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The heart-protective mechanism of Qishaowuwei formula on murine viral myocarditis induced by CVB3

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ABSTRACT

Aim of study: The heart-protective effect and mechanism of Qishaowuwei formula (QSW), a Traditional Chinese Medicine formula composed of *Radix Astragali*, *Radix Paeoniae Rubra* and *Fructus Schisandrae* was investigated on murine model of viral myocarditis (VMC) induced by Coxsackievirus B3 (CVB3).
Materials and methods: Mice were randomly divided into infected control group, QSW high dose group, QSW medium dose group, QSW low dose group and Vitamin C plus Ribavirin treatment group. 50 mice were included in each group. The day of virus inoculation was defined as day 0 and the drug treatment continued once a day for 14 days. Mice were sacrificed on days 3, 7, 14, 21 postinoculation (p.i.). The histopathological changes of myocardium, CVB3 RNA copies in the myocardium, cardiomycytic apoptosis, the serum level of superoxide dismutase (SOD) and maleic dialdehyde (MDA) and the phenotype of T lymphocytes subsets in peripheral blood was analyzed.
Results: QSW treatment significantly increase the survival rate ($p < 0.05$) in VMC model. Histopathology and flow cytometry inspection revealed low ratio of cardiomyocytes necrosis and apoptosis in QSW treated mice with dose dependent manner. The cardiomyocytic ultra-structure observed by transmission electron microscope also supported the above results. The ameliorated tissue damage was consistent with reduced CVB3 copy numbers detected by real-time PCR in the myocardium of QSW treated mice. The antioxidant effect of QSW was proved by elevated activity of SOD and reduced level of MDA in the serum. Furthermore, the disturbed balance of CD4+ and CD8+ subsets in peripheral blood was restored.
Conclusion: These results demonstrated QSW had potent protective effect against CVB3-induced heart injury and this effect might be mediated by its inhibition on viral replication, antioxidant activity and immunoregulation mechanism.

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1. Introduction

Viral myocarditis (VMC), clinically defined as inflammation of the heart muscle caused by viral infection, is an insidious disease and a major cause of youth sudden death, accounting for approximately 20% of cases in adults less than 40 years of age and myocardial inflammation could be detected in 1–9% of routine postmortem examinations (Drory et al., 1991; Feldman and McNamara, 2000). The clinical features of myocarditis are varied. Endomyocardial biopsy specimens reveal evidence of both myocytolysis and infiltrating inflammatory cells. Although the pathogenesis mechanism of VMC has not been completely understood, the physiopathology can be disaggregated into the direct injury by infectious viruses invade, resulting in death or dysfunction of myocytes and subsequent acute and chronic autoimmune reaction. Coxsackievirus B (CVB, enterovirus) is among the most common pathogens of VMC (Feldman and McNamara, 2000; Magnani and Dec, 2006). They could directly destruct the structural integrity of myocytes by viral protease cleaving dystrophin and induce necrosis after receptor-mediated endocytosis (Badorff et al., 1999; Bergelson et al., 1995). VMC frequently precedes the development of dilated cardiomyopathy (DCM) and heart failure (Feldman and McNamara, 2000; Magnani and Dec, 2006). Increasing evidence supports the role of cardiomyocytes apoptosis in the development of VMC and myocardium remodeling (Kawano et al., 1994; Ellis and Di Salvo, 2007) and free radicals relate to the pathogenesis (Hiraoka et al., 1992; Suzuki et al., 1993; Chen and Zhou, 2001; Xie et al., 2002).

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Multiple treatments have been under investigation for treating VMC, including antiviral agents, immunoglobulins, immunosuppressants, etc. (Anandasabapathy and Frishman, 1998; Feldman and McNamara, 2000). However, no effective clinical therapy has been available so far. Traditional Chinese Medicine (TCM) is distinguished in treatment of cardiovascular diseases including viral myocarditis, either as compound formula or single herb by study *in vivo* and *in vitro*, such as Radix Astragali (Meng et al., 2005); Sheng-Mai-San (Wang et al., 2002), Salviae miltiorrhizae injection and Shengmai injection (Liu et al., 2004), etc. No serious adverse effect was reported. Although quite a lot of herb formulas have been clinically adopted for VMC in China based on TCM theory and empirical education, the pharmacological evaluation is still lacking.

Qishaowuwei formula (QSW) is a Chinese herbal prescription for treating viral myocarditis (VMC) and consists of three Chinese herbs: Radix Astragali (Huang-Qi in Chinese), Radix Paeoniae Rubra (Chi-Shao in Chinese) and Fructus Schisandrae (Wu-Wei-Zi in Chinese). The treatment mechanism of QSW on murine myocarditis model was investigated in the present study to support its clinical application and facilitated our understanding of TCM in treating viral diseases.

2. Materials and methods

2.1. Animals and drugs

Inbred male 6–8 week-old BALB/c mice (18–22 g) were supplied by Shandong University Laboratory Animal Center (Jinan, Shandong, China). Animals were allowed free access to standard diet and sterile water in a restricted access room with controlled temperature (22–26 °C), humidity (50–60%) and 12 h light/dark cycle in Laboratory Animal Center of Provincial Hospital affiliated to Shandong University. The experimental protocol was performed in accordance with the Guidelines for Animal Experiments of Shandong University and approved by the Institutional Authority for Laboratory Animal Care.

QSW was provided by Pharmaceutical Research Institute, China Academy of Chinese Medical Sciences (number of voucher specimens 040922, 040924, 040926 respectively). The herbal origin and nature were authenticated by the Pharmaceutical Research Institute. The quality of plant materials and purity were according with Pharmacopoeia of the People's Republic of China. The clinical prescription of QSW contains 15 g of Radix Astragali, 10 g of Radix Paeoniae Rubra and 7.5 g of Fructus Schisandrae per day. 10 g powdered extract of QSW is derived from 21.7 g crude drug. The purity of extract was evaluated by high efficiency liquid chromatography. The human clinical dose was converted to medium dose for mouse in the study based on the body surface area (Sun et al., 2002): 10.8 g/kg for high dose group, 5.4 g/kg for medium dose group and 2.7 g/kg for low dose group. QSW was made into even suspension of corresponding concentration using sterile drinking water for mice and mice accepted 0.1 ml of QSW by oral administration. Vitamin C (Hefeng Medicine Co. Ltd., Shanghai, China) was given with 1.5 g/kg by oral administration and Ribavirin (Lukangchengxin Medicine Co. Ltd., Shandong, China) with 0.1 g/kg by intramuscular injection.

2.2. CVB3-induced myocarditis

Mice were intraperitonially (i.p.) injected with 0.15 ml Coxsackievirus B3 (CVB3) (TCID50 10^9/ml, Nancy strain, from Virology Department, Shandong Academy of Medical Sciences) diluted in RPMI 1640 medium (Invitrogen, Beijing, China) to induce acute viral myocarditis as suggested previously (Hiraoka et al., 1992) and our pilot experiments. They were randomly divided into five groups: group B (Infected control group), group C (high dose group), group D (medium dose group), group E (low dose group) and group F (Vitamin C plus Ribavirin treatment group). 50 mice were included in each group. 40 mice were injected i.p. with 0.15 ml RPMI 1640 medium, as normal control group (group A). The day of virus inoculation was defined as day 0 and the drug treatment continued once a day for 14 days. Mice in normal control group and infected control group accepted 0.9% saline of 0.1 ml as placebo.

2.3. Sampling havseting

After the day of virus inoculation (day 0), mice were carefully observed daily and the body weight was recorded till the end of the experiment. 10 surviving mice in each group were sacrificed on days 3, 7 and 14 postinoculation (p.i.). On day 21 p.i., 9 surviving mice of group B and 10 mice of every other group were sacrificed. The heart was taken out aseptically and cut along the middle line of left ventriculus cordis. Part of heart tissue was fixed in 10% neutral formaldehyde for histopathological examination. Two mice of each group were randomly chosen and the cardiac specimens of 1–2 mm in diameter were fixed in 3% glutaraldehyde for electron microscope examination. Part of heart tissue was immediately frozen in liquid nitrogen and stored at −80 °C for total RNA extraction. Fresh blood samples were sent to flow cytometry detection. Aliquot of serum samples were stored at −20 °C for superoxide dismutase (SOD) and maleic dialdehyde (MDA) detection.

2.4. Histopathology and ultra-microstructure examination

The myocardial samples were embedded in paraffin. 5 μm of slices were cut along heart axes from cardiac apex. Sections were stained with hematoxylin and eosin (H&E) and examined at 100× and 200× for evidence of cardiopathological alterations. Then five high power fields (HPF) (400× magnification) were selected to calculate pathologic scores of necrosis and mononuclear cell infiltration by the ratio between involved area and total view area as described previously (Rezkalla et al., 1988): zero, no lesions; one, lesions involving <25%; two, lesions involving 25–50%; three, lesions involving 50–75%; four, lesions involving >75%. The histopathological study was performed in Pathology Department of Provincial Hospital affiliated to Shandong University and the examiner was blinded for the presence of myocarditis under light microscope. The electron microscope specimen were processed in the Electron Microscope Institute of Medical College of Shandong University and observed under transmission electron microscope (JEM-1200E, JEOL, Tokyo, Japan).

2.5. Analysis of apoptosis and necrosis by flow cytometry

On the sacrifice day, heart samples containing both left and right cardiac tissue were made into cell suspension by trypsin (Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China) for apoptosis and necrosis detection by flow cytometer with Annexin V-FITC kit (Jingmei BioTech Co. Ltd., Shenzhen, China). Briefly, (1) wash cells twice with cold PBS and then resuspend cells in 1× binding buffer at a concentration of 1×10^6 cells/ml; (2) transfer 100 μl of the solution (1×10^5 cells) to a 5 ml culture tube; (3) add 5 μl of Annexin V-FITC and 5 μl of propidium iodide; (4) vortex the cells gently and incubate for 15 min at RT (25 °C) in the dark; (5) add 400 μl of 1× binding buffer to each tube; (6) analyze by flow cytometry within 1 h. The percentage analysis was finished on BECKMAN COULTER EPICS XL-4 Flow Cytometer with SYSTEM II^{TM} software (Beckman Coulter, Inc., Fullerton, USA).

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