ISSN 0095-4527, Cytology and Genetics, 2022, Vol. 56, No. 2, pp. 185–195. © Allerton Press, Inc., 2022.

Melatonin Improves Left Ventricular Mitochondrial Dynamics in Rats

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Received September 23, 2020; revised March 2, 2021; accepted March 18, 2022

Abstract—There is increasing awareness that efficient and regular mitochondrial dynamics improvement cardiac function and affects the quality of life. Melatonin is a main pineal gland hormones and ameliorates mitochondrial dynamics in many cardiac disorders. For that purpose, we administrated melatonin to healthy rats all day long in order to investigate change in left ventricle mitochondrial dynamics both in the end of the nighttime and daytime. Twenty male Wistar rats (3–4 months age) were randomly assigned into Control (C; $n = 10$) and Melatonin groups (MEL; 10 mg/kg melatonin added drinking water, $n = 10$). On the 5th day of the study, 5 rats from the groups were randomly selected and euthanized at 08:00 AM and the remaining 5 rats were euthanized at 20:00 PM from each groups and samples of left ventricle (LV) tissue were harvested. Quantitative real-time PCR and western blot analysis demonstrated that melatonin acts preventive role on mitochondrial fusion and mitophagy through the DRP1/FIS1 and BNIP3/NIX axis, respectively. Additionally, melatonin administration significantly reduced P21 activation, induced cell cycle arrest, P27, finally regulated caspase-depended mitochondrial apoptosis signals in a time dependent manner. Our results suggest that melatonin may emerge as a therapeutic candidate to protect the bioenergetic dynamics of mitochondria in hearth.

Keywords: melatonin, heart, apoptosis, mitochondrial dynamics, mitophagy

DOI: 10.3103/S0095452722020116

INTRODUCTION

Cardiac diseases (CD), with their high morbidity, mortality, and hospitalization rates, comprise some of the greatest problems for public health throughout the world (Benjamin et al., 2017). Heart failure (HF) is the end stage of CD and myocardial energy metabolism remodeling has been proposed as the one of the main reason for HF (Qui et al., 2019).

In recent years, maintaining of healthy mitochondrial activity accepted as an important factor for healthy cardiac function. The heart is rich in mitochondria due to its own tremendous energy requirements for maintaining excitation-contraction coupling (Dorn et al., 2015). Therefore, the mitochondrial dynamics in the myocardium are suggested to be one of the essential reasons for HF (Qui et al., 2019). The disorder in mitochondrial dynamics is related to various CD's, such as: cardiac hypertrophy, myocardial I/R injury, and drug-induced cardiac toxicity (Ikada et al., 2015; Vasquez-Trincado et al., 2016; Zhou et al., 2018a; Zhang et al., 2019b; Wang et al., 2020). However, despite intense research, there is paucity of knowledge pertaining to the underlying mechanism between mitochondrial dynamics and heart failure.

The changes in shape, distribution and movement of mitochondria depend on fusion-fission frequency, which is defined as mitochondrial dynamics (Ong et al., 2015). Mitochondrial fusion and fission are controlled by large guanosine triphosphatases (GTPases) in the dynamin family (Scott and Youle, 2010). The increase in mitochondrial fission may cause the fragmentation of mitochondria and triggers mitophagy and apoptosis process. Which, in turn, results in a decrease of mitochondrial function and, finally, cardiomyocyte death (Moyzis et al., 2015; Marin-Garcia and Akhmedov, 2016). However, mitochondrial fusion may lead to the repairing of mitochondrial DNA (mtDNA) damage, the maintaining of membrane potential, the maximizing of oxidative capacity, the declining of the effects of environmental damage, and, ultimately, increasing cell survival (Redy et al., 2011; Youle and van der Bliek, 2012; Zhang and Ma,

2018). Typically, Dynamic-related peptide-1 (DRP1) and Human fission protein-1 (FIS1) have been defined as mitochondrial fission related molecules, whereas Optic Atrophy 1 (OPA1), Mitofusins 1 and 2 (MFN1 and MFN2) are seen as regulatory molecules in mitochondrial fusion (Vasquez-Trincado et al., 2016). However, beyond the molecules mentioned above, BCL-2/adenovirus EIB 19kD-interacting protein 3 (BNIP3), Nip3-like protein X (NIX), PTEN-induced kinase 1 (PINK1), tumour suppressor (P53) , B-cell CLL/lymphoma2 (BCL-2), BCL-2-associated X protein (BAX), Apoptotic peptidase activating factor 1 (APAF1), Cytochrome C (Cyt-C), and Caspases are also regarded as molecules related to mitochondrial dynamics (Zhang et al., 2019a; Morales et al., 2020).

It has been known for many years that CD's exhibit circadian rhythm and occur especially in the first three hours of the day (Willich et al., 1992). Moreover, mitochondrial dynamics also have a circadian pattern (Canaple et al., 2006; Liu et al., 2007; Kohsaka et al., 2014). Furthermore, pineal melatonin secretion also has diurnal rhythm (Tan et al., 2002). Melatonin can enter the cell easily and it has been known for a long time that mitochondria have a considerable level of melatonin. It has been reported that mitochondrial melatonin content exceeds that blood melatonin content (Venegas et al., 2012; Acuna-Castroviejo et al., 2014). The cardioprotective effects of melatonin have been well studied (Zhou et al., 2018b). However, to the best of our knowledge, there is no research investigating the effects of the daytime exposure of melatonin on left ventricular myocardial dynamics in healthy rats. Here we aimed to further characterize mitochondrial dynamics in left ventricle tissue of rats, determine whether melatonin also exerts a beneficial effect on mitochondrial dysfunction, and assess mitochondrial fusion, fission, mitophagy and apoptosis process for its beneficial effect on cardiac dysfunction in vivo.

MATERIALS AND METHODS

Animals

Twenty male Wistar rats (between 3 and 4 months age) were provided by Canakkale Onsekiz Mart University Experimental Research Center (COMUDAM). During all of the experimental procedures, the rats were kept at constant temperature (22 ± 2°C) in a 12 h light/dark cycle. The animals were fed with a commercial diet and allowed to drink water ad libitum.

Experimental Design

The rats were randomly assigned into 2 distinct groups.

(1) *Control group* (C; n= 10);

(2) *Melatonin group* (MEL; n = 10).

Control group (C). Ethyl alcohol (used to dissolve melatonin) was added to the drinking water of the rats as vehicle in this group for 5 days. The average water consumption of each rat was calculated and 1 ml vehicle was added into the daily drinking water freshly every day. On the 5th day of the study, 5 rats in this group were randomly selected and euthanized at 08:00 AM (C-8h) and samples of the left ventricle (LV) were harvested. The remaining 5 rats were euthanized at 20:00 PM (C-20h) and samples of LV tissue were obtained.

Melatonin group (MEL). Melatonin dissolved in ethyl alcohol solution (final concentration of ethanol was 2%) was added to the drinking water of the rats in this group for 5 days (10 mg/kg/day). As in the control group, the average water consumption of each rat was calculated and 1 ml 2% ethanol solution (contain 10 mg/kg/day melatonin for each rats) was added into the daily drinking water freshly every day. On the 5th day of the study, 5 rats in this group were randomly selected and euthanized at 08:00 AM (MEL-8h) and samples of the LV were harvested. The remaining 5 rats were euthanized at 20:00 PM (MEL-20h) and samples of LV tissue were obtained.

The doses of melatonin and ethyl alcohol were determined by calculating the average water consumption of the groups. The dose was adjusted by assuming that each animal consumes a similar amount of water to each other.

Anesthesia and euthanasia. The rats were euthanized with cervical dislocation under ketamine (80 mg/kg) and xylazine (5 mg/kg) anesthesia.

Following euthanasia, the heart was removed and LV tissue was isolated. Tissue samples were stored at −80°C, until analysis. All tissue acquisition and storage procedures were performed under red light.

Genetic Analysis

Chemicals. The RIPA Lysis Buffer System, 50 mL (sc-24948) was obtained from Santa Cruz Biotechnology (USA), The PureLink® RNA Mini Kit, High Capacity cDNA Reverse Transcription Kit, Power SYBR® Green Master Mix, and molecular grade water were obtained from Thermo Fisher Scientific (USA).

Antibodies. The antibodies used were: BCL-2 antibody (N-19), sc-492; caspase-3 monoclonal (E-8), sc-7272; Actin Monoclonal Antibody (ACTN05 (C4)), (ThermoFisher Sci); and the secondary horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies were supplied by WesternBreeze™ Chemiluminescent Kit, anti-mouse and anti-rabbit (Thermo Fisher, Sci.).

Isolation of Total RNA

The total RNA was isolated from samples of 5–10 mg of LV from each group (five replicates) using a PureLink® RNA Mini Kit (Thermo Fisher Scientific, USA) as per the manufacturer's instructions. The

extracted RNA concentrations were measured using a Qubit® Fluorometer (Life Technologies, USA). The concentration of total RNA was adjusted to 200 ng/μL for the synthesis of first-strand cDNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA).

cDNA Synthesis

The cDNA synthesis was performed using an Applied Biosystems® Proflex® thermal cycler (Step 1: 25°C, 10 min; Step 2: 37°C, 120 min; Step 3: 85°C, 5 min). The cDNA was stored at −20°C for subsequent steps of the analysis procedure.

Quantitative Real-Time PCR (qRT-PCR) Analysis

The relative expression levels of the genes in the mitochondrial dysfunction pathway in LV tissues were analysed by qRT-PCR using Applied Biosystems™ Power SYBR® Green Master Mix (Thermo Fisher Scientific, USA) in an ABI Quant Studio 5 Real-Time PCR system. The primer sequences were shown in Table 1 and PCR conditions are as follows: 1 cycle of 2 min at 50°C and 10 min at 95°C, followed by 42 cycles of denaturation at 95°C for 15 s, a cycle annealing and extension at 60°C for 1 min.

Western Blot Assay

After melatonin or the vehicle treatments, LV tissue was randomly selected for each replicate and were homogenized 2 times for 5 min intervals with Daihan 15D high speed homogenizator (27.000 rpm) in 500 μL RIPA protein isolation kit with 2 μL PMSF, 2 μL sodium orthovanadate and 2 μL protease inhibitor cocktail (sc-24948, Santa Cruz, USA) at ice cold conditions. Lysate was centrifuged at 14000 *g* for 20 min and in range of 2.1–5.4 mg/mL total protein was determined by Nanodrop spectrophotometer (Optizen Nano Q, Mecasys, Korea). After denaturation, proteins were loaded onto a NuPAGE® Bis-Tris polyacrylamide gel (12%), electrophoresed, and transferred to PVDF membranes (Life Technologies, USA). The membranes were incubated in blocking solution. Antibodies were diluted according to band intensity in antibody binding buffer overnight in darkroom conditions (+4°C) and then incubated in the secondary antibody solution containing anti-mouse IgG-HRP and anti-rabbit IgG-HRP for 1 hour on washed and enhanced immunoblots. Protein bands were observed with a chemiluminescence Micro ChemiDoc, (DNR Bio-Imaging Systems Ltd., USA) gel imaging system. The bands were quantified by Gel-Quant software.

Statistical Analysis

The relative fold change in gene expression due to melatonin treatment was compared between vehicle treated control and experimental groups using a one-way-ANOVA and Tukey HSD test or Dunnet T3 test. The normality of each variable was assessed before the appropriate statistical test was used. Molecular data was represented as the mean ± standard error (*SE*), unless otherwise noted. Statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, N.Y., USA), and $P \leq 0.05$ was considered significant.

RESULTS

Mitochondrial Dysfunction Gene Expression Change

Both DRP1 and FIS1 gene expressions were decreased in the melatonin group compared to the control group. It was understood that this decrease was similar to each other at 08:00 and 20:00. Melatonin administration did not affect BNIP3 and NIX gene expression levels at 08:00, but there was a decrease at 20:00. On the other hand, the application of melatonin led to an increase in OPA1 gene expression levels, with a higher level in the MEL-8h group. MFN2 gene expression levels decreased at both times (Fig. 1).

Tumor Suppressor and Cell Cycle Arrest Gene Expression Change

We evaluated P21, P27, and P53 gene expression levels as tumor suppressor and cell cycle arrest genes. In the melatonin group, a significant decrease in P21 gene expression levels and a significant increase in P27 were observed both in 08:00 and 20:00 in spide of P53 gene expression levels did not show any significant alteration (Fig. 2).

Apoptosis Related Gene Expression Change

It has been found that administering melatonin significantly reduced APAF1, CASP3, and CASP8 gene expression in both hours. On the other hand, both BCL-2 and Cyt-C gene expression level in the melatonin group did not significantly change at 20:00 but BAX gene expression level showed statistically significant increases at 08:00 (Fig. 3).

Western Blot Results

Melatonin administration reduced the BCL-2 and CASP3 level in both of the MEL-8h and MEL-20h groups compared to C-8h and C-20h groups. The western blot analysis of the BCL-2 and CASP3 levels in the left ventricle is given in Fig. 4.

Table 1. Genes, primer sequences and PCR conditions belonging to the qRT-PCR assay

Gene ID	Name	Primer sequence (5'–3') F:R
BNIP3	BCL-2/adenovirus EIB 19kD-interacting protein 3	CCGGGATGCAGGAGGAGAG TTATAAATAGAAACCGAGGCTGGAAC
DRP1	DRP1 (dynamic-related peptide-1)	CGGGAAGTGGATCCAGATGGTC ATAAGCTGCAATAAAGTGGCACT
FIS1	Human fission protein-1	GCCCCTGCTACTGGACCAT CCCTGAAAGCCTCACACTAAGG
NIX	Nip3-like protein X	GGACAGAGTAGTTCCAGAGGCAGTTC GGTGTGCATTTCCACATCAAACAT
OPA1	Optic Atrophy 1	TGACAAACTTAAGGAGGCTGTG CATTGTGCTGAATAACCCTCAA
MFN2	Mitofusins 2	ACAGCCTCAGCCGACAGCAT TGCCGAAGGAGCAGACCTT
P53	Tumor Supressor P53	GAGGTTGGCTCTGACTGTACC TCCGTCCCAGTAGATTACCAC
P21	Cyclin Dependent Kinase Inhibitor 1A (P21, Chip1)	TGTCCGTCAGAACCCATGC AAAGTCGAAGTTCCATCGCTC
P27	Cyclin-dependent kinase inhibitor 1B (P27, Kip1)	AACGTGCGAGTGTCTAACGG CCCTCTAGGGGTTTGTGATTCT
BCL-2	B-cell CLL/lymphoma 2	GGTGGGGTCATGTGTGTGG CGGTTCAGGTACTCAGTCATCC
BAX	BCL-2-associated X protein	CCCGAGAGGTCTTTTTCCGAG CCAGCCCATGATGGTTCTGAT
Cyt-C	Cytochrome C	CTTTGGGCGGAAGACAGGTC TTATTGGCGGCTGTGTAAGAG
APAF1	Apoptotic peptidase activating factor 1	AAGGTGGAGTACCACAGAGG TCCATGTATGGTGACCCATCC
CASP3	Caspase 3	CATGGAAGCGAATCAATGGAC CTGTACCAGACCGAGATGTCA
CASP8	Caspase 8	ACACAGTCGAGTAGACTCTCAAA AGGAAGTGATGCTCGTTCAGA
β-Actin	β-Actin;beta cytoskeletal actin	CATGTACGTTGCTATCCAGGC CTCCTTAATGTCACGCACGAT

DISCUSSION

The results of this study have shown that the administration of melatonin augments mitochondrial dynamics both during the day and night periods in healthy rats. Melatonin improves mitochondrial dynamics through: (a) the decrease in the gene expression levels of proteins, such as DRP1 and FIS1, that cause mitochondrial fission, (b) the decrease in the gene expressions of proapoptotic proteins such as APAF1 and CASP3, (c) the increase of the OPA1 gene expression levels causing mitochondrial fusion. Our data suggests that the protective effects of melatonin on the heart muscle also occur via mitochondrial dynamics which correlates with current research.

Cardiac diseases exhibit a circadian rhythm and occur especially in the first three hours of the day (Willich et al., 1992). Moreover, mitochondrial dynamics also have a circadian pattern. PPARa and PGC-1a, encoded by clock genes, have important roles in mitochondrial dynamics and bioenergetics (Canaple et al., 2006; Liu et al., 2007). Kohsaka et al. (2014), investigated the effects of disrupting the circadian rhythm on Bmal1 knockout mice on both genes

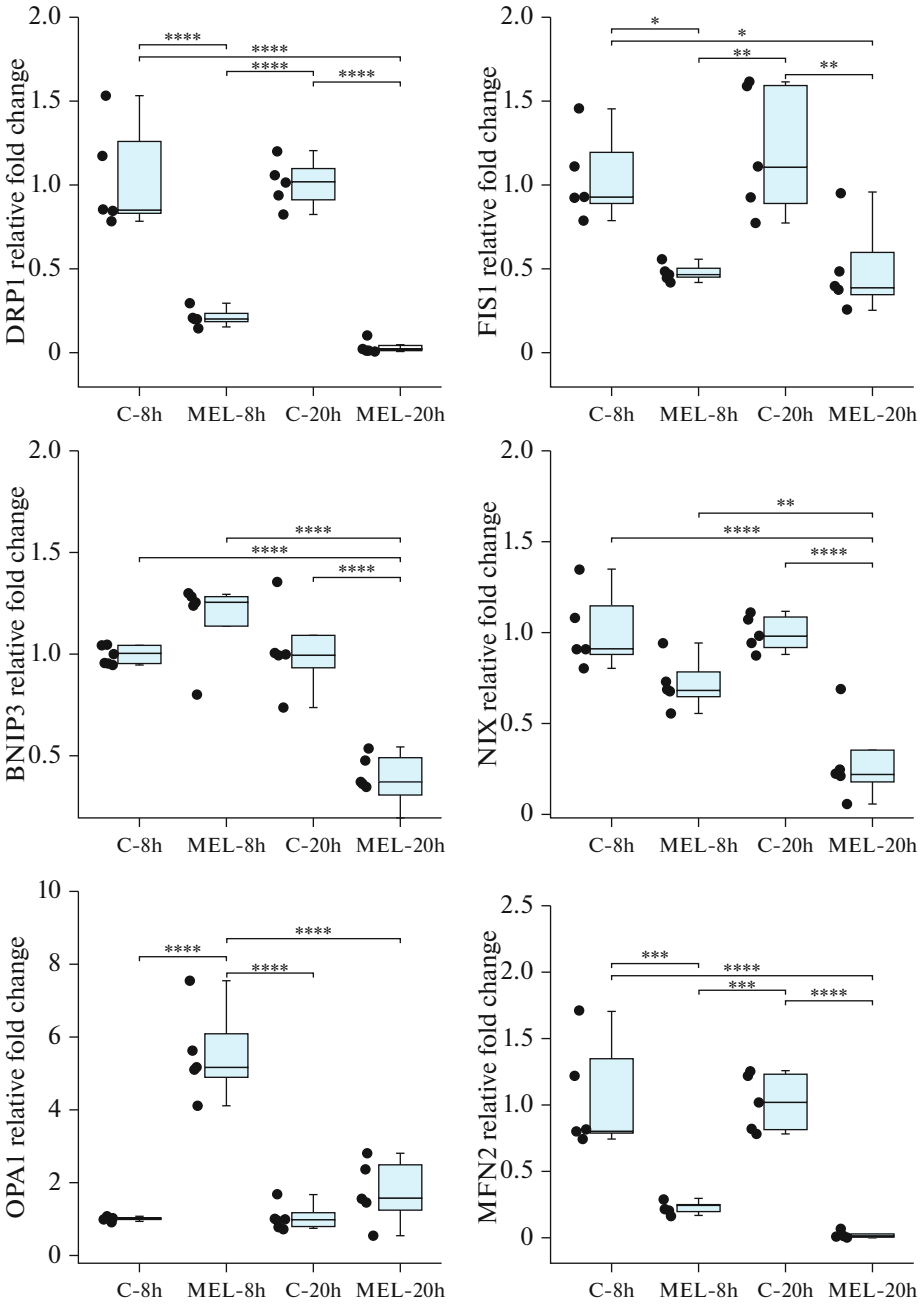


Fig. 1. Relative fold change determined by quantitative real-time PCR (qRT-PCR) analysis of mitochondrial fission (DRP1, FIS1), mitophagy (BNIP3, NIX) and fusion (OPA1, MFN1-2) in Control and Melatonin administrated LV tissue of healthy rat. All data were normalized with β-Actin expression and given as relative to control; Data were represented mean ± *SE*. * Showed significantly different value $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

and mitochondrial dynamics. They observed that reductions in the expression of PPARa and PPAR1a genes encoded in Bmal1 knockout mice by 50 and 40%, respectively. They also recorded a reduction in MFN1 and OPA1 genes, which have roles in mitochondrial quality control (Kohsaka et al., 2014). Due to the fact that both CD and mitochondrial dynamics have a diurnal rhythm, we investigated the left ventricular mitochondrial dynamics at the end of the night (08:00) and day (20:00). In our study, rats were kept in the dark for the period between 20:00–08:00 and in the light for a period between 08:00–20:00. In this way, we examined whether administering melatonin had any effect on mitochondrial dynamics during the

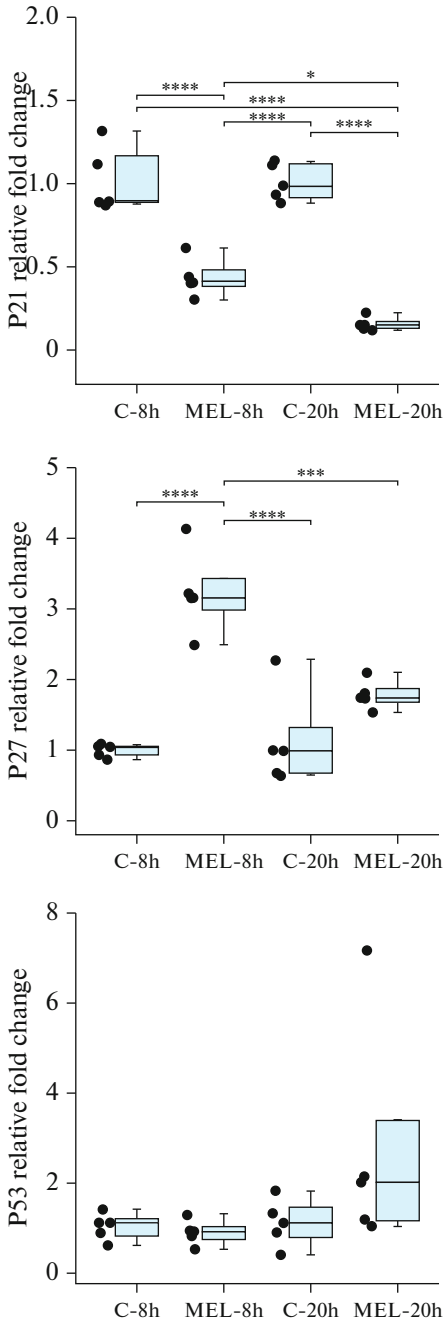


Fig. 2. Relative fold change determined by quantitative real-time PCR (qRT-PCR) analysis of cell cycle arrest (P21 and P27) and tumor suppressor (P53) genes in Control and Melatonin administrated LV tissue of healthy rat. All data were normalized with β-Actin expression and given as relative to control; Data were represented mean ± *SE*. * Showed significantly different value $P \leq 0.05$; ** $P \leq 0.01$,*** $P \leq 0.001$, **** $P \leq 0.0001$.

day-to-night cycle in the LV. Thus, a relationship between melatonin and mitochondrial dynamics has been revealed during both day and night periods.

Melatonin is a hormone secreted from the pineal gland and its antioxidant properties are well known (Tan et al., 2002). The cardioprotective effects of melatonin are also well studied (Lochner et al., 2018). Melatonin can enter the cell easily and it has been known for quite some time that mitochondria have a considerable level of melatonin, and the mitochondrial melatonin content is higher than that of the blood melatonin content (Venegas et al., 2012, Acuna-Castroviejo et al., 2014). Mitochondria is defined as an organelle that synthesis and metabolizes melatonin. Owing to this feature, mitochondria utilize antioxidant capacity of melatonin. Moreover, its reported that the MT1 receptors are located in outer membrane of mitochondria (Tan et al., 2019).

On the other hand, the heart is highly dependent on mitochondrial functions due to its high energy requirement and, as such, mitochondria cover about 30% of the heart muscle cells. It is also known that there is a relationship between changes in factors known as mitochondrial dynamics and CD. These factors (such as mitochondrial fission, fusion, and mitophagia) regulate morphology, quality, and number of mitochondria (Paradies et al., 2015; Vasquez-Trincado et al., 2016). Along with all these scientific data, studies on melatonin and its own cardioprotective effects have focused on mitochondrial dynamics. However, the studies are mostly directed towards investigating the protective effects of melatonin on damaged myocardium through the change of mitochondrial dynamics. There are limited data on the effect of giving a certain dose of melatonin to individuals without any cardiac disease on cardiac mitochondrial dynamics. In addition, there is no research that investigates the effects of daytime exposure to melatonin on left ventricle myocardial dynamics in healthy rats.

In our study, administering melatonin to the rats revealed significant reductions in left ventricular DRP1, PINK1, and FIS1 gene expression levels at both 08:00 and 20:00. Melatonin therapy also caused significant increases in OPA1 gene expression level, especially at 08:00. The balance between mitochondrial fission and fusion is very important for healthy myocardium. Unrecoverable mitochondria can be insulated with the help of the mitochondrial fission so that they can be eliminated via autophagy or mitophagy, even though mitochondrial fusion enables mitochondria to maintain membrane potential by fusing depolarized mitochondria to intact others (Twig et al., 2008). The communication of mitochondria with fission and fusion warnings plays an important role both in maintaining the functions of healthy mitochondria and repairing damaged mitochondria (Cao et al., 2019). The outer mitochondrial membrane proteins of MFN1-2 and the inner mitochondrial membrane protein of OPA1 are essential regulators of mitochondrial fusion. Conversely, FIS1 and DRP1 are known as mitochondrial fission proteins (Okamoto et al., 2005; Otera et al., 2011). The myocardium contains all of the mitochondrial fission and fusion proteins (Doll et al., 2017). It has been demonstrated that changes in

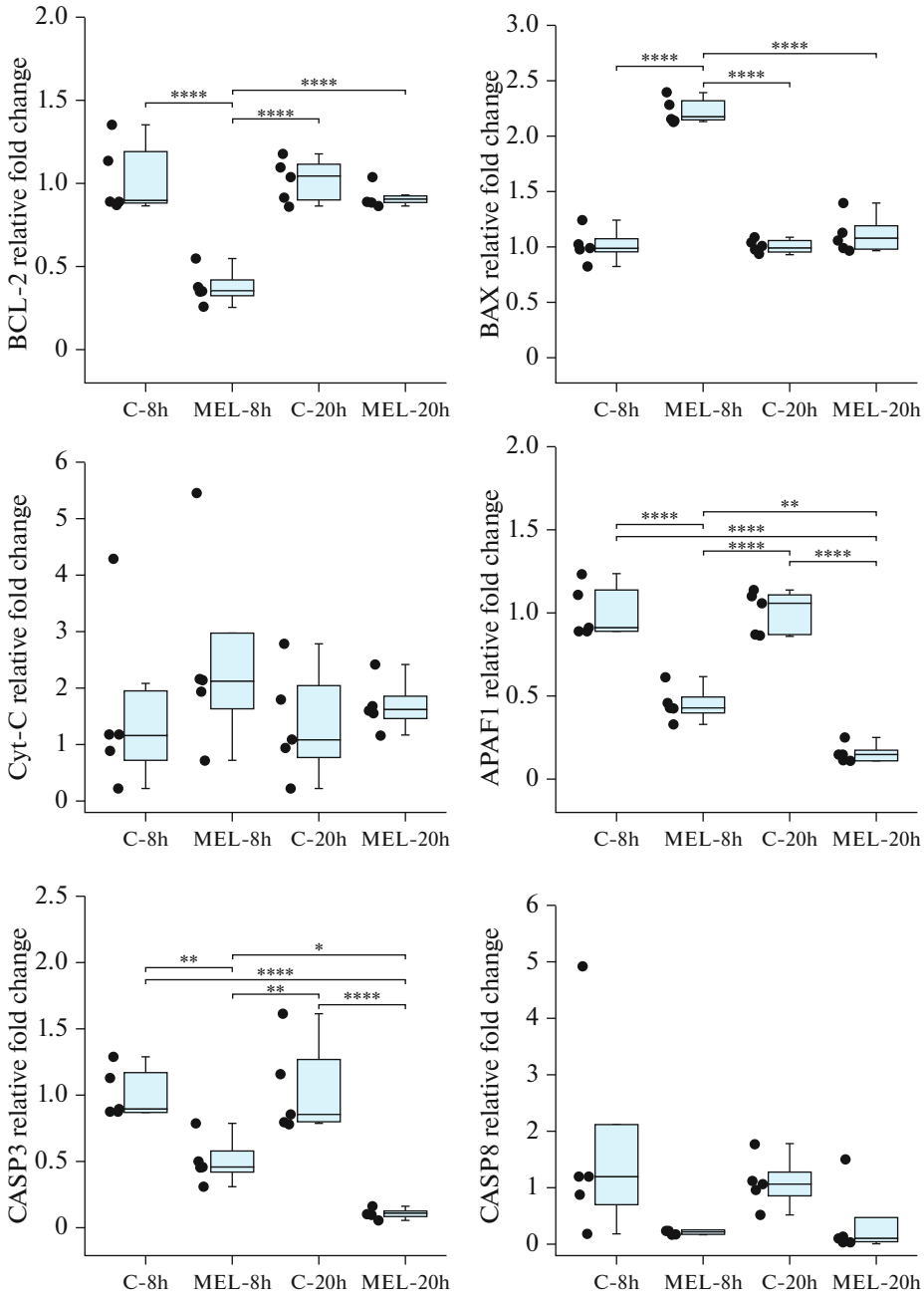


Fig. 3. Relative fold change determined by quantitative real-time PCR (qRT-PCR) analysis of mitochondrial apoptosis genes in Control and Melatonin administrated LV tissue of healthy rat. All data were normalized with β-Actin expression and given as relative to control; Data were represented mean ± SE. * Showed significantly different value $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

mitochondrial morphologies occur in myocardial diseases due to the alterations in fission/fusion controlling proteins. Thus, the change in mitochondrial morphologies affects the progression of the disease. For instance, it was reported that chronic pressure overload can lead to changes in OPA1 (Piquereau et al., 2012). In addition, DRP1-related mitochondrial fission causes myocardial death during ischemia/reperfusion (Ong et al., 2010) and the decreasing of MFN1-2 level results in cardiac dysfunction without mitochondrial fusion (Papanicolaou et al., 2012). It has been demonstrated that the administration of melatonin in the heart exposed to mechanical trauma prevents the fission by decreasing the DRP1 gene expression levels, thereby reducing the damage in the myocardium (Ding et al., 2018a). Furthermore, melatonin administration has been found to cause decreased DRP1 expression, leading to inhibited

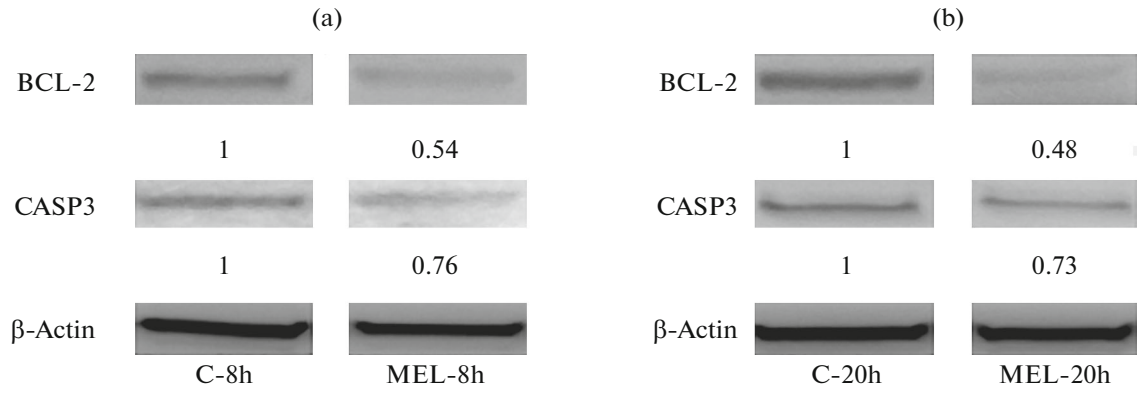


Fig. 4. The determination of the BCL-2 and CASP3 levels in the left ventricle at the 08:00 (a) and 20:00 (b) hours by western blot analysis.

mitochondrial fragmentation and reduced cardiomyocyte apoptosis in streptozotocin-induced diabetic mice (Ding et al., 2018b). Melatonin excerts its protective effect on myocardial ischemia/reperfusion on stimulating fusion. It was reported that, OPA1-related mitochondrial fusion and mitophagy are essential to maintain mitochondrial dynamics under stress conditions. Hence, it has been observed that melatonin administration in myocardial ischemia/reperfusion increases OPA1 gene expression (Zhang et al., 2019b).

When taken together, the data obtained during our study and the information above, it is apparent that administering melatonin improved mitochondrial dynamics both in the morning and in the evening. The ameliorative effects of melatonin on mitochondrial dynamics are understood from reduction in the gene expressions of the proteins (DRP1 and FIS1) that stimulate fission and the increase in the gene expressions of the proteins (OPA1) that activate fusion. Surprisingly, melatonin did not increase MFN1-2 gene expression level in our study, although it was expected. These results indicate to us that melatonin administration has stronger effects on the reduction in fission then the effects on the promotion of fusion. Moreover, it can be said that melatonin may be considered as an adjuvant molecule that decreases heart diseases, more commonly in the morning hours, and that melatonin application throughout the day will also serve a protective function for the heart muscle.

In our study, we also investigated effects of melatonin on other molecules involved in the cell mitophagy, cycle arrest and apoptosis pathway. In this regard, our study was the first to investigate the effects of administering melatonin on the molecules in the left ventricle at two different times of the day under physiological conditions. At the end of our study, it was determined that administering melatonin decreased BNIP3 and NIX gene expression levels in the heart muscle only during in the night. The hypoxia sensor genes BNIP3 and NIX implicate in promoting mitophagy (Zhang et al., 2009). In oxidative stress or hypoxia, over-expression of both BNIP3 and NIX act clearance of damaged-mitochondria response to stressful condition via mitophagic process (Chinnadurai et al., 2009; Novak et al., 2012). But high rate of mitophagy is a principle component of mithocondrial dysfunction and promote cell death (Boland et al., 2013). Our result show that the reduction in gene expression of these two molecules with melatonin administration reduces mitochondrial damage and regulates the formation of mitophagia compared to control. The cell cycle arrest, P21 and P27 play a significant role in regulate cell death (Coqueret, 2003). P27 prevent apoptosis when overexpressed, possibly through the inhibition of cytochrome c release (Eymin et al., 1999). Moreover, P27 regulates the expression of the anti-apoptotic protein MCL-1, and reduced death signals but this effect is cell-type specific (Eymin et al., 1999; Woltman et al., 2003). However, P21 over-expression is related with apoptotic cell death via P53 depended mitochondrial apoptosis pathway (He et al., 2005; Kim et al., 2017). In this study whereas melatonin administration caused upregulation of $P27^{Kip1}$ and downregulation of $P21^{Chip1}$, the main initial factor of apoptosis gene P53 did not exhibit significant change in control and experimental groups. Consistent with previous finding, in this study, melatonin regulate cell cycle and prevent P53 depended apoptotic signals in experimental groups. In the current study, melatonin has a strong inhibitory effect on both APAF1, CASP-3, and CASP-8 gene expression levels at 08:00 and 20:00. Also, in western blot analyses, we observed the decreasing of the BCL-2 and CASP3 levels by melatonin treatment. We observed that melatonin had no significant effect on BCL-2, Bax, and Cytc-c levels at 20:00. BNIP3 belongs to BCL-2 family, and it localized in the outer mitochondrial membrane which has proapoptotic effect. When BNIP3 and BNIP3L/Nix bind to BCL-2, proapoptotic proteins such as BAX and Bak are stimulated, cytochrome c is delivered due to mitochondrial outer membrane via a permabilization increase. This is fol-

lowed by caspase-dependent cell death (Gustafsson et al., 2011; Hamacher-Brady and Brady, 2016). NIX induction in the heart muscle is stimulated by Gaq in ischemic/hypoxic conditions and has been determined to lead the cell to apoptosis. It has been reported that the stimulus which initiates NIX upregulation is the PKCα molecule (Galvez et al., 2006). On the other hand, NIX levels in the myocardium are known to induce both intrinsic and mitochondrial apoptosis and are localized in the mitochondria (Yussman et al., 2002). Another molecule that stimulates mitochondrial apoptosis is P53. In our study, melatonin did not significantly change P53 gene expression levels in both hours. Li et al., reported that melatonin has protective effect on chromium-induced cardiac damage via activating the AMPK/Nrf2 pathway (Li et al., 2019). In this study, it was determined that melatonin treatment in in vivo conditions also reduced P53 gene expression levels and apoptosis. However, our study was conducted on healthy rats. In the study mentioned above, it was determined that P53 levels increased after melatonin chromium, while P53 levels did not decrease statistically when compared with MT only group. In this regard, our findings seem compatible with each other. The reason for this may be the MT application time.

In conclusion, it was observed that mitochondrial dynamics were significantly improved in rats that had melatonin added to the drinking water. These results showed that the administration of melatonin will improve cardiac mitochondrial dynamics among those without any previous heart disease. Our results also suggested that the administration of melatonin has protective efficiency on heart function as it regards to the amelioration of mitochondrial dynamics. Our findings support the hypothesis that melatonin may be defined as a cardioprotective adjuvant molecule.

FUNDING

This study was supported by Canakkale Onsekiz Mart University Scientific Research Projects with project number TSA-2018-1346.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. All the animal procedures were approved by the Local Animal Care and Use Committee of Canakkale Onsekiz Mart University (Protocol number: 2017/10-08).

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