Noricandil pretreatment inhibits myocardial apoptosis and improves cardiac function after coronary microembolization in rats

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Abstract

Background Nicorandil (NIC) is a vasodilatory drug used to treat angina. However, its efficacy of cardioprotection in coronary microembolization (CME) is largely unknown. This study was undertaken to determine whether nicorandil pretreatment could attenuate myocardial apoptosis and improve cardiac function after CME in rats.

Methods Forty-five rats were randomly divided into a Sham group, a CME group and a CME + NIC (NIC) group (n = 15 per group). CME was established by injecting plastic microspheres (42 μm in diameter) into the left ventricle of the rats in all of the groups except the Sham group. The NIC group received nicorandil at 3 mg/kg per day for seven days before the operation. Cardiac function was assessed by echocardiography, the expression levels of cleaved caspase-9/8/3 were detected by Western blot, microinfarction area was measured by haematoxylin-basic fuchsin picric acid staining, and myocardial apoptosis was detected by TUNEL staining.

Results Compared to that in the Sham group, cardiac function in the CME group was significantly decreased (P < 0.05). However, compared to the CME group, the NIC group showed improved cardiac function (P < 0.05). The expression levels of cleaved caspase-9/8/3 protein and myocardial apoptosis were dramatically increased in the CME group compared to those in the Sham group (P < 0.05), while the NIC pretreatment group had significantly decreased expression levels of cleaved caspase-9/8/3 protein as well as a decreased apoptotic rate (P < 0.05).

Conclusions NIC pretreatment inhibited CME-induced myocardial apoptosis and improved cardiac function through blockade of the mitochondrial and death receptor-mediated apoptotic pathways.

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1 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 or therapeutic percutaneous coronary interventions.[1,2] CME can cause periods of transient interruption or reduction in cardiac circulation, which is an independent predictor of poor long-term prognosis in patients who have suffered from acute myocardial infarction.[3] Moreover, clinical studies have shown that CME can induce contractile dysfunction, malignant arrhythmias, and reduction in coronary flow reserve which even lead to death.[4,5] However, the mechanism of CME-induced myocardial injury is complicated. Previous studies have revealed that myocardial apoptosis plays a crucial role in the mechanism of CME-induced myocardial damage.[6-9] Therefore, it is reasonable to propose that interventions that attenuate myocardial apoptosis could protect the heart from CME-related cardiac dysfunction.

Nicorandil (NIC) is an adenosine triphosphate (ATP)-sensitive potassium channel opener and nicotinamide nitrate that has been shown to have multiple protective effects on the reduction of myocardial damage after acute myocardial infarction in animal and clinical studies.[10-12] In ischaemia reperfusion models, nicorandil prevents apoptosis due to oxidative stress and reduces infarct size.[13,14] Ishii, et al.[12] found that NIC has a preconditioning-like cardioprotective effect against ischaemic injury in myocardial infarction patients. Chen, et al.[15] also demonstrated that NIC can improve chest pain symptoms and myocardial function during periods of reduced coronary flow in clinical studies. Studies in cultured cardiac myocytes suggested that NIC exerts anti-apoptotic effects mediated by oxidative stress and selective activation of mitochondrial K⁺-ATP channels.[14] However, the potential role of NIC in the treatment of CME remains unclear.
Therefore, in this study, we sought to determine whether NIC pretreatment could attenuate myocardial apoptosis and improve cardiac function after coronary microembolization in rats. The results showed that NIC pretreatment could significantly inhibit CME-induced myocardial apoptosis and improve cardiac function and that the cardioprotective effect was mediated by the blockade of the mitochondrial and death receptor-mediated apoptotic pathways. Together, the data imply that therapeutic strategies are needed to treat CME-induced myocardial injuries.

2 Methods

2.1 Animals

Forty-five healthy Sprague Dawley (SD) rats (14–16 weeks old, 250–300 g) were purchased from the Experimental Animal Center of Guangxi Medical University. The animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals and approved by the Ethics Committee for Animal Use of Guangxi Medical University, Nanning, China.

2.2 Modelling and grouping

Forty-five SD rats were randomly assigned to one of three groups: a sham-operated (Sham) group, a CME group and a CME + NIC group, with 15 rats in each group. The CME model was successfully established as described previously.[6–8] Briefly, general anaesthesia was induced with sodium pentobarbital at a dose of 30–40 mg/kg after endotracheal intubation and connection to a respirator. A left lateral thoracotomy was performed through the third and fifth intercostal space. The pericardium was opened, and the ascending aorta was occluded for 10 s with a vascular clamp while the left ventricle was injected with 0.1 mL of a saline suspension containing 3000 polyethylene microspheres (42 μm in diameter, Biosphere Medical Inc. Rockland, USA). After injection of the microspheres, the vascular clamp was removed, and the chest was sutured after the heart rate and breathing returned to normal. Using the same procedure, the sham-operated control group was administered saline only. The NIC group received NIC powder (Nipro Pharma Corporation, Japan) dissolved in saline (3 mg/kg per day) orally for seven days before the operation.[16] The rats were sacrificed 6 h after the operation (n = 15 in each group).

2.3 Echocardiography

Our previous research showed that cardiac function was lowest at 6 h after CME; therefore, we evaluated cardiac parameters at that time point.[6]

All transthoracic echocardiographic studies were blinded and performed by the same experienced investigator. The rats were lightly anaesthetized with an intraperitoneal injection of sodium pentobarbital (30–40 mg/kg) and were laid in a supine position on the experimental platform. The left anterior chest wall was imaged by ultrasound using a Hewlett Packard Sonos 7500 (Philips Technologies, USA) equipped with a 12.0 MHz transducer to obtain the left ventricle ejection fraction (LVEF), the left ventricle end-diastolic dimension (LVEDd), the fractional shortening (FS) and the cardiac output (CO). Measurements represent the mean of three consecutive cardiac cycles.

2.4 Tissue sampling and sample treatment

After echocardiography was performed, the hearts were arrested in diastole by injecting 2 mL of 10% KCl into the tail vein of the rats, and the hearts immediately isolated. The atria were removed, and the ventricles were rinsed of blood with cold saline. Then, the left ventricle was cut from the mid-point of the left ventricle long axis in a plane parallel to the atrioventricular groove to separate the apex and base of the heart. The apex was rapidly frozen and stored at −80°C until analysis by Western blot. The base was fixed in 4% paraformaldehyde for 12 h, embedded in paraffin and sectioned into 4-μm sections for terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining, HE staining and haematoxylin-basic fuchsin picric acid (HBFP) staining.

2.5 Myocardial microinfarction area

The early myocardial ischaemic or infarct regions can be detected by HBFP (haematoxylin-basic fuchsin-picric acid) staining. Red staining indicates ischaemic myocardium, and yellow or brown staining indicates normal myocardium. Pattern analysis using a DMR-Q550 imager (Leica, Wetzlar, Germany) was used to analyse HBFP-stained slices. Briefly, five visual fields (× 100) from each section were randomly sampled using the Qwin analysis software (Leica), and the planar area method was used to measure the infarction zone, expressed as an average area percentage of bulk analysis slices.[17] The percent relative ischaemic area was calculated according to the following formula: ischaemic area/total area ×100.

2.6 Apoptosis assay

Myocardial apoptosis was quantified using the TUNEL apoptosis detection kit (Roche, USA) according to the manufacturer’s instructions. The apoptotic nuclei were
stained yellow-brown (TUNEL-positive), while the normal nuclei were stained light blue. In each specimen, a total of 40 non-overlapping zones (> 400) from the infarct zone, the marginal zone and the remote zone were quantified. The number of apoptotic cardiomyocytes and the overall cell number were determined, and the myocardial apoptotic rate was calculated as follows: number of apoptotic cells/total number of cells × 100.

2.7 Western Blot analysis

Total myocardial protein from the apex of the left ventricle was extracted using protein lysis buffer, and the Lowry method was used to determine the protein concentration. Equal amounts of protein (50 μg) were fractionated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, Mass, USA). The membranes were blocked for 1 h at room temperature using 5% non-fat milk in Tris-buffered saline supplemented with Tween-20 (TBST) and incubated overnight at 4°C with the following primary antibodies: anti-cleaved caspase-9, anti-cleaved caspase-3, anti-cleaved caspase-8 and anti-glycerdehyde-3-phosphate dehydrogenase (anti-GAPDH; all obtained from Abcam, Cambridge, Mass, USA). The membranes were washed with TBST and incubated with goat anti-rabbit IgG HL secondary antibodies (1: 10000 Abcam, Cambridge, Mass, USA). The membranes were incubated with goat anti-rabbit IgG HL secondary antibodies (1: 10000 Abcam, Cambridge, Mass, USA) at room temperature for 2 h. The immuno-reactive bands were detected by exposing the blots in an Odyssey infrared imaging system (LI-COR). GAPDH was used as the internal control.

2.8 Statistical analysis

Data were expressed as the mean ± SD and were analyzed using SPSS software (IBM Corporation, USA). Comparisons were made using Student’s t test (two groups) or an analysis of variance (ANOVA) followed by Tukey’s post hoc tests. Differences were considered statistically significant at P < 0.05.

3 Results

3.1 NIC pretreatments improved cardiac function after CME

The echocardiographic examination results are summarized in Table 1. Briefly, the CME group exhibited significantly decreased cardiac systolic function when compared with the Sham group, as indicated by the significantly reduced LVEF, FS and CO, as well as increased LVEDd in the CME group (P < 0.05). Pre-treatment with NIC significantly improved all indices of cardiac function after CME, as reflected by the increased LVEF, FS and CO, as well as decreased LVEDd in the NIC group compared to that in the CME group (P < 0.05). These results suggest that pretreatment with NIC improves cardiac function in this rat model of CME.

Table 1. Effect of NIC on cardiac function after CME.

| Group | n  | LVEF, % | FS, % | CO, L/min | LVEDd, mm |
|-------|----|---------|-------|-----------|-----------|
| Sham  | 15 | 87.59 ± 2.32 | 54.75 ± 3.24 | 0.18 ± 0.006 | 5.25 ± 0.1 |
| CME   | 15 | 53.76 ± 1.55 | 22.80 ± 1.05 | 0.13 ± 0.007 | 6.45 ± 0.2 |
| NIC   | 15 | 71.79 ± 1.56 | 37.71 ± 0.99 | 0.15 ± 0.004 | 5.83 ± 0.07 |

Data are expressed as the mean ± SD. *P < 0.05, compared to the Sham group; **P < 0.05, compared to the CME group. CO: cardiac output; CME: coronary microembolization; FS: left ventricular fractional shortening; LVEDd: left ventricular end diastolic diameter; LVEF: left ventricular ejection fraction; NIC: nicorandil.

3.2 Effects of NIC on myocardial infarction size after CME

CME was induced by injecting microspheres directly into the coronary arteries and was confirmed by histology (Figure 1). Myocardial infarction was assessed by HBF staining (Figure 2). Myocardial microinfarcts were observed in the CME and NIC groups, but no obvious microinfarcts were observed in the Sham group. However, there was no significant difference (P > 0.05) in myocardial infarct size in the NIC group compared with that in the CME group. Infarct areas in the Sham, CME and NIC groups were 0, 7.32 ± 3.27% and 6.98 ± 2.72%, respectively, indicating that NIC pretreatment has no significant effect on myocardial infarct size caused by CME in rats.

3.3 Effect of NIC on myocardial apoptosis after CME

Myocardial apoptosis was assessed by TUNEL staining (Figure 3). In the Sham group, we occasionally observed apoptosis in the subendocardium and papillary muscles. However, an increased number of TUNEL-positive (yellow-brown) cells was found in CME-treated hearts compared to that in the Sham group (P < 0.05), and the apoptotic cells were mostly located near the myocardial microinfarction foci and in the peripheral zones. Interestingly, the number of TUNEL-positive cells in the infarct and peri-infarct regions was significantly lower in the NIC group that in the CME group (P < 0.05). The myocardial apoptotic rates in the Sham, CME and NIC groups were 1.45% ± 0.39%, 19.66 ± 2.65% and 9.80 ± 2.79%, respectively. Taken together, these data indicate that NIC pretreatment significantly reduces the apoptotic rate.

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Figure 1. The histopathology of myocardial tissue (×200). HE staining of tissue samples from the Sham group (A), the CME group (B) and the NIC group (C). Red cytoplasmic staining indicates microinfarction region with myocardial karyolysis and hypochromatosis. Moreover, peripheral cardiac muscle edema and denaturation and peripheral inflammatory cell infiltration was also detected in the CME group and the NIC group but not in the Sham group. The arrow indicates the presence of a 42-μm microsphere in CME group. CME: coronary microembolization; NIC: nicorandil.

Figure 2. Effect of NIC on myocardial infarction size after CME. HBFP staining was performed to identify the myocardial infarct size in each group. As shown, no microinfarct was noted in the Sham group (A); however, multiple microinfarcts (arrows) were observed in the CME group (B) and the NIC group (C). Original magnification, ×100. Normal myocardial cytoplasm is stained yellow, the nuclei are stained blue, and the ischaemic myocardium is stained red. CME: coronary microembolization; HBFP: haematoxylin-basic fuchsin-picric acid; NIC: nicorandil.

Figure 3. Effect of NIC on myocardial apoptosis after CME. (A): TUNEL staining was used to assess myocardial apoptosis in each group. As shown, almost no apoptotic cells were observed in the Sham group (a); in contrast, a large number of apoptotic cells were found in the CME group (b), and NIC pretreatment significantly reduced CME-induced myocardial apoptosis (c). (B): Changes in the apoptotic rate in each group. Original magnification, ×400. The data (n = 15) are expressed as the means ± SD. *P < 0.05 compared to the sham group, †P < 0.05 compared to the CME group. CME: coronary microembolization; NIC: nicorandil.
3.4 Effect of NIC on the protein expression of cleaved caspase-9, cleaved caspase-8 and cleaved caspase-3 after CME

Western blot analysis was performed to detect the expression levels of cleaved caspase-9, cleaved caspase-8 and cleaved caspase-3 (Figures 4–6). Compared to that in the Sham group, the expression level of cleaved caspase-9, cleaved caspase-8 and cleaved caspase-3 in the CME group and the NIC group were significantly increased ($P < 0.05$). However, the levels of cleaved caspase-9, cleaved caspase-8 and cleaved caspase-3 in the NIC group were remarkably decreased compared to that in the CME group ($P < 0.05$). Furthermore, pre-treatment with NIC before CME modelling significantly inhibited the activation of cleaved caspase-9, cleaved caspase-8 and cleaved caspase-3 compared to that in the CME group.

4 Discussion

CME is a frequent and serious event in patients with ischaemic heart disease.\cite{1} It may occur spontaneously in patients with acute coronary syndromes or during thrombolytic therapy and percutaneous coronary interventions (PCI). It is a common clinical pathological-physiological process that can result in malignant arrhythmias, contractile dysfunction, coronary flow reserve reduction and even death.\cite{4,5,18} Thielmann, et al.\cite{17} demonstrated that during the acute phase after CME, the myocardium exhibited microinfarction foci, myocardial cells underwent necrosis and apoptosis, and cardiac function progressively declined. Interestingly, intravenous nicorandil treatment can protect the heart during...
ischaemia/reperfusion injury and reduce myocardial apoptosis.\textsuperscript{[21]}

In our preliminary studies, we found that apoptosis plays a crucial role in myocardial dysfunction and that peak myocardial apoptosis and the expression of the cleaved caspase-9/8/3 appeared 6 h after CME.\textsuperscript{[8,22]} Therefore, we chose to observe cardiac effects 6 h after CME in this study. Notably, our data show that at 6 h post CME, compared to that in the sham group, multiple microinfarction foci appeared in the myocardium, myocardial cells became apoptotic and necrotic, and cardiac function decreased.

In the present study, we observed TUNEL-positive cells in the ischaemic region and along the borders of the ischaemic region in CME animals. In comparison, NIC-treated hearts had a significant attenuation of TUNEL-positive cells. The TUNEL assay data clearly demonstrated that NIC pretreatment could significantly inhibit CME-induced myocardial apoptosis. This conclusion was also supported by a dramatic improvement in cardiac function in NIC-treated animals. As shown by echocardiography, LVEF, FS and CO in the NIC group were significantly increased, while LVEDd was significantly decreased compared to that in the CME group.

NIC is a vasodilatory drug that has been proposed to have multiple cardiovascular benefits in addition to its anti-angina effect, including anti-oxidative, anti-inflammatory and anti-apoptotic properties.\textsuperscript{[16,23]} In recent years, many studies have validated its anti-apoptotic effect in animal models of myocardial ischaemia-reperfusion injury.\textsuperscript{[21]} doxorubicin-induced heart failure\textsuperscript{[24]} and doxorubicin-induced cardiotoxicity.\textsuperscript{[25]} Additional studies have shown that NIC may regulate the mitochondrial and cell surface death receptor apoptotic pathways. Caspases are a family of aspartic acid-specific proteases that are critical in the induction of apoptosis.\textsuperscript{[26]} Caspase-3 catalyses a terminal step in apoptosis, and its activation is required for apoptosis.\textsuperscript{[27,28]} The active form of caspase-9 is an initiator caspase in the mitochondrial apoptosis pathway.\textsuperscript{[8,29]} The active form of caspase-8 is an initiator caspase in the cell surface death receptor apoptosis pathway.\textsuperscript{[6]} Interestingly, in the present study, we showed that NIC pretreatment significantly improved the cardiac function and decreased the expression levels of cleaved caspase-9, cleaved caspase-8 and cleaved caspase-3 as well as the degree of apoptosis in rat cardiac myocytes after CME. Consistent with previous studies, there was no difference in myocardial infarct size compared to that in the CME group, indicating that myocardial infarct size was not a major contributor to the cardiac dysfunction caused by CME.\textsuperscript{[30]} Therefore, NIC mediates the inhibition of cardiac myocyte apoptosis, possibly through the blockade of the mitochondria-mediated and death receptor-mediated apoptotic pathways. Because cardiac myocytes are terminally differentiated, inhibition of cardiac myocyte apoptosis may be one of the most critical role by which NIC pretreatment improves CME prognosis.

In conclusion, pretreatment with NIC can effectively ameliorate CME-induced myocardial apoptosis and improve cardiac function, which might be due to a blockade in the mitochondria-mediated or death receptor-mediated apoptotic pathways. This may be of potential clinical significance in CME therapy, and may be valuable to patients with acute coronary syndrome or undergoing thrombolytic or PCI therapy.

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### Competing interests

All authors have read and approved the final version and have agreed to submit this manuscript. There was no ethical/legal conflicts involved in the article.

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