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# Chloride channel blocker 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid inhibits nitric oxide-induced apoptosis in cultured rat hippocampal neurons★

Jinbao Yin<sup>1,2</sup>, Lijuan Xu<sup>1</sup>, Shuling Zhang<sup>1</sup>, Yuanyin Zheng<sup>1</sup>, Zhichao Zhong<sup>1</sup>, Hongling Fan<sup>1</sup>, Xi Li<sup>1</sup>, Quanzhong Chang<sup>1</sup>

1 Department of Physiology, Zhuhai Campus of Zunyi Medical College, Zhuhai 519041, Guangdong Province, China

2 Department of Pathology, Dongguan Campus of Guangdong Medical College, Dongguan 523808, Guangdong Province, China

## Abstract

Apoptosis in cultured rat hippocampal neurons was induced using the nitric oxide donor 3-morpholinosydnonimine, and cells were treated with the chloride channel blocker, 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid. Results showed that the survival rate of neurons was significantly increased after treatment with 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid, and the rate of apoptosis decreased. In addition, the expression of the apoptosis-related proteins poly(adenosine diphosphate-ribose)polymerase-1 and apoptosis-inducing factor were significantly reduced. Our experimental findings indicate that the chloride channel blocker 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid can antagonize apoptotic cell death of hippocampal neurons by inhibiting the expression of the apoptosis-related proteins poly(adenosine diphosphate-ribose)polymerase-1 and apoptosis-inducing factor.

Jinbao Yin★, Master.

Corresponding author:  
Quanzhong Chang,  
Professor, Ph.D., Master's  
supervisor, Department of  
Physiology, Zhuhai Campus  
of Zunyi Medical College,  
Zhuhai 519041, Guangdong  
Province, China,  
cqzchang@sina.com.

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## Key Words

neural regeneration; brain injury; chloride channel; 3-morpholinosydnonimine; hippocampus; poly(adenosine diphosphate-ribose)polymerase-1; apoptosis inducing factor; neuronal apoptosis; grants-supported paper; photographs-containing paper; neuroregeneration

## Research Highlights

- (1) 3-morpholinosydnonimine (1.0 mM) can significantly induce apoptosis in rat hippocampal neurons.
- (2) When poly(adenosine diphosphate-ribose)polymerase-1 and apoptosis-inducing factor were simultaneously expressed, neuronal apoptosis was evident.
- (3) Poly(adenosine diphosphate-ribose)polymerase-1 and apoptosis-inducing factor contribute to neural damage following ischemia.
- (4) 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid (0.1 mM) can antagonize the expression of poly(adenosine diphosphate-ribose)polymerase-1 and apoptosis-inducing factor via a caspase independent signaling pathway.
- (5) The chloride channel blocker 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid can protect neurons by inhibiting the caspase-dependent and -independent signaling pathways.

## Abbreviations

PARP-1, poly(adenosine diphosphate-ribose)polymerase-1; AIF, apoptosis-inducing factor; DIDS, 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid; NO, nitric oxide; SIN-1, 3-morpholinosydnonimine

## INTRODUCTION

Several signaling molecules are involved in neuronal apoptosis following ischemic injury<sup>[1-2]</sup>. Following injury, secondary apoptotic signaling pathways are triggered, which include caspase-dependent and -independent pathways.

Poly(adenosine diphosphate-ribose)polymerase-1 (PARP-1) activation can lead to the activation of apoptosis-inducing factor (AIF), resulting in apoptosis<sup>[3-5]</sup>. The chloride channel blocker 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) has been shown to reduce caspase-3 activity and inhibit nitric oxide (NO)-induced neuronal apoptosis in rat hippocampal neurons<sup>[6]</sup>. However, there is little evidence regarding the influence of DIDS on PARP-1/AIF, which is a key signaling molecule of the caspase-independent apoptosis pathway<sup>[6]</sup>.

This study aimed to explore the influence of the chloride channel blocker DIDS on hippocampal apoptotic cell death, and monitor the expression of the apoptosis-related proteins PARP-1 and AIF in cultured rat hippocampal neurons following treatment with the NO donor 3-morpholinosydnonimine (SIN-1).

## RESULTS

### Identification of *in vitro* cultured rat hippocampal neurons

At 12 days after culture, rat hippocampal neurons were subjected to Hoechst 33258 and anti-NeuN staining (a neuronal marker). Results showed that the majority of cultured cells (more than 90%) were neurons (Figure 1).

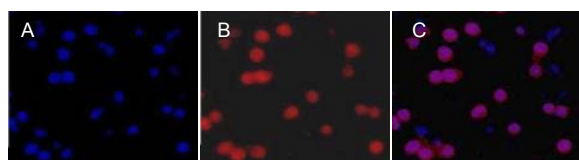


Figure 1 Morphology of rat hippocampal neurons 12 days after culture (inverted fluorescence microscope,  $\times 200$ ).

(A) Hoechst 33258 staining shows that normal neurons stained blue (Hoechst 33258 in blue).

(B) The neuronal antibody anti-NeuN was indicated by red staining (Cy3 labeling in red).

(C) Merged image of A and B. Neurons were simultaneously positive for Hoechst 33258 and the neuron-specific antibody NeuN.

### Effect of DIDS on SIN-1-induced neuronal apoptosis

After rat hippocampal neurons were cultured for 12 days *in vitro*, they were randomly divided into three groups, namely a normal control group (normal culture), SIN-1 group (ischemic neuronal apoptosis model), and a SIN-1 + DIDS group (ischemic neuronal apoptosis model treated with DIDS). The results of a MTT assay demonstrated that the survival rate of neurons in the SIN-1 + DIDS group was significantly higher than that of the SIN-1 group ( $P < 0.05$ ; Figure 2).

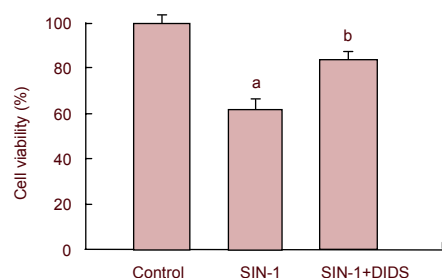


Figure 2 Change in survival rate of rat hippocampal neurons following 3-morpholinosydnonimine (SIN-1) treatment.

<sup>a</sup> $P < 0.05$ , vs. control group; <sup>b</sup> $P < 0.05$ , vs. SIN-1 group. Data are expressed as mean  $\pm$  SD,  $n = 6$ , by one-way analysis of variance and the least significant difference *t*-test. The experiment was repeated in triplicate. The survival rate (%) = (absorbance value of experimental group – absorbance value of blank control group) / (absorbance value of control group – absorbance value of blank control group)  $\times 100\%$ .

### Effect of DIDS on SIN-1-induced neuronal apoptosis as assessed by Hoechst 33258 staining

Hoechst 33258 staining revealed very few apoptotic neurons in the control group, but a large number of apoptotic neurons in the SIN-1 group. The number of apoptotic neurons in the SIN-1 + DIDS group was significantly lower than that in the SIN-1 group ( $P < 0.05$ ; Figure 3).

### Expression of PARP-1-positive neurons in rat hippocampus

Immunostaining revealed minimal expression of PARP-1 in control neurons, whereas strong expression was evident following SIN-1 treatment. PARP-1 expression significantly decreased following treatment with DIDS (Figure 4).

### Effect of DIDS on AIF expression in rat hippocampal neurons

Immunostaining revealed that the expression of AIF in rat hippocampal neurons was significantly increased in the SIN-1 group, but was significantly decreased after treatment with the chloride channel blocker DIDS (Figure 5).

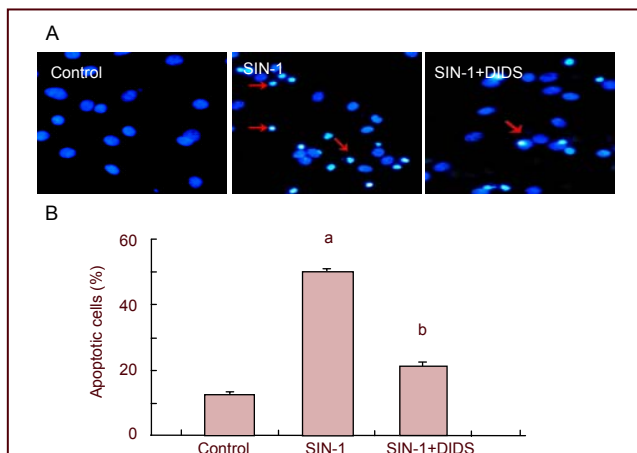


Figure 3 Apoptotic neurons in rat hippocampus.

(A) Morphology of cultured rat hippocampal neurons following DNA fluorescent staining (Hoechst 33258 staining, inverted fluorescence microscope,  $\times 200$ ). Arrows indicate apoptotic neurons (Hoechst 33258-positive cells displayed strong blue fluorescence). Apoptotic cells were evident in the 3-morpholinosydnonimine (SIN-1; 1.0 mM) group, but fewer were visible in the SIN-1 + DIDS group.

(B) Quantification of apoptotic cells. <sup>a</sup> $P < 0.05$ , vs. control group; <sup>b</sup> $P < 0.05$ , vs. SIN-1 group. Data are expressed as mean  $\pm$  SD, the experiment was repeated in triplicate, by one-way analysis of variance and least significant difference *t*-test. In total, 200 cells were randomly selected within three fields of view to calculate the rate of apoptosis (percentage).

SIN-1: 3-morpholinosydnonimine; DIDS: 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid.

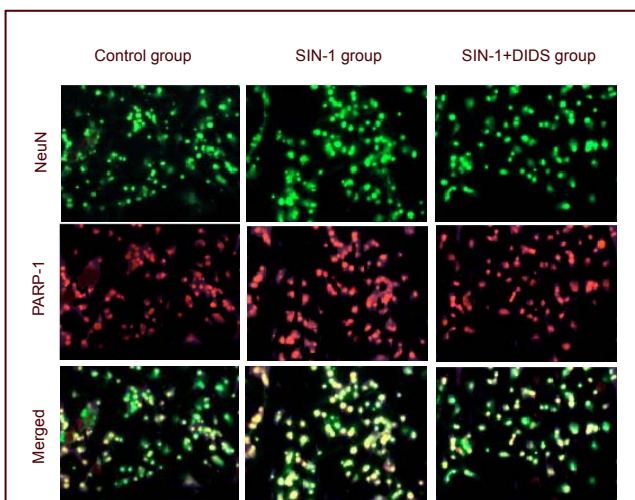


Figure 4 Expression of poly(adenosine diphosphate-ribose)polymerase-1 (PARP-1) in rat hippocampal neurons (inverted fluorescence microscope,  $\times 200$ ).

The specific neuronal antibody anti-NeuN was green (FITC labeled) and PARP-1 was red (Cy3 labeled). PARP-1 expression was significantly increased in the SIN-1 group, and was significantly decreased after treatment with the chloride channel blocker DIDS.

SIN-1: 3-morpholinosydnonimine; DIDS: 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid.

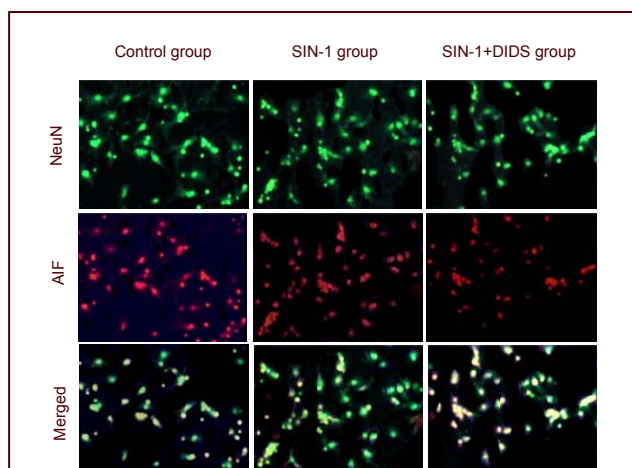


Figure 5 Immunofluorescence staining of apoptosis-inducing factor (AIF) in rat hippocampal neurons (inverted fluorescence microscope,  $\times 200$ ).

The specific neuronal antibody anti-NeuN was green (FITC labeled), AIF expression was significantly increased in the SIN-1 group (Cy3 labeled), and significantly decreased after treatment with the chloride channel blocker DIDS.

SIN-1: 3-morpholinosydnonimine; DIDS: 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid.

### PARP-1 and AIF protein expression in rat hippocampal neurons

Western blot analysis revealed that PARP-1 and AIF protein expression was significantly upregulated in the SIN-1 group compared with the control group. DIDS was able to downregulate PARP-1 and AIF protein expression (Figure 6).

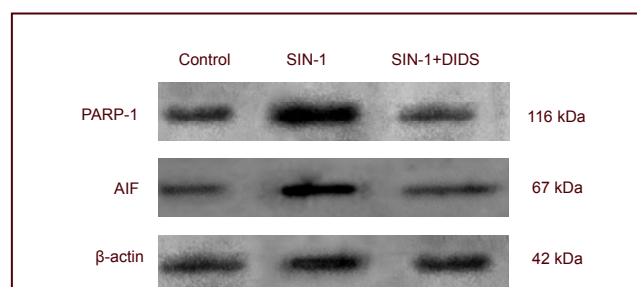


Figure 6 Poly(adenosine diphosphate-ribose) polymerase-1 (PARP-1) and apoptosis-inducing factor (AIF) protein expression in rat hippocampal neurons.

Expression levels in the SIN-1 group were significantly higher than those in the control group. DIDS downregulated PARP-1 and AIF expression.

SIN-1: 3-morpholinosydnonimine; DIDS: 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid.

## DISCUSSION

Apoptosis is the main form of neuronal death following brain ischemia, and toxicity caused by the

overproduction of NO is one of the mechanisms that underlie neuronal apoptosis<sup>[7-8]</sup>. SIN-1 is the main NO donor, and can be decomposed to produce NO and O<sup>2-</sup> in aqueous solution. In addition, it can form OONO<sup>-</sup>. NO and OONO<sup>-</sup> at physiological concentrations cannot induce apoptosis, but excessive NO and OONO<sup>-</sup> or antioxidant depletion can generate neuronal toxicity<sup>[9]</sup>. In this experiment, 1.0 nM SIN-1, an NO donor, was found to induce neuronal apoptosis, as assessed by cell survival rate and DNA fluorescent staining. Our results are consistent with previous findings<sup>[9]</sup>, indicating the success of our apoptosis model. PARP-1 plays an important role in the repair of DNA fracture and the maintenance of genome integrity<sup>[10]</sup>. However, under some pathological conditions, excessive activation of PARP-1 may aggravate cell injury and lead to apoptosis. Therefore, PARP-1 is a potential therapeutic target in the treatment of neuronal apoptosis following brain injury<sup>[11-12]</sup>. AIF can be released from the mitochondrial inner membrane by stressors such as cerebral ischemia, after which its molecular weight is reduced from 62 kDa to 57 kDa (soluble form), and thus participates in cell apoptosis<sup>[13-14]</sup>. Increasing evidence indicates that PARP-1 can induce AIF translocation from the mitochondrion to the nucleus. AIF then combines with DNA to induce chromatin condensation and generate a large number of DNA fragments, thus leading to caspase-independent apoptosis<sup>[10]</sup>. DIDS is a chloride channel blocker, and functions to protect SIN-1-induced apoptosis of hippocampal neurons in rats. However, its mechanism of action remains unclear<sup>[6, 15]</sup>.

In this study, cell immunofluorescence staining and immunoblotting revealed that PARP-1 and AIF protein expression was minimal in control neurons, but significantly higher in the SIN-1 (1.0 mM) group. This increase in protein expression was accompanied by significant neuronal apoptosis, which is evidence that PARP-1 and AIF are involved in ischemic neuronal damage, and can be considered as one of the important factors leading to apoptosis. Compared with the SIN-1 group, PARP-1 and AIF protein expression was lower; however, the survival rate was higher in the SIN-1 (1.0 mM) + DIDS (0.1 mM) group, which suggested that DIDS can protect neurons by inhibiting PARP-1 and AIF expression. DIDS (0.1 mM) can antagonize the expression of caspase-3, an apoptotic protein, in the caspase-dependent apoptotic pathway<sup>[15]</sup>. Our experimental results revealed that DIDS (0.1 mM) can downregulate the expression of PARP-1 and AIF protein in the caspase independent apoptotic pathway. Therefore, the chloride channel blocker DIDS can protect

neurons by inhibiting the caspase-dependent and-independent apoptotic signaling pathways.

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## MATERIALS AND METHODS

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### Design

An *in vitro* randomized controlled experiment.

### Time and setting

Experiments were performed from August 2010 to July 2011 at the laboratory in Zhuhai Campus of Zunyi Medical College, China.

### Materials

A total of 60 male and female Sprague-Dawley rats, 1 day old, weighing  $12.31 \pm 2.15$  g, were provided by the Animal Experimental Center of Sun Yat-sen University, China. All animal experiments were approved by the Experimental Animal Ethics Committee of Sun Yat-sen University, and complied with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China<sup>[16]</sup>.

### Methods

#### **Culture of primary rat hippocampal neurons**

According to previously described methods<sup>[6]</sup>, 1 day old Sprague-Dawley rats were sacrificed and the brain tissue was harvested. The bilateral hippocampi were separated in cold D-Hank's solution (Guangzhou Whiga Technology Co., Ltd., Guangzhou, Guangdong Province, China), blood vessels and meninges were eliminated, and the specimens were cut into 1-mm<sup>3</sup> pieces and digested with 0.25% (w/v) trypsin at 37°C for 15 minutes. The digestion was terminated with DMEM/Ham's F12 basic culture medium (Gibco, Carlsbad, CA, USA). Cells were dispersed by mechanical trituration, filtered using a 500-mesh filter, and prepared into a single cell suspension. The suspension was centrifuged at 1 000 r/min at room temperature for 10 minutes and added to DMEM/Ham's F12 culture medium after the supernatant was discarded. Cells were prepared into a  $4 \times 10^5$ /mL single cell suspension. Cells plated on 96-well culture plates, which were previously coated with polylysine, were maintained in a 95% air and 5% CO<sub>2</sub> saturated humidified incubator at 37°C. Culture medium consisted of 90% (v/v) DMEM/Ham's F12, 10% (v/v) fetal bovine serum (Hangzhou Sijiqing, Hangzhou, Zhejiang Province, China), 2 mM glutamine, and 100 U/mL penicillin and streptomycin. At 48 hours, culture medium was replenished with  $1 \times 10^{-5}$  M cytosine arabinoside for an additional 48 hours, to inhibit glial cell proliferation. The



medium was changed twice per week. Neurons were identified using Hoechst 33258 staining (Sigma, St. Louis, MO, USA) and the neuron specific antibody anti-NeuN (Sigma)<sup>[6]</sup>. Specimens were observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

#### **Establishment of neuronal apoptosis model**

According to previous studies<sup>[6, 9]</sup>, hippocampal neurons cultured for 12 days were randomly divided into three groups, namely a normal control group, SIN-1 group and a SIN-1 + DIDS group (Sigma). Thirty-six wells of cells in each group (same number of cells per well) were used for the identification of neurons, determination of cell survival rate, morphological observation, PARP-1 and AIF immunofluorescence staining and western blot assay. The control group was only cultured with DMEM/Ham's F12 complete culture medium. The SIN-1 group was exposed to SIN-1 (1.0 mM) for 18 hours to induce apoptosis *in vitro* and to establish an ischemic neuronal apoptosis model. Neuronal apoptosis was identified using the Hoechst 33258 DNA fluorescence staining kit (Wuhan Boster, Wuhan, Hubei Province, China). Under the inverted fluorescence microscope, Hoechst 33258 staining appeared light blue in control neurons. In apoptotic neurons, Hoechst staining exhibited strong fluorescence in nuclei, and fragmented DNA was evident. In the SIN-1 + DIDS group, 0.1 mM DIDS (Sigma) and 1.0 mM SIN-1 (Sigma) were simultaneously added to the culture medium for 18 hours. The concentration of drugs and intervention methods were used according to a previously described study<sup>[6]</sup>.

#### **Determination of hippocampal neuron survival**

According to previously described methods<sup>[17]</sup>, hippocampal neurons cultured in 96-well culture plates were incubated with MTT (Sigma; final concentration 0.5 mg/mL) at 37°C for 4 hours, and then oscillated with dimethyl sulfoxide for 10 minutes, after the culture medium was discarded, to completely dissolve the particles. Cell-free culture medium served as a control. The absorbance value at 570 nm was read using an enzyme labeling instrument (Bio-tek, Hercules, CA, USA). The survival rate (%) = (absorbance value of experimental group – absorbance value of blank control group)/(absorbance value of normal control group – absorbance value of blank control group) × 100%.

#### **Determination of the rate of hippocampal cell apoptosis**

According to previously described methods<sup>[6]</sup>, hippocampal neurons were rinsed with 0.01 M PBS (pH 7.4), fixed with 4% (w/v) paraformaldehyde for 30

minutes, rinsed with PBS three times, blocked with goat serum, and stained with Hoechst 33258 (0.25 µg/mL; Sigma) for 15 minutes. The morphology of cell nuclei was observed under an inverted fluorescence microscope (Olympus), and 200 cells were randomly selected from three fields of view to calculate the percentage of apoptotic cells. Normal cells exhibited oval or circular nuclei and refracted light blue fluorescence, while apoptotic neurons exhibited contracted nuclei and refracted strong blue light fluorescence. The percentage of apoptotic cells represented the rate of apoptosis.

#### **PARP-1 and AIF expression in rat hippocampal neurons by immunohistochemical detection**

Based on previously described methods<sup>[18-19]</sup>, PARP-1 and AIF immunofluorescence staining was performed. In brief, cells were rinsed with 0.01 M PBS (pH 7.4), fixed in 4% (w/v) paraformaldehyde for 30 minutes, rinsed with PBS three times, blocked with goat serum, and incubated with rabbit anti-mouse PARP antibody (Sigma; 1:800) and rabbit anti-mouse AIF antibody (Sigma; 1:800) at 4°C overnight, and then with secondary fluorescence agents (FITC and Cy3; Wuhan Boster Biological Technology Ltd., Wuhan, Hubei Province, China) labeled goat anti-rabbit IgG (1:200; Wuhan Boster) for an additional 30 minutes. Cells were observed under a fluorescence microscope (Olympus).

#### **Detection of PARP-1 and AIF protein expression in rat hippocampal neurons by western blot assay**

In accordance with previous methods<sup>[20-21]</sup>, hippocampal neurons were rinsed with cold 0.1 M PBS twice and cells were collected in 85°C sampling buffer solution, followed by sodium dodecyl sulfate polyacrylamide electrophoresis, transfer onto polyvinylidene fluoride and blocking with 3% (v/v) fetal bovine serum for 2 hours. The membrane was incubated with rabbit anti-mouse PARP-1 antibody (Sigma; 1:1 000) at 4°C overnight, rinsed, and incubated with rabbit anti-mouse AIF antibody (Sigma; 1:1 000) at 4°C overnight. The membrane was rinsed with triethanolamine-buffered saline containing 0.05% (v/v) Tween-20 three times, 10 minutes each, and finally incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:500; Wuhan Boster) at room temperature for 2 hours. After rinsing with triethanolamine-buffered saline containing 0.05% (v/v) Tween-20 three times, 5 minutes each, the membrane was subjected to ECL detection of protein.

#### **Statistical analysis**

Data were expressed as mean ± SD and analyzed with one-way analysis of variance using SPSS 11.5 statistical

software (SPSS, Chicago, IL, USA). Comparison between two groups was performed using the least significant difference *t*-test. A *P* value less than 0.05 was considered statistically significant.

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**Author contributions:** Jinbao Yin designed the study and drafted the manuscript. Lijuan Xu performed the study. Shuling Zhang evaluated the study. Yuanyin Zheng, Zhichao Zhong, Hongling Fan, and Xi Li provided experimental data. Quanzhong Chang was responsible for funds, design of the study and validated the manuscript. All authors approved the final version of the manuscript.

**Conflicts of interest:** None declared.

**Ethical approval:** All animal experiments described in this study were approved by the Experimental Animal Ethics Committee of Sun Yat-sen University in China.

**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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