Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

The effects of melatonin on oxidative stress and prevention of primordial follicle loss via activation of mTOR pathway in the rat ovary

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Received October 10, 2016; **Accepted** February 20, 2017; **Published** February 28, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.2.16

Abstract: Mammalian Target of Rapamycin (mTOR) signaling pathway has important roles in the regulation of puberty onset, gonadotropin secretion, follicular development and ovulation. Melatonin (N-acetyl-5-methoxytryptamine) is a lipophilic hormone has multiple functions in regulating the fertility. Recent studies have shown that melatonin affected the number or maturation of follicles in the ovary. The aim of this study was to investigate the effects of melatonin on mTOR expression and quantity of follicle in rat ovary. In the present study, a total of 45 female rats were randomly divided into three groups. Group 1; Control (C), Group 2: Vehicle (V) and Group 3; Melatonin (M). Melatonin was administered intraperitoneally at a dose of 50 mg/kg/day for 30 days in Melatonin group. The effects of Melatonin on the expression of mTOR and downstream components were determined by Western Blot and Reverse Transcriptase PCR analysis. Upon Western Blot and RT-PCR evaluations, we detected higher expression and activation of mTOR, P70S6K, PKCalpha, PCNA and higher numbers of primordial follicles in melatonin group compared with V and C group. In addition to this results, melatonin decreased oxidative stress markers, such as MDA, on the contrary, levels of antioxidative markers, such as CAT and GPx, were increased by melatonin in rat ovary. This study indicated that melatonin may have a significant protective effect on primordial follicles and increase the expression of mTOR and downstream components in rat ovary. Melatonin treatment may have a beneficial effect on fertility.

Key words: Melatonin; mTOR; Ovary; Follicle; Infertility.

Introduction

Infertility is essentially the failure to conceive offspring. Gametes from both sexes are necessary for sexual reproduction. 30% of the couples incapable of conceiving have no identifiable reason have been classified as idiopathic infertility. The treatment is contingent upon the infertility duration and the female partner's ovulation. Many researchers have contributed to clarifying the general mechanisms that drive ovarian folliculogenesis in females and they showed that complex endocrine connection between the reproductive system, the pituitary and the ovary integrated by various intraovarian paracrine messages (1).

Melatonin (N-acetyl-5-methoxytryptamine), a small lipophilic hormone which is secreted by the pineal gland, has multiple functions in regulating the fertility, which involves in lipid metabolism that adjusts biological rhythms. Melatonin also inhibits cancer growth, acting as an antioxidant molecule displaying effects on many metabolic processes (2-4). Melatonin receptors have been detected in the ovary. The interrelationship between melatonin and the reproductive hormones are suggested by many studies (5-7). Ovarian function may be affected by melatonin. Melatonin is present in ovarian follicular fluid at higher levels compared to the level in plasma and its levels change during folliculogenesis in human (8, 9). MT1 and MT2 melatonin receptors have been found in the membrane fractions of human granulosa cells in rat ovaries (antral follicles and corpus luteum) (10). Melatonin levels increase at different stages of ovarian follicular maturation and lead to the production of sex steroids (11). Positive effects of antioxidant therapy on reproductive functions in rats have been detected (5, 12). Melatonin leads to complete follicular maturation and ovulation, playing roles as an antioxidant and free radical scavenger, protecting follicles from oxidative stress (13).

mTOR signaling has pivotal roles in the integration of cellular signals from mitogens, stress and nutritions . It also regulates gonadotropin secretion and involves in the control of puberty onset (14). In recent years, researchers have focused on how the mTOR regulates the cellular processes such as cell survival, growth and proliferation. There is a crosstalk between many cellular signaling pathways to control ovarian function, specifically regulating follicular development and ovulation, in granulosa cells (15). As a mechanism of mTOR combines signals from growth factors and nutrients in many cells (16) proper signaling function during the follicle maturation may be fundamental for granulosa cells.

The effects of melatonin on the number or maturation of follicles in the ovary (16-18) and expression/activation of mTOR in the brain (12), hepatocellular carcinoma HepG2 cells have been studied in few earlier types of research (19). Primary, the focus of some of these studies in expressions of mTOR in the ovary (16-18). In this research, a similar paradigm and treat-

ment of melatonin, expression of mTOR and numbers of follicles have been focused on. This research aimed to determine the effects of melatonin upon the quantity of follicle and expression profiles of mTOR signaling pathway components in rat ovary, and to ensure a better insight on the role of melatonin in fertility.

Materials and Methods

Animals

45 female *Wistar* rats, aged between 8 to 10 weeks, with body weights from 150 to 200 grams were used. We housed the animals in eight cages (5 animals each), placing them under controlled light (12-hour light/dark cycle) and temperature (25°C) conditions. The present study was performed in accordance with the Akdeniz University Institutional Animal Care and Use Committee Policies for Animal Use under an approved animal. Rats had access to food and water *ad libitum*. 45 rats in total were randomly allocated into the following three groups, each containing 15 rats: Group 1: Control (C), Group 2: Vehicle (V) and Group 3: Melatonin (M) (Table 1).

Melatonin exposure

Melatonin (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol and diluted with normal saline. The ethanol concentration in the final solutions was 10%. Being adjusted to a dose of 50 mg/kg/day which is the optimal dose we determined in our previous study (20) and administered in the same light period (10:00 a.m.). Melatonin was intraperitoneally injected for 30 days in M group. 10% ethanol was administered to the vehicle treated animals (Group V).

Animals were sacrificed by cervical dislocation, after cessation of the 30-day melatonin treatment . For immunostaining, the ovaries were instantly fixed with 4% paraformaldehyde for 24 hours. As we obtained the blood for antioxidant analysis through cardiac puncture from after cervical dislocation, blood samples were centrifuged at 1500×g for 20 mins, separating plasma in plastic tubes stored at -20°C until analyzed.

Histomorphometric analysis of folliculogenesis and follicle count in ovaries

After cutting fifteen ovaries from each group into 5 µm cryosections by cryostat, in order to study the effect melatonin on the amount of follicle, serial sections were stained with hematoxylin and eosin (DAKO Corporation, Carpinteria, CA, USA) to count the number of follicles. By assessing every 5th section, the total number of follicles for each ovary was detected by counting the follicles that contain oocytes with a visible nucleus. Follicles were classified as primordial, primary, secondary, early antral and antral follicles (21). Primordial follicles, in brief, were identified as an oocyte surrounded by a layer of squamous granulosa cells and primary follicles have defined an oocyte surrounded by a single layer of cuboidal granu losa cells. Secondary follicles being surrounded by two or three layers of cuboidal granulosa cells with no visible antrum, early antral layers of granulosa cells that form the follicular antrum. Antral follicles contained a clearly defined single antral space.

Western blot analysis

Using RIPA tissue protein extraction reagent which contained protease inhibitor cocktail, total protein from t he ovary lysates from each rat were prepared. The protein concentrations were evaluated by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was carried out as defined earlier (22). In short, 20-µg of protein lysates were loaded into each lane, proteins separated electrophoretically by SDS–PAGE, using 7.5% Tris–HCl gels. After electrophoresis the proteins were electroblotted onto PVDF membrane (Bio-Rad Laboratories). Tris-buffered saline (TBS) that contained 5% nonfat milk for 1 h at the room temperature and incubated with primary antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Membranes have been washed in TBS-T for 15 minutes to remove unbonded primary antibodies and after the membranes were incubated with peroxidase-labeled goat anti-rabbit IgG (1:1000 dilution; Pierce; USA) and consecutively washed with TBS-T three times for 20 minutes . Using chemiluminescence detecting reagents (Perkin-Elmer Life Sciences, Boston, MA, USA) and exposure of the membrane to BioMax film (Kodak, Rochester, NY, USA), signals were detected. The same procedure was repeated for mTOR (Serin- 2448), p-mTOR, P70S6K, p- P70S6K, PKCalpha (Serin-2481), p-PKCalpha and β-actin which was used for internal control.

RT-PCR

RNA was extracted from whole ovaries in order to obtain reverse transcriptase polymerase chain reaction (RT-PCR) as shown (n=15). Following RNA extraction, total ovary tissue homogenized in 1 ml of Trizol Reagent (Invitrogen). The reverse transcription was performed, using Superscript II Reverse Transcriptase according to the manufacturer protocol. PCR reaction was carried out upon 25-µl reaction, using 2 µl of each RT sample within a reaction mix containing 1X Buffer D, 10 pmol of each primer, and Taq Polymerase. Thermal cycling conditions for all genes were 5 minutes at 95°C and PCR cycles were carried out for all target genes and control β-actin. All analyses were applied duplicated. Primers that were employed for the amplification of gene mTOR, P70S6K and PKCalpha were as follows (oligonucleotide sequences listed 5' to 3' orientation and product size follow gene name). PCR primers were prepared such that a genomic exon was expanded, and mock reversed-transcribed control samples were used versus every experimental sample (Table 2).

Table 1. Animal groups.

	n	Etanol administered	Melatonin treatment
Group 1 (Control)	15	-	-
Group 2 (Veicle)	15	10% mg/kg/day	-
Group 3 (Melatonin)	15	-	50 mg/kg/day

Table 2. Primers list.

mTOR	20 bp	sense,	TTG GAG TGG CTG GGT GCT GA
	20 bp	antisense,	AAG GGC TGA ACT TGC TGG AA
P70S6K	20 bp	sense,	CTT GGC GAA TTA AGG GCT GC
	20 bp	antisense,	GCA TAG GCC AGT TCT ACA AT
PKCalpha	20 bp	sense,	GTC CTG CAC CGG TTG GCG AA
	20 bp	antisense,	GAC CCA CAG TGA TCA CAG AA
ß- aktin	21 bp	sense,	GAT GAC GAT ATC GCT GCG CTG
	21 bp	antisense,	GTA CGA CCA GAG GCA TAC AGG

Biochemical analysis

SOD, CAT and GPx activity assay the enzymatic activity of SOD (Cayman-706002) (24), CAT (Cayman- 707002) (25) and GPx (Sigma CGP-1) (26) in the plasma, assay kits and spectrophotometric analyses were used to measure according to the manufacturer protocols, and stated in units per milliliter.

TBARS assay

Using 1,1,3,3-tetramethoxypropane as a standard (27). TBARS levels were determined by fluorometric analysis, by adding samples (50 µl) into a tube which contains 1 ml of distilled water that was then mixed with equal volumes of 29 mol/l 2-thiobarbituric acid (TBA) in acetic acid (8.75 mol/l) and placing in a water bath at 95 ºC for 1 h. 25 µl of 5 mol/l hydrochloric acid (HCl) was added to the mixture after the samples got cooled. Having been extracted with 3.5 ml of n-butanol, the final reaction mixture, was vortexed and centrifuged at 3000g for 10 min.

Data analysis

Having been compared by one –way ANOVA, groups were analyzed by post hoc Holm-Sidak test as the statistical calculations were carried out using Sigma Stat for Windows, version 3.0 (Jandel Scientific Corp., San Rafael, CA), considering $p< 0.05$ statistically significant.

Results

The results are presented in three main parts; number of the follicle, expression and activation of mTOR and downstream components and antioxidant activity.

Effect of melatonin treatment on ovarian follicular reserve

We carried out a histochemistry method in order to figure out if melatonin treatment affected the number of follicle rat ovary. Significant differences *(*$p<0.05$) were observed between groups treated with melatonin, vehicle and control as a result of the follicle count whereas no difference was detected between the control and vehicle ovaries in the number of primordial, primer, seconder, early antral, antral and degenerated follicles. We observed that the numbers and percentage of primordial follicles have reached the two -fold higher in the melatonin group *(*$p<0.05$) compared to the control group and it was also observed that many primordial follicles were added together in the melatonin group which indicated that melatonin treatment suppressed primordial follicle development, preserving the foll icular reserve. We found that %10 ethanol delivery did not affect the numbers of follicles in the vehicle group . In addition to the these results, it was also observed that the numbers and per centage of degenerated follicles in the melatonin group were 3,5 fold less than the control group, indicating that the melatonin treatment reduces follicular degeneration (Figure1).

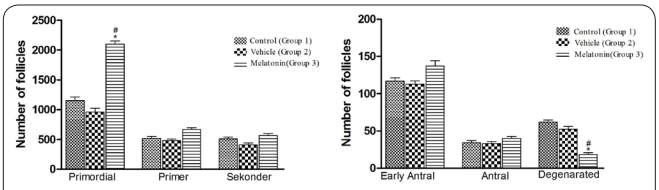

Figure 1. Follicles numbers. Comparison of the percentage of the total number of follicles between the three groups. Data are shown as means ± S.E.M.; n = 15 rat/group. *, $p< 0.05$ indicates significance from respective vehicle values.#, $p<0.05$ indicates significance from respective control values.

Morphological evaluation

It was detected that melatonin, control, and vehicle groups appeared histologically normal. The general appearance of ovaries from all groups had normal histology with a peripheral cortex, containing large numbers of follicles in various stages of development. Follicular developmental synchronization and compact stromal tissue were observed between the developing follicles (Figure 2). Oocytes in the follicles presented normal healthy histology as medullar region showed normal vessels network areas in the center of ovary. Corpus Luteum structure was also detected in all groups ovaries (Figure 2).

Expression and Activation of mTOR and its downstream components

Melatonin, vehicle and control group ovaries expressed mTOR, p-mTOR and downstream components (Figure 3). β-actin were used as internal control. Our results indicated that the expression of mTOR, phosphorylated mTOR and downstream components in the melatonin group increased compared to the control group. It was further observed that the melatonin treatment led to increase activation of mTOR signaling in ovaries. mTOR is composed of two distinct complexes, mTORC1 and mTORC2. mTORC1 ensures cell growth and autophagy largely through activation of P70S6K and through phosphorylation p-P70S6K (by phosphorylation of P70S6K Threonine 389) while mTORC2 regulates cytoskeletal dynamics and acti n organization through activation of PKCα and through phosphorylation p-PKCα (by phosphorylation of PKCα/beta (Thr638/641)) (15, 27, 28).

All the data evaluated statistically and there were

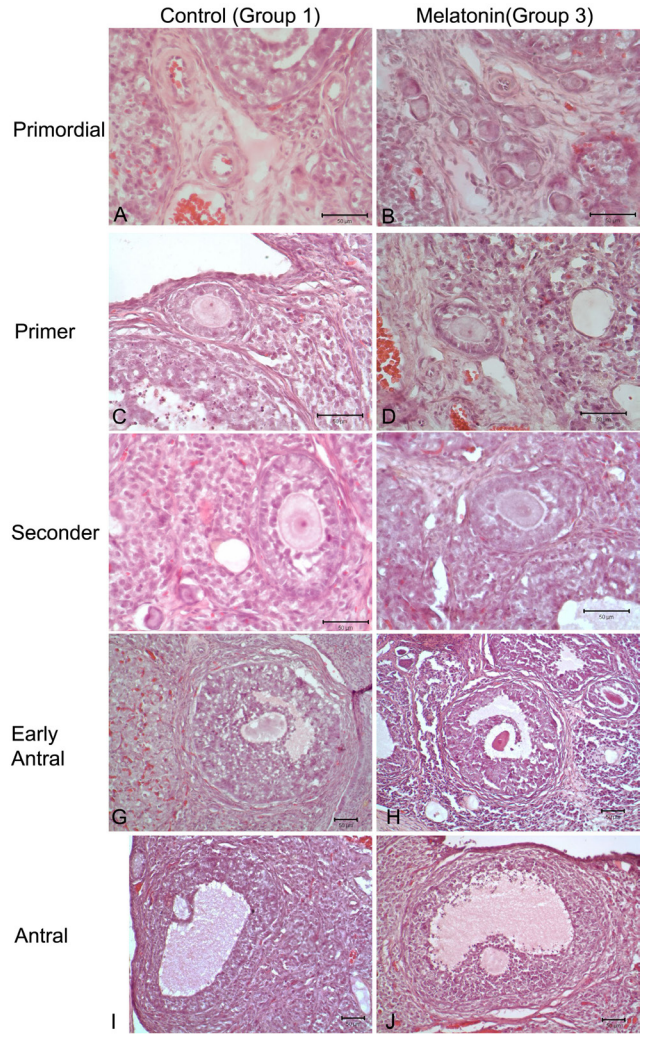

Figure 2. Histomorphometric analysis. Histomorphometric assessment of melatonin-treated rat ovary. Follicle development was evaluated in ovaries of melatonin and control rats. A, B, C, D, E, F, G, H, I, J represent high magnification micrographs of follicles from control and melatonin groups at different developmental stages. Scale bars represent 50µm.

significant differences between control and melatonin group ($p< 0.05$). mTOR and p-mTOR (serine 2448) protein levels were considerably increased in melatonin group compared to the control group (Figures 3) ($p< 0.05$). mTORC2 is activated by phosphorylation of serine-2481 region of mTOR. P70S6K protein levels showed difference among three groups (Figure 3) and mTORC1- dependent phosphorylation of P70S6K (p-P70S6K1) was increased in melatonin group (Figure 3) ($p< 0.05$). There were differences of PKCα and p-PKCα/beta (Thr638/641 phosphorylation) signaling (Figure 3) ($p< 0.05$). PKCα and p-PKCα/beta (Thr638/641 phosphorylation) were increased in the melatonin-treated group.

Expression of PCNA (Set 8)

For the detection of differences in proliferation between three groups western blot analysis used with PCNA antibody were applied (Set 8). We observed that PCNA signaling in melatonin group was significantly increased compared to the control and vehicle groups. (Figure 3) ($p< 0.05$).

Reverse Transcriptase- PCR (RT-PCR)

Reverse transcription polymerase chain reaction analysis of mTOR, p-mTOR, P70S6K, p-P70S6K, PK-Calpha, p-PKCalpha genes showed that the key components of both mTORC1 and mTORC2 were expressed in three groups of ovaries. (3 unique repeats, Ov1-3). β-actin were used as internal control (Figure not shown).

Effects of melatonin treatment on ovary antioxidant enzymes activities and MDA levels

The summary of the antioxidant enzyme activities and MDA values measured in the plasma of experimental groups are shown in Figure 4.

Melatonin group was observed to have a 78 % reduction tendency in plasma MDA level compared to control and vehicle group. Melatonin treatment significantly decreased the levels of MDA in melatonin group ($p<0.05$) (Figure 4).

The plasma's antioxidant enzyme activity analysis showed a beneficial effect of melatonin treatment. A significant increase in plasma SOD activity was observed in melatonin group compared to control and vehicle group. However, there were no detectable differences in SOD activity levels between control and vehicle groups (Figure 4).

CAT enzyme activity was observed significantly different between the study groups. The CAT level was higher in melatonin group than control and vehicle groups ($p<0.05$). Additionally, no significant difference was detected in CAT enzyme activity of control group compared to the vehicle group ($p> 0.05$) (Figure 4).

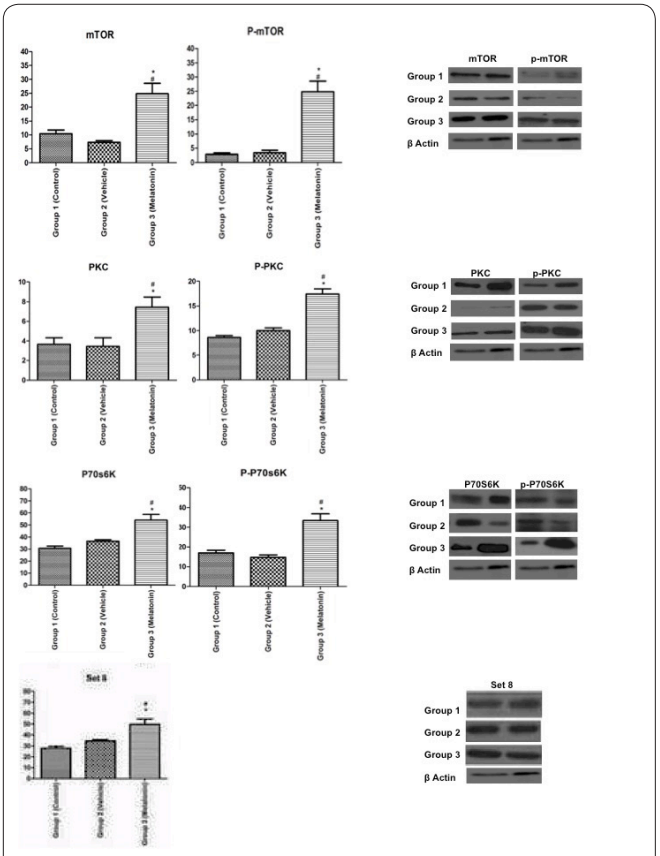

Figure 3. PCNA (Set 8), mTOR and downstream components protein expression. Expressions and activations of mTOR and its downstream components PKC, p70S6K and PCNA (Set8) expression in group 1 (Control), group 2 (Vehicle), group 3 (Melatonin). Data are shown as means ± S.E.M.; n = 15 rats/group.*, $p<0.05$ indicates significance from respective control values. #, $p<0.05$ indicates significance from respective vehicle values.

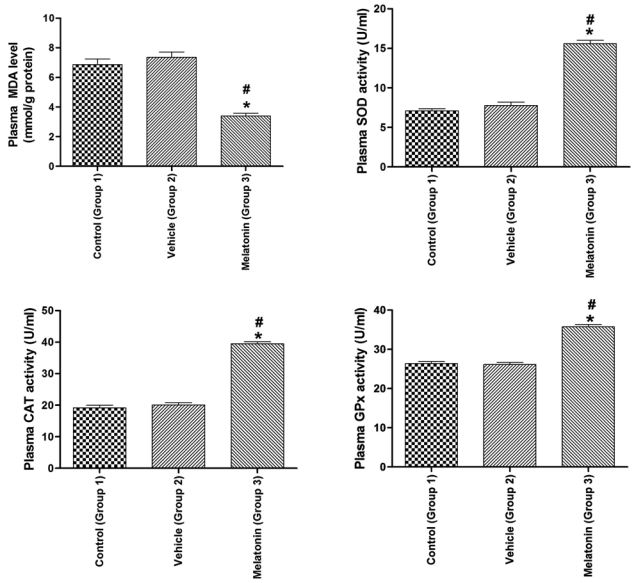

Figure 3. Antioxidant enzymes and MDA levels. Diagram displaying antioxidant enzymes and MDA levels of animal groups. Note that MDA levels were significantly reduced while antioxidant enzymes were significantly increased after melatonin administration in both experimental paradigms.Data are shown as means ± S.E.M.; n = 15 rat/group. *, $p<0.05$ indicates significance from respective control values, #, $p<0.05$ indicates significance from respective vehicle values

The GPx activity levels of the experimental groups were shown in Figure 4. We detected that GPx levels were significantly higher in the melatonin group than control and vehicle groups. ($p<0.05$). However, the GPx level in the control group was not remarkably different from the vehicle group.

Discussion

In earlier studies (29,30), The anti-inflammatory, antioxidant, antiapoptotic effects of melatonin have been displayed findings reached after the clinical use of melatonin has been studied, reveal new methods for managing several diseases including, diabetes, cancer, immunological diseases, Alzheimer's disease and viral infections (31, 32). It has been found that the safety of exogenous melatonin has been revealed along with the fertilization rate increase and oxidative stress decrease in follicular fluid (10). Along with the findings such as the fertilization rate increase and oxidative stress decrease in follicular fluid (10), exogenous melatonin has been observed safely. Melatonin, therefore, seems to be effective as an agent for the treatment of such diseases and their oxidative stress related complications in experimental models.

Oxidative stress is the result of disturbance of balance between the formation of free radicals and the scavenging of these radicals in the body. The MDA levels measurement which is a product of lipid peroxidation determine the damage of these radicals in the cells. Our results were in line with the literature in which melatonin treatment was shown to decrease lipid peroxidation in rats ovary (33, 34). In addition, we observed no significant change MDA level in vehicle and control group ($p>0.05$) while the level of MDA in melatonin group was significantly decreased ($p<0.05$). Depending on given results, we think that melatonin improves the circulating biomarkers of oxidative stress in rat ovary.

Oxidative status of the ovary undergoing seasonal growth has been determined by measuring the levels of different enzymatic agents (SOD, CAT, GPx). In antioxidant defense, antioxidative enzymes, such as SOD, CAT, GPx play a significant role in metabolizing radicals or ROS into non-radical products (35). Melatonin has been shown to enhance the activity of these antioxidant enzymes in animal tissues in which oxidative stress was induced by different agents (36,37). By raising the mRNA for SOD (38), first Antolin et al. demonstrated that melatonin has a direct cytoprotective effect; then supporting the idea that melatonin treatment stimulates the activity of antioxidative enzymes in rats (39,40) several researches followed. Klepac et al., recently reported that the activity of SOD (19%) and CAT (4%) in plasma of rats increased by melatonin. Adding Chang et al.'s results which stated that lipid peroxidation levels were reduced by 25% and 57.25% with 10 mg/kg and 50 mg/kg doses of melatonin, respectively, our findings were also in line with all the other researches given above. We, therefore, agreed with the hypothesis that melatonin reduces oxidative stress effectively in pharmacological amounts and it may also protect cells against oxidative damage by increasing SOD, CAT, and GPx activities.

The results of our study showed that melatonin treatment causes a two-fold increase in the number and percentage of primordial follicles in addition to the primordial follicle aggregation which suggest that melatonin may inhibit the activation of primordial follicles. Much as the number of primary and secondary follicles was not significantly affected ($p>0.05$), melatonin was observed to be decreasing the number and percentage of degenerated follicles, suggesting melatonin may inhibit follicular atresia.

In this research, along with the stated studies above, we investigated mTOR and its downstream effectors in melatonin -treated rat model. The mTORC1 and mTORC2 have vital roles in different pathways as nutrient sensing and energy, cell growth, metabolism, and differentiation (41, 42). Yaba at all. formerly indicated that mTOR is ubiquitously expressed in mouse ovary with predominantly cytoplasmic and perinuclear expression in granulosa cells, whereas the p-mTOR (serine 2448) is strongly enriched within mitotic granulosa cells and localizes in the region of the mitotic spindle and also near actin filament containing structures that include the contractile ring of cytokinesis (43). In our study, we observed that expression and activation of mTOR were elevated in the melatonin group than control and vehicle group. In this given situation we claim that serine-2448 phosphorylated form of mTOR may be responsible for increased granulosa and theca cell proliferation in melatonin-treated rat ovary. Our PCNA western blot results confirmed that melatonin-treated rat ovary showed increased proliferation compared the other groups. mTOR regulates cell growth and proliferation functions through cytoplasmic targets such as P70S6K, which is well defined downstream signal protein of mTORC1. As our results claimed that activation of mTORC1 has a st imulatory effect on P70S6K and p-P70S6K protein level showed no difference as compared to P70S6K. In our research, we detected p-

P70S6K protein expression increase in melatonin group and claimed that P-P70S6K might increase melatonin-treated rat ovary due to functional folliculogenesis and that insufficient P70S6K activation causes the arrest of follicular development.

PKCalpha is a crucial protein in cell proliferation, differentiation and apoptosis. mTORC2 is responsible for the phosphorylation of PKCalpha and in post-translational processing (27, 28). In our research, we found that PKCalpha and its phosphorylated form p-PKCα/beta (Thr638/641) displayed the same protein level in control and vehicle groups. In addition to this, difference between melatonin-treated and control group for PKCalpha and p-PKCα/beta (Thr638/641) protein levels was detected. We consequently indicated that, in melatonin-treated rat ovary, mTORC2 may also use downstream signal proteins in addition to PKCalpha.

It is observed that more women experience premature ovarian failure. Women diagnosed with premature ovarian failure experience anger, depression, anxiety, loss and sadness as the diagnosis is unexpected and upsetting, and this point is often underestimated. The diagnosis gets even more upsetting if the woman or couple has no child (44). Therefore curing premature ovarian failure with an effective way would have significant benefit. Indicating that melatonin treatment might be an effective on treating the female follicular reserve. This study optimistically suggests the melatonin use an effective treatment for premature ovarian failure in the future.

As a conclusion, melatonin treatment could be suggested to promote the ovarian lifespan of female rats by suppressing the activation of primordial follicles, the development of follicles at different stages and the follicle maturation and degeneration through modulating activation and expression of mTOR pathway. We believe that the understanding of the inter-relationship between melatonin and mTOR signaling will prove to be effective to design the rapeutic strategies for ovarian associated diseases.

Acknowledgments

The authors would like to thank Necdet Demir, Prof. Dr, and Aylin Yaba, Asst. Prof. Dr. for his valuable contributions to this project. The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was partly supported by Scientific and Technological Research Council of Turkey (TUBITAK)—Project 213S049.

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