

Rapamycin prevents apoptosis of myocardial cells in heart failure rats through up-regulating Akt.

Chunxiao Zhang^{1,2}, Aifen Liu², Guohai Su^{1,3*}, Yudong Chen^{2*}

¹Shandong University, Jinan, China

²Department of Cardiology, the Central Hospital of Shengli Oil Field, Dongying, PR China

³Department of Cardiology, Jinan Central Hospital Affiliated to Shandong University, Jinan, PR China

Abstract

Objectives: Heart Failure (HF) progression could be prevented by an inhibitor of the mTOR and an autophagy enhancer rapamycin. This study aimed to investigate the effect of rapamycin on HF progression and myocardial cells apoptosis.

Methods: HF rats were injected with low-, middle-, and high-dose rapamycin. Echocardiography, HE staining, plasma Brain Natriuretic Peptide (BNP), myocardial cells apoptosis and Akt activation in rapamycin treated rats were detected.

Results: HF rats showed reduced cardiac functions, destructive pathological changes in myocardium, enhanced Akt activation and myocardial cells apoptosis. However, rapamycin reversed all the changes in a dose-dependent manner. Cardiac functions were enhanced by rapamycin. Myocardial cells apoptotic percentage, Akt expression, and pathological changes in HF rats myocardium were inhibited by rapamycin administration.

Conclusions: Rapamycin protected against myocardial hypertrophy and myocardial cells apoptosis in HF rats in a dose-dependent manner.

Keywords: Heart failure, Rapamycin, Akt, Apoptosis.

Accepted on August 23, 2017

Introduction

Heart Failure (HF) is a clinical syndrome with inability of heart pumping blood of which the derived metabolic dysfunction contributes to high rates of disability [1]. Though numerous studies focus on HF, the mechanisms of heart failure are still unclear and the treatment of HF still needs to be improved.

The well-known phosphatidylinositol 3-phosphate kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway involves in the regulation of autophagy and apoptosis in mammal cells [2]. PI3K pathway plays an essential role in cell, organ, and body size determination [3]. Despite of Akt's beneficial effects on contractility, prolonged Akt activation could result in contractile dysfunction, including heart failure [4]. The excessive cardiac growth induced by Akt or others, rather than long-term Akt activation itself, is detrimental for the HF or myocardial hypertrophy [4]. On contrary, reports showed that Akt activation inversely preserved cardiac function and prevented injury after transient cardiac ischemia [5]. The overexpressed Akt in mesenchymal stem cells dramatically repaired infarcted myocardium and improved cardiac function [6].

Rapamycin is a lipophilic, macrolide antibiotic. Rapamycin can effectively attenuate myocytes hypertrophy induced by growth factors *in vitro* [3]. Moreover, HF progression and the associated long-term Akt activation could be prevented by rapamycin [4,7]. The effect of rapamycin on cell apoptosis is duality too. Rapamycin exhibits apoptotic effect on cancer cells, or protects against apoptotic neuronal death [8,9]. In cancer cells, rapamycin administration induced cancer cell apoptosis and blocked cell proliferation [10-14]. In male germ cells, rapamycin could inhibit chemicals induced apoptosis *via* impairing ROS-derived dysfunction in mitochondria [15]. In human neuronal SH-SY5Y cells, rapamycin showed neuroprotective against rotenone-induced apoptosis through enhancing autophagy to clear damaged mitochondria [16]. As reported by Shioi et al. rapamycin could attenuate load-induced compensated hypertrophy *via* an mTOR-dependent mechanism [3].

Studies also showed that rapamycin is an inhibitor of the mTOR and an autophagy enhancer [7,16-18]. mTOR negatively regulates autophagy, and rapamycin could induce autophagy by inactivating mTOR [17]. However, the effect of rapamycin on myocardial cells apoptosis in HF progression had not been well reported.

To our best of knowledge, no study focused on mechanism of rapamycin on heart failure as well as the role of AKT in this progress. This study was designed to investigate the effect and mechanism of rapamycin on HF progression. HF rats were injected with rapamycin to evaluate rapamycin's effect on myocardial function, myocardial cells apoptosis, and regulation of Akt. The present study would provide new information on rapamycin protective effect on myocardial cells and in HF progression.

Methods and Materials

Rat HF model

Rat HF model was established as previously described [19,20]. After acclimation for 7 d (under a 12 h light/dark cycle with free access to food and water), 40 male Sprague-Dawley (SD) rats (280 ± 30 g) received proximal coronary ligation for the induction of chronic myocardial infarction. Rats were standardized feeding for 14 w followed with rapamycin interferences. According to the rapamycin dose, 40 rats model were randomly divided into 4 groups (n=10, for each group), including model group (HF), HF model+1 mg/kg (body weight)/d rapamycin (tail vein injection, low-dose (L) every two weeks), HF model+2.5 mg/kg (body weight)/d (middle-dose, M) rapamycin and HF model+4.5 mg/kg (body weight)/d rapamycin (high-dose, H). Sham ligated animals (n=10) served as non-failing Controls (CTR). Four weeks after rapamycin interference, animals were anaesthetized and hearts were separated, pieced and prepared immunohistochemistry, and western blot analysis, respectively. Tail vein blood samples were collected for plasma BNP (Brain Natriuretic Peptide) measurement using ELISA kits. All experimental procedures were approved by the Institutional Animal Care Committee of The Central Hospital of Shengli Oil Field, Dongying, China.

Echocardiography

The assessment for heart function was performed using twinkling color Doppler ultrasound machine before anaesthetization [21,22]. The Left Ventricular (LV) end diastolic diameter (LVIDd, mm), Left Ventricular end systolic diameter (LVIDs, mm), Left Ventricular Ejection Fraction (LVEF, %), and Left Ventricular Fractional Shortening (LVFS, %) were recorded or calculated.

Brain natriuretic peptide (BNP) measurement

The plasma BNP level in rat model after 4 w rapamycin interferences were measured according to the ELISA kits [21].

HE staining

Immunohistochemistry were performed on the xenograft myocardial tissues. Tissues were fixed, transparent, and embedded. For Hematoxylin-Eosin (HE) staining, 5 µm

sections were cut, dehydrated, deparaffinized, and rehydrated. Then sections were stained with HE staining solution (Nanjing Jiancheng Biotechnology Institute). Images were captured using a Olympus microscopy (BX60, Olympus, Japan).

TUNNEL assay

The apoptosis of myocardial cells was detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNNEL) assay (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The apoptotic myocardial cells were stained brown coloration. The average percentage (%) of apoptotic cell number from five arbitrarily selected fields was calculated (under magnification X40) using microscopy (Olympus, Japan).

Western blotting analysis

Xenograft myocardial tissues from rats were homogenized in ice-cold RIPA buffer, and the supernatants were collected for protein determination. Proteins of 35 µg were separated by 10% SDS-PAGE, and then transferred to PVDF membrane (Invitrogen Corp., Carlsbad, CA, USA). After blocking with Bovine Serum Albumin (BSA, Sigma, USA), membranes were incubated with primary antibodies against GAPDH (dilution 1: 2000), Akt (dilution 1: 5000) and phosphorylated (p)-Akt (dilution 1: 1000, Cell Signal Technology Inc. Danvers, MA, USA) at 4°C overnight. Membranes were then incubated with HRP-conjugated secondary antibodies for 1 h in dark at 37°C. At last, the polypeptide bands were analysed using an Enhanced Chemiluminescence (ECL) detection system.

Statistical analysis

Statistical analysis was performed using SPSS 19.0. Data were expressed as mean ± SD. Statistical analysis was performed using a one-way Analysis of Variance (ANOVA) test for difference among groups more than three. P<0.05 was considered to be statistically significant.

Results

Rapamycin repairs cardiac function in HF rats

Statistical analysis of echocardiography detection showed rapamycin administration significantly prevented HF progression (p<0.05, Table 1). Echocardiography indicators LVIDd (mm) and LVIDs (mm) were significantly increased in HF model (p<0.01), while LVEF (%) and LVFS (%) were dramatically reduced in HF model (p<0.01), as compared to Sham rats. Moreover, the administration of rapamycin into HF model inversed the changes in echocardiography indicators in a dose-dependent manner. Rats received high-dose rapamycin administration showed the lowest LVIDd and LVIDs values and the highest LVEF and LVFS values among the rapamycin-administrated groups (p<0.01). Echocardiography indicators

assessment showed rapamycin administration prevented the HF progression.

Table 1. Echocardiography indicators in rats (Mean ± SD).

Group	LVIDd (mm)	LVIDs (mm)	LVEF (%)	LVFS (%)
Sham	5.46 ± 0.34	2.89 ± 0.21	88.46 ± 3.14	57.21 ± 1.29
HF model	7.24 ± 0.16**	4.09 ± 0.11**	62.18 ± 2.19**	30.40 ± 1.01**
HF+L	6.98 ± 0.43**#	3.79 ± 0.24**#	69.22 ± 2.21**#	37.52 ± 1.57**#
HF+M	6.42 ± 0.11**#^	3.33 ± 0.54*#	74.04 ± 1.64**#^	45.75 ± 0.89**##^
HF+H	5.97 ± 0.32**##^	3.04 ± 0.19*##^	80.18 ± 2.89*##^	48.32 ± 1.12**##^

LVIDd: Left Ventricular (LV) End Diastolic Diameter; LVIDs: Left Ventricular End Systolic Diameter; LVEF: Left Ventricular Ejection Fraction; LVFS: Left Ventricular Fractional Shortening. *, and ** indicates p<0.05, and p<0.01, vs. Sham, respectively. #, and ## indicates p<0.05, and p<0.01, vs. HF model, respectively. ^, and ^^ indicates p<0.05, and p<0.01, vs. HF+L, respectively.

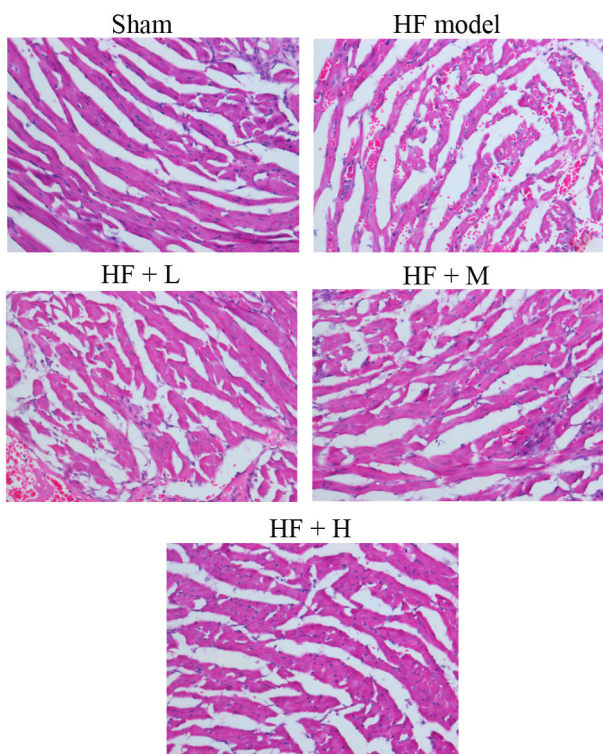


Figure 1. HE staining for pathological changes in myocardium of HF rats. Magnification X200.

Rapamycin suppresses pathological changes in myocardium in HF rats

Using HE staining, we found the myocardium tissue of HF rats showed pathological changes including disorganization of cardiac myocytes, hypertrophy of cardiac myocytes, and increased intercellular collagen fibers. HF model showed numerous vacuoles of varying sizes and shapes of myocardial cells. However, all the pathological changes could be suppressed by rapamycin administration. Moreover, these rapamycin suppressed the pathological changes in in myocardium of HF rats in a dose-dependent manner. No obvious differences were observed in pathological changes in

myocardium tissues between the Sham and high-dose rapamycin administrated HF rat (Figure 1).

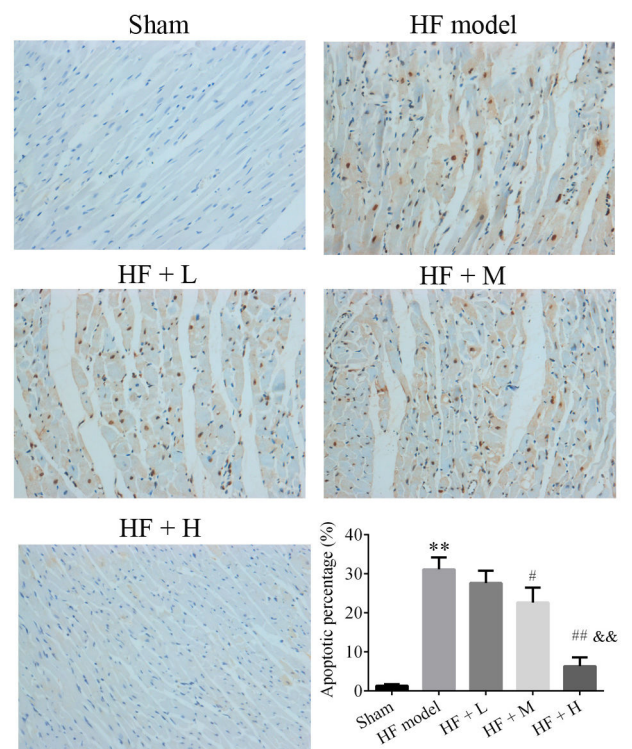


Figure 2. TUNNEL assay. Percentage (%) of TUNEL positive cell number was calculated as positive cells × 100/total number of cells. ** indicates p<0.01, vs. Sham. #, and ## indicates p<0.05, and p<0.01, vs. HF model, respectively. && indicates p<0.01, vs. HF+L model. Magnification X200.

Rapamycin inhibits apoptosis of myocardial cells in HF rats

We performed the TUNNEL assay to analysis the apoptosis of myocardial cells in HF rats and to evaluate rapamycin’s antiapoptotic effect on myocardial cells. Results showed significant apoptotic myocardial cells percentage in HF rats in comparison with that in Sham rats (p<0.01, Figure 2). The

percentage of apoptotic myocardial cells was reduced by rapamycin in a dose-dependent manner. HF rats received high-dose rapamycin showed the lowest percentage of apoptotic myocardial cells (Figure 2).

Rapamycin reduced plasma BNP level

The plasma BNP concentration in rats was detected to evaluate the effect of rapamycin on BNP expression. Results showed that BNP level were significantly upregulated in HF model in comparison with the sham controls ($p < 0.01$, Figure 3). However, the BNP levels in HF model were significantly reduced by rapamycin administration, as compared to the HF model ($p < 0.05$), in a dose-dependent manner. The BNP level in high-dose rapamycin treated rats was lower than low- or middle-dose rapamycin treated rats ($p < 0.01$). We demonstrated that rapamycin administration reduced plasma BNP level in HF rats.

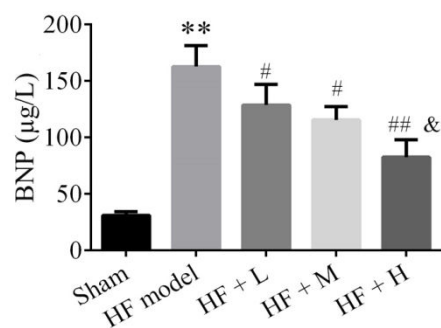


Figure 3. BNP ELISA assay. ** indicates $p < 0.01$, vs. Sham. #, and ## indicates $p < 0.05$, and $p < 0.01$, vs. HF model, respectively. & indicates $p < 0.05$, vs. HF+L.

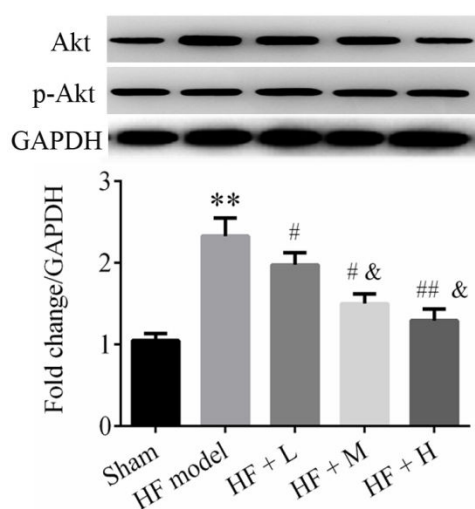


Figure 4. Western blot analysis for Akt activation. ** indicates $p < 0.01$, vs. Sham. #, and ## indicates $p < 0.05$, and $p < 0.01$, vs. HF model, respectively. & indicates $p < 0.05$, vs. HF+L.

Rapamycin impedes Akt activation

As reported long-term Akt activation induces contractile dysfunction [4]. Heart failure progression induced by long-

term Akt activation could be prevented by rapamycin [4]. We detected the activation of Akt and found the upregulation of Akt in HF rats ($p < 0.01$, Figure 4). However, the activation of Akt could be inhibited by rapamycin administration in a dose-dependent manner. Significant reduction in Akt expression were observed in high-dose rapamycin administrated rats, versus rats treated with low- or middle-dose rapamycin administrated rats ($p < 0.05$, Figure 4).

Discussion

The present study demonstrated that rapamycin administration to HF rats could prevent HF progression *via* inhibiting apoptosis of myocardial cells through inactivation of Akt.

Studies showed that Akt is a crucial factor in the regulation of autophagy and apoptosis [2]. The chronic activation of Akt could induce feedback inhibition of PI3K [23]. The up-regulation of Akt plays a key role in the inhibition of cardiomyocyte apoptosis or autophagy in hypoxia/reoxygenation or ischaemia/reperfusion injury rat [24,25]. During HF progression, long-term Akt activation induces contractile dysfunction [4]. Heart failure progression induced by long-term Akt activation could be prevented by rapamycin [4,7]. In the present study, we demonstrated the dramatically Akt activation and apoptosis of myocardial cells in HF rats as compared to the normal control. After rapamycin administration, however, the expression of Akt as well as myocardial cells apoptosis was significantly inhibited, in a dose-dependent manner. These demonstrated that rapamycin could inhibit the apoptosis *via* reducing overexpression of Akt in HF progression.

Shioi et al. previously reported that rapamycin attenuated load-induced compensated hypertrophy *via* an mTOR-p70 ribosomal S6 kinase (p70S6K) protein-dependent mechanism [3]. In further study by McMullen et al. they demonstrated that rapamycin administration blocked S6 phosphorylation and concluded that mTOR-p70S6K1 signaling cascade might be the major pathway activated during compensated hypertrophy [26]. In this presents study, we demonstrated that rapamycin alleviated pathological changes including suppressing cardiomyocyte hypertrophy in myocardium. This fact suggested mTOR-p70S6K1 signaling cascade might be the major pathway mediated by rapamycin in HF progression.

The effect of rapamycin on cell apoptosis is duality. Rapamycin exhibited apoptotic effect and blocked cancer cells proliferation on one hand [8-14]. On the other hand, rapamycin could inhibit chemicals induced excess death or apoptosis *via* enhancing autophagy [15,16]. In the presents study, we demonstrated that rapamycin protected against HF-derived myocardial cells apoptosis and inhibited Akt activation in HF myocardium. These results showed the rapamycin protective effect against myocardial cells apoptosis in HF rats *via* inhibiting Akt overexpression.

Conclusions

We concluded that rapamycin protected against myocardial cells apoptosis in HF rats in a dose-dependent manner. HF rats received rapamycin showed ameliorative heart functions, reduced plasma BNP level and myocardial cells apoptosis in comparison with controls. These demonstrated rapamycin protective effect against HF progression and the therapeutic potential for HF therapy.

Acknowledgment

There is no Funding. All of the authors have no conflict of interest in this research.

Ethics, Consent and Permissions

Ethical approval was given by the medical ethics committee of Department of Cardiology, The Central Hospital of Shengli Oil Field.

Consent to Publish

All of the authors have consented to publish this research.

Author's Contribution

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript.

Interest Conflict

All of the authors have no conflict of interest in this research.

References

1. Miller MS, VanBuren P, LeWinter MM, Braddock JM, Ades PA, Maughan DW. Chronic heart failure decreases crossbridge kinetics in single skeletal muscle fibres from humans. *J Physiol* 2010; 588: 4039-4053.
2. Hou X, Hu Z, Xu H, Xu J, Zhang S, Zhong Y. Advanced glycation endproducts trigger autophagy in cardiomyocyte via RAGE/PI3K/AKT/mTOR pathway. *Cardiovasc Diabetol* 2014; 13: 78.
3. Shioi T, McMullen JR, Tarnavski O, Converso K, Sherwood MC, Manning WJ. Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation* 2003; 107: 1664.
4. Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest* 2005; 115: 2108-2118.
5. Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* 2001; 104: 330-335.
6. Noiseux N, Gnecci M, Lopez-Illasaca M, Zhang L, Solomon SD, Deb A. Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. *Mol Ther* 2004; 14: 840-850.
7. McMullen JR, Jennings GL. Differences between pathological and physiological cardiac hypertrophy: novel therapeutic strategies to treat heart failure. *Clin Exp Pharmacol Physiol* 2007; 34: 255-262.
8. Ding K, Wang H, Wu Y, Zhang L, Xu J, Li T. Rapamycin protects against apoptotic neuronal death and improves neurologic function after traumatic brain injury in mice via modulation of the mTOR-p53-Bax axis. *J Surg Res* 2015; 194: 239-247.
9. Saqcena M, Patel D, Menon D, Mukhopadhyay S, Foster DA. Apoptotic effects of high-dose rapamycin occur in S-phase of the cell cycle. *Cell Cycle* 2015; 14: 2285-2292.
10. Alayev A, Berger SM, Kramer MY, Schwartz NS, Holz MK. The combination of rapamycin and resveratrol blocks autophagy and induces apoptosis in breast cancer cells. *J Cell Biochem* 2015; 116: 450-457.
11. Morad SA, Schmid M, Buchele B, Siehl HU, El Gafaary M, Lunov O. A novel semisynthetic inhibitor of the FRB domain of mammalian target of rapamycin blocks proliferation and triggers apoptosis in chemoresistant prostate cancer cells. *Mol Pharmacol* 2013; 83: 531-541.
12. Frost P, Berlinger E, Mysore V, Hoang B, Shi Y, Gera J. Mammalian target of rapamycin inhibitors induces tumor cell apoptosis in vivo primarily by inhibiting VEGF expression and angiogenesis. *J Oncol* 2013; 2013.
13. Wang YD, Su YJ, Li JY, Yao XC, Liang GJ. Rapamycin, an mTOR inhibitor, induced apoptosis via independent mitochondrial and death receptor pathway in retinoblastoma Y79 cell. *Int J Clin Exp Med* 2015; 8: 10723.
14. Nekova TS, Dotterweich J, Schütze N, Einsele H, Stuhler G. Small molecule enhancers of rapamycin induce apoptosis in myeloma cells via GSK3A/B preferentially within a protective bone marrow microenvironment. *Br J Haematol* 2014; 167: 272-274.
15. He X, Song W, Liu C, Chen S, Hua J. Rapamycin inhibits acrolein-induced apoptosis by alleviating ROS-driven mitochondrial dysfunction in male germ cells. *Cell Proliferat* 2014; 47: 161-171.
16. Pan T, Rawal P, Wu Y, Xie W, Jankovic J, Le W. Rapamycin protects against rotenone-induced apoptosis through autophagy induction. *Neurosci* 2009; 164: 541-551.
17. Ravikumar B, Berger Z, Vacher C, Okane CJ, Rubinsztein DC. Rapamycin pre-treatment protects against apoptosis. *Human Mol Gene* 2006; 15: 1209-1216.
18. Cui Y, Kam K, Sherman D, Janczewski WA, Zheng Y, Feldman JL. Defining preBöttinger complex rhythm- and pattern-generating neural microcircuits in vivo. *Neuron* 2016; 91: 602-614.
19. Kumarswamy R, Lyon AR, Volkmann I, Mills AM, Bretthauer J, Pahuja A. SERCA2a gene therapy restores

- microRNA-1 expression in heart failure via an Akt/FoxO3A-dependent pathway. *Eur Heart J* 2012; 33: 1067-1075.
20. Lyon AR, Bannister ML, Collins T, Pearce E, Sepehrpour AH, Dubb SS. SERCA2a gene transfer decreases SR calcium leak and reduces ventricular arrhythmias in a model of chronic heart failure. *Circ Arrhythm Electrophysiol* 2011.
21. Wang F, Li D, Chen J, Hu WJ, Liu Y. Heart protective effects of luteolin on rats with doxorubicin-induced heart failure. *Latin Am J Pharm* 2013; 32: 1-8.
22. Li S, Guo K, Wu J, Guo Z, Li A. Altered expression of c-kit and nanog in a rat model of Adriamycin-induced chronic heart failure. *Am J Cardiovasc Dis* 2017; 7: 57.
23. Nagoshi T, Matsui T, Aoyama T, Leri A, Anversa P, Li L. PI3K rescues the detrimental effects of chronic Akt activation in the heart during ischemia/reperfusion injury. *J Clin Investig* 2005; 115: 2128-2138.
24. Wang L, Lu Y, Liu X, Wang X. Ghrelin protected neonatal rat cardiomyocyte against hypoxia/reoxygenation injury by inhibiting apoptosis through Akt-mTOR signal. *Mol Biol Rep* 2017; 1-8.
25. Fan G, Yu J, Asare PF, Wang L, Zhang H, Zhang B. Danshensu alleviates cardiac ischaemia/reperfusion injury by inhibiting autophagy and apoptosis via activation of mTOR signalling. *J Cell Mol Med* 2016; 20: 1908-1919.
26. McMullen JR, Sherwood MC, Tarnavski O, Zhang L, Dorfman AL, Shioi T. Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. *Circulation* 2004; 109: 3050-3055.

*Correspondence to

Guohai Su

Department of Cardiology

Jinan Central Hospital Affiliated to Shandong University

PR China

Department of Cardiology

The Central Hospital of Shengli Oil Field

PR China