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Neuroprotective effects of total saponins from *Rubus parvifolius* L. on cerebral ischemia/reperfusion injury in rats[☆]

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Abstract

This study examines the neuroprotective effects and mechanisms of action of total saponins from *Rubus parvifolius* L. (TSRP) on focal cerebral ischemia and reperfusion injury in rats. Focal cerebral ischemia and reperfusion injury was performed in rats using the suture method. The results indicate that intragastric injection of TSRP, at 5, 10 and 20 mg/kg, could decrease neurological impairment, reduce cerebral infarct volume, diminish pathological changes, and significantly inhibit the apoptosis of neurons surrounding the ischemic area. In addition, TSRP upregulated the expression of the anti-apoptotic factor Bcl-2, at the protein and mRNA levels, and it downregulated the expression of the pro-apoptotic factor Bax, at the protein and mRNA levels. These findings indicate that TSRP protects against cerebral ischemia/reperfusion injury, and that it may do so by regulating the expression of Bcl-2 and Bax.

Key Words: total saponins of *Rubus parvifolius* L.; cerebral ischemia/reperfusion; apoptosis; Bcl-2; Bax

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INTRODUCTION

Rubus parvifolius L. is an herb used in traditional Chinese medicine. The neuroprotective effects of this plant have been demonstrated in a variety of models of acute cerebral ischemia, including transient and permanent middle cerebral artery occlusion (MCAO). It significantly reduces infarct size and edema, and improves neurological outcome^[1]. Based on a preliminary pharmacological screening of the bioactive components of the shrub, we found that a total saponin extract of *Rubus Parvifolius* L. (TSRP) protects against cerebral ischemia^[2]. In addition, previous experiments demonstrated that TSRP can reduce apoptosis and scavenge free radicals^[3-4]. However, the mechanisms underlying the neuroprotective effects of TSRP on focal cerebral ischemia remain unclear. In this study, we employed the MCAO model of ischemia/reperfusion injury in rats. Using this model, we investigated the neuroprotective effects of TSRP at the behavioral, tissue, cellular and molecular levels—we assessed neurological severity, cerebral infarct volume, brain water content (BWC) and apoptosis. To better understand the mechanisms mediating apoptosis, immunohistochemistry was used to examine

the expression of Bcl-2 and Bax proteins, and in situ hybridization was used to examine the expression of Bcl-2 and Bax transcripts.

RESULTS

Quantitative analysis of experimental animals

A total of 120 rats were included in this study and they were utilized for three different assays. One batch was used for neurological function evaluation and cerebral infarct volume measurement, another batch was used for BWC determination, and the remaining animals were used to examine apoptosis. The rats in each batch were randomly divided into six groups: sham-surgery group ($n = 3$); cerebral ischemia/reperfusion group (CIR group, $n = 6$); TSRP at 5, 10 and 20 mg/kg groups ($n = 6$ each); and Nimodipine (Nim) at 10 mg/kg group ($n = 6$). There were 21 deaths during the course of the experiments. In the end, 99 rats were involved in the final analyses.

Neurological function assessment 24 hours after MCAO

Neurobehavioral testing was performed on all rats before MCAO, at 60 minutes and 24 hours after MCAO. Neurological function was graded as previously described^[5]. All

rats had a total neurological score of 0 before ischemia and a high-grade neurological deficit at 60 minutes after MCAO. The neurological deficits were significantly alleviated in rats that received TSRP treatment compared with CIR animals at 24 hours after MCAO (Table 1).

Table 1 Neurological severity scores at 24 hours after middle cerebral artery occlusion

Group	n	Neurological severity scores
Sham-surgery	3	0
Cerebral ischemia/reperfusion	6	3.46±1.02
TSRP 5 mg/kg	6	2.10±0.98 ^a
TSRP 10 mg/kg	6	1.62±0.68 ^b
TSRP 20 mg/kg	6	1.24±0.58 ^b
Nimodipine 10 mg/kg	6	1.40±0.42 ^b

Data are expressed as mean ± SD. Differences between groups were determined with a one-way analysis of variance and Student's *t*-test. ^a*P* < 0.05, ^b*P* < 0.01, vs. cerebral ischemia/reperfusion group. TSRP: Total saponins of *Rubus parvifolius* L. *n* indicates the number of samples analyzed.

Infarct volume at 24 hours after MCAO

Infarct volume was determined using 2, 3, 5-triphenyltetrazolium chloride (TTC) staining^[6-8]. Dye staining at 24 hours after MCAO is shown in Figure 1A; the infarct area appears white. Compared with the CIR group, the infarct volume of the TSRP group was significantly reduced (Figure 1B).

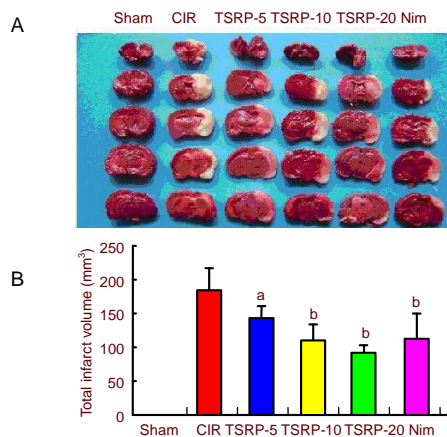


Figure 1 Cerebral infarct volume at 24 hours after middle cerebral artery occlusion. CIR: Cerebral ischemia/reperfusion; TSRP: total saponins of *Rubus parvifolius* L.; TTC: 2, 3, 5-triphenyltetrazolium chloride; TSRP-5, -10, -20: TSRP 5, 10, 20 mg/kg groups.

(A) TTC dyeing results, the infarct area is outlined in white.

(B) Infarct volume quantitative analysis results. Data are expressed as mean ± SD of three independent experiments. Differences between groups were determined with a one-way analysis of variance and Student's *t*-test. ^a*P* < 0.05, ^b*P* < 0.01, vs. CIR group.

BWC at 24 hours after MCAO

BWC was measured for each group using the dry/wet weight method^[9]. BWC in the CIR group was significantly higher than in the control group (*P* < 0.01). The infusion of TSRP significantly attenuated brain edema at 24 hours after reperfusion (*P* < 0.01 or *P* < 0.05; Table 2).

Table 2 Effect of TSRP on total brain water content (%)

Group	n	Brain water content
Sham-surgery	3	77.43±0.83
Cerebral ischemia/reperfusion	6	82.45±0.94 ^c
TSRP 5 mg/kg	6	81.31±0.87 ^a
TSRP 10 mg/kg	6	81.06±0.63 ^a
TSRP 20 mg/kg	6	79.95±0.65 ^b
Nimodipine 10 mg/kg	6	79.25±0.83 ^b

Data are expressed as mean ± SD of three independent experiments. Differences between groups were determined with a one-way analysis of variance and Student's *t*-test. ^a*P* < 0.05, ^b*P* < 0.01, vs. cerebral ischemia/reperfusion group; ^c*P* < 0.01, vs. sham-surgery group. TSRP: Total saponins of *Rubus parvifolius* L. *n* indicates the number of samples analyzed.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining for cell apoptosis at 24 hours after MCAO

TUNEL staining results at 24 hours after MCAO are shown in Figure 2A. TUNEL-positive cells are stained dark brown, exhibiting apoptotic bodies and condensed nuclei. TSRP, at 5, 10 and 20 mg/kg, significantly decreased the percentage of apoptotic cells compared with the CIR group (*P* < 0.01 or *P* < 0.05; Figure 2B).

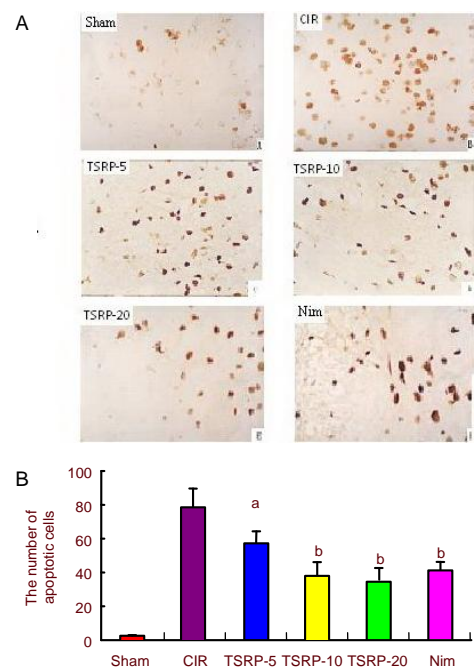


Figure 2 Apoptosis in rats at 24 hours after middle cerebral artery occlusion. CIR: Cerebral ischemia/reperfusion; TSRP: total saponins of *Rubus parvifolius* L.; Nim: nimodipine; TUNEL: transferase-mediated dUTP nick end-labeling; TSRP-5, -10, -20: TSRP 5, 10, 20 mg/kg groups.

(A) TUNEL staining for each group (× 400).

(B) Apoptosis quantitative analysis results. No positive staining was seen in the sham-surgery group. Apoptosis increased significantly in the CIR group. Apoptosis in the TSRP groups and in the Nim group were significantly reduced. Data are expressed as mean ± SD of three independent experiments. Differences between groups were determined using a one-way analysis of variance and Student's *t*-test. ^a*P* < 0.05, ^b*P* < 0.01, vs. CIR group.

Protein expression of Bcl-2 and Bax in rat brains at 24 hours after MCAO

Brain paraffin sections from each group of rats were used for immunohistochemical staining to examine Bcl-2 and Bax protein expression, because these proteins are crucial regulators of apoptosis (Table 3). Bcl-2 and Bax were expressed at low levels in the sham-surgery group. Compared with the CIR group, Bcl-2 expression was significantly higher in the TSRP groups and in the Nim group, while Bax expression was significantly lower in the TSRP 10 and 20 mg/kg groups and in the Nim group ($P < 0.01$ or $P < 0.05$; Table 3, supplementary Figures 1, 2 online).

Table 3 Change in Bcl-2 and Bax protein expression (absorbance) in rats at 24 hours after middle cerebral artery occlusion

Group	n	Bcl-2	Bax
Sham-surgery	3	0.03±0.05	0.01±0.06
Cerebral ischemia/reperfusion	6	0.16±0.03	0.28±0.06 ^c
TSRP 5 mg/kg	6	0.19±0.02 ^a	0.25±0.03
TSRP 10 mg/kg	6	0.30±0.05 ^b	0.22±0.08 ^a
TSRP 20 mg/kg	6	0.35±0.03 ^b	0.18±0.04 ^b
Nimodipine 10 mg/kg	6	0.36±0.04 ^b	0.17±0.07 ^b

Data are expressed as mean ± SD of three independent experiments. Differences between groups were determined with a one-way analysis of variance and Student's *t*-test. ^a $P < 0.05$, ^b $P < 0.01$, vs. cerebral ischemia/reperfusion group; ^c $P < 0.01$, vs. sham-surgery group. TSRP: Total saponins of *Rubus parvifolius* L. *n* indicates the number of samples analyzed.

mRNA expression of bcl-2 and bax in rat brains at 24 hours after MCAO

Brain paraffin sections from each group of rats were used for *in situ* hybridization assays to examine bcl-2 and bax mRNA expression (Table 3). Compared with the CIR group, the expression of bcl-2 mRNA in the TSRP groups and in the Nim group were significantly higher, while the expression of bax mRNA in the TSRP groups and in the Nim group were significantly lower (Table 4, supplementary Figure 3 online).

Table 4 Change in bcl-2 and bax mRNA expression (absorbance) in rats at 24 hours after middle cerebral artery occlusion

Group	n	bcl-2 mRNA	bax mRNA
Sham-surgery	3	0.02±0.02	0.03±0.01
cerebral ischemia/reperfusion	6	0.22±0.02 ^d	0.36±0.11 ^d
TSRP 5 mg/kg	6	0.26±0.05 ^{ac}	0.32±0.08 ^a
TSRP 10 mg/kg	6	0.32±0.09 ^b	0.28±0.09 ^b
TSRP 20 mg/kg	6	0.35±0.04 ^b	0.25±0.14 ^b
Nimodipine 10 mg/kg	6	0.34±0.10 ^b	0.26±0.07 ^b

Data are expressed as mean ± SD of three independent experiments. Differences between groups were determined with a one-way analysis of variance and Student's *t*-test. ^a $P < 0.05$, ^b $P < 0.01$, vs. cerebral ischemia/reperfusion group; ^c $P < 0.05$, ^d $P < 0.01$, vs. sham-surgery group. TSRP: Total saponins of *Rubus parvifolius* L. *n* indicates the number of samples analyzed.

DISCUSSION

Rubus parvifolius L. is a raspberry plant in the Rosaceae family. The roots of this plant are used as a traditional Chinese medicine for a number of different conditions^[10]. Extracts of the roots contain various terpenoids, ester compounds, and glycosides, of which total saponins have been found to have neuroprotective properties, including against ischemic injury. However, the underlying mechanisms are still unclear. Numerous studies have shown that ischemic damage disrupts numerous critical processes, perturbing cerebral homeostasis^[11-12]. The excessive production of reactive oxygen species, nitric oxide and tumor necrosis factor may initiate neuronal apoptosis^[13-14] after CIR injury^[15-17]. Apoptosis results from a cascade of cellular events that are mediated by several apoptosis-regulating genes^[18-19]. Among these, Bcl-2 and Bax are widely regarded as the most important^[20-21]. Bcl-2 is an anti-apoptotic protein whose overexpression can reduce ischemic injury. In contrast, Bax is a proapoptotic antagonist of Bcl-2^[22-23]. In this study, we demonstrate that treatment with TSRP rapidly increases bcl-2 mRNA and protein levels in cerebral neurons, whereas it decreases Bax mRNA and protein levels. The possibility that the reduction in Bax levels was a consequence of cell death can be excluded, since TSRP treatment decreased the number of apoptotic neurons. Therefore, TSRP appears to down-regulate Bax in cerebral neurons. This finding, however, contrasts with the recent observation of increased Bcl-2 immunoreactivity in neurons after transient ischemia^[24-25]. It is possible that cytokines or other signaling molecules released from glial cells following injury (perhaps during reperfusion) may modulate neuronal Bcl-2 expression. Recent studies have shown that the ratio of Bax to Bcl-2 is increased in the CA1 region^[26]. In the present study, the ratio of Bax to Bcl-2 increased during hypoxia-induced neuronal death, which supports the hypothesis that the balance in protein expression of these two crucial factors determines cell survival or death following an apoptotic stimulus^[27-28]. The temporal expression profile during hypoxia-induced neuronal death in the present study consisted of an initial decrease in Bcl-2 protein levels. Recent studies have demonstrated that overexpression of Bcl-2 protects against hypoxia-induced cell death in non-neuronal cells^[29]. Furthermore, the regulation of endogenous Bcl-2 expression by extrinsic stimuli affects cell survival in non-neuronal cells^[30]. Thus, based on these observations, the results of the present study suggest that the down-regulation of Bcl-2 expression triggers apoptosis in ischemia/reperfusion injury. In this study, neuronal death following hypoxia was accompanied by changes characteristic of apoptosis, such as cytoplasmic shrinkage, condensation or fragmentation of nuclei and the degradation of DNA into

oligonucleosomal fragments. The first detectable signs of apoptosis were observed 24 hours after hypoxic treatment^[31]. The number of condensed or fragmented nuclei detected by TUNEL staining increased. Concomitantly, DNA breaks were detected by *in situ* DNA end labeling. Morphological features of apoptotic cell death, such as cell shrinkage and loss of neurites, appeared 18 hours after hypoxic treatment. These observations indicate that the apoptotic process initiated by hypoxia starts 12 hours after hypoxic treatment at the latest^[32]. Before the appearance of apoptotic cell death, neurons undergo acute necrotic cell death, while the nuclei remain intact, there is a loss of membrane integrity^[33]. Hypoxia induces both apoptosis and necrosis in cerebral neuron cultures. In summary, TSRP protects against ischemia/reperfusion injury. It reduces cerebral infarct volume; and it may do so by increasing Bcl-2 expression and decreasing Bax expression, consequently inhibiting apoptosis.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

This experiment was performed at the Chongqing University of Medical Science, China, from 2006 to 2009.

Materials

A total of 120 healthy male adult Sprague-Dawley rats, weighing 280–350 g were acclimatized for 3 days prior to surgery. They were housed in an animal room at the second-class Experimental Animal Center, Daping Hospital, the Third Military Medical University of Chinese PLA, China, with license No. SCXK (Yu) 2001003. The laboratory was humidity (50–60%) and temperature (22–25°C) controlled under a 12-hour day/night cycle. All animals were housed in plastic cages with free access to standard food and tap water throughout the experiments. All experimental protocols were approved by the Local Experimental Ethics Committee and were in accordance with the *Regulations for the Administration of Affairs Concerning Experimental Animals*, formulated by the Ministry of Science and Technology of China^[34]. TSRP was supplied by the Pharmaceutical Analysis Department of Chongqing University of Medical Sciences, with a purity of 95%. Nim was supplied by Corina Chongqing Pharmaceutical Company Limited, China (batch No. 2004081).

Methods

Experimental groups and interventions

Ischemia was produced by occluding the middle cerebral artery with an intraluminal thread advanced rostrally into the internal cerebral artery and up to the origin of the middle cerebral artery. Both the sham-surgery and CIR model groups were given normal saline, while the others were gavaged TSRP or Nim. These treatments were administered before the ischemia/reperfusion operation, once daily for 3 days. All rats were killed 24 hours after

reperfusion.

MCAO rat model preparation

Ischemia in the middle cerebral artery of the CIR, TSRP (5, 10 and 20 mg/kg) and Nim (10 mg/kg) groups was induced as previously described^[35]. Animals were anesthetized with chloral hydrate (350 mg/kg). Rectal temperature was maintained between 36.5°C and 37.5°C. After implanting catheters into the left femoral vein, focal ischemia was induced. Briefly, a longitudinal incision of 1.5 cm length was made in the midline of the ventral cervical skin. The right common carotid artery, right internal carotid artery and right external carotid artery were identified and separated from the vagus nerve. The distal portion of the external carotid artery was ligated with two sutures and the external carotid artery was cut between the two sutures. A 4-0 silk suture was tied loosely around the origin of the external carotid artery. The right common carotid artery and internal carotid artery were temporarily clamped using microvascular clips. A small puncture was made on the wall of the external carotid artery with a pair of spring scissors. A 4-0 surgical nylon suture, 18.5–19.5 mm length, determined by body weight, with a tip rounded by heating near a flame, was inserted into the internal carotid artery. After releasing the clip at the internal carotid artery, the suture was advanced into the proximal portion of the middle cerebral artery for 2 hours. A heat lamp was used during and for 2 hours after surgery. Each group of animals was subjected to ischemia/reperfusion, except for the sham-surgery group, which did not have the nylon suture constricting the blood vessels.

Behavioral testing

Neurobehavioral tests were performed in all rats before MCAO, during occlusion at 60 minutes and 24 hours after MCAO. Neurological function was graded as previously described^[5]. Briefly, postural reflex, visual placing in the forward and sideways directions, tactile placing of the dorsal and lateral paw surfaces, and proprioceptive placing were tested. These 6 tests were each scored from 0 to 2, and the behavioral deficit was calculated as the sum of the scores of the individual tests ranging from 0 (normal) to 12 (maximum deficit). Neurological deficits were assessed by an observer blinded to the identity of the treatment.

Measurement of infarct volume

The ischemic area was evaluated using the TTC method^[6-8]. Briefly, after an overdose of chloral hydrate, the brains were quickly removed and placed in ice-cold saline for 5 minutes and then cut into 2-mm coronal slices. Sections were incubated in 2% TTC in normal saline at 37°C for 20 minutes and then stored in 4% paraformaldehyde. The infarct area was outlined in white. Ischemic lesion volumes were quantified using standard computer-assisted image analysis techniques. To avoid overestimating infarct size, the infarct volume was calculated using the following formula: infarct volume = [the volume of the left hemisphere – (the volume of the right hemisphere – measured infarct volume)]/the volume

of the left hemisphere. The infarct volume was expressed as a percentage of the total volume of the contralateral hemisphere.

Determination of BWC

BWC was determined by the dry/wet weight method as previously described^[9]. Briefly, rats were decapitated under chloral hydrate deep anesthesia 24 hours after reperfusion. Brains were immediately removed and weighed on precooled aluminum foil to obtain the wet weight. The tissue was then dried in a 100°C oven for 48 hours and reweighed to obtain the dry weight. Brain water content was calculated as (wet weight–dry weight)/wet weight × 100%.

TUNEL staining

The rats in each group were sacrificed under deep anesthesia and transcardially perfused with 4% phosphate-buffered paraformaldehyde. Paraffin-embedded sections were examined for TUNEL reactivity using an *in situ* cell death detection kit (Roche Diagnostics, Penzberg, Germany)^[36-37]. Paraffin sections were dewaxed, rehydrated, treated with proteinase K (20 µg/mL), and blocked for endogenous peroxidase activity with 3% H₂O₂. Subsequent end-labeling was performed with TdT enzyme at 37°C for 1 hour. Anti-digoxigenin peroxidase conjugate was applied to the tissue for 30 minutes at room temperature. A dark brown color indicating DNA breaks developed after incubation with diaminobenzidine tetrachloride (DAB). Apoptotic cells were counted as TUNEL-positive, in addition to the apoptotic bodies and condensed nuclei through the ischemic core and in the border region of the infarct. Four random and non-overlapping regions (500 µm × 500 µm) were sampled in the ischemic and penumbral area of the cerebral cortex and striatum of animals. TUNEL-positive cells within each subregion were counted under a light microscope using 20 × magnification objective lens (Olympus, Tokyo, Japan). The mean number of positive cells was calculated for each section in each animal.

Immunohistochemical staining for Bcl-2 and Bax protein expression

Six rats in each of the groups were sacrificed under deep anesthesia and transcardially perfused with 4% phosphate-buffered paraformaldehyde. Every paraffin embedded sample was cut into ten sections and two sections were picked randomly for immunohistochemistry. Tissues were cut into 4-µm thick sections and transferred onto polylysine-coated slides. Immunohistochemistry was performed according to the manufacturer's protocol. At first, paraffin sections were deparaffinized, dehydrated through graded alcohols and incubated in H₂O₂ (0.3%) for 15 minutes. The sections were heated in a microwave oven for 11 minutes at 98°C in citrate buffer at pH 6 for antigen retrieval, then blocked in normal goat serum (1: 10) for 30 minutes (Zhongshan Golden Bridge, Beijing, China). The sections were incubated with primary monoclonal goat anti-mouse Bcl-2 or Bax (1: 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at 36°C, followed by

incubation with biotinylated anti-goat secondary antibody for 30 seconds at 36°C. Immunoreactivity was observed with DAB. Negative controls were obtained by substituting the primary antibody with phosphate buffered saline (PBS). Using the PM20 automatic microscope (Olympus, Tokyo, Japan) and the TC-FY-2050 pathology image acquisition system (Yuancheng INC, Wuhan, China), immunopositivity was assessed semiquantitatively based on the optical densities of every random visual field. The latter was performed using the automated Motic Med 6.0 CMIAS pathology image analysis system (Beihang Motic Inc, Beijing, China).

In situ hybridization of Bcl-2 and Bax mRNA expression

Six rats from each group were sacrificed under deep anesthesia and transcardially perfused with 4% phosphate-buffered paraformaldehyde (1% DEPC-treated). Paraffin-embedded sections were placed in 4% paraformaldehyde/PBS for 5 minutes at room temperature, rinsed in PBS four times for 20 minutes each, digested with 2 µg/mL protease K at 37°C for 15 minutes, and treated with 1% phosphate-buffered paraformaldehyde for 10 minutes. The sections were hybridized with the probe in hybridization buffer for 20 hours at 37°C, followed by a rinse in 2 × saline sodium citrate (SSC) buffer for 30 minutes, twice, then in 0.5 × SSC buffer for 15 minutes, twice, at 37°C, and finally in 0.2 × SSC buffer for 15 minutes, twice, at 37°C. The sections were allowed to equilibrate in Buffer I for 2 minutes and in Buffer II for 30 minutes, and were incubated with the anti-digoxigenin antibody solution (1: 2 000) for 1 hour at room temperature. The sections were then rinsed in Buffer I for 30 minutes twice and Buffer III for 5 minutes once, and stained with DAB for 10 minutes^[38]. For the negative control, PBS was used in place of the probe, or sections were digested with RNAase before hybridization.

Statistical analysis

Data were analyzed using SPSS 11.0 software (SPSS, Chicago, IL, USA). All data were analyzed by an independent investigator blinded to the experimental conditions. Differences between groups were determined using a one-way analysis of variance or the Student's *t*-test. All the data are presented as mean ± SD. Differences were considered significant at *P* < 0.05.

Author contributions: Jisheng Wang was responsible for study design, study assessment, manuscript authorization and the funding process. Fang Zhang participated in implementing the experiments and statistical analysis. Li Tang was in charge of manuscript writing and obtaining experimental data. Liqiong Sun, Xiaolin Song, Lisha Cao, Zongyin Qiu and Chenglin Zhou provided technical instruction.

Conflicts of interest: None declared.

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Ethical approval: All experimental protocols that involved the

use of animals in this study were approved by the Institutional Animal Care and Use Committee of Chongqing University of Medical Sciences, China.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrroonline.org, and entering Vol. 7, No. 3, 2012 after selecting the "NRR Current Issue" button on the page.

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