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Neuroprotective effects of the Chinese Yi-Qi-Bu-Shen recipe extract on injury of rat hippocampal neurons induced by hypoxia/reoxygenation

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ABSTRACT

Objective: To explore the protective effects of the Chinese Yi-Qi-Bu-Shen recipe (YB) against neuronal injury induced by hypoxia–reoxygenation, which has shown beneficial effect in improving the brain function of type 2 diabetics likely through its antihyperglycemic, antioxidant activity, and investigate its mechanisms.

Methods: The bilateral hippocampus was collected from newborn rats to establish single cell suspension. On the 10th day, the primarily cultured hippocampal neurons were randomly divided into five groups: the normal group (NG), the hypoxia/reoxygenation group (HG), and groups protected with small, medium and large dosages of YB (SG, MG and LG, respectively). The YB-protected groups were treated with different concentrations of YB containing serum before reoxygenation. The metabolic rate of MTT, the malondialdehyde (MDA) content, and the activity of superoxide dismutase (SOD) and lactate dehydrogenase (LDH) were measured with assay kits. The apoptosis rate of hippocampal neurons were tested using flow cytometry analysis. RT-PCR was used to evaluate the mRNA expressions of bcl-2 and bax genes.

Results: The SOD activity, the cell survival rate, the bcl-2/bax ratio, and the bcl-2 mRNA expression in the HG group were significantly lower (all $P < 0.01$), but the levels of MDA and LDH, the apoptosis rate, and the bax mRNA expression were higher (all $P < 0.01$) than those in the NG group. The SOD activity, the cell survival, the bcl-2 mRNA expression, and the bcl-2/bax ratio were significantly higher in all of the YB-protected groups (all $P < 0.01$), but the level of MDA and LDH, the apoptosis rate, and the bax mRNA expression were lower ($P < 0.01$, $P < 0.05$) than those in the HG group in a dose dependent manner.

Conclusion: The YB extract has a protective effect on hippocampal neurons against injury induced by hypoxia/reoxygenation, through its antioxidant activity and the regulation of apoptosis.

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1. Introduction

Oxygen-derived free radicals doctrine plays an important role in the mechanisms of cerebral ischemia injury; the oxyradical results in a tissue's structure damage leading to cellular necrosis, programmed cell death and functional disorder (Feng et al., 2008; Olmez and Ozyurt, 2012). Numerous studies have demonstrated that hypoxia and oxidative stress are associated with diabetic complications and neurodegenerative disorders (Sayre et al., 2001; Catrina et al., 2004; Jomova et al., 2010; Wright et al., 2010; Tang et al., 2012). In recent years, natural products with antioxidant properties have drawn increasing attention as potential candidates for the prevention or treatment of neurodegenerative disorders caused by oxidative damage (Noda et al., 1997). Natural herbal products have been used in traditional Chinese medicine (TCM) for thousands of years. Though the active ingredient has not been fully identified in most of these mixed herbal medications, it is widely recognized that synergism among components is an important characteristic (Cheng, 2000). The Yi-Qi-Bu-Shen recipe (YB) is a traditional Chinese herbal formula that is commonly used for the treatment of diabetes especially diabetic encephalopathy. It is composed of nine herbs: *Astragalus membranaceus* (Fisch.) Bge., *Rehmannia glutinosa* Libosch., *Polygonatum sibiricum* Red., *Ligusticum chuanxiong* Hort., *Lycium barbarum* L., *Epimedium brevicornu* Maxim., *Atractylodes lancea* (Thunb.) DC., *Pueraria lobata* (Willd.) Ohwi, *Coptis chinensis* Franch. It has been previously demonstrated that the traditional Chinese medicine, with the function of invigorating Qi and tonifying kidneys, displays anti-oxidation and anti-apoptosis

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effects on cerebral ischemia injury (He et al., 2003; Tian et al., 2003). Recent studies have reported that the YB and its constitutive extracts have plenty of obvious therapeutic effects such as promoting the insulin sensitivity, decreasing blood glucose level, resisting free radical, inhibiting hippocampal neuronal apoptosis, increasing learning and memory functions of diabetics, and thus improving clinical symptoms of diabetic brain function (Liu et al., 2004, Liu et al., 2005, Liu et al., 2006, 2011b). We found the YB could also treat ischemic stroke in clinical practice and the therapeutic effect was notable but lack of enough experimental evidences. Anti-oxidation can be an important factor both in the treatment of ischemic stroke and improving diabetic brain function. However, whether this formula can treat more neurodegenerative diseases and the exact mechanism is still unknown. In this study, an injured model induced by hypoxia/reoxygenation was established to study the antioxidant and anti-apoptosis effects of YB extract.

2. Materials and methods

2.1. Materials

The experiment was carried out in the Institute of Basic Medical Sciences, Qilu Hospital of Shandong University from February to July, 2008. Neonatal Wistar rats (1-day-old, both male and female with a mean body mass of 5 g) and mature male Wistar rats (mean body mass of 250 g) were provided by the Experimental Animal Center of Shandong University (certification: SCXK20030004). All rats received humane care, and the study protocol was approved by the Ethics Committee of Qilu Hospital of Shandong University. The inverted phase contrast microscope CKX41 was provided by the Olympus Corporation of Japan. Culture medium of various volumes, 96-well and 24-well culture plates, MTT, Trypsin, arabinosyl cytosine, and poly-L-lysin were Sigma products. DMEM/F12 and Trizol were products of GIBCO Company. Fetal bovine serum was provided by Hangzhou Sijiqing Company. SOD, LDH, and MDA test kits were purchased from Nanjing Jiancheng Bio-engineering Institute. Rabbit anti-rat MAP-2 antibody and goat anti-rabbit IgG labeled by TRITC were products of Beijing Zhongshan Golden Bridge Biotechnology Company. MML-V reverse transcriptase, Taq DNA polymerase, and Oligo (dt) were provided by Fermentas Company. Other reagents were national analytical pure, and the primer was synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd.

2.2. Preparation of serum with YB

The composition and preparation of the YB extract are identical with our previous studies (Liu et al., 2011a, b). Preliminary quality control was established as described previously (Zhong et al., 2010). All crude drugs were the products of Jianlian Traditional Chinese Medicine Co. Ltd. (Jinan, China). They were chopped finely and extracted with 20 times the amount of distilled water at 100 °C for 2 h three times. The filtrate was evaporated under reduced pressure and dried out in a vacuum drying oven (yield: 28.06%) after filtering the insoluble matter. In the YB, the polysaccharide content was determined by sulfuric acid–phenol method, and the content of Astragaloside IV was determined by HPLC–ELSD, and other compounds (including puerarin, icariin, berberine and 5-hydroxymethylfurfural) were determined by HPLC-UV. Polysaccharides, Astragaloside IV, puerarin, icariin, berberine and 5-hydroxymethylfurfural content in the YB were determined to be 5.01%, 0.008%, 0.434%, 0.062%, 0.108% and 0.022%, respectively.

The various concentrations of YB extract (2.5%, 5%, 10%) were prepared with distilled water. The concentrations of YB extract were selected on the basis of the previous studies and our preliminary experiment (Liu et al., 2011a, b). A total of 16 male Wistar rats were randomly divided into four groups, the former three groups were orally administered different concentrations of YB extract (10 mL/kg B.W./d), respectively and the control group was given the same dosage of normal sodium. Ninety min after the last administration on the third day, all the rats were anesthetized with 10% Chloral Hydrate (350 mg/kg). The blood was drawn from atrium dextrum and centrifuged for 8 min (3000 rpm) after coagulation. The serum was put in 56 °C water bath to destroy infectivity and stored at −20 °C. Then the various concentrations of serum containing YB were prepared successfully.

2.3. Primary culture of hippocampus neurons

Newborn rats were frozen for 5–10 min and sterilized with 75% alcohol. Their heads were cut and their brains harvested. Then, the bilateral hippocampus was separated and digested with a 0.125% Trypsin for 15 min at 37 °C. Digestion was terminated by fetal bovine serum (FBS), and the brain tissue was delivered with Pasteur pipettes to establish single cell suspension. Extracted cells (1×10^6 /L) were cultured on poly-L-lysin coated plates in DMEM/F12 media supplemented with 10% FBS for 24 h in sterile conditions (37 °C, 5% CO_2, 100% humidity). From the third to the fourth day, 5 mg/L of arabinosyl cytosine was added to the culture medium to suppress overpopulation of non-neurocytes and increase the purification rate of neurons. 50% media was changed every two days and cells were check periodically using an inverted phase contrast microscope (Olympus CKX41, Tokyo, Japan). Neurons were identified by MAP-2 Immunofluorescence staining on the seventh day (Fig. 1A). On the tenth day, hippocampal neurons were rounded or elliptic, smooth and glossy with good stereo and strong light refraction. As cells mature and had stable cell morphology (Fig. 1B), they were used in experiments.

2.4. Grouping and intervention

On the tenth day, the primarily cultured neonate rat hippocampal neurons were randomly divided into five groups: the normal group, the hypoxia/reoxygenation group, and the large, medium, and small dosage of YB-protected groups. The hypoxia/reoxygenation group and the YB-protected groups were treated with non-serum DMEM solution and cultured in a hypoxic environment (37 °C, 95% N_2, 5% CO_2, oxygen content under 1%) for 6 h. Various concentrations of YB containing serum were added to the small, medium and large dosages of YB-protected groups within 24 h of reoxygenation, respectively. Then the hypoxia/reoxygenation injury model in rat hippocampal neurons was established successfully (Wu et al., 2003; Zhu et al., 2009b). All cell culture studies were performed in nonuplicate.

2.5. Survival rate of cells detected with MTT method

Neurons in the wells were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium romide (MTT) (500 mg/ml) for 4 h at 37 °C. Subsequently, the culture medium was deprived and cells were rinsed twice with ice-cold 0.1 mol/L PBS. 200 μl DMSO was then added to each well to dissolve the blue deposit after the above treatment was finished. Optical density (OD) was measured at 570 nm. The survival rate = OD assay/OD normal × 100%.

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