Establishment and Evaluation of Isoproterenol Induced Chronic Heart Failure and Cardiac Remodeling Model in Rats: An Experimental Study

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Abstract Objective: To assess the efficacy of isoproterenol (ISO) in the establishment of chronic heart failure (CHF) and cardiac remodeling model in rats. Methods: Twelve Wistar rats were randomly divided into two groups: control group (n=3) and model group (n=9). Rats in model group were hypodermic injected with ISO 5mg/kg/d for 10 days. Ultrasonic cardiogram, HE staining, immunohistochemistry of collagen I and Masson staining were performed to evaluate the cardiac function and myocardial fibrosis. Besides, ventricular mass/body mass ratio, collagen volume fraction (CVF), perivascular collagen area (PVCA), hydroxyproline (HYP) concentration and relative expression of transforming growth factor $\beta 1$ (TGF- $\beta 1$) mRNA were also detected. Results: Compared to the controls, rats in model group had a marked enlargement of cardiac dilatation and reduction of ejection fraction ($57.00 \pm 3.61\%$ vs. $44.67 \pm 3.06\%$, $P=0.011$). Ventricular mass/body mass ratio (3.60 ± 0.31 vs. 4.88 ± 0.34 , $P=0.020$), CVF ($5.65 \pm 0.68\%$ vs. $27.62 \pm 4.89\%$, $P=0.020$), PVCA ($11.22 \pm 3.40\%$ vs. $28.50 \pm 4.52\%$, $p=0.001$) and HYP level ($0.24 \pm 0.08 \mu\text{g}/\text{mg}$ wet weight vs. $0.62 \pm 0.11 \mu\text{g}/\text{mg}$ wet weight, $P=0.001$) were significantly increased in model group. Remarkably cardiac fibrosis were also observed in the Masson staining and immunohistochemistry. The expression of TGF- $\beta 1$ mRNA was increased significantly in the model group ($P=0.003$). The mortality rate in the model group was 33.3% during the whole procedure. Conclusion: ISO can successfully induce a CHF and cardiac remodeling rat model with a low mortality rate.

Keywords Isoproterenol, Heart Failure, Cardiac Remodeling, Myocardial Fibrosis

1. Introduction

Chronic heart failure (CHF) is a complicated clinical

syndrome with impaired ventricular filling and ejection function caused by the abnormality of structure or function. CHF is one of the most important cardiovascular diseases [1]. Therefore, the study on CHF and cardiac remodeling is constantly the focus of cardiovascular territory [2]. The methods for establishment of CHF and cardiac remodeling rat model include coronary artery ligation or embolization, high capacity load, high pressure load, drug-induced method and viral infection method. Drug-induced method is preferred because of easy operation and low cost. Previous study [3] suggests large dose of isoproterenol (ISO) could cause myocardial impairment in rats, which induced CHF and cardiac remodeling. However, there is relatively short of the systematic study on the effect of modeling establishment. This experiment was conducted to assess the efficacy of ISO on the establishment of CHF and cardiac remodeling model in rats.

2. Materials and Methods

2.1. Animals and Reagents

Twelve 8-week-old male Wistar rats (weight, 200~230g) used in this study were obtained from Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). ISO was purchased from Bo Yuan Pharmaceutical Co., Ltd. (Jinan, China). Other reagents include Masson staining kit (PanEra, Guangzhou, China), TRIzol (Invitrogen, CA, USA), All-in-One TM first strand cDNA synthesis kit (GeneCopoeia, Rockville, MD, USA) and All-in-One TM qPCR mix (GeneCopoeia, Rockville, MD, USA). Ultrasound System (GE Vivid 7, New York, USA), Fluorescence microscopy (ZEISS Axio Scope A1, Oberkochen, Germany) and Quantitative PCR instrument (EPPENDORF Mastercycler ep realplex2, Hamburg, Germany) were also used in this study.

2.2. Modeling Method

Rats were randomly divided into two groups using computer: control group (n=3) and model group (n=9). In the model group, rats were hypodermic injected with ISO 5mg/kg/d from the first day to tenth day. Accordingly, the controls were injected with normal saline. Rats were fasted for 24 hours after the last injection. All of them were executed on the eleventh day.

2.3. Ultrasonic Cardiogram (UCG)

Rats were anaesthetized with 2% pentobarbital sodium (50mg/kg). And then, the chest area was shaved for UCG examination. The left ventricular internal diameter at end-systole (LVIDs), left ventricular internal diameter at end-diastole (LVIDd), left ventricular fractional shortening fraction (FS) and left ventricular ejection fraction (EF) were measured. Three consecutive cardiac cycles was saved for each measurement and the average of them was taken for final analysis.

2.4. Ventricular Mass/Body Mass Ratio (VW/BM)

Rats were sacrificed after UCG examination. Their hearts were immediately got out and washed with cold normal saline to remove the blood. Cardiac atrium, vessels and adipose tissue were removed and only ventricular tissues were retained. The samples were dried with filter papers and weighed with electronic balance. The VW/BM = ventricular mass (mg)/ body mass (g).

2.5. Histopathology and Immunohistochemistry

The ventricular samples were fixed with 10% formaldehyde solution and embedded with paraffin. Then, the paraffin embedded tissues were sectioned perpendicularly to the longitudinal axis of the heart. The sections were used for HE staining and Masson staining to assess the severity of cardiac fibrosis. In Masson staining, collagen fibers would be stained blue and myocardial tissue would be stained red. Immunohistochemistry of collagen I was also performed. The expression of Collagen I would be observed under microscope. The rest of ventricular tissues were retained in ultra-low temperature freezer (-80°C).

2.6. Collagen Volume Fraction (CVF) and Perivascular Collagen Area (PVCA)

Masson staining sections were also used to calculate the CVF and PVCA. Based on the positive stained area, which scattered between the surviving myocytes and around the blood vessels, collagen volume fraction (CVF) and perivascular collagen area (PVCA) were measured (3). CVF= collagen area/total area. PVCA= collagen area around the arterial lumen/arterial lumen area. Six visions under microscope of each sample were randomly chosen and the average of them was taken for analysis.

2.7. Hydroxyproline (HYP) Concentration

Put 30~100mg (wet weight) ventricular tissue into 1ml hydrolytic agent, 95 °C constant temperature bath for 20min, adjust the PH to 6.0~6.8. And then, according to the instruction of HYP testing kit (Alkaline method) provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China), optical density (OD) of each sample was measured. HYP concentration in ventricular tissue (µg/mg) = (sample tube OD-blank tube OD) × standard sample concentration (5µg/ml) × total volume (10ml)/ [(standard tube OD- blank tube OD) × tissue wet weight (mg)].

2.8. Real-time Quantitative PCR

Total RNA was prepared from ventricular tissue by using TRIzol reagent and reverse transcribed to cDNA by using the All-in-One TM first strand cDNA synthesis kit. Two microliter cDNA was amplified by real-time quantitative PCR using the All-in-One TM qPCR mix (SYBR Green method). The primers used in this study were provided by GENEWIZ Co., Ltd. (Suzhou, China). The sequences were shown in Table 1. Each experiment was performed at least three times.

2.9. Statistical Analysis

All data were analyzed with IBM SPSS software (Version 20.0). The data were presented as mean ± standard deviation. Student t test or Wilcoxon test was used according to the result of normality test and homogeneity of variance test. A two-tail P<0.05 was considered to indicate a significant difference.

Table 1. Primers used in the study

Primer	Sequence
TGF-β1(F)	5'-TGCTTCAGCTCCACAGAGAA-3'
TGF-β1(R)	5'-TGGTTGTAGAGGGCAAGGAC-3'
β-actin(F)	5' -AGGGAAATCGTGCGTGACAT-3'
β-actin(R)	5'-GAACCGCTCATTGCCGATAG-3'

Table 2. Comparison of cardiac function and structure

	Control	Model	P
LVIDd(mm)	4.75±1.19	8.91±0.22	0.023
LVIDs(mm)	3.52±0.79	7.19±0.32	0.002
EF (%)	57.00±3.61	44.67±3.06	0.011
FS (%)	25.67±2.52	19.33±1.53	0.020
VM(mg)	965.40±86.21	1249.07±79.51	0.002
BM(g)	268.00±3.00	256.50±12.74	0.179
VM/BM(mg/g)	3.60±0.31	4.88±0.34	0.020

LVIDd=left ventricular internal diameter at end-diastole; LVIDs=left ventricular internal diameter at end-systole;
EF=ejection fraction; FS=fractional shortening fraction; VM=ventricular mass; BM=body mass

3. Results

3.1. General Data

During the whole procedure, three rats in model group died. All of them were died within two hours after injection of ISO. No rats died in control group. Compared to the controls, rats in model group became depressive, inactive and shaggy.

3.2. Comparison of UCG Results

The LVIDd (4.75±1.19mm vs. 8.91±0.22mm) and LVIDs (3.52±0.79mm vs. 7.19±0.32mm) were increased in model group. Besides, their EF (57.00±3.61% vs. 44.67±3.06%) and FS (25.67±2.52% vs. 19.33±1.53%) levels decreased significantly, compared to the control group (Table 2).

3.3. Comparison of Ventricular Mass/Body Mass Ratio

As shown in Table 2, the ventricular mass (965.40±86.21 mg vs. 1249.07±79.51 mg) and VM/BM ratio (3.60±0.31 vs. 4.88±0.34) in model group increased significantly as compared to the controls. No statistical difference of body mass was found between the two groups (268.00±3.00 g vs. 256.50±12.74 g).

3.4. Comparison of HE Staining and Masson Staining

As shown in Figure 1, the myocardial tissues of control group were arranged in order and stained well (A). In the model group, an irregular arrangement of myocardial tissues was found. The tissues were infiltrated with inflammatory cells. The major lesions were located in the subendocardial myocardium area (B). In Masson staining, collagen fibers were stained blue and myocardial tissue

were stained red. Compared to the controls (C), the collagen fibers were significantly increased in model group, especially in subendocardial myocardium and perivascular areas (D).

3.5. Comparison of Immunohistochemistry of Collagen I

In the immunohistochemistry of collagen I, the positive tissue was stained brown. According to Figure 2, collagen I was found thinly distributed in control group (A). However, the positive area was increased significantly in the model group (B).

3.6. Comparison of CVF and PVCA

Compared to the controls, the CVF (5.65±0.68% vs. 27.62±4.89%, P=0.020) and PVCA (11.22±3.40% vs. 28.50±4.52%, p=0.001) were found higher in the model group (Figure 3).

3.7. Comparison of HYP Concentration

The HYP concentrations in myocardial tissues were measured. A significant increase of HYP (0.24±0.08μg/mg wet weight vs. 0.62±0.11μg/mg wet weight, P=0.001) was found in model group as compared to the control group.

3.8. Comparison of Relative Expression of TGF-β1 mRNA

In this study, β-actin was used as a reference gene and ΔΔCt method was used to analyze the relative expression of TGF-β1 mRNA in myocardial tissue. As shown in Figure 4., compared to the controls, the relative expression of TGF-β1 mRNA was found increased significantly in the model group (P=0.003).

Figure 1. Comparison of histopathology.(A) Myocardial tissues of control group were arranged in order and stained well in control group (HE staining, 100×); (B) Myocardial tissues were arranged irregularly and infiltrated with inflammatory cells, especially in the subendocardial myocardium area (HE staining, 100×); (C) Collagen fibers were stained blue and myocardial tissue were stained red. Only few collagen fibers were found in control group (Masson staining, 100×); (D) Collagen fibers were significantly increased in the model group (Masson staining, 100×).

Figure 2. Comparison of Immunohistochemistry of Collagen I . (A) Myocardial tissues were arranged in order and few collagen I was found in control group (Immunohistochemistry, 200×); (B) Collagen I was found increased significantly in the model group (Immunohistochemistry, 200×).

Figure 3. Comparison of CVF and PVCA

Figure 4. Comparison of Relative Expression of TGF-β1 mRNA

4. Discussion

Our study established the CHF and cardiac remodeling rat model with hypodermic injection with ISO for 10 days successfully. Compared to the control group, hearts in model group were enlarged and the cardiac function sharply decreased. Histopathology suggested an irregular array of myocardium cells, infiltration of inflammatory cells and increase of collagen I in the model group. The HYP concentration and relative expression of TGF-β1 mRNA were significantly increased.

A good animal model is essential for the basic research on CHF and cardiac remodeling [4]. At present, coronary artery ligation or embolization is a relatively common method to set up the animal model of CHF by cutting off blood flow in coronary artery and inducing myocardium cells' necrosis. But this method is more suitable for big animals such as pig and dog, because it has strict requirements for the experimental environment, device and operation skills. Moreover, there is a high risk of death for

animals after operation. The rat model of CHF and cardiac remodeling can be also accomplished with drugs, such as adriamycin and catecholamine [5]. ISO is an agitation to β-receptor of adrenaline, which has a positive function to heart rate and strength of myocardium. Previous studies [4, 6] suggested that large dose of ISO with hypodermic injection could induce necrosis, hyperplasia and myocardial fibrosis. It could also increase the expression of angiotensin II and impair pump function of myocardium in rats. The possible mechanism might be acute diffuse necrosis of myocardium caused by sharp increase of catecholamine. ISO can also cause a relatively lack of blood flow for myocardium. In addition, chronic inflammatory reaction and activation of renin-angiotensin-aldosterone system may also be involved in the progress of CHF and myocardial remodeling [7]. Consequently, compared to coronary artery ligation or embolization, ISO method is preferred by many researchers because of its convenience, lower cost and no need for extra device [8-10].

However, there is no consensus yet for the accurate

method using ISO in the establishment of CHF model. Firstly, the dose of ISO varies greatly. Previous studies [7, 11-12] suggested that high dose of ISO (85~170mg/kg/d) could set up CHF model in rats, but acute death rate was up to 55%~66%. The mortality was related to the doses. While some other studies suggested that low dose of ISO (5~15mg/kg/d) could also set up the model and death ratio was around 30% [9, 13-15]. Secondly, the program steps of ISO injection are different. Garjani and Li [13, 15] used ISO 5mg/kg/d for 10days to establish CHF model. However, Li [14] used ISO 10mg/kg/d with hypodermic injection for 3 days, and then 5mg/kg/d for another 11days to build the model. All of them reported a successful result. To exam the actual efficiency of ISO in the establishment of CHF and cardiac remodeling rat model, we use ISO 5mg/kg/d by hypodermic injection for 10days with a 24-hour interval between every two injections in this study. The death rate in model group is 33.3% (3/9). The hearts were enlarged and pump function were decreased apparently. Besides, indicators of cardiac remodeling (HYP and TGF- β 1) were markedly increased. Therefore, the method we use can build the model of CHF and cardiac remodeling successfully and the death rate of rats is low, which is worthy of reference and spread.

However, the study has some limitations. Firstly, the number of rats is relatively low and can cause some bias. Secondly, we didn't set up groups with different doses to make a parallel comparison, so that we can ensure the best dose of ISO. Thirdly, there is no comparison with different combination groups, with which we can find a best medicine combination to minimize the death rate. Future studies are still needed.

In conclusion, ISO can induce CHF and cardiac remodeling rat model successfully with great efficacy and low death rate.

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