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Original Article

Effects of rapamycin on autophagy of myocardial cells in rats with early heart failure

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Abstract: Objective: To investigate the effect of rapamycin on autophagy of myocardial cells in rats with early heart failure. Methods: A total of 48 male SD rats were used to establish rat models with heart failure induced by abdominal aorta constriction (AAC) and then randomly divided into the following 3 groups: the control group treated intraperitoneally with normal saline (n=16), the treatment group treated intraperitoneally with rapamycin (n=16) and the sham group (n=16). Echocardiography was performed at 4, 8, and 12 weeks. Morphological changes of myocardium and autophagy rate of myocardial cells were observed by HE staining and transmission electron microscopy respectively; the expression levels of protein Cathepsin D and Beclin-1 were detected by western blotting. Results: Echocardiography showed the presence of ventricular hypertrophy at 4 weeks after modeling and the presence of heart failure at 8 weeks. At 4 weeks after rapamycin intervention, significantly higher left ventricular ejection fraction (LVEF) was observed in the treatment group compared to the control group (P<0.05); but there were substantial reductions in the left ventricular posterior wall thicknessatend-systole (LVPWs), the interventricular septum thickness atend-systole (IVSTs), the left ventricular posterior wall thicknessatend-diastole (LVPWd) and in the interventricular septal thickness atend-diastole (IVSd) (P<0.05). The autophagy rate of myocardial cells was significantly lower in the treatment group versus the control group (P<0.05). Compared to the sham group, the levels of proteins Beclin-1 and Cathepsin D expression significantly improved in the control group (P<0.05), but reduced markedly in the treatment group (P<0.05). Conclusion: Reductions in the autophagy of myocardial cells and improvements in the cardiac functions of rats with heart failure by rapamycin may be attributable to regulation of the expression levels of proteins Beclin-1 and Cathepsin D.

Keywords: Rapamycin, heart failure, autophagy

Introduction

Chronic heart failure (HF) is the end stage in the progression of cardiovascular diseases, which has brought huge psychological, physiological and economic burdens to patients and their families [1]. With the accelerated aging in the world, the incidence of HF is increasing gradually. Epidemiological studies show HF 1%-2% of the European and American adults suffer from HF and 0.9% of Chinese adults do [2]. HF has become an important public health care concern in the field of cardiovascular disease. Therefore, how to treat chronic HF effectively has become a common issue to most scholars who study cardiovascular diseases.

In recent years, autophagy has been considered as a self-protective function of cells. A lot of stimulating factors can induce autophagy in cells while many signaling molecules are involved in the regulation of autophagy in cells. Some studies have shown that the pathogenesis of infectious diseases, tumors and other diseases may be due to uncontrolled autophagic activity [3, 4]. Autophagy has found to play a crucial role in the pathogenesis of HF, regulating autophagy of dysfunctional myocardial cells, which can reduce the apoptosis of the myocardial cells [5, 6]. Therefore, we suppose that the regulation of autophagy in myocardial cells would have beneficial effect on the treatment of HF. In addition, Beclin-1 and Cathepsin D are autophagy-related proteins in the myocardia, and their expression levels are indicative, to some extent, of the autophagy of the myocardia.

The recent studies have found that rapamycin is protective to myocardial cells and has been used in drug eluting stents [7, 8]. However, its effect in the pathogenesis of chronic HF remains unclear. Therefore, we aimed to investigate the therapeutic effect of rapamycin on rats with early HF and observed the autophagy rates of myocardial cells, as well as the expression levels of Beclin-1 and Cathepsin D (autophagy-related proteins), so as to explore the effect and mechanism of rapamycin on anti-HF. The results of this study will bring new insights and approaches to the prevention and treatment of chronic HF.

Materials and methods

Materials

Experimental animals: Forty-eight male SD rats at SFP level, aged 8 to 12 weeks and weighed 240 ± 10 g, were provided by the Experimental Animal Center of Shandong University.

Reagents

Rapamycin (3SBio Inc., China); rabbit anti-rat Cathepsin D monoclonal antibody (primary antibody) and rabbit anti-rat Beclin-1 monoclonal antibody (primary antibody; Abcam, US); goat anti-rabbit IgG antibody (secondary antibody; Santa Cruz, US); TUNEL kit (Sigma, US); and GAPDH antibody (ProteinTech Group, US).

Methods

Establishment of rat models with HF: Abdominal aorta constriction (AAC) was used to establish animal models of chronic HF in 32 rats. The procedures are as follows: rats were anesthetized with 10% chloral hydrate (3 mL/kg, intraperitoneally) after 24 h fasting, followed by exposure of their abdominal cavity and dissection of the abdominal aorta. A No.7 needle was inserted into the site near the separated abdominal aorta and then a 4-0 silk thread went through the separated abdominal aorta and the needle. Ligation was slowly conducted until there was a sense of tight binding when the needle was pulled out. The abdominal cavity was sutured until the abdominal aorta was narrowed to about its original 60%-70%. Penicillin anti-infective therapy was performed on all the rats after operation. The procedures for the sham operation on the 16 rats were the same as described above except that the abdominal aorta was not ligated narrowly.

Experimental group allocation

The SD rats were randomized into three rat models with abdominal aorta constriction-induced HF (the control group, n=16; the treatment group, n=16 and the sham group, n=16). At 8 weeks after modelling, the rats in the treatment group were injected intraperitoneally with rapamycin (3000 U/kg) 3 times (1 min once) weekly (Monday, Wednesday and Friday) for 4 weeks whereas those in the control group were injected intraperitoneally with physiologic saline under the same conditions (identical injection time, times and duration as the treatment group). The rats in sham group were not treated intraperitoneally.

Echocardiography

Myocardial function of rats was examined by echocardiography on the last day of 4, 8, and 12 weeks after operation, respectively. The procedures are as follows: the SD rats were anesthetized with 10% chloral hydrate (3 mL/kg intraperitoneally) 24 h after fasting. Cardiac examination was conducted using high frequency linear probe and PHILPS IE33 echocardiography. The detected parameters included the interventricular septum thicknessatend-diastole (IVSTd), the interventricular septum thickness atend-systole (IVSTs), the left ventricular ejection fraction (LVEF), the left ventricular posterior wall thickness atend-systole (LVPWs), and the left ventricular posterior wall thickness atend-diastole (LVPWd).

Collection of myocardial tissue specimens

Eight rats from each group were anesthetized with 10% chloral hydrate (3 mL/kg intraperitoneally), and the apical myocardial tissues of heart were quickly collected via thoracotomy and swiftly stored in liquid nitrogen for future use. The rest of myocardial tissues were perfused with pre-cooled saline solution for approximately 2-3 min, fixed with 4% paraformaldehyde solution, and then treated by regular HE staining. The changes in the pathophysiological structure of myocardial tissues and the infiltration of inflammatory cells in each group were detected under light microscope (200×), and then four slices were randomly selected.

Table 1. Comparison of results of echocardiography across groups at 4, 8, and 12 weeks after modeling

Groups	Wk	n	LVPWs (cm)	IVSs (cm)	LVPWd (cm)	IVSd (cm)	LVEF
S group	4	8	0.266 ± 0.006*	0.276 ± 0.004*	0.135 ± 0.004*	0.166 ± 0.005*	0.647 ± 0.002
C group	4	8	0.346 ± 0.007	0.356 ± 0.003	0.235 ± 0.005	0.278 ± 0.006	0.638 ± 0.004
S group	8	8	0.267 ± 0.003*	0.335 ± 0.006*	0.165 ± 0.006*	0.169 ± 0.002*	0.825 ± 0.005*
C group	8	8	0.357 ± 0.001	0.367 ± 0.007	0.268 ± 0.002	0.237 ± 0.004	0.624 ± 0.002
S group	12	8	0.336 ± 0.002#	0.346 ± 0.015#	0.239 ± 0.011#	0.201 ± 0.002#	0.819 ± 0.127#
C group	12	8	0.439 ± 0.003	0.387 ± 0.013	0.344 ± 0.011	0.251 ± 0.013	0.618 ± 0.036
T group	12	8	0.346 ± 0.001#	0.362 ± 0.018#	0.229 ± 0.017#	0.217 ± 0.002#	0.716 ± 0.002#

Note: S Group denotes Sham Group; C Group, Control Group; T Group, Treatment Group; Wk, Week; for the comparison with the control group, *P<0.05, #P<0.05.

Detection of autophagy rate of myocardial cells

Ultra-thin slices (50-100 nm) of apical myocardial tissues harvested as described above were prepared. The autophagy of myocardial cells was observed under transmission electron microscope. Four non-overlapping views were selected. If autophagosomes are present in the cytolymph of cells, the cells are positive. Autophagic index (AI) of myocardial cells = number of autophagic positive cells/ total cell count*100%.

Detection of expression levels of beclin-1 and Cathepsin D protein

Novel myocardial tissues were cut from 8 rats from each group anesthetized with 10% chloral hydrate (3 mL/kg intraperitoneally) and then total proteins were extracted from the tissues. The expression levels of proteins Cathepsin D and Beclin-1 in myocardial tissue were detected by Western blotting. The procedures are as follows: the extracted proteins from homogenized and lysed myocardial tissues were separated by SDS-PAGE gel electrophoresis, and then were transferred to PVDF membranes. The targeted bands obtained by cutting the membranes were blocked in 5% skimmed milk dissolved TBS-T solution for 2 h at room temperature, followed by an overnight incubation with primary antibody at 4°C. Membranes were fully washed with subsequent 2 h incubation with secondary antibody, and then added with developer for exposure and film scanning. With GAPDH as an internal reference, the optical density (OD) of the targeted bands (proteins Cathepsin D or Beclin-1) was compared across groups.

Statistical analysis

All the data analyses were performed with Software SPSS 16.0 package. The count data were expressed as probability, and the comparisons across groups were made by chi-square test; the measurement data were expressed as $\bar{x} \pm s$, the comparisons across groups were made by the T-test. P<0.05 was considered statistically significant.

Results

Results of echocardiography in each group

Compared to the sham group, the control groupimproved significantly in IVSs (P=0.025), LVPWs (P=0.015), IVSd (P=0.018) and LVPWd (P=0.005) at 4 weeks after operation, but no markedly difference showed in LVEF between the two groups (P>0.05). At 8 week, the control group showed significantly higher IVSs (P= 0.004), LVPWs (P=0.008), IVSd (P=0.012) and LVPWd (P=0.011) but markedly lower LVEF (P=0.002). Likewise, at 12 weeks, the control group also showed significantly higher IVSs (P=0.011), LVPWs (P=0.014), IVST (P=0.016) and LVPWd (P=0.003) but markedly lower LVEF (P=0.001). Conversely, the treatment group showed significantly lower IVSd (P=0.006), LVPWs (P=0.004), IVSs (P=0.027) and LVPWd (P=0.003) but markedly higher LVEF (P=0.009) versus the control group, as shown in **Table 1**.

HE staining showed orderly-arranged intact cardiac muscle fibers, no edema, and normal intercellular gaps in the sham group; damaged myocardial tissues, myocardial cells with partial edema, disorderly-arranged and partly ruptured cardiac muscle fibers, and significantly-

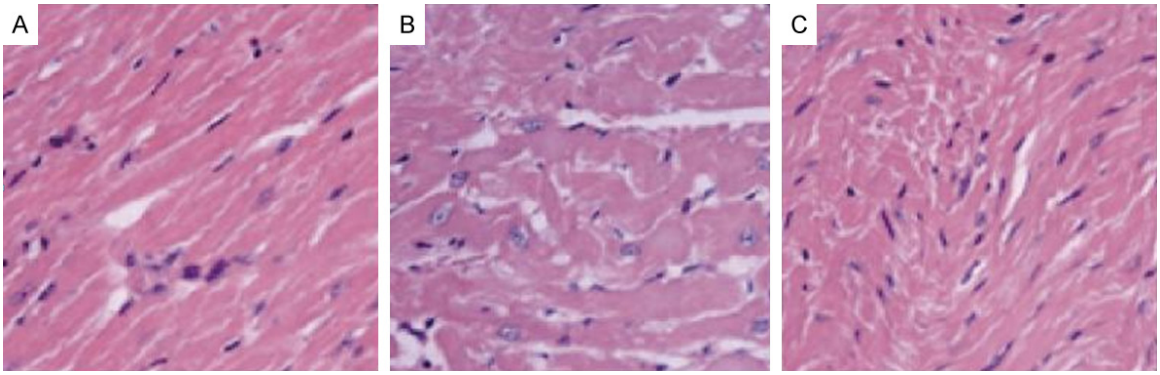

Figure 1. HE staining of heart tissue of rats in each group (400×). A: Sham group; B: Control group; C: Treatment group.

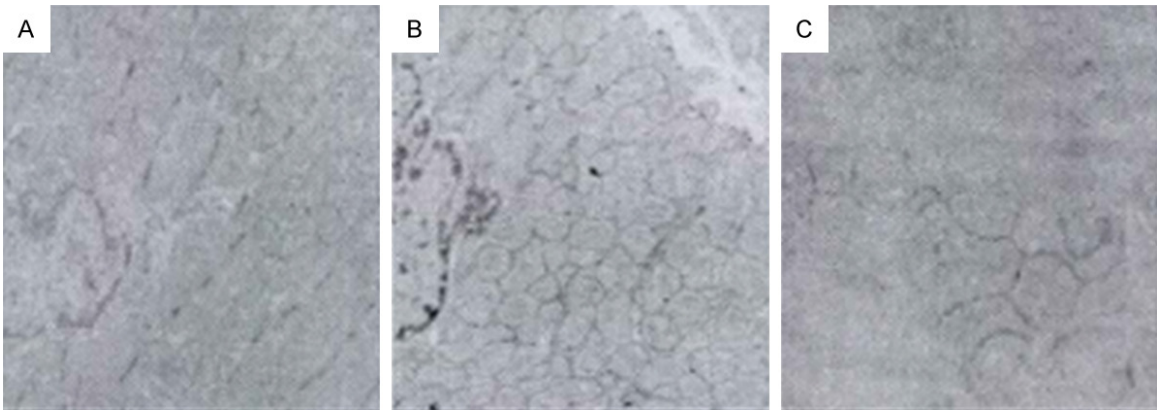

Figure 2. Structure of myocardial cells under transmission electron microscope. A: Sham group; B: Control group; C: Treatment group.

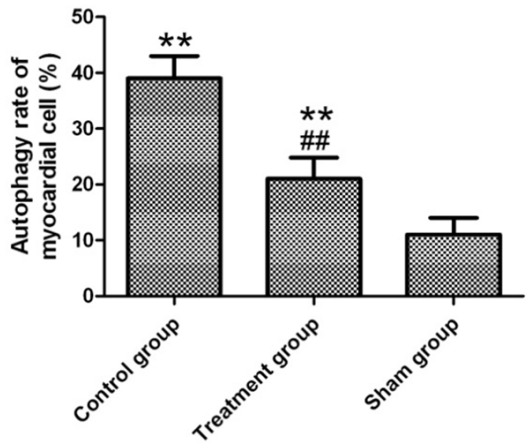

Figure 3. Comparison of autophagy rate of myocardial cells across groups. **For the comparison with the sham group, P<0.01; ##For the comparison with the control group, P<0.01.

widened gaps between myocardial cells in the control group. Less severe damage in myocardial fibers was found in the treatment group than in control group, as shown in **Figure 1**.

Results of autophagy of myocardial cells

The sham group showed clear nuclei with large volumes and distinct boundary under transmission electron microscope (**Figure 2A**). The control group showed a large number of visible bubble-like structures, which are cardiomyocyte-specific autophagosomes at the presence of autophagy (**Figure 2B**). Compared to the control group, the phagocytotic vesicles and autophagosomes in the treatment group were significantly reduced (**Figure 2C**). The autophagy rate of myocardial cells significantly increased in the control group compared to the sham group (P=0.000). The autophagy rate in the treatment group was higher than that in the sham group (P=0.002), but lower than that in the control group (P=0.001), as shown in **Figures 2** and **3**.

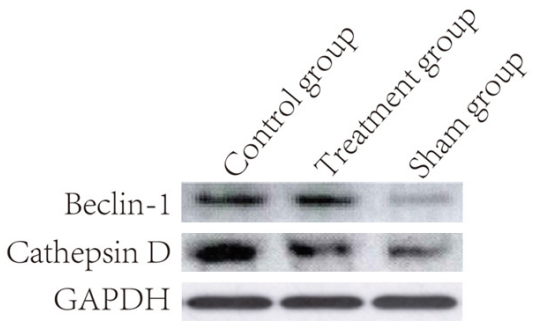

Figure 4. Expression levels of proteins Cathepsin D and Beclin-1 of myocardial cells in each group by western blot.

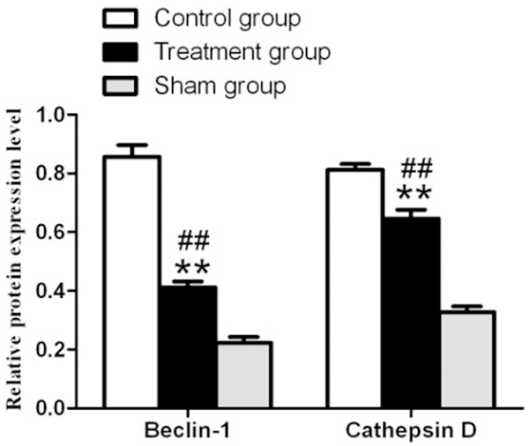

Figure 5. The comparison of the relative protein expression in each group. **For the comparison with the control group, P<0.01; ##For the comparison with the sham group, P<0.01.

Expression levels of proteins Cathepsin D and beclin-1 in myocardial cells detected by western blotting

Figures 4 and **5** show significantly increases in the levels of proteins Cathepsin D and Beclin-1 in the control group versus the sham group (P<0.01); markedly lower levels of proteins Cathepsin D (P=0.007) and Beclin-1 (P=0.002) in the treatment group versus the control group but higher levels in the treatment group versus the sham group (P=0.000).

Discussion

HF is the final result of myocardial damage induced by various cardiovascular diseases. Its pathophysiological changes are very complicated. The studies in recent years have shown the important role of autophagy of myocardial cells in the pathogenesis of HF [9, 10]. Rapamycin is a macrolide immunosuppressant that exerts its effect by blocking the cell cycles of T-lymphocytes [11]. Clinical trials have found that rapamycin drug eluting stents can significantly reduce the incidence of stent thrombosis and of restenosis, thereby markedly improving the clinical efficacy of percutaneons coronary intervention [12]. Another study has found that rapamycin has a protective effect on myocardial cells [13]; however, no extensive reports on the effect of rapamycin on chronic HF have been found in China and abroad.

In the present study, we investigated the therapeutic effects and potential mechanisms of rapamycin on rats with chronic HF by establishing the rat models with chronic HF. We applied AAC to narrow the abdominal aorta, which increased the aorta pressure, cardiac afterload and compensation, and finally led to heart failure because of decompensation. Meanwhile, we observed the changes in myocardial morphology and cardiac function to determine the heart failure model. Cardiac pathological slices showed that the myocardial fibers in the sham group were neatly arranged without destruction or edema and the cell gap was normal. However, in the control group, the myocardia were damaged, the partial myocardial cells showed edema and the myocardial fibers were disordered, as well as myocardial cell gap was significantly widened. But, the damage to myocardial fibers in the treatment group was significantly less severe than that in the control group. Meanwhile, the result of the examination of cardiac function showed that there was no significant difference in LVEF between the control group and the sham group at 4 weeks after modeling. The parameters of IVSs, LVPWs, IVSd and LVPWd in the control group improved significantly, suggesting that the myocardial hypertrophy occurred in the rats in control group, but such changes had no effect on cardiac function. At 8 weeks after modeling, significantly higher IVSs, LVPWs, IVSd and LVPWd but markedly lower LVEF were observed in the control group than in the sham group (P<0.05), which indicates the presence of progressive myocardial hypertrophy had had adverse effects on cardiac functions. Therefore, the rat models with HF had been completely established at 8 weeks after AAC, so at that time we began to inject intraperitoneally the rats in each group with apamycin or normal saline. The

treatment lasted for 4 weeks, namely till 12 weeks after AAC, when echocardiography showed significantly higher IVSs, LVPWs, IVSd and LVPWd but markedly lower LVEF in the control group versus the sham group, suggesting the symptoms of HF aggravated. However, the rapamycin treated rats in the treatment group had significantly lower IVSs, IVSs, LVPWd and LVPWd but markedly higher LVEF than did the control group ($P<0.05$). This shows that rapamycin can reverse ventricular hypertrophy and improve the cardiac function in rats.

Autophagy is a process mediated by lysosomes and induced by such factors as intracellular hypoxia, pressure, starvation and stress, and it is a mechanism allows recycling proteins and organelles in cells [14, 15]. Essentially, it is a normal physiological stress, but some recent studies have reported that the uncontrolled autophagic response plays a crucial role in pathophysiological changes of various diseases [16]. For example, autophagy reduces the number of myocardial cells in cardiovascular disease, resulting in thinner walls and dilated cavities, thus further deteriorating HF [17, 18]. In this trial, the results of transmission electron microscope showed that the concentrations of phagocytic vesicles and autophagosomes in the myocardial cells significantly decreased in the treatment group compared to those in the control group. The autophagy rate of myocardial cells in the control group was significantly higher than that in the sham group, while it was lower in the treatment group than that in the control group ($P<0.05$, **Figures 2**, **3**), indicating that apoptosis of the autophagic cells among myocardial cells can lead to the loss of the cells and reductions in the number of myocardial cells, thus resulting in thinner walls and dilated cavities. Apoptosis of the autophagic cells may play an important role in the presence and progression of HF, which is an important mechanism for apoptosis of the myocardial cells. However, after rapamycin intervention, the autophagy in the setting of HF can be reduced, which could significantly improve the cardiac functions in rats.

Beclin-1 and Cathepsin D are two proteins closely associated with cell autophagy. The expression levels of Beclin-1 and Cathepsin D have reported to be extremely low in normal cells, but significantly higher in autophagy cells [19, 20]. The results in the present study showed that the levels of Beclin-1 and Cathepsin D were significantly elevated in the control group, compared to the sham group; but they markedly decreased in the treatment group, compared to the control group, though they were still higher than that in the sham group ($P<0.05$), which shows that the levels of autophagy proteins in cells were significantly elevated in the presence and progression of chronic HF in rats, but rapamycin can decrease the levels of proteins Beclin-1 and Cathepsin D as well as the occurrence of autophagy in cells, thus attenuating HF and improving the ventricular remodeling and cardiac functions. Therefore, rapamycin may have the effect of regulating the process of autophagy in myocardial cells.

In conclusion, apoptosis of autophagic cells among myocardial cells significantly increased in the rat models with HF induced by AAC. Apoptosis of autophagic cells is related to the ventricular remodeling and left ventricle dysfunction, which has also played a critical role in the occurrence and development of HF. Rapamycin can attenuate the reductions in autophagy-related proteins Beclin-1 and Cathepsin D concomitant with apoptosis of myocardial cells, indicating that rapamycin may reverse ventricular remodeling and improve cardiac functions by regulating the autophagic process of myocardial cells. However, additional studies are required to explore how HF induces the autophagy, how it promotes the presence and development of diseases after the inducement, and how it regulates the autophagy process. If these problems can be explained, new ideas of studying the occurrence and developing mechanisms of HF may be provided; and also, the effective target for preventing and treating HF may be provided by studying regulation of autophagy pathway.

Disclosure of conflict of interest

None.

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