Original Paper

Local Delivery of β -Elemene Improves Locomotor Functional Recovery by Alleviating Endoplasmic Reticulum Stress and Reducing Neuronal Apoptosis in Rats with Spinal Cord Injury

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Key Words

β -elemene • Spinal cord injury • Motor neurons • ER stress • Apoptosis

Abstract

Background/Aims: Spinal cord injury (SCI) is a serious global problem that leads to permanent motor and sensory deficits. This study explores the anti-apoptotic and neuroprotective effects of the natural extract β -elemene *in vitro* and in a rat model of SCI. **Methods:** CCK-8 assay was used to evaluate cell viability and lactate dehydrogenase assay was used to evaluate cytotoxicity. A model of cell injury was established using cobalt chloride. Apoptosis was evaluated using a fluorescence-activated cell sorting assay of annexin V-FITC and propidium iodide staining. A rat SCI model was created via the modified Allen's method and Basso, Beattie, and Bresnahan (BBB) scores were used to assess locomotor function. Inflammatory responses were assessed via enzyme-linked immunosorbent assay (ELISA). Apoptotic and surviving neurons in the ventral horn were respectively observed via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and Nissl staining. Western blotting was used to measure protein expression. **Results:** β -elemene (20 μ g/ml) promoted cell viability by activating phosphorylation of the PI3K-AKT-mTOR pathway. β -elemene reduced CoCl_2 -induced cellular death and apoptosis by suppressing the expression levels of CHOP, cleaved-caspase 12, 78-kilodalton glucose-regulated protein, cleaved-caspase 3, and the Bax/Bcl-2 ratio. In the rat model of SCI, Nissl and TUNEL staining showed that β -elemene promoted

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motor neuron survival and reduced neuronal apoptosis in the spinal cord ventral horn. BBB scores showed that β -elemene significantly promoted locomotor behavioral recovery after SCI. In addition, β -elemene reduced the ELISA-detected secretion of interleukin (IL)-6 and IL-1 β . **Conclusion:** β -elemene reduces neuronal apoptosis by alleviating endoplasmic reticulum stress *in vitro* and *in vivo*. In addition, β -elemene promotes locomotor function recovery and tissue repair in SCI rats. Thus, our study provides a novel encouraging strategy for the potential treatment of β -elemene in SCI patients.

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Introduction

Spinal cord injury (SCI) is a serious global problem that usually leads to permanent motor and sensory deficits, generating an enormous burden for patients, their relatives, and society [1, 2]. The number of people living with SCI around the world is estimated to be about 3 million, with 130,000 new patients reported each year [3, 4]. After the initial traumatic insult, secondary injury mechanisms begin, including neuronal apoptosis, edema, oxidative stress, vascular dysfunction, glutamate-mediated excitotoxicity, and inflammation, which result in continued and extensive tissue damage [1, 5-7]. Multiple studies in recent decades have demonstrated that neuronal apoptosis occurs in the early stage of SCI, resulting in massive neuronal loss [8-10]. Although the exact mechanisms of secondary injury are unclear, inhibition of the extensive neuronal death induced by apoptosis may become an effective therapy for SCI.

During SCI, protein misfolding appears due to alteration of the microenvironment in the injured area [11-13]. Then, three main stress sensors consisting of protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring protein 1 α (IRE1 α), and activating transcription factor 6 (ATF6) on the endoplasmic reticulum (ER) are stimulated by the accumulation of misfolded proteins, which induce ER stress [14]. Recent studies have indicated that prolonged activation of PERK signaling promotes cellular apoptosis while activation of ATF6 and IRE1 α promote cellular survival under ER stress conditions [15, 16]. Downregulation of ER stress might be able to reduce neuronal apoptosis and thereby encourage nerve function recovery and lessen pathological damage post-SCI [17-19].

β -elemene extracted from *Curcuma phaeocaulis* plant is well known as an antitumoral drug [20-23]. Although β -elemene has been widely used in the treatment of gliomas, its effects on neurons during the therapeutic course are unclear [24, 25]. Therefore, we investigated the effects of β -elemene on neurons. Intriguingly, our preliminary work showed that β -elemene significantly increased the cell viability of ventral spinal cord 4.1 (VSC4.1) motor neurons at concentrations lower than 40 μ g/ml. In addition, because β -elemene has cytoprotective effects against oxidative damage in human umbilical vein endothelial cells [26, 27], we next explored the effect of β -elemene on cobalt chloride (CoCl₂)-induced ER stress damage in VSC4.1 motor neurons. We also investigated whether β -elemene promotes the recovery of motor deficits in rats after SCI and explored the molecular mechanisms associated with the neuroprotective effects of β -elemene.

Materials and Methods

Cell culture and treatment

The VSC4.1 motor neuron cell line was used as previously described [28]. VSC4.1 motor neurons were grown in RPMI 1640 medium with fetal bovine serum (10%, v/v; Gibco, Invitrogen, Shanghai, China) and 1% penicillin and streptomycin at 37°C with 5% CO₂ in a fully humidified incubator, as recommended by the suppliers. For certain experiments, VSC4.1 motor neurons were plated in 96-well plates at a concentration of 5×10^3 cells per well. At first, β -elemene (Sigma-Aldrich, St. Louis, MO) was tested for its independent effects at six doses (5, 10, 20, 40, 80, and 160 μ g/ml) for 24 h and 48 h. The phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 (Sigma) and protein kinase B (Akt) inhibitor MK2206 (Sigma) were dissolved

in dimethyl sulfoxide. The working concentrations of LY294002 and MK2206 were 10 μ M and 1 μ M, respectively, as previously described [29-31]. With or without pretreatment with the above inhibitors for 1 h, VSC4.1 motor neurons were exposed to β -elemene (20 μ g/ml) for 24 h. Chemical hypoxic injury of VSC4.1 motor neurons was then induced by CoCl_2 (Sigma) in a dose-dependent manner (150, 300, 400, 500, 600, and 1200 μ M) for 24 h. Then, VSC4.1 motor neurons were pretreated with 20 μ g/ml β -elemene for 4 h to observe its protective effects on CoCl_2 (400, 500, and 600 μ M)-induced cellular damage. For western blotting, VSC4.1 motor neurons were treated with 20 μ g/ml β -elemene for 6 h, 12 h, 24 h, and 48 h. Additionally, with or without β -elemene (20 μ g/ml) pretreatment for 4 h, VSC4.1 motor neurons were exposed to CoCl_2 for 12 h or 24 h.

Cell Counting Kit-8 and lactate dehydrogenase release assays

The viability of VSC4.1 motor neurons was measured in a 96-well plate using a Cell Counting Kit-8 (CCK-8) kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions and as previously described [28]. After the designated treatments, the cells were cultured with 100 μ l CCK-8 mixture comprising 90 μ l medium and 10 μ l CCK-8 at 37°C for 2 h. Cell viability was determined by the absorbance at a 450-nm wavelength using a TECAN Infinite M5 microplate reader (Molecular Devices LLC, San Jose, CA). In addition, cultured supernatants were collected to measure the level of lactate dehydrogenase (LDH) using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Jiancheng Bioengineering Institute, Jiangsu, China).

Flow cytometry assay of apoptosis

Apoptosis of VSC4.1 motor neurons was detected by flow cytometry using annexin V/propidium iodide (PI) staining as previously described [32]. VSC4.1 motor neurons were plated at 1×10^5 cells per well in 6-well plates and pretreated with 20 μ g/ml β -elemene to observe its protective effects on CoCl_2 treatment (400, 500, and 600 μ M). After the treatment, VSC4.1 motor neurons were detached, washed, and incubated in 500 μ l binding buffer, 5 μ l annexin V-FITC, and 5 μ l PI (Invitrogen) at room temperature for 15 min in the dark. Cells were then detected through fluorescence-activated cell sorting with a Becton-Dickinson FACScan (Immunocytometry Systems, San Jose, CA). Annexin V-positive cells were labeled as apoptotic cells and annexin V-negative but PI-positive cells were marked as necrotic cells. The sum of the PI percentage and the annexin V percentage was used as the cell death percentage.

Spinal cord injury model

Adult female Sprague-Dawley rats (age, 6–8 weeks; weight range, 235–275 g) were obtained from the Animal Center of Zhejiang University (Zhejiang, China). The rats were housed in groups of 3 to 5 per cage at a temperature of $24 \pm 1^\circ\text{C}$ with a 12-h light/dark cycle and ad libitum access to water and food. The rats ($n = 75$) were randomly divided into five experimental groups as follows: Sham, Sham + high dose β -elemene, SCI, SCI + low dose β -elemene (80 μ g/kg), and SCI + high dose β -elemene (320 μ g/kg). All experimental procedures were approved by the Ethics Committee of the Second Affiliated Hospital of Medical School, Zhejiang University, and conducted in accordance with the Care and Use of Laboratory Animals guidelines of the National Science Council of the Republic of China.

The SCI rat model was established as described previously [32]. Briefly, experimental rats were deeply anesthetized with 2% isoflurane fixed with 100% O_2 at 400 ml/min. The dura was exposed at the T10 vertebral level via vertebral laminectomy. Subsequently, a 2-mm-diameter impactor (10 g) was dropped on the exposed spinal cord from a height of 50 mm to produce a moderate contusion via the New York University (NYU) II struck instrument model. The sham group rats were subjected to the same procedure without the contusion. After the injury, the contusion area of treatment groups was covered by gelatin sponges ($2 \times 2 \times 2 \text{ mm}^3$; Xiang En Medical Technology Development Company, Jiang Xi, China) containing β -elemene; control animals were administered sponges with an equal volume of normal saline.

Tissue processing

Two days after β -elemene treatment, for western blotting, 15 rats ($n = 3$ per group) were euthanized and the spinal cords at the T10 vertebral level were isolated rapidly and flash-frozen at -80°C . For Nissl and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, 20 rats ($n = 4$ per group) were intracardially perfused with 50 ml phosphate-buffered saline followed by 150 ml of 4%

paraformaldehyde solution. Subsequently, the spinal cords were dissected out and a 1-cm length of segment centered on and enclosing the injured site was removed and post-fixed in 4% paraformaldehyde at 4°C overnight. The cords were dehydrated in 30% sucrose solution at 4°C before being embedded in optimal cutting temperature compound (Sakura Finetech, Tokyo, Japan) and frozen. All samples were then cut into 10- μ m-thick sagittal sections via a cryostat (Thermo Fisher Scientific, Waltham, MA); these sections were mounted onto poly-L-lysine-coated glass slides.

Motor functional scale

Another 40 rats (n = 8 per group) were assessed after surgery for hind limb motor function on days 1, 3, 5, 7, 14, 21, and 28 post-injury using the Basso, Beattie, and Bresnahan (BBB) open field locomotor score, which comprises 21 different criteria for hind limb movement [33]. This scale is based upon accurate observation of hind limb stepping, joint movements, and coordination for 3 min in open field locomotion by a trained observer blinded to experimental conditions.

In situ TUNEL staining assay

Apoptotic neurons in the ventral horn of the spinal cord were analyzed by using a One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions and as described previously [32]. DNA fragments in the injured section were stained by the TUNEL method. Apoptotic neurons showed green fluorescence and the green fluorescent area as a proportion of the total area was used to calculate the percentage of apoptotic cells. Quantitative analysis was performed blindly by counting the number of TUNEL-positive cells in three cross sections per rat.

Nissl staining

Frozen sections (10- μ m-thick) of spinal cord at 2 days after surgery were washed twice with distilled water and immersed in Nissl staining solution (C0117; Beyotime Institute of Biotechnology) for 5 min as described previously [34]. The sections were then washed twice with distilled water, dehydrated in 95% ethanol, and mounted. Quantitative analysis was performed blindly by counting the number of Nissl-positive motor neurons in the anterior horn of the spinal cord from three cross sections per rat. Any neurons defined as non-basophilic neurons with both pale nuclei and discrete nuclei with nucleolar fragments larger than one-half of the average nucleolar diameter were included in the count, as well as neurons with intact neuronal bodies.

Western blot analysis

Tissue triturated via TissuePrep (Gering Scientific Instruments, Beijing, China) was dissolved in RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration (3 mg/ml) was quantified by a BCA kit (Beyotime Institute of Biotechnology). Cells were dissolved in western and IP lysis buffer (Beyotime Institute of Biotechnology) containing 1% protease/phosphatase inhibitor cocktail and 1% phenylmethanesulfonyl fluoride and centrifuged at 13,000 $\times$ g rpm for 10 min at 4°C. All samples (20 μ l) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were incubated at 4°C with primary antibodies overnight after being blocked in 5% skimmed milk powder dissolved in tris-buffered saline with Tween 20 (TBST) for 1 h. The following primary antibodies were used: caspase 12 (ab18766; 1:1000; Abcam, USA), CHOP (2895s; 1:1000; Cell Signaling Technology, Danvers, MA), Bcl-2 (2870s; 1:1000; Cell Signaling Technology), Bax (14796s; 1:1000; Cell Signaling Technology), ATF4 (11815s; 1:1000; Cell Signaling Technology), p-PERK (3179s; 1:1000; Cell Signaling Technology), p-eIF2 α (3179s; 1:1000; Cell Signaling Technology), cleaved-caspase 3 (9664s; 1:1000; Cell Signaling Technology), p-AKT (4060s; 1:1000; Cell Signaling Technology), AKT (9272s; 1:1000; Cell Signaling Technology), p-mTOR (5536s; 1:1000; Cell Signaling Technology), mTOR (2972s; 1:1000; Cell Signaling Technology), p-PI3K (ab182651; 1:1000; Abcam), PI3K (4292s; 1:1000; Cell Signaling Technology), HSPA5/78-kilodalton glucose-regulated protein (GRP78) (PB0669; 1:500; Boster; Wuhan, China), and β -actin (1:5000; Sigma-Aldrich). After washes with TBST, membranes were incubated with infrared-labeled secondary antibodies (Li-COR Biosciences, Lincoln, NE). An Odyssey infrared imaging system (LI-COR® Biosciences) was used to visualize the immunoblot bands. Band intensity was analyzed with Image Studio Ver. 5.2 and compared with the β -actin internal standard as described previously [28].

Enzyme-linked immunosorbent assay

For enzyme-linked immunosorbent assay (ELISA), rats ($n = 18$) were randomly divided into three experimental groups ($n = 6$ per group) as follows: Sham, SCI, and SCI+ high dose β -elemene ($320 \mu\text{g/kg}$). Segments of the spinal cord (15 mm) including the lesion epicenter at the center were collected 24 h after SCI and homogenized in lysis buffer containing protease inhibitors at 4°C . After 30 min, they were centrifuged at $13,000 \times g$ at 4°C for 10 min. Then, the protein concentrations of the tissue lysates were measured using a BCA protein assay kit and the lysates were diluted to a final concentration of 1 mg/ml. The ELISA kits for interleukin (IL)-1 β (RLB00) and IL-6 (R6000B) were purchased from R&D Systems (Minneapolis, USA). The content of each segment was obtained according to the standard curve at 450 nm minus that at 570 nm.

Statistical analysis

All data are presented as the mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) with Newman-Keuls post-hoc test or two-way repeated measures ANOVA with Bonferroni post-hoc test were used for comparison of multiple groups. Statistical significance was set at $p < 0.05$.

Results

Effects of β -elemene on the cell viability of VSC4.1 motor neurons under normal conditions

To assess the effect of β -elemene on the viability of VSC4.1 motor neurons, neurons were incubated with different concentrations of β -elemene (5, 10, 20, 40, 80, and $160 \mu\text{g/ml}$) for 24 h and 48 h. A CCK-8 assay showed that β -elemene treatment at concentrations lower than $40 \mu\text{g/ml}$ increased the viability of VSC4.1 motor neurons (Fig. 1A and B) and decreased cellular death as assessed by a LDH release assay (Fig. 1C and D). β -elemene at $20 \mu\text{g/ml}$ showed a maximal effect. The cell viability of VSC4.1 motor neurons was increased by $24.67\% \pm 1.32\%$ after 24 h ($p < 0.01$; Fig. 1A) and by $88.82\% \pm 13.51\%$ after 48 h ($p < 0.01$; Fig. 1B). On the other hand, cell death was significantly decreased by $27.99\% \pm 1.23\%$ after 48 h ($p < 0.01$; Fig. 1D). Moreover, β -elemene at the concentration of 80 or $160 \mu\text{g/ml}$ greatly decreased cell viability compared with the control group ($p < 0.01$). All of these results indicated that β -elemene significantly increased the cell viability of VSC4.1 motor neurons at concentrations lower than $40 \mu\text{g/ml}$ and was toxic at equal to or higher than $40 \mu\text{g/ml}$.

Fig. 1. Effects of β -elemene on the cell viability of VSC4.1 motor neurons under normal conditions. With different concentrations of β -elemene (5, 10, 20, 40, 80, and $160 \mu\text{g/ml}$) for (A, C) 24 h and (B, D) 48 h, (A, B) the cell viability of VSC4.1 motor neurons was assessed by a CCK-8 assay and (C, D) the cytotoxicity of β -elemene was measured by a LDH assay. Experiments were performed at least in triplicate. The given values are the mean $\pm$ SEM. $n = 4$, $*p < 0.05$, $**p < 0.01$ vs the control group.

Fig. 2. β -elemene activates the PI3K-AKT-mTOR signaling pathway in VSC4.1 motor neurons under normal conditions. With or without pretreatment with the PI3K inhibitor LY294002 (1 μ M) and the AKT inhibitor MK2206 (10 μ M) for 1 h, VSC4.1 motor neurons were exposed to β -elemene (20 μ g/ml) for 24 h. (A, B) Cell viability was assessed by a CCK-8 assay. (C) Expression levels of PI3K, AKT, mTOR, p-PI3K, p-AKT, and p-mTOR protein were analyzed by western blot in VSC4.1 motor neurons treated with β -elemene (20 μ g/ml) for 6 h, 12 h, 24 h, and 48 h. Corresponding densitometric analyses of the protein bands measured in the immunoblots and

normalized to the signal of β -actin are also shown. (D-F) Expression levels of proteins in each group are expressed as percentages of the control values. Experiments were performed at least in triplicate. Given values are the mean $\pm$ SEM. * p <0.05, ** p <0.01 vs the control group; ## p <0.01 vs the 20 μ g/ml β -elemene group.

β -elemene activates the PI3K-AKT-mTOR signaling pathway in VSC4.1 motor neurons under normal conditions

The promotive effect of β -elemene on the viability of VSC4.1 motor neurons was partially blocked by MK2206 and LY294002 ($114.20\% \pm 2.03\%$ vs $131.60\% \pm 2.65\%$; $122.10\% \pm 2.79\%$ vs $138.60\% \pm 2.75\%$, $p < 0.01$) (Fig. 2A and B). In addition, western blotting was performed to detect the expression levels of PI3K, p-PI3K, AKT, p-AKT, mTOR, and p-mTOR proteins in VSC4.1 motor neurons. β -elemene significantly increased the expression levels of p-PI3K, p-AKT, and p-mTOR proteins. The phosphorylation of PI3K, AKT, and mTOR peaked at 12 h and lasted until 48 h (5.82 ± 0.71 vs control; 2.77 ± 0.12 vs control; 4.24 ± 0.81 vs control, $p < 0.01$), whereas the expression levels of total PI3K, AKT, and mTOR were not significantly altered (Fig. 2C-F).

β -elemene protects VSC4.1 motor neurons against CoCl_2 -induced cell death

To establish a cell injury model, VSC4.1 motor neurons were exposed to different concentrations of CoCl_2 (0, 150, 300, 400, 500, 600, and 1200 μ M) for 24 h. A CCK-8 assay showed that cell viability was significantly decreased in a dose-dependent manner by CoCl_2 ($p < 0.01$; Fig. 3A and B). VSC4.1 motor neurons were then pretreated with 20 μ g/ml β -elemene for 4 h and then with varying concentrations of CoCl_2 (400, 500, and 600 μ M) for 24 h. The CCK-8 assay of the three CoCl_2 treatment groups showed that cell viability in the β -elemene pretreatment groups significantly increased from $50.98\% \pm 2.46\%$ to $86.89\% \pm 1.25\%$, from $39.14\% \pm 4.13\%$ to $75.23\% \pm 6.22\%$, and from $31.37\% \pm 3.14\%$ to 67.73%

Fig. 3. β -elemene protects VSC4.1 motor neurons against CoCl_2 -induced cell death. Different concentrations of CoCl_2 (0, 150, 300, 400, 500, 600, and 1200 μM) were added to the VSC4.1 motor neuron culture medium to establish a hypoxic injury model. (A) Cell viability was assessed by a CCK-8 assay and (B) cytotoxicity was measured by a LDH assay. With or without β -elemene (20 $\mu\text{g}/\text{ml}$) pretreatment for 4 h, VSC4.1 motor neurons were exposed to CoCl_2 (400, 500, and 600 μM) for 24 h. (C) Cell viability was assessed by a CCK-8 assay. Experiments were performed at least in triplicate. Given values are the mean $\pm$ SEM. $n = 4$, ** $p < 0.01$ vs the control group; ## $p < 0.01$ vs the 400 μM CoCl_2 group; && $p < 0.01$ vs the 500 μM CoCl_2 group; \$\$ $p < 0.01$ vs the 600 μM CoCl_2 group.

$\pm 6.10\%$, respectively ($p < 0.01$; Fig. 3C). These data indicated that β -elemene was able to protect VSC4.1 motor neurons from cell death induced by CoCl_2 .

β -elemene protects VSC4.1 motor neurons against CoCl_2 -induced apoptosis

VSC4.1 motor neurons were stained with annexin V-FITC/PI after pretreatment with or without β -elemene for 4 h and then exposed to CoCl_2 for 24 h. Flow cytometry analysis revealed a significant increase in the apoptosis of VSC4.1 motor neurons subjected to CoCl_2 . VSC4.1 motor neurons pretreated with β -elemene showed a marked reduction in apoptosis compared with the CoCl_2 group (400, 500, and 600 μM) ($136.60\% \pm 5.09\%$ vs $227.30\% \pm 10.70\%$; $191.20\% \pm 3.20\%$ vs $375.10\% \pm 7.67\%$; $317.10\% \pm 7.36\%$ vs $514.60\% \pm 7.38\%$, respectively; $p < 0.01$; Fig. 4).

β -elemene alleviates ER stress in VSC4.1 motor neurons treated with CoCl_2 through inhibition of the PERK-eIF2 α -ATF4 pathway

As shown in Fig. 5, CoCl_2 induced ER stress by activating the phosphorylation of the PERK-eIF2 α -ATF4 pathway. After 12 h of CoCl_2 stimulation, the expression levels of p-PERK, p-eIF2 α , and ATF4 proteins were significantly increased to 12.02 ± 2.38 -, 7.76 ± 0.48 -, and 6.71 ± 0.63 -fold of the control group. However, the expression levels of p-PERK, p-eIF2 α , and ATF4 were significantly lower in the β -elemene pretreatment group than in the CoCl_2 group (6.01 ± 0.71 vs 13.13 ± 2.55 ; 6.00 ± 0.59 vs 8.81 ± 0.41 ; 4.29 ± 1.06 vs 7.57 ± 0.82 , respectively; $p < 0.01$). Similarly, the expression levels of cleaved-caspase 12, CHOP, and GRP78 (a marker protein that reflects the severity of ER stress) showed the same trend between the β -elemene pretreatment group and the CoCl_2 group (3.21 ± 0.77 vs 6.31 ± 1.07 ; 1.43 ± 0.16 vs 1.86 ± 0.15 ; 2.19 ± 0.14 vs 3.29 ± 0.19 , respectively; $p < 0.05$). Subsequently, after 24 h of CoCl_2 stimulation, the increased expression levels of cleaved-caspase 3 and Bax in the CoCl_2 group were significantly attenuated by β -elemene pretreatment, from $4.58 \pm$

Fig. 4. β -elemene protects VSC4.1 motor neurons against CoCl_2 -induced apoptosis. (A) With or without β -elemene (20 $\mu\text{g}/\text{ml}$) pretreatment for 4 h, VSC4.1 motor neurons were exposed to CoCl_2 (500 μM) for 24 h and cellular apoptosis was assessed by flow cytometry. (B) Data from experiments performed at least in triplicate are presented, showing the percentage of apoptosis (early apoptosis percentage plus late apoptosis percentage). Given values are the mean $\pm$ SEM. ** $p < 0.01$ vs the control group; ## $p < 0.01$ vs the 400 μM CoCl_2 group; && $p < 0.01$ vs the 500 μM CoCl_2 group; \$\$ $p < 0.01$ vs the 600 μM CoCl_2 group.

0.24-fold of control to 2.77 ± 0.31 -fold of control and from 2.62 ± 0.12 -fold of control to 2.01 ± 0.10 -fold of control, respectively ($p < 0.05$). In addition, the decreased expression level of Bcl-2 in the CoCl_2 group was significantly increased by β -elemene pretreatment, from 0.16 ± 0.05 -fold of control to 0.47 ± 0.04 -fold of control ($p < 0.05$).

β -elemene decreases neuronal apoptosis in the ventral horn after SCI

To explore the effect of β -elemene on motor neurons in the spinal cord after traumatic injury, Nissl staining and TUNEL assay were performed on spinal cord sections obtained on day 2 after SCI. The percentage of apoptotic cells was significantly higher in the SCI group than in the sham group ($78.41\% \pm 2.86\%$ vs $0.46\% \pm 0.26\%$, $p < 0.01$). In addition, the number of surviving motor neurons was significantly lower in the SCI group than in the sham group ($50.00\% \pm 4.36$ vs $126.70\% \pm 6.49$, $p < 0.01$). After β -elemene treatment, the β -elemene-treated group showed more surviving motor neurons and a lower percentage of apoptotic cells compared with the SCI group ($79.67\% \pm 6.69\%$ vs $50.00\% \pm 4.36\%$; $34.24\% \pm 4.38\%$ vs $78.41\% \pm 2.86\%$, respectively; $p < 0.01$) (Fig. 6 and 7).

Fig. 5. β -elemene alleviates ER stress in VSC4.1 motor neurons induced by CoCl_2 by inhibiting the activation of the PERK-eIF2 α -ATF4 pathway. (A) Expression levels of p-PERK, p-eIF2 α , ATF4, GRP78, CHOP, cleaved-caspase 12, cleaved-caspase 3, Bcl-2, and Bax were analyzed by western blot. (B-D) Quantification of the optical densities of the protein bands to determine the levels of protein expressed as percentages of control values. Experiments were performed at least in triplicate. Given values are the mean $\pm$ SEM. ** $p < 0.01$ vs the control group; ## $p < 0.01$ vs the 500 μM CoCl_2 group.

Fig. 6. β -elemene enhances the survival of motor neurons in the ventral horn after SCI in rats. (A) Nissl staining of motor neurons of the ventral horn in all groups at 2 days after treatment. Magnification: 100 $\times$ (A-up) and 200 $\times$ (A-down). Scale bar: 200 μm . (B) Data from multiple experimental replicates are presented, indicating the percentage of surviving motor neurons. The given values are the mean $\pm$ SEM. ** $p < 0.01$ vs the sham group; ## $p < 0.01$ vs the SCI + vehicle group.

Fig. 7. β -elemene decreases neuronal apoptosis in the ventral horn after SCI. (A) Spinal cord sections in the injured region were stained with TUNEL (green) and DAPI (blue). Magnification: 100 $\times$. Scale bar: 100 μ m. (B) Insert shows the region of interest. (C) Data from multiple experimental replicates are presented, indicating the percentage of neuronal apoptosis. Given values are the mean $\pm$ SEM. ** $p < 0.01$ vs the sham group; ## $p < 0.01$ vs the SCI + vehicle group.

β -elemene alleviates ER stress induced by SCI in the spinal cord

After SCI, ER stress occurs in the spinal cord lesion. Western blotting was performed to detect the expression levels of cleaved-caspase 12, CHOP, GRP78, Bcl-2, Bax, and cleaved-caspase 3 in the spinal cord. The expression levels of cleaved-caspase 12, CHOP, GRP78, Bax, and cleaved-caspase 3 were significantly increased in the SCI group compared with the sham group, whereas the expression level of Bcl-2 was significantly reduced (Fig. 8). However, the levels of cleaved-caspase 12, CHOP, GRP78, Bax, and cleaved-caspase 3 were reduced compared with the SCI group (2.42 ± 0.43 vs 4.35 ± 0.49 ; 2.05 ± 0.17 vs 3.18 ± 0.34 ; 3.71 ± 0.52 vs 5.54 ± 0.59 ; 2.11 ± 0.29 vs 3.46 ± 0.49 ; 2.22 ± 0.35 vs 4.34 ± 0.95 , respectively; $p < 0.01$) and the expression level of Bcl-2 was significantly increased (0.36 ± 0.05 vs 0.69 ± 0.05 , $p < 0.01$) in the high-dose β -elemene-treated group.

β -elemene reduces the inflammatory response and promotes the recovery of locomotor function in rats with SCI

To assess the effect of β -elemene on neuroinflammation, ELISA was performed to evaluate the secretion of IL-6 and IL-1 β in rats after SCI. As shown in Fig. 9A and B, β -elemene markedly reduced the secretion of IL-6 and IL-1 β . Furthermore, locomotor function recovery was evaluated using the BBB locomotor scores (Fig. 9C). All rats had a normal score of 21 before SCI induction, and the score dropped to zero at day 1 after SCI. However, the BBB score showed a significantly improved locomotor functional recovery in the 320 μ g/kg β -elemene-treated group compared with the SCI group from 14 days to 28 days of the post-treatment period time (9.69 ± 0.73 vs 5.13 ± 0.59 , 10.06 ± 0.64 vs 5.56 ± 0.59 , 10.25 ± 0.59 vs 6.00 ± 0.53 , respectively; $p < 0.01$).

Fig. 8. β -elemene alleviates ER stress induced by SCI in the spinal cord. (A) Expression levels of GRP78, CHOP, cleaved-caspase 12, cleaved-caspase 3, Bcl-2, and Bax after SCI with or without β -elemene treatment were analyzed by western blot. (B–D) Corresponding densitometric analyses of the protein bands measured in the immunoblots and normalized to the signal of β -actin are shown. The expression levels of proteins in each group are expressed as the percentage of the control value. The given values are the mean $\pm$ SEM. ** $p < 0.01$ vs the sham group; ## $p < 0.01$ vs the SCI + vehicle group.

Fig. 9. β -elemene reduces the inflammatory response and promotes the recovery of locomotor function after SCI. (A, B) Expression levels of IL-6 and IL-1 β were evaluated via ELISA. (C) BBB open field scores 1, 3, 5, 7, 14, 21, and 28 days after SCI in rats with or without β -elemene (80 and 320 μ g/kg). The BBB score was significantly improved in the 320 μ g/kg β -elemene-treated rats from 14 days to 28 days after SCI. The given values are the mean $\pm$ SEM. $n = 8$ /group. * $p < 0.05$, ** $p < 0.01$ vs the SCI + vehicle group.

Discussion

Previous studies have indicated that β -elemene has cytoprotective effects against oxidative damage in HUVECs [26]. Nevertheless, no *in vivo* or *in vitro* data had yet been reported for motor neurons. Therefore, we investigated the use of an *in vitro* model of VSC4.1 motor neuron cultures damaged by CoCl_2 and an *in vivo* model of spinal cord contusion injury to examine whether β -elemene has neuroprotective effects.

We first examined the effect of β -elemene on VSC4.1 motor neurons. The results showed that the appropriate concentration of β -elemene promotes the viability of motor neurons, which can be blocked by PI3K or AKT inhibitors. Numerous studies have demonstrated that cell proliferation can be induced through the PI3K-AKT-mTOR signaling pathway [35, 36]. Similarly, our results showed that the phosphorylation of PI3K, AKT, and mTOR is induced by β -elemene, revealing that β -elemene promotes VSC4.1 motor neuronal proliferation through the PI3K-AKT-mTOR signaling pathway under normal conditions.

ER stress is one of the main molecular events underlying the pathology of SCI, activating three main sensors consisting of PERK, IRE1, and ATF6 to remove misfolded or unfolded proteins and rebalance the intercellular homeostasis as part of the unfolded protein response

[37]. After the activation of ER stress, PERK dimerizes and phosphorylates, resulting in the phosphorylation of the eukaryotic translation initiator factor 2 α (eIF2 α), which can arrest protein synthesis and eventually alleviate the overload of misfolded proteins inside the ER [38]. Then, phosphorylation of eIF2 α promotes the translation of ATF4, which is a pivotal transcription factor involved in the regulation of genes related to protein folding, apoptosis, and autophagy [39, 40]. Finally, CHOP controlled by ATF4 can be upregulated, promoting cellular apoptosis [14, 41]. Moreover, caspase 12 as a key protein activated by ER stress eventually activates caspase 3 to induce the cellular apoptosis. Cobalt is appropriate for mimicking hypoxic conditions in cultured cells due to its ability to activate hypoxic signals by stabilizing the expression of hypoxia-inducible factor-1 α [42, 43]. Thus, CoCl₂ was used to mimic hypoxic conditions in VSC4.1 motor neurons. Using this system, we demonstrated that CoCl₂ was able to induce VSC4.1 motor neuronal ER stress and cause apoptosis in a dose-dependent manner. Our results are consistent with previous reports that the PERK-eIF2 α -ATF4 pathway was activated by CoCl₂ [44]. Then, we explored the effect of β -elemene on neuronal apoptosis induced by ER stress, with our results suggesting that β -elemene reduces VSC4.1 motor neuronal apoptosis by inhibiting the phosphorylation of PERK and eIF2 α and the expression of ATF4. Furthermore, we investigated whether β -elemene affects the expression of the ER stress-related proteins (cleaved-caspase 12, CHOP, and GRP78) and downstream mitochondria-related proteins (Bax, Bcl-2, and cleaved-caspase 3), which determine susceptibility to apoptosis [45, 46]. Our results showed that β -elemene reduces caspase 3 and caspase 12 activity and increases the Bcl-2/Bax ratio, indicating that β -elemene inhibits VSC4.1 motor neuronal apoptosis. The expression of GRP78, which is responsible for targeting misfolded protein for degradation, is reduced by β -elemene, indicating that β -elemene can reduce the severity of ER stress in VSC4.1 motor neurons induced by CoCl₂. Regrettably, we have not yet explored the effect of β -elemene on primary motor neurons.

SCI is a common central nervous system condition without an effective therapeutic strategy. The secondary injury, particularly neuronal apoptosis, leads to severe disability [1, 6, 47]. No therapy can completely improve the motor and sensory capabilities lost after SCI [48]. We demonstrated that there were fewer apoptotic and more surviving motor neurons in the ventral horn of the spinal cord in the β -elemene treatment group compared with the SCI group, suggesting that β -elemene can protect motor neurons against apoptosis after SCI. Indeed, activation of ER stress has been described in animal models of SCI [49]. For instance, activation of ATF6 processing and phosphorylation of eIF2 α were reported in a rat model of contusion SCI, and nuclear localization of CHOP has been shown in motor neurons and oligodendrocytes, but not in astrocytes [17]. Induction of ER stress has also been observed in motor neurons after SCI [50]. Therefore, we explored whether β -elemene has neuroprotective effects *in vivo* against motor neuronal apoptosis by alleviating the severity of ER stress after SCI. Our study showed that β -elemene can protect motor neurons against apoptosis after SCI by reducing the expression of activated caspase 12 and CHOP and by increasing the Bcl-2/Bax ratio. In addition, abundant evidence has shown that neuroinflammation rapidly occurs during SCI, which further results in tissue damage and becomes a catalyst for further damage [51, 52]. Thus, we performed ELISA to show the change in the expression of inflammatory factors (IL-6, IL-1 β), with our results showing that β -elemene alleviates the inflammatory response in rats after SCI. In future work, we will focus on the effects of β -elemene on neuroinflammation. Ultimately, we used BBB scores to evaluate the behavioral performances of the rat hindlimbs after SCI. A significant improvement in BBB scores was discovered from days 7 to 28 in the 320 μ g/kg β -elemene treatment group compared with the SCI group, suggesting that β -elemene treatment may improve behavioral performance in the acute stage of SCI.

Conclusion

In summary, this study reveals the neuroprotective effects of β -elemene in the treatment of SCI *in vivo* and *in vitro*. The appropriate concentration of β -elemene can promote the proliferation of VSC4.1 motor neurons by activating the PI3K-AKT-mTOR pathway. β -elemene protects VSC4.1 motor neurons against apoptosis induced by CoCl_2 by downregulating the PERK pathway during ER stress, as well as by downregulating CHOP, grp78, cleaved-caspase 12, cleaved-caspase 3, and the Bax/Bcl-2 ratio. In addition, β -elemene alleviates the motor neuronal apoptosis *in vivo* by downregulating caspase 12, CHOP, and Bax and upregulating Bcl-2 and promotes the recovery of locomotor function in SCI rats. The BBB scores suggest that β -elemene promotes behavioral recovery after SCI. These results offer insight into the role of β -elemene in regulating neuron survival of the cellular pathology associated with SCI.

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Disclosure Statement

The authors declare that they have no conflict of interests.

References

- 1 Silva NA, Sousa N, Reis RL, Salgado AJ: From basics to clinical: a comprehensive review on spinal cord injury. *Prog Neurobiol* 2014;114:25-57.
- 2 Zhu LL, Li MQ, He F, Zhou SB, Jiang W: Mitochondria Targeted Peptide Attenuates Mitochondrial Dysfunction, Controls Inflammation and Protects Against Spinal Cord Injury-Induced Lung Injury. *Cell Physiol Biochem* 2017;44:388-400.
- 3 Dobkin BH, Havton LA: Basic Advances and New Avenues in Therapy of Spinal Cord Injury. *Ann Rev Med* 2004;55:255-282.
- 4 Anna Z, Katarzyna J-W, Joanna C, Barczewska M, Joanna W, Wojciech M: Therapeutic Potential of Olfactory Ensheathing Cells and Mesenchymal Stem Cells in Spinal Cord Injuries. *Stem Cells Int* 2017;2017:3978595.
- 5 Tohda C, Kuboyama T: Current and future therapeutic strategies for functional repair of spinal cord injury. *Pharmacol Ther* 2011;132:57-71.
- 6 Zhang D, Ma G, Hou M, Zhang T, Chen L, Zhao C: The Neuroprotective Effect of Puerarin in Acute Spinal Cord Injury Rats. *Cell Physiol Biochem* 2016;39:1152-1164.
- 7 He J, Zhao J, Peng X, Shi X, Zong S, Zeng G: Molecular Mechanism of MiR-136-5p Targeting NF-kappaB/A20 in the IL-17-Mediated Inflammatory Response after Spinal Cord Injury. *Cell Physiol Biochem* 2017;44:1224-1241.
- 8 Nicola FD, Marques MR, Odorczyk F, Arcego DM, Petenuzzo L, Aristimunha D, Vizuete A, Sanches EF, Pereira DP, Maurmann N, Dalmaz C, Pranke P, Netto CA: Neuroprotector effect of stem cells from human exfoliated deciduous teeth transplanted after traumatic spinal cord injury involves inhibition of early neuronal apoptosis. *Brain Res* 2017;1663:95-105.

- 9 He Z, Zhou Y, Huang Y, Wang Q, Zheng B, Zhang H, Li J, Liu Y, Wu F, Zhang X, Tong S, Wang M, Wang Z, He H, Xu H, Xiao J: DL-3-n-butylphthalide improves functional recovery in rats with spinal cord injury by inhibiting endoplasmic reticulum stress-induced apoptosis. *Am J Transl Res* 2017;9:1075-1087.
- 10 Yeo JE, Kim JH, Kang SK: Selenium attenuates ROS-mediated apoptotic cell death of injured spinal cord through prevention of mitochondria dysfunction; *in vitro* and *in vivo* study. *Cell Physiol Biochem* 2008;21:225-238.
- 11 Balch WE, Morimoto RI, Dillin A, Kelly JW: Adapting proteostasis for disease intervention. *Science* 2008;319:916-919.
- 12 Gao Y, Yang H, Chi J, Xu Q, Zhao L, Yang W, Liu W, Yang W: Hydrogen Gas Attenuates Myocardial Ischemia Reperfusion Injury Independent of Postconditioning in Rats by Attenuating Endoplasmic Reticulum Stress-Induced Autophagy. *Cell Physiol Biochem* 2017;43:1503-1514.
- 13 Chong WC, Shastri MD, Eri R: Endoplasmic Reticulum Stress and Oxidative Stress: A Vicious Nexus Implicated in Bowel Disease Pathophysiology. *Int J Mol Sci* 2017;18:pii:E771.
- 14 Cornejo VH, Pihan P, Vidal RL, Hetz C: Role of the unfolded protein response in organ physiology: lessons from mouse models. *IUBMB Life* 2013;65:962-975.
- 15 Hetz C, Chevet E, Oakes SA: Proteostasis control by the unfolded protein response. *Nat Cell Biol* 2015;17:829-838.
- 16 Wang M, Kaufman RJ: Protein misfolding in the endoplasmic reticulum as a conduit to human disease. *Nature* 2016;529:326-335.
- 17 Penas C, Guzman MS, Verdu E, Fores J, Navarro X, Casas C: Spinal cord injury induces endoplasmic reticulum stress with different cell-type dependent response. *J Neurochem* 2007;102:1242-1255.
- 18 Ohri SS, Maddie MA, Zhang Y, Shields CB, Hetman M, Whittemore SR: Deletion of the Pro-Apoptotic Endoplasmic Reticulum Stress Response Effector CHOP Does Not Result in Improved Locomotor Function after Severe Contusive Spinal Cord Injury. *J Neurotrauma* 2012;29:579-588.
- 19 Bi Y, Zhu Y, Zhang M, Zhang K, Hua X, Fang Z, Zhou J, Dai W, Cui Y, Li J, You T: Effect of Shikonin on Spinal Cord Injury in Rats Via Regulation of HMGB1/TLR4/NF-kB Signaling Pathway. *Cell Physiol Biochem* 2017;43:481-491.
- 20 Wu J, Tang Q, Yang L, Chen Y, Zheng F, Hann SS: Interplay of DNA methyltransferase 1 and EZH2 through inactivation of Stat3 contributes to beta-elemene-inhibited growth of nasopharyngeal carcinoma cells. *Sci Rep* 2017;7:509.
- 21 Li X, Lin Z, Zhang B, Guo L, Liu S, Li H, Zhang J, Ye Q: beta-elemene sensitizes hepatocellular carcinoma cells to oxaliplatin by preventing oxaliplatin-induced degradation of copper transporter 1. *Sci Rep* 2016;6:21010.
- 22 Zhu T, Li X, Luo L, Wang X, Li Z, Xie P, Gao X, Song Z, Su J, Liang G: Reversion of malignant phenotypes of human glioblastoma cells by beta-elemene through beta-catenin-mediated regulation of stemness-, differentiation- and epithelial-to-mesenchymal transition-related molecules. *J Transl Med* 2015;13:356.
- 23 Liang D, Yang M, Guo B, Yang L, Cao J, Zhang X: HIF-1 α induced by beta-elemene protects human osteosarcoma cells from undergoing apoptosis. *J Cancer Res Clin Oncol* 2012;138:1865-1877.
- 24 Mu L, Wang T, Chen Y, Tang X, Yuan Y, Zhao Y: beta-Elemene enhances the efficacy of gefitinib on glioblastoma multiforme cells through the inhibition of the EGFR signaling pathway. *Int J Oncol* 2016;49:1427-1436.
- 25 Yao YQ, Ding X, Jia YC, Huang CX, Wang YZ, Xu YH: Anti-tumor effect of beta-elemene in glioblastoma cells depends on p38 MAPK activation. *Cancer Lett* 2008;264:127-134.
- 26 Chen J, Duan W, Bai R, Yao H, Shang J, Xu J: Design, synthesis and antioxidant activity evaluation of novel beta-elemene derivatives. *Bioorg Med Chem Lett* 2014;24:3407-3411.
- 27 Chen JC, Duan WL, Bai RR, Yao HQ, Wu XM, Shang J, Xu JY: Synthesis of 13-beta-elemene ester derivatives and evaluation of their antioxidant activity in human umbilical vein endothelial cells. *Chin J Nat Med* 2015;13:618-627.
- 28 Gu C, Li H, Wang C, Song X, Ding Y, Zheng M, Liu W, Chen Y, Zhang X, Wang L: Bone marrow mesenchymal stem cells decrease CHOP expression and neuronal apoptosis after spinal cord injury. *Neurosci Lett* 2017;636:282-289.
- 29 Taiyab A, Korol A, Deschamps PA, West-Mays JA: beta-Catenin/CBP-Dependent Signaling Regulates TGF-beta-Induced Epithelial to Mesenchymal Transition of Lens Epithelial Cells. *Invest Ophthalmol Vis Sci* 2016;57:5736-5747.

- 30 Ying C, Chen L, Wang S, Mao Y, Ling H, Li W, Zhou X: Zeaxanthin ameliorates high glucose-induced mesangial cell apoptosis through inhibiting oxidative stress via activating AKT signalling-pathway. *Biomed Pharmacother* 2017;90:796-805.
- 31 Ji S, Lin W, Wang L, Ni Z, Jin F, Zha X, Fei G: Combined Targeting of mTOR and Akt Using Rapamycin and MK-2206 in The Treatment of Tuberous Sclerosis Complex. *J Cancer* 2017;8:555-562.
- 32 Wang C, Shi D, Song X, Chen Y, Wang L, Zhang X: Calpain inhibitor attenuates ER stress-induced apoptosis in injured spinal cord after bone mesenchymal stem cells transplantation. *Neurochem Int* 2016;97:15-25.
- 33 Basso DM, Beattie MS, Bresnahan JC: A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995;12:1-21.
- 34 Ooigawa H, Nawashiro H, Fukui S, Otani N, Osumi A, Toyooka T, Shima K: The fate of Nissl-stained dark neurons following traumatic brain injury in rats: difference between neocortex and hippocampus regarding survival rate. *Acta Neuropathol* 2006;112:471-481.
- 35 Zhang J, Ye J, Yuan C, Fu Q, Zhang F, Zhu X, Wang L, Gao P, Shu G, Jiang Q, Wang S: Exogenous H₂S exerts biphasic effects on porcine mammary epithelial cells proliferation through PI3K/Akt-mTOR signaling pathway. *J Cell Physiol* 2018; DOI:10.1002/jcp.26630.
- 36 Yao X, Yan C, Zhang L, Li Y, Wan Q: LncRNA ENST00113 promotes proliferation, survival, and migration by activating PI3K/Akt/mTOR signaling pathway in atherosclerosis. *Medicine (Baltimore)* 2018;97:e0473.
- 37 Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D: Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2000;2:326-332.
- 38 Harding HP, Zhang Y, Ron D: Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 1999;397:271-274.
- 39 Ameri K, Harris AL: Activating transcription factor 4. *Int J Biochem Cell Biol* 2008;40:14-21.
- 40 Lange PS, Chavez JC, Pinto JT, Coppola G, Sun CW, Townes TM, Geschwind DH, Ratan RR: ATF4 is an oxidative stress-inducible, prodeath transcription factor in neurons *in vitro* and *in vivo*. *J Exp Med* 2008;205:1227-1242.
- 41 Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D: CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev* 1998;12:982-995.
- 42 Lopez-Sanchez LM, Jimenez C, Valverde A, Hernandez V, Penarando J, Martinez A, Lopez-Pedraza C, Munoz-Castaneda JR, De la Haba-Rodriguez JR, Aranda E, Rodriguez-Ariza A: CoCl₂, a mimic of hypoxia, induces formation of polyploid giant cells with stem characteristics in colon cancer. *PLoS One* 2014;9:e99143.
- 43 Yang G, Xu S, Peng L, Li H, Zhao Y, Hu Y: The hypoxia-mimetic agent CoCl₂ induces chemotherapy resistance in LOVO colorectal cancer cells. *Mol Med Rep* 2016;13:2583-2589.
- 44 Fu P, Wu Q, Hu J, Li T, Gao F: Baclofen Protects Primary Rat Retinal Ganglion Cells from Chemical Hypoxia-Induced Apoptosis Through the Akt and PERK Pathways. *Front Cell Neurosci* 2016;10:255.
- 45 Garcia de la Cadena S, Massieu L: Caspases and their role in inflammation and ischemic neuronal death. *Focus on caspase-12. Apoptosis* 2016;21:763-777.
- 46 Sadidi M, Lentz SJ, Feldman EL: Hydrogen peroxide-induced Akt phosphorylation regulates Bax activation. *Biochimie* 2009;91:577-585.
- 47 Kucera KL, Yau RK, Register-Mihalik J, Marshall SW, Thomas LC, Wolf S, Cantu RC, Mueller FO, Guskiewicz KM: Traumatic Brain and Spinal Cord Fatalities Among High School and College Football Players - United States, 2005-2014. *MMWR Morb Mortal Wkly Rep* 2017;65:1465-1469.
- 48 Barnabe-Heider F, Frisen J: Stem cells for spinal cord repair. *Cell Stem Cell* 2008;3:16-24.
- 49 Aufenberg C, Wenkel S, Mautes A, Paschen W: Spinal cord trauma activates processing of xbp1 mRNA indicative of endoplasmic reticulum dysfunction. *J Neurotrauma* 2005;22:1018-1024.
- 50 Liu S, Sarkar C, Dinizo M, Faden AI, Koh EY, Lipinski MM, Wu J: Disrupted autophagy after spinal cord injury is associated with ER stress and neuronal cell death. *Cell Death Dis* 2015;6:e1582.
- 51 David S, Kroner A: Repertoire of microglial and macrophage responses after spinal cord injury. *Nat Rev Neurosci* 2011;12:388-399.
- 52 Bell MT, Puskas F, Agoston VA, Cleveland JC, Jr., Freeman KA, Gamboni F, Herson PS, Meng X, Smith PD, Weyant MJ, Fullerton DA, Reece TB: Toll-like receptor 4-dependent microglial activation mediates spinal cord ischemia-reperfusion injury. *Circulation* 2013;128:S152-156.