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Influence of Tanshinone IIa on heat shock protein 70, Bcl-2 and Bax expression in rats with spinal ischemia/reperfusion injury☆

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Abstract

Tanshinone IIa is an effective monomer component of *Danshen*, which is a traditional Chinese medicine for activating blood circulation to dissipate blood stasis. Tanshinone IIa can effectively improve brain tissue ischemia/hypoxia injury. The present study established a rat model of spinal cord ischemia/reperfusion injury and intraperitoneally injected Tanshinone IIa, 0.5 hour prior to model establishment. Results showed that Tanshinone IIa promoted heat shock protein 70 and Bcl-2 protein expression, but inhibited Bax protein expression in the injured spinal cord after ischemia/reperfusion injury. Furthermore, Nissl staining indicated a reduction in nerve cell apoptosis and fewer pathological lesions in the presence of Tanshinone IIa, compared with positive control *Danshen* injection.

Key Words

Tanshinone IIa; *Danshen*; spinal ischemia/reperfusion injury; heat shock protein 70; Bcl-2; Bax; cell apoptosis; Chinese medicine; neural regeneration

Research Highlights

Tanshinone IIa reduced nerve cell apoptosis and effectively improved rat spinal ischemia/reperfusion injury by influencing heat shock protein 70, Bcl-2 and Bax expression.

Abbreviations

HSP70, heat shock protein 70; ELISA, enzyme linked immunosorbent assay

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INTRODUCTION

After ischemia/reperfusion injury, the spinal cord restores for a period of time, however function is greatly damaged, often resulting in irreversible, delayed death of spinal neurons[1]. Studies have shown that cell apoptosis is common in spinal nerve cells after spinal cord ischemia/reperfusion injury[3-7]. Heat shock protein 70 (HSP70) is a stress-protecting protein. Mitochondrial HSP70 may promote apoptosis by repairing or preventing degradation of cell apoptosis inhibitory protein, Bcl-2[8-11]. Bcl-2 and Bax are important cell apoptosis-regulatory genes. Bcl-2 inhibits apoptosis and Bax promotes apoptosis. The ratio of Bcl-2 to Bax controls the occurrence of apoptosis, whereby an increased ratio of Bcl-2 to Bax promotes cell survival and a decreased ratio promotes apoptosis[12-14].

In vitro studies have demonstrated that the required rate of *Danshen* injection is 65% to clear hydroxy radicals in the H_2O_2-Fe^{2+} system and 100% to clear superoxide anions in the xanthine-xanthine oxidase system[15].

Tanshinone IIa is an effective monomer component of Danshen and a representative liposoluble constituent. It participates in various biochemical events in organisms and exhibits bioactivities to antagonize oxidation and blood coagulation[16]. Bi *et al* [17] report that Tanshinone IIa rapidly distributes to tissues within 15 minutes after intravenous injection. Another study showed that Tanshinone IIa is slowly metabolized *in vivo* and remains detectable in tissues 20 hours after administration[18]. In addition, Tanshinone IIa has been shown to promote HSP70 expression, induce upregulation of Bcl-2 protein expression and inhibit Bax protein expression, thereby reducing nerve cell apoptosis and protecting the brain against ischemia/reperfusion injury[19-20].

Based on the important regulatory effects of HSP70, Bcl-2 and Bax in nerve cell apoptosis, the present study investigated the influence of Tanshinone IIa on apoptosis-related protein HSP70, Bcl-2 and Bax expression in spinal nerve cell apoptosis after ischemia/reperfusion injury, to explore the neuroprotective effect and mechanism of action for Tanshinone IIa.

RESULTS

Quantitative analysis of experimental animals

A total of 120 Sprague Dawley rats were used and randomly assigned to sham-surgery, model, *Danshen* (positive control) and Tanshinone IIa groups. The model, *Danshen* and Tanshinone IIa groups were subjected to spinal ischemia/reperfusion injury, and the *Danshen* and Tanshinone IIa groups were intraperitoneally injected with *Danshen* and sodium Tanshinone IIa sulfonate injection respectively, 0.5 hour prior to model establishment. Six rats were excluded due to failed model establishment or death. After supplementation, 120 rats were included in the final analysis, and six from each group were selected at 0.5, 1, 4, 8 and 12 hours after reperfusion for observation.

Tanshinone IIa improved spinal cord pathology of rats with spinal ischemia/reperfusion injury

Nissl staining showed no significant pathological changes in the spinal cord of the sham-surgery group. Spinal neuron volume was diminished or deformed with mild swelling at 0.5 and 1 hour post ischemia/reperfusion, accompanied by decreased Nissl bodies, but unchanged nuclei. At 4 hours, neuron morphologies were altered and some cells were swollen or broken, observed with an obscure outline, unclear boundary and disorder arrangement, Nissl bodies were significantly decreased, in some cases absent, or with light stain and karyopyknosis. At 8 and 12 hours, the number of neurons was gradually reduced and those remaining had an incomplete appearance with dissolved Nissl bodies in the cytoplasm. The boundary between the nuclei and cytoplasm was unclear, processes were decreased or absent and spaces were detected around the neurons. These pathological changes were significantly attenuated following intraperitoneal injection of *Danshen* and sodium Tanshinone IIa sulfonate injection. Moreover, the number of Nissl bodies was reduced to a greater extent in the Tanshinone IIa group compared with the *Danshen* group (Figure 1).

Influence of Tanshinone IIa on HSP70, Bcl-2 and Bax expression in the spinal cord of rats with spinal ischemia/reperfusion injury

Enzyme linked immunosorbent assay (ELISA) showed low expression of HSP70 and Bcl-2 and no expression of Bax in the spinal cord of the sham-surgery group. At 0.5 hours post ischemia/reperfusion, HSP70, Bcl-2 and Bax expression were increased in the spinal cord compared with the sham group ($P < 0.01$), with gradual increase over time. HSP70, Bcl-2 and Bax expression were significantly greater in the *Danshen* and Tanshinone IIa groups compared with the model group ($P < 0.01$; Tables 1–3, Figure 2).

DISCUSSION

Under normal physiological conditions, Nissl bodies are abundant and large in nerve cells, reflecting their predominant function of synthesizing protein. However, following neuronal injury, the number of Nissl bodies is significantly reduced and in some cases absent[21]. Results from the present study showed no obvious pathological changes in the spinal cord of the sham-surgery group. Motor neurons displayed a complete appearance, Nissl bodies were abundant in the neuronal body with dark blue staining and irregular size, blue-stained axons were detected and nuclei were round, stained light blue with clear nucleoli. After spinal cord injury, neuron size was reduced, mild swelling of deformations was observed and Nissl bodies were dissolved, with unclear boundaries between the nucleus and cytoplasm. Axons were reduced or absent and spaces appeared around the neurons. Cell morphology was gradually restored and the number of Nissl bodies gradually increased after treatment with *Danshen* and Tanshinone IIa. In particular, the treatment effect was superior in the presence of Tanshinone IIa.

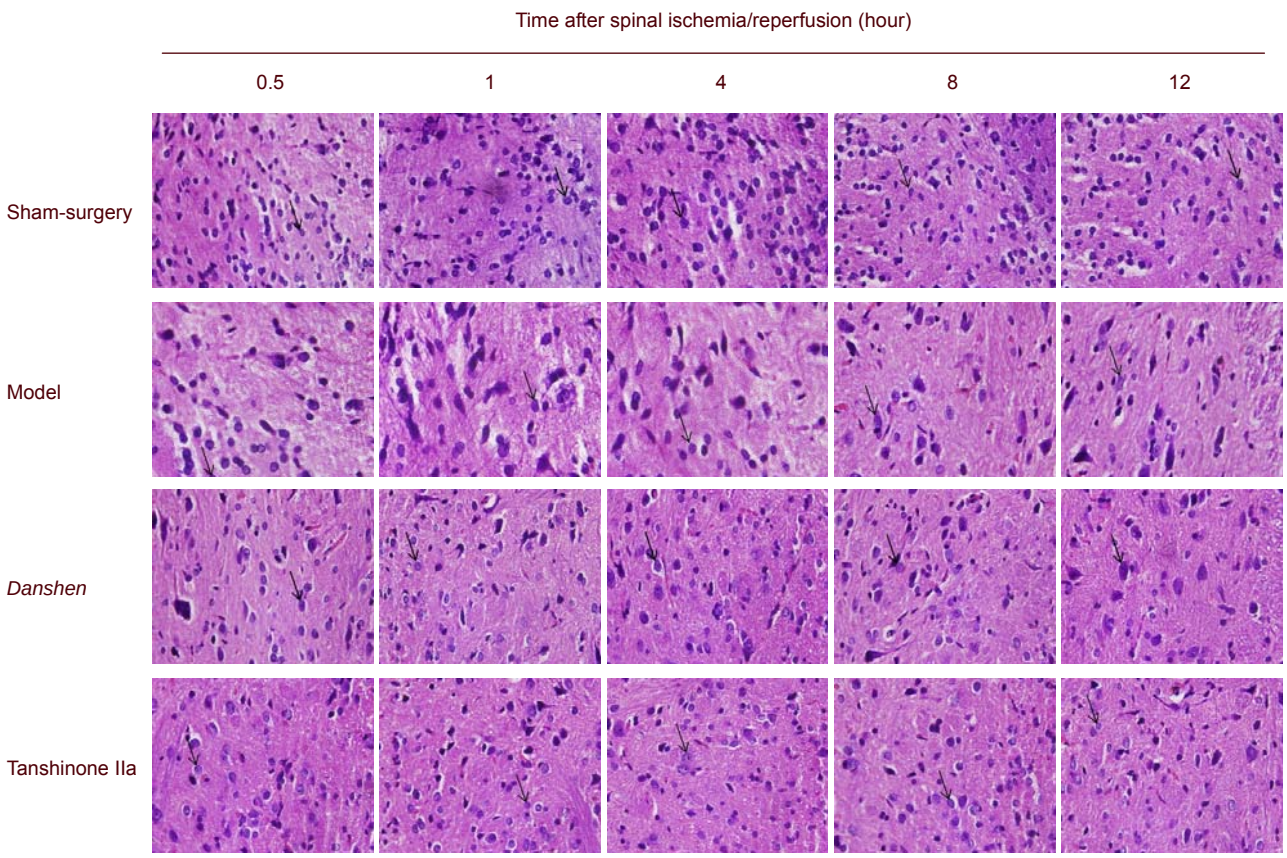

Figure 1 Spinal cord morphology (Nissl staining, light microscope, × 400).

At 0.5 and 1 hour after ischemia, the volume of some neurons was diminished or mildly deformed, and Nissl body number was slightly reduced, with nuclei remaining unchanged. With increasing time, pathological changes worsened. *Danshen* and sodium Tanshinone IIa sulfonate injection significantly improved neuronal morphology.

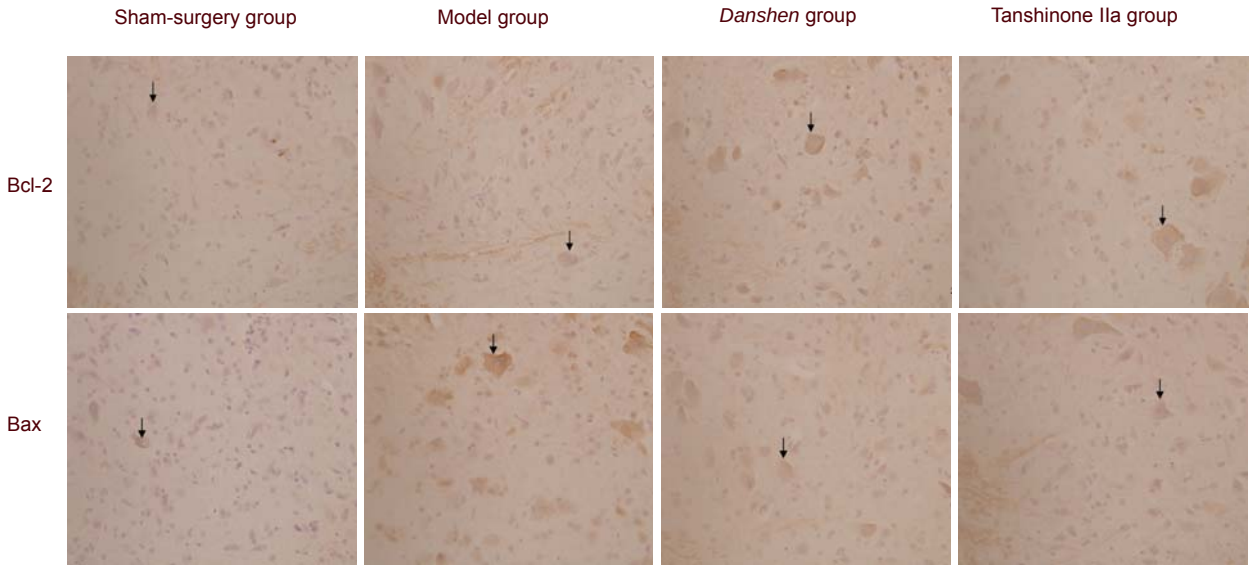

Figure 2 Bcl-2 and Bax expression in spinal cord of rats after ischemia/reperfusion for 12 hours (× 400). Arrows represent positive expression.

Table 1 Heat shock protein 70 expression (μg/mg) in the spinal cord of rats with ischemia/reperfusion injury

Group	Time after model establishment (hour)		
	0.5	1	4
Sham-surgery	333.0±34.8	346.1±36.1	350.2±28.7
Model	466.6±23.2[a]	617.2±41.6[a]	996.5±60.2[a]
Danshen	587.3±28.8[ab]	748.8±50.2[ab]	1 253.6±60.9[ab]
Tanshinone IIa	728.6±39.2[abc]	920.2±55.1[abc]	1 459.7±121.1[abc]

Group	Time after model establishment (hour)	
	8	12
Sham-surgery	336.1±27.8	339.1±31.00
Model	1 287.7±47.4[a]	1 479.3±59.9[a]
Danshen	1 497.2±62.2[ab]	1 711.8±55.2[ab]
Tanshinone IIa	1 701.5±66.6[abc]	1 897.9±65.3[abc]

Data are expressed as mean ± SD of six rats from each group at each time point. Intergroup differences were compared using a two-sample *t*-test. [a]*P* < 0.01, *vs*. sham-surgery group; [b]*P* < 0.01, *vs*. model group; [c]*P* < 0.01, *vs*. *Danshen* group.

Table 2 Bcl-2 expression (μg/mg) in the spinal cord of rats with ischemia/reperfusion injury

Group	Time after model establishment (hour)		
	0.5	1	4
Sham-surgery	63.5±6.0	61.61±5.8	59.9±7.12
Model	124.8±17.2[a]	221.6±20.5[a]	418.8±17.3[a]
Danshen	183.6±11.5[ab]	313.6±13.2[ab]	507.8±18.2[ab]
Tanshinone IIa	256.3±12.0[abc]	368.6±38.1[abc]	584.8±15.4[abc]

Group	Time after model establishment (hour)	
	8	12
Sham-surgery	61.7±5.9	62.2±5.8
Model	528.1±19.9[a]	615.1±18.6[a]
Danshen	610.9±14.5[ab]	703.0±18.9[ab]
Tanshinone IIa	733.3±25.4[abc]	781.7±26.6[abc]

Data are expressed as mean ± SD of six rats from each group at each time point. Intergroup differences were compared using a two-sample *t*-test. [a]*P* < 0.01, *vs*. sham-surgery group; [b]*P* < 0.01, *vs*. model group; [c]*P* < 0.01, *vs*. *Danshen* group.

HSP70 can inhibit cell apoptosis[22]. HSP70 expression can be used to identify neuronal injury in the central nervous system and to evaluate efficacy of some prevention or treatment methods for central nervous system injury[23-24]. Studies show HSP70 can facilitate protein degradation and reduce activation of various proteases and nucleate endonuclease through ion channels inhibition of cell apoptosis[25]. Results from the present study showed that 0.5 hours after ischemia/reperfusion, HSP70, Bcl-2 and Bax expression were observed in the spinal cord and gradually increased with time, significantly higher than in the sham-surgery group. Moreover, HSP70, Bcl-2 and Bax expression in the spinal cord, following *Danshen* and Tanshinone IIa injection, were significantly higher than in the model group.

In conclusion, Tanshinone IIa may attenuate spinal ischemia/reperfusion injury by promoting HSP70 and Bcl-2 expression and downregulating Bax expression.

Table 3 Bax expression (μg/mg) in the spinal cord of rats with ischemia/reperfusion injury

Group	Time after model establishment (hour)		
	0.5	1	4
Sham-surgery	0.64±0.09	0.62±0.09	0.58±0.07
Model	6.55±0.45[a]	8.75±0.51[a]	14.42±1.31[a]
Danshen	4.59±0.12[ab]	6.62±0.44[ab]	11.20±0.85[ab]
Tanshinone IIa	2.60±0.12[ab]	4.63±0.33[abc]	8.59±0.52[abc]

Group	Time after model establishment (hour)	
	8	12
Sham-surgery	0.59±0.08	0.57±0.09
Model	21.36±1.19[a]	26.32±1.89[a]
Danshen	15.67±1.78[ab]	21.80±1.46[ab]
Tanshinone IIa	11.07±1.09[abc]	16.36±1.57[abc]

Data are expressed as mean±SD of six rats from each group at each time point. Intergroup differences were compared using a two-sample *t*-test. [a]*P* < 0.01, *vs*. sham-surgery group; [b]*P* < 0.01, *vs*. model group; [c]*P* < 0.01, *vs*. *Danshen* group.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was performed in the Laboratory of College of Life Science, Fujian Normal University, China from December 2010 to June 2011.

Materials

Animals

A total of 126 Sprague Dawley rats, of clean grade, either female or male, aged 4 months, weighing 250 ± 10 g, were provided by the Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China (license No. 2007000508444). Animals were housed for adaptability for 1 week at 25 ± 2°C and fed with common food. All experiments 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[25].

Drugs

Tanshinone IIa injection was purchased from Shanghai

No.1 Biochemical Pharmaceutical Co., Ltd., Shanghai, China (No. H19999195; 100213). The main component was sodium Tanshinone IIa sulfonate, with the chemical formula R-C_6H_4-SO_3Na (R = C_{10}–C_{13}) and molecular weight 340–352. *Danshen* injection was purchased from Chiatai Qingchunbao Pharmaceutical Co., Ltd., Shanghai, China (No. Z33020177; 0911053) and stored at 4°C. 1 mL *Danshen* injection was equal to 1 g *Danshen*.

Methods

Establishment of spinal ischemia/reperfusion injury model

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 g/kg). Rats were fixed in the supine position on the sterile operating table. The middle of the abdomen was shaved, disinfected with iodophor, deiodinated with alcohol and a median-right incision was made at the abdomen. The abdominal aorta to the level of the renal artery was separated and the right renal artery at the proximal end was clamped with a Scoville-Lewis clip to occlude the abdominal aorta blood flow. The incision was sutured layer by layer and the abdominal cavity was temporarily closed. The artery was declamped to restore blood flow after 0.5 hour and the abdominal cavity was closed (Figure 3). Rats were observed using digital subtraction radiography (Siemens, Berlin, Germany) and the model was identified as successful with complete spinal ischemia[26]. The abdominal aorta to the level of the renal artery was separated alone in the sham-surgery group.

Intraperitoneal injection of Danshen and Tanshinone IIa injection

Rat dosage was determined according to the following administration formula: rat dose = human dose × human conversion factor (36.8)/rat conversion factor (6.9)[27]. The *Danshen* and Tanshinone IIa groups were injected with 0.891 5 mL/kg *Danshen* and 3.565 2 mg/kg Tanshinone IIa injection respectively, 0.5 hours prior to model establishment.

Sampling

Rats were anesthetized and fixed in the prone position. The back was shaved and a median incision was made at the lumbar vertebra. Under sterile conditions, the bilateral sacrospinal muscles were cut and separated, and the spinous process and vertebral plate were removed. The nerve root was cut using a sharp blade to fully expose and harvest the spinal cord at $L_{2\text{-}5}$ levels. The spinal cord was divided into two segments for Nissl staining and ELISA.

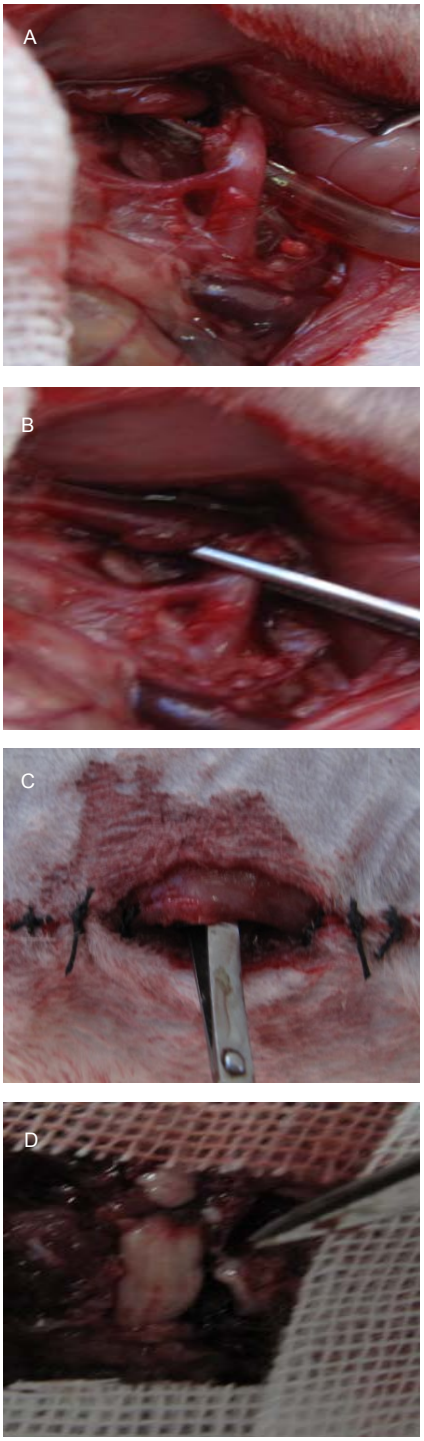

Figure 3 Establishment of spinal cord ischemia/reperfusion model.

(A) The abdominal aorta to the level of the renal artery was separated.

(B) The right renal artery at the proximal end was clamped.

(C)The abdominal cavity was temporarily closed.

(D) The spinous process and vertebral plate were removed, the nerve root was dissected to expose $L_{2\text{-}5}$ segments.

Nissl staining for morphology of spinal cord nerve cells

Spinal cord tissues were fixed in 4% paraformaldehyde for 24 hours, washed with distilled water for 4 hours to remove the paraformaldehyde solution, dehydrated, cleared and embedded. Samples were embedded by immersion in a mixture of Creosote, benzene and paraffin wax at 45°C, followed by twice immersion in paraffin wax at 54°C, for 2 hours each time. The embedded tissues were sectioned at a thickness of about 7 μm, adhered to gelatin-treated glass slides and incubated at 65°C overnight. Sections were dewaxed, mixed with 1% toluidine blue solution at 50°C, stained at 56°C and washed with water prior to differentiation with alcohol. Samples were then stained with eosin, dehydrated, cleared and mounted with neutral gum prior to observation using a light microscope (Suzhou Easymicro, Suzhou, China).

ELISA for HSP70, Bcl-2 and Bax expression in the spinal cord

Spinal cord tissues were prepared into a homogenate, diluted five times and incubated at 37°C for 30 minutes. Coated wells were washed for four times, mixed with 50 μL of enzyme conjugate (Wuhan Boster, Wuhan, China) and incubated at 37°C for 30 minutes. The wells were washed four times, mixed with 50 μL of chromogenic agent B solution (Wuhan Boster), shaken gently for 30 seconds and colorized in the dark at 37°C for 15 minutes. The reaction was terminated with stop buffer (Wuhan Boster). The blank well was zeroed. Absorbance of each well at 450 nm was determined using a microplate reader (BioTek, Winooski, VT, USA) within 15 minutes. The linear regression equation of the standard curve was calculated according to the standard sample concentration and corresponding absorbance. Sample concentration was calculated using the regression equation according to sample absorbance. Terminal concentration was then determined by multiplying the sample concentration by the dilution multiple, 5.

Statistical analysis

Data were expressed as mean ± SD and analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA). Intergroup mean differences were compared using one-way analysis of variance and a two-sample *t*-test. A value of $P < 0.05$ was considered statistically significant.

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Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethics.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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