

Traditional Chinese Medicine Column

Influence of *Xiaochaihu* Decoction on Myocardial Cytopathic Effect and Enzyme-histochemistry in Myocarditis in Vitro

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Abstract

Objective: To evaluate the therapeutic effects of a traditional medicine *Xiaochaihu* Decoction on viral myocarditis. **Methods:** Newborn rat myocardial cell cultures were infected with Coxsackievirus B₃ (CVB₃) and divided into two groups. One group was treated with *Xiaochaihu* Decoction while the others acted as controls. Cytopathic changes of the myocardial cells in the two groups were compared at 24, 48, 72, and 96 hours after infection. The two groups were also compared for the Cytochrome C Oxidase (CCO) activities at these time points, using enzyme-histochemical method and computerized microphotography. **Results:** In the controls, progressive cytopathic changes were observed since 24h after infection, which was associated with a progressive decline in CCO activities. In the *Xiaochaihu* Decoction-treated group, the cells exhibited cytopathic changes and decrease in CCO activities which were also first noticed at 24h but to a much lesser extent than those in the controls. The cells however recovered quickly to regain normal morphology and near normal CCO activities. **Conclusion:** The findings suggested that *Xiaochaihu* Decoction has beneficial effects on myocardial cells infected by Coxsackievirus in vitro.

Key words

Coxsackievirus B₃ (CVB₃); Cytochrome C Oxidase (CCO); Cytopathic Effect (CPE); Traditional Chinese Medicine (TCM)

Introduction

Coxsackie viral myocarditis frequently occurs in children especially in the newborns. The fatality rate was high, and there is so far no effective specific treatment. In an endeavor to explore for a medicine effective in the

treatment of this condition, we examined in-vitro the protective effects of a traditional Chinese medicine, *Xiaochaihu* Decoction, on newborn rat myocardial cells infected with Coxsackievirus. Myocardial enzyme-histochemistry and cytopathic changes were used as the markers of cell injury.

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Materials and Methods

Xiaochaihu Decoction

The *Xiaochaihu* Decoction contained chaihu, astragalum, panax ginseng, pinellia tuberifera, licorice root, ginger and jujube in a concentration of 100 mg/ml. The decoction was sterilized under high pressure and stored at 4°C.

Viruses

Coxsackievirus B₃ (CVB₃), Nancy Group, was prepared by the Capital Pediatric Institute, and stored at -20°C.

Myocardial Cell Culture¹

The culture fluid contained 20% growth-promoting media-DMEM (Dulbecco's Modified Eagle Medium, Life technologies, Inc, USA), 20% Fetal Bovine Serum (FBS), routine Glutamine and antibiotics (Penicillin and Streptomycin).

Myocardial tissues were obtained under aseptic condition from the cardiac ventricle of 1 to 3-day old newborn Sprague-Dawley rats. The tissues were rinsed in Phosphate Buffered Saline (PBS), cut to 1 mm³ pieces, and then rinsed in PBS for 3 more times. The tissues were then digested in 0.08% trypsin, and mixed well by magnetic stirring (100 RPM) at 37°C for 4 times, each lasting for 15 minutes. The fluid containing the cells was centrifuged at 800 rpm for 8 minutes, and the supernatant was discarded. Cold culture fluid was then added to terminate trypsin digestion. After centrifugation, 20% growth-promoting media was added and the mixture was stirred repeatedly. Subsequently, the cellular fluid was placed in equal parts into the culture flasks and culture plates with cover slips and incubated at 37°C. The culture fluid was replaced daily until the myocardial cells grew to monolayer cells in 3-4 days.

Determination of the Highest Non-lethal Dose of Xiaochaihu Decoction to Myocardial Cells

The Xiaochaihu Decoction was diluted with 20% growth-promoting media serially to 2.0, 1.0, 0.5, 0.25, and 0.125 mg/ml. 2 ml of the decoction at each of these concentrations was added to each culture flask containing mono-layer myocardial cells, and incubated at 37°C. The specimens were observed daily and the highest non-lethal dose of Xiaochaihu Decoction was determined.

Fifty Percent Tissue Culture Infective Dose (TCID₅₀) of CVB₃ for Rat Myocardial Cells

The TCID₅₀ of CVB₃ Nancy Group on rat myocardial cells was determined by titration, and found to be 10⁻⁶.

Cytopathic Effect (CPE) of Rat Myocardial Cells

Nineteen mono-layer myocardial cell flasks were divided randomly into 4 groups: Group A (normal control group, n₁=2), group B (drug control group, n₂=2), group C (virus-infected non-treatment group, n₃=8), and group D (virus-infected treatment group n₄=7). In Group C and D, 2.5 ml TCID₅₀ of CVB₃ were added to each of the culture flasks, whereas 2.5 ml of 20% growth-promoting media were added to each of the culture flasks in Group A and B, The

mixtures were adsorbed for 1 hour at 37°C. After discarding the culture fluid, 2.5 ml of 0.5 mg/ml Xiaochaihu Decoction were added to each of the culture flasks in group B and D, and 2.5 ml of 'non-drug' 20% growth-promoting media were added to each of the flasks in group A and C. All the flasks were incubated at 37°C, and CPE was observed daily under a Nikon TMS inverted microscope.

Activities of CCO (Cytochrome C Oxidase)

Pores of monolayer myocardial cell plates were allocated randomly into 4 groups, treated in the same manner as that described above for the culture flasks. At 24, 48, 72, and 96 hours after viral infection, 8 cover-slips in each group were removed and color -developed histochemically for the estimation of CCO activities: the cover-slips were placed in 3% paraformaldehyde in 1% PBS (PH7.4), fixed at 4°C for 1 hour, rinsed in 0.1 mol/L PBS (PH7.4), containing 0.2 mol/L sucrose, and incubated in the reaction media of Seligman CCO at 37°C for 2 hours. Subsequently, the cover slips were rinsed in PBS and mounted with Glycerogelation. Cytochrome C Oxidase activity was estimated using an Image Analyzer (Luzex-F, Japan). Two randomly chosed optical fields in every group were measured.

Statistical Analysis

All data were expressed as mean±SEM. Comparisons between experimental groups were performed with analysis of variance (ANOVA) and Post Hoc Tests with SPSS 10.0. Differences were considered significant if the probability value was less than 0.05.

Results

Growth of Rat Myocardial Cells

By 24h, myocardial cells began to appear and were stuck on the flask wall. The cells, which were spindle-shape in the beginning, grew to the monolayer after 3-4 days, and changed their morphology to being broad and irregular in shape, with indistinct nuclei (Figure 1).

Non-lethal Dose of Xiaochaihu Decoction to Myocardial Cells

The non-lethal dose of Xiaochaihu Decoction was found to be ≤0.5 mg/ml. At doses higher than 0.5 mg/ml, the myocardial cells shrand in size, became detached (Figure 2). The cells treated with lower doses (≤0.5 mg/ml) remained normal in their morphology.

CPE of Rat Myocardial Cells

By 24h of incubation with the virus, cytopathic changes were present in the virus-infected non-treatment group C. The myocardial cells shrank in size, became detached, and formed a suspension in the fluid. The cell membranes and nuclei became distinct. By 72h, vacuolation of cytoplasm and reticular necrosis occurred (Figure 3). In the virus-infected treatment group D, cytopathic changes were also present by 24h of incubation with the virus, but at a significantly smaller magnitude when compared to those in group C. In contrast to group C, the cytopathic process did not progress. There was a small amount of cell shrinkage, but quickly recovered their normal shape. The cell bodyline was clear, the nuclei remained indistinct and the cell bodies remained full (Figure 4). There were no

cytopathic changes in the normal control group A and the drug control group B.

Alteration of Activities of Myocardial CCO (Table 1)

Clear brown granules representing active products of CCO could be seen microscopically in the cellular cytoplasm in all except the virus-infected non-treatment group C, where the granules were faint and sparse. Image analysis showed an obvious decline in the CCO activities in group C, being significantly lower than those in group A at 24, 48, 72, and 96 hours after incubation with the virus. In group D, the activities of CCO recovered to near normal levels which were significantly higher than those in group C and similar to those in group A at 24, 48, 72, and 96 hours. The CCO activities in group A and B did not show any significant differences at all the time points.

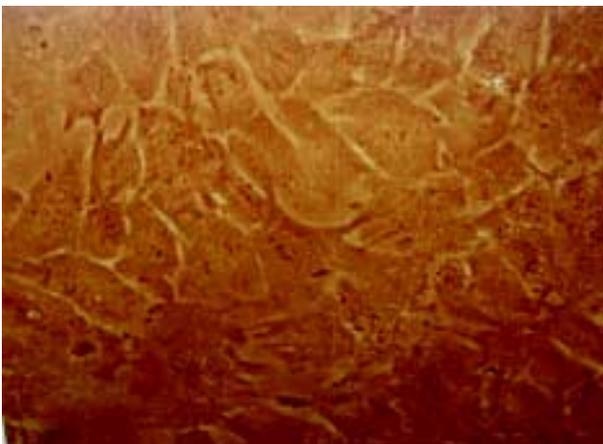


Figure 1 Normal myocardial cells.



Figure 2 Reaction of drug poison.

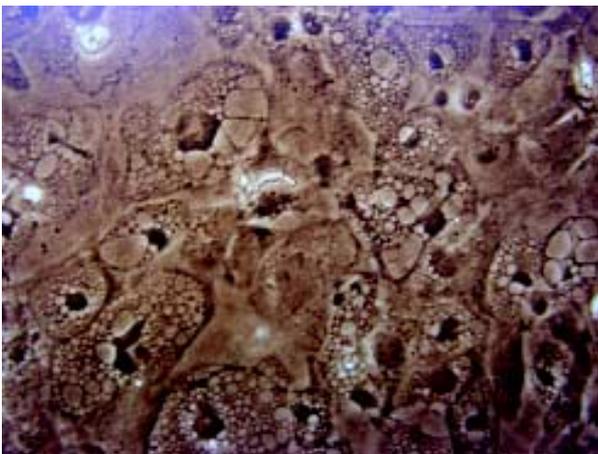


Figure 3 Virus-infected non-treatment group.

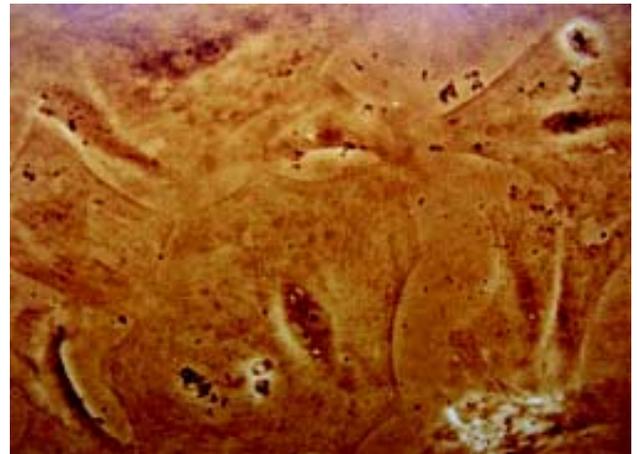


Figure 4 Virus-infected treatment group.

Table 1 Activities of CCO of rat myocardial cells in different periods

Group	CCO ($\bar{x}\pm s$)			
	24h	48h	72h	96h
A	121.96±5.51*	118.93±8.65*	117.13±8.50*	115.49±5.38*
B	120.35±4.15	120.33±7.35	120.82±6.25	117.59±4.91
C	104.30±10.88	96.00±6.99	91.79±7.07	88.32±4.00
D	118.81±3.41*	117.83±7.90*	117.25±6.22*	113.71±5.98*

A: Normal control group

B: Drug control group

C: Virus-infected non-treatment group

D: Virus-infected treatment group

In different periods B to A, D to A, $P>0.05$ * to C, $P<0.05$

Discussion

Group B Coxsackie virus (CVB), a single-stranded RNA virus that belongs to the enterovirus genus Picornaviridae, is a major pathogen causing viral myocarditis. Serological and epidemiological evidence showed that 36-65% of patients with acute myocarditis had either recent or recurrent CVB infection.² The incidence of viral myocarditis in children was presumably even higher. Coxsackievirus infection is a well-known complication in newborns, presenting mainly as myocarditis and sometimes aseptic meningitis. To date there is no effective specific treatment for viral myocarditis and its case-fatality rate remains high especially in newborns.

Xiaochaihu Decoction contains chaihu, astragalus, panax ginseng, pinellia tuberifera, licorice root, ginger and jujube. It has been reported that panax ginseng could induce myocardial cells to produce interferon, increase myocardial contractility, improve cellular metabolism in the myocardium and enhance the immunity against viral infection.³ In Balb/C mouse model of myocarditis, it has been demonstrated that *Xiaochaihu* Decoction had anti-inflammatory effect, was able to regulate T-cell subpopulation and stimulate the myocardial cells to produce antibodies. In addition, the *Xiaochaihu* Decoction-treated animals were able to eliminate the viruses at a faster rate.⁴ There was also evidence that *Xiaochaihu* Decoction could modulate the immune function by improving T-cell responses in children with viral myocarditis, and improve their left heart function.⁵

Viral myocarditis is an acute non-suppurative inflammatory disorder characterized by myofiber necrosis and infiltration of the myocardium with inflammatory cells.^{6,7} The necrosis of myofibers may be

either patchy or diffuse and may occur at any site of the heart with a predilection for the left ventricle. Necrotic myofibers have been observed as early as 2 days after the onset of illness.⁸ In our study, the typical CVB₃-infected rat myocardial cells showed cytopathic changes of cell atrophy and necrosis. In the *Xiaochaihu* Decoction-treated group, myocardial CPE was inhibited, suggesting a protective effect of the *Xiaochaihu* Decoction against CVB₃ infection in vitro.

Mitochondrion is one of the most important intracellular organelles. The inner mitochondrial membrane is an important site of cellular respiration and energy production. In any myocardial lesion, structural and functional disturbances of the mitochondria are earliest features of cell injury. Cytochrome C is one of the key enzymes that participate in cell energy metabolism, and its respiration is closely related to mitochondrial function. Thus serial estimation of the activities of CCO by enzyme-histochemical method is a sensitive way to monitor mitochondrial function and cell metabolism.^{9,10}

In our experiment, at 24, 48, 72 and 96 hours after infection with CVB₃, the untreated myocardial cells showed significantly diminished activities of CCO. Treatment with *Xiaochaihu* Decoction resulted in rapid recovery of these activities to near normal levels. This observation strongly suggested that *Xiaochaihu* Decoction have a therapeutic effect on CVB₃ myocarditis.

In conclusion, we have observed in this study treatment of myocardial cell cultures with *Xiaochaihu* Decoction protected the cells from CVB₃ infection, resulting in less cellular damage and more rapid recovery of mitochondrial function. These observations suggest that *Xiaochaihu* Decoction have a therapeutic effect in the management of the CVB₃ myocarditis.

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