Research Paper

Effects of Melatonin on Cardiac Function, Metabolic Stress and Apoptosis of Cardiomyocytes in Rats with Heart Failure after Myocardial Infarction

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To research the effects of melatonin on cardiac function, myocardial cell metabolic stress and apoptosis in heart failure after myocardial infarction rats. Thirty specific-pathogen-free male Sprague Dawley rats were randomly divided into sham operation group (n=10), model group (n=10) and melatonin group (n=10). The model group and the melatonin group were constructed by ligating the left anterior descending coronary artery, while not in the sham operation group. Melatonin group (10 mg/kg) was intraperitoneally injected with melatonin (Sigma, United States of America) 30 min before operation. The sham operation group and the model group were injected intraperitoneally with the same amount of normal saline. The cardiac function of rats was measured by left ventricular ejection fraction, left ventricular fractional shortening, left ventricular end diastolic diameter and left ventricular end-systolic diameter. The myocardial cell apoptosis in ischemic areas of each group was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling staining. The expression of related proteins, measure myocardial fibrosis and determine the oxidative stress parameters (nicotinamide adenine dinucleotide phosphate oxidases activity, superoxide anion, malondialdehyde and superoxide dismutase) were detached *via* Western blot. Left ventricular fractional shortening % and left ventricular ejection fraction % in the melatonin group were significantly increased than those in the model group ($p<0.05$), while left ventricular end diastolic diameter and left ventricular end-systolic diameter in the melatonin group were significantly decreased ($p<0.05$). The apoptotic cardiomyocytes number in melatonin group was significantly less in model group ($p<0.05$). The levels of B-cell lymphoma 2 associated X-protein and caspase-3 protein in rat cardiomyocytes in melatonin group were significantly lower than those in model group ($p<0.05$). The proportion of sirius red staining in the melatonin group was significantly less than that in model group ($p<0.05$). Collagen I/III and transforming growth factor of rat cardiomyocytes in melatonin group-beta was significantly down-regulated than that in the model group ($p<0.05$). Nicotinamide adenine dinucleotide phosphate oxidase activity, superoxide anion and malondialdehyde level of rats in melatonin group were significantly decreased than those in model group, while superoxide dismutase activity was significantly increased than that in model group ($p<0.05$). Melatonin can improve heart failure and cardiac fibrosis by restraining oxidative stress in heart failure rats. Nicotinamide adenine dinucleotide phosphate oxidases 1 play an important role in regulating the role of melatonin in reducing cardiac fibroblasts fibrosis.

Key words: Melatonin, heart failure, myocardial infarction, cardiac function, metabolic stress, apoptosis

Heart Failure (HF) is caused by the structural or functional cardiac disease and it is featured as the cardiac fibrosis[1]. Cardiovascular fibrosis is a significant driving factor for Chronic Heart Failure (CHF) and the excessive cardiac fibrosis can lead to the large infarct scar, cardiac insufficiency and heart dilation[2]. The Cardiac Fibroblasts (CF) are currently considered to be the main source of cardiac fibrosis after ischemic injury[3]. CF has important role in the remodeling after infarction, which can eventually lead to pathological cardiac fibrosis and HF[4]. CF is a sign of HF and there is no efficient therapeutic drug. Melatonin is one kind of neurohormones and maintains the Circadian Rhythm (CR)[5]. Abundant researches indicate that it has the antioxidant activity[6]. Clinical evidence showed that melatonin plays a protective role in regulating hypertension, pulmonary hypertension, HF, atherosclerosis, arrhythmia and Ischaemia-

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Reperfusion Injury (IRI)[7]. Melatonin is known to protect the heart by activating the G protein coupled membrane receptor, tumor necrosis factor receptor, toll like receptor, riboretinoic acid receptor and others[8]. Oxidative stress is considered to play a key role in pathological cardiac remodeling and the transformation of HF. Collagen production induced by the Angiotensin II (Ang II) is caused by the production of the Reactive Oxygen Species (ROS) in the adult rat CF. Ang II activates ROS-sensitive kinase, a key protein in cardiac fibrosis remodeling. However, it is not clear whether the melatonin administration inhibits the oxidative stress to alleviate the HF mice's cardiac fibrosis induced by Myocardial Infarction (MI). This research aims to discuss the anti-fibrosis effect of melatonin on HF rats induced by MI and whether oxidative stress participated in melatonin in reducing HF.

MATERIALS AND METHODS

Experimental animals and grouping:

Thirty Specific-Pathogen-Free (SPF) male Sprague Dawley (SD) rats were purchased from the Experimental Animal Center of Guangzhou University of Chinese Medicine, weighing 250-300 g, adaptive feeding for 7 d under SPF environment with humidity (45 %±5 %) and temperature (20°±2°). Regular CR was provided, as well as feeding and drinking freely. The rats were fasted for 12 h and they were divided into sham-operated group (n=10), model group (n=10) and melatonin group (n=10). The model group and melatonin group established rat MI model by ligating the left anterior descending coronary artery, while it was not in the sham-operated group. Melatonin (Sigma, United States of America (USA)) was injected into the abdominal cavity for the melatonin group (10 mg/kg) 30 min before the operation. An equal amount of saline was injected intraperitoneally in other two groups.

Establishment of HF rat model:

The experimenters adopted the pentobarbital sodium to anesthetize the rats through the intraperitoneal injection and it was fixed in the supine position, connected the limbs to the Electrocardiogram (ECG), cut the neck skin, separated the muscle layer, exposed the trachea and connects the small animal ventilator (Chengdu Teichmann). The thorax was opened along the left 3rd and 4th intercostal spaces. Then, the 0 gauge guide wire was used to ligate the coronary artery along the left artery at 1 mm below the left atrial appendage. Heart beat weakened, myocardium turned white, myocardial tissue appeared cyanosis with ST elevation. It meant successful occlusion of the anterior coronary descending artery. Stitch the chest and anti-infection treatment was performed *via* intramuscular injection of penicillin within 12 h after surgery to record the 7 d survival of rats.

Cardiac function test:

The rat cardiac function was determined by M-type echocardiography with the small animal ultrasound instrument. Left Ventricular Ejection Fraction (LVEF), Left Ventricular Fractional Shortening (LVFS), Left Ventricular End Diastolic Diameter (LVEDD) and Left Ventricular End-Systolic Diameter (LVESD) were measured *via* BL-420E+.

Determination of myocardial apoptosis:

The experimenters killed the rats immediately after the experiment, removed their hearts separated the left ventricle. Myocardial apoptosis in the ischemic area was detected with the Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) kit by strictly following the instructions strictly. The experimenters observed and photographed the sections under a fluorescence microscope. TUNEL positive cells, which were apoptotic cells, showed the yellow green fluorescence, whereas TUNEL negative cells were normal without the fluorescence. The apoptosis level in myocardial cell was calculated for each group.

Detection of related protein expression by Western blotting:

The rats were killed and the left ventricle was isolated. According to the instructions of TUNEL staining kit (Beyotime Biotechnology), the number of cardiomyocyte apoptosis in ischemic area was detected. The sections were observed and photographed under a fluorescence microscope. The level of cardiomyocyte apoptosis in each group was calculated.

Sirius red staining:

The heart slice (5 μm) was inspected through the sirius red staining, to measure the fibrosis of the myocardial cell. 3-5 slices were selected for each animal (30-50 cells at each vision) to observe under the optical microscope. The Image-Pro Plus software was adopted to analyze the image.

Parametric measurement of oxidative stress:

We detached the Nicotinamide Adenine Dinucleotide

Phosphate (NADPH) Oxidases (NOX) activity through enhanced chemiluminescence of lucigenin in CF. For the measurement of the superoxide anion, the superoxide anion level in CF was determined by chemiluminescence derived from glossin. An Enzyme-Linked Immunosorbent Assay (ELISA) kit was adopted to measure the level of Malondialdehyde (MDA). The micro plate reader was used to measure Superoxide Dismutase (SOD) according to the description.

Statistical method:

The data of the present study were analyzed by Statistical Package for Social Sciences (SPSS) 22.0. All data with normal distribution were compared and represented by ($\bar{x}\pm s$). One-way Analysis of Variance (ANOVA) was adopted for comparisons among multiple groups and the Student–Newman–Keuls (SNK)-q test for pairwise comparisons. The enumeration data was expressed in percent (%) and the comparison between the groups was made by Chi-square (χ^2)-test. The statistical results were statistically significant if $p<0.05$.

RESULTS AND DISSCUSION

After 7 d modelling, both LVEF % and LVFS % in the model group were lower than those of the sham operated group. However, LVEDD and LVESD increased significantly in the model group ($p<0.01$). LVEF % and LVFS % in the melatonin group were higher than those in the model group ($p<0.05$), while LVEDD and LVESD in the melatonin group were lower ($p<0.05$) as shown in Table 1.

Compared to the model group, the number of apoptotic cardiomyocytes in the sham operated group was decreased ($p<0.05$); meanwhile it was less in the melatonin group ($p<0.05$) as shown in Table 2.

The B-Cell Lymphoma-2 (BCL-2) protein of rat cardiomyocytes in the model group was obviously down-regulated than that in the sham operated group ($p<0.05$). The BCL-2 protein of rat cardiomyocytes in the melatonin group was significantly up-regulated than that in the model group ($p<0.05$). BAX and caspase-3 protein of rats in the model group were significantly higher than those in the sham operated group ($p<0.05$); BAX and caspase-3 protein in the melatonin group were decreased compared with model group ($p<0.05$) as shown in Table 3.

Compared to model group, the proportion of sirius red staining was increased in the sham operated group and model group ($p<0.05$) as shown in Table 4.

Collagen I/III and the Transforming Growth Factor Beta (TGF-β) protein of the rat cardiomyocyte in the model group were significantly raised from the sham-operated group ($p<0.05$); collagen I, collagen III and TGF-β protein of rat cardiomyocyte in the melatonin group were significantly reduced than those in the model group ($p<0.05$) as shown in Table 5.

The NOX activity, superoxide anion and MDA level of the rat's cardiomyocyte in the model group were raised than that of the sham-operated group and the SOD activity was significantly lower than the operation group ($p<0.05$); NOX activity, superoxide anion and MDA level of the rats in the melatonin group were reduced than that of the model group and the SOD activity was up-regulated than that of the model group ($p<0.05$) as shown in Table 6.

TABLE 1: CARDIAC FUNCTION INDICATORS OF RATS ($\bar{x}\pm s$)

Grouping	n	LVEF (%)	LVFS (%)	LVEDD (cm)	LVESD (cm)
Sham operated group	10	84.85±4.36	46.65±3.65	0.72±0.06	0.37±0.05
Model group	10	45.48±3.29[a]	20.53±2.18[a]	0.94±0.05	0.76±0.08
Melatonin group	10	63.64±3.42[ab]	28.82±23.25[ab]	0.83±0.06	0.49±0.06
t		13.482	12.658	5.625	6.352
p		0.001	0.001	0.001	0.001

Note: [a]$p<0.05$ when compared to the sham-operated group and [b]$p<0.05$ when compared to the model group

TABLE 2: COMPARISON OF THE NUMBER OF TUNEL-STAINED APOPTOTIC CARDIOMYOCYTES OF RATS AMONG GROUPS

Grouping	n	Number of TUNEL apoptotic cells (n/mm^2)
Sham operated group	10	15.65±1.14
Model group	10	326.84±39.85[a]
Melatonin group	10	107.55±8.62[ab]
t		365.71
p		0.001

Note: ([a]) indicates that compared to the sham operated group, $p<0.05$ and ([b]) indicates that compared to the model group, $p<0.05$

TABLE 3: EXPRESSION COMPARISON OF BCL-2, BAX AND CASPASE-3 PROTEIN TABLES IN RAT CARDIOMYOCYTES (x̄±s)

Grouping	n	BCL-2	BAX	Caspase-3
Sham operated group	10	0.13±0.06	0.17±0.03	0.42±0.08
Model group	10	0.06±0.03[a]	0.52±0.16[a]	1.09±0.13
Melatonin group	10	0.09±0.04[ab]	0.33±0.14[ab]	0.69±0.09
t		1.65	4.58	5.47
p		0.045	0.001	0.001

Note: ([a]) indicates that compared to the sham operated group, p<0.05 and ([b]) indicates that compared to the model group, p<0.05

TABLE 4: CARDIAC FIBROSIS OF EACH GROUP OF THE MICE

Grouping	n	Proportion of sirius red staining (%)
Sham operated group	10	6.57±1.05
Model group	10	38.72±6.84[a]
Melatonin group	10	18.26±3.24[ab]
t		17.65
p		0.001

Note: ([a]) indicates that compared to the sham operated group, p<0.05 and ([b]) indicates that compared to the model group, p<0.05

TABLE 5: EXPRESSION OF COLLAGEN I/III AND TGF-β OF RAT CARDIOMYOCYTES IN EACH GROUP (x̄±s)

Grouping	n	Collagen I	Collagen III	TGF-β
Sham-operated group	10	0.48±0.06	0.39±0.05	0.29±0.05
Model group	10	1.08±0.11[a]	0.89±0.23[a]	0.84±0.10
Melatonin group	10	0.78±0.10[ab]	0.56±0.17[ab]	0.56±0.09
t		6.58	5.64	5.18
p		0.001	0.001	0.001

Note: ([a]) indicates that compared to the sham operated group, p<0.05 and ([b]) indicates that compared to the model group, p<0.05

TABLE 6: COMPARISON OF OXIDATIVE STRESS LEVELS IN RAT CARDIOMYOCYTES

Grouping	n	NOX activity (MLU/min/mg)	Superoxide anion (MLU/min/mg)	MDA (nmol/mg)	SOD (U/mg)
Sham operated group	10	8.97±0.86	8.62±1.20	76.54±8.21	375.65±40.61
Model group	10	17.65±1.95[a]	17.55±2.01[a]	213.28±32.69	176.53±18.64
Melatonin group	10	12.43±1.34[ab]	11.27±1.32[ab]	131.54±14.12	234.73±26.23
t		9.546	9.875	18.273	16.584
p		0.001	0.001	0.001	0.001

Note: ([a]) indicates that compared to the sham operated group, p<0.05 and ([b]) indicates that compared to the model group, p<0.05

Melatonin is a hormone. It not only maintains CR, but protects the heart from the continuous ischemia-reperfusion injury[9]. It is observed from clinical research that the reduction in serum melatonin level is related to the increase of the heart attack and other acute cardiovascular events[10]. The acute MI is related to the lack of serum melatonin at night and the increase of oxidative stress, which indicates that melatonin, clears free radicals formed during acute MI at night. The patients with MI have the high-level oxidized Low-Density Lipoprotein (LDL), but the serum melatonin level is relatively low. It also shows that the heart attack is related to the level of melatonin in the serum[11]. Before operation, increased melatonin level leads to IRI and the decrease of inflammatory markers intercellular adhesion molecule 1, Interleukin 8 (IL-8), Troponin I (TnI). Wherein, the 20 d melatonin treatment may produce significant anti-ischemic and anti-angina effects and normalize oxidative stress in individuals with Cardiovascular Disease (CVD)[12]. It is also reported that people with the vascular diseases have a significantly reduced level of melatonin at

night and the reduced melatonin secretion is associated with a raised risk of high Body Mass Index (BMI) in women[13]. In addition, supplemented melatonin could improve the ejection fraction and reduce cardiac TnI and IL-1 in plasma β and caspase-3 enzyme, which significantly inhibits IRI in the myocardium IRI. It indicates that the melatonin could alleviate the IRI related to the cardiac surgery[14]. In addition, compared with the control group, the circulating melatonin levels are also low in patients with acute MI and dilated cardiomyopathy, which may be associated with the myocardial injury and cardiac output of the patients with the dilated cardiomyopathy[15]. The HF impairs the ability of the ventricle filling or ejection to resulting in progressive loss of contractility and ejection fraction, ventricular wall thinning, humoral homeostasis, activation of cytokines and neurohumors[16]. In this research, the LVEF % and LVFS % of the mice in the modeling group after modeling for 7 d are significantly lower than that of the sham operated group, LVEDD and LVESD increase significantly in the model group, while melatonin administration inhibits the increase. These results indicate that melatonin could alleviate the HF caused by MI and improve cardiac insufficiency of HF, for the formation of CF in HF, it could develop to HF[17]. At present, it is still unclear for the relationship between the melatonin and cardiac fibrosis of HF. In the current research, the result indicates that collagen I/III and TGF-β level increase in the MI rats' heart, while the melatonin alleviates the increase. These results indicate that the CF of MI mice increases and the melatonin improves the cardiac fibrosis of HF rat.

As an important molecule in the organism, ROS is involved in lots of signal pathways. As a key factor of the organic damage, the oxidative stress is associated with various diseases[18], including CF[19]. Increased apoptosis and mitochondrial ROS production in hypoxic and deoxygenated cells reduce the exposure to short N-terminal gelatin fragments 2-15[20]. The antioxidant has been shown to improve cardiac function and produce the antifibrosis effect[21,22]. Currently, it is found that the NOX activity, superoxide anion and MDA levels increase in the hearts of MI rats and CFs and the SOD level decreases, while melatonin reverses these changes. Melatonin could reduce oxidative stress to inhibit the cardiac fibrosis in HF. To sum up, the melatonin could inhibit the HF rat's oxidative stress, to improve the HF and cardiac fibrosis. NOX1 plays an important role in melatonin of CF fibrosis.

Conflict of interests:

The authors have no conflict of interest to report.

REFERENCES

1. Wang L, Liu C, Chen X, Li P. Alamandine attenuates long-term hypertension-induced cardiac fibrosis independent of blood pressure. Mol Med Rep 2019;19(6):4553-60.
2. Kong P, Christia P, Frangogiannis NG. The pathogenesis of cardiac fibrosis. Cell Mol Life Sci 2014;71(4):549-74.
3. Tallquist MD, Molkentin JD. Redefining the identity of cardiac fibroblasts. Nat Rev Cardiol 2017;14(8):484-91.
4. Philip JL, Xu X, Han M, Akhter SA, Razzaque MA. Regulation of cardiac fibroblast-mediated maladaptive ventricular remodeling by β-arrestins. PloS One 2019;14(7):e0219011.
5. Lochner A, Marais E, Huisamen B. Melatonin and cardioprotection against ischaemia/reperfusion injury: What's new? A review. J Pineal Res 2018;65(1):e12490.
6. Aslan G, Gül HF, Tektemur A, Şahna E. Ischemic postconditioning reduced myocardial ischemia-reperfusion injury: The roles of melatonin and uncoupling protein 3. Anatolian J Cardiol 2020;23(1):19-27.
7. Randhawa PK, Gupta MK. Melatonin as a protective agent in cardiac ischemia-reperfusion injury: Vision/Illusion? Eur J Pharmacol 2020;885:173506.
8. Han D, Wang Y, Chen J, Zhang J, Yu P, Zhang R, *et al.* Activation of melatonin receptor 2 but not melatonin receptor 1 mediates melatonin-conferred cardioprotection against myocardial ischemia/reperfusion injury. J Pineal Res 2019;67(1):e12571.
9. Lochner A, Genade S, Davids A, Ytrehus K, Moolman JA. Short and long-term effects of melatonin on myocardial post-ischemic recovery. J Pineal Res 2006;40(1):56-63.
10. McMullan CJ, Rimm EB, Schernhammer ES, Forman JP. A nested case–control study of the association between melatonin secretion and incident myocardial infarction. Heart 2017;103(9):694-701.
11. Dominguez-Rodriguez A, Abreu-Gonzalez P, Garcia-Gonzalez M, Ferrer-Hita J, Vargas M, Reiter RJ. Elevated levels of oxidized low-density lipoprotein and impaired nocturnal synthesis of melatonin in patients with myocardial infarction. Atherosclerosis 2005;180(1):101-5.
12. Zaslavskaia RM, Shcherban EA, Lilitsa GV, Logvinenko SI. Melatonin in the combined treatment of cardiovascular diseases. Klin Med 2010;88(3):26-30.
13. McMullan CJ, Rimm EB, Schernhammer ES, Forman JP. A nested case–control study of the association between melatonin secretion and incident myocardial infarction. Heart 2017;103(9):694-701.
14. Dwaich KH, Al-Amran FG, Al-Sheibani BI, Al-Aubaidy HA. Melatonin effects on myocardial ischemia–reperfusion injury: Impact on the outcome in patients undergoing coronary artery bypass grafting surgery. Int J Cardiol 2016;221:977-86.
15. Misaka T, Yoshihisa A, Yokokawa T, Sato T, Oikawa M, Kobayashi A, *et al.* Plasma levels of melatonin in dilated cardiomyopathy. J Pineal Res 2019;66(4):e12564.
16. González A, Schelbert EB, Díez J, Butler J. Myocardial interstitial fibrosis in heart failure: Biological and translational perspectives. J Am Coll Cardiol 2018;71(15):1696-706.
17. Guimaraes DA, Batista RI, Tanus-Santos JE. Nitrate and

nitrite-based therapy to attenuate cardiovascular remodelling in arterial hypertension. Basic Clin Pharmacol Toxicol 2021;128(1):9-17.

18. Honda T, Hirakawa Y, Nangaku M. The role of oxidative stress and hypoxia in renal disease. Kidney Res Clin Pract 2019;38(4):414-26.
19. Kura B, Szeiffova Bacova B, Kalocayova B, Sykora M, Slezak J. Oxidative stress-responsive microRNAs in heart injury. Int J Mol Sci 2020;21(1):358.
20. Pisarenko O, Timotin A, Sidorova M, Studneva I, Shulzhenko V, Palkeeva M, *et al.* Cardioprotective properties of N-terminal galanin fragment (2-15) in experimental ischemia/reperfusion injury. Oncotarget 2017;8(60):101659-71.
21. Wang XB, Cui H, Du JB. Potential therapeutic effect of SO_2 on fibrosis. Histol Histopathol 2019;34(12):1289-97.
22. Liu Y, Li M, Du X, Huang Z, Quan N. Sestrin 2, a potential star of antioxidant stress in cardiovascular diseases. Free Radic Biol Med 2021;163:56-68.