

Trimetazidine ameliorates myocardial ischemia-reperfusion injury

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Abstract: Aim of this study was to investigate the effects of trimetazidine attenuating the myocardial ischemia-reperfusion injury to myocardium in rats and the underlying mechanisms. A model of myocardial ischemia reperfusion was established via ligating the left anterior descending coronary artery in 30 rats, and then they were randomly assigned to model group (n=10), low dose group (n=10) and high dose group (n=10). Moreover, additional 10 rats were collected and allocated to sham operation group, which was served as control group. Then, rats in the low dose group and high dose group were given trimetazidine with the dose of 10mg/kg and 30mg/kg respectively by intragastric administration, while rats in the control group and model group were given the equivalent volume saline. The dose was given once a day for consecutive 4 weeks in all rats. Echocardiography was applied to evaluate cardiac function, including left ventricular end-systolic dimension (LVESD), left ventricular end diastolic dimension (LVEDD) and left ventricular ejection fraction (LVEF). Next, myocardial tissue was collected, and Bax and Bcl-2 mRNA and the protein levels in the four groups were detected by RT-PCR and Western blot respectively. The level of malonaldehyde (MDA) and super oxide dismutase (SOD) activity in rat myocardium in each group were detected by colorimetric methods, while the variables of apoptosis were measured by TUNEL methods. In comparison with the control group, LVEDD, LVESD of rats increased significantly, LVEF decreased obviously, as well as Bax level, MDA level and the apoptotic variables in myocardial tissue increased ($P<0.05$), but Bcl-2 level and SOD activity decreased significantly in low dose, high dose and model group ($P<0.05$). Compared with model group, LVEDD, LVESD of rats decreased obviously, LVEF increased significantly, as well as Bax level, MDA level and the apoptotic variables in myocardial tissue decreased ($P<0.05$), but Bcl-2 level and SOD activity increased significantly in low dose group, high dose group ($P<0.05$). The regulatory role of trimetazidine on above indicators of rats was in a dose-dependent manner. Conclusion: Trimetazidine can ameliorate rat myocardium following ischemia-reperfusion injury by effectively attenuating the injury from myocardial cell apoptosis; meanwhile, it can resist cell apoptosis through regulating Bax and bcl-2 expression, which exhibits guiding significance for the treatment of myocardial ischemia and reperfusion.

Keywords: Trimetazidine, myocardial ischemia reperfusion, acute myocardial infarction, apoptosis.

INTRODUCTION

Ischemia-reperfusion injury can generate damage to organs and four types of tissues during and after ischemic organs and tissues regaining oxygen supply or perfusion. Myocardial ischemia and reperfusion can bring about myocardial structural damage, cardiomyocyte death, and even can lead to infarct expansion; meanwhile, its mechanisms is complicated. More importantly, apoptosis is one of crucial mechanisms of ischemia-reperfusion injury to myocardium, since it triggers the generation a lot of reactive oxygen segments in tissue, which can induce apoptosis through multiple pathways (Li *et al.*, 2015; Dwaich *et al.*, 2016; Suchal *et al.*, 2016). It has been considered that mitochondrial pathway is the most important signaling pathway of apoptosis, and Bax is a pro-apoptotic genes, which can activate Caspase enzyme cascade to apoptosis. Obviously, the occurrence of ischemia-reperfusion injury to myocardium significantly reduces the therapeutic efficacy. Therefore, effective prevention and treatment of ischemia-reperfusion injury has become a hot spot in related medical researches.

Numerous studies have found that medicine administration can inhibit myocardial apoptosis and significantly reduce infarct size to improve cardiac function and protect the heart following ischemia and reperfusion (Liang *et al.*, 2014; Zeng *et al.*, 2016). Therefore, suppression of apoptosis and medicine intervention of some links of apoptosis may provide new therapeutic tools for ameliorating the myocardial reperfusion injury.

Trimetazidine (TMZ) is an anti-ischemic medicine, and significantly reduces intracellular acidosis and cell apoptosis to protect mitochondrial function and myocardium. The TMZ has been widely used in the treatment of hypertrophic cardiomyopathy, hypertension, myocardial infarction and other ischemic heart diseases (Martins *et al.*, 2011, Zhang *et al.*, 2015). In this study, the establishment of a rat model of myocardial infarction and reperfusion followed by trimetazidine administration was carried out to observe the effects of trimetazidine on oxygen free radical damage and cell apoptosis after ischemia reperfusion, and to explore its underlying mechanisms.

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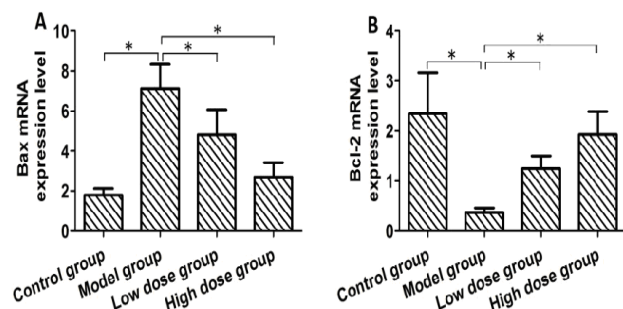


Fig. 1: Comparison of Bax and Bcl-2 mRNA levels of rats in each group. A. Bax mRNA level of rats in each group; B. Bcl-2 mRNA level of rats in each group

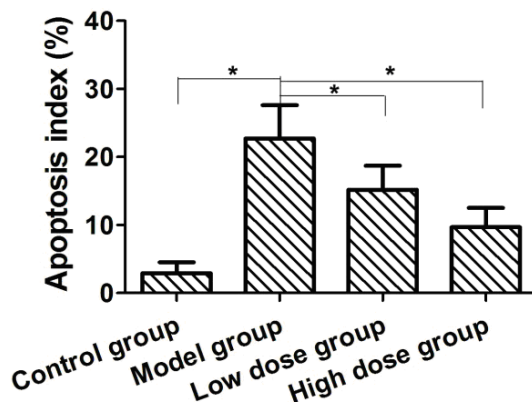


Fig. 4: Comparison of apoptosis variables in myocardium of rats in each group.

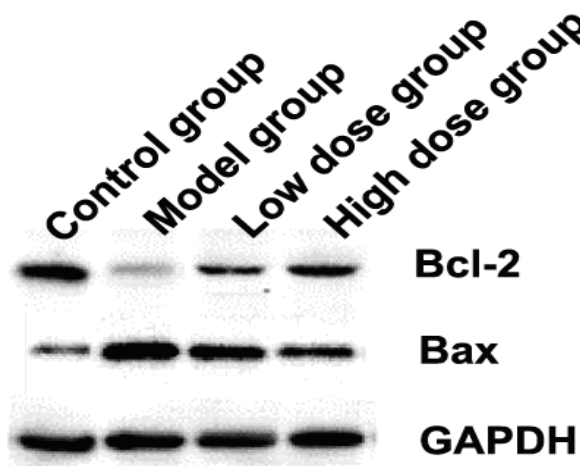


Fig. 2: Comparison of Bax and Bcl-2 protein levels of rats in each group.

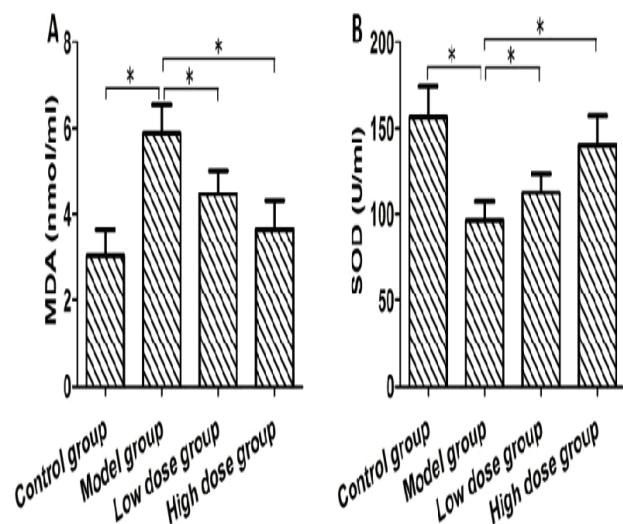


Fig. 3: Comparison of MDA content and SOD activity in myocardium of rats in each group. A. MDA content in myocardium of rats in each group. B. SOD activity in myocardium of rats in each group.

MATERIALS AND METHODS

Establishment of rat model of myocardial ischemia reperfusion and administration

40 male Wistar rats (SPF grade, Jiake Biotechnology, Shanghai, China), average weight of 250±15g, were selected for our study and a model of myocardial ischemia-reperfusion was induced as follows. Rats were weighed, anesthetized by administration of sodium pentobarbital (50mg/kg) intraperitoneally and fixed on the board at supine. After routine disinfection, they were tracheostomized and mechanically ventilated to control breathing. Left common carotid artery was subjected to blunt dissection for connecting electrophysiological signal recorder (Yi Lian Medicine, Shanghai, China) to monitor hemodynamics. After arterial puncture and stability for 30 min, thoracotomy was performed at the left of sternum between 3-4 rib, contributing to heart exposure. Then the pericardium was cut open, coronary left anterior descending artery was found between the pulmonary infundibulum and the left atrium, followed by threading at about 2mm under the left atrial appendage, and then a thin hose was placed between the coronary and ligatures. The coronary artery was occluded completely with ligatures for 30min ischemia and then reperused for 120min after loosening up ligatures. Whereas, controls in sham group were not subjected to ligation and other groups were ligated for 30min ischemia, and then reperused for 120 min. After the model of myocardial ischemia-reperfusion was constructed successfully, low dose group and high dose group received 10mg/kg and 30mg/kg trimetazidine (Servier, Tianjin, China; Code No. Approved by SFDA: 5450206H5; Specification: 20mg) by intragastric administration accordingly, while rats in control group and model group were given the same volume saline, and after administration of trimetazidine once a day for consecutive 4 weeks, the heart function indicators were detected in all rats. Finally, these rats were sacrificed, followed by collection of the myocardial tissue. We conducted this study in line with the references in the

Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). Eighth Edition, 2010. And this study was approved by the Institutional Animal Care and Use Committee (IACUC) of People's Hospital of Beijing Daxing District.

Evaluation of cardiac function

After consecutive intragastric administration for 4 weeks, all rats were intraperitoneally anesthetized with 1.5% sodium pentobarbital. Skin preparation was performed from upper left chest to infraclavicular section and in the lower edge of the rib. Ultrasonic detector for small animal rats (SonoStar, Guangzhou, China) was applied to detect LVESD, LVEDD and LVEF.

RT-PCR analysis

0.1g myocardial tissue was drawn from rats in each group, cut into piece with an ophthalmic scissor into a mortar, and ground quickly with liquid nitrogen. Then total RNA was extracted using TRIZOL reagent (Invitrogen, CA, USA) in strict accordance with the protocols. The precipitate was dissolved with RNase-free water, and automated enzyme immunoassay analyzer (Mettler-Toledo, Ohio, USA) was applied to measure OD values of total RNA OD_{260}/OD_{280} to calculate its purity. Reverse transcription of RNA into single-stranded cDNA using reverse transcription kit (TaKaRa, Dalian, China) according to the manufacturer's protocols, and that is the template for PCR. Finally, the primers were designed following the sequence of Bax and Bcl-2 genes. Bax-F: 5'-CTCAAGGCCCTGTGCACTAA-3', Bax-R: 5'-TAGGAAAGGAGGCCATCCCA-3'; Bcl-2-F: 5'-GAACTGGGGAGGATTGTGG-3', Bcl-2-R: 5'-GGGGTGACATCTCCCTGTTG-3'; β -Actin-F: 5'-GCGGGAAATCGTGCGTGAC-3', β -Actin-R: 5'-CGTCATACTCTGCTTGCTG-3'. The PCR amplification were performed for 35 cycles of pre-denaturation at 92°C for 3min, denaturation at 92°C for 30s, annealing at 55°C for 45s and extension at 72°C for 30s with cDNA and SYBR Green Universal qPCR Master Mix (Roche, Basel, Switzerland). Data from RT-PCR instrument (ABI7500; Applied Biosystems, Foster City, CA, USA) was recorded for quantitative analysis.

Western blot analysis

1g myocardial tissue was drawn from rats in each group, cut into piece in a small beaker with an ophthalmic scissor; after addition of 200 μ l tissue lysis buffer, the tissue was homogenized fully for 5min, following ice bath for 15min, and centrifuged at 15,000g for 15min. Then supernatant was gathered, and BCA assay kit (Boster, Wuhan, China) was applied to detect protein concentration. After sample buffer was added, the solution was boiled in boiling water for 30min. Total protein was performed for SDS-PAGE electrophoresis, and then grafted on PVDF membrane. Next, they were sealed with 5% skim milk. Bax and Bcl-2 monoclonal antibodies (Santa, USA) were coated

overnight respectively, then washed with PBST three times, each time for 5min; HRP-goat anti-mouse secondary antibody (Boster bio-engineering, Wuhan, China) was coated for 1h, and then washed three times in PBST, each time for 5min. Finally, luminescent liquid was used for development and photograph. In addition, with β -actin as internal control, the band of target protein was analyzed, and relative expression of the protein was calculated.

Determination of MDA content and SOD activity

Rat myocardial tissue was drawn from rats in each group, and the supernatant was collected described above. And OD value at 532nm and 550nm wavelength was measured respectively using MDA and SOD assay kit (Yilian Medicine, Shanghai, China) in line with the production instruments. All procedures were performed in triplicates. MDA content and SOD activity of rats were calculated according to the averages.

Tunel assay

Rat myocardial tissues in the four groups were embedded in paraffin and cut into sections, and rinsed with xylene for deparaffinage. After rehydrated in a graded series of alcohol, above samples were fixed in 4% paraformaldehyde for 15min, and washed twice in PBS, each time for 5min. Then all sections were soaked in proteinase K for 15min, and washed twice in PBS, each time for 5min. Above slices were repaired with citrate buffer, and then washed with PBS 2 times, each time 5 min again. Next, 50 μ l TUNEL solution was dropped on each slice, and incubated at 37°C in wet box for 1h. After washed twice in PBS, each time for 5min, with converting agent added dropwise, the sections were washed twice in PBS, each time for 5min, followed by incubation in DAB solution at room temperature for 10min in dark, then washed twice in PBS again, each time 5min. The slides were dehydrated in gradient ethanol, with xylene as clearing medium, and mounted by neutral resin. Five fields around infarction area under light microscope were randomly selected and observed, and increased uptake of nuclear chromatin, marginalization and formation of brown apoptotic bodies were considered as apoptotic cells, namely TUNEL positive staining. TUNEL-positive cells under each field were counted, and the cell numbers from five fields were collected for calculating the average.

STATISTICAL ANALYSIS

All data were analyzed using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). Measurement variables were expressed as $X \pm S$, while data among groups were compared using analysis of variance. And LSD methods were applied for pair wise comparison of multiple samples. $P < 0.05$ was statistically significant.

Table 1: Comparison of cardiac function level in rats in four groups

Groups	Cases (n)	LVEDs/cm	LVEDd/cm	LVEF/%
Control group	10	0.31±0.25	0.62±0.15	80.63±8.12
Model group	10	0.79±0.31 ^o	0.91±0.23 ^o	45.21±11.13 ^o
Model group	10	0.59±0.28 ^{o*}	0.80±0.26 ^{o*}	59.05±10.37 ^{o*}
Model group	10	0.48±0.21 ^{o*}	0.71±0.22 ^{o*}	72.35±12.09 ^{o*}

Note: When compared with control group, ^oP <0.05; when compared with model group, ^{*}P <0.05.

RESULTS

Comparison of cardiac function of rats in each group

As shown in table 1, in comparison with control group, rats in low dose group, high dose group and model group showed elevated LVEDD and LVESD (P<0.05), but descending LVEF (P<0.05); and in comparison with model group, rats in low dose group, and high dose group presented decreased LVEDD and LVESD (P<0.05), but increased LVEF (P<0.05). The regulatory effect of trimetazidine on cardiac function of rats was in a dose-dependent manner (P<0.05, table 1).

Comparison of Bax and Bcl-2 mRNA levels in rat myocardium in all groups

In comparison with control group, the results showed that the expression of Bax mRNA was up-regulated, but the expression of Bcl-2 mRNA was down-regulated in myocardial tissue in low dose group, high dose group and model group (P<0.05); while rats in low dose group, and high dose group presented decreased Bax mRNA level, but increased Bcl-2 mRNA level in myocardial tissue when compared with the model group (P<0.05). The regulatory effect of trimetazidine on Bax and Bcl-2 mRNA levels in myocardium of rats was in a dose-dependent manner (P<0.05, fig. 1).

Comparison of Bax and Bcl-2 protein in rat myocardium in all groups

In comparison with the control group, the results showed that Bax protein expression was up-regulated, but Bcl-2 protein expression was down-regulated in low dose group, high dose group and model group (P<0.05); while rats in low dose group, and high dose group presented down-regulated Bax protein expression, but up-regulated Bcl-2 protein level in myocardial tissue when compared with the model group (P<0.05). And the regulatory effect of trimetazidine on Bax and Bcl-2 protein expression in rat myocardium was in a dose-dependent manner (P<0.05, fig. 2).

Comparison of MDA content and SOD activity in rat myocardium in all groups

In comparison with control group, the results showed increased MDA content, but decreased SOD activity in myocardial tissue in low dose group, high dose group and model group (P <0.05); while rats in low dose group and

high dose group presented decreased level of MDA level, but ascending SOD activity in myocardial tissue (P<0.05). And the regulatory effect of trimetazidine on MDA level and SOD activity in myocardium of rats was in a dose-dependent manner (P<0.05, fig. 3).

Comparison of apoptotic variables in rat myocardium in all groups

As shown in fig. 4, the results showed that apoptotic variables were markedly ascending in low dose group, high dose group and model group in comparison with control group (P<0.05); whereas they were dramatically decreased in low dose group and high dose group when compared with model group (P<0.05, fig. 4). The regulatory effect of ligustrazine on apoptotic variables was in a dose-dependent manner.

DISCUSSION

Myocardial ischemia reperfusion has been considered as a common pathological process in clinical anesthesia procedures; in detail, after acute myocardial infarction, blood supply of the heart is restored as soon as possible, but the myocardial injury in ischemic area is surprisingly more serious when compared with ischemia period, and this phenomenon is regarded as myocardial ischemia-reperfusion injury (Chen *et al.*, 2016). During period of ischemia reperfusion, there are a large number of free radical generation in the ischemic area, and it has been recognized an important factor now in ischemia-reperfusion injury. In addition, intracellular redox homeostasis state is dependent on the oxygen free radical scavenging enzyme system, including superoxide dismutase (SOD), catalase, and glutathione, and its imbalance will result in the accumulation of reactive oxygen free radical, thereby triggering oxidative stress, which is one of the characteristics of ischemic tissue reperfusion (Guo and Sun, 2014; Goto *et al.*, 2016; Pasqualin *et al.*, 2016). In the present study, we carried out the establishment of myocardial ischemia-reperfusion model to detect MDA content and SOD activity in rat myocardium via colorimetric methods and the results showed that rats in model group presented significant increased level of MDA content and decreased SOD activity, indicating that the accumulation of oxygen free radicals existed in model rats.

In recent years, as the understanding of apoptosis in

ischemia-reperfusion injury to myocardium deepens gradually, the importance of apoptosis to myocardial ischemia-reperfusion injury has aroused more and more attention, which has become a new subject, exhibiting important theoretical and clinical values. Study has shown that myocardial apoptosis around peripheral infarct region increases the infarct size, promoting ventricular remodeling, which has an adversely effect on the heart, so reduction of myocardial apoptosis can significantly improve heart function (Xing *et al.*, 2015, Yu *et al.*, 2016; Chen *et al.*, 2016). Additional study has also shown that Bax and Bcl-2 are two major genes to regulate apoptosis, and the level of Bax and Bcl-2 proteins is directly related to apoptotic regulation, as well as the ratio of Bcl-2 to Bax is a key element for the regulation of apoptosis, and increased Bax level can up-regulate cell apoptosis, while increased Bcl-2 level can suppress cell apoptosis. Furthermore, Caspases is a key enzyme of apoptosis, and Caspases-3 is the most critical enzyme in Caspase S cascade downstream; importantly, Bax protein can activate Caspases-3, leading to apoptosis (Fan *et al.*, 2015; Wang *et al.*, 2016). In this study, RT-PCR and Western bolt were applied to analyze Bax and Bcl-2 mRNA and protein in myocardium of rats with myocardial ischemia-reperfusion, and the results showed a significant increase in Bax protein level, but a obvious decrease in Bcl-2 protein level; meanwhile, TUNEL assays showed that myocardial apoptosis in the model rats was significantly increased, indicating apoptotic pathway was activated in rats with myocardial ischemia-reperfusion to lead to myocardial apoptosis.

Other study has reported that apoptosis will further aggravate reperfusion injury, so there is a vicious cycle between them, and how to inhibit myocardial apoptosis during reperfusion attaches vital importance to myocardial ischemia-reperfusion injury. Trimetazidine is firstly used in clinical as an anti-angina medicine and recent practices and extensive studies have shown that this drug has a better therapeutic effect in the treatment of coronary artery disease and heart failure, of which the mechanism is that trimetazidine can increase glucose and lactic acid metabolism by inhibiting fatty acid oxidation, reducing the accumulation of oxygen free radicals, and accelerating the activation of pyruvate dehydrogenase (PDH) to ensure normal metabolism of adenosine triphosphate (ATP), achieving the aims of the optimization of myocardial energy metabolism, reduction of myocardial intracellular acidosis, inhibition of myocardial apoptosis, and maintenance myocardial intracellular environment stability (Liu *et al.*, 2016, Wen *et al.*, 2014, Şenturk *et al.*, 2014). In this study, intragastric administration of trimetazidine in the treatment of rats with myocardial ischemia-reperfusion, and the results of post-treatment test showed that LVEDD and LVEDS of rats decreased, but LVEF increased significantly; moreover Bax level, MDA level and

apoptosis index decreased in myocardial tissue, but level of Bcl-2 content and SOD activity increased significantly, which indicated that trimetazidine had the scavenging effects on oxygen free radicals and inhibition effects on apoptosis to protect cardiomyocytes and reduce the damage to myocardial tissue. This result is consistent with the findings of Ma *et al.* that trimetazidine protects against cardiac ischemia/reperfusion injury via effects on the Bcl-2/Bax pathway (Ma *et al.*, 2016). In addition, the regulatory effect of ligustrazine on above variables was in a dose-dependent manner.

CONCLUSION

Trimetazidine has obvious protective effects on myocardial tissue of rats with ischemia-reperfusion injury, and it can effectively ameliorate the level of injury following ischemia and reperfusion, as well as reduce myocardial cell apoptosis through regulating the expression of Bax and Bcl-2, which has a guiding significance in the treatment of ischemia-reperfusion injury to myocardium.

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