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Cardioprotective effects of combined therapy with diltiazem and superoxide dismutase on myocardial ischemia-reperfusion injury in rats

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ABSTRACT

Aims: Our experiments were designed to study the effect of diltiazem (DIL) combined with superoxide dismutase (SOD) on myocardial ischemia-reperfusion (MIRI) injury in a rat model.
Main methods: Fifty rats were randomly separated into sham, ischemia-reperfusion (IR), DIL (5 mg/kg), SOD (10,000 U/kg) and combinatorial therapy (DIL plus SOD) groups. MIRI was induced by ligating the left anterior descending coronary artery for 30 min and then reperfusing for 60 min. The cardioprotective effects of combinatorial therapy were evaluated using hemodynamics, biochemical indices, histopathology and apoptotic-related proteins and gene expression.
Key findings: Compared with the IR group, combinatorial therapy significantly improved cardiac function and decreased arrhythmia, myocardial infarction area and release of myocardial enzyme. In addition, combinatorial therapy protected the myocardial cell structure as well as markedly alleviated oxidative stress, resulting in upregulation of Bcl-2 and adenine nucleotide transporter-1 expression as well as downregulation of Bax, caspase-3 and cleaved caspase-3 expression.
Significance: Our results indicated that DIL combined with SOD can provide protection against MIRI in rats, and these effects may be attributed to a reduction in oxygen stress damage, attenuation of calcium overload, and inhibition of cell apoptosis.

1. Introduction

Acute myocardial infarction is a major cause of death in Western countries [1]. Experimental and clinical studies have demonstrated that early reperfusion of ischemic myocardium attenuates such injuries and reduces infarct size [2]. However, reperfusion can also cause further severe damage to myocardial cells. This phenomenon has been defined as myocardial ischemia-reperfusion injury (MIRI) [3,4]. Until recently, there have been several mechanisms to explain its occurrence. One widely acknowledged theory is the generation of oxygen free radicals. Several enzymes may contribute to the production of oxygen free radicals: xanthine oxidase, lipoxygenase, cyclooxygenase, mitochondrial cytochrome oxidase, and oxidation of catecholamines [5,6]. If the level of oxidative stress exceeds the capacity of endogenous free radical scavenging, then oxygen free radicals can interact with cell membrane lipids and protein and contribute to myocardial cell damage and cardiac dysfunction [7]. Intracellular calcium overload is another hypothesis proposed to cause myocardium necrosis. Due to the dysfunction of the sarcoplasmic reticulum induced by myocardial ischemia followed by reperfusion, the heart loses the ability to maintain intracellular calcium ions while extracellular calcium ions access the cell via leaky myocardial cells [7]. High cytosolic calcium ions result in uncontrolled activation of the contractile machinery [8]. Importantly, cellular ATP will rapidly exhaust during ischemia. Furthermore, all cross bridges between actin and myosin may maintain a fixed state, and contracture may then develop [9]. In addition, it has been confirmed that both ischemic and reperfused rats undergo apoptotic cell death and reperfusion accelerated nonsalvageable cells apoptosis [10]. Thus, therapies involving attenuation of oxidative stress, calcium overload and blockade of the apoptotic process will have great potential to minimize cardiac injury by MIRI as well as delay the occurrence of heart failure.

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Superoxide dismutases (SODs) are a class of enzymes that catalytically scavenge superoxide radicals to form hydrogen peroxide and molecular oxygen [11]. In an organism, three SOD isoforms exist, namely, copper/zinc-containing enzyme (CuZnSOD), manganese-containing enzyme (MnSOD), and extracellular SOD (ECSOD). Extensive evidence has demonstrated the effectiveness of exogenous SOD in protecting against ischemic injury [12,13]. Considering its half-life in blood circulation [14], many modifications, including the addition of cell penetrating molecules and targeting sequences, have been made to the SOD protein to improve its pharmacokinetics and delivery system [15,16]. It has been reported that CuZnSOD-encapsulated with polyketal microparticles can reduce both extracellular and intracellular superoxide levels and improve cardiac function following MIRI [17]. Using a transgenic model, it has been observed that CuZnSOD overexpression abolished the cellular injury associated with reperfusion of ischemic tissues [18,19]. In addition, two heat shock proteins, HSP 70 and HSP 25, are well known markers of MIRI resistance, and heart function and myocardial infarction were improved in a model of transgenic mice [20]. Recently, it was found that as a predominant isoform in the cardiovascular system, ECSOD exhibits a cardioprotective effect on cases of MIRI by affecting intracellular signal transduction and the apoptotic pathway [21,22].

Our previous laboratory studies showed that as a calcium antagonist, diltiazem (DIL) demonstrated a cardioprotective effect and contributed to further mechanisms related to reducing oxygen stress damage, correcting energy metabolism, improving endothelial function and modulating cell apoptosis [23]. In addition, clinical trials found that DIL significantly decreased cardiac events [24]. The beneficial effect of DIL was predominantly the improvement of LV systolic function [25], increasing coronary flow dynamics in patients with myocardial ischemia [26], and preventing reperfusion arrhythmia [26] in patients with ST-elevation myocardial infarction during percutaneous coronary intervention.

In our current study, a MIRI model that ligated the left anterior descending (LAD) artery was explored to determine whether DIL combined with SOD exhibited a prolonged protective effect on hemodynamics and the infarction area as well as to determine the underlying mechanisms involved in the calcium overload and apoptotic pathway. In addition, combinatorial therapy was also evaluated by comparison with SOD or DIL treatment alone.

2. Material and methods

2.1. Animals

SD rats of both sexes, weighing 180–220 g, were provided by the Experimental Animal Center of Guangxi Medical University (Certificate No. SYXK 2009-0002). The research was performed according to protocols approved by our institutional ethical committee (approval no.: 20110501202) and U.S. guidelines (NIH publication No. 85-23, revised in 1996) for laboratory animal use and care. The animals were housed under controlled temperatures at 25 ± 2 °C and humidity of 60 ± 10% on a 12 h light-dark cycle. Food and water were provided ad libitum.

2.2. Myocardial ischemia-reperfusion model and experimental protocol

The ischemia-reperfusion model was established as previously described [23]. Briefly, SD rats were anesthetized via i.p. with sodium pentobarbital (Sigma, St. Louis, USA, 30 mg/kg) and restrained in the supine position. The animals had an intratracheal cannula inserted and were mechanically ventilated using a rodent ventilator (Shanghai Alcott Biotech Co., Ltd., respiration rate 70 min^{-1}, respiration-to-expiration ratio 1:2, and tidal volume 50 mL/kg) during the surgical procedures. A left parasternal incision was performed through the third and fourth intercostal space, and the pericardium was then opened to expose the heart. Myocardial ischemia was induced by placing a 5–0 silk suture with a slipknot around the left anterior descending coronary artery (LAD). After 30 min of ischemia, the slipknot was released and rats received 60 min of reperfusion. Fifty rats were randomly assigned to five experiment groups (10 rats in each group) as follows:

(1) Sham group: LAD was encircled by a silk suture, but not ligated. Rats received normal saline (2 mL/kg) 5 min before reperfusion.
(2) IR group: LAD was ligated for 30 min then allowed 60 min reperfusion. Rats were received normal saline (2 mL/kg, i.v.) 5 min before reperfusion.
(3) SOD group: LAD was ligated for 30 min and then allowed 60 min reperfusion. SOD (10,000 U/kg, i.v., Sigma-Aldrich, Inc., USA) was administered 5 min before reperfusion.
(4) DIL group: LAD was ligated for 30 min and then allowed 60 min reperfusion. DIL (1 mg/kg, i.v., Shanghai Sine Wanxiang Pharmaceutical Co., China) was administered 5 min before reperfusion.
(5) Combinatorial therapy group: LAD was ligated for 30 min and then allowed 60 min reperfusion. SOD (10,000 U/kg, i.v.) followed by DIL (1 mg/kg, i.v.) were administered 5 min before reperfusion.

At the end of the experiment, blood samples were collected prior to sacrificing the animals. The necrosis size was measured immediately after sacrifice. In addition, myocardial samples were obtained for further measurement.

2.3. Hemodynamics and arrhythmia recording

After rats were anesthetized with pentobarbital, a polyethylene catheter filled with heparinized saline was passed through the right carotid arteries into the left ventricle (LV). The LV pressure was processed via a transducer. The LV function, including the left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximal rates of the rise and decline of LV pressure (±dp/dtmax), and heart rate (HR), were collected and measured by a MS 4000 biological signal quantitative analytical system (Longfeida Technology Co., Ltd.) [27]. The hemodynamic parameters were measured at baseline, after 30 min of ischemia and after 60 min of reperfusion. In addition, an electrocardiogram was monitored continuously throughout the ischemia-reperfusion process. According to the guidelines from the Lambeth conventions [28], ventricular arrhythmias were classified as ventricular premature beat (VPB), ventricular tachycardia (VT), and ventricular fibrillation (VF). The incidence and episode of ventricular arrhythmias were recorded and then scored in 60 min of reperfusion. The classical criteria for scoring the arrhythmias included 6 grades [29]: no VPB, VT or VF (0), VPB (1), 1–5 episodes of VT (2), >5 episodes of VT or 1 episode of VF or both (3), 2–5 episodes of VF (4), and >5 episodes of VF (5). If there was more than one type of arrhythmia in one sample, the highest grade of arrhythmia was analyzed.

2.4. Measurement of myocardial infarction area

According to the previous method [30], the myocardial infarct size was determined using Evans blue and tetrazolium chloride (TTC) staining (Shanghai Chemical Reagent Co., China). Briefly, after 60 min of reperfusion, five rats in each group were treated with 1.5 mL of 0.5 g/L Evans blue solution through the thoracic aorta. Next, the heart was removed, washed with saline and stored at −70 °C. The left ventricle was section into parallel 1- to 1.5-mm-thick myocardial sections along the coronary sulcus from the apex to the base of the heart. The sections were placed into 1% TTC in PBS (pH 7.4) and incubated at 37 °C for 15 min. The area of the white zone (unstained by Evans blue and TTC) was determined as the infarct size (IS), while the area unstained by Evans blue was estimated to be area at risk (AAR). The extent of ischemic myocardium was calculated as a percentage of AAR/LV and extent of infarct myocardium was presented as a percentage of IS/AAR [31].