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The Effect of Anaerobic Exercise with Melatonin Consumption on HSP60 and HSP70 in Rat Myocard After Ischemic-Reperfusion

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Abstract: *Introduction: Physical activity and foods containing antioxidants seem to be beneficial for cardiovascular disease prevention. The present research aims to examine the effect of anaerobic exercise with melatonin consumption on the expression of HSP60 and HSP70 markers in rat myocard after ischemic-reperfusion. Methods: 30 male Wistar rats weighted approximately 200-250 g and aged two to three months old were used. The pilot group (N = 14) was divided into healthy group without isoprenaline induction (N=7) and the isoprenaline-induced group with (N = 7). In following, rats in melatonin group (N = 4) were gavaged every day for one month using a dose of 10 mg/kg BW. Meanwhile, rats in anaerobic group and melatonin anaerobic group were exposed tredmill training course with frequency of three times per week for one month. Finally, rats were sacrificed after confirmation of infarct and expression of HSP60 and HSP70 gene were studied by real-time method. Results: There was a significant difference between the experimental groups in terms of changes in HSP70 genes expression. It was found that there was no significant difference between the two anaerobic and melatonin anaerobic groups (sig.=0.989). There was no significant difference between the experimental groups in terms of changes in HSP70 genes expression because the significance was estimated 0.297 which was greater than 0.05. Conclusion: Melatonin therapy and anaerobic training have negligible effect on HSP60 and HSP70 gene expression. But, anaerobic exercise with consuming melatonin can decrease and increase HSP60 and HSP70 gene expression respectively but don't show a significant effect compared to melatonin therapy alone.*

Keywords: *Apoptosis, Anaerobic Training, Melatonin, HSP60 and HSP70*

INTRODUCTION

Before 1900, infectious diseases and malnutrition were the most common causes of deaths in the world; and today, cardiovascular disease (CVD) is responsible for the most deaths in many regions of the world. Accordingly, by 2020, the coronary heart disease (CHD) is projected to be the most important cause of deaths (Gudarzinezhad et al., 2012).

Hypoxia or oxygen deficiency is one of the causes of heart muscle damages, and ultimately the programmed heart cell death called apoptosis; in which the cells and mitochondria of that area become swollen and lose their membrane integrity and consequently, intracellular substance releases. Then, inflammatory responses

take place and primary damage is exacerbated. However, there are some processes that inhibit cell death and, as a result, prevent the formation of apoptosis complex (Sepehri et al., 2011).

However, several factors can affect the severity of coronary artery lesions and cardiac apoptosis and most important of them are physical activity and nutritional habits. Also, a study has indicated that doing exercises improves the elderly's heart rate recovery after functional ischemia through limiting protein oxidation, increasing the synthesis of nitric oxide and HSP70 (Le Page et al., 2009). So, it can be said that regular physical activity can improve the aerobic capacity of coronary patients as it increases cardiac output, oxygen extraction and cell death resistance. Of course, more physical activity would be more beneficial (Daryanoush et al., 2012). In other words, those do regular physical activity for a longer time or more intensely, will probably get more benefits than those who do less physical activity (Gaeini et al., 2014).

Also, during taking acute anaerobic training, the left ventricular contractility increases significantly compared to resting mode. But during maximal anaerobic training, left ventricular contractility is significantly higher in comparison with the values obtained from acute maximal aerobic training, partly due to increased systolic and diastolic blood pressure and a lower reduction in overall peripheral resistance. Moreover, lower reduction in overall peripheral resistance is caused by an increased concentration of vasoactive substances that occurs due to acidic conditions and tissue hypoxia. Therefore, less reduction in the overall peripheral resistance and increased afterload during taking maximal anaerobic exercises may require ventricles to pump blood against a relatively high pressure (Daryanoush et al., 2012). Therefore, anaerobic exercise, like aerobic exercise, seems to be a good pre-preparer for cardiovascular events.

There is also a lower risk of coronary artery disease in people who have a good diet because the studies have shown that low consumption of vegetables and fruits, as well as high cholesterol levels can result in loss of health and heart disease (Gudarzinezhad et al., 2012). In this regard, some studies have been conducted on the effects of dietary supplements consumption on the improvement nutritional habits. For example, consuming melatonin to improve cardiovascular disease can be mentioned. Because melatonin has a protective effect against ischemic injury through reducing sympathetic tone, rhythmic changes in heart rate, blood pressure and cardiac output during the day. It seems that melatonin may play a role in reducing heart failure. Also, the studies on melatonin have shown low levels of blood circulation in patients with heart failure, and noted that reduced melatonin causes vascular stenosis (Alizadeh et al., 2011). A 90% decrease in the diameter of the vein causes blood flow restriction even at resting mode, in which myocardium need normal oxygen (Farouni et al., 2011). Eventually, this process leads to ischemia or hypoxia. In this regard, a study has shown that apoptosis factors such as Bax and Bad increase with aging, but they are reversed by melatonin therapy (Forman et al., 2010). A study has shown that increase in melatonin in pancreatic cells leads to a significant increase in HSP70 in this group of cells (Bonior et al., 2005). Finally, it can be said that melatonin increases the heart cell resistance to cell death.

Most of the studies have emphasized on the importance of treating patients with cardiovascular diseases to slow or stop the progression of atherosclerosis and thrombosis through the ways such as modifying risk factors and changing lifestyles. Moreover, it has been stated that pharmaceutical treatment is important in preventing or eliminating angina (Farouni et al., 2011). Given that in many studies, taking exercise and consuming melatonin are considered as two anti-apoptotic or anti-cardiac agents; and on the other hand, physical activity and melatonin secretion decreases as one ages, so that more myocardial infarction takes place in individuals older than 65 years; in present study, studying the effectiveness of active lifestyle and melatonin consumption on pre-preparation against myocardial infarction seems necessary.

Method:

In present research, 30 male Wistar rats aged 2-3 months old and weighted 200-250 gr were used. The pilot group (N = 14) was divided into healthy group without isoprenaline induction (N=7) and the isoprenaline-

induced group (N = 7). In following, rats in melatonin group (N = 4) were gavaged every day for one month using a dose of 10 mg/kg BW Meanwhile, rats in anaerobic group and melatonin anaerobic group were familiar with running on treadmill for one week, and then they were exposed training course with frequency of three times per week for one month (Høydal et al., 2007), but the melatonin anaerobic group (n = 4) was gavaged with melatonin, in addition to the training. Until the end of the 4 weeks, the control group (n = 4) was with other groups (Table 1). The Melatonin used in present study was purchased from Sigma with the M5250 product code.

Table 1. Anaerobic training protocol

Training phases Training component	Warming up	Main anaerobic training body (acute aerobic training)	Cooling down
Training duration(min)	6	15-23	6
Severity of training (VO_{2max})	50-60%	80-approximately 100%	50-60%
Rate of running (m/min)	15-20	30-approximately 30	15-20
Total distance(m)	90-120	690-750	90-120
Treadmill slope (degree)	0	5-20	0

All rats were resting for two consecutive days after a month of training and injected isoprenaline with dose of 125 and 150 mg/kg BW with 24 hours in two consecutive days. (Azamian Jazi et al., 2016). The Isoprenaline Code. I5627 was bought from Sigma. In the following, the Trichromo staining technique was used and it was found that this amount of drug injection causes infarct in the pilot group because the results of independent t-test showed that there is significant difference between the rats in the group with isoprenaline injection and healthy rats in the fibrosis obtained from software j and the mean fibrosis of the ischemic reperfusion group was significantly higher than that of the healthy group (Table 2); therefore, the result showed that injection of isoprenaline with doses of 125 and 150 mg/kg BW in two consecutive days causes fibrosis in the rats' hearts, indicating infarct (Figure 1).

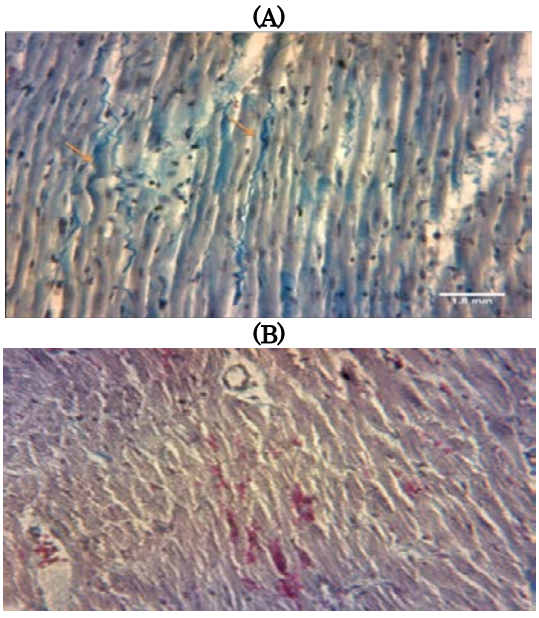

Figure 1. Images of Trichromo staining
A: isoprenaline-induced left ventricle with 40x magnification
B: Left ventricle of healthy rat with 40x magnification

RNA extraction, cDNA synthesis and design of primers

The RNA of left ventricular samples was extracted with liquid nitrogen and using mortar by manual extraction method and the standard TRIzoL protocol (code: ETI01, Civic Bioscience limited.). According to the protocol, 1 ml TRIzoL solution per 30-60 mg tissue was added to a micro-tube containing the tissue. The samples were frozen for one day and then, 200 µl chloroform (code 102445, Merck, Germany) was added to the solution and the solution was centrifuged at 12000 rpm and at 4 °C for 15 minutes. Then, the supernatant was removed after centrifugation and the same amount of isopropanol (code 109634, Merk Co., Germany) was added to it. After freezing the samples at -80 °C overnight, they were centrifuged at 12000 rpm and at 4 °C for 10 minutes, then the precipitate formed at the bottom of the micro-tube, along with a 75% alcohol, was centrifuged at 7500 rpm and at 4 °C for 5 minutes. Finally, 33 µl RNA stabilizing water was added to the precipitate at 55-60 ° C to dissolve the RNA in water. The cDNA was prepared using the protocol for Vivantis cdna synthesis kit (code: RTPL12, Malaysia) Also, beta-actin, HSP60 and HSP70 primers were designed and used by Primer3 software (Shi et al., 2016; Yang et al., 2006).

Real-time preparation

In order to prepare the real-time PCR materials, 4 λ cDNA from each research group was poured into the wells of the real-time device; then, 6 λ master mix solution (1 λ forward and reverse primer and 5 λ Cyber Green, Yekta Co. equipped with code: YT2551) was prepared. Then, the real-time device was launched for 15 minutes at 95 °C (initial denaturation), 15 seconds at 95 °C (secondary denaturation), 20 seconds at 60 °C (primer binding) and 20 seconds at 72 °C (duplication). After the second phase, the reaction was repeated for 40 cycles. The Cts of the reaction were extracted and recorded using the software of real-time device.

Results

The fold change results of HSP60 and HSP70 genes showed that expression of HSP60 gene in melatonin, anaerobic, melatonin anaerobic groups, compared to ischemia, became 1.09, 0.78, and 0.38 times greater, respectively, while the expression of HSP70 gene in the melatonin, anaerobic, melatonin anaerobic groups became 1.4, 1.57 and 1.77 times greater, respectively (Fig. 2). These results indicated that melatonin consumption for 4 weeks did not change the expression of HSP60 and HSP70 genes, but anaerobic exercise reduced and increased the expression of HSP60 and HSP70 genes, which was more significant in the melatonin anaerobic group.

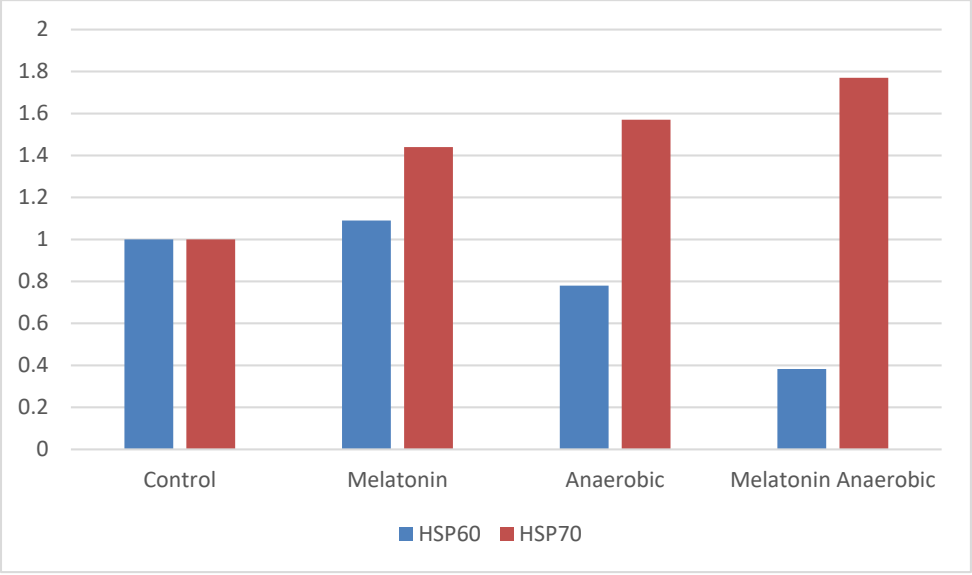

Figure 2: Expression of HSP60 and HSP70 genes in control, melatonin, anaerobic and melatonin anaerobic groups

In order to evaluate the significance level, $2^{-\Delta\Delta CT}$ values of HSP60 and HSP70 genes were used. Since according the statistics of leven test, the variances of HSP60 and HSP70 data (0.017 and 0.669, respectively) were significant and insignificant respectively, Anova and Tukey post hoc tests for HSP70 gene and Kruskal-Wallis test for HSP60 gene were used to compare the research groups (Table 2 and 3).

Table 2: Independent t-test results on fibrosis in pilot group

Statistical index	N	Mean fibrosis	SD.	Leven	Sig.
Ischemic reperfusion	7	107.85	11.95	0.098	0.00
Healthy	7	24.57	17.17		

Table 3: Variance analysis for comparison of control, melatonin, anaerobic, melatonin anaerobic groups in terms of expression of HSP60 and HSP70 genes

Research group	$2^{-\Delta\Delta CT}$HSP70	$2^{-\Delta\Delta CT}$HSP60	Fold change HSP70	Fold change HSP60	SD. HSP70	SD. HSP60	Sig HSP70	Sig HSP60
Control	0.04422775	0.03904163	1	1	1.076	0.843	0.989	0.297
	2.16009650	3.683703892						
	0.12423088	0.129511947						
	1.671444863	0.14774253						
Melatonin	0.71752239	0.582834061	1.44	1.09	1.314	0.843		
	0.42960889	0.285419755						
	1.29333478	1.190161288						
	3.34288972	2.31523253						
Anaerobic	0.678817548	0.940276585	1.57	0.78	1.68	0.843		
	1.157567053	1.405571747						
	4.058919606	0.51804779						
	0.395320799	0.259023895						
Melatonin anaerobic	2.363779995	0.248472328		0.38	1.56	0.843		
	0.301681005	0.046751521						
	3.70916839	0.702785874						
	0.737694695	0.536316706						

According to the results shown in Table 3, the expression of HSP70 gene was significantly changed in experimental groups. The results of Tukey post hoc test showed that there was no significant difference between the melatonin group and the melatonin anaerobic group (sig.=0.989). In other words, melatonin therapy and anaerobic exercises did not have a significant effect on HSP70 gene expression.

The results of Kruskal-Wallis test used to compare the groups in HSP60 gene expression showed that there was no significant difference between the research groups because the significance level was 0.297 which is greater than 0.05. In other words, independent variables of melatonin therapy and aerobic exercises have no significant effect on HSP60 gene expression.

Discussion

The results of present study showed that melatonin consumption increases the expression of HSP60 and HSP70 apoptosis genes, but this increase was not significant. These result are consistent with previous studies in terms of increased expression of HSP60 and HSP70 genes, but they are inconsistent with them in terms of significance. These results are inconsistent with the study by Forman et al. (2010) because they investigated the beneficial effects of melatonin on the heart rate changes in the old rats, while the present study was carried out on the rats aged 2 to 3 months. It seems that in the study by Forman et al. (2010), melatonin had antioxidant effect while in the present study, melatonin had hypnotic and vasodilatory effects in 2 to 3-month-old rats, which resulted in lipid accumulation in the myocardium, and thereby causing ischemic injury to myocardium. In confirmation of this, Forman et al. (2010) stated that after melatonin

therapy, inflammatory parameters, oxidative stress and apoptosis were more reversed in older rats than in young rats. Melatonin seems to play a double role with its antioxidant, vasodilatory, and hypnotic properties, so that it can play a useful role if its antioxidant properties prevail, and it can play harmful role if its vasodilatory, and hypnotic properties prevail (Forman et al., 2010).

On the other hand, the results of present study are different from those of Russell et al. (2009), which is that in the present study, melatonin has been introduced as a pre-preparation, but in Russell at al.'s study, it was seen as a drug used ischemic reperfusion. Since inflammatory and oxidative factors increase after ischemic reperfusion, melatonin can act as an antioxidant and remover of ischemic toxicity and play its useful role. But in the present study, melatonin was not useful because with precipitation of lipid indices such as LDL and VLDL, and consequently an increase in inflammatory and oxidative factors, it caused more injury to the myocardium (Russel & Tan, 2009).

On the other hand, a study showed that melatonin decreases and increases the level of VEGF and ROS in the ovary, respectively; therefore, in the present study, melatonin therapy seems to reduce the level of myocardial angiogenesis due to lack of mobility and reduced stress, and this may lead to left ventricles of the rats more prone to ischemic reperfusion injury, because the oxygen delivery decreases in the myocardium and, on the other hand, lipid infiltration, I.e. inflammatory substances, increases; that is why the myocardium become more prone to injury (Li et al., 2016).

Other findings of the study showed that aerobic and anaerobic exercises reduced and increased the expression of HSP60 and HSP70 genes, respectively. However, this reduction was not significant compared to ischemic reperfusion group (the sig. of HSP60 was 0.279). These results are consistent with the studies by Le Pege et al. (2010), Juliana and Marcos (2015) and ShirinBayan et al. (2011), but they are inconsistent in terms of significance. In the study by ShirinBayan et al. (2011), the groups 1 and 2 were familiar with running on treadmill for 3 weeks (5 times a week) at a rate of 17-15 m / min and for 25 to 39 minutes, and groups 3 and 4 for 6 weeks at 20 -15 m / min and for 25-54 minutes; while in the present study, the rats were taking exercises for 4 weeks (3 times a week). It seems that increasing training duration can be useful for adapting the characteristics of exercises. If the training duration increases or its frequency per week increases, it can affect the expression of HSP70 markers, because through adapting exercises, the oxidant and inflammatory factors, lipid profiles and ultimately the apoptotic factors, such as Caspase 3, decrease. In contrast to antioxidant agents, cellular pre-existence such as the IGF1-R / PI3K / AKT pathway increases, which in turn can reduce heart apoptosis (Le Page et al., 2009; Borges, Lessa, 2015; ShirinBayan et al., 2011). Therefore, it seems that increasing the training duration can reduce the pre-apoptotic factors of myocardium.

Other results of present study showed that aerobic and anaerobic exercises with melatonin consumption reduced and increased the HSP60 and HSP70 expression, respectively which were not significant compared to the ischemic reperfusion group (the sig. value for HSP60 and HSP70 genes were 0.297 and 0.989, respectively); these results indicate that aerobic and anaerobic exercises with melatonin consumption has reduced the expression of HSP60 gene more than other groups (Veneroso et al., 2009).

To confirm this, Kumar et al. (2002) examined the effects of oral melatonin and exercise-induced oxidative stress in healthy people. In this study, healthy people were allowed to go on a treadmill for 12 minutes based on the Bruce Protocol. The superoxide dismutase and glutathione peroxidase decreased while there was no change in the total activity of plasma antioxidant or the catalase activity in RBC before and after exercise. The basic level of lipid peroxidation products in patient under melatonin therapy significantly decreased compared those without melatonin therapy. Moreover, reduction of superoxide dismutase and glutathione peroxidase induced by taking exercises was prevented after melatonin therapy (Kumar Kv. Naidu, 2002).

Conclusion

According to the results of present and previous studies, melatonin therapy along with exercise can prevent cardiovascular disorders such as increased free radicals, ROS, RNA, oxidants and apoptotic factors such as HSP60, and, on the other hand, increase antioxidant factors such as superoxide dismutase, glutathione, as well as anti-apoptotic factors such as HSP70 and heat shock proteins such as HSP72 and ion channel proteins such as calcium and potassium. So, it is recommended to reduce the amount of apoptosis induced by ischemic reperfusion by increasing the training duration and the frequency of training per week, with melatonin consumption.

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