Melatonin Does Not Affect Progesterone Basal Secretion but Suppresses the Luteinizing Hormone Receptor Expression in Granulosa Cells of the Japanese Quail

Guang-Min Yu[1], Naoki Isobe[1,2] and Teruo Maeda[1,2]

[1] Department of Bioresource Science, Graduate School of Biosphere Science,
Hiroshima University, Higashi-Hiroshima 739−8528, Japan
[2] The Research Center for Animal Science, Hiroshima University, Higashi-Hiroshima 739−8528, Japan

The aim of this study was to evaluate the potential effect of melatonin on progesterone production by granulosa cells of the Japanese quail. For *in vitro* experiments, granulosa cells were isolated from pre-ovulatory follicles (F1−F3) when the F1 follicles were predicted to be either immature or mature (at 3−6 or 18−21 h after oviposition, respectively). Granulosa cells were cultured for 12 h with or without melatonin concentration gradients of 0.0001−100 μg/mL, thereby averting luteinizing hormone (LH) stimulation. The concentration of progesterone in culture medium was measured using an enzyme immunoassay. The expression of melatonin receptor subtypes in granulosa cells from F1 follicles was detected by reverse transcription-PCR. The LH receptor (LHCGR) mRNA level in cultured granulosa cells of the F1 follicles was analyzed using quantitative real-time PCR. Six quails were used in each of four groups for *in vivo* experiments. Each group received intraperitoneal injection of melatonin (0.67 mg/kg body weight) or mock-vehicle at 3 or 18 h after oviposition, respectively. The birds were decapitated to collect serum 3 h later (at 6 or 21 h after oviposition, respectively). The serum progesterone level was also measured using an enzyme immunoassay. We observed that melatonin receptor subtypes (Mel-1a, 1b, and 1c) were expressed in the granulosa cells of the F1 follicles of the Japanese quail. Melatonin suppresses the LHCGR mRNA expression in granulosa cells of F1 follicles but does not affect the basal secretion of progesterone in cultured granulosa cells of the F1−F3 follicles. In addition, melatonin treatment has no influence on the serum progesterone concentration at 6 h post-oviposition, but suppresses progesterone level 21 h after oviposition in the Japanese quail.

Key words: granulosa cell, Japanese quail, luteinizing hormone receptor, melatonin, progesterone

Introduction

Melatonin (N-acetyl-5-methoxytryptamine), a major pineal secretary product, has been regarded as a neurohormone for a long time (Davis, 1997; Pang *et al*., 1998; Vanecek, 1998). This generally accepted view has recently been challenged by the discovery that melatonin is involved in various physiological processes, such as female reproduction (Wang *et al*., 2014), innate immunity (Zhou *et al*., 2016), neuroprotection (Tyagi *et al*., 2010), abiotic stress resistance (Zhang *et al*., 2015), and anti-inflammation (Hardeland *et al*., 2015), antiradiation (Fernández-Gil *et al*., 2017), anticancer (Moreira *et al*., 2015; Söderquist *et al*., 2016), and antioxidant proc-

esses (Mehaisen *et al*., 2015), in both plants and animals. Besides melatonin, its metabolites, including cyclic-3-hydroxymelatonin, N-acetyl-N-formyl-5-methoxykynuramine, and N-acetyl-5-methoxykynuramine, also have the ability to scavenge reactive oxygen species and reactive nitrogen species (Galano *et al*., 2013; Galano *et al*., 2014). These findings indicate that melatonin is a more universal molecule than previously thought, with functions in various tissues and organs. In mammals, melatonin acts on the ovaries, with direct effects on granulosa cell steroidogenesis and follicular functions (Wang *et al*., 2012). Melatonin directly stimulates progesterone production by human and bovine granulosa cells *in vitro* (Webley and Luck, 1986). Perfusion with melatonin markedly stimulated progesterone secretion by the marmoset corpus luteum (Webley and Hearn, 1987). Melatonin also improves the concentration of plasma progesterone in sheep and rats (Abecia *et al*., 2002; Chuffa *et al*., 2013). However, there are some notable differences between birds

and mammals with regard to ovarian function and divergent characteristics of the biosynthesis and role for progesterone. Poultry birds maintain a strict follicular hierarchy consisting of approximately 2 to 6 pre-ovulatory follicles, and ovulate a single follicle almost every day (McDerment *et al*., 2012). In nearly all species of birds, the synthesis of progesterone within the follicular granulosa is a requirement for ovulation, comparable to the site of synthesis and role of estradiol in mammals (Johnson, 2014). Murayama *et al*. (1997) indicated the direct action of melatonin on hen ovarian granulosa cells to lower their responsiveness to luteinizing hormone (LH) *in vitro*. However, reports on the effects of melatonin on progesterone production under basal conditions and the expression of the LH receptor (LHCGR) in birds are not available.

To fill this gap in our knowledge, we conducted a series of experiments to determine whether melatonin affects progesterone production by the follicular granulosa in Japanese quail. We investigated the effect of melatonin on progesterone production by granulosa cells *in vitro* at a wide range of concentrations in F1−F3 follicles without LH stimulation. We also examined the expression of melatonin receptor subtypes and the level of LHCGR mRNA in cultured granulosa cells from F1 follicles stimulated by melatonin. Finally, we investigated the effect of melatonin on progesterone production *in vivo*.

Materials and Methods

Experimental Birds

In total, 36 female Japanese quails, 15−30 weeks of age, were used. All quails were reared in individual cages under a lighting regimen of 14 h light: 10 h dark and were provided with feed and water *ad libitum*. For the *in vitro* experiment, birds were decapitated to collect pre-ovulatory follicles when the F1 follicles were predicted to be immature or mature, 3−6 or 18−21 h after oviposition, respectively (Reece, 2004). F1−F3 follicles were collected at the same time. For the *in vivo* experiment, animals were intraperitoneally injected with melatonin (0.67 mg/kg body weight; a concentration similar to that used for rat injection [Abd-Allah *et al*., 2003; Chuffa *et al*., 2013]) or vehicle at 3 and 18 h after oviposition. The serum level of melatonin after administration was estimated as 8.38 μg/mL, based on the method of Ito *et al*. (2011). The birds were decapitated to collect serum 3 h later (at 6 and 21 h after oviposition). All animals used in this study were handled in accordance with the regulations of the Animal Experiment Committee of Hiroshima University for animal experiments.

Cell Culture and Treatment

Granulosa cells were isolated as previously described (Rangel *et al*., 2009). In brief, pre-ovulatory F1−F3 follicles were placed in cell-culture dishes containing preheated Dulbecco's phosphate-buffered saline (DPBS; Nissui Pharmaceutical, Tokyo, Japan). After the yolk was drained out through an incision made in the follicular wall, the follicle was inverted and shaken in sterile DPBS. The granulosa layers detached from the hierarchical follicles were disaggre-

gated at 37℃ for 5 min under continuous agitation in 500, 300, and 200 μL of dissociation solution (CTK; ReproCELL, Yokohama, Japan). The enzymatic reaction was quenched by the addition of 2 mL of Dulbecco's modified Eagle's medium (DMEM) /F12 medium (Gibco BRL/Invitrogen, Karlsruhe, Germany) containing 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). Cells pellets were flushed with a pipette, and centrifuged. Cells were washed three times by resuspension in 5 mL DPBS and centrifuged for 5 min at $500 \times g$. Granulosa cells were seeded at a concentration of 2×10^5 cells/mL in DMEM/F12 medium containing 10% (v/v) FBS, 1% (v/v) nucleosides (Millipore, Billerica, MA, USA), 1% (v/v) non-essential amino acids (Gibco BRL/Invitrogen), 1 mM sodium pyruvate (Gibco BRL/Invitrogen), and 1% (v/v) antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan). Cells were cultured at 39℃ in a humidified atmosphere with 5% CO_2 and 95% air.

A stock solution of melatonin was prepared by dissolving 10 mg of melatonin (Sigma-Aldrich) in 200 μL of ethanol. Working solutions were prepared by dilution in cell culture medium to give a concentration gradient of 0.0001−100 μg/mL. Granulosa cells were treated with different concentrations of melatonin for 12 h, a culture time used previously (Taketani *et al*., 2011). As controls, cells were incubated with ethanol at the highest concentration used for melatonin treatment.

RNA Extraction

At least 5×10^5 cultured granulosa cells from F1 follicles were used for total RNA extraction. The cell culture medium was aspirated. Adherent cells were washed once with 2 mL ice-cold DPBS, which was then aspirated as much as possible. The cells were used for total RNA extraction with the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instruction. The obtained RNA pellet was treated with DNase I (Macherey-Nagel) and dissolved in diethyl pyrocarbonate-treated water (Nacalai Tesque). The total RNA was quantified by measuring the optical density at a wavelength of 260 nm using an OD_{260} unit equivalent to 40 μg/mL of RNA. The RNA purity was determined by measuring the ratio of absorbance at 260 and 280 nm. Only samples with a ratio between 1.8 and 2.2 were used.

Reverse Transcription (RT)-PCR

The RNA solution was incubated at 65℃ for 5 min and kept on ice afterwards. The total RNA was reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) to obtain the cDNA. The reaction mixture consisted of 240 ng of total RNA, 5× reverse transcription buffer, 0.5 μL reverse transcription enzyme mixture, and 0.5 μL primer mixture, and nuclease-free water was added to a total volume of 10 μL. Reverse transcription was performed at 37℃ for 15 min, followed by heat inactivation for 5 min at 98℃ using an Applied Biosystems GeneAmp PCR System 9700 (Life Technologies, Darmstadt, Germany).

The RT-PCR mixture consisted of 0.25 μL TaKaRa Ex Taq, 5 μL 10× Ex Taq buffer, 4 μL dNTP mixture, 0.5 μM

each of forward and reverse primers, 1 μL template, and double distilled water to a total volume of 20 μL. The reaction procedure was as follows: initial denaturation at 98℃ for 2 min, followed by 50 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s, and extension at 72℃ for 30 s. A final extension step was performed at 72℃ for 7 min. cDNA products were resolved on 2% (w/v) agarose gels containing 0.025% (w/v) ethidium bromide. The analysis was repeated thrice.

Analysis of the Difference in LHCGR Expression

The LHCGR mRNA levels in cultured granulosa cells of F1 follicles was analyzed using quantitative real-time PCR and the SYBR Premix Ex Taq II (Takara Bio., Shiga, Japan) on an Applied Biosystems StepOne real-time PCR system (Life Technologies) according to the method described previously (Guangmin et al., 2015). In brief, the PCR mixture (20 μL) consisting of 10 μL SYBR Premix Ex Taq II, 0.4 μM each of forward and reverse primers, 0.4 μL ROX reference dye, 2 μL template, and 6 μL double distilled water were mixed in PCR tubes (Life Technologies). The thermal protocols for PCR were as follows: initial denaturation at 95℃ for 30 s followed by 50 cycles of denaturation at 95℃ for 5 s, annealing and extension at 60℃ for 34 s, and a melting curve from 60 to 95℃, increasing in increments of 0.5℃ every 5 s. Normalization was performed using the *GAPDH* housekeeping gene as a control. Primer sequences are listed in Table 1. Real-time PCR data were analyzed using the $2^{-\Delta\Delta ct}$ method.

Measurement of Progesterone Levels

Cell culture medium or blood sample was pre-cleared by centrifugation at $3,000 \times g$ for 20 min to remove cells and then stored at −20℃ until further use. For the progesterone assay, cell-free culture medium or serum was extracted in advance as described previously (Isobe et al., 2005). The culture medium or serum was mixed with 2 mL petroleum ether (Kanto Chemical Co., Tokyo, Japan) and shaken for 15 min. After decantation, the ether phase was evaporated in a glass tube. Borate buffer, consisting of 0.05 M boric acid (Kanto Chemical Co.), 0.2% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1 mg/mL thimerosal (Kanto Chemical Co.), was added into tubes for reconstitution, followed by an enzyme immunoassay with a rabbit anti-

progesterone antibody (Isobe and Nakao, 2003). Cross reactivity of the anti-progesterone antibody with progesterone, 5α-pregnanedione, 20β-hydroxyprogesterone, deoxycorticosterone, pregnenolone, 5β-pregnane-3α-ol-20-one, and 17α-hydroxyprogesterone were 100, 5.8, 0.7, 0.62, 0.2, 0.1, and 0.05%, respectively. Horseradish peroxidase (Sigma-Aldrich) was conjugated with progesterone carboxymethyloxime (Sigma-Aldrich) using a mixed anhydride reaction. The sensitivity of the assay was 0.0055 ng/mL. Intra- and inter-assay coefficients of variation were 9.6−10.9% and 10.8−16.6%, respectively. Recovery rate ranged between 73% and 84%. Samples from six quails were measured in duplicates.

Statistical Analysis

Continuous variables are expressed as the mean±standard deviation (SD) of at least three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test with the Statview software (Abacus Concepts, Berkeley, CA, USA). Differences were considered statistically significant when the P value was less than 0.05.

Results

Fig. 1 shows the effect of melatonin, follicle size, and collection time post-oviposition on the secretion of progesterone by pre-ovulatory granulosa cells cultured for 12 h. No statistical differences were observed between progesterone secretion by granulosa cells with or without melatonin administration ($P > 0.05$). When F1 follicle granulosa cells were cultured with 0.1 and 1 μg/mL melatonin, progesterone secretion by cells collected 3−6 h after oviposition was significantly less than that of cells collected 18−21 h after oviposition ($P < 0.05$); however, no differences between the two collection times were observed at other melatonin concentrations (Fig. 1a). Progesterone secretion by the granulosa cells of the F2 follicles was remarkably less for cells collected at 3−6 h than by those collected at 18−21 h after oviposition when they were cultured with 0, 0.0001, and 1 μg/mL melatonin ($P < 0.05$); however, this difference disappeared in the presence of other concentrations of melatonin (Fig. 1b). Progesterone secretion by F3 granulosa cells was significantly lower at 3−6 h than for those collected 18−21 h

Table 1. **Primers used for PCR**

Gene	Primer sequence (5′−3′)	Accession no.	Product size (bp)
Mel-1a	Forward: CAATGGATGGAATCTGGGA	NM_205362.1	333
	Reverse: GCTATGGGAAGTATGAAGTGG		
Mel-1b	Forward: TTTGCTGGGCACCTCTAAAC	NM_001293103.1	259
	Reverse: CGCTTGCTCTTCTGTCCATC		
Mel-1c	Forward: AGATAAGTGGGTTCCTGATGG	NM_205361.1	237
	Reverse: GCAAAGGTGCAAGAGTAAATC		
LHCGR	Forward: TTGCACATTGAGGACGGAGC	NM_204936.1	194
	Reverse: GATTCGTTGCTCATGCCCTG		
GADPH	Forward: ATCACAGCCACACAGAAGACG	M11213	124
	Reverse: TGACTTTCCCCACAGCCTTA		

(a)

Fig. 2. **The expression of melatonin receptor subtypes in cultured granulosa cells from F1 follicles of Japanese quail.** The PCR products were electrophoresed on 2% agarose gel containing ethidium bromide ($n=3$). M=marker.
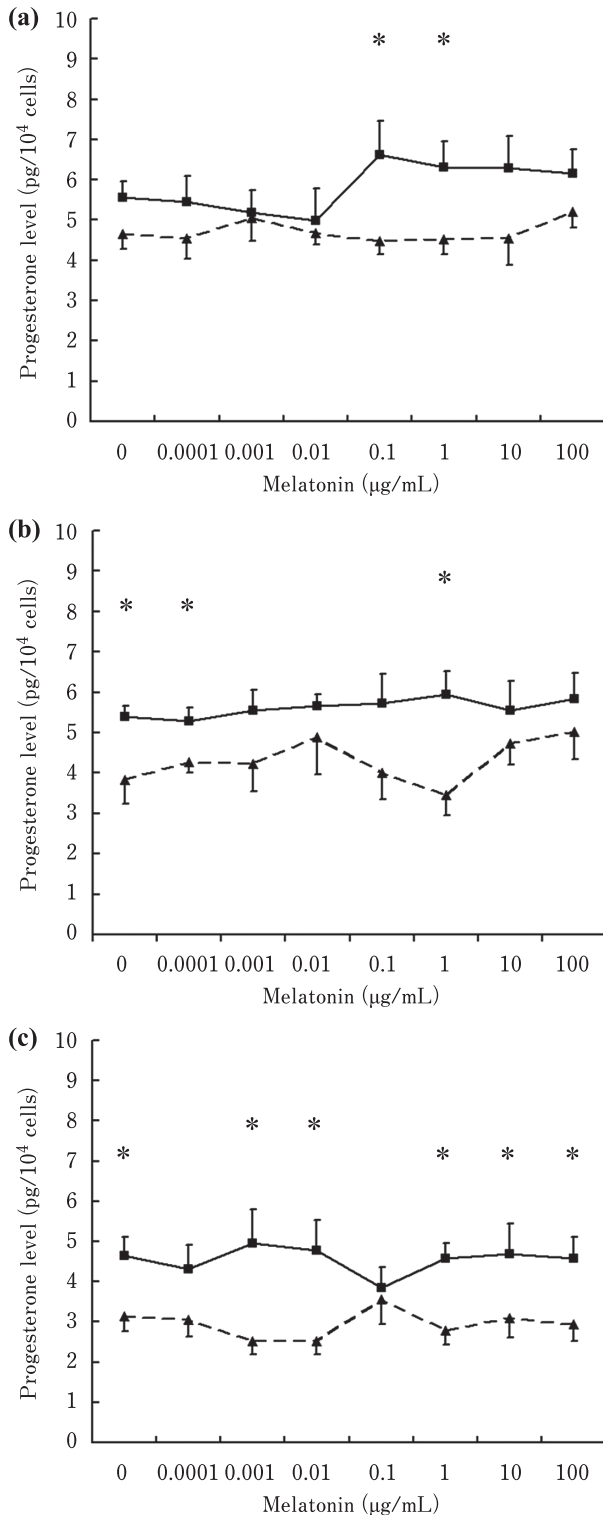
(b)

(c)

Fig. 1. **Progesterone secretion by granulosa cells of Japanese quail cultured for 12 h with or without melatonin stimulation.** Granulosa cells were obtained from (a) F1, (b) F2, or (c) F3 follicles, at 3–6 h (triangles, dashed line) or 18–21 h (squares, solid line) after oviposition. Data are the mean±SD ($n=6$). * Values are significantly different between 3–6 h group and 18–21 h group ($P<0.05$).
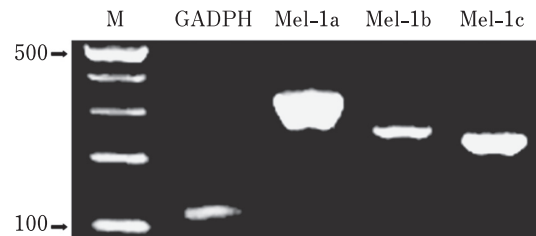
after oviposition at nearly all melatonin concentrations, except 0.0001 and 0.1 µg/mL melatonin.

The expression profile of melatonin receptor subtypes in cultured granulosa cells of the F1 follicles showed that melatonin receptor subtypes (Mel-1a, 1b, and 1c) were expressed in granulosa cells of the Japanese quail (Fig. 2).

The effect of melatonin on the expression of LHCGR mRNA in cultured granulosa cells of the F1 follicles is shown in Fig. 3. Melatonin at two doses (0.0001 and 0.001 µg/mL) significantly suppressed LHCGR mRNA expression after culturing for 12 h ($P<0.05$) in cells collected at both 3–6 h and 18–21 h after oviposition.

With regard to the *in vivo* experiment, no statistical differences were observed between serum progesterone levels of quails at 6 h after oviposition with or without melatonin administration ($P>0.05$). However, for animals injected with melatonin, the serum progesterone level was significantly less at 21 h after oviposition ($P<0.05$) (Fig. 4).

Discussion

Progesterone plays a key role in regulating the reproductive activity (Yu and Maeda, 2017). In birds, ovarian follicles maintain a monolayer of granulosa cells (Diez-Fraile *et al*., 2010). The pre-ovulatory follicles grow in size due to yolk incorporation and the granulosa cells produce progressively greater amounts of progesterone (Onagbesan *et al*., 2009; Sechman, 2013; Johnson, 2014). Granulosa cells can be collected from pre-ovulatory follicles of poultry bird and cultured *in vitro*, which provides a means for the study of progesterone biosynthesis in avian species. In the present study, we investigated the effect of melatonin on progesterone secretion by granulosa cells collected from F1–F3 preovulatory follicles at 3–6 h and 18–21 h after oviposition without LH stimulation *in vitro*. We demonstrated that melatonin does not affect the basal secretion of progesterone in cultured granulosa cells of the Japanese quail.

The pre-ovulatory progesterone surge is predominantly derived from the granulosa layer of the largest mature preovulatory follicle (F1) (Etches, 1994). The pre-ovulatory F1 follicle produces 30 times more progesterone than the F2 and F3 follicles in the absence of exogenous LH *in vitro* (Yu *et al*., 1992) and granulosa cells of the F1 follicle are more
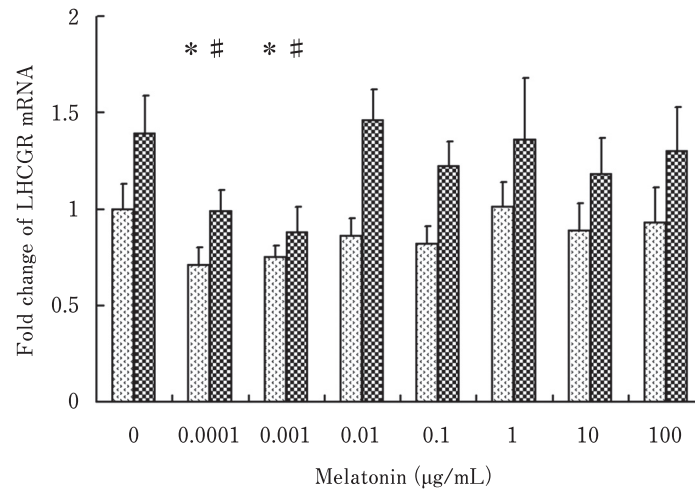
Fig. 3.   **The effect of melatonin on the expression of LHCGR mRNA in cultured granulosa cells from F1 follicles of Japanese quail.** Histograms filled with arrowheads present data of 3–6 h, while filled with squares present data of 18–21 h. Data are the mean±SD (n=6). *,# Values are significantly different compared to the values of 0 μg/mL (P<0.05).
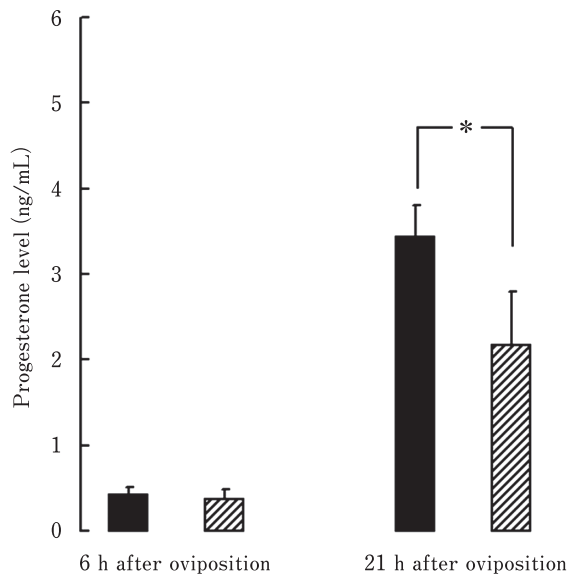

Fig. 4.   **Progesterone level in serum of Japanese quail with or without melatonin administration.** Black histograms present data of the controls, while histograms filled with oblique lines present data of melatonin treatment. Data are the mean±SD (n=6). * Values are significantly different at P<0.05.

responsive to LH stimulation than granulosa cells of the F3 follicle (Robinson *et al*., 1988). In addition, the pre-ovulatory release of LH is stimulated by the positive feedback action of progesterone in avian species (Johnson *et al*., 1985; Johnson

et al., 2002). Finally, adequate amounts of granulosa cells can be isolated from F1 follicles of the Japanese quail. Thus, we investigated the expression of melatonin receptor subtypes and the effect of melatonin on LHCGR mRNA in granulosa cells of the F1 follicles. We found that melatonin significantly suppressed LHCGR mRNA expression in the relatively lower concentrations used in our study. Moreover, decreased LHCGR expression suggests a concordant decrease in the responsiveness of melatonin-treated granulosa cells to LH.

To substantiate the above assumption, we investigated the effect of melatonin on progesterone production *in vivo*. There were no statistical differences between serum progesterone levels of Japanese quails at 6 h after oviposition with or without melatonin administration, which is similar to the basic conditions observed *in vitro*. The release of LH from the pituitary relates to the degree of maturation of the pre-ovulatory follicles (Etches, 1994). LH is not released from the pituitary when the F1 follicles are predicted to be immature (at 3–6 h after oviposition), maintaining LH at low basic levels in circulating blood (Hrabia *et al*., 2014). At 18–21 h after oviposition, the F1 follicles are predicted to mature, and the pituitary starts releasing LH into circulating blood (Reece, 2004). The progesterone level in blood reaches a peak with a surge in LH levels (Nakagawa-Mizuyachi *et al*., 2010). In the present study, at 21 h after oviposition, the serous progesterone level was significantly lower in quails injected with melatonin, which is in agreement with an earlier *in vitro* study performed by Murayama *et al*. (1997). In their study, the dose-response curve for LH-stimulated progesterone production of hen granulosa cells shifted to a higher concentration of LH to attain ED50 in the

presence of melatonin. Therefore, these effects of melatonin on progesterone production are consistent with the decreased LHCGR mRNA expression demonstrated by our study. The findings in the present study and published information can explain, at least in part, the phenomenon of longer nocturnal hours being de-stimulatory in poultry bird reproductive cycle. Prolonged nocturnal hours stimulate melatonin biosynthesis in the pineal gland, which subsequently increases melatonin release in the bloodstream. The elevated concentration of melatonin in circulating blood inhibits LHCGR mRNA expression in the ovarian granulosa cells, which thereby reduces the LH responsiveness of granulosa cells in the pre-ovulatory follicles. With the decrease in LH-stimulated progesterone production by granulosa cells, the serum progesterone level decreases, which weakens the positive feedback action of progesterone on the pre-ovulatory release of LH. Thus, decreased serum LH levels ulteriorly reduce progesterone production by granulosa cells. These findings can also explain why short-day photoperiod or administration of melatonin decreases the ovary weight of developing avian species (Darre, 2011). Accumulating evidence may account for the fact that poultry birds produce well on long days and short nights.

In summary, we found that melatonin receptor subtypes (Mel-1a, 1b, and 1c) were expressed in granulosa cells of F1 follicles of the Japanese quail. Melatonin does not affect the basal secretion of progesterone in cultured granulosa cells of the F1−F3 follicles but suppresses LHCGR mRNA expression in the granulosa cells of the F1 follicles. In addition, melatonin treatment has no influence on the serum progesterone concentration at 6 h post oviposition; however, it suppresses progesterone level at 21 h post oviposition in the Japanese quail.

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