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Protective effect of 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride on myocardial ischemia-reperfusion injury in rats

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Received November 19, 2004, accepted March 3, 2005

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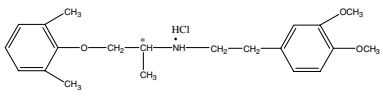
Pharmazie 60: 934–938 (2005)

The aim of the present study was to investigate the protective effect of 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride (DDPH) on myocardial ischemia-reperfusion (I/R) injury in rats and the mechanism of its myocardial protection. For this purpose, 50 Wistar rats were divided into five groups: sham group, control group, verapamil treated group, and two DDPH treated groups (20 and 40 mg/kg, respectively). Myocardial I/R injury model was established by reperfusion for 120 min after 40 min ischemia induced by the ligation of left descending coronary artery in rats. The influence of DDPH on myocardial infarction size was observed and the levels of myocardial enzymes in serum were measured. The activities of oxygen free radical scavenging enzymes and the content of malondialdehyde (MDA) in myocardium and serum were determined. The pathological changes of myocardial tissue were observed. The results showed that DDPH significantly diminished myocardial infarction size, reduced the release of myocardial creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and glutamic oxaloacetic aminotransferase (GOT), protected the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and decreased the content of MDA in myocardium and serum as compared with the control group. The degree of myocardial injury was slighter in DDPH treated groups than in control group. These results suggest that DDPH produces a cardioprotective effect during myocardial I/R injury, which may be related to blocking calcium channels and inhibiting the formation of the oxygen free radical and subsequent peroxidation of lipid by DDPH.

1. Introduction

Myocardial ischemia-reperfusion (I/R) occurs spontaneously in patients with coronary variant, transient coronary spasm, or spontaneous coronary thrombolysis, and generally accompanies clinical interventions associated with local or global attenuation of coronary flow, such as coronary artery bypass surgery, cardio-pulmonary bypass, percutaneous transluminal coronary angioplasty, and heart transplantation. A modified form of reperfusion may accompany the removal of critical coronary stenosis by thrombolysis or angioplasty. However, it is important to recognize that the reperfusion subsequent to restoration of blood flow into the previously ischemic vascular bed causes a myocardial I/R injury (Flaherty and Weisfeldt 1988). This so-called reperfusion injury is known to aggravate the damage induced by the ischemic episode alone and may in fact be more broadly considered as a syndrome consisting of several negative pathological consequences: extension of the mass of tissue injury (infarction), low coronary reflow, enhancement of the vascular permeability (edema), acute myocardial stunning (transient left ventricular dysfunction), and reperfusion arrhythmias (Bolli et al. 1989; Kloner 1993; Piper et al. 1998). Myocardial I/R is a multifactorial situation and the detailed comprehensive review of etiologic and pathophysiologic mechanisms mediating cardiac I/R injury has not been presented clearly. But there is considerable evidence that intracellular calcium overload and generation of oxygen free radical have been strongly implicated in the pathogenesis of myocardial I/R injury (Gunther et al. 1999; Steenbergen et al. 1990). The cardioprotective effects of some Ca^{2+} channel blockers such as verapamil and mibefradil (Sandmann et al. 2000; Wang et al. 1992), free radical scavenger such as superoxide dismutase (Kashimoto et al. 1999), and some medical plants which have antioxidant properties such as proanthocyanidin and mognolol have been proven to prevent the myocardium from the damage due to I/R (Huang et al. 2000; Sato et al. 1999).

1-(2,6-Dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride (DDPH) is a novel compound, which is designed and synthesized by China Pharmaceutical



DDPH

Table 1: Effect of DDPH on serum CPK, LDH and GOT activities in rats

Group	CPK/U · mL^{-1}	LDH/U · mL^{-1}	GOT/U · mL^{-1}
Sham	0.27 ± 0.09	0.12 ± 0.08	0.15 ± 0.07
Control	78 ± 35$^{\#\#}$	2.1 ± 0.7$^{\#\#}$	1.1 ± 0.3$^{\#\#}$
Ver 1.25 mg/kg	6.1 ± 1.3**	1.3 ± 0.9*	0.76 ± 0.27*
DDPH 20 mg/kg	5.8 ± 2.5**	1.4 ± 0.8*	0.73 ± 0.24*
DDPH 40 mg/kg	4.4 ± 1.7**$^{\triangle}$	1.1 ± 0.5**	0.55 ± 0.16**

Ver: verapamil. CPK: creatine phosphokinase; LDH: lactate dehydrogenase; GOT: glutamic oxaloacetic aminotransferase.
($^{\#\#}$P < 0.01 vs sham group. *P < 0.05, **P < 0.01 vs control group. $^{\triangle}$P < 0.05 vs Ver group). n = 10 (5 male, 5 female). Mean ± SD.

University according to the structural characteristic of mexiletine (Xia et al. 1984). One part of its structure is similar to mexiletine, the other is chemically related to verapamil. The previous investigations have shown that DDPH remarkably decreased blood pressure with the reduction of heart rate, prevented I/R arrhythmias, and reversed cardiac hypertrophy (Du et al. 1990; Ni et al. 1988; Zhang et al. 1997). A highly selective α_1 adrenoceptor blocking action with weak calcium antagonistic effect was suggested to be involved in the mechanism of its anti-hypertensive and anti-arrhythmic effects (Hu and Qian 2002; Lu et al. 2000). On the basis of the weak calcium channel blocking properties of DDPH, in the present paper we have further evaluated the cardioprotective effect of DDPH in an open-chest anesthetized rat model of myocardial I/R injury and its possible mechanism.

2. Investigations and results

2.1. Activities of myocardial enzymes in serum

The serum activities of creatine phosphokinase (CPK), lactate dehydrogenase (LDH), and glutamic oxaloacetic aminotransferase (GOT) were significantly increased in the control group as compared with the sham group. The release of these enzymes was markedly decreased in groups treated with DDPH (20 or 40 mg/kg) or verapamil (1.25 mg/kg) as compared with control group (Table 1).

2.2. Activity of superoxide dismutase (SOD) and content of malondialdehyde (MDA) in serum

The activity of SOD and the content of MDA in serum were measured in all groups. I/R evidently caused a decrease of SOD activity and an increase of MDA content in serum of control group as compared with the sham group. The activity of SOD was increased and the content of MDA was apparently decreased in groups treated with the DDPH (20 or 40 mg/kg) or verapamil (1.25 mg/kg) as compared with the control group (Table 2).

2.3. Activities of antioxidant enzymes and MDA content in myocardium

The measurement of SOD, glutathione peroxidase (GSH-Px) activities and MDA content in myocardial tissue showed that I/R gave rise to a noticeable decrease in the activities of SOD and GSH-Px and a marked increase in the content of MDA in control group as compared with the sham group. Similarly, pretreatment with DDPH (20 or 40 mg/kg) or verapamil (1.25 mg/kg) significantly increased the activities of SOD and GSH-Px and decreased the content of MDA as compared with the control group (Table 3).

Table 2: Effect of DDPH on serum activity of SOD and content of MDA in rats

Group	SOD/U · mL^{-1}	MDA/nM · mL^{-1}
Sham	481 ± 59	6.3 ± 1.1
Control	429 ± 30$^{\#}$	13.9 ± 5.6$^{\#\#}$
Ver 1.25 mg/kg	473 ± 47*	8.1 ± 3.6*
DDPH 20 mg/kg	476 ± 60*	9.5 ± 3.6*
DDPH 40 mg/kg	480 ± 35**	8.1 ± 3.6*

Ver: verapamil. SOD: superoxide dismutase; MDA: malondialdehyde.
($^{\#}$P < 0.05, $^{\#\#}$P < 0.01 vs sham group. *P < 0.05, **P < 0.01 vs control group). n = 10 (5 male, 5 female) Mean ± SD.

2.4. Myocardial infarction size

In control group and all drug-treated groups, the clear area of infarction was displayed by the nitroblue-tetrazolium chloride staining technique. A significant reduction in myocardial infarction size was noted in groups treated with DDPH (20 or 40 mg/kg) or verapamil (1.25 mg/kg) compared with control group (Fig. 1).

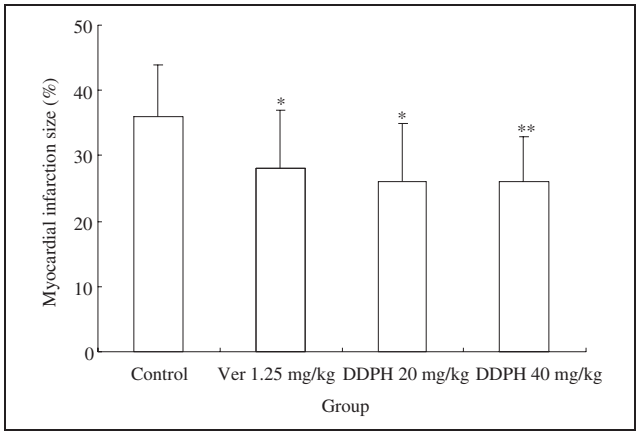


Fig. 1: Effect of DDPH on myocardial infarction size (*P < 0.05, **P < 0.01 vs control group). n = 10 (5 male, 5 female). Mean ± SD. Ver: verapamil

Table 3: Effect of DDPH on myocardial SOD, GSH-Px activities, and MDA content in rats

Group	SOD/U · mg^{-1} Pro	GSH-Px/U · mg^{-1} Pro	MDA/nM · mg^{-1} Pro
Sham	382 ± 129	159 ± 37	28 ± 8
Control	231 ± 60$^{\#\#}$	76 ± 18$^{\#\#}$	39 ± 9$^{\#}$
Ver 1.25 mg/kg	343 ± 121*	105 ± 30*	31 ± 6*
DDPH 20 mg/kg	359 ± 140*	131 ± 24**	28 ± 8*
DDPH 40 mg/kg	384 ± 185*	138 ± 26**$^{\triangle}$	27 ± 5**

Ver: verapamil. SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; MDA: malondialdehyde; Pro: protein.
($^{\#}$P < 0.05, $^{\#\#}$P < 0.01 vs sham group. *P < 0.05, **P < 0.01 vs control group. $^{\triangle}$P < 0.05 vs Ver group). n = 10 (5 male, 5 female) Mean ± SD.

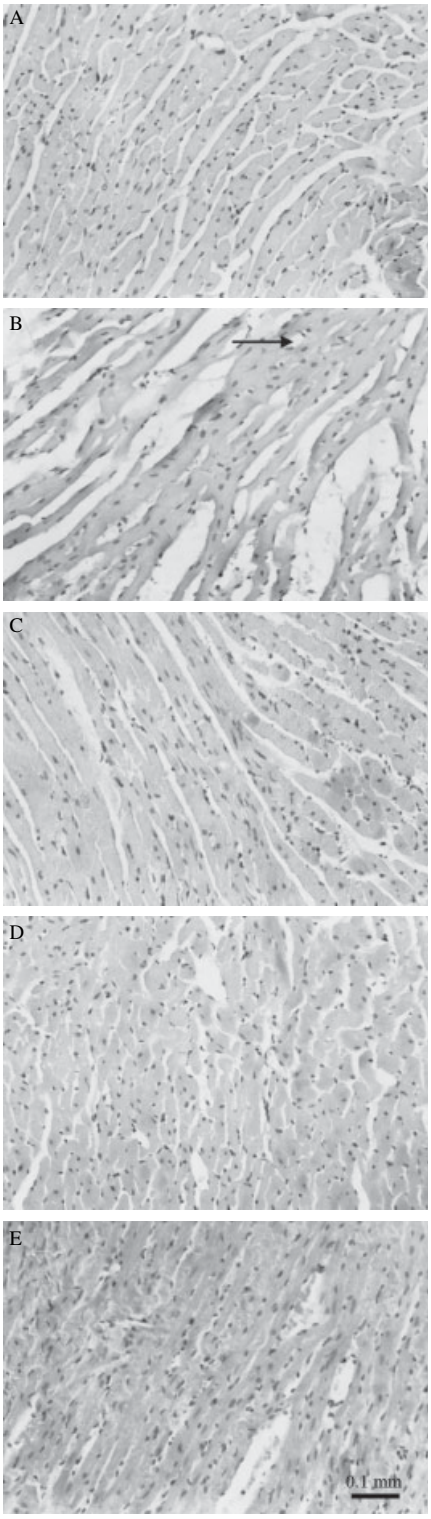


Fig. 2: Hematoxylin-eosin (H-E) stain of rat myocardium after 40 min of ischemia followed by 120 min of reperfusion (×100). A: sham group; B: control group (arrow displays intracellular edema and myofibrilla destruction); C: verapamil group; D: DDPH 20 mg/kg group; E: DDPH 40 mg/kg group. Scale bar, 0.1 mm

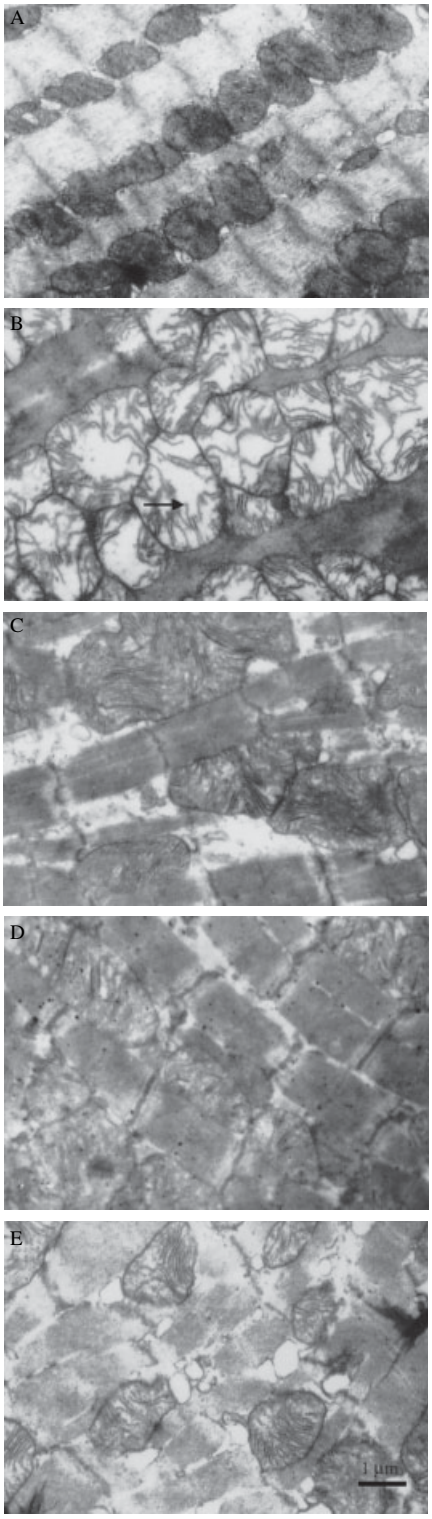


Fig. 3: Ultrastructural examination of rat myocardium (×10000). A: sham group; B: control group (arrow displays swollen mitochondria and ruptured mitochondrial cristae); C: verapamil group; D: DDPH 20 mg/kg group; E: DDPH 40 mg/kg group. Scale bar, 1 μm

2.5. Myocardial histology

The hearts in the sham group showed normal myocardial structure (Fig. 2A). After the I/R, the marked myocardial damage was found in the myocardial tissues of the control group, which consisted of the apparent intracellular edema and myofibrilla destruction (Fig. 2B). The myocardial damage was not found in the myocardial tissues of verapamil treated group (Fig. 2C) and DDPH treated groups (Figs. 2D, E).

2.6. Myocardial ultrastructure

The myocardium from the sham-operated hearts had a typical ultrastructural morphology. Myofibrilla appeared regular with distinct transverse tubules, and abundant mitochondria in rows were alongside the myofibrilla (Fig. 3A).

In contrast, the myocardial tissues after I/R had a severe loss of the normal myofibrilla structure characterized by the disruption and hypercontraction and the disorganized mitochondria were enormously swollen and greater in size with the ruptured mitochondrial cristae (Fig. 3B). However, these abnormalities were slighter in I/R hearts treated with verapamil or DDPH, in which the ultrastructural features of myocardial cells were similar to those of the sham-operated hearts, and the mitochondria were slightly abnormal in shape with most of the normal structures preserved (Fig. 3C, D, E).

3. Discussion

Reperfusion of myocardium after a period of ischemia may lead to cell death and increase the release of intracellular enzymes from the injured myocytes. Cell death determines myocardial infarction size. Myocardial enzymes such as CPK, LDH, and GOT are often used as the markers of the myocardial damage (Jiang et al. 2000). So enzyme analysis has proved considerably valuable in the diagnosis of myocardial infarction.

The results of the present study demonstrated that DDPH similar to verapamil in chemical structure obviously reduced myocardial infarction size and the serum activities of CPK, LDH and GOT. Meanwhile, pathomorphological studies indicated that the myocardial destruction caused by I/R was improved in rats treated with DDPH or verapamil. These results and previous observations that DDPH reduced the incidence of ventricular arrhythmias induced by reperfusion suggested that DDPH provides a significant cardioprotection against I/R injury (Du et al. 1990).

Myocardial calcium overload is assumed to be one of the critical reasons for the development of irreversible tissue injury induced by I/R. The increase in concentration of cytosolic calcium may occur during ischemia or reperfusion (Jeremy et al. 1992; Silverman and Stern 1994). There are various mechanisms described in the intracellular Ca^{2+} overload, including the activation of $Na^{+}-H^{+}$ exchange and/or $Na^{+}-Ca^{2+}$ exchange systems in ischemic myocardium (Allen and Xiao 2000; Tani and Neely 1990). Cytosolic Ca^{2+} overload is believed to have several kinds of harmful effects on myocardial cells by triggering some deleterious processes. It exhausts ATP by activating Ca^{2+}-activated ATPases and inhibiting high-energy phosphate production in mitochondria, degrades cellular and subcellular membrane which contain the large amounts of phospholipids and proteins by activating Ca^{2+}-dependent phospholipases and proteases, and speeds up the generation of oxygen free radical via the xanthine oxidase system (Opie 1993). Finally structural and metabolic alterations subsequent to lipid peroxidation of cell membrane systems lead to cell death and tissue injury (Kukreja and Hess 1992).

Previous investigations showed that DDPH displayed calcium channel blocking properties and reduced the intracellular calcium concentration in a concentration-dependent manner through blocking L-type calcium channel in myocytes (Hu and Qian 2001). Thus, in the condition of I/R, DDPH ensured the integrity of myofibrilla membrane and mitochondria and minimized ATP exhaustion through inhibiting calcium overload in myocytes. In addition, it is reported that DDPH selectively blocks the alpha 1-adrenoceptor (Lu et al. 2000), inhibits the contraction of aorta and slows the heart rate (Ni et al. 1988). This implies that it may reduce the myocardial oxygen consumption and ATP utilization. Therefore, DDPH has a beneficial effect on the hearts of rat with I/R. However, the precise mechanism responsible for the cardioprotection of DDPH against the injury caused by I/R needs to be further elucidated.

The abnormal formation of oxygen-derived free radicals such as superoxide, hydroxyl, and hydrogen peroxide is thought to be another major factor in cardiac I/R injury. The burst of reactive oxygen species production that mainly occurs upon the reperfusion in the heart leads to a decrease in SOD and GSH-Px activities as a consequence of their consumption and cellular lysis during oxidative stress (Singal et al. 1993). This reduction increases the cellular damage by the free radical attack. Meanwhile, the generation of MDA, the metabolite of lipid peroxidation, increases. The present study showed that DDPH strongly preserved the activities of SOD and GSH-Px, and reduced the content of MDA in serum and/or myocardium. The results indicated that the protective effect of DDPH on myocardial I/R injury could be related to the antioxidation. But, we could not confirm whether the efficacious antioxidation action of DDPH was exerted directly or indirectly through its calcium antagonistic properties, and it needs to be further investigated.

In conclusion, the present findings indicate that DDPH protects the myocardium against injury due to I/R in rats, and its protective effect may rely on blocking calcium channels and inhibiting formation of the oxygen free radical and subsequent peroxidation of lipid.

4. Experimental

4.1. Reagents

DDPH (Department of Organic Chemistry, China Pharmaceutical University), chemical purity more than 99%, was dissolved in distilled water when it was in use. Verapamil was obtained from Hefeng Pharmaceutics Co Ltd (Shanghai, China). Sodium pentobarbital was obtained from China National Pharmaceutical Co Ltd (Beijing, China). Nitroblue-tetrazolium chloride was obtained from Qianjin Reagent Factory (Shanghai, China). CPK assay kit, LDH assay kit and GOT assay kit were purchased from Zhongsheng Bioengineering Co (Beijing, China). SOD assay kit, GSH-Px assay kit and MDA assay kit were purchased from Jiancheng Bioengineering Co (Nanjing, China).

4.2. Animals

A total of fifty adult Wistar rats of either sex (Grade II, Certificate No 14-006) weighing 250 ± 23 g were provided by the Experimental Animal Center of Lanzhou Medical College. The animals were housed in individual cages with free food and water in a room maintained on a 12 h light/12 h dark cycle with controlled temperature (25 ± 2) °C for a week before the experiment. All of animal experiments were approved by the College Committee on the Use and Care of animals for experiment procedures.

4.3. Surgical preparation

All rats were anesthetized by intraperitoneal (i.p.) administration of sodium pentobarbital (30 mg/kg), and then were mechanically ventilated with room air by using a positive pressure rodent ventilator connected to a cannula inserted into the rat's trachea. The ventilation rate was synchronized with the rat's spontaneous rate at 65–70 strokes per min with a tidal volume of approximately 15 mL/kg body weight.

A left-sided thoracotomy was performed at the level of the fourth intercostal space and the pericardium was opened to let the heart expose. A ligature (5–0 silk suture) was placed around the left descending coronary artery approximately 1–2 mm from its origin, and a small polyethylene tubing was co-ligated along with the coronary artery to facilitate the successive removal of the suture. Ischemia was induced by tightening the suture for 40 min. Subsequently the ligature around the coronary artery was released to allow reperfusion of the previous ischemic vascular beds for 120 min. A lead II electrocardiogram (ECG) connected to the limbs was recorded on a computer for continuous ECG monitoring throughout the experiment. The criteria of the successful surgical models were confirmed by the ST segment elevation and the T wave inversion or tallness in ECG induced by I/R.

All the rats were assigned into five groups at random. There was an even spread of the sexes among the groups. (1) Sham group: the rat was treated with distilled water of corresponding volume as DDPH treated groups 30 min before ischemia and then underwent the same surgical procedures

without tightening the coronary suture; (2) control group: the same as sham group except that coronary artery was occluded; (3) verapamil group: the rat treated with verapamil (1.25 mg/kg, i.p.) 30 min before ischemia (Li et al. 1988) underwent I/R; (4) DDPH 20 mg/kg group: the rat treated with DDPH (20 mg/kg, i.p.) 30 min before ischemia underwent I/R; (5) DDPH 40 mg/kg group: the same as (4) except for DDPH (40 mg/kg, i.p.) (Qu et al. 2003; Wang et al. 2001).

4.4. Measurement of activities of myocardial enzymes in serum

Blood were taken from the abdominal aorta at the end of 120 min reperfusion and promptly centrifuged at 3000 × g at 4 °C for 10 min to separate serum. The serum was stored at −20 °C until the assay. The activities of CPK, LDH and GOT in serum were separately determined using CPK, LDH and GOT kits.

4.5. Measurement of activity of SOD and content of MDA in serum

The serum samples were obtained as mentioned above. SOD activity and the content of MDA in serum were determined by the use of SOD and MDA kits.

4.6. Measurement of myocardial infarction size

At the end of 120 min reperfusion, the heart was promptly excised and washed with cold saline at 4 °C. Both atria and the roots of the great vessels were removed. The left ventricle was isolated and sliced from the apex to the base into four 1–2 mm thick transverse slices and incubated in 0.1% nitroblue-tetrazolium chloride phosphate buffer solution at 37 °C for 10 min. The normal myocardium was stained purple while the infarction portion of myocardium unstained in the solution (Amsterdam et al. 1995). Each slice was scanned by a color image scanner, and the infarction size on the surface of each slice was measured by image analysis with computer. Myocardial infarction size was expressed as a percentage of total left ventricle.

4.7. Light microscope and electronic microscope studies

After the analysis of infarction size, the two central slices were taken from each group samples, and several left ventricle tissue blocks (1–2 mm^3) were cut longitudinally from the ischemic areas in the two slices for microstructural and ultrastructural evaluations. Some blocks (2 mm^3) were fixed by immersion in 4% poly-formaldehyde. Standard hematoxylin-eosin (H−E) stain was used for histomorphological evaluation under a light microscope (Olympus, Japan). Other small blocks (1 mm^3) of myocardium were fixed in 2% glutaraldehyde fixative and stored at 4 °C until processed. The blocks were post-fixated with 2% osmium tetroxide, dehydrated in a graded series of alcohol, treated with propylene oxide, and embedded in epoxy. After polymerization, 0.5 μm sections were examined under a light microscope, and representative areas of tissue samples were chosen for ultrathin sectioning (0.1 μm). The ultrathin sections were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and viewed under transmission electron microscope (MODEL JEM-1200EX/S, Japan).

4.8. Measurement of activities of antioxidant enzymes and content of MDA in myocardium

The other two left ventricle tissue were homogenized with tissue homogenizer in saline at 4 °C and centrifuged at 3000 × g at 4 °C for 10 min. Then, the supernatant fluid was removed and stored at −20 °C until the assay. SOD and GSH-Px activities and the content of MDA in myocardium were determined using SOD, GSH-Px and MDA kits respectively.

4.9. Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed by Student's t-test, which was provided by SPSS 10.0 statistical software. Statistical significance was assessed as $P<0.05$.

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