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# Diazoxide preconditioning antagonizes cytotoxicity induced by epileptic seizures★

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## Abstract

Diazoxide, an activator of mitochondrial ATP-sensitive potassium channels, can protect neurons and astrocytes against oxidative stress and apoptosis. In this study, we established a cellular model of epilepsy by culturing hippocampal neurons in magnesium-free medium, and used this to investigate effects of diazoxide preconditioning on the expression of inwardly rectifying potassium channel (Kir) subunits of the ATP-sensitive potassium. We found that neuronal viability was significantly reduced in the epileptic cells, whereas it was enhanced by diazoxide preconditioning. Double immunofluorescence and western blot showed a significant increase in the expression of Kir6.1 and Kir6.2 in epileptic cells, especially at 72 hours after seizures. Diazoxide pretreatment completely reversed this effect at 24 hours after seizures. In addition, Kir6.1 expression was significantly upregulated compared with Kir6.2 in hippocampal neurons after seizures. These findings indicate that diazoxide pretreatment may counteract epileptiform discharge-induced cytotoxicity by suppressing the expression of Kir subunits.

## Key Words

neural regeneration; ATP-sensitive potassium channel; activator of mitochondrial ATP-sensitive potassium channel; epilepsy; diazoxide; inwardly rectifying potassium channel subunit; hippocampal neuron; cytotoxicity; neuroprotection; grants-supported paper; neuroregeneration

## Research Highlights

(1) We induced epilepsy *in vitro* by culturing hippocampal neurons in magnesium-free medium, and tested the influence of ATP-sensitive potassium channel activator diazoxide preconditioning on the expression of inwardly rectifying potassium channel (Kir) subunits of the ATP-sensitive potassium channel.
(2) Diazoxide improved the viability of hippocampal neurons exposed to magnesium-free medium and prevented seizure-induced increases in Kir6.1 and Kir6.2 expression.
(3) Kir6.1 expression was significantly upregulated compared with Kir6.2 after seizures.
(4) Diazoxide pretreatment may exert neuroprotective effects by inhibiting seizure-induced cytotoxicity, maintaining mitochondrial and cellular physiological functions, and ensuring normal metabolic balance and excitability.

## INTRODUCTION

Epilepsy is a common neurological disorder. Continued epileptic discharges could cause many changes at the cellular level including oxidative stress, cytokine activation, activation of glutamate receptors, and activation of subsequent cell death pathways[1]. Sustained epileptic seizures cause a decline in ATP content and change the redox potential, which may lead to mitochondrial dysfunction and energy failure[1-4]. The hippocampus is especially vulnerable, and tends to suffer selective neuronal loss in the CA1 and CA3 regions[4].

The ATP-sensitive potassium channel can adjust membrane potential-dependent functions according to cellular energetic demands[5]. ATP-sensitive potassium channels are widely represented in metabolically active tissues throughout the body, including the brain. Activation of ATP-sensitive potassium channels hyperpolarizes brain cells, reducing activity and energy consumption, and thereby linking the metabolic state to excitability[6-7]. With functions ranging from glucose regulation to neuroprotection, ATP-sensitive potassium channels play an important role in the adaptive response to pathophysiological stress[5]. ATP-sensitive potassium channels are composed of pore-forming inwardly rectifying potassium channel (Kir) subunits, Kir6.2 or Kir6.1, and modulatory sulfonylurea receptor subunits, sulfonylurea receptor 1 or sulfonylurea receptor 2[5]. Different combinations of ATP-sensitive potassium channel subunits can form functional ATP-sensitive potassium channels with different susceptibility to hypoxia, oxidative stress, toxicity or changes in blood glucose[7]. It was reported that 60 minutes of myocardial ischemia followed by 24–72 hours of reperfusion specifically upregulated Kir6.1 mRNA[8]. In another study, Kir6.1 mRNA was increased in the rat spinal cord at 4 and 24 hours after acute spinal cord injury[9]. However, the effect of epilepsy on Kir subunit expression in cultured cells remains unclear.

It was reported that diazoxide can induce mild oxidative stress and preconditioning-like neuroprotection[10]. Diazoxide has been reported to provide protective effects for neurons and astrocytes against necrosis and apoptosis in animal models of stroke and Parkinson's disease, as well as in cultured cells[10-12]. However, the effect of diazoxide preconditioning on Kir subunit expression in cultured cells is also unclear. In this study, we used double immunofluorescence and immunoblotting to investigate the effects of epilepsy and diazoxide preconditioning on the expression of Kir subunits in cultured rat hippocampal neurons. To simulate epileptic conditions *in vitro*, cultured hippocampal neurons were exposed to magnesium-free media for 3 hours, which can induce a permanent change in the neuronal culture physiology as a permanent "epileptiform" phenotype[13-14].

## RESULTS

### Influence of diazoxide preconditioning on viability of hippocampal neurons

The cells were treated with magnesium-free medium for 3 hours to induce epilepsy, and were then returned to normal culture medium for 24 hours (Ep24 group) or 72 hours (Ep72 group). The diazoxide + Ep24 group and diazoxide + Ep72 group were pretreated with diazoxide (1 mM) for 1 hour before the 3-hour incubation in magnesium-free medium, and then returned to normal medium for 24 hours (diazoxide + Ep24 group) or 72 hours (diazoxide + Ep72 group). The control group was treated with an equal volume of PBS.

An MTT reduction assay showed that the epileptiform activity induced cell damage and significantly reduced cell viability ($P < 0.05$), by 32.2% in the Ep24 group and by 59.7% ($P < 0.01$) in the Ep72 group, compared with control group. Pretreatment of cells with diazoxide resulted in a reduction of seizure-induced cytotoxicity and significantly increased cell viability (Figure 1). These results demonstrate that diazoxide can protect against epilepsy-induced cell loss.

### Influence of diazoxide preconditioning on Kir6.1 and Kir6.2 expression in hippocampal neurons

Double immunofluorescence analysis was used to detect expression of Kir6.1 and Kir6.2. Kir6.1 was stained red whereas Kir6.2 was stained green (the merged image of Kir6.1 and Kir6.2 was yellow or brown). In the Ep24 and Ep72 groups, especially in the latter, Kir6.1 and Kir6.2 expression was upregulated compared with the control group. No obvious changes were found in the diazoxide + Ep24 group. However, in the diazoxide + Ep72 group, expression of Kir6.1 and Kir6.2 was decreased compared with the Ep72 group (Figure 2). The overlay of Kir6.1 and Kir6.2 expression indicated that the expression of the two subunits was not parallel: Kir6.1 showed a greater increase than Kir6.2 (Figure 2). These results suggest that expression of Kir subunits may be regulated by diazoxide.

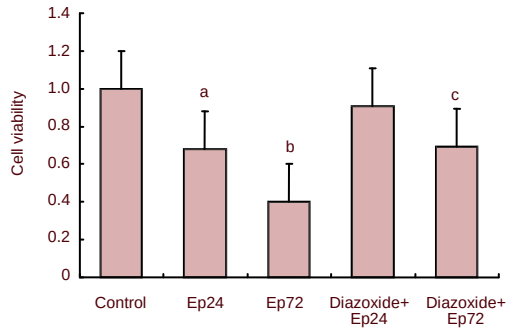


Figure 1 Effect of diazoxide pretreatment on the viability of hippocampal neurons with epilepsy.

Cellular viability was measured by a quantitative colorimetric MTT assay. The cells were treated with magnesium-free medium for 3 hours, and then returned to normal culture medium for 24 hours (Ep24 group) or 72 hours (Ep72 group). The diazoxide + Ep24 group and diazoxide + Ep72 group were pretreated with diazoxide (1 mM) for 1 hour, then exposed to the magnesium-free medium for 3 hours before being returned to normal culture medium for 24 hours (diazoxide + Ep24 group) or 72 hours (diazoxide + Ep72 group). The control group was treated with an equal volume of PBS.

Results are expressed as mean ± SEM of six wells from each group. The experiment was repeated three times. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. control group; $^{c}P < 0.05$, *vs*. epilepsy group at the same time point (analysis of variance followed by Student's *t*-test). Cellular viability was calculated as a percentage of control value (absorbance at 570 nm).

Western blot analysis showed that the expression of Kir6.1 and Kir6.2 was significantly increased in the Ep24 group compared with the control group. This increase was completely prevented by pretreatment with diazoxide (diazoxide + Ep24 group; Figure 3). In the Ep72 group, the up-regulation of Kir6.1 and Kir6.2 was partially reversed by pretreatment with diazoxide (diazoxide + Ep72 group). The increase of Kir6.1 expression in the Ep72 group was particularly large with a 2.47-fold increase compared with the control group. Kir6.2 expression in the Ep72 group was upregulated to 1.88 times the control levels (Figure 3).

## DISCUSSION

Epilepsy is the second most common neurodegenerative disease after stroke[15]. Brain injury resulting from seizures is a dynamic process that comprises multiple factors that contribute to neuronal cell death. These may involve oxidative stress, altered cytokine levels, genetic factors, excitotoxicity-induced mitochondrial dysfunction, and energy failure[16-19]. Many reports have shown that ATP-sensitive potassium channels can recognize changes in the cellular metabolic state and translate this information into changes in membrane excitability[5].

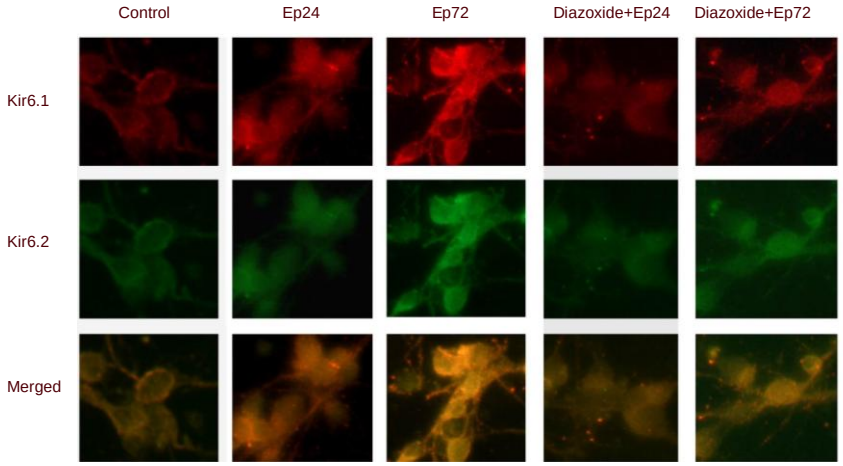


Figure 2 Effects of diazoxide pretreatment on Kir6.1 and Kir6.2 expression in hippocampal neurons with epilepsy (double immunofluorescence staining, fluorescence microscopy, × 400).

Kir subunit expression in each group was observed by double immunofluorescence. Kir6.1 (rhodamine-labeled, red) and Kir6.2 (fluorescein isothiocyanate-labeled, green) were upregulated after epileptiform discharges, and this was prevented by diazoxide pretreatment. The color of the merged images (yellow) indicates that the changes in expression were not parallel in the two subunits: Kir6.1 showed a greater increase than Kir6.2.

Ep24: Treatment with magnesium-free medium for 3 hours followed by normal medium for 24 hours; Ep72: treatment with magnesium-free medium for 3 hours followed by normal medium for 72 hours; Diazoxide + Ep24: pretreatment with diazoxide for 1 hour, magnesium-free medium for 3 hours and normal medium for 24 hours; Diazoxide + Ep72: pretreatment with diazoxide for 1 hour, magnesium-free medium for 3 hours and normal medium for 72 hours; control: treatment with an equal volume of PBS.

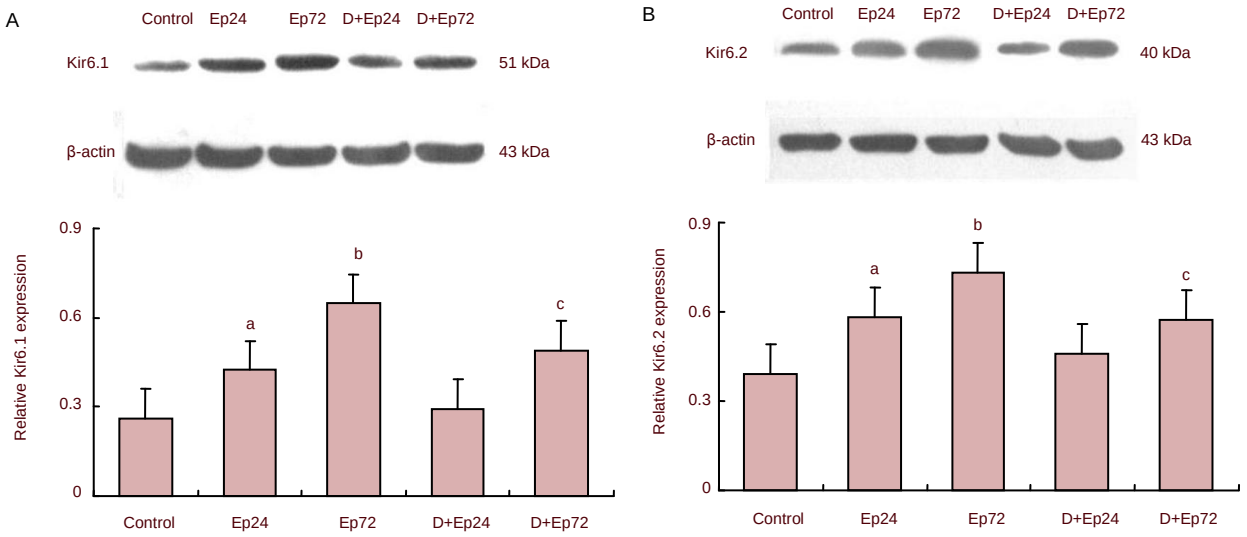


Figure 3 Effects of diazoxide pretreatment on Kir6.1 and Kir6.2 protein expression in hippocampal neurons with epilepsy.

Kir6.1 (A) and Kir6.2 (B) expression changes were observed by western blot analysis. Results are expressed as mean ± SEM (absorbance ratio of Kir protein to β-actin). Three dishes from each group were analyzed, and the experiment was repeated three times. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs*. control group; $^{c}P < 0.05$, *vs*. epilepsy group at the same time point (analysis of variance followed by Student's *t*-test).

Ep24: Treatment with magnesium-free medium for 3 hours followed by normal medium for 24 hours; Ep72: treatment with magnesium-free medium for 3 hours followed by normal medium for 72 hours; Diazoxide (D) + Ep24: pretreatment with diazoxide for 1 hour, magnesium-free medium for 3 hours and normal medium for 24 hours; D + Ep72: pretreatment with diazoxide for 1 hour, magnesium-free medium for 3 hours and normal medium for 72 hours; control: treatment with an equal volume of PBS.

ATP-sensitive potassium channels are made up of four Kir6 pore-forming subunits (Kir6.1 or Kir6.2) associated with four sulfonylurea receptor subunits (sulfonylurea receptor 1 or sulfonylurea receptor 2)[20-22]. The mitochondrial ATP-sensitive potassium channels may have a functionally important role in neurons because Kir subunits are more concentrated in neurons than in whole brain tissue[23-25].

Diazoxide is the most commonly used mitochondrial ATP-sensitive potassium channel opener. Many reports have supported that diazoxide preconditioning exhibits potent neuroprotective effects against ischemic neuronal injury, oxidative stress and epilepsy[26-29]. Recently, diazoxide was shown to protect against status epilepticus-induced neuronal damage during diabetic hyperglycemia[30]. Flagg *et al* [31] reported that PI3K/Akt signaling may be involved in diazoxide preconditioning that protects against hippocampal neuronal death after pilocarpine-induced seizures in rats. Consistent with previous results, our study in hippocampal cell culture demonstrated massive neuronal loss at 24 and 72 hours after seizures, and a significant attenuation of cell death by diazoxide.

ATP-sensitive potassium channels in different brain regions show different subunit compositions, which determine their varying susceptibility to hypoxia, oxidative stress, toxicity and change of blood glucose[32]. A recent study[33] showed that both Kir6.1 and Kir6.2 proteins are present on synaptic membranes of terminals and spines as well as in vesicular structures within the synaptic cytoplasm. Kir6.1 subunits were located predominantly in the pre-synaptic membrane, whereas Kir6.2 subunits were most likely to be located in the perisynaptic area of terminals. Melamed-Frank *et al* [33] reported that hypoxia upregulates the expression of Kir6.1 mRNA *in vivo* and *in vitro*, which in turn can change the composition of ATP-sensitive potassium channels. We previously found a significant increase in Kir6.1 expression in cultured neurons exposed to amyloid beta (1–42) for 24 hours, whereas Kir6.2 showed no significant change. After treatment with amyloid beta (1–42) for 72 hours, the expression of both Kir6.1 and Kir6.2 was significantly increased compared with the control group[34-35]. In this study, we found that the expression of Kir6.1 and Kir6.2 was significantly increased at 24 and 72 hours after seizures. The effect on Kir6.1 expression was especially significant. Diazoxide completely prevented the changes seen at 24 hours, and partly attenuated the effects at 72 hours. Both amyloid beta (1–42) and

seizures can cause oxidative stress and mitochondrial dysfunction. Based on the present data and previous studies, we suggest that oxidative stress and mitochondrial dysfunction may change the expression of ATP-sensitive potassium subunits, and that diazoxide has antioxidant properties.

Kir6.1 and Kir6.2 belong to the same subfamily of Kir subunits and are highly homologous with 70% identity in the primary amino acid sequence. Both form functional ATP-sensitive potassium channels when expressed with sulfonylurea receptors. The unitary conductance of the Kir6.1/sulfonylurea receptor channel is ~35 pS and that of the Kir6.2/sulfonylurea receptor channel is ~80 pS[36-37]. In our study, Kir6.1 increased more significantly than Kir6.2, indicating that the composition of ATP-sensitive potassium channels changed after epileptiform activity. The increased ratio of Kir6.1/Kir6.2 may make the channel more sensitive to the metabolic state of neurons and may be helpful in coordinating the electrophysiological function with oxidative stress and the inflammatory reaction.

However, it might also contribute to the disturbance of membrane excitability that results in sustained epileptic seizures and neuronal loss. Diazoxide pretreatment may counteract seizure-induced cytotoxicity and maintain mitochondrial and cellular function. The differential regulation of Kir subunits may alter the composition of ATP-sensitive potassium channels, causing changes in channel properties.

In summary, epileptiform activity in hippocampal cells increased the expression of Kir6.1 and Kir6.2, the former in particular. These changes were attenuated by pretreatment with diazoxide, indicating a possible neuroprotective action of this drug.

## MATERIALS AND METHODS

### Design

A parallel controlled *in vitro* study.

### Time and setting

The experiments were performed at the Laboratory of Linyi People's Hospital, Shandong Province, China, from July 2011 to April 2012.

### Materials

Twenty healthy pregnant Wistar rats at 17 or 19 days of gestation, weighing 270 ± 20 g, of clean grade, were provided by the Laboratory Animal Center of Shandong University (License No. SCXK (Lu) 2007-0004). Animals were housed with a 12-hour light/dark cycle and allowed free access to standard diet and water. Animal protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[38].

### Methods

#### *Isolation and culture of hippocampal neurons from fetal rats and preparation of neuronal epilepsy model*

Embryos were removed from pregnant Wistar rats at 17 or 19 days of gestation. The hippocampus was dissected and digested with 0.125% trypsin at 37°C for 20 minutes. Digestion was ended with proliferation growth medium composed of a 1:9 mixture of fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) and Dulbecco's modified Eagle's medium (Gibco-BRL), and the cells were mechanically dissociated with a fire-polished pipette. The density of the cells was $6.0 \times 10^5$ cells/mL on 12-well plates for double immunofluorescence analysis, or $2.0 \times 10^6$ cells/mL on culture dishes for western blot analysis. During the 3–5 days of culture, cells were treated with cytarabine (5 μM) to inhibit the proliferation of gliocytes. To induce epileptiform activity, cells were placed in magnesium-free medium (145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 10 mM glucose, and 0.002 mM glycine, pH 7.3, adjusted to 325 mOsm with sucrose) for 3 hours[39].

#### *Treatments*

Diazoxide (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (Sigma) to a concentration of 0.08%, and then diluted in serum-free medium prior to experiments.

#### *MTT reduction assay for cell viability*

Cellular viability was measured in a 96-well plate with a quantitative MTT colorimetric assay[40]. The culture medium was changed, and MTT (final concentration 0.5 mg/mL) was added to the cells. The cells were treated with MTT solution for 4 hours. The absorbance (570 nm) was measured in a multiwell plate reader after 30 minutes (Bio-Tek Instruments, Inc., Winooski, VT, USA). The results were expressed as a percentage of the control value.

#### *Double immunofluorescence analysis of Kir subunit expression*

Cells were fixed with 4% paraformaldehyde for 30 minutes, washed with PBS and incubated with 0.3%

Triton X-100 for 20 minutes. They were then blocked with 10% bovine serum albumin for 30 minutes at room temperature, incubated overnight at 4°C with polyclonal goat anti-Kir6.1 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and washed with PBS. The cells were serially incubated with polyclonal rabbit anti-Kir6.2 (1:50; Santa Cruz Biotechnology) at room temperature for 1 hour, rhodamine-conjugated mouse anti-goat IgG (1:100; Santa Cruz Biotechnology) at room temperature for 30 minutes in the dark, and fluorescein isothiocyanate-conjugated mouse anti-rabbit IgG (1:100; Santa Cruz Biotechnology) at room temperature for 30 minutes in the dark. PBS washes were performed between each step. Fluorescent signals were analyzed with a Leica DM IRE2 fluorescence microscope (Nussloch, Germany) and DP Manager software (Olympus, Tokyo, Japan).

### *Western blot analysis of Kir subunit expression*

Cells were collected, washed in ice-cold PBS, and lysed in 250 μL of lysis buffer per dish. After incubation for 20 minutes on ice, cell lysates were centrifuged at 10 000 × *g* for 10 minutes at 4°C, and the protein concentration in the extracts was determined using a BCA Protein Assay Kit (Shenneng Bocai Company, Shanghai, China). Twenty microliters of solubilized total cell lysate (50 μg protein) was loaded per lane for sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% (w/v) polyacrylamide gel. The proteins were transferred onto a polyvinylidene fluoride membrane by a Mini Trans-Blot Cell apparatus (Bio-Rad Laboratories, Shanghai, China) at 100 V for 120 minutes at 4°C. Membranes were blocked at room temperature (25°C) for 60 minutes with 5% (w/v) dried milk in Tris-buffered saline, and then incubated with polyclonal goat anti-Kir6.1 or polyclonal rabbit anti-Kir6.2 (1:400; Santa Cruz Biotechnology) overnight at 4°C. The blots were incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) diluted in Tris-buffered saline and Tween 20 (1:3 000) at room temperature for 1 hour. Signal detection was performed with an enhanced chemiluminescence kit (Beijing Zhongshan Company). Immunoreactive bands were quantified using AlphaImager 2200 (Alpha Innotech, Santa Clara, CA, USA). Values were normalized to the absorbance of β-actin. The β-actin was detected with mouse anti-rat monoclonal antibody (Santa Cruz Biotechnology).

### *Statistical analysis*

Data were expressed as mean ± SEM. The statistical significance of the difference between control and samples treated for different times was determined by analysis of variance followed by Student's *t*-test using SPSS 13.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

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**Author contributions:** Qingxi Fu and Zhiqing Sun were in charge of funds, designed the study, provided technical support, and validated the final version of the manuscript. Qingxi Fu was responsible for data acquisition, integration and analysis, statistical management, and drafting of the manuscript. Jinling Zhang, Naiyong Gao, Faying Qi, Fengyuan Che and Guozhao Ma participated in the study and provided technical support. All authors read and approved the final manuscript.

**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Institutional Animal Care and Use Committee of Shandong University, China.

**Author statements:** The manuscript is original, has not been submitted to and 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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