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Short Communication

Protection by 3'-Methoxypuerarin of Rat Hippocampal Neurons Against Ischemia/Reperfusion Injury

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Abstract

3'-Methoxypuerarin (3'-MOP) is an isoflavone extracted from radix puerariae. The aim of this study was to investigate the role and the mechanism of 3'-MOP in the protection of hippocampal neurons against cerebral ischemia/reperfusion (I/R) injury in rats. I/R injury was induced by a modified four-vessel occlusion model. Rats were randomly divided into an I/R group, an I/R + 3'-MOP group and a control group. Histological changes in the neurons of the hippocampal CA1 region were observed with hematoxylin and eosine (H&E) staining. The apoptotic neurons in the hippocampal CA1 area were counted with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. The results showed that compared with the I/R group, 3'-MOP increased the number of surviving neurons in the hippocampal CA1 region ($P < 0.001$) and markedly reduced the number of apoptotic pyramidal neurons ($P < 0.001$) after I/R injury. In conclusion, 3'-MOP can protect hippocampal neurons against I/R injury by inhibiting apoptosis.

Key Words: 3'-methoxypuerarin, hippocampal neurons, ischemia/reperfusion injury, apoptosis, rat

Introduction

Puerarin is an isoflavone compound which is the main active component of radix puerariae. Puerarin has been demonstrated to ameliorate cerebral circulation and protect neurons against cerebral I/R injury (1-4, 11). The neuroprotective function of puerarin is associated with prevention of apoptosis in rats (14). Furthermore, puerarin has long been used clinically in the treatment of cerebral ischemia (6, 12, 13). 3'-MOP is another isoflavone extracted from radix puerariae and is highly similar with puerarin in its chemical structure and physical-chemical characteristics. Nevertheless, 3'-MOP has always been discarded after the purification of puerarin. Recently, several studies have demonstrated that the neuroprotection of 3'-MOP against focal cerebral I/R injury is associated with anti-oxidant and vasodilating effects in rats (5, 16, 17). However, the potential anti-apoptosis effect of 3'-MOP involved in the neuroprotective mechanisms has not been reported.

In the present study, we investigated the role and the mechanism of 3'-MOP in the protection of hippocampal neurons against acute cerebral I/R injury in rats.

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Materials and Methods

Animals

Male Wistar rats, weighing 260~320 g, were provided by the center of laboratory animals in Shandong University. The license is SCXK (Lu) 2003-0004. All procedures in this study were approved by the Institutional Animal Care and Use Committee of Shandong University.

Drugs

3'-MOP, 99% pure, was provided by Ruidemaier Biotechnology, Beijing, China. TUNEL Staining Kit was obtained from Zhongshan Biotechnology, Beijing, China. Sodium pentobarbital and sodium heparin were provided by Tianjin Biochemical Laboratory, Tianjin, China. Paraformaldehyde (P6148- 1KG) and proteinase K were obtained from Sigma, Saint Louis, USA.

Equipment

An M-6000 multiple channel recorder (KOHDEN), an OPMI 99 surgical microscope (Zeiss, Germany), T 100 electric forceps (DOLLEY S.A), a CM3050 S paraffin microtome (Leica, Germany) and a DM RXA optical microscope (Leica, Germany) were used in this study.

Cerebral Ischemia/Reperfusion Model

Forebrain ischemia was induced according to the modified Pulsinelli four-vessel occlusion method (9, 10). The vertebral arteries were electrocauterized in the rats under anesthesia with 1% sodium pentobarbital (40 mg/kg) through intraperitoneal (i.p.) injection, and threads were placed around the common carotid arteries. After 1 day, the rats were re-anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and the common carotid arteries were occluded with aneurysm clips for 8 min. After 3 days of reperfusion, the rats were anesthetized and killed for histology studies. The electroencephalogram (EEG) was recorded during the operation. The rats in which the EEG became isoelectric in 1 min after four-vessel occlusion were retained. The rectal temperature was measured and maintained above 37°C.

Experimental Design

The rats were randomly divided into three groups (n = 8 per group). [1] I/R+3'-MOP group: the rats underwent 8 minutes of ischemia and then 3 days of reperfusion. The 3'-MOP (60 mg/kg i.p.) was administrated at the moment of clamping of the carotid arteries. The rats received daily i.p. injection of 3'-MOP for 3 days. [2] I/R group: the rats underwent 8 minutes of ischemia and 72 h of reperfusion. The same volume of solvent (35% polyethylene glycol solution) was administrated in the same way as 3'-MOP. [3] Control group: the rats underwent a sham operation. Vertebral and carotid arteries were exposed but not occluded. The rats received a daily i.p. injection of 35% polyethylene glycol solution for 3 days.

Tissue Preparation

At 72 h after reperfusion, the rats were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and then transcardially perfused for 5 min with 200 ml saline containing 10 U/ml sodium heparin and then with 4% cold formaldehyde in 100 mM sodium phosphate buffer (pH 7.4) for 25 min. Brains were removed and coronal samples (3-4 mm) were obtained from 6.5 mm anterior to 2.5 mm anterior of the interaural axis, then postfixed with the same formalin solution. After post fixation for 24 h at 4°C, the brains were embedded in paraffin and sections were cut at 5 μm.

Neuropathology

Pathological damage of hippocampal cells in CA1 region was examined with standard H&E staining. The damage was evaluated by counting the number of surviving neurons of the CA1 pyramidal cell layer per × 200 field examined under light microscopy.

TUNEL Staining

After deparaffinization and rehydration, the sections were digested for 15 min in proteinase K (20 mg/ml). The reaction was terminated with tap water, and the tissue was treated with buffer A (25 mM Tris, pH 6.6, containing 200 mM potassium cacodylate and 0.25 mg/ml bovine serum albumin) for 5 min. Sections were incubated at 37°C with labeling solution containing 0.3 U/ml Tdt, 20 mM biotinylated-16-dUTP, and 1.5 mM cobalt chloride in buffer A for 1 h in a humidified chamber. The reaction was terminated with 2 × SSC. After vigorous washing with 100 mM Tris, pH 7.4, the sections were blocked with 10% goat serum in 100 mM Tris for 15 min. The DNA was visualized by treating the tissue with a 1:40 dilution of strepta-vidin-conjugated alkaline phosphatase and reacted with 0.05% 3-3'-diaminobenzidine tetrahydrochloride (DAB) for 10 min. The sections were then stained by hematoxylin. TUNEL-positive cells were expressed as the number of brown stained

Table 1. Comparison of surviving and apoptotic neurons in the control, I/R and I/R+3'-MOP groups (n = 8 per group)

Groups	Surviving neurons	Apoptotic neurons
Control group	84.91 ± 2.588	2.32 ± 0.59
I/R group	6.39 ± 0.80$^{\#}$	33.03 ± 0.82$^{\#}$
I/R+3'-MOP group	55.81 ± 2.19$^{\#*}$	12.06 ± 0.51$^{\#*}$

Data were means ± SE. Neuronal counting was expressed (means ± SE) as the number of surviving neurons/ × 200 field under light microscopy. Apoptotic neurons were expressed (means ± SE) as the number of brown stained cells/ × 200 field under light microscopy. Five × 200 images were taken from one of the 4 sections of each rat for the statistics. $^{\#}P < 0.001$, *vs*. Control; $^{*}P < 0.001$, *vs*. I/R.

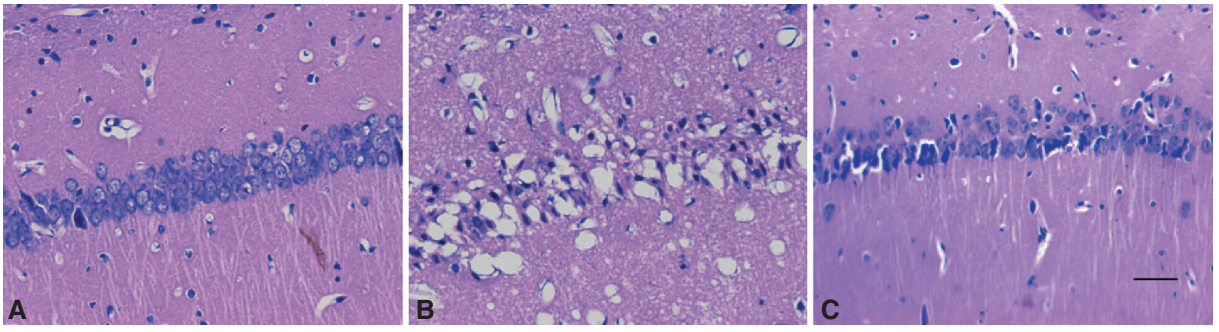

Fig. 1. H&E staining of neurons in the hippocampal CA1 region. A: Control group, B: I/R group, C: I/R+3'-MOP group. After fixation by perfusion *in situ* with formalin, standard 5 μm paraffin sections of the brains were made and treated by a standard histological method. Dramatic neuronal loss occurred in B compared with A whereas there were many more surviving neurons in C. Scale bar, 30 μm.

cells per × 200 field under light microscopy.

Statistical Analysis

The data were analyzed by SPSS10.0 and expressed as means ± SE. Comparisons of the three groups were made by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered statistically significant when $P < 0.05$.

Results

Neuropathological Changes of Hippocampal CA1 Neurons

Compared with the I/R+3'-MOP group and the control group, severe neuronal losses occurred in the CA1 area of the I/R group. Dying cells showed shrunken cytoplasm and degeneration of the nuclei and the number of surviving neurons was significantly decreased ($P < 0.001$, Table 1) (Fig. 1B). In the 3'-MOP-treated group (Fig. 1C), most of the neurons survived in the same brain area, and the number of intact neurons was much greater than that in the I/R group ($P < 0.001$) but was still less than that in the control group ($P < 0.001$) (Fig. 1A). These results imply that 3'-MOP exerts neuroprotective effects on the I/R damage of pyramidal cells in the CA1 region of hippocampus in rats.

TUNEL Staining Observations

No TUNEL-positive neurons were detected in the CA1 region of the normal control rats (Fig. 2A). Extensive TUNEL-positive neuronal staining appeared in the CA1 region of the I/R group (Fig. 2B). The TUNEL-positive neurons showed chromatin condensation in the perinuclear regions which are generally accepted as hallmarks of apoptosis (arrows). In the I/R+3'-MOP group, only occasional TUNEL-positive neurons were observed (Fig. 2C). The apoptotic cells in the I/R+3'-MOP group were more numerous than in the control group ($P < 0.001$) (Table 1) but were much fewer than in the I/R group ($P < 0.001$). These findings demonstrate that 3'-MOP treatment greatly reduced the number of TUNEL-positive neurons.

Discussion

Radix puerariae can be used as medicine and food. The main active components of radix puerariae are compound of isoflavones which include puerarin, 3'-MOP and soy daidzein. Many basic research and

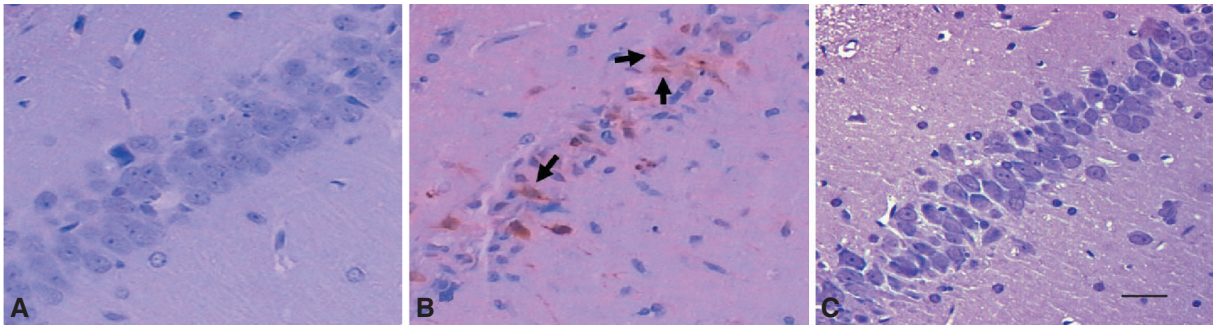

Fig. 2. TUNEL staining to demonstrate neuronal apoptosis in the hippocampal CA1 region. A: Control group, B: I/R group, C: I/R+3'-MOP group. After fixation by perfusion *in situ* with formalin, standard 5 μm paraffin sections of the brains were first made. Following deparaffinization and rehydration, the standard protocol of R&D systems for staining the dislocated DNA was applied (see methods) with revelation by DAB (dark brown staining). Counterstaining was performed with hematoxylin. Arrows indicate apoptotic cells. There were no apoptotic cells in the hippocampal CA1 region in A, a large number in B and clearly a reduced number in C. Scale bar, 30 μm.

clinical studies have demonstrated that puerarin can lower blood pressure (4, 12) and blood lipid (4) and blood glucose concentrations (4, 6). Our previous study demonstrated that puerarin administration at 50 mg/kg i.p. significantly ameliorated cerebral blood flow (3). Furthermore, puerarin has a significant therapeutic effect on cerebral ischemic injury in clinical use (6, 12, 13). More recently, many studies have been done on the development and utilization of puerarin (6, 7).

In the course of extraction of puerarin from radix puerariae by advanced technologies, puerarin can be separated well from 3'-MOP, and the purity of both products can attain 99%. However, 3'-MOP has always been discarded after the purification of puerarin. Even now, 3'-MOP is only used as a related substance, or as a marker, in the process of measuring puerarin from puerarin preparations. By modulating the amount of 3'-MOP, the technique can be controlled and the production of puerarin can be improved (11). Examination of 3'-MOP with two dimensional nuclear magnetic resonance (NMR) has indicated that it is an isoflavone highly similar to puerarin in chemical structure (15) and could have strong biological activities. It has been reported that the neuroprotective function of 3'-MOP is associated with its anti-oxidant and vasodilating effects (5, 16, 17). Our preliminary experiment on the dose-effect relationship of 3'-MOP showed that administration of 3'-MOP at 60 mg/kg i.p. improved hemodynamics in rat. Recent data from our study group also showed that 3'-MOP had the property of blocking β-adrenergic receptors, regulating the hemodynamics and increasing the blood flow in the hippocampus (unpublished). Thus, we chose the administration of 60 mg/kg i.p. in this study. In the current study, H&E staining demonstrated that neuronal damage of the hippocampal CA1 region in the 3'-MOP treated group was significantly decreased compared to the same brain area in the I/R group indicating that 3'-MOP exerted neuroprotective effects on the cerebral I/R injury. TUNEL staining demonstrated that the apoptotic cells in the I/R+3'-MOP group were much fewer than those in the I/R group. Our previous work has found that apoptosis through mitochondrial pathway is involved in the cerebral I/R-induced neuronal damage in the rat hippocampus (9). Thus, we speculate that 3'-MOP has a role in the inhibition of apoptosis through the mitochondrial pathway after cerebral I/R injury.

In conclusion, our data reported here demonstrate for the first time that 3'-MOP may protect against cerebral I/R injury through the prevention of apoptosis. This finding is important in the development of radix puerariae as a natural traditional medicine and the enlargement of its clinical value in the treatment of cerebral I/R injury. Further studies will need to be performed on the other mechanisms that may be involved in the protective action of 3'-MOP against cerebral I/R injury.

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