

Original Article

Effect of metoprolol on myocardial apoptosis after coronary microembolization in rats

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BACKGROUND: Coronary microembolization (CME) is a serious complication following percutaneous coronary intervention (PCI) in patients with acute coronary syndromes. The use of metoprolol before PCI can significantly protect ischemic myocardium from myocardial damage, but the function of metoprolol in the treatment of CME is not entirely clear. This study was to explore the effect and significance of metoprolol on myocardial apoptosis and caspase-3 activation after CME in rats.

METHODS: Thirty rats were randomly divided into three groups including sham-operation (control group), CME plus saline (CME group), CME plus metoprolol (metoprolol group), 10 rats for each group. The CME group was induced by injecting 3 000 polyethylene microspheres (42 μ m) into the left ventricle during a 10-second occlusion of the ascending aorta; the control group was injected with physiological saline instead of microembolization ball; the metoprolol or saline group was given three intravenous bolus injections before CME. Echocardiography, TUNEL staining, and Western blotting were used to evaluate cardiac function, proportion of apoptotic cells and activation of caspase-3 respectively at 6 hours after operation.

RESULTS: Echocardiographic parameters displayed that the metoprolol group improved cardiac function significantly compared with the CME group ($P < 0.05$). The myocardial apoptotic rate of the CME group as well as the contents of activated caspase-3 increased significantly ($P < 0.05$), both of which were ameliorated significantly by metoprolol treatment ($P < 0.05$).

CONCLUSIONS: This study demonstrates that metoprolol can protect the myocardium during CME in rats by inhibiting apoptosis and improving cardiac function. These results suggest that the inhibition of apoptosis can be a potential therapeutic strategy for the treatment of CME.

KEY WORDS: Coronary microembolization; Apoptosis; Caspase-3; Metoprolol

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INTRODUCTION

Coronary microembolization (CME) is a spontaneous event caused by atherosclerotic plaque rupture in patients with acute coronary syndromes and a potential iatrogenic complication in patients undergoing thrombolytic therapy and coronary interventions.^[1–3] The incidence is 15%–20%, even up to 30%–45% in great saphenous vein grafts PCI. CME causing transient "no blood flow" or "slow flow" is the independent predictor of long-term poor prognosis in patients with acute myocardial infarction.^[2] Furthermore, CME can also lead to progressive myocardial malfunction, including overt myocardial infarction,

contractile dysfunction, arrhythmias and coronary reserve reduction.^[4,5] Once CME occurs, clinical outcomes of such patients are not improved by using coronary thrombolysis agents, nitroglycerin and platelet GPIIb/IIIa receptor antagonists or direct thrombectomy.^[6,7] Therefore, therapy for CME remains an issue and furthermore, the underlying molecular mechanisms of pathophysiology in CME remain to be elucidated.

Clinically, intracoronary β -adrenergic blocker administration before coronary intervention reduces complications caused by CME.^[8] The use of metoprolol before PCI can significantly protect the ischemic

myocardium from myocardial damage.^[9] The intracoronary use of metoprolol can also reduce the occurrence of "no reflow" or "show reflow" phenomenon and improve the short-term and long-term prognosis of patients.^[10] Clinical findings show that metoprolol could play its roles of anti-inflammation and anti-apoptosis to improve cardiac function in condition of ischemic cardiomyopathy.^[8,11,12] The function of metoprolol in the treatment of CME is not entirely clear, but myocardial apoptosis certainly occurs after CME^[13] and metoprolol can inhibit myocardial apoptosis. Therefore, this study aims to observe the effect of metoprolol on myocardial apoptosis and caspase-3 activation after CME in rats, and to explore how metoprolol can prevent myocardial injury caused by CME, and to provide theoretical basis for the clinical prevention and treatment of CME.

METHODS

Animals and reagents

Male Sprague-Dawley rats (200–250 g) were purchased from the Animal Center of Guangxi Medical University. All procedures were performed in accordance with institutional guidelines for animal care and the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). Microembolization ball (lyophilized power) was purchased from Dynal Corporation, Norway; the diameter of balls was 42 μm , there was 2×10^7 balls in each gram of powder, and the final density was 3×10^4 balls/mL. Metoprolol was provided by AstraZeneca Laboratories. The monoclonal anti-cleaved caspase-3 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit was purchased from Roche (Indianapolis, IN, USA).

Modeling and grouping

The 30 rats were randomized into sham-operation (control group), CME plus saline (CME group), and CME plus metoprolol (metoprolol group), 10 rats for each group. The CME model was performed as described previously.^[14,15] Briefly, the rats were anesthetized with 10% chloral hydrate (3 mL/kg, i.p.) and the trachea was intubated to maintain ventilation with a respirator. The chest was opened and the ascending aorta was isolated. Then, 3 000 microspheres (42 μm , Dynal, Norway) suspended in 0.1 mL 0.9% saline were rapidly injected into the left ventricle during a 10-second occlusion of the ascending aorta. The occlusion device was removed following injection, and the chest was closed with

sutures after the heart rate and breathing rate returned to normal. The rats in the sham-operated control group were given normal saline without microspheres. The rats in the metoprolol group were intravenously given three bolus injections of metoprolol (2.5 mg/kg) at approximately 5-minute intervals at 30 minutes before CME induction; while the CME group was given the same volume of saline as the metoprolol group.

Echocardiography

The rats were lightly anesthetized with an intraperitoneal injection of sodium pentobarbital (30–40 mg/kg) and the left ventricle was examined using echocardiography. Briefly, a 12 MHz transducer was placed on the left anterior chest wall to obtain left ventricle ejection fraction (LVEF), left ventricle end-diastolic dimension (LVEDd), fractional shortening (FS), and cardiac output (CO). All echocardiography was performed by an experienced physician using a Philips Sonos 7500 system (Philips, Andover, USA). Cardiac function evaluation was performed three times, and the average was used for each index.

Tissue sampling

After echocardiography was performed, the heart was arrested in diastole by injecting 2 mL 10% KCl into the tail of the rats. Immediately, the rat heart was detached, the atrial appendage and atrium cordis were removed, and the heart ventricle was segmented into the apex and base parts from the long axis midpoint of the left ventricle along the direction paralleling to the atrioventricular groove. The apex part underwent fast freezing in liquid nitrogen, then was stored at -80°C , waiting for western blot assay. The base part underwent fixation using 4% paraformaldehyde for 12 hours, paraffin embedding, and serial sectioning (4- μm thick; at least 12 pieces of slices), three pieces of slices containing the same number of microinfarction foci were selected for conducting TUNEL assay to detect myocardial apoptosis, and HE staining and HBFP (hematoxylin basic fuchsin picric acid) staining were also performed on triplicate slices to detect myocardial microinfarction area.

Measuring myocardial microinfarction area

HBFP staining was an important method for the early diagnosis of myocardial ischemia, the ischemic cardiac muscle and red cells were dyed into red color, and the normal myocardial cytoplasm and nuclei were dyed into yellow and blue colors, respectively. Using DMR+Q550 pathological image pattern analysis instrument to analyze slices stained by HBFP, five microscopic visual fields ($\times 100$) were randomly sampled from each slice

for observation, using Leica Qwin analysis software (Germany) and the planar area method to measure the infarction zone, expressing as area percent of bulk analysis slice and averaging.^[13]

Detecting myocardial apoptosis by TUNEL assay

The procedure strictly accorded with the description of Kit specification. The optical microscopic apoptotic nuclei displayed yellow brown color (TUNEL positive), a total of 40 non-overlapping zones (400 ×) from each slice were randomly observed, respectively belonging to the microinfarction zone, the marginal zone and the zone far from infarction.^[16] The myocardial apoptotic number and overall myocardial cell number were counted using the following equation: the myocardial apoptotic rate = apoptotic number/overall cell number × 100%.

Western blot assay

To detect the contents of activated caspase-3 in myocardial tissues, antibodies recognizing shear-activated caspase-3 were used in western blot. Using lysis, we extracted buffer myocardial total proteins and the Lowry method was used to measure protein concentration. 10% SDS-PAGE was performed with 50 μg of loading samples. Membrane (PVDF membrane) transfer was conducted at 100 mA current for 2 hours. Membrane was incubated in blocking solution at room temperature (RT) for 1 hour, rinsed by TBST, followed by incubation with primary antibody (cleaved caspase-3, 1:1 000) overnight at 4 °C and then with horseradish peroxidase(HRP) conjugated secondary antibodies (1:2 000) at room temperature for 1 hour. HRP-labeled anti-GAPDH (1:1 000) served as inner control. Following rinsing, the membrane was incubated with the corresponding secondary antibodies for 1 hour. The Bio-Rad Gel Doc 2000 imaging system and software were used to calculate the integrated absorbance (IA) of identified bands: IA = area × average density. After normalizing to GAPDH levels, the ratios of caspase-3 IA to GAPDH IA were used to represent the relative levels of activated caspase-3.

Statistical analysis

Data were expressed as mean ± standard deviation. Comparisons were made using an unpaired Student's *t* test (two groups) or the analysis of variance (ANOVA, three or more groups) followed by Tukey's post-hoc

test. For linear correlation analysis, Pearson's product-moment correlation coefficient analysis was made using SPSS software (SPSS, Chicago, Illinois, USA). Statistical significance was set at *P*<0.05.

RESULTS

General outcomes

No statistically significant differences (*P*>0.05) were found in body weight or heart rate between all groups (data not shown).

Effect of metoprolol treatment on cardiac function in CME rats

Initially, we measured cardiac function by using echocardiography to determine the effect of metoprolol treatment 6 hours after CME-induced cardiac dysfunction (Table 1). Compared with the control group, the rats in the CME group showed a significant decrease in LVEF, FS, and CO, but an increase in LVEDd (*P*< 0.05), suggesting that cardiac function in rats with CME was impaired. Cardiac function in the metoprolol group was impaired compared with the control group, but it was less impaired compared with the CME group, i.e. LVEF, FS, and CO significantly increased, and LVEDd significantly decreased (*P*<0.05), showing that metoprolol treatment can somehow improve the cardiac function in CME rats.

Effect of metoprolol treatment on myocardial infarction size in CME rats

Microspheres were injected directly into the coronary arteries, and effective CME was evident by histology (Figure 1). However, there was no significant difference (*P*>0.05) in myocardial infarct size in the metoprolol group compared with the CME group (Figure 2). Infarct areas in the CME and metoprolol groups were 8.32±3.27 and 7.98±2.72, respectively, indicating that metoprolol treatment has no significant effect on myocardial infarction size caused by CME in rats.

Effect of metoprolol treatment on myocardial apoptosis in CME rats

As there was no significant difference in myocardial infarction size in the metoprolol group compared with CME group, apoptosis in the rat heart tissues

Table 1. Cardiac function after coronary microembolization

Groups	Number	LVEF (%)	FS (%)	CO (L/min)	LVEDd (mm)
Control	10	82.67±3.50	42.97±3.44	0.164±0.009	5.11±0.33
CME	10	72.88±3.27*	37.59±2.49*	0.102±0.007*	6.20±0.16*
Metoprolol	10	79.97±4.26 [#]	41.64±2.08 [#]	0.157±0.014 [#]	5.34±0.29 [#]

Compared with the control group, **P*<0.05; compared with the CME group, [#]*P*<0.05.

was assessed using TUNEL staining. Compared with the control group, there were many TUNEL positive (brown) cells in rat tissues of the CME group ($P<0.05$). Metoprolol treatment significantly decreased ($P<0.05$) the relative apoptotic cell proportion following CME. The myocardial apoptotic percentages in the control, CME, and metoprolol groups were 0.20 ± 0.15 , 3.17 ± 1.26 , and 1.32 ± 0.28 , respectively (Figures 3 and 4), indicating

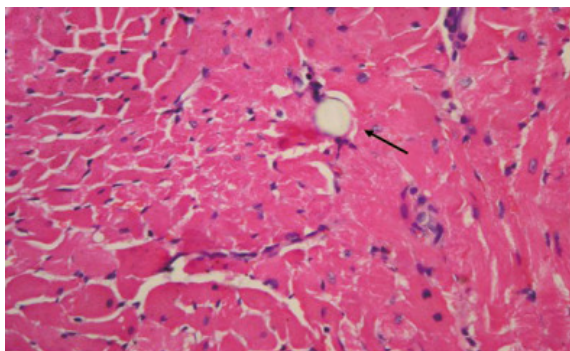


Figure 1. Histopathology of post-CME myocardial microinfarcts. HE staining of tissue samples from the coronary microembolization group revealed myocardial microinfarcts with a low level of inflammatory cell infiltration. However, a portion of cardiomyocyte nuclei is not visible. The arrow indicates the presence of a 42 μm microsphere following the experimental protocol (HE staining, original magnification $\times 400$).

that metoprolol treatment can significantly reduce myocardial apoptosis caused by CME in rats.

Effect of metoprolol on myocardial expression of caspase-3

Since metoprolol treatment decreased apoptosis in CME rats. Western blot analysis was made of mitochondrial apoptotic pathway proteins, including activated caspase-3 (Figure 5). Compared with the control group, the CME group showed the significantly enhanced expression of activated caspase-3 in myocardial cells of rats ($P<0.05$). Compared with the CME group, the metoprolol group significantly decreased the contents of activated caspase-3 ($P<0.05$). Obviously, metoprolol treatment can reduce apoptosis in CME rats by inhibiting the expression of activated caspase-3.

Linear correlation analysis

Linear correlation analysis indicated that the activated caspase-3 is positively correlated with relative apoptotic cell proportion ($r=0.856$, $P=0.029$), demonstrating that caspase-3 is activated during apoptosis and positively correlated with apoptosis in CME rats.

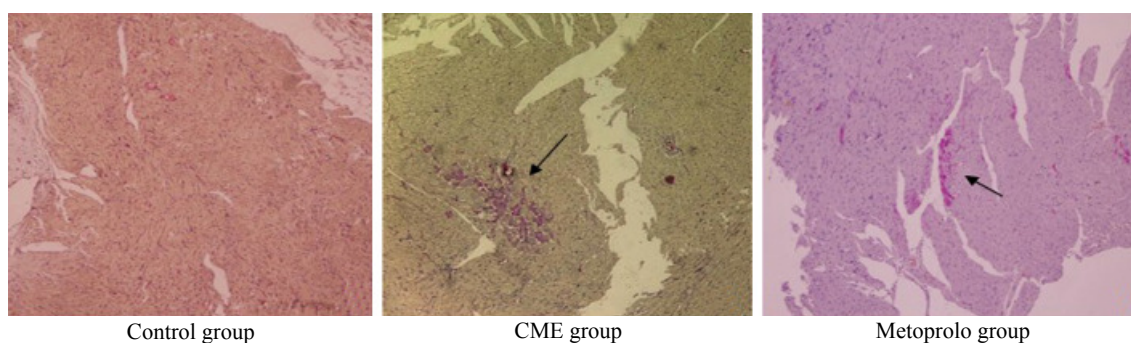


Figure 2. Histopathology of myocardial microinfarcts. Hematoxylin basic fuchsin picric acid (HBFP) staining of tissue samples revealed no infarct in the control group. Microinfarcts in the tissue samples from the CME and metoprolol groups are indicated by arrows. Normal myocardial cytoplasm is stained yellow, the nuclei are stained blue, and the ischemic myocardium is stained red (HBFP staining, original magnification $\times 100$).

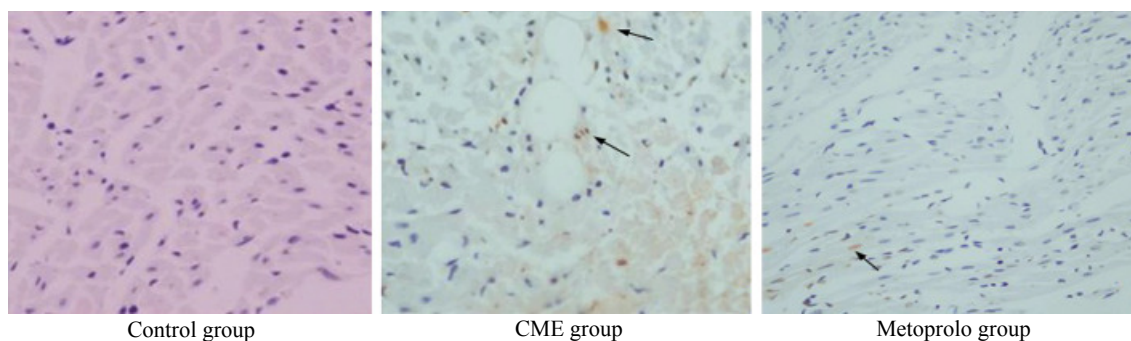


Figure 3. Photomicrographs showing effect of metoprolol on apoptosis following CME. TUNEL assay revealed the myocardial apoptosis in the control, CME, and metoprolol groups. Normal cell nuclei are stained pale blue while the apoptotic cardiomyocyte nuclei (arrows) are stained brown (original magnification $\times 400$).

DISCUSSION

Apoptosis plays an important role in myocardial dysfunction. It has been reported that the microembolized myocardium is characterized by perfusion-contraction mismatch, reduced contractile function and unchanged or possibly elevated blood flow.^[17,18] Since cardiomyocytes are non-renewable cells, the loss of cardiomyocytes via apoptosis may play an important role in the progression of myocardial contractile dysfunction. The mechanisms of myocyte cell death include apoptosis and necrosis, with early research confirming that myocyte cell death by apoptosis is likely in the beginning hours of ischemia, and that myocyte cell death by necrosis is evident after long-term ischemia.^[15] Although cardiomyocyte apoptosis may directly lead to myocardial dysfunction,

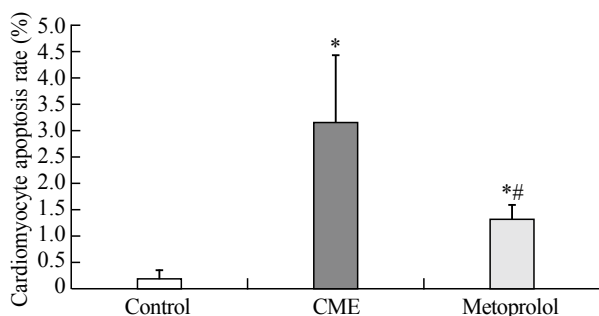


Figure 4. Graph showing effect of metoprolol on apoptosis following CME. Myocardial apoptosis after CME (TUNEL assay) was detected primarily in the myocardial microinfarction foci and the peripheral zones. In control animals, myocardial apoptosis was occasionally found in the subendocardium and papillary muscles. The data ($n=10$) are expressed as means \pm SD. Compared with the control group, * $P<0.05$; compared with the CME group, # $P<0.05$.

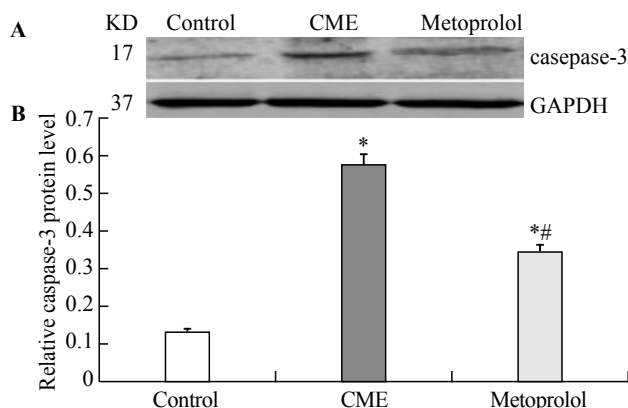


Figure 5. Effect of metoprolol on caspase-3 expression following CME. A: The relative expression levels of activated caspase-3 protein were determined by using western blot; the IA values were normalized to GAPDH expression levels. Lanes 1, 2, and 3 in the representative gel show caspase-3 expression in the control, CME (after 6 hours), and metoprolol groups, respectively. B: The data obtained ($n=10$) were expressed and compared between the groups as means \pm SD values, herein presented graphically. Compared with the control group, * $P<0.05$; compared with the CME group, # $P<0.05$.

it remains unknown whether inhibition of apoptosis is beneficial in CME treatment. Intravenous β -blocker treatment before coronary artery occlusion is capable of reducing myocardial injury.^[19] Clinically, administration of intravenous metoprolol could significantly produce heart protective effect in ischemia-reperfusion.^[9]

In our preliminary experiment, caspase-3 was activated after CME. The level of caspase-3 peaked at six hours after CME, thereby, this study adopted 6 hours after CME as the time point of observation. Notably, at 6 hours after CME, multiple microinfarction foci appeared in the myocardium, myocardial cells became apoptotic and necrotic, and the cardiac function was decreased.

Caspases, a family of aspartic acid-specific proteases, are the major effectors of apoptosis. Caspases exist as inactive zymogens in cells and undergo a cascade of catalytic activation at the onset of apoptosis.^[20] Caspase-3 mediates the pathway to apoptosis protease cascade, as the key protease to mammalian cell apoptosis, the main apoptosis effect factor and the most important apoptosis performer.^[21,22] Intravenous injection of metoprolol before surgery could significantly improve the myocardial apoptosis and systolic dysfunction caused by CME. Molecular biology showed the significantly inhibited caspase-3 protein activation in myocardial tissue, and reduced myocardial apoptosis rate. Cardiac echocardiography revealed systolic dysfunction and the left ventricular dilation manifested by a decrease in LVEF, FS and CO as well as an increase in LVEDd. Since myocardial cells are non-differentiating, inhibition of myocardial apoptosis may be one of the most important mechanisms by which metoprolol therapy improves the prognosis of CME.

In the present study, metoprolol pretreatment significantly improved the cardiac function in CME rats; however no difference in myocardial infarction size was found as compared with the CME group. We determined the effect of metoprolol treatment on myocardial apoptosis in CME rats, and found that metoprolol can significantly reduce myocardial apoptosis rate caused by CME and inhibit the expression of activated caspase-3, which were activated during CME in rats, indicating that metoprolol-related improvement of cardiac function after CME is associated with the blockade of myocardial apoptosis and with the inhibition of the expression of activated caspase-3. Our study is the first to illustrate the effect of metoprolol on myocardial apoptosis and caspase-3 activated after coronary microembolization.

LIMITATIONS

First, there is a need for a suitable animal model

that mimics the real clinical scenario of CME as closely as possible. The polymer microspheres do not have the biochemical characteristics of natural microemboli including platelets, endothelial cells, and plaque debris with cholesterol crystals. Second, an open-chest operation will also generate an impact on apoptosis.

In summary, in our study myocardial apoptosis was increased in CME rat lesion tissues, as evidenced by TUNEL staining and activation of caspase-3. Treatment with metoprolol significantly attenuated CME induced myocardial injury and improved cardiac function. Thus, metoprolol could protect the myocardium through the inhibition of the apoptosis and provide theoretical basis for the clinical prevention and therapy of CME.

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Ethical approval: The present study was approved the Animal Care and Use Committee of First Affiliated Hospital of Guangxi Medical University, Nanning, China.

Conflicts of interest: The authors have no competing interests relevant to the present study.

Contributors: Su Q proposed and wrote the paper. All authors contributed to editing the final manuscript for content and style.

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