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Original Article

Chronic resveratrol treatment improves cardiac function in a rat model of diabetic cardiomyopathy via attenuation of mitochondrial injury and myocardial apoptosis

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Abstract: Incidence of diabetic cardiomyopathy (DCM) is associated with increased mortality in diabetic patients. Mitochondrial dysfunction has been indicated to play a key role in the pathogenesis and development of diabetic cardiomyopathy. Accumulating evidence suggests that resveratrol, a polyphenol, exerts cardiovascular protective effects. In this study, we aimed to explore the effects of chronic resveratrol administration on mitochondrial injury and cardiac function in a streptozotocin-induced rat model of DCM. After the successful induction of hyperglycemia for 8 weeks, resveratrol was intraperitoneally injected (2.5 mg/Kg/day) as treatment for 8 weeks. Echocardiography, histological analyses, myocardial succinate dehydrogenase and cytochrome c oxidase activities, and mitochondrial membrane potential were analyzed to evaluate the changes of cardiac structures and functions, myocardial apoptosis, as well as mitochondrial function following resveratrol treatment. Results of above analyses indicated that chronic resveratrol administration was associated with relieved mitochondrial swell and membrane loss, increase mitochondrial enzymes activities, and mitochondrial membrane potential, suggesting a potential role of resveratrol for the attenuation of mitochondrial injury. Moreover, chronic resveratrol administration was also related to significantly improved cardiac systolic dysfunction and reduced myocardial fibrosis, demonstrating a beneficial effect of resveratrol on cardiac remodeling. Overall, results of our study suggest that chronic resveratrol treatment may improve cardiac function and cardiac remodeling in rat model of DCM possibly via attenuation of myocardial mitochondrial injury and apoptosis.

Keywords: Diabetic cardiomyopathy, resveratrol, cardiac dysfunction, mitochondrial injury, myocardial apoptosis

Introduction

Results from epidemiologic studies suggest that the prevalence and incidence of diabetes mellitus (DM) have been increasing rapidly both in the eastern and western countries during the past decades [1-3]. Moreover, DM as a clinical syndrome has been recognized as one of the major risk factors for many cardiovascular diseases, such as coronary heart disease, stroke, peripheral artery disease, heart failure and atrial fibrillation [3]. Patients with DM has been indicated to be with approximately 2 folds risk for the development of cardiovascular diseases [4], which has been proved to be the leading cause of death in diabetic patients. Particularly, patients with DM are vulnerable for myocardial injuries independent of hypertension, vascular or valvular pathology and other causes, which were defined as diabetic cardiomyopathy (DCM) [5]. Previous studies in DM patients and animal models suggest that DCM is characterized by diastolic and systolic cardiac function, and subsequent development of chronic heart failure [6-8]. Multiple pathophysiological mechanisms have been suggested to be involved in the development of DCM, of which mitochondrial dysfunction and myocardial apoptosis have been considered to be the important ones [9]. Indeed, myocardial mitochondrial dysfunction may cause energy metabolic abnormalities and oxidative stress injury of cardiomyocytes, which may further accelerate myocardial apoptosis and loss of functional cardiomyocytes, leading to cardiac dysfunction [10, 11]. Therefore, treatment that attenuation of mitochondrial injury

may preserve cardiac functions in patients with DCM.

Resveratrol (trans-3,5,49-trihydroxystilbene, RES), a natural polyphenol mostly found in red grape skins, red wine, and some other vegetables [12], has been considered to exert potential cardiovascular protective effect possibly because of the phenomena named as French paradox, characterized by the improved cardiovascular outcomes despite of a high-fat diet in French people [13]. Subsequent analyses indicated that RES may exert its cardioprotective effect via many mechanisms, such as anti-inflammation, antioxidant, and alleviating of ischemia-reperfusion injury effects [10, 14, 15]. Interestingly, results of recent studies suggest that RES may be beneficial in DCM via mechanisms such as reducing oxidative stress, activating Sirt-1, and upregulating sarcoplasmic calcium ATPase [16-18]. Moreover, some studies even suggest that RES is also a mitochondrial nutrient [19], in view of its benefits on reducing oxidative stress, increasing mitochondrial membrane potential, preventing the opening of mitochondrial permeability transition pore and regulating mitochondrial biogenesis in ischemia/reperfusion injury or other pathologic conditions [20-22]. However, whether RES administration was associated with attenuated myocardial apoptosis and preserved cardiac function via alleviating mitochondrial injury has not been determined. Therefore, in this study, by establishment of a streptozotocin induced DCM in rat, we evaluated the potential benefits of chronic RES administration on mitochondrial function, myocardial apoptosis, and cardiac function. Results of our study may provide further evidence for use of RES for the prevention and treatment of DCM in patients with DM.

Material and methods

Animal model and grouping

30 female Sprague-Dawley (SD) rats, body weight 247±19 g, were obtained from Animal Experimental Center of Xi'an Jiaotong University and were fed in a room at 23±1°C with an alternating 12 h light-dark cycle. After one week, the fasting blood-glucose levels were detected with Johnson OneTouch® UltraEasy (Johnson, America) by tail vein puncture blood sampling to ensure the fasting blood-glucose levels of 4.1-6.3 mmol/L. Then rats were randomly divided into 2 groups, control group (n=8) and DCM group (n=22). The DCM rats were given streptozotocin (65 mg/kg, (Sigma) dissolved in 0.1 mol/L citric acid/sodium citrate buffer PH=4.3) by a single intraperitoneal injection. The controls were given an equivalent volume of citrate buffer alone with the same routine. Blood glucose levels were measured at 3, 4 and 5 days after streptozotocin injection via tail vein blood sampling. Rats with blood glucose levels ≥16.7 mmol/L at all of the three times were defined as diabetic and used in the subsequent study. Two rats of STZ-injected rats were excluded from subsequent experiment because they did not reach this glucose threshold. Three DCM rats died at the end of the third week after induction of hyperglycemia. 8 weeks after induction of hyperglycemia, the remaining 17 DCM rats were randomly divided into 2 groups which were expose to different interventions, DCM group (n=9) and DCM+RES group (n=8). DCM rats were untreated, while the DCM+RES rats received chronic intraperitoneal injection of trans-RES 2.5 mg/Kg/day (Sigma, Milan, Italy) (from 9th week to 16th week) according to Delucchi et al [12]. RES was dissolved in ethanol as a stock solution (12.5 mg/mL) and stored in the dark at 4°C. An appropriate stock solution for each animal (2.5 mg/Kg) was diluted in PBS to reach a final volume of 200 mL as a working solution for intraperitoneal injection. The animal experimental protocols were approved by the Institutional Animal Research and Ethics Committee of Xi'an Jiaotong University before performance.

Echocardiographic evaluation

Cardiac structure and function were evaluated by echocardiography. Briefly, rats were anesthetized by intraperitoneal injection of chloral hydrate (4%, 1 ml/100 g bodyweight). Echocardiographic studies were performed with an echocardiographic system (Philips iE33) equipped with a 12-4 MHz transducer (Philips, Holland) by an experienced investigator blinded to the grouping of the animals. M-mode recordings of the left ventricle were obtained at the level of the papillary muscles. A series of cardiac data including morphological and functional parameters such as left ventricular end-diastolic dimension (LVEDd), left ventricular end-systolic dimension (LVESd), left ventricular end-

diastolicvolume(LVEDV),leftventricularend-systolic volume (LVESV), and Tei index were measured and calculated. Each measurement was obtained with an average of three consecutive heart beats in order to minimize the effects of noise and respiratory variation.

Histologic analyses

After satisfied anesthesia, the apex of the left ventricle (LV) was dissected from rat heart and cut into pieces 1 mm^3, then fixed in 2.5% glutaraldehyde. The chloral hydrate with lethal dose was given to rats after hearts were isolated. Ultrathin sections were placed on 400 mesh grids and double-stained with uranyl acetate and lead citrate, then observed with a transmission electron microscope (TEM, HITACHI-H7650, Japan). Then left LV fiber bundles were separately put into 4% paraformaldehyde and liquid nitrogen. The LV fibers fixed in paraformaldehyde were processed for histological analysis according to standard procedure. Then paraffin sections were cut and stained with hematoxylin-eosin and picrosirius red.

TUNEL assay

The tissue that was put into liquid nitrogen was embedded by opti-mum cutting temperature (OCT) compound (SAKURA, America). Frozen sections were cut on the cryoultramicrotome (MICROM-505E, Germany), and fixed in cool acetone for 15 min. DNA fragmentation of myocardial cells was detected in situ byterminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) with the One Step TUNEL Apoptosis Assay Kit (KeyGEN Bio TECH) as the manufacturer's instructions. Briefly, the sections were permeabilized in 0.1% Triton X-100, then incubated in freshly prepared TdT Enzyme reaction mixture for 60 min at 37°C in the dark. Subsequently, the sections were incubated in Streptavidin-Fluorescein working solution for 30 min at 37°C in the dark and washed with PBS and counterstained with 4',6'-diamidino-2-phenylindole (DAPI). Finally the sections were washed again with PBS and observed with a fluorescence microscope (Leica Company, Germany). Three high power fields were randomly sampled and positive cells were calculated for every section and five sections from each group.

Mitochondrion isolation

Cardiac mitochondria were isolated from rat hearts with differential centrifugation via the mitochondrial isolation kit according to the instructions provided by Genmed (Shanghai). In briefly, myocardial tissues were grinded in glass homogenizer on ice. Nuclei and unbroken cells were pulled down by centrifugation at 1500 g for 10 min at 4°C. Then, the mitochondrial fraction was obtained by centrifugation of supernatant at 10000 g for 10 min at 4°C, suspended in mitochondrial storage fluid.

Mitochondrial membrane potential (MMP) measurements

Mitochondrial membrane potential (MMP) was detected by the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,10,3,30-tetraethylbenzimidazolylcarbocyanine iodide probe (JC-1, Beyotime, Shanghai). The isolated mitochondria were mixed with dilute JC-1 working solution, and then scanned with a fluorescence microplate reader (Tecan Infinite M200, Switzerland) at 490 nm excitation and 590 nm emission to detect green and red JC-1 fluorescence. The ratio of red and green fluorescence represented the level of MMP.

Mitochondrial enzyme activities measurements

Cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activities were measured using commercialized COX assay kit (Genemed, Shanghai) and SDH assay kit (Nanjing Jiancheng, Nanjing) following the manufacturer's instructions. COX and SDH activities of cardiac mitochondria were detected with spectrophotometer. At 550 nm, the change of reduced cytochrome c was catalyzed into oxidized cytochrome c reflected the COX activity. Then the COX activity was quantitatively determined due to the changes of absorbance at 550 nm under the spectrophotometer. Since SDH catalyzed succinate oxidization, and the electron was delivered by FAD in this reaction which were accompanied by a reduction of 2,6-DCIP. Therefore, the SDH activity of the mitochondrion could be reflected by the rate of 2,6-DCIP reduction in this study.

Statistical analysis

Data of continuous variables were expressed as mean ± standard deviation (SD) and ana-

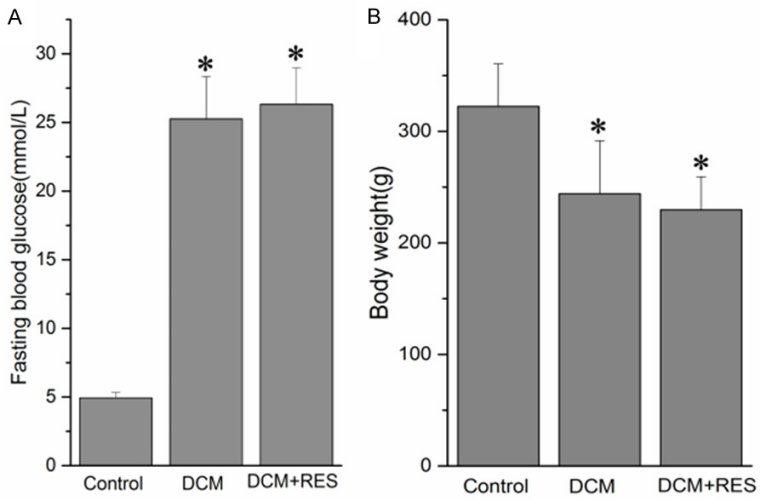

Figure 1. Effects of RES treatment on blood glucose and body weight in DCM. A: The plasma blood glucose level of rats in control, DCM and DCM+RES group. B: Body weight of rats in control, DCM and DCM+RES group. The values were presented as Mean ± SD (n=8 in control and DCM+RES groups and n=9 in DCM group), **P*<0.05 compared with control group.

lyzed with SPSS 17.0. The differences between groups were evaluated using one-way ANOVA and subsequent LSD test. A P<0.05 was set as statistically significant.

Results

General results

After administration streptozotocin for 11 days, we observed that there was significantly polydipsia, polyuria feature in DCM rats as compared with controls. From 8 weeks after induction of hyperglycemia, DCM+RES rats were treated with low doses of RES, and the symptoms of increased food and water intake and polyuria were observed to be relieved in DCM+RES group as compared with DCM group. At 16 weeks after inducing of hyperglycemia, most of DCM rats showed inanimate behavior, and decreased physical activity. The symptoms of polydipsia and polyuria in DCM rats were significantly alleviated.

Body weight and blood glucose levels

Bodyweight of rats in DCM and DCM+RES group were significantly decreased compared with those of controls, as shown in **Figure 1B**. The fast blood glucose of rats from DCM and DCM+RES group was significantly increased as compared with controls. Furthermore, the blood glucose of DCM+RES rats was mildly increased as compared with DCM group (*P*>0.05) as shown in **Figure 1A**. These results suggest that RES treatment did not significantly affect body weight or glucose blood level in DCM rats.

Effects of RES on cardiac structure and function in DCM rats

Echocardiography studies showed that the ventricular dimension was significantly increased and left ventricle ejection fraction significantly decreased in DCM rats as compared with controls. LVEDd, LVESd, LVEDV, LVESV, and Tei index were significantly increased in DCM rats as compared with controls. Moreover, LVEF and LVFS were also significantly decreased. These results indicated that chronic RES administration could improve cardiac function and remodeling following hyperglycemia in DCM as shown in **Figure 2**.

Effects of RES on histological features of myocardium in DCM

Myocardial hypertrophy, swelling of cardiomyocytes, and disorganization of myofibrils were observed in myocardium of DCM rats. Compared with control group, the myocardial pattern of DCM was disordered. Interestingly, RES administration seemed to reverse the unfavorable pathological changes to some extent, as shown in **Figure 3A**. Moreover, the amount of collagen accumulation in myocardium was significantly increased in rats from DCM group compared with those from the control group. However, for myocardium of rats from RES treated DCM groups, the extent of fibrosis seemed to be similar to controls. Further quantitative analysis showed that collagen volume fraction in the DCM group was significantly greater than that in the control and DCM+RES groups, as shown in **Figure 3B** and **3C**, indicating that chronic treatment with RES may attenuate myocardial fibrosis in DCM.

Effects of RES on cardiomyocytes apoptosis in DCM

TUNEL assay demonstrated notable myocardial apoptosis occurred in the DCM rats. And the change could be attenuated significantly by

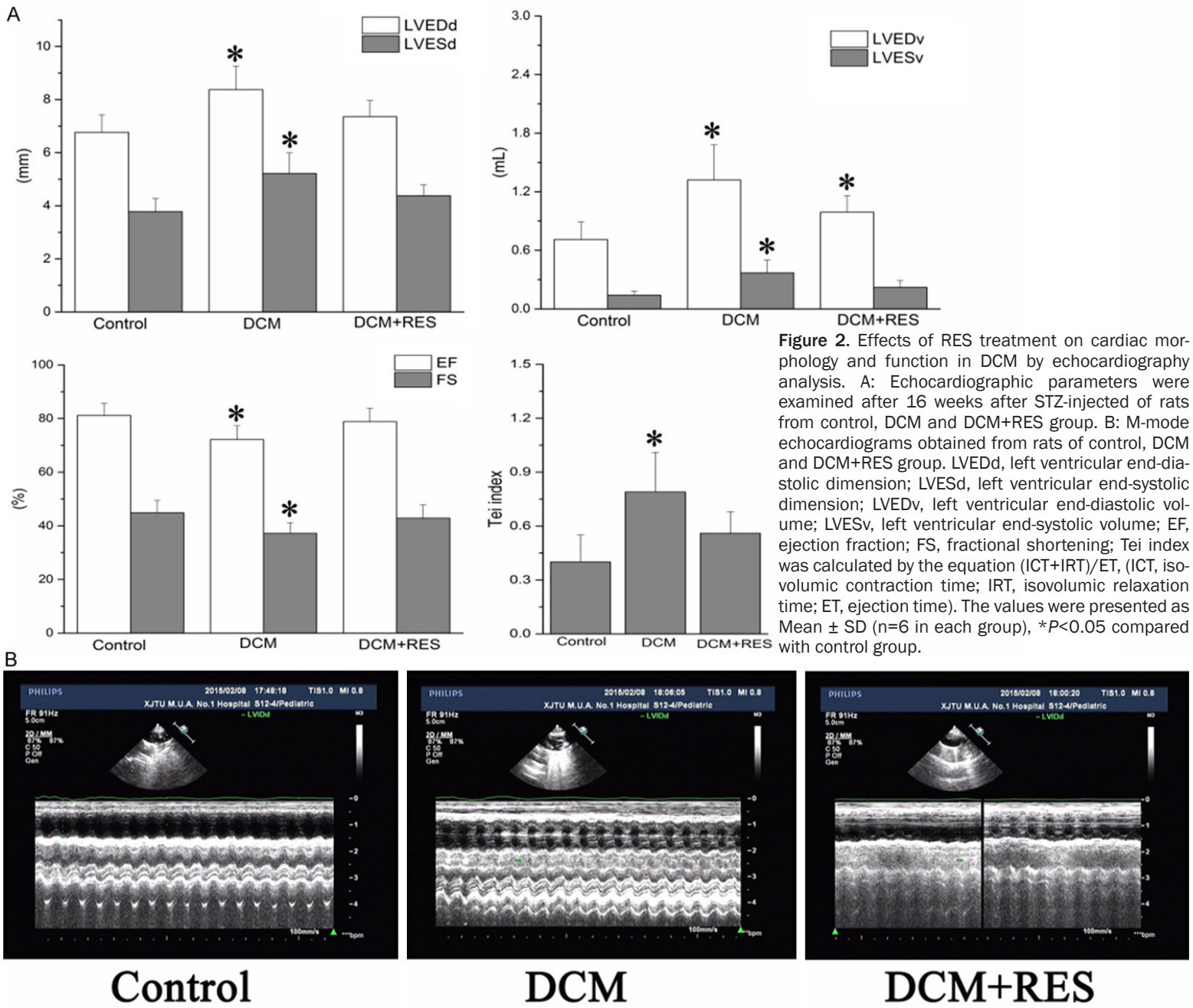

Figure 2. Effects of RES treatment on cardiac morphology and function in DCM by echocardiography analysis. A: Echocardiographic parameters were examined after 16 weeks after STZ-injected of rats from control, DCM and DCM+RES group. B: M-mode echocardiograms obtained from rats of control, DCM and DCM+RES group. LVEDd, left ventricular end-diastolic dimension; LVESd, left ventricular end-systolic dimension; LVEDv, left ventricular end-diastolic volume; LVESv, left ventricular end-systolic volume; EF, ejection fraction; FS, fractional shortening; Tei index was calculated by the equation (ICT+IRT)/ET, (ICT, isovolumic contraction time; IRT, isovolumic relaxation time; ET, ejection time). The values were presented as Mean ± SD (n=6 in each group), *P<0.05 compared with control group.

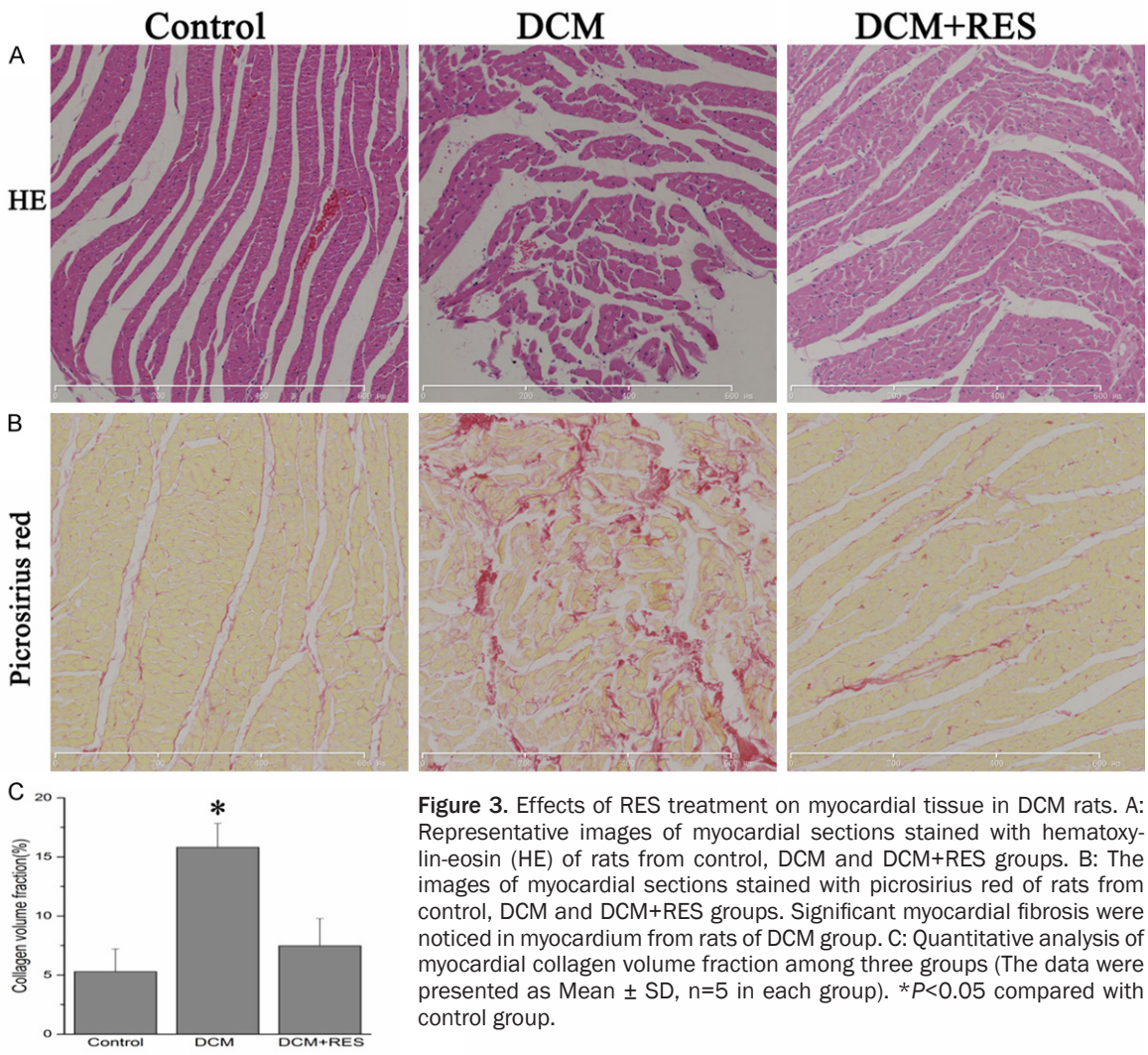

Figure 3. Effects of RES treatment on myocardial tissue in DCM rats. A: Representative images of myocardial sections stained with hematoxylin-eosin (HE) of rats from control, DCM and DCM+RES groups. B: The images of myocardial sections stained with picrosirius red of rats from control, DCM and DCM+RES groups. Significant myocardial fibrosis were noticed in myocardium from rats of DCM group. C: Quantitative analysis of myocardial collagen volume fraction among three groups (The data were presented as Mean ± SD, n=5 in each group). *P<0.05 compared with control group.

chronic RES treatment as seen in DCM+RES group, as shown in **Figure 4**, which indicated that chronic treatment with RES was associated with decreased myocardial apoptosis following DCM.

Effects of RES on the myocardial mitochondrial morphology and function in DCM

The cardiac ultrastructure in the control group showed regular myofibril arrangement and integrated mitochondria under the electron microscopy. However, abnormal changes in myocardial ultrastructure could be detected in DCM rats, including the rupture or dissolve of myofiber, proliferation and swelling of mitochondrion with loss of membrane integrity, destruction of intercalated disc, as well as deposition of lipid and edema of circum nuclear. Moreover, these pathological changes could be alleviated by chronic RES treatment (**Figure 5A** and **5B**). On the other hand, compared with the control group, the respiratory enzyme activities of SDH and COX were significantly decreased in DCM rats (P<0.05), while the enzyme activities of DCM+RES rats were increased as compared with DCM rats. However, there still was prominent different on SDH activity between the control group and DCM+RES group (P<0.05). In addition, MMP level of the isolated fresh cardiac mitochondria in the DCM rats significantly decreased compared with controls (P<0.05). Moreover, chronic RES administration could preserve the MMP level, as shown in **Figure 5C**. These results suggest that RES was associated with preserved mitochondrial function in myocardium of DCM rats.

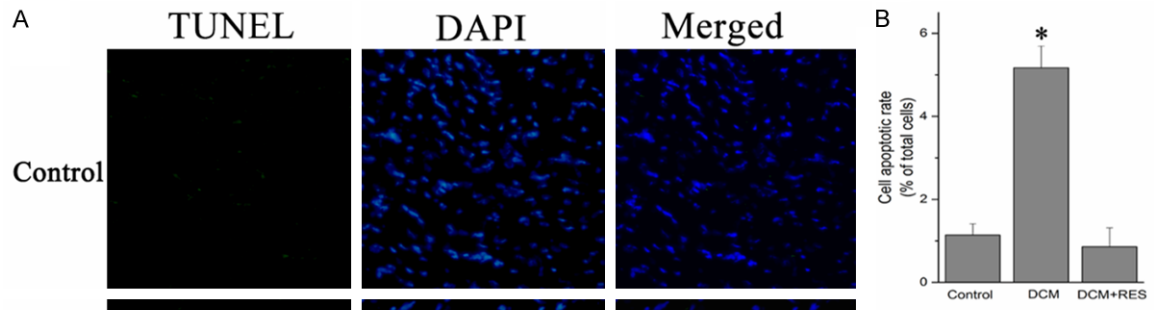

Figure 4. RES attenuates apoptosis in myocardium of DCM. A: Representative TUNEL images (100×) obtained from myocardium of rats from control, DCM and DCM+RES groups. B: Quantitative analysis of TUNEL- positive cardiomyocytes among the three groups (The values were presented as Mean ± SD, n=5 in each group). *P<0.05 compared with control group.

Discussion

Although DCM has been recognized an important cause of decompensated heart failure and mortality in diabetic populations, there remains no effectively therapeutic strategy to prevent the development of the disease and mortality. Thus, development of effective and safe treatment strategies for DCM is of important clinical significance. Accumulating evidence suggests that mitochondrial dysfunction accelerates structural and functional abnormalities of the myocardium in DM patients and thereby contributes to the pathogenesis and progression of DCM [23]. In our study, with a rat model of streptozocin-induced DCM, we found that chronic RES administration could improve cardiac function and reducing cardiac remodeling in DCM, and these benefits seemed to be related to their effects of preventing mitochondrial injury and myocardial apoptosis. These results suggest that RES may be a potential treatment for early prevention of DCM.

RES is a naturally existing phytoalexin which has extensive biological activities, especially cardiovascular protective effects, such as anti-atherosclerosis and that against myocardial ischemia/reperfusion injury [24]. Results of some studies have shown that RES may exert anti-diabetic effects by the attenuation of oxidative stress and inflammation, as well as the regulatory actions of metabolism [25-30]. In our study, we observed the effects of RES on alleviating myocardial mitochondrial injury and further improving cardiac dysfunction. From present data, we found that chronic RES treatment could significantly reverse the dilation of LV in DCM and improve the systolic function as indicated by echocardiography. These results were consistent with previous studies [17, 31]. Then we used Tei index to assess the global cardiac dysfunction which is considered to be independent of ventricular geometry, heart rate, atrioventricular valve regurgitation arterial pressure, and other external conditions [32]. We found that Tei index was also significantly reduced in rats from DCM+RES group as compared with those from DCM group, suggesting a benefit of RES treatment on global cardiac function in DCM. Previous studies have indicated that Tei index is one of indicators that reflect the disease severity in heart failure and patients with markedly increased Tei index were always associated poor prognosis [33,

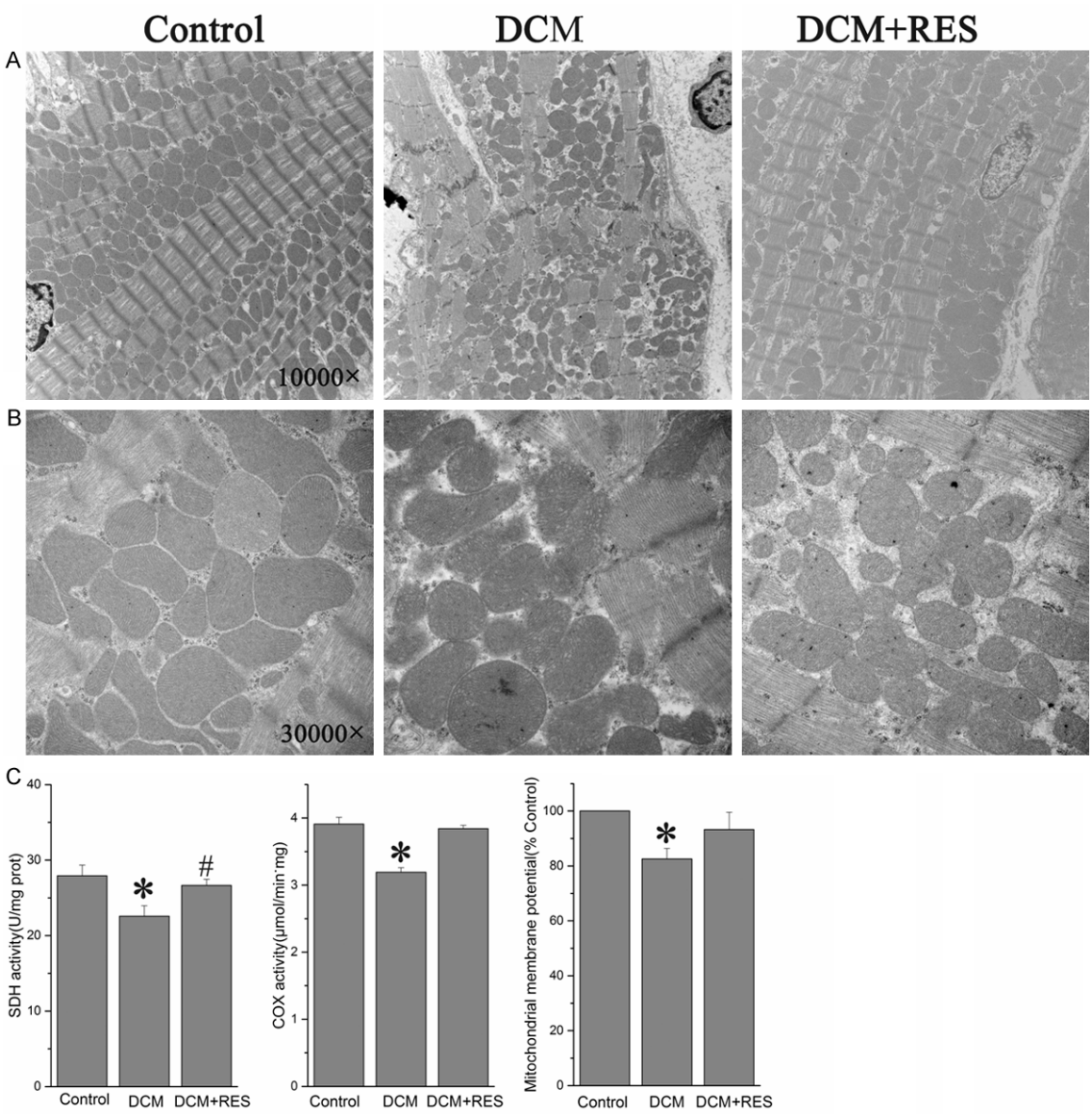

Figure 5. Effects of RES treatment on mitochondrial structure and function in DCM. A: Ultrastructure of the myocardium in rats from control, DCM and DCM+RES groups observed by TEM (10000×). B: Mitochondrial morphology and structure of the myocardium in control, DCM and DCM+RES rats observed by TEM (30000×). C: Columns indicate the values of SDH and COX activities and mitochondrial membrane potential levels of the isolated cardiac mitochondria in control, DCM and DCM+RES rats. SDH, succinate dehydrogenase; COX, cytochrome c oxidase. The values were presented as Mean ± SD, n=5 in each group. **P*<0.05 compared with control group; #*P*<0.05 compared with control group and DCM group.

34]. In this regarding, RES may improve the prognosis of patients with DCM by improve the Tei index, although futher studies are needed to confirm these results.

Apoptosis of cardiomyocytes and proliferation of cardiac fibroblasts resulting in fibrosis have been considered as critical pathological changes in DCM [35, 36]. These changes may lead to loss of cardiac contractile function and abnormalities of cardiac structure, such as ventricular hypertrophy, and finally cause severe clinical events such as heart failure and arrhythmia. Apoptosis of cardiomyocytes in DCM has been considered at least partly as the results of the initial mitochondrial injury. The reduction of MMP and opening of mitochondrial permeability transition pore (MPTP) has been related to

the increase of caspase-3 and caspase-9 activities in STZ-induced diabetic myocardium [37]. Besides, mitochondrial oxidative stress has also been suggested to play an important role in myocardial apoptosis in DCM, and strategies for suppression of mitochondrial oxidative stress had been proved to attenuate the myocardial apoptosis against DCM [38]. Furthermore, the destruction of mitochondrial membrane integrity also contributed to mitochondrial induced apoptosis in DCM [39]. On the other hand, mitochondrial dysfunction was associated with myocardial fibrosis, which may be caused by increased ROS via activating ROS/ERK/TGF-β pathway, increasing MMP activity and regulating NF-κB to facilitate fibroblasts proliferation and extracellular matrix increase [40-42]. Indeed, Lagouge demonstrated that RES may favorably affect mitochondrial function of skeletal muscle in metabolic disorder [43], which means RES could bring benefits to mitochondrial dysfunction. In the current study, RES treatment protected myocardial mitochondrial injury in DCM, as reflected by maintaining mitochondrial membrane integrity, rescuing decreased MMP and preserving the respiratory enzyme activities. The mechanism underlying the mitochondrial protective effect of RES might involve the followings according to previous studies (1) RES is a SIRT-1 activitor, potent antioxidant, which could reduce ROS production, and relieve oxidative stress related mitochondrial dysfunction and further prevent cardiac dysfunction by reducing myocardial apoptosis and fibrosis. (2) RES might increase mitochondrial biogenesis via upregulating SIRT-1/PGC-1 pathway. Interestingly, although this hypothesis has been proved in skeletal muscle metabolic disease and Duchenne muscular dystrophy [44], but little is known regarding the role of RES in mitochondrial biogenesis and SIRT-1/PGC-1 pathway in DCM myocardium and apperantly, more evidence are needed. (3) RES may exert the effect of activating AMP-activated protein kinase (AMPK), and subsequently result in the increase uptake and utilization of glucose, which improves substrate flexibility limitation and decreases the oxygen consumption to protect gradual development cardiac dysfunction. These also need to be confirmed in future studies.

Blood glucose levels are proved to relate to cardiovascular disease, so normalized blood glucose in diabetes is expected to decrease the incidence of cardiovascular events. However, studies regarding the effects of RES on blood glucose retrieved inconsistent results [45, 46]. In our study, we found that chronic RES treatment did not significantly affect blood glucose levels in diabetic rats. These results suggest that RES protects cardiac function in DCM via mechanisms other than glucose-lowering. Also, these results should be confirmed in the future.

In conclusion, results of our study suggest that chronic resveratrol treatment may improve cardiac function and cardiac remodeling in rat model of DCM possibly via attenuation of myocardial mitochondrial injury and apoptosis. These results suggest that RES may be a potential treatment for early prevention of DCM.

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Disclosure of conflict of interest

None.

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