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The influence and significance of rapamycin on cardiomyocyte autophagy level of early heart failure rat.

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Abstract

Objective: To explore the influences of rapamycin on cardiomyocyte autophagy level of early Heart Failure (HF) rat.

Methods: HF rat model was built by abdominal aorta surgery, and then they were randomly divided into the treatment group and the control group. The treatment group was given 3000 U/kg intraperitoneal injection of rapamycin, 3 times per week, for 4 weeks; the control group given the same amount of saline solution. Respectively 4 weeks, 8 weeks, 12 weeks after surgery (i.e., dosing 4 weeks), heart colour ultrasound examination was given for rat cardiac function. After 24 h fasting of all rats at the end of 12 weeks, rats were killed, then to observe the myocardial tissue cell morphology, cell apoptosis and Apoptosis Index (AI); Using Western blot method to detect myocardial *Beclin 1*, *Cathepsin D* protein expression.

Results: Echocardiography showed ventricular hypertrophyat 4 weeks , HF at 8 weeks in the model group; rapamycin intervention in the control group after 4 weeks, Left Ventricular Ejection Fraction (LVEF) increased significantly (P<0.05), interventricular septal thickness at end-systole (IVSs), left ventricular posterior wall thickness at end-systole (LVPWs), Interventricular Septum thickness at end-diastole (IVSd), Left Ventricular Posterior Wall Thickness at end-Diastole (LVPWd) significantly decreased after treatment (P<0.05). Compared with the sham group, *mTOR* expression decreased significantly, *cTnT* and *pmTOR* expression increased obviously in the control group (P<0.05); compared with the control group, *mTOR* expression increased significantly, cTnT and pmTOR expression decreased obviously in the treatment group (P<0.05). AI of the treatment group was significantly lower than Control (25.55% ± 2.56% *vs.* 34.66% ± 1.46%, P<0.01); compared with the control group (0.234 ± 0.001 and 0.001 ± 0.001), *Beclin 1* and *Cathepsin D* OD value of the control group (0.867 ± 0.005 and 0.867 ± 0.003) increased significantly, the treatment group (0.423 ± 0.002 and 0.002 ± 0.001) was lower than the control group (P<0.01).

Conclusion: Rapamycin can effectively improve cardiac function in heart failure rats, inhibit cardiomyocyte autophagy.

Keywords: Rapamycin, Heart failure, Autophagy.

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Introduction

Chronic Heart Failure (CHF) is the ultimate result of most Cardiovascular Disease (CVD), which influences patients' health even life severely, also brings about heavy economic burden on family and society [1]. Therefore, treatment on CHF is still a hot spot of research in cardiovascular field. Recent years, that rapamycin has the function of protecting cardiomyocyte has been found in pharmacological experiment and clinical experiment, and it has been applied to Drug Eluting Stent (DES) [2,3]. At present, a few of reports have been seen about the effects of Rapamycin treatment on CHF. However, the effect on Chinese citizens is still unknown. This paper is aimed at setting up rat model with CHF treated with Rapamycin through observing Ultrasonic Cardiogram (UCG), autophagic rate of cardiomyocyte and the expression of *Beclin-1* and *Cathepsin D*, then to explore whether Rapamycin has the autophagic function of resisting cardiomyocyte of CHF or not in Chinese people, and its possible mechanism, finally it provides new thinking for treatment on CHF.

Materials and Methods

Animals

Choosing of SPF level of 30 male SD rats, the average age was 8 to 12 weeks, the average weight was 241.55 ± 17.70 g, which were bought from The Animal Center in West China Center of

Medical Sciences of Sichuan University. All rats were fed with food and water freely in same condition. Fodder was provided by The Animal Center in West China Center of Medical Sciences of Sichuan University. This research was approved by the Animal Committee of People's Hospital of Laizhou City.

Drug and reagent

Rapamycin was bought from shenyang 3sBio Inc. TUNEL kits from Sigma company, *Beclin-1* and *Cathepsin D* antibody from Cell Signalling Technology (USA) and GAPDH antibody from ProteinTech Group, Inc. (USA).

Grouping and modelling

30 rats were randomly divided into the model group (n=24) and the sham group (n=6). CHF model was built with abdominal aorta surgery in the model group. The specific methods as follows, water was forbade before 1d of surgery, 10% chloralic hydras (3 ml/kg) was given for anesthesia by intraperitoneal injection, the abdominal cavity was opened and veins in left kidney were exposed, abdominal aorta was separated bluntly about 10 to 15 mm apart from upper part above left kidney veins, then using 4-0 suture passed through abdominal aorta which has been separated, no.7 (diameter 0.7) needle was located beside abdominal aorta, which paralleled to abdominal aorta, after ligation, needle was pulled out, the tightness feeling was proper when needle extraction, then stenosis of abdominal aorta reached to 60% to 70%, finally abdominal cavity was closed. Amikacin was given for anti-infection 3 d by intraperitoneal injection after surgery, rats were observed and fed for 8 weeks, and then model was built. The sham group, the same part with the model group was given threading but no ligation, other groups were same with the model group.

Grouping and intervention after modelling

16 rats survived in the model group after 8 weeks of surgery, then 16 rats were randomly divided into two groups: the treatment group (n=8); 10000 U/ml. Rapamycin was diluted into 1000 U/ml by normal saline, rats were given 3000 U/kg intraperitoneal injection of Rapamycin, 3 times per week, for 4 weeks. The control group (n=8) were given the same amount of saline solution by intraperitoneal injection, 3 times per week, for 4 weeks. The sham group was same with the control group. At the end of 12 weeks, 2 died in the control group, there was no death in the sham group and the treatment group.

Examination of heart colour ultrasound

Respectively 4 weeks, 8 weeks, 12 weeks after surgery (i.e., dosing 4 weeks), heart colour ultrasound examination was given for rat cardiac function. After rats fasting for 24 h, 10% chloral hydrate (3 ml/Kg) was given for anesthesia and skin preparation. Using ultrasound cardiotachograph (i.e. 33 type, PHILIPS Company) and 7.5 HZ high frequency linear control probe (PHILIPS Company) to detect LVEF, IVSs, LVPWs, IVSd and LVPWd.

Intracardiac blood collection and sample disposal of myocardium

At the end of 12 weeks, all rats were given UCG examination after 24 h fasting, 10% chloral hydrate (3 ml/Kg) was given for anesthesia and thoracotomy by intraperitoneal injection, collecting blood by intracardiac puncture, cutting myocardial tissue in apex of heart about 5 mm × 5 mm × 5 mm, which was put into precooling PBS solution to be washed to no red color, then located into ultra-low temperature freezer of-80°Cafter quick-freezed in liquid nitrogen for molecular biology experiment; other heart tissues were perfused by precooling saline solution for 3 min, then perfused and fixed by 4% Paraformaldehyde (PA) solution, the heart was took out and located into PA buffer solution to be fixed, and embedded with routine paraffin, then made into 3~4 μm slices of thick paraffin for stand-by use.

Pathological examination

3 slices were randomly selected in each group from paraffin slices has been made, using HE staining and light microscope with 200 folds to observe morphological structure of myocardial tissue and condition of inflammatory cell infiltration in each group.

Detection on cell apoptosis by TUNEL

3 slices were randomly selected in each group from paraffin slices has been made, detecting cardiomyocyte apoptosis according to operational approach of TUNEL kits, fluorescence coloration of cell apoptosis was observed under fluorescent microscope and image was adopted, then with DAB coloration, each slice randomly read 6 underlap visions under high power lens (X400), the positive cell was cell nucleus with brown color. After counting, AI=single vision positive apoptosis cell/single vision total cells × 100%, the average value was counted and analysed.

Detection of western blot on beclin-1 and cathepsin D protein expression

Protein concentration of myocardial tissue was detected by ELISA after grinded and splited, total protein quality of upper sample according to protein concentration was 80 μg, after electrophoretic separation by SDS-PAGE hydrogel, using electro-blotting to transfer protein to PVDF membrane and Ponceaus to stain. Cutting membrane according to required target bands, after skim milk powder with 5% mass concentration of TBS-T solution closed at room temperature for 2 h, rabbit-anti *Beclin-1*, monoclonal antibody *Cathepsin D* and GAPDH antibody were added into TBS-T solution, incubated overnight under 4°C, after membrane washed by TBS-T solution at room temperature, rabbits (rats) secondary antibody incubation were added for 2 h, using TBS-T solution to wash membrane, then it reacted with ECL reagent, film was exposed and scanned, using Image J software to calculate OD value, then using OD ratio between AKT, P-AKT and GAPDH

bands to evaluate expression level of *Beclin-1*, *Cathepsin D* protein.

Statistical treatment

Using software spss 17.0 to do statistical analysis on data, all measurement data expressed by x ± s, selecting chi-square test for enumeration data comparison and t-test, one-way ANOVA and SNK-q test for between-group average comparison, Spearman Wilcoxon correlation analysis for correlation analysis and dichotomy Logistic regression for multi-factor analysis. There has statistical difference (P<0.05).

Results

Index changes of ultrasonic cardiogram before and after modelling and rapamycin intervention

After 4 weeks of modelling, compared with LVEF of rats in the model group, there was no statistical difference (P>0.05); LVPWs, IVSs, LVPWd and IVSd increased obviously in the model group (P<0.05), it indicated myocardial hypertrophy of rats in the model group. After 8 weeks of modelling, LVEF of rats in the model group decreased significantly (P<0.01), LVPWs, IVSs, LVPWd and IVSd were still higher than the sham group (P<0.05), it indicated successful modelling of CHF rats in the model group. After 12 weeks of modelling (the Rapamycin group given drugs for 4 weeks), compared with the sham group, LVEF of rats in the control group decreased furtherly (P<0.05), LVPWs, IVSs, LVPWd and IVSd increased furtherly (P<0.05), it indicated CHF became more severe of rats in the control group; LVEF of rats increased obviously (P<0.05) and LVPWs, IVSs, LVPWd and IVSd decreased obviously (P<0.05) in the treatment group compared with the control group, it indicated Rapamycin can improve cardiac function of rats with heart failure and reverse ventricular hypertrophy (Table 1).

Table 1. *Comparison of echocardiography 4, 8 and 12 weeks of operation.*

Group	Weeks	n	LVPWs (cm)	IVSs (cm)	LVPWd (cm)	IVSd (cm)	LVEF
Sham	4	6	0.277 ± 0.007	0.287 ± 0.005	0.146 ± 0.005	0.177 ± 0.006	0.878 ± 0.001
Model	4	16	0.357 ± 0.007	0.367 ± 0.004	0.246 ± 0.006	0.288 ± 0.007	0.846 ± 0.005
t-value			5.455**	2.565*	3.566*	2.577*	1.576
Sham	8	6	0.277 ± 0.003	0.346 ± 0.007	0.175 ± 0.007	0.178 ± 0.001	0.845 ± 0.006
Model	8	16	0.368 ± 0.001	0.377 ± 0.008	0.277 ± 0.001	0.246 ± 0.004	0.623 ± 0.001
t-value			5.234**	3.245**	3.135**	2.535*	8.355**
Sham	12	6	0.335 ± 0.001	0.345 ± 0.017	0.245 ± 0.013	0.199 ± 0.001	0.824 ± 0.134
Control	12	6	0.445 ± 0.002	0.394 ± 0.013	0.345 ± 0.011	0.245 ± 0.012	0.624 ± 0.041
Treatment	12	8	0.345 ± 0.002	0.374 ± 0.023	0.234 ± 0.025	0.215 ± 0.001	0.714 ± 0.001
t-value			10.355**	5.456*	13.245**	7.345**	32.876**

*P<0.0, **P<0.01; [a]Compared with Sham group, [b]compared with Control group, P<0.05.

Expression level comparison of cTnT, mTOR and pmTOR

Compared with the sham group, *mTOR* expression decreased significantly, *cTnT* and *pmTOR* expression increased obviously in the control group (P<0.05); compared with the control group, *mTOR* expression increased significantly, *cTnT* and *pmTOR* expression decreased obviously in the treatment group (P<0.05) (Table 2).

Table 2. *Comparison of the expression of cTnT, mTOR, pmTOR between three groups.*

Group	n	*cTnT* (ng/ml)	*mTOR*	*pmTOR*
Sham group	6	9.554 ± 0.005	1.151 ± 0.001	1.245 ± 0.023
Control group	6	13.367 ± 0.005	1.381 ± 0.003	0.566 ± 0.006
F		11.355**	12.455**	13.565**

Changes of cardiomyocyte autophagy

Cell nucleus was clearly visible, relatively big, the border of it was clear and stained to blue in the sham group. There was a large quantity bubble-like structure, which was specific autophagic vaculoe in autophagic cardiomyocyte. Phagocytic vaculoe and autophagic vaculoe decreased obviously in the treatment group compared with the control group. Compared with the sham group, the autophagic rate of cardiomyocyte increased significantly in the control group; compared with the control group, the autophagic rate of cardiomyocyte decreased in the treatment group, but it was still higher than the sham group (P<0.01), Table 3 as follows.

Detection of western blot on beclin-1 and cathepsin d protein expression of cardiomyocyte

Compared with the sham group, *Beclin-1* and *Cathepsin D* expression increased obviously in the control group; compared with the control group, *Beclin-1* and *Cathepsin D* expression increased obviously in the treatment group, but it was still higher than the sham group (P<0.01), Table 3 as follows.

Table 3. *Comparison of the expression of Beclin-1, Cathepsin D protein (OD) and myocardial Autophagy Index (AI) between three groups.*

Group	n	*Beclin-1*	*Cathepsin D*	AI (%)
Sham group	6	0.234 ± 0.001	0.331 ± 0.001	8.45 ± 0.89
Control group	6	0.867 ± 0.005	0.821 ± 0.003	34.66 ± 1.46
Treatment group	8	0.423 ± 0.002	0.655 ± 0.001	25.55 ± 2.56

F	13.455**	14.458**	53.541**

Discussion

Heart Failure (HF) is the ultimate result of myocardial damage caused by multiple CVD. Its pathophysiological changes are very complex. Recent years, study finds that cardiomyocyteautophagy plays an important role in occurrence and development of HF [4-6]. Autophagy is a highly conserved cellular mechanism of intracellular protein and organelle recycle mediated by lysosome. Autophagy can be induced by pathological factors of intracellular anoxia, starvation, pressure and stress. But in recent years, the role of uncontrolled factor in occurrence and development of many diseases begin to be taken seriously [7-10]. Cardiomyocyte autophagy can cause reduction of cardiomyocyte, thinness of ventricular wall and enlargement of ventricular cavity, making HF get worse.

Study finds that mTOR is the key regulatory protein of autophagic regulatory pathway of mammals [10-12]. Study shows that when myocardium is hypoxic-ischemic and phosphorylation of *mTOR*, then forming *pmTOR*, which activates cardiomyocyte autophagy and causes cardiomyocyteautophagy furtherly [12-15]. Autophagy can protect cardiomyocyte; however, HF will cause excessive autophagy, finally causing injury of cardiomyocyte [16]. The results show, compare with the sham group, mTOR expression decreases significantly, *cTnT* and *pmTOR* expression increase obviously in the control group ($P<0.05$); compare with the control group, *mTOR* expression increases significantly, *cTnT* and *pmTOR* expression decrease obviously in the treatment group ($P<0.05$), it shows that Rapamycin can inhibit excessive autophagy of cardiomyocyte through lowering *pmTOR* expression to a certain degree.

This study shows, cardiac function of HF rats decreases, and autophagic relevant protein of *Beclin-1*, *Cathepsin D* expression of myocardial tissue increase significantly compare with the sham group, after Rapamycin treatment, cardiac function of HF rats improves greatly. And autophagic relevant protein of *Beclin-1*, *Cathepsin D* expression decrease significantly. The results point out autophagy plays an important role in occurrence and development of HF; Rapamycin can relieve cardiomyocyte autophagy of HF and improve cardiac function. Therefore, autophagy may become potential target for HF treatment; regulating myocardial cell autophagy is new strategy for HF treatment. Regulating uncontrollable cardiomyocyteautophagy of HF can reduce cardiac cell death caused by autophagy, which will be benefit for HF treatment. At present, the relevant mechanism of overloaded cardiac pressure causing cardiomyocyte autophagy is still not clear, which needs further study [17-20]. Searching for a kind of drug which can be applied to clinic and regulate cardiomyocyteautophagy, which may be benefit for HF treatment.

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