, cytochrome c expression was detected in the brain tissues surrounding the injury site 1 hour post-blast injury. This suggests that the blast induced ischemia and hypoxia in the brain tissues, leading to the production of active oxygen and increased intracellular Ca^{2+} concentrations. In turn, this would open the mitochondrial permeability transition pore and alter mitochondrial permeability. As a result, the mitochondrial membrane potential would be reduced, affecting normal respiratory chain function and leading to cytochrome c release into the cytoplasm^[25].

Cytochrome c expression peaked at 12 hours post-blast injury, possibly because the mitochondrial permeability transition pore was opened further and the ATP substrate in the mitochondrial matrix was transferred to the cytoplasm, resulting in the collapse of the

membrane potential, respiratory chain decoupling, ATP synthesis reduction and outer membrane rupture.

Cytochrome c levels were similar to those of the control group at 3 days post-blast injury, this indicates that its release occurred soon after blast injury. Indeed, cytochrome c was released into the cytoplasm prior to the peak in caspase-3 levels, which is consistent with its role in inducing caspase activation and cell apoptosis^[19-20, 26-29]. Both cytochrome c and caspase-3 expression in the brain tissues around the injury site was significantly reduced following HBOT, indicating that HBOT suppresses apoptosis^[21].

Factors that stimulate apoptosis can induce Bax to form a dimer and bind with the mitochondrial membrane to promote cytochrome c release^[30], while Bcl-2 stably binds the surface of the mitochondrial membrane at its C-end transmembrane domain and inhibits cytochrome c mitochondrial release^[31-32]. In the present study, Bax and Bcl-2 expression was detected

1 hour post-blast injury, and Bcl-2 expression peaked earlier than that of Bax. It is likely that Bcl-2 protein expression was enhanced post-blast injury in response to stress to protect neurons. However, over time, increasing apoptosis stimulating factors enhanced Bax expression, leading to apoptosis. After HBOT administration, Bax expression was reduced while Bcl-2 expression increased, suggesting that HBOT suppresses Bax but increases Bcl-2 in the early post-injury stage. Early HBOT may inhibit cell apoptosis through increasing Bcl-2 expression, reducing Bax expression, inhibiting cytochrome c release from mitochondria and suppressing caspase activation.

In summary, HBOT administered in the early stage post-injury can block mitochondrial cytochrome c release into the cytoplasm to some extent and block the mitochondrial pathway to suppress cell apoptosis. This mechanism is associated with its effects of inhibiting Bax expression and increasing Bcl-2 expression.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The study was performed at the Animal Experimental Center, Daping Hospital, Third Military Medical University of Chinese PLA, China between February and October 2009. Immunohistochemistry was conducted 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). They were housed at $26 \pm 1.5^\circ\text{C}$ with a humidity of 60%. Animal experimental 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^[33].

Methods

Establishment of blast brain injury model

Rabbits in model and HBOT groups were placed under a self-made explosion frame. A paper detonator (Chongqing 845 Factory, China) equivalent to 600 mg TriNitro Toluene (TNT) was fixed 6.5 cm above the rabbits' brains, 1.5 cm to the median, 2 cm anterior to the line of both ears to induce an explosion. The explosion

pressure was detected using a 113A31 sensor (PCB, Buffalo, NY, USA) and a Wavebook/516A data acquisition system^[34] (Japan; supplementary Video 1 online).

HBOT

Rabbits in the HBOT group underwent HBOT in a transparent oxygen animal experimental chamber, and were exposed to 2 atmosphere absolute, equal to 2.03 MPa. The rabbits were compressed with pure oxygen for 5 minutes, boosting pressure for 30 minutes, stabilizing pressure for 1 hour and reducing pressure for 30 minutes. This was performed twice a day, once in the morning and once in the afternoon, for 2 hours each time. Control and model group rabbits were exposed to air of normal pressure.

Preparation of brain tissue paraffin sections

Ten rabbits from each group were selected 1, 6, 12, and 24 hours, and 3, 7 and 14 days post-blast injury, anesthetized and sacrificed (supplementary Figure 1 online). Brain tissues surrounding the injury site (0.5 cm from the margin of injury, approximately 3.0 mm^3) were harvested, fixed in 4% paraformaldehyde, dehydrated, cleared, immersed in paraffin, embedded, and sectioned ($4 \mu\text{m}$ thick).

Immunohistochemistry for caspase-3, cytochrome c, Bax and Bcl-2 expression in brain tissues surrounding the injury site

The sections were fixed in paraformaldehyde for 15 minutes, washed with phosphate buffered saline (PBS), for 3×3 minutes, mixed with 3% H_2O_2 and left at room temperature for 10 minutes. They were then washed again with PBS for 3×3 minutes, mixed with normal goat serum blocking solution at room temperature for 30 minutes, and incubated with rabbit anti-caspase-3, cytochrome c, Bax and Bcl-2 polyclonal antibody (1:200; Boster, Wuhan, China) overnight at 4°C . The sections were rewarmed at room temperature for 10 minutes, washed with PBS for 3×5 minutes, then incubated with 100 μL goat anti-rabbit IgG (1:100; Boster) per section at 37°C for 30 minutes. They were again washed with PBS for 3×5 minutes, treated with 100 μL streptavidin-biotin complex solution (Boster) per section, washed with PBS for 3×5 minutes, 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 by light microscope (Nikon, Tokyo, Japan). Ten fields of view from each section ($400 \times$ magnification) were randomly selected and the mean

absorbance value was calculated using the JEDA 801D morphologic image analysis system (Molecular Devices, Sunnyvale, CA, USA) and MetaMorph software (Molecular Devices)^[35].

Statistical analysis

Data were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Differences at different time points between groups were compared using one-way analysis of variance, and paired comparisons were performed by the Student-Newman-Keuls method. A value of $P < 0.05$ was considered statistically significant.

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

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics Committee of 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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