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Methyl 3,4-dihydroxybenzoate promotes neurite outgrowth of cortical neurons cultured *in vitro*★

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Abstract

Cerebral cortical neurons from neonatal rats were cultured in the presence of methyl 3,4-dihydroxybenzoate (MDHB; 2, 4, and 8 μM). Results showed that MDHB significantly promoted neurite outgrowth and microtubule-associated protein 2 mRNA expression, and increased neuronal survival in a dose-dependent manner. Moreover, MDHB induced brain-derived neurotrophic factor expression. These findings suggest that MDHB has a neurotrophic effect, which may be due to its ability to increase brain-derived neurotrophic factor expression.

Key Words

methyl 3,4-dihydroxybenzoate; neurite outgrowth; brain-derived neurotrophic factor; neurodegenerative diseases; neural regeneration

Abbreviations

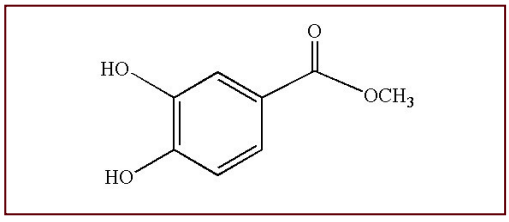
BDNF, brain-derived neurotrophic factor; MDHB, Methyl 3,4-dihydroxybenzoate; NSE, neuron specific enolase; MAP2, microtubule-associated protein 2

INTRODUCTION

Neurodegenerative diseases are characterized by marked loss of neurons[1]. Neurotrophic factors such as nerve growth factor and brain-derived neurotrophic factor (BDNF) are very important for neuronal survival and physiological functions[2]. They are widely expressed in the central and peripheral nervous system. A previous study has shown that the level and activity of neurotrophic factors decreased in neurodegenerative diseases[3]. However, neurotrophic factors have been shown to have poor effects in clinical trials because they have difficulty in crossing the blood-brain barrier[4]. Therefore, investigating small molecules that have neurotrophic effects may provide new drug developments for neurodegenerative diseases.

Methyl 3,4-dihydroxybenzoate (MDHB, $C_8H_8O_4$, molecular weight: 168.15) is a derivative of phenolic acid that is extracted from plants such as *Kalimerisindica*[5]. Previous studies have shown that MDHB has an antioxidant activity[6-7]. Therefore, we aimed to investigate the neurotrophic effects of MDHB to provide a theoretical and experimental basis for drug developments of neurodegenerative diseases.

The chemical structure of MDHB is as follows:



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RESULTS

Morphology and phenotype of cultured cortical neurons

Neonatal rat cortical neurons were cultured in serum-free Dulbecco's modified eagle's medium (DMEM)/F12 supplemented with 0.4% (v/v) B27 to prevent the proliferation of gliocytes. Neuron specific enolase (NSE) is an enzyme that is encoded by the *ENO2* gene in humans, which can be used to identify neuronal cells[8]. Microtubule-associated protein 2 (MAP2) is a neuron-specific cytoskeletal protein that is enriched in the cell body and dendrites[9]. NSE and MAP2 staining can distinguish neurons from other cells. After cortical neurons were cultured for 3 days, NSE immunostaining and MAP2 immunofluorescence staining was performed. Results showed that more than 95% of cultured cells were neurons (Figure 1).

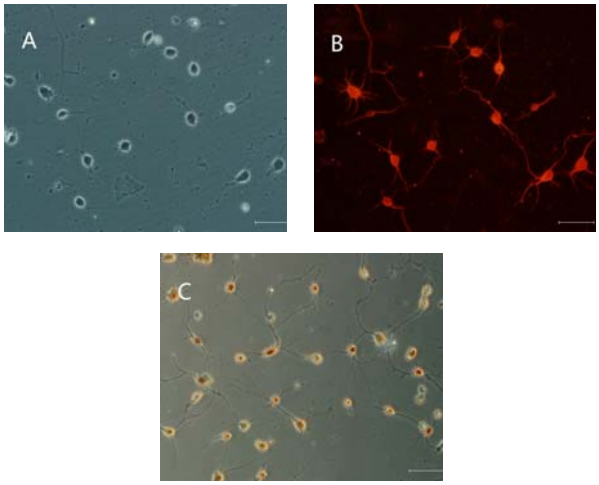

Figure 1 Identification of neurons cultured for 3 days using neuron specific enolase immunostaining and microtubule-associated protein 2 immunofluorescence staining (scale bars: 50 μm).

(A) Cells cultured for 3 days.

(B) Immunofluorescent staining for microtubule-associated protein 2: the cell bodies, axons and dendrites were stained red (Cy3).

(C) Cells stained for neuron specific enolase: neurons were stained brown (3,3'-diaminobenzidine).

MDHB promoted neurite outgrowth in a dose-dependent manner

To investigate the effects of MDHB on neurite outgrowth, cortical neurons were cultured in DMEM/F12 supplemented with 0.4% (v/v) B27 medium containing MDHB (2, 4, 8 μM). BDNF (20 ng/mL), which has been shown to promote neuron survival and neurite outgrowth in cell culture[10], was applied to alternate culture wells as a positive control. After 3 days *in vitro*, neurite outgrowth of cortical neuron cells increased compared with that of the vehicle control (dimethyl sulfoxide; 0.1% (v/v)). MAP2 immunofluorescence staining results (Figures 2, 3) showed that MDHB significantly promoted neurite outgrowth in a dose-dependent manner at concentrations ranging from 2 to 8 μM compared with the vehicle control ($P < 0.05$ or $P < 0.01$).

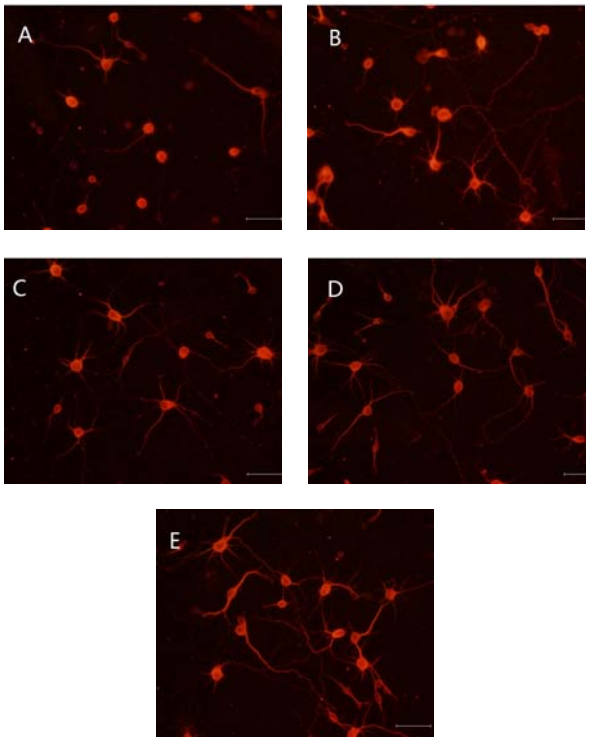

Figure 2 Visualization of morphological effects of methyl 3,4-dihydroxybenzoate on cultured cortical neurons following microtubule-associated protein 2 immunostaining (Cy3-labelled; scale bars: 50 μm).

After cortical neurons were treated for 3 days, microtubule-associated protein 2 immunostaining showed that neurite outgrowth was significantly promoted by methyl 3,4-dihydroxybenzoate.

(A) Neurons treated with vehicle (dimethyl sulfoxide).

(B) Neurons treated with 20 ng/mL brain-derived neurotrophic factor as a positive control.

(C–E) Neurons treated with 2, 4 and 8 μM methyl 3,4-dihydroxybenzoate, respectively.

MDHB increased neuronal viability

The 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay (Figure 4) showed that MDHB significantly increased neuronal viability at concentrations ranging from 2 to 16 μM ($P < 0.01$). This result was consistent with the morphological effects seen above (Figures 2, 3).

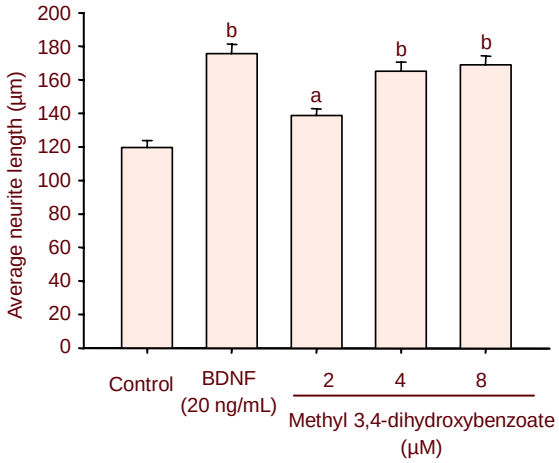

Figure 3 Quantitative analysis of neurite length after cortical neurons were treated with the vehicle control (dimethyl sulfoxide; control), 20 ng/mL brain-derived neurotrophic factor (BDNF) and various concentrations of methyl 3,4-dihydroxybenzoate. More than 200 neurons were measured in random fields for each group (× 400 magnification). Data were expressed as mean ± SEM, derived from one of three repeated experiments with similar results. $^aP < 0.05$, $^bP < 0.01$, *vs.* vehicle control (one-way analysis of variance followed by Dunnett's *t*-test).

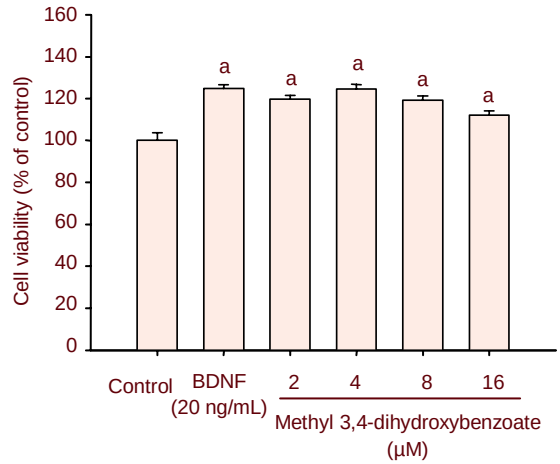

Figure 4 Methyl 3,4-dihydroxybenzoate enhanced neuronal viability.

The MTT assay was performed after cortical neurons were treated for 3 days. Values are expressed as mean ± SEM, derived from one of three repeated experiments with similar results. The absorbance was normalized as a percent of the control. $^aP < 0.01$, *vs.* vehicle control (one-way analysis of variance followed by Dunnett's *t*-test). BDNF: Brain-derived neurotrophic factor.

MDHB increased MAP2 mRNA expression

Reverse transcription-PCR was used to determine MAP2 mRNA expression and to confirm the effect of MDHB on neurite outgrowth. Results (Figure 5) showed that MDHB promoted MAP2 mRNA expression ($P < 0.05$ or $P < 0.01$), which corresponded with quantitative data obtained from neurite outgrowth analyses shown in Figure 2.

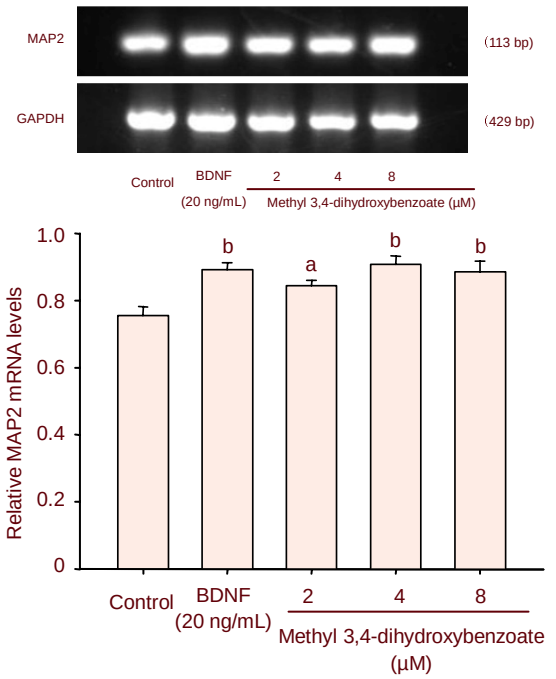

Figure 5 MAP2 mRNA expression as measured by reverse transcription-PCR and agarose gel electrophoresis.

Values are expressed as mean ± SEM, derived from one of three repeated experiments with similar results. The absorbance was normalized to GAPDH. $^aP < 0.05$, $^bP < 0.01$, *vs.* vehicle control (one-way analysis of variance followed by Dunnett's *t*-test). MAP2: Microtubule-associated protein 2; BDNF: brain-derived neurotrophic factor.

MDHB increased BDNF mRNA expression

Real-time PCR was performed to quantify whether MDHB could increase BDNF mRNA expression in cultured cortical neurons. Results showed that MDHB at concentrations of 4 μM and 8 μM could significantly promote mRNA expression ($P < 0.05$ or $P < 0.01$; Figure 6).

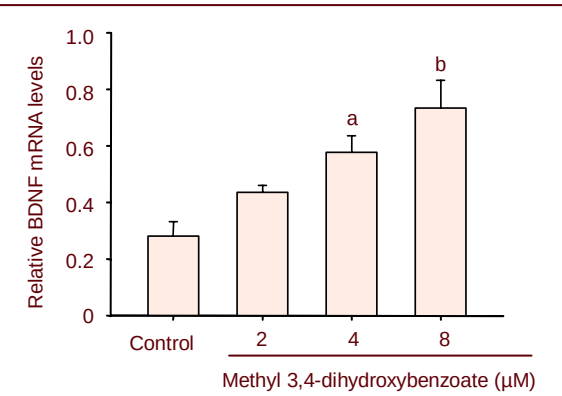

Figure 6 Expression levels of brain-derived neurotrophic factor (BDNF) in cortical neurons treated with different concentrations of methyl 3,4-dihydroxybenzoate as detected by real-time PCR.

Data are expressed as the mean ± SEM, derived from at least three independent experiments. Results are normalized to glyceraldehyde 3-phosphate dehydrogenase. $^aP < 0.05$, $^bP < 0.01$, *vs.* vehicle control (one-way analysis of variance followed by Dunnett's *t*-test).

DISCUSSION

In the present study, MDHB, a derivative of phenolic acid, induced neurite outgrowth of cortical neurons. In recent years, neurotrophic factors have been used to treat neurodegenerative diseases. Neurotrophic factors have been reported to promote survival and neurite outgrowth *in vitro* and *in vivo*. For example, nerve growth factor can prevent the loss of neurons in an Alzheimer's disease model[11], over-expression of BDNF in the forebrain ameliorates Huntington's disease phenotypes in mice[12], and glial cell line-derived neurotrophic factor enhances the survival of dopaminergic neurons *in vivo*[13]. Many compounds have been reported to have neurotrophic effects or induce expression of endogenous neurotrophic factors. For instance, Honokiol, 4-O-methylhonokiol, Ginkgolide B, Tripchlorolide, Ptychopetalumolacoides, and Russujaponols G-L, which are all extracted from medicinal plants, have neurotrophic effects in cell culture[14-20].

In the present study, MDHB promoted neurite outgrowth in a dose-dependent manner. The length and number of neurite branches significantly increased compared with that of the vehicle control. Different cell densities were used for assaying neurite outgrowth and cell viability. To easily observe neurite outgrowth, the cell density used was lower than that used for MTT assays[21-22]. Results showed that MDHB significantly altered neuronal survival as well and neurite extension. MAP2 is very important in the stabilization and function of microtubules of the nerve cell cytoskeleton. It is widely distributed in the cell body, dendrites, and the axon[23-24]. MAP2 can act as a neurite outgrowth marker protein for neurons[25]. MDHB also induced the expression of MAP2 mRNA, which corresponded with neurite outgrowth results and MAP2 immunofluorescence.

The production and release of neurotrophic factors are important in regulating neuronal survival, and promoting neurite outgrowth and differentiation[26]. BDNF plays a central role in survival, differentiation of neurons and synaptic plasticity[27-29]. BDNF can bind to and activate the TrkB receptor[30]. Trk receptors mediate cell development through three main pathways including extracellular signal-regulated kinase, phosphatidylinositol 3-kinase and phospholipase Cγ pathways. A good deal of evidence shows that activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase promotes neuronal survival and neurite outgrowth[31-33]. It has been reported that some compounds exert neurotrophic effects by enhancing the expression of neurotrophic factors. 4-O-methylhonokiol isolated from Magnolia officinalis induces the release of nerve growth factor and BDNF into cell culture medium[17]. An extract of Chinese herb Tripterygium Wilfordii Hook F, Tripchlorolide, has neurotrophic effects by stimulating the expression of BDNF[19]. Ginkgolide B has neuroprotective effects because it can up-regulate BDNF expression in hippocampal neurons[18]. In the present study, MDHB promoted BDNF expression at a concentration of 4 and 8 μM. These results showed that the neurotrophic effect of MDHB may be associated with an increase in BDNF expression.

In summary, MDHB has a strong neurotrophic effect that may encourage the development of new drugs. However, further studies need to focus on receptor interactions and the downstream signaling pathways activated by MDHB to understand the mechanism of this effect.

MATERIALS AND METHODS

Design

A controlled *in vitro* study.

Time and setting

The study was performed at the Laboratory of Neuropharmacology, Jinan University Medical College, China in October 2011.

Materials

Animals

Neonatal Sprague-Dawley rats were purchased from the Center of Laboratory Animal of Guangdong (license No. SCXK (Yue) 2008-0002). The care of animals and animal experimental operation 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[34].

Drugs

MDHB (> 97%; lot No. 10131708) was obtained from the Alfa Aesar company (Ward Hill, MA, USA).

Methods

Isolation and culture of cortical neurons

Cerebral cortical cells were obtained from neonatal rats. The neonatal rats were sacrificed 24 hours after birth. The brains were removed quickly and the meninges were removed in D-Hank's solution (8.0 g NaCl, 0.4 g KCl, 0.13 g $Na_2HPO_4 \cdot 12H_2O$, 0.06 g KH_2PO_4, 0.35 g $NaHCO_3$, 1 L deionized water, filtrated by 0.22 μm filter). Cerebral neocortices were separated without the olfactory bulbs and hippocampus. The tissue was minced with an eye scalpel for 1 minute, and dissociated into a single-cell suspension using 0.25% (w/v) trypsin digestion for 10 minutes. Digestion was terminated using DMEM/F12

(Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco). Neurons were centrifuged at 1 000 r/min for 5 minutes and the pellet was resuspended to the desired concentration with DMEM/F12 containing 0.4% (v/v) B27 (Gibco). Neurons were seeded on poly-L-lysine coated culture plates. Subsequently, these cells were cultured at 37°C in a humidified atmosphere of air with 5% CO_2. After 3 days *in vitro*, cells were collected for subsequent experiments.

Identification of cortical neurons

Cortical neurons were identified by immunostaining for NSE and MAP2, specific neuron markers. After 3 days *in vitro*, cells were fixed with 4% (w/v) paraformaldehyde at room temperature for 20 minutes, and blocked with 5% (w/v) bovine serum albumin for 30 minutes. Subsequently, cells were incubated with rabbit polyclonal antibodies against NSE (1:80 dilution; Boster, Wuhan, China) overnight at 4°C. After three washes using 0.1 M phosphate buffered saline, cells were incubated with goat anti-rabbit IgG (1:500 dilution; Boster) for 20 minutes at room temperature. Finally, cells were colorized using the 3,3'-diaminobenzidine kit (MAIXIN biocompany, Fuzhou, China). The mouse anti-MAP2 polyclonal antibody (1:800 dilution in blocking solution; Sigma, St. Louis, MO, USA) were detected using Cy3 AffiniPure Goat Anti-Mouse IgG (1:500 dilution; Proteintech Group company, Chicago, IL, USA). Images were taken using the Olympus fluorescence microscope (model IX-71; × 400 magnification; Tokyo, Japan)

Neurite outgrowth measurement

Neurons were cultured in 24-well plates at a density of 3.5×10^4 cells/cm^2. After 6 hours, the medium was changed to DMEM/F12 + 0.4% (v/v) B27 containing different concentrations (2, 4, 8 μM) of MDHB or 20 ng/mL recombinant human BDNF (R&D Systems, Minneapolis, Minnesota, USA) as a positive control. After 3 days, 12 fields were randomly selected from each well, and photographs were taken using the Olympus fluorescence microscope (model IX-71; × 400 magnification). The neurites which are twice longer than the diameter of the cell body were evaluated using Image-pro plus 6.0 software (Olympus).

MTT assay for neuronal viability

The MTT assay was used to assess cell viability[35]. Briefly, neurons were cultured in 96-well plates in DMEM/F12 + 0.4% (v/v) B27 at a density of 1.2×10^5 cells/cm^2 for 6 hours. The medium was changed to either DMEM/F12 + 0.4% (v/v) B27 containing 0.1% (v/v) dimethyl sulfoxide (vehicle control), different concentrations (2, 4, 8, and 16 μM) of MDHB, or 20 ng/mL BDNF as a positive control. After 3 days' culture, the medium was removed, and DMEM/F12 containing MTT (MTT: 0.5 mg/mL in PBS, MTT: DMEM/F12 = 1:10; Sigma) was added. After incubation for 4 hours, the medium was gently aspirated. Dimethyl sulfoxide (150 μL) was added to each well to dissolve the MTT formazan product. The absorbance was determined at 570 nm using a Bio-rad 400 microplate reader (Bio-rad, Hercules, CA, USA).

Quantitative reverse transcription-PCR for MAP2 mRNA expression in cortical neurons

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control for normalization. Cortical neurons were cultured in 24-well plates at a density of 1.2×10^5 cells/cm^2 and treated with or without different concentrations (2, 4, and 8 μM) of MDHB and 20 ng/mL BDNF as a positive control. Three days later, total RNA was extracted from cerebral cortical neurons with RNAiso Plus (Takara, Tokyo, Japan) and reverse transcribed using a kit (Takara). Resulting cDNAs were then subjected to PCR for amplification of the MAP2 fragment. Sequences used for reverse transcription-PCR were designed according to Genbank (Table 1). The first denaturation step lasted 3 minutes at 94°C, and the amplification cycle was repeated 38 times (30 seconds at 94°C, 30 seconds at 58°C, and 60 seconds at 72°C), and held at 16°C when finished. The PCR product was detected by agarose gel electrophoresis (100 mL 2% (w/v) gel stained with 10 μL GelRed) and photographed under ultraviolet light (590 nm; Ultra-Lum. Inc, Ontario, CA, USA).

Table 1 MAP2, BDNF and GAPDH primer sequences

Gene	Primer sequence	Products size (bp)
MAP2	Sense: 5'-TGT ACC TGG AGG TGG TAA CGT AA-3' Antisense: 5'-ACC TGC TTG GCG ACT GTG TG-3'.	113
BDNF	Sense: 5'-AGC CTC CTC TGC TCT TTC TG-3' Antisense: 5'-ATG GGA TTA CAC TTG GTC TCG-3'	259
GAPDH	Sense: 5'-GGC AAG TTC AAC GGC ACA-3' Antisense: 5'-CCA CAG CTT TCC AGA GGG-3'	429

MAP2: Microtubule-associated protein 2; BDNF: brain-derived neurotrophic factor.

Real-time PCR analysis of BDNF mRNA expression in cortical neurons

Cells were treated with vehicle control and different concentrations of MDHB for 3 days. Total RNA was

extracted and reversely transcribed as mentioned above. Real-time PCR was performed using the LightCycler 480 Real-Time PCR System (Roche, Basal, Switzerland). The primers are in Table 1. The conditions of the PCR reactions are described as follows: pre-denaturation at 95°C for 30 seconds; 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, extension at 72°C for 10 seconds. All the samples were tested in triplicate. The melting curves for BDNF/GAPDH primers and amplification curves for BDNF/GAPDH mRNA are shown in supplementary Figure 1 online.

Statistical analysis

All data are expressed as mean ± SEM. The evaluation of statistical significance was analyzed by one-way analysis of variance followed by the Dunnett's *t*-test using SPSS 16.0 (SPSS, Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.

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Author contributions: Zheng Zhang and Huanmin Luo designed the study. Zheng Zhang, Xing Zhou, Xiaowen Zhou and Xiao Xu conducted the experiments. Zheng Zhang, Minjing Liao, Li Yan and Ruohua Lv integrated and processed data. Zheng Zhang wrote the manuscript. Huanmin Luo revised the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of Jinan University.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol.7, No. 13, 2012 after selecting the "NRR Current Issue" button on the page.

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