Melatonin regulates the ovarian function and enhances follicle growth in aging laying hens via activating the mammalian target of rapamycin pathway

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ABSTRACT The signal pathway of target of rapamycin (**TOR**) plays an important role in regulating cell growth and proliferation, follicular development, and ovulation. Melatonin (N-acetyl-5-methoxytryptamine) (**MT**) is involved in the regulation of many physiological functions in animals. Recent studies have shown that MT affects the number and the degree of maturation of follicles in the ovary, but there are few studies concerning its mechanism. Therefore, the aim of this study was to investigate the mechanism of TOR signal pathway in the regulation of ovarian function by MT in aging laying hens. In the present study, a total of 60 hens (70-week-old) were randomly divided into 2 groups: control group and melatonin group (**M**). Melatonin was administered intraperitoneally at a dose of 20 mg/kg/D for 28 D in the M group. The results showed that MT significantly increased the levels of the antioxidant enzymes superoxide dismutase and total antioxidant capacity ($P < 0.01$) as well as levels of immunoglobulin (IgA, IgG, and IgM) ($P < 0.05$) and the reproductive hormones estradiol and luteinizing hormone ($P < 0.01$) in the plasma and also increased the numbers of middle white follicles and small white follicles ($P < 0.05$) and decreased the level of reactive oxygen species in plasma ($P < 0.01$) in laying hens. There were higher expression levels in MT receptor A ($P < 0.05$), melatonin receptor B ($P < 0.01$), and tuberous sclerosis complex 2 ($P < 0.01$). Activation of TOR, 4E binding protein-l (4E-BP1), and ribosomal protein 6 kinase ($P < 0.01$) was found in the M. The levels of mTOR and p-mTOR protein were increased in the M ($P < 0.05$). The mTORC1-dependent 4E-BP1 and p-4E-BP1 were increased in the M ($P < 0.05$). This study indicated that MT may enhance follicle growth by increasing levels of antioxidant enzymes and reproductive hormones and by activating the mTOR and downstream components in aging laying hens.

Key words: melatonin, mTOR, aging, follicle, hen

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INTRODUCTION

In the avian ovary, aging and the associated dysfunction may be the main reason for the decline in the rate of egg production. It is well known that laying hens have a series of problems during the later laying period, including lower laying performance (Roland, 1979; K.C. and Williams, 1992), lower numbers of follicles in the ovary (Zakaria, et al., 1983), higher rates of egg breaking (Albatshan, et al., 1994), as well as apoptosis and follicular atresia (Lillpers and Wilhelmson, 1993) and longer average intervals between egg production (Grossman, et al., 2000). In addition, the follicle pool is depleted during the later laying period (Faddy, 2000). How to extend the laying period of laying hens and achieve the goal of 500 eggs by 100 wk of age has been one of the areas of focus for foreign breeding companies in recent years. However, the mechanism by which the ovary ages at an accelerated rate during the later laying period is still not fully understood. Therefore, protecting ovarian aging and preventing ovarian dysfunction are the primary goals.

Melatonin (N-acetyl-5-methoxytryptamine; MW = 232) (**MT**) was first discovered in the pineal gland in the 1950s (Lerner, et al., 1958). Melatonin is involved in the regulation of many physiological functions in animals (Reiter, 1991; Morgan, et al., 1994), including female reproduction (Wang, et al., 2014), innate immunity (Zhou, et al., 2016), and the capacity of scavenging reactive oxygen species (**ROS**) (Zhang, et al., 2006); in

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addition, MT possesses antiradiation (Fernández-Gil, et al., 2017), anticancer (Söderquist, et al., 2016), and antioxidant properties (Reiter, 1993; Mehaisen, et al., 2015). Ovarian function may be affected by MT. The previous studies have reported that MT-binding sites are present in the ovaries of chickens (Ayre, et al., 1992; Sundaresan, et al., 2009). The concentration of MT in the blood was inversely proportional to the age of chickens, and positive effects of antioxidant therapy on reproductive functions in chickens have been reported (Jia, et al., 2016). Melatonin and its metabolites promote follicle maturation and ovulation because of the scavenging of free radicals (Ressmeyer, et al., 2003; Annia, et al., 2011) and the associated antioxidant properties (Roland, 1979; K.C. and Williams, 1992).

The signal pathway of mammalian target of rapamycin (**mTOR**) plays a pivotal role in the integration of cellular signals from mitogens, stress, and nutrition. Mammalian target of rapamycin has effects on regulating cell growth and proliferation (Cheng, et al., 2015). The signal pathway of mTOR can integrate growth factors and nutrients in many cells, so it may be the basis of follicle maturation in granulosa cells. Currently, researchers have found that the signal pathways of mTOR regulate ovarian function and granulosa cell development and ovulation (Dowling, et al., 2009). The effects of MT on the number or maturation of follicles in the ovary (Meredith, et al., 2000; Tamura, et al., 2017) and expression/activation of mTOR in the brain (Ding, et al., 2014) as well as its effects on hepatocellular carcinoma HepG2 cells have been reported previously (Bennukul, et al., 2014). The specific regulation mechanism of TOR on chicken granulosa cells is still unclear.

Previous studies concerning MT in chickens focused on the period of egg-laying peak or earlier (Moore and Siopes, 2002; Rozenboim, et al., 2002; Maddineni, et al., 2008; Mcguire, et al., 2011). However, there are no studies related to the effects of MT on laying hens beyond the egg-laying peak. In addition, delaying ovarian aging and reducing the excessive consumption of the primordial follicle pool after the egg production peak are particularly important for improving the performance of aging laying hens. Therefore, we examined whether MT treatment would defer ovarian aging in laying hens, and we investigated whether the signal pathway of TOR is involved in the regulation of follicular development by MT. We explored the mechanism of the signal pathway of TOR in MT-regulated ovarian aging in laying hens.

MATERIALS AND METHODS

Animals

Sixty birds (initial weight = 1.4 ± 0.17 kg) aged 70 wk from Breeding Poultry of Hebei Dawu Group (Baoding, China) were divided into 2 groups. The 2 groups were as follows: (1)control group (**CON**) and (2) melatonin group (**M**). The experiment lasted 28 D. All birds were individually maintained in cages. The birds were housed under standard conditions of temperature (23°C ± 2°C), relative humidity (65% ± 10%), and light (16:8 h light/dark), which is the light time of the laying hens in the late laying period. Water and diets were available ad libitum during the entire experimental period. Before the beginning of the experiment, all birds were housed in cages for 7 D to habituate before all drug injection and behavioral testing. All experimental procedures took place in the same room in which the habituation took place.

During the experiment, all animal procedures strictly followed the recommendations of the relevant national and local animal welfare bodies. Protocols were approved by the Animal Care and Use Committee of Hebei Agricultural University (permit number: 2,019,010).

Melatonin Exposure

Melatonin (Sigma, St. Louis, MO) was dissolved in absolute ethanol and diluted with normal saline. The ethanol concentration was 10% in the final solutions. Birds of the M were intraperitoneally injected with a dose of 20 mg/kg/D at the same time every day (16:00 h) for 28 D.

Determination of Melatonin, Total Antioxidant Capacity, Superoxide Dismutase, Reactive Oxygen Species, Estradiol, Progesterone, Follicle-Stimulating Hormone, and Luteinizing Hormone as Well as Immunoglobulin A, G, and M Concentrations in Plasma by ELISA

At day 1, 7, 14, 21 and 28, all chickens had 5 mL blood collected from under the wing vein. Blood samples were centrifuged at 3,000 r/min for 15 min. Plasma was separated from the blood and was stored at −20°C to determine blood parameters. Levels of MT, ROS, total antioxidant capacity (**T-AOC**), superoxide dismutase (**SOD**), estradiol (**E2**), progesterone (**P4**), follicle-stimulating hormone (**FSH**), and luteinizing hormone (**LH**) as well as immunoglobulin A, G, and M (**IgA**, **IgG**, and **IgM**) were measured by ELISA using commercial kits (Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China), in accordance with the manufacturer's instructions. The assay limits of detection, limits of quantification, intra-assay coefficients of variation (%), and inter-assay coefficients of variation (%) were MT (0.1 pg/mL, 2.5 pg/mL, 10, 15%), ROS (1.0 U/mL, 15 U/mL, 10, 15%), T-AOC (0.1 U/mL, 0.75 U/mL, 10,15%), SOD (0.1 ng/mL, 0.15 ng/mL, 1 0, 15%), FSH (0.1 mIU/mL, 0.25 mIU/mL, 10, 15%), LH (1.0 ng/mL, 5 ng/mL, 10, 15%), E2 (1.0 pg/mL, 10 pg/mL, 10, 15%), P4 (1.0 pmol/mL, 37.5 pmol/mL, 10, 15%), IgA (1.0 μg/mL, 10 μg/mL, 10, 15%), IgG (1.0 μg/mL, 25 μg/mL, 10, 15%), and IgM (1.0 μg/mL, 10 μg/mL, 10, 15%), respectively.

Ovarian Follicle Counting

At the end of the experiment, 10 chickens randomly selected from each group were euthanized by cervical dislocation, and the ovaries were removed. The number and size (diameter) of the follicles were recorded. Follicle sizes were determined based on the following categories: (1) preovulatory follicle, **POF**: >10 mm; (2) small yellow follicle, **SYF**: 8 to 10 mm; (3) large white follicle: 6 to 8 mm; (4) medium white follicle, **MWF**: 4 to 6 mm; (5) small white follicle, **SWF**: 2 to 4 mm; and (6) primary follicle: < 2 mm (Johnson, 2015).

RNA Extraction and Real-Time PCR Analysis

At the end of the experiment, 2 chickens from each treatment replicate were euthanized, and the ovaries were immediately removed and frozen at −80°C. Expression levels of *TOR, 4E-BP1, S6K, PKC, TSC1, TSC2, MELRA,* and *MERLB* genes in the tissue samples were measured using real-time RT-PCR. Total RNA was isolated from ovaries using a total RNA extraction kit (Invitrogen, 12,183-555, Waltham, MA) in accordance with the manufacturer's instructions. The quality and concentration of extracted RNA were determined by agarose gel electrophoresis and nucleic acid quantification, respectively. The latter was performed using a nucleic acid quantification analyzer (Smart Spec Plus BIO-RAD). The total cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, 11,752-050) in accordance with the manufacturer's instructions. Successful cDNA synthesis was confirmed by amplifying the β-actin amplicon via PCR. The synthesized cDNA was amplified using a 20-μL PCR reaction system containing 1 μL cDNA, 10 μL Power SYBR Green PCR Master Mix (Roche, 4,913,914,001, CH,GER), 0.5 μL PCR Forward Primer, 0.5 μL PCR Reverse Primer (Huada Biological Engineering Technology & Service, Beijing, China), and 8 μL ddH_2O. PCR products were verified by electrophoresis on 1% agarose gels and subsequent DNA sequencing. Standard curves were generated using pooled cDNA. The sequences of primers for qPCR are listed in Table 1. The relative expression level of each gene was calculated in triplicate for each sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western Blot Analysis

Total protein from the ovary lysates of each hen was extracted by T-PER tissue protein extraction reagent (Thermo Pierce, 78,510, Rockford, IL) that contained a protease inhibitor cocktail (Thermo Pierce, 78,440). The protein concentrations were evaluated by the BCA Protein Assay (Angle Gene Technologies, VB0002, CHN). That is, 20 μg protein lysate was loaded into each lane, and proteins were separated electrophoretically by SDS–PAGE using 7.5% Tris–HCl gels. After electrophoresis, protein was electroblotted onto a PVDF membrane (Merck Millipore, IPVH00010, Burlington, MA). Tris-buffered saline (**TBS**) that contained 5% nonfat milk was placed for 1 h at room temperature and then was incubated with primary antibodies overnight at 4°C. Membranes were washed in TBS-T for 15 min to remove unbonded primary antibodies (Cell Signaling Technology, Danvers, MA), and then, the membranes were incubated with Goat Anti-Rabbit IgG H&L (HRP) (Abcam, ab6721, Cambridge, MA, 1:5,000 dilution) and consecutively washed with TBS-T 3 times for 20 min. Chemiluminescence ECL detecting reagents (GE, RPN810, Chicago, IL) and exposure to the Super Signal West Dura Extended Duration Substrate (Thermo Pierce, 34,075) signals were detected by chemiluminescence ECL detecting reagents (GE, RPN810, USA) and exposure to the Super Signal West Dura Extended Duration Substrate (Thermo Pierce, 34,075). The same procedures were repeated for analysis of mTOR (CST, 2,972, 1:1,000 dilution), P-mTOR (CST, 2,971, 1:500 dilution), 4EBP1 (CST, 9,452, 1:1,000 dilution), p-4EBP1 (CST, 9,451, 1:1,000 dilution), PKC (Abcam, ab31, 1:500 dilution), p-PKC

Table 1. Primers list.

Gene	GenBank accession	Primer sequences (5′to3′)	Size (bp)
TOR	XM_417614	CACAACCACTGCTCGCCACAA CCATAGGATCGCCACACGGATTAG	124
4E-BP1	XM_424384	GCGAATGTAGGTGAAGAAGAG AACAGGAAGGCACTCAAGG	146
S6K1	AW_239721	CAATTTGCCTCCCTACCTCA AAGGAGGTTCCACCTTTCGT	176
PKC	NM_00102804.1	GGCGGACAGGAAGAATACAGAGG GAAGCTGTGTCAGGAATGGTGGTT	146
MELRA	NM_205,62.1	ATGTTGGTCTTATCTGGGTC ATGGGAAGTATGAAGTGGAA	160
MELRB	NM_001,293,103.1	ATTCATTTCATCGTCCCTAT TTTCAGTCTTGGCTTTGTTT	111
TSC1	XM_415449.6	CTGAGCCCGCAGTCAATAAA AGGTTGGTGGAGTGGTCATA	94
TSC2	XM_015,294,446.2	CTTACGTGTTGGTGCCTTAGA GTGTCTGTGGAGCAGGATTAG	125
β-actin	XM_424,84	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	152

(CST, 2,261, 1:500 dilution), and β-actin (Abcam, ab8226, 1:1,000), which was used for an internal control. In addition, the antibodies were all validated previously for use with chicken samples (Young, et al., 2010; Liu, et al., 2015).

Data Analysis

Data were analyzed using SPSS 19.0 (IBM Corporation, Armonk, NY). Differences in parameters were analyzed by independent sample t tests. Data were expressed as means ± SD A level of $P < 0.05$ was set as the criterion for statistical significance.

RESULTS

Effects of Melatonin on Antioxidant Enzymes Activities and ROS of Plasma in Aged Laying Hens

The antioxidant enzyme activities and levels of MT and ROS in the plasma of aged laying hens injected with or without MT are shown in Figure 1. The results indicated that MT concentration in the plasma under MT treatment was significantly increased compared with the CON ($P < 0.05$). Melatonin had a beneficial effect on antioxidant enzyme activity in the plasma of aged laying hens. At day 21 and 28, significant increases in SOD and T-AOC activities in the plasma of hens were observed in the M compared with the CON ($P < 0.05$). At day 14, 21, and 28, MT significantly decreased the levels of ROS in the plasma of aged laying hens ($P < 0.05$).

Effects of Melatonin on Reproductive Hormones

The concentrations of reproductive hormones in the plasma of aged laying hens for each treatment are shown in Figure 2. The results showed that at day 7 and 14, the concentration of E2 of plasma in the MT-treated group was higher than in the CON ($P < 0.01$). The LH concentration of plasma was significantly increased at day 28 in the M compared with the CON ($P < 0.01$). Melatonin-treatment did not influence the plasma levels of P4 or FSH in aged laying hens ($P > 0.05$).

Effects of Melatonin on Immunoglobulins

The concentrations of immunoglobulins in the plasma of aged laying hens injected with or without MT are shown in Figure 3. IgA concentration in the plasma had increased at day 28 in the M compared with the CON ($P < 0.05$). At day 14 and 28, the concentrations of IgG and IgM in the plasma were higher in the MT-treated group ($P < 0.05$).

Effect of Melatonin on Laying Rate and Ovarian Follicular Numbers

The effect of MT on laying rate in aged laying hens is shown in Figure 4A. We found that the laying rate of the CON showed a decreasing trend with the increase of the

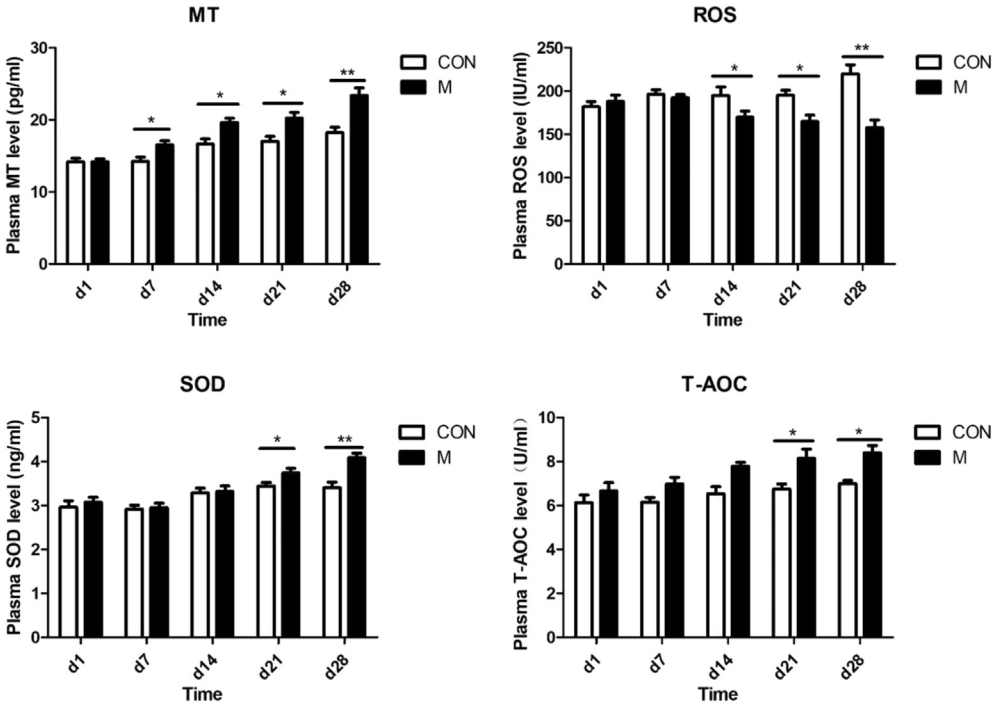

Figure 1. Effects of melatonin treatment on MT , ROS levels, and antioxidant enzymes in the plasma. Diagram displaying MT , ROS levels, and antioxidant enzymes of animal groups. Data are shown as means ± SD; n = 12 chicken/group. *$P < 0.05$ indicates significance from respective control values, **$P < 0.01$ indicates extremely significance from respective control values. MT, melatonin; ROS, reactive oxygen species; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; CON, control group; M, melatonin group.

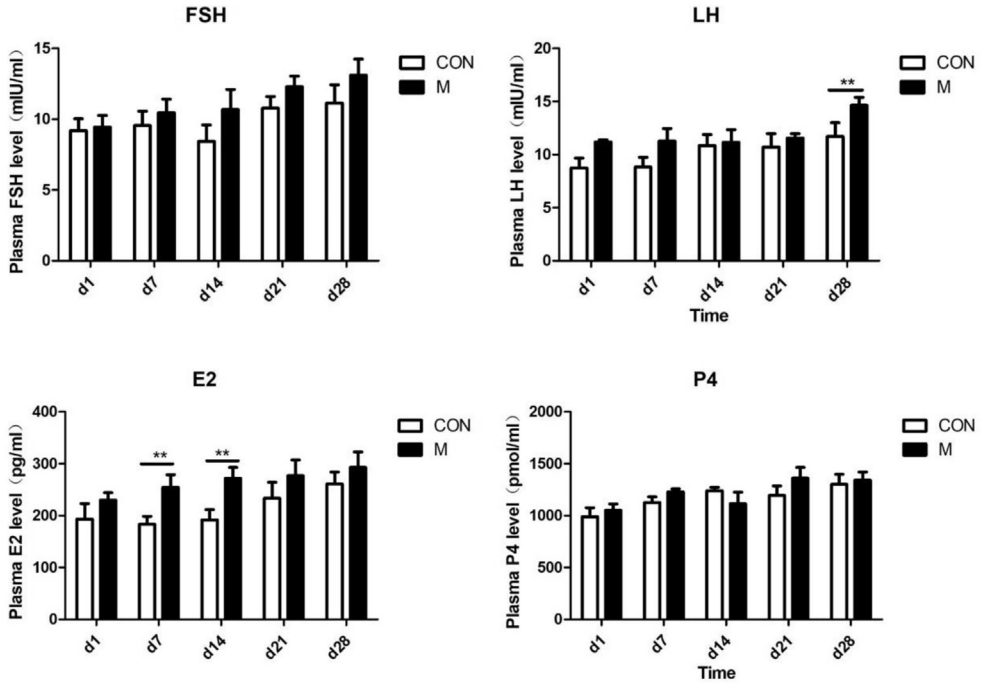

Figure 2. Effects of melatonin treatment on reproduction index in the plasma. Diagram displaying FSH, LH, E2, and P4 levels of animal groups. Data are shown as means ± SD; n = 12 chicken/group. $^{**}P < 0.01$ indicates extremely significance from respective control values. E2, estradiol; P4, progesterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; CON, control group; M, melatonin group.

test period. Although the laying rate of the MT treatment group decreased at day 7, it increased with the extension of the test cycle and was significantly higher than that of the CON at day 28 ($P < 0.05$).

The numbers of ovarian follicles in aged laying hens for each treatment are shown in Figure 4B. The results indicated that the numbers of MWF and SWF were significantly different ($P < 0.05$) in aged laying hens between the CON and the MT-treatment group.

Reverse Transcriptase-PCR

Effects of MT on mRNA expression of *MELRA, MELRB, TSC1, TSC2, TOR, 4E-BP1, S6K,* and *PKC* in the ovary are shown in Figure 5. Melatonin significantly increased the expression of MT receptors MELRA and MELRB in the ovary ($P < 0.05$) (Figure 5A and B). Melatonin significantly increased TOR upstream components of TSC2 in the ovary compared with the CON ($P < 0.01$) (Figure 5D). The relative mRNA expression of TOR and its downtown components had significantly elevated mTORC1 in the MT-treated group, whereas this was not observed in mTORC2. The expression levels of *TOR, 4E-BP1,* and *S6K* were significantly increased in the MT-treated group ($P < 0.01$) (Figure 5E−G). The expression level of PKC in the ovary was significantly decreased in the M compared with the CON ($P < 0.01$) (Figure 5H). It was further observed that MT led to increased activation of mTORC1 signaling in the ovaries.

Western Blot

Effects of MT on mTOR and protein expression of downstream components in the ovary are presented in Figure 6. There were significant differences between

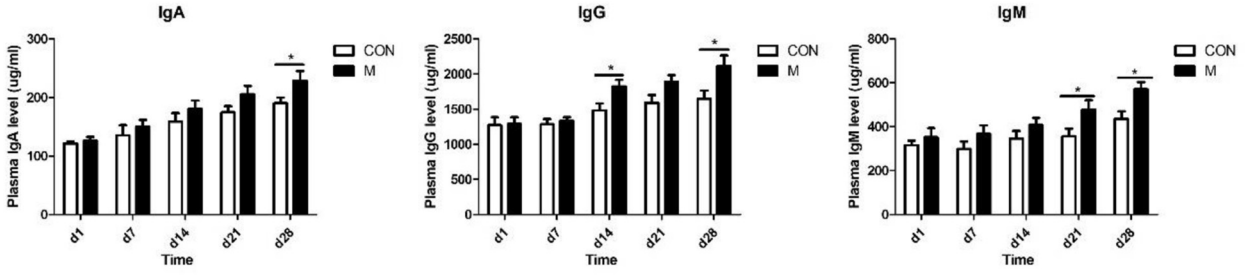

Figure 3. Effects of melatonin treatment on immune index in the plasma. Diagram displaying IgA, IgG, and IgM of animal groups. Data are shown as means ± SD; n = 12 chicken/group. $^{*}P < 0.05$ indicates significance from respective control values. IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.

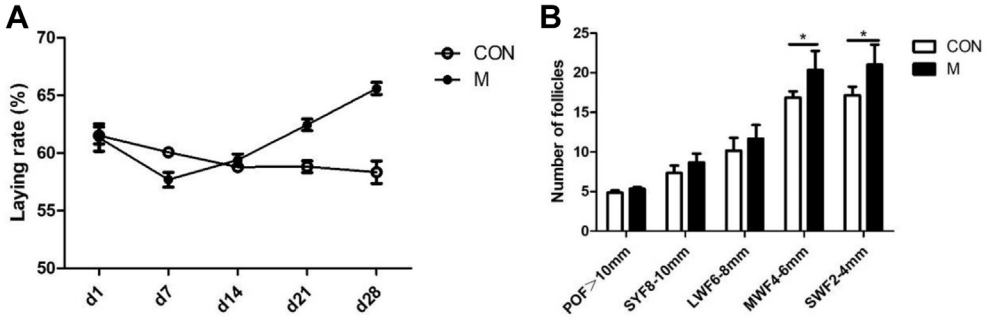

Figure 4. Effect of melatonin treatment on laying rate and ovarian follicular numbers. Comparison of the laying rate (A) and number of follicles (B) between the control groups (CON) and melatonin groups (M). Data are shown as means ± SD; Laying rate: n = 30 chicken/group, numbers of follicle: n = 6 chicken/group. *$P < 0.05$ indicates significance from respective control values.

the CON and M ($P < 0.05$). The mTOR and p-mTOR protein levels were considerably increased in the M compared with the CON ($P < 0.05$) (Figure 6A and B). The mTORC1-dependent 4E-BP1 and phosphorylation of 4E-BP1 (p-4E-BP1) were increased in the M ($P < 0.05$) (Figure 6C and D). However, there were no differences for PKC and p-PKC in the MT-treated group compared with the CON (Figure 6E and F).

DISCUSSION

The present study demonstrated the mechanism of mTOR signaling pathway in MT regulation of ovarian function in aging laying hens. Melatonin could enhance laying rate and ovarian follicle growth in aging chickens and could prolong the physiological egg-laying peak by increasing the levels of oxidase and reproductive hormones and by activating mTOR and the associated downstream mRNA and protein expression.

Ovarian aging is characterized by gradual declines in ovarian follicle quantity and quality (Zakaria, et al., 1983; Lillpers and Wilhelmson, 1993; Faddy, 2000). The mechanism by which the ovary ages at an accelerated rate during the later laying period is still not fully understood. Previous research found that MT levels of plasma decreased with age in chickens (Jia, et al., 2016) and in mice (Lahiri, et al., 2010), which imply the hypothesized that the decrease in MT levels with age contributes to the ovarian aging process (Hardeland, 2012). Excessive amounts of ROS caused oxidative stress and damaged oocytes and granulosa cells in 43-week-old rats, which was considered to be the most likely factor aggravating ovarian aging (Toshio, et al., 2004; Grondahl, et al., 2010). In the present study, we found that the MT levels in the plasma of laying hens under MT treatment were significantly increased compared with the CON, which suggested that exogenous injection of MT significantly increased MT levels in laying hens. Melatonin is known as a highly efficient free radical scavenger and a broad-spectrum antioxidant (Zavodnik, et al., 2006; Tamura, et al., 2013; Tan, et al., 2015). Melatonin plays an important role as an antioxidant in the follicle (Taniguchi, et al., 2009). Additionally, the SOD activity is an important indicator of the antioxidant capacity of the body (Ragel, et al., 2006), and the T-AOC reflects the total antioxidant capacity of the organism. In the present research, it was clear that MT treatment significantly increased the SOD and T-AOC activities and reduced ROS levels in the plasma compared with the CON. Our results were in agreement with others studies in which administration of 10 mg/kg of MT significantly increased the SOD activity in rats (Ozturk, et al., 2000; Behram, et al., 2017), or MT administration (715 g/kg) increased T-AOC and SOD levels in rats under CsA treatment (Kim, et al., 2002). Our study indicated that MT had a high capacity to detoxify ROS and that it suppressed the oxidative effect indirectly by enhancing the production of endogenous antioxidants.

The regulation of gonadotropin secretion is very complex. Many studies have shown that MT participates in animal reproduction. It is reported that high levels of MT could increase the levels of LH and FSH in the plasma, whereas low levels of LH and FSH could inhibit ovulation (Odell and Swerdloff, 1968). In the present study, we found that the E2 level of serum in the MT-treated group was generally higher than in the CON at day 7 and 14. The LH level was significantly elevated at day 28, and this did not influence the levels of P4 in the blood in the M. These results were consistent with the finding that MT (10 mg) implanted elevated the serum E2 content at 360 D of age (Jia, et al., 2016). As a result, these activities of MT may also help the hens preserve their egg-laying rate after their peak age.

Immune performance is an important parameter for the maintenance of healthy poultry, and there are few reports on the direct effects of immunization in avian poultry. There are studies reported that the immune performances of pigeons (Rodriguez and Lea, 2010) and Japanese quail (Moore, et al., 2002) were reduced after excision of the pineal gland, which suggested that immune functions may be partially regulated by MT. The role of immunoglobulins in the immune status in poultry is well established (Yang, et al., 2012). In the present study, the MT-treatment group had significantly increased levels of IgA, IgG, and IgM in the plasma at day 28. Previous studies found that MT improved the overall performance of turkeys, including

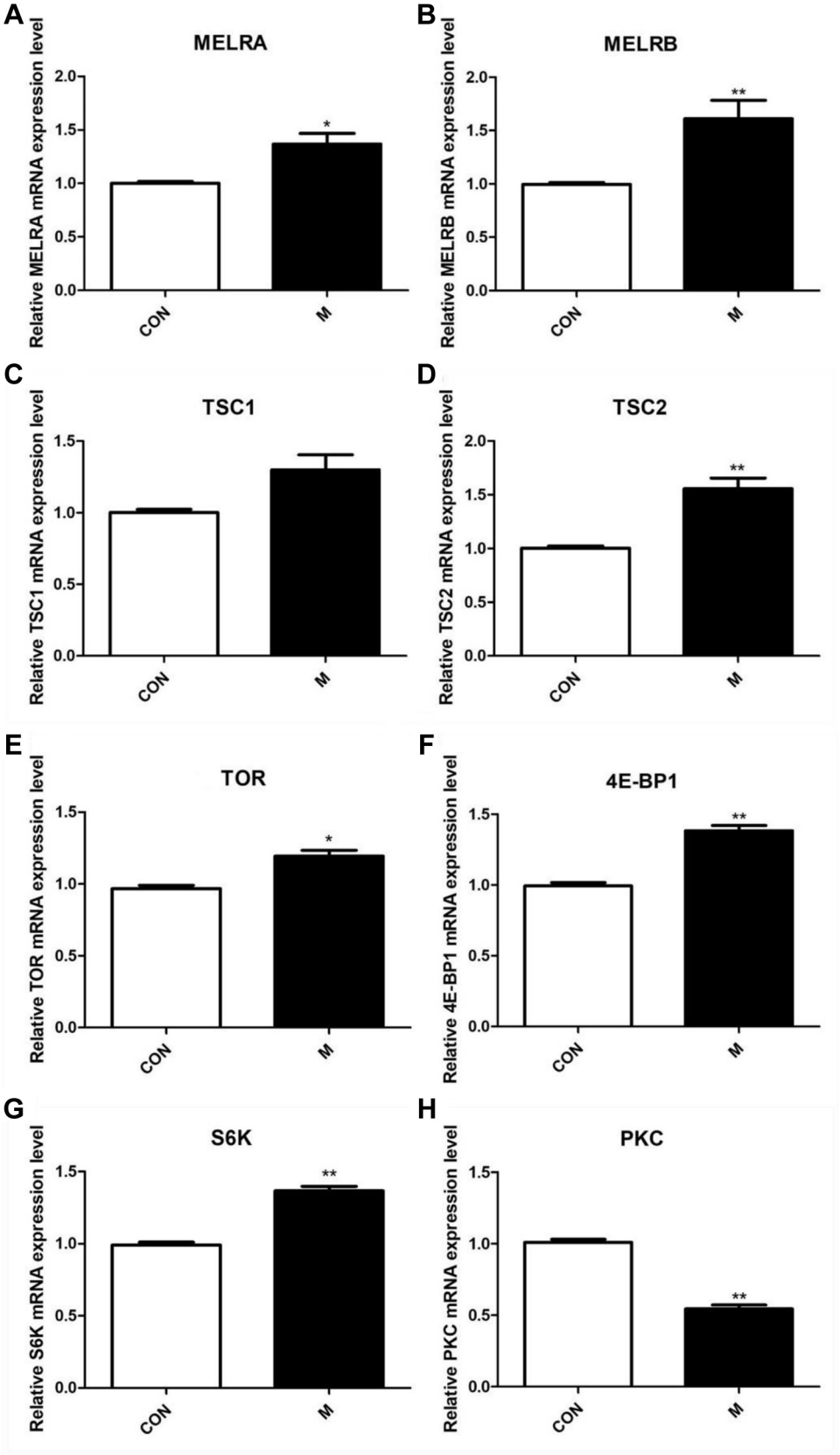

Figure 5. Effects of melatonin treatment on *MELRA, MELRB, TSC1, TSC2, avTOR,* and *4E-BP1, S6K* and *PKC mRNA* expression in the ovary. Expressions melatonin receptors (A and B)and activations of TOR upstream components TSC1, TSC2(C and D) and its downstream components PKC, 4E-BP1, and S6K (E-H) mRNA expression between the control groups (CON) and melatonin groups (M). N = 6 chicken/group. $^{*}P < 0.05$ indicates significance from respective control values, $^{**}P < 0.01$ indicates extremely significance from respective control values.

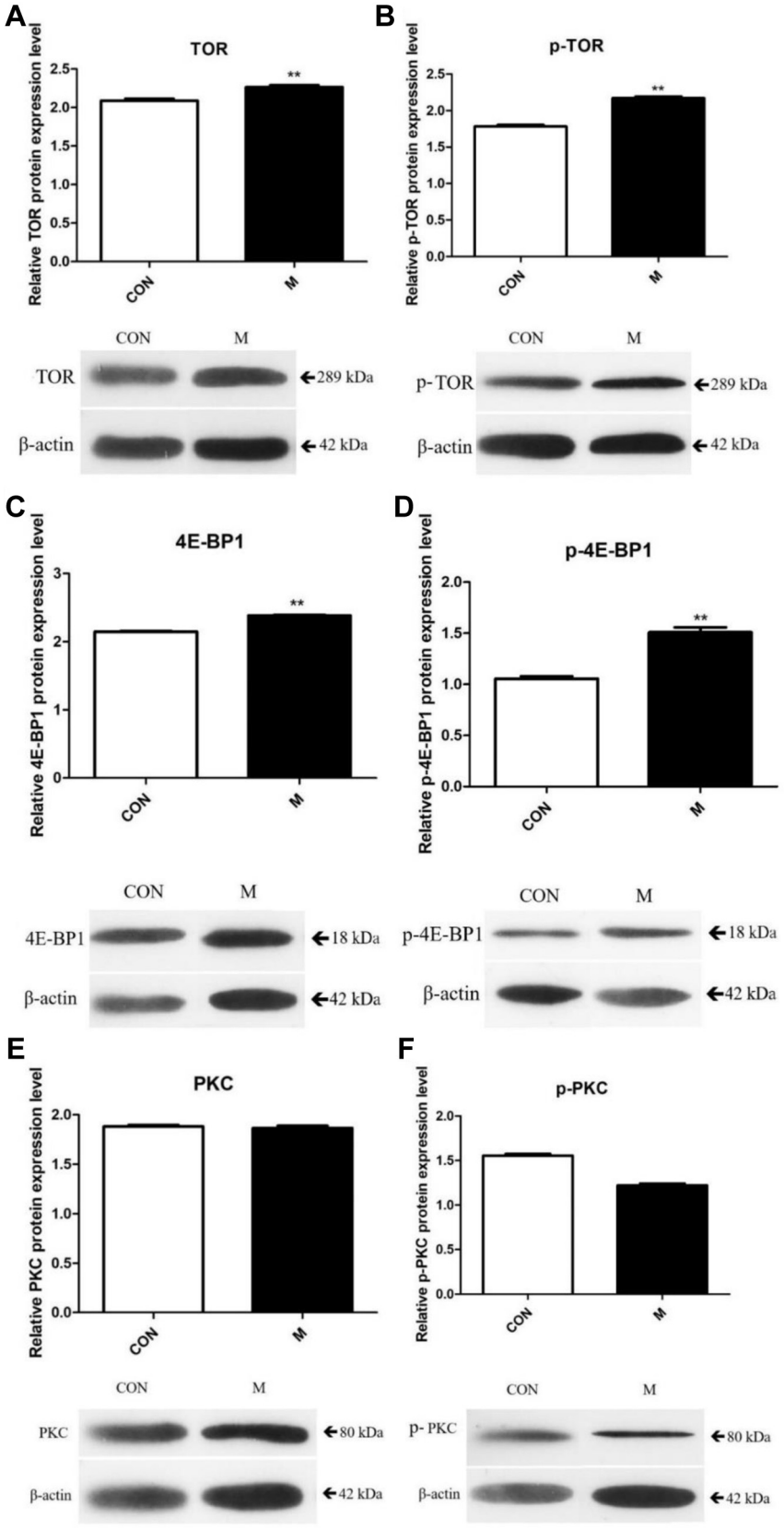

Figure 6. Effects of melatonin treatment on TOR and downstream components protein expression in the ovary. Expressions and activations of TOR (A and B) and its downstream components 4E-BP1 (C and D) and PKC (E and F) expression between the control groups (CON) and melatonin groups (M). N = 6 chicken/group. $^{**}P < 0.01$ indicates extremely significance from respective control values.

immune and growth parameters (Moore and Siopes, 2002). The immunomodulatory influence of MT may be mediated via direct interactions with its receptors in the immunocytes. Because MT receptors are present in the spleen and lymphoid tissues (Pang, et al., 1995), MT reacts with receptors 2(1,25I) iodomelatonin binding sites, stimulating immune cells to produce immunoopioids and lymphokines that mediate the immunomodulatory functions of MT. Our findings were consistent with the results of those studies, suggesting that MT may directly act on the immune system.

Ovaries of poultry consist of follicles as basic functional units. The growth and development of follicles are very complex processes with a strictly ordered hierarchy. Generally, only a dominant SYF (diameter < 8 mm) enters the POF (diameter $8-40$ mm) and finally results in an ovulated egg. The remaining SYFs are recruited to atresia (Gilbert, et al., 1983). The number of follicles in the chicken determines the laying rate. Previous studies have found that the numbers of ovarian follicles decrease with increasing age in laying hens (Zakaria, et al., 1983). Our results showed that the laying rate of the CON decreased with increasing age, but exogenous MT treatment significantly increased the laying rate. Because MT receptors were expressed in ovarian and granulosa cells, it was thought that MT could directly regulate the ovaries through the MT receptors. The present research observed that the numbers of MWF and SWF were significantly increased in the group treated with MT. The numbers of SYF and large white follicle were also increased compared to the CON. Melatonin played an antioxidant role in improving the quality of mouse oocytes and increasing the numbers of ovulation in aging mice (Tamura, et al., 2017). These results suggest that MT treatment reduces ovarian aging by ameliorating declines in POF quantity.

Melatonin and its receptors play a crucial role in the regulation of the animal reproductive process (Talpur, et al., 2018). In the present research, MT treatment significantly increased the expression of MT receptors in the ovary compared with the CON. The mTOR signaling pathway is important in regulating cell growth and proliferation. There are 2 protein complexes in mTOR, mTORC1 and mTORC2. The mTORC1 ensures cell growth and autophagy, whereas mTORC2 regulates cytoskeletal dynamics and actin organization (Dowling, et al., 2010; Ikenoue, et al., 2014). There are 2 main mTOR upstream signaling pathways, PI3K/AKT and TSC1/2, and the mTOR downstream signaling pathways are 4E-BP1 and S6K1 (Gorre, et al., 2014; Zhang, et al., 2016). Previous research found that the follicular pool was overly activated, and the follicles were depleted in mice lacking TSC1 and TSC2, and all mice suffered from POF (Deepak, et al., 2009). In the present research, we found that MT treatment significantly increased the expression of TSC2. The TSC/mTORC1 signaling in oocytes is equally important for preservation of the primordial follicles in their dormant state, and this controls the activation of primordial follicles. The present study found that the mRNA and protein expression levels of *TOR, 4E-BP1,* and *S6K* were significantly increased in the MT-treated group. The results of the present experiment were consistent with previous reports that MT treatment (50 mg/kg) significantly increased the mRNA and protein expression levels of mTOR and P70S6k after 30 D (Behram, et al., 2017). PKC is a crucial protein involved in cell proliferation, differentiation and apoptosis. The mTORC2 is responsible for the phosphorylation of PKC and post-translational processing (Ikenoue, et al., 2014). However, we found that PKC mRNA and protein were significantly decreased in the M compared with the CON in the present study. It was further observed that the MT treatment increased activation of mTORC1 signaling in the ovaries.

In the current study, we observed that MT (20 mg/kg) significantly increased the plasma levels of SOD and T-AOC, decreased ROS, and upregulated the gene expression *of TOR, S6K*, and *4E-BP1* and the protein expression of mTOR, p-mTOR, 4E-BP1, and p-4E-BP1 in 70-week-old laying hens. The results indicated that MT treatment may enhance follicle growth and prolong the physiological egg-laying peak through activating the mTOR pathway of mTORC1.

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