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Protective effects of melatonin on ischemia-reperfusion induced myocardial damage and hemodynamic recovery in rats

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Abstract. – AIM: To investigate the mechanism of melatonin (MT) protection of adult rate myocardial ischemia-reperfusion injury and its influence on rat's hemodynamic recovery.

MATERIALS AND METHODS: 48 rats were randomly divided into MT group (n=36) and the control group (n=12), MT group was divided into three sub-groups according to different dosages: Group I (n=12) was administered with 2.5 mg/kg MT; Group II (n=12) was administered with 5 mg/kg MT; Group III (n=12) was administered with 10 mg/kg MT. The electrocardiogram of four groups was observed with the left coronary artery blocked for 10min at first and then reperfused for 15min. Hemodynamic evolving was observed and changes in energy metabolism of rat myocardium were monitored. TUNEL and immunohistochemistry were applied to detect the cell apoptosis index, protein expression of Bcl-2 and Bax.

RESULTS: LVDP (left ventricular developed pressure) and ± dp/dt in MT group presented better recovery at various time points than the control group. Among them, Group III had the optimal recovery degree ($p < 0.05$). After MT administration, ATP content in myocardial cells in MT group was significantly higher than the control group. Compared with the control group, the concentration of mitochondrial MDA and Ca2+ in myocardial cells in MT group showed a downward trend. But its GSH concentration was significantly higher than the control group ($p < 0.05$). The improvement degree of ATP, MDA, GSH and Ca2+ concentration in Group II over-performed Group I ($p < 0.05$). MT-intervened myocardial apoptosis index (AI) and Bax positive expression index declined while Bcl-2 positive expression index increased ($p < 0.01$).

CONCLUSIONS: MT effectively inhibited myocardial apoptosis during the myocardial ischemia-reperfusion of rats, protected the structural integrity of mitochondria in myocardial cells, promoted ATP synthesis, and avoided heart damage in many ways. This protection mechanism was related with anti-oxidative damage. Meanwhile, MT could promote the hemodynamic recovery after myocardial ischemia-reperfusion in rats.

Key Words:
Melatonin, Myocardial ischemia-reperfusion injury, Myocardial protection, Hemodynamics.

Introduction

With the advancing of techniques like percutaneous transluminal coronary angioplasty, thrombolytic therapy and coronary artery bypass grafting, the prognosis of patients with coronary heart disease has been greatly improved. Under most circumstances, ischemia-reperfusion effectively restores blood circulation in tissues, contributing to extensive repair of damaged structure and effectively controlled condition of disease. Yet, some patients would suffer from enhanced tissue damage led by ischemia reperfusion, even irreversible damages, that is, ischemia-reperfusion injury[1,2]. Myocardial ischemia-reperfusion injury is currently the most difficult problem to solve while commonly found clinically. Its main pathogenesis is as follows: more oxygen free radicals are generated in cells and free radicals could trigger enhanced lipid peroxidation, damage the normal structure of the cell membrane and lead to muscle fiber rupture dissolution[3]. Mitochondria lipid peroxidation damages the mitochondrial structure and reduces the myocardial ATP generation[4]. Free radicals also lead to endonuclear base hydroxylation or DNA breakage, chromosomal aberration and abnormal cell death. Melatonin (MT) is an indole neuroendocrine hormone synthesized and secreted by the pineal. It is a strong antioxidant that could regulate the nervous system, en-

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docrine system, immune system and reproductive system[5]. Recent research pointed out that MT is a highly effective free radical scavenger that could efficiently inhibit doxorubicin's toxic effects on heart and greatly alleviate oxidative damage in myocardial cells due to ischemia/hypoxia[6]. In addition, MT not only inhibits the formation of free radicals but also eliminates the presence of existing free radicals, destroying its activity reactants[7,8]. However, the influence of MT on heart functions after acute myocardial ischemia-reperfusion and whether it protects heart are less studied. Hence, this study investigated the mechanism of MT's role in rat myocardial ischemia-reperfusion injury from various ways.

Materials and Methods

Experimental Animals

48 adult rats, weighed 350 g to 400 g, were provided by Laboratory Animal Center of Suzhou University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Affiliated Hospital of Soochow University.

Experimental Groups

48 adult rats were kept in clean environment or normal breeding environment. They were randomly divided into MT group (n=36) and the control group (n=12). 10 min before coronary artery ligation, two groups were administered with intraperitoneal injection of MT (Sigma, San Francisco, CA, USA) and normal saline respectively. MT group was divided into three subgroups according to different dosages: Group I (n=12) was administered with 2.5 mg/kg MT; Group II (n=12) was administered with 5 mg/kg MT; Group III (n=12) was administered with 10 mg/kg MT. The control group was administered with 1.0 ml/kg normal saline.

Model Preparation

With intraperitoneal anesthesia using 5 ml/kg 20% urethane, rats were anesthetized and then fixed on the test bench. Subcutaneous clicks were placed for ECG. The right cephalic artery was separated to deliver heparin normal saline to the left ventricle. And then the pressure sensor was connected to monitor changes in left ventricular pressure. Next, rats were intubated and thoracotomy was conducted to expose the heart after the breath was stabilized. The pericardium was cut open to quickly identify the position of left anterior descending, and ligation was managed by suturing. After 10 min of blocking, 15 min of reperfusion was managed.

Hemodynamic Monitoring

Using MS2000 multimedia recording and analyzing system (Longfei Technology Co. Ltd., Shenzhen, China), we carried out simultaneous monitoring to record Left Ventricular Developed Pressure (LVDP) and intraventricular pressure rate of change in isovolumic phase (dp/dt) 10 min, 30 min and 60 min before ischemia and after reperfusion respectively.

Specimen Preparation

The bottom parts of the heart that linked with blood vessels were separated and the heart was removed. After removing left and right atrium, the antetheca of left ventricle was cut off. Ice cold normal saline was applied for rapid rinsing of removed parts. These parts were wiped dry for further processing.

Preparation of Cardiac Muscle tissue Slices

The infarcted zone in antetheca of left ventricle was cut off and embedded in paraffin. Several slices with the thickness of 5 μm were made for every 1 mm, and examined by HE staining, detection of apoptosis (Boster Biological Engineering Co., Ltd., Wuhan, China), protein expression of Bcl-2 and Bax. CS-930 thin-layer chromatographic scanner (Shimadzu Corporation, Tokyo, Japan) was used for image analysis. 5 visions of each slice were collected to score positive cell number, and to calculate apoptosis index (AI) = (average optical density × positive cell number/total cell number) × 100%, positive expression index (PEI) = (average optical density × positive cell number/total cell number) × 100%.

Preparation of Myocardial Homogenate

After reperfusion, 50 mg of cardiac muscles in lower parts of ligature was collected and placed in liquid nitrogen. After the processing with perchloric acid, capillary electrophoresis device (Waters Corporation, Milford, MA, USA) was used to identify the myocardial ATP concentration. The rest was made into myocardial homogenate. Mitochondria were extracted by dif-

ferential centrifugation. Next, PE-2000 atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT, USA) was used to detect the total amount of calcium. MDA (malondialdheyde) and GSH (reduced glutathione) were detected according to the kit instructions.

Electron Microscope Examination

One specimen from each group was selected for histological slice, fixed by 2.5% glutaraldehyde and the ultrastructure was examined.

Statistical Analysis

SPSS17.0 statistical software (SPSS Inc, Chicago, IL, USA) was used for data analysis. The measurement data were expressed as mean ± standard deviation. Comparison between groups was conducted by *t*-test, and $p < 0.05$ indicated statistically significant difference.

Results

Hemodynamic Recovery of Rats in Different Groups at Different Time Points

LVDP and ±dp/dt in MT group presented better recovery at various time points than the control group. Among them, Group III had the optimal recovery degree ($p < 0.05$). However, the difference between Group I and Group II was not significant ($p > 0.05$) (Table I).

Influence of MT on ATP Concentration in Reperfused Myocardial Cells and MDA, GSH, Ca^{2+} in its Mitochondria

After MT administration, ATP content in myocardial cells in MT Group was significantly higher than the control group. Compared with the control group, the concentration of mitochondrial MDA and Ca^{2+} in myocardial cells in MT group showed a downward trend. But its GSH concentration was significantly higher than the control group, with the differences statistically significant ($p < 0.05$). The improvement degree of ATP, MDA, GSH and Ca^{2+} concentration in Group II over-performed Group I ($p < 0.05$) (Table II).

Myocardial Apoptosis Index, Protein Expressions of Bcl-2 and Bax

MT-intervened myocardial apoptosis index (AI) and Bax positive expression index declined while Bcl-2 positive expression index increased ($p < 0.01$). AI and positive expressions of Bax and Bcl-2 among three sub-groups of MT group were not significantly different ($p > 0.05$) (Table III).

Comparison of Myocardial Ultrastructure Examination Results Between Different Groups

Under an electron microscope, myocardial ultrastructure of the control group showed swelling cell nucleus, nucleus membrane perforation, myofibril dissolution fracture and other damages. However, in MT group, we only observed slight

Table I. Hemodynamic recovery degrees of rats in various groups at different time points.

Items	Groups	Before ischemia	After reperfusion		
			10 min	30 min	60 min
LVDP (mmHg)	Group I	108.50 ± 3.25	62.65 ± 11.23$^{*\#}$	72.70 ± 14.25$^{*\#}$	81.46 ± 10.45$^{*\#}$
	Group II	107.25 ± 3.55	68.36 ± 12.25$^{*\#}$	77.32 ± 15.00$^{*\#}$	88.32 ± 11.23$^{*\#}$
	Group III	107.40 ± 4.05	88.15 ± 10.58*	97.65 ± 12.10*	102.34 ± 10.56*
	Control group	109.30 ± 3.85	55.82 ± 14.40	66.50 ± 14.85	70.15 ± 12.25
+dp/dt (mmHg/s)	Group I	2356.30 ± 231.20	846.58 ± 110.56$^{*\#}$	876.12 ± 115.86$^{*\#}$	925.32 ± 146.60$^{*\#}$
	Group II	2423.50 ± 189.40	1205.23 ± 185.24$^{*\#}$	1315.64 ± 175.33$^{*\#}$	1456.64 ± 112.34$^{*\#}$
	Group III	2516.60 ± 224.50	2246.05 ± 237.80*	2250.56 ± 226.30*	2334.15 ± 226.35*
	Control group	2356.10 ± 210.00	565.15 ± 26.40	456.50 ± 18.75	340.25 ± 13.75
-dp/dt (mmHg/s)	Group I	2143.50 ± 158.60	812.25 ± 110.48$^{*\#}$	876.34±111.42$^{*\#}$	927.60 ± 142.32$^{*\#}$
	Group II	2058.10 ± 110.50	889.12 ± 142.45$^{*\#}$	1024.45±148.84$^{*\#}$	1102.54 ± 186.65$^{*\#}$
	Group III	2120.20 ± 140.30	1702.25 ± 133.14*	1863.45±145.00*	1967.32 ± 156.32*
	Control group	2180.50 ± 136.00	485.30 ± 12.65	366.25±14.50	312.45 ± 12.85

Note: Compared with the control group, $^{*}p < 0.01$; Compared with the group III, $^{\#}p < 0.05$.

Table II. Influence of MT on ATP concentration in reperfused myocardial cells and MDA, GSH and Ca2+ in mitochondria.

Groups	ATP (μmol/g)	MDA (μmol/g)	GSH (μmol/g)	Ca^{2+} (μmol/g)
Groups	ATP (μmol/g)	MDA (μmol/g)	GSH (μmol/g)	Ca^{2+} (μmol/g)
Group I	$5.12 \pm 1.14^{*\#}$	$12.85 \pm 3.45^{*\#}$	$0.05 \pm 0.01^{*\#}$	$180.15 \pm 21.35^{*\#}$
Group II	$6.74 \pm 1.23^{*\#}$	$11.34 \pm 3.75^{*\#}$	$0.06 \pm 0.01^{*\#}$	$162.75 \pm 28.74^{*\#}$
Group III	$9.38 \pm 1.28^{*}$	$8.60 \pm 1.51^{*}$	$0.09 \pm 0.01^{*}$	$134.25 \pm 18.35^{*}$
Control group	3.23 ± 0.84	16.10 ± 3.25	0.03 ± 0.01	225.37 ± 28.95

Note: Compared with the control group, $^{*}p < 0.01$; Compared with the group III, $^{\#}p < 0.05$.

swelling cell nucleus. Except, the rest parts of myocardial ultrastructure were normal (Figure 1).

Discussion

Oxidative stress is the main mechanism of myocardial ischemia-reperfusion injury in organism. According to Yapca et al[9], MT additions in the cardioplegic solution could effectively restore myocardial function. In our research, LVDP and ±dp/dt in MT group showed better recovery degrees at various time points than the control group, indicating that MT effectively promoted the functional restoration of left ventricle after ischemia, significantly reduce myocardial ischemia-reperfusion injury and effectively protect cardiac structure and function. These were consistent with the results of Yapca et al[9]. This was related with the cleaning channels of MT free radicals. For one thing, MT can directly bind with free radicals and prevent chain reactions of free radicals. For another, MT protects the activity of some important enzymes in antioxidant reactions. For example, glucose-6-phosphate dehydrogenase can reduce the generation of oxygen free radicals[10]. This study observed MT's protection of cells, finding that MT has an important role in maintaining mitochondria and energy metabolism. Mitochondria are the most important and sensitive organelles for reperfusion injury. The primary injury is expressed by transmembrane potential dissipation and clogged cell energy metabolism[11,12]. Therefore, reducing and taking precautions of mitochondria injury is of indispensible meanings to protect myocardial cells injured by ischemia reperfusion[13]. In this experiment, mitochondria mainly showed significant rise of MDA and Ca^{2+} concentration during ischemia reperfusion while the decline of GSH, indicating that in myocardial ischemia-reperfusion injury, mitochondria injury is mainly attributed to the increase of oxygen free radicals and calcium overload. Besides, in mitochondria, the antioxidant GSH decreased, which enhanced the degree of injury. Mitochondria injury substantially weakened the oxidative phosphorylation of cells, ultimately leading to the severely inhibited ATP synthesis[14]. However, after the injection of MT, the ability of myocardial cells for ATP synthesis was restored. MDA and Ca^{2+} concentration in mitochondria declined while GSH concentration greatly increased, indicating MT could alleviate calcium overload, increase GSH concentration

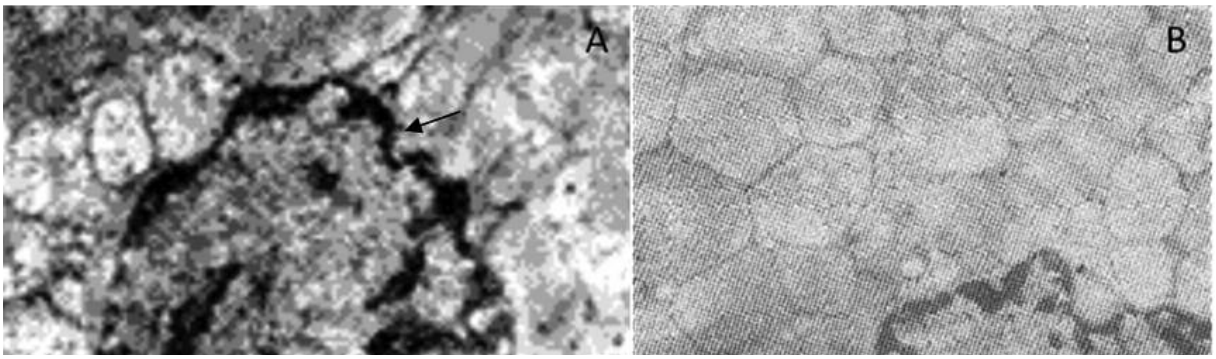

Figure 1. ***(A)*** Ultrastructure of myocardial cells in the control group (×5000); ***(B)*** Ultrastructure of myocardial cells in MT group (×5000).

Table III. Protein expressions of AI, Bcl-2 and Bax in myocardium of different groups

Groups	AI (%)	Bcl-2 PEI (%)	Bax PEI (%)
Group I	15.56 ± 8.12*	10.82 ± 4.01*	13.15 ± 3.95*
Group II	14.23 ± 7.74*	12.48 ± 3.75*	11.78 ± 3.56*
Group III	12.17 ± 6.50*	14.58 ± 3.15*	10.25 ± 2.95*
Control group	23.56 ± 8.15	7.23 ± 2.15	20.12 ± 5.69

Note: Compared with the control group, $^{*}p < 0.01$.

and restore the ability of myocardial cells for ATP synthesis. This could maintain the structural integrity of cells and alleviate the degree of myocardial injury[15]. This was verified in ultrastructural examination.

The study also found that ischemia-induced cellular calcium overload and increase of oxygen free radical led to extensive apoptosis of myocardial cells. MT, however, could effectively reduce myocardial apoptosis index (AI), Bax positive expression index, but increase Bcl-2 positive expression index. These were consistent with findings of Mohseni et al[16]. Apoptosis is a death process of cells co-regulated by several kinds of genes stimulated by internal and external experiments. Bcl-2 and Bax are apoptosis inhibiting gene and apoptosis-promoting gene respectively. The two functions together to regulate apoptosis[17]. Changes in Bcl-2 and Bax expressions in myocardial cells, to a great extent, showed that Bcl-2 was inhibited by a certain factor during ischemia reperfusion so that the imbalance of Bcl-2 and Bax co-regulation happened, finally leading to the dominant apoptosis-promoting role of Bax and the excessive apoptosis of myocardial cells[18]. MT's role of alleviating myocardial apoptosis may be attribute to the following factors: (1) MT is a strong antioxidant that can effectively remove hydroxyl free radicals, change the imbalanced co-regulation of Bcl-2 and Bax, and reduce the degree of myocardial cell injury after the reperfusion[19]; (2) MT's ability to maintain the calcium ion concentration balance in and outside cells protects the activity of Ca^{2+}-ATP enzyme, thus, blocking the apoptosis induced by calcium overload[20]. However, Group III in MT group, improvement of hemodynamics, myocardial cells energy metabolism and apoptosis over-performed Group I and II, indicating that in research of myocardial reperfusion injury of rats. 10 mg/kg MT could effectively protect myocardium without influencing other physiological functions of rats. This could reduce experimental errors and be in stark contrast with that before the administration, thus facilitating the research[21,22].

Conclusions

During the myocardial ischemia-reperfusion of rats, MT effectively inhibits myocardial apoptosis, protects the structural integrity of mitochondria in myocardial cells, promotes ATP synthesis, and avoids heart damage in many ways. This mechanism is related with anti-oxidative damage. Besides, MT can promote the hemodynamic recovery after myocardial ischemia-reperfusion in rats.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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