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Resveratrol protects myocardial apoptosis induced by ischemia-reperfusion in rats with acute myocardial infarction *via* blocking P13K/Akt/e-NOS pathway

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Abstract. – OBJECTIVE: To elucidate the protective role of resveratrol (RSV) in myocardial apoptosis induced by ischemia-reperfusion injury in rats with acute myocardial infarction (AMI), and to explore its underlying mechanism.

MATERIALS AND METHODS: The AMI rat model was successfully established by ligation of the left anterior descending coronary a tery. Rat cardiomyocytes were isolated and cu Cells were divided into four groups, ing: control group (no specific treatmen MI group (acute ischemia-reperfusion treatn AMI+RSV group (RSV pretreatment for 2 before acute ischemia-reperfusion) and AM RSV+LY group (RSV pretrea combined with 40 μmol/L phosphatid -kinases (PI3K) pathway inhibito 2940 r 24 h before acute ischemia-r fusion) olo-gy of apoptotic cardion es in was observed by Ho hst The prolif-eration, apoptosis cell progression of cardiomyocyte ere deter by MTT (3-(4,5-dimethyl 2-yl)-2,5-dip tetrazolium bromid minal dexyn leotidyl transferase(-medi UTP nick end labeling (TUNE assay and f tometry, respectively. Fi the protein lev genes relative to PI3 t/eNOS pathway w e detected by West lot.

R LTS: H chst staining showed a large num f otic c cell retraction, enhance ve in and enlarged cell gap MI gr sm umber of necrotic cells und i V group, which was signifi-can ewer tha at of AMI group. Meanwhile, rem g cells presented normal morphology. Ho number of necrotic cells were o ved i MI+RSV+LY group, which was ob-sly more than that of AMI+RSV group. Com-d with control group, cells in AMI group s significantly decreased proliferative rate, reased early phase, late phase and total op ptosis. In AMI oup, the ratio of G0/ hase was remarkably increased, whereas se of S and /M phases were decreased. reover, the e ession levels of phosphor-d Akt (p-A and phosphorylated e-NOS (p OS) were nificantly downregulated in AM MI+RSV group, cell apoptosis, cell cy gression and levels of p-Akt and p-eNOS showed the opposite trends as those AMI group. However, LY294002 pretreatment he protective role of RSV in cellular ha s of cardiomyocytes.

CONCLUSIONS: RSV protects cardiomyocyte apoptosis from ischemia-reperfusion injury through regulating phosphorylation levels f proteins relative to PI3K/Akt/e-NOS pathway.

Key Words
Resveratrol, Ischemia-reperfusion, Cardiomyocytes, PI3K/Akt/eNOS, Apoptosis, Proliferation, Cell cycle progression.

Introduction

Ischemic heart disease is one of the most fateful diseases in the world. It seriously threatens human health, especially in the middle-aged and elderly population[1]. The imbalance of coronary blood flow and myocardial demand caused by changes in coronary circulation is the major pathogenic factor of ischemic heart disease[2]. Surgical approaches, including thrombolytic therapy, percutaneous transluminal coronary angioplasty and coronary artery bypass grafting, mainly contribute to the immediate restoration of blood flow in ischemic myocardium[3]. Hypoxia-reoxygenation injury frequently occurs during the treatment of ischemic heart diseases. The main

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characteristics include abnormalities in morphology, structure and function of cardiomyocytes[4]. Therefore, prevention of hypoxia-reoxygenation injury is the key to improve the successful therapeutic rate of ischemic heart disease.

Resveratrol (RSV) is a natural polyphenolic substance extracted from plants. It has strong biological activity, which can prevent free radicals, oxidative stress, and tumor diseases. Meanwhile, it can also enhance immune response in the body. RSV exerts a relatively small side effect, which is frequently applied in clinical treatment[5]. RSV pretreatment has been proved to alleviate hypoxia-reoxygenation injury[6]. It is known to all that hypoxia-reoxygenation injury leads to a cascade of myocardial injury involving multiple factors and pathways[7]. Based on this finding, inhibition or activation of certain pathways is identified to inhibit cell apoptosis, thus efficiently protecting myocardial injury. Previous works have reported that phosphatidylinositide 3-kinases/protein kinase B (PI3K/Akt) pathway is an important intracellular pathway preventing against ischemia-reperfusion injury. This may eventually reverse the occurrence, development and prognosis of h[illegible]ia-reoxygenation injury *via* certain inter[illegible] Various cytokines are produced by the stim[illegible]on of myocardial ischemia, hypoxia and reperfu[illegible] Thereafter, this can initiate PI3K/Akt pathway[illegible] protect the impaired myocardium[9]. In this study, we investigated the function[illegible]mediated PI3K/Akt/eNOS pathway i[illegible]t ca[illegible]myocyte apoptosis after ischemia-[illegible]fusion [illegible]ry. We aimed to provide a nove[illegible]ence[illegible] ment of hypoxia-reoxy[illegible]nat[illegible]

Mate[illegible]nd Method

Experime[illegible]tal Rats and Ca[illegible]myocyte Cul[illegible]

Thi[illegible]wo 10-day-old neonatal Sprague-Dawley (S[illegible]rats w[illegible]ng 10-20 g were enrolled in thi[illegible]y. Th[illegible]MI mo[illegible] was successfully established[illegible]tion of[illegible]eft anterior descending coronary [illegible][10]. In[illegible], rats were anesthetized [illegible]aperit[illegible]ction of 1% pentobarbital sodi[illegible] Next, t[illegible]ats were immersed in 75% etha[illegible] for 5 min and transferred to a clean bench. U[illegible]ditions, rat heart was harvested to [illegible]ct large blood vessels, valves and surrounding [illegible]nective tissues. Left ventricle was isolated, cut i[illegible]ue blocks of about 1 mm^3, followed by diges[illegible]d with 0.25 g/L trypsin at 37°C for 5 min. After centrifugation at 1000 r/min for 5 min, the precipitate was incubated with 0.2% t[illegible] lagenase for 10 min. Subsequently, [illegible]recipi[illegible] was centrifuged again at 1000 r[illegible] for 5 min. After that, the precipitate was[illegible]lated into a 25 cm^2 culture bottle and culture[illegible] Dulbecco's Modified Eagle' Medi[illegible] (DMI[illegible]ibco, Rockville, MD, USA) c[illegible]ning 15% [illegible] vine serum (FBS) (Gib[illegible] Rockv[illegible], MD, [illegible] Non-adherent cells [illegible] remov[illegible]t 12 h. After cell adherence, cult[illegible]di[illegible]as rep[illegible]d every 1-2 days. Thi[illegible]udy [illegible]proved [illegible]he Animal Procedur[illegible]ics Co[illegible] of [illegible] hospital.

In Vitro [illegible]-Reperfusio[illegible] in Car[illegible]myoc[illegible]

Cardiomyocytes [illegible]cultured in a 6-well plat[illegible]ensity of 1×[illegible]ls/well for 24 h in a[illegible]oxic-reoxygenation environment. Next, the [illegible]emia-reperfusi[illegible]n model was simulated. Cells [illegible]e maintained[illegible] three-gas incubator (JVAN. [illegible] with 94% N[illegible]% CO_2 and 1% O_2 for 24 h. S[illegible]uently, [illegible] were changed to a normal incu[illegible]CO_2 for 24-h reoxygenation.

Experimental Groups

[illegible]were divided into four groups, in[illegible]ding control group (no specific treatment), AMI group (acute ischemia-reperfusion treatment), AMI+RSV group (RSV pretreatment for 24 h before acute ischemia-reperfusion) and AMI+RSV+LY group (RSV pretreatment combined with 40 μmol/L PI3K pathway inhibitor LY294002 (Beyotime, Shanghai, China) for 24 h before acute ischemia-reperfusion). The dosage of RSV was selected as previous literature reported and was confirmed without any adverse events[11]. Hoechst staining was performed to observe nucleus of normal and apoptotic cells.

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide] Assay

Cardiomyocytes were seeded into 96-well plates at a density of 5×10^4 cells per well. Marginal wells were added with PBS. 5 replicates were set in each group. After specific treatment, 20 μL of MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were added to each well for 4-h incubation. Subsequently, 150 μL of dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, followed by low-speed oscillation for 10 min. Finally, the optical density at the wavelength of 490 nm was detected by a micro-plate reader.

Terminal Dexynucleotidyl Transferase(TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining

According to the instructions of TUNEL Fluorescence Kit (Promega, Madison, WI, USA), fixed cardiomyocytes were immersed in 0.2% Triton X-100 solution for 5 min to enhance cell membrane permeability. Then, the cardiomyocytes were incubated with deoxynucleotide terminal transferase (rTdT) at 37°C for 1 h. Positive cells were stained brown in cell nucleus. 5 fields of vision were randomly selected for each section. Apoptotic rate = number of apoptotic positive cells / total number of cells × 100%.

Flow Cytometry

Flow cytometry (FCM) (BD Biosciences, FranklinLakes, NJ, USA) was used to detect the apoptotic rate using Annexin V-fluorescein isothiocyanate (FITC) / Propidium Iodide (PI) staining. Briefly, cells were first seeded into 6-well plates and digested with 200 μL of Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin per well (Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation at 1000 r/min for 10 m[illegible] the cells were stained with Annexin V-FITC[illegible] nally, the apoptotic rate was calculated. Ap[illegible]tic rate = (B2+B4) quadrant cell number / tota[illegible] number × 100%. Cells in early phase of apopt[illegible] were located in the lower right quadrant, whi[illegible] those in late phase of apopto[illegible]cated in the upper right quadrant. Via[illegible] cells [illegible] necrotic cells were located in the l[illegible] left q[illegible]nt and the upper left quadrant, [illegible]ively.

Western Blot

Total protein w[illegible]rst extracted[illegible] cell lysate. The concen[illegible] extracted pro[illegible] sample was quantifie[illegible] the [illegible]chroninic acid (BCA) method (Pi[illegible]ce, Rockford, [illegible]USA). Subsequently, protei[illegible]mples were sepa[illegible]by sodium dodecyl s[illegible]ate-polyacrylamide g[illegible] electrophoresis (SDS[illegible]GE) ge[illegible]ectrophoresis and transferred ont[illegible]mbra[illegible] After [illegible]cking with 5% skim milk, [illegible]branes [illegible]e incubated with pri-[illegible]ary ant[illegible] (Al[illegible]d eNOS; β-actin as the [illegible]ce). O[illegible] day, the membranes were incu[illegible]d with [illegible]sponding secondary antibodies. [illegible]muno-reactive bands were developed by en[illegible]uminescence (ECL) method.

[illegible]tistical Analysis

[illegible]stical Product and Service Solutions (SPS[illegible]22.0 software (IBM, Armonk, NY, USA) was utilized for all statistical analysis. Quantitative data were represented as mean[illegible] deviation ($\bar{x}\pm s$). *t*-test was used to [illegible]pare differences between two groups[illegible]e-way analysis of variance (ANOVA) w[illegible]rformed to compare the difference among d[illegible] groups, followed by post-hoc test. [illegible]05 wa[illegible]dered statistically significant.

Re[illegible]

Morpholo[illegible]f Cardiomy[illegible] in Each

Hoec[illegible]stain[illegible]owed a large number of necrotic cells, cell [illegible]ion, enhanced refractive[illegible] and enlarged [illegible]gap in AMI group. A[illegible]an number of necrotic cells were found in [illegible]I+RSV group, which was significantly fewer [illegible]n that of AM[illegible]roup. Meanwhile, remaining [illegible] presented [illegible]al morphology. However, a g[illegible]umber [illegible]crotic cells were observed in AM[illegible]roup, which was much more than that of AMI+RSV group (Figure 1).

[illegible]f RSV on the Proliferative [illegible]al of Cardiomyocytes After Ischemia-Reperfusion

Cell viability was determined by MTT assay. Compared with control group, the proliferative rate of AMI group was markedly decreased ($p<0.05$). The proliferative rate of AMI+RSV group was significantly higher than that of AMI group, which was inhibited in AMI+RSV+LY group ($p<0.05$, Table I). The above data suggested that PI3K inhibitor reversed the protective role of RSV in cardiomyocyte viability.

Effect of RSV on Cardiomyocyte Apoptosis After Ischemia-Reperfusion

TUNEL staining indicated that the apoptotic rate of cardiomyocytes in AMI group was significantly increased when compared with control group. RSV pretreatment markedly reduced apoptotic rate, while was further reversed by LY294002 treatment ($p<0.05$, Figure 2A and 2B). Annexin V-FITC/PI staining demonstrated that there were a great number of necrotic cells in AMI group, showing remarkable cardiomyocyte injury. Moreover, AMI+RSV+LY group showed significantly more apoptotic cells than AMI+RSV group, especially in late phase ($p<0.05$, Figure 3A and 3B).

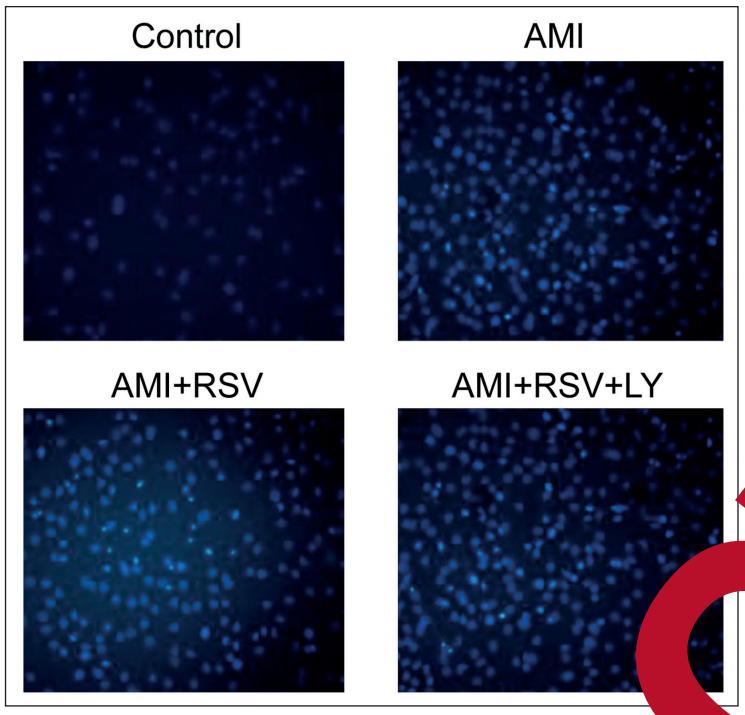

Figure 1. Morphology changes in 4 different group. Control group; AMI group group and AMI+RSV+LY group

Effect of RSV on Cell Cycle Progression of Cardiomyocyte After Ischemia-Reperfusion

Cell cycle progression in each group determined by FCM. In comparison with con group, cell proportion in G0/G1 phase increase remarkably in AMI group, wh S phase and G2/M phase decreased s sug ted that cardiomyocytes in AMI p were sted in G0/G1 phase. RSV pre ent cantly alleviate such ll cy . However, AMI+RSV+LY gro owed antly higher proportion in G1 phase. portion of S and G2/M was significa lower than S and G pha ble II). These results suggested t at RSV prom ell cycle progression, wh was arrested a hibiting PI3K pathwa

Ef f RS n the Expressions of Re enes 3K/Akt/e-NOS

Table Prolifera of cardiomyocytes

Gr	Proliferative rate
	0.232±0.034
MI	0.170±0.058
+RSV	0.228±0.025
V+LY	0.165±0.055

Pathway

No significant changes in the protein expres- t and e-NOS were found among dif- ent groups. However, the protein expression of phosphorylated Akt (p-Akt) and phosphorylated e-NOS (p-eNOS) in AMI group was marked- ly downregulated when compared with control group. AMI+RSV group presented significantly higher levels of p-Akt and p-eNOS relative to AMI group, which was then reversed by LY294002 treatment (Figure 4A and 4B). These results indicated that RSV protected cardiomyocyte apoptosis by altering the phosphorylation levels of Akt/e-NOS through PI3K/Akt/e-NOS pathway. However, LY294002 could reverse this protective effect.

Discussion

Due to the extremely high incidence and mortality of ischemic heart disease, we explored whether the protective role of RSV in rat cardiomyocytes induced by hypoxia-reoxygenation injury was achieved *via* activating PI3K/Akt/e-NOS pathway[12]. This study aimed to provide references for enhancing the success of surgery for this disease. In the present work, we first observed cardiomyocyte morphology by Hoechst staining. As the results indicated, abundant necrotic cells and abnormal morphol-

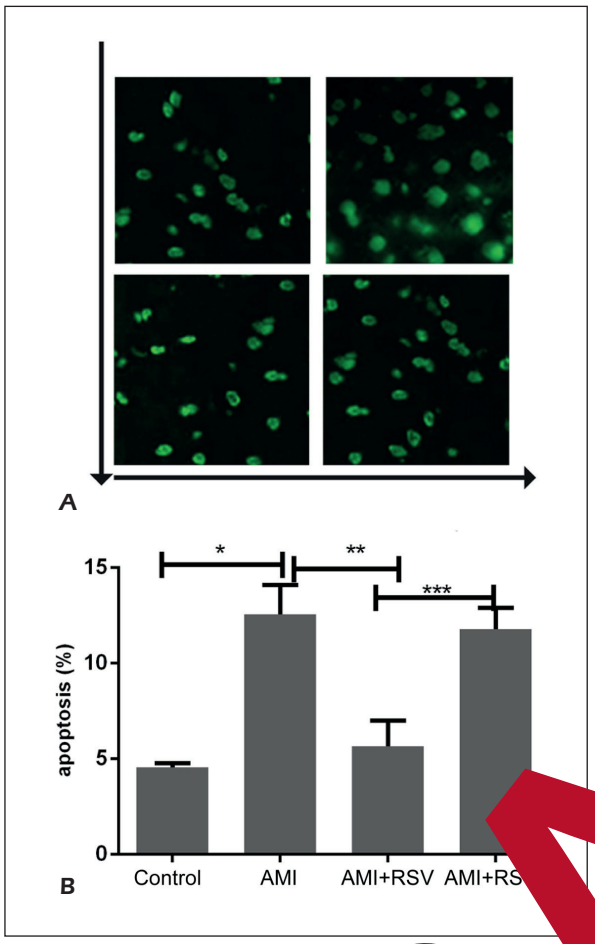

Figure 2. Cell apoptosis in each ... rmined by TUNEL assay. **A**, TUNEL staining ... t and upper to bottom: 1. Control group; 2 ... group; ... MI+RSV group; 4. AMI+RSV+LY grou ... poptotic ... group, AMI group, AMI+RS ... and ... group. *Control group vs ... MI ... MI group vs. AMI+RSV group; ***A ... SV gro ... MI+RSV+LY group. *p<0.05, **p<0 ... nd ***p<0.05.

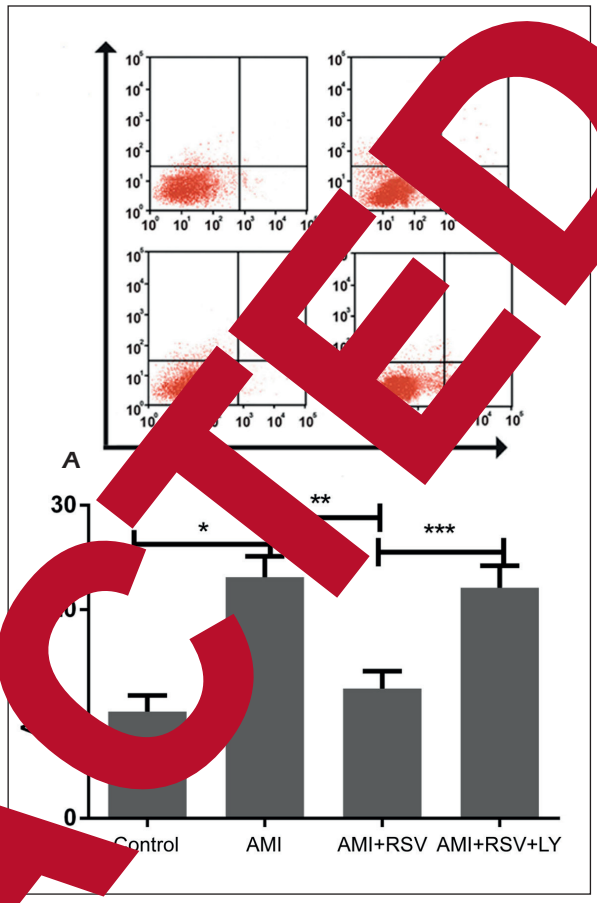

Figure 3. Cell apoptosis in each group determined by FCM. **A**, FCM images from left to right and upper to bottom: 1. Control group; 2. AMI group; 3. AMI+RSV group; 4. AMI+RSV+LY group. **B**, Apoptotic rate in control group, AMI group, AMI+RSV group and AMI+RSV+LY group. *Control group vs. AMI group; **AMI group vs. AMI+RSV group; ***AMI+RSV group vs. AMI+RSV+LY group. *p<0.05, **p<0.05 and ***p<0.05.

ogy of car ... myocytes ... bserved in AMI group. R ... pretreatment re ... bly protected the ap ... sis and necrosis of ... ardiomyocytes, whic ... uld be ... ersed by LY294002 pretreatme ... e mi ... conclu ... that ischemia-reperfusion ... rdiomy ... e injury, which could ... alleviat ... RS ... owever, PI3K pathway ... e agg ... ellular damage. Based on thes ... sults, w ... esumed that the protective role ... RSV in ischemic cardiomyocytes might be ... K/Akt/e-NOS pathway. Subsequently, we highlighted the potential roles of ... and LY294002 in regulating cell survival, c ... le progression of ischemic cardiomyocyte ... nd PI3K/Akt/e-NOS pathway involvement. Compared with control group, cells in AMI group showed significantly decreased proliferative rate, increased early phase, late phase and total one of apoptosis. The ratio of cells in G0/G1 phase increased, whereas those in S

Table II. Cell cycle progression of cardiomyocytes

Group	Cell cycle		
	G0/G1	S	G2/M
Control	57.33±3.91	27.10±3.22	10.04±2.93
AMI	76.19±4.18	16.82±2.65	8.82±1.21
AMI+RSV	65.72±3.73	24.41±1.72	17.60±1.62
AMI+RSV+LY	80.90±4.13	15.14±1.66	10.11±1.60

*p<0.05, **p<0.05 and ***p<0.05.

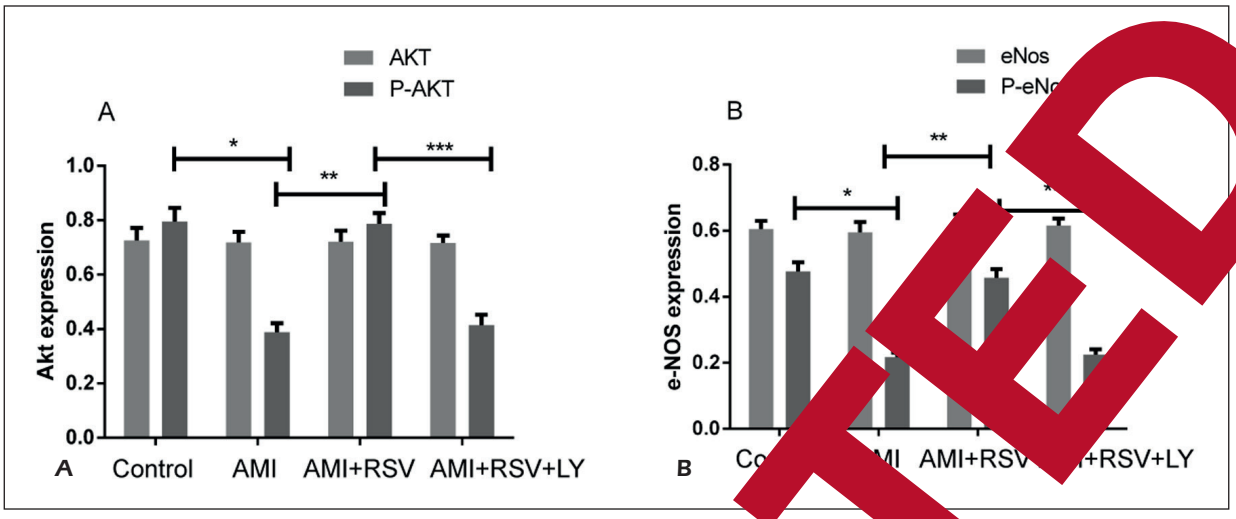

Figure 4. Expression changes of Akt, p-Akt, e-NOS and p-eNOS in each group. **A**, Expre vels of Akt and p-Akt in control group, AMI group, AMI+RSV group and AMI+RSV+LY gro ression levels of S and p-eNOS in control group, AMI group, AMI+RSV group and AMI+RSV+LY group. * group vs. AMI group; ** AMI group vs. AMI+RSV group; ***AMI+RSV group vs. AMI+RSV+LY group. *p<0.05, * .05 and ***p<0.0

and G2/M phases were significantly decreased in AMI group. Moreover, the expression levels of p-Akt and p-eNOSAkt were downreg d in AMI group than control group. H AMI+RSV group presented the opposite ds to AMI group in cell apoptosis, cell cycle gression and the phosphorylation levels of and e-NOS. However, LY294002 pretreatme could reverse the protective V in cellular behaviors of cardiom tes. olars[13,14] have found that RSV h gnifica harmacological effects, such nti-i vasodilation promot uscle cell proliferation inhibi , lipid olism promotion, anti-tum bility, and o e stress inhibition. The y effect of R on abnormal proli on ular smooth muscle cells allow it to be appl the prevention of cardiova r diseases, suc oronary heart disease d hyp rtension[15]. In addition, RSV can ate c gnal transduction, improve en ial i ity, re e the expressions of vascu dhesio olecules, and inhibit ular th m e cell hypertrophy by ring Akt pathway[16]. An *in vivo* rese has cla d that RSV alleviates hypoxia-re genation injury in rat cardiomyocytes, ex ective effect on ischemic and xic myocardium[17]. Meanwhile, our findings indicated that the anti-apoptotic function might be related to the promotion of RNA plication and protein synthesis in G0/G1 p thereby moting cell mitosis[18]. It has bee at RSV inhibits cell apoptosis through blocking cell cycle progression[19]. Moreover, another research has suggested that RSV inapoptosis through suppressing the activ- es of ey protein tyrosine kinases and promoting cell proliferation[20]. The activation of PI3K/Akt/e-NOS pathway regulates the expressions of apoptosis-associated proteins, which in turn mediates cell apoptosis[21]. Activated Akt induces multiple targets in the downstream pathway, which also inhibits the opening of mitochondrial permeability transition pores. Maintaining mitochondrial extracellular stability contributes to reduce the activation of pro-apoptotic factors, thereby impeding cell apoptosis[22]. Meanwhile, the activation of e-NOS helps to improve vascular function and endothelial function[23]. It is believed that RSV protects myocardial function and suppresses apoptosis through PI3K/Akt/e-NOS pathway. As PI3K inhibitor, LY294002 blocks the activation of PI3K/Akt pathway through suppressing the phosphorylation of downstream genes[24]. Inhibited activities of Akt threonine site and serine site lead to reduction in enzyme activity of e-NOS. This can in turn downregulate the phosphorylated levels of Akt and e-NOS[25]. PI3K/Akt/e-NOS pathway transmits a variety of pleiotropic effects, such as cell proliferation and apoptosis, hematopoiesis, and immune responses[26]. Current investigations have showed that RSV can attenuate the apoptosis of rat cardiomyocytes caused by ischemia-reperfusion[27]. In this study we

observed that RSV reduced cardiomyocyte apoptosis *via* PI3K/Akt/e-NOS pathway. Some limitations should be pointed out here. Firstly, *in vitro* effect of RSV on protecting cardiomyocytes after hypoxia-reoxygenation injury should be further elucidated. Secondly, we only choose a single dose of RSV, and the dosage effect of RSV is required for specific exploration as well.

Conclusions

We found that resveratrol protects cardiomyocyte apoptosis from ischemia-reperfusion injury through regulating phosphorylation levels of proteins relative to PI3K/Akt/e-NOS pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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