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

Guang-Min Yu¹, Naoki Isobe¹,² and Teruo Maeda¹,²

¹Department of Bioresource Science, Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan
²The 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

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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; Vanecck, 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., 2014). 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.
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 (McDermont 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 (LHCRG) 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 LHCRG 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 14h light: 10h dark and were provided food 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 18–21h 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 disaggregated at 37°C 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)/F2 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×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°C in a humidified atmosphere with 5% CO2 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 OD260 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°C for 5 min and kept on ice afterwards. The total RNA was reverse-transcribed using ReverTra Acc (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°C for 15 min, followed by heat inactivation for 5 min at 98°C 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
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 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°C for 30 s followed by 50 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 34 s, and a melting curve from 60 to 95°C, increasing in increments of 0.5°C every 5 s. Normalization was performed using the GADPH housekeeping gene as a control. Primer sequences are listed in Table 1. Real-time PCR data were analyzed using the 2−ΔΔct method.

Measurement of Progesterone Levels

Cell culture medium or blood sample was pre-cleared by centrifugation at 3,000 × g for 20 min to remove cells and then stored at −20°C 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-pregnanediene, 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 carboxymethylloxime (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.

| Gene  | Primer sequence (5′–3′) | Accession no. | Product size (bp) |
|-------|-------------------------|---------------|------------------|
| Mel-1a| Forward: CAATGGAGTGAATCTGGGA  
|       | Reverse: GCTATGGGAAGTATGAAGTG  
|       |                  | NM_205362.1  | 333 |
| Mel-1b| Forward: TTGTCTGGGACACCCTCAAAC  
|       | Reverse: CGCTTGGTCTTCGTCATC  
|       |                  | NM_001293103.1  | 259 |
| Mel-1c| Forward: AGATAAGTGGGGTTCTGATGG  
|       | Reverse: GCAAAGGTGCAAGAGTAAATC  
|       |                  | NM_205361.1  | 237 |
| LHCGR | Forward: TTGACATTGGACAGCGGAGC  
|       | Reverse: GATTCTGGTCTCATGGCCCTG  
|       |                  | NM_204936.1  | 194 |
| GADPH | Forward: ATCAGAGCCACACAGAGAGCG  
|       | Reverse: TGACTTCCCCACACGGCTTA  
|       |                  | M11213  | 124 |
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 12h ($P<0.05$) in cells collected at both 3–6h and 18–21h after oviposition.

With regard to the in vivo experiment, no statistical differences were observed between serum progesterone levels of quails at 6h after oviposition with or without melatonin administration ($P>0.05$). However, for animals injected with melatonin, the serum progesterone level was significantly less at 21h 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 pre-ovulatory follicles at 3–6h and 18–21h 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 pre-ovulatory 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
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

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**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.
presence of melatonin. Therefore, these effects of melatonin on progesterone production are consistent with the decreased LH/CGR 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 LH/CGR 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 LH/CGR 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.

References

Abd-Allah AR, El-Sayed el SM, Abdel-Wahab MH and Hamada FM. Effect of melatonin on estrogen and progesterone receptors in relation to uterine contraction in rats. Pharmacological Research, 47: 349–354. 2003.

Abecia JA, Forcada F and Zúñiga O. The effect of melatonin on the secretion of progesterone in sheep and on the development of ovine embryos in vitro. Veterinary Research Communications, 26: 151–158. 2002.

Chuffa LG, Seiva FR, Favaro WJ, Amorim JP, Teixeira GR, Mendes LO, Fiorucci-Fontanelli BA, Pinheiro PF, Martinez M and Martinez FE. Melatonin and ethanol intake exert opposite effects on circulating estradiol and progesterone and differentially regulate sex steroid receptors in the ovaries, oviducts, and uteri of adult rats. Reproductive Toxicology, 39: 40–49. 2013.

Darre MJ. University of Connecticut Web. http://web.uconn.edu/poultry/poultrypages. Accessed on May 24, 2011.

Davis FC. Melatonin: Role in development. Journal of Biological Rhythms, 12: 498–508. 1997.

Díez-Fraile A, Musche S, Vanden Berghe T, Espeel M, Vandenabeele P and D’Herde KG. Expression of calcium-sensing receptor in quail granulosa explants: a key to survival during folliculogenesis. Anatomical Record (Hoboken), 293: 890–899. 2010.

Etches RJ. Maturation of ovarian follicles. In: Reproductive Biology of Poultry (Cunningham FJ, Lake PE and Hewitt D eds.). pp. 29–49. Cambridge Press. Cambridge. 1994.

Fernández-Gil B, Moneim AEA, Ortiz F, Shen YQ, Soto-Mercado V, Mendivil-Perez M, Guerra-Libero A, Acuña-Castroviejo D, Molina-Navarro MM, García-Verdugo JM, Sayed RKA, Florido J, Luna JD, López LC and Escames G. Melatonin protects rats from radiotherapy-induced small intestine toxicity. PLOS ONE, 12: e0174474. 2017.

Galano A, Tan DX and Reiter RJ. On the free radical scavenging activities of melatonin’s metabolites, AFMK and AMK. Journal of Pineal Research, 54: 245–257. 2013.

Galano A, Tan DX and Reiter RJ. Cyclic 3-hydroxymelatonin, a key metabolite enhancing the peroxyl radical scavenging activity of melatonin. RSC Advances, 4: 5220–5227. 2014.

Guangmin Y, Haq IU, Khan SH and Zeng M. Actin filaments are necessary for FSH-induced CYP19A1 transcription in bovine granulosa cells in vitro. Pakistan Veterinary Journal, 35: 53–57. 2015.

Hardeland R, Cardinali DP, Brown GM and Pandi-Perumal SR. Melatonin and brain inflammation. Progress in Neurobiology, 127–128: 46–63. 2015.

Hrabia A, Sechman A and Rzasa J. Effect of growth hormone on basal and LH-stimulated steroid secretion by chicken yellow ovarian follicles. An in vitro study. Folia Biologica (Kraków), 62: 313–319. 2014.

Ito T, Yoshizaki N, Tokumoto T, Ono H, Yoshimura T, Tsukada A, Kansaku N and Sasanami T. Progesterone is a sperm-releasing factor from the sperm-storage tubules in birds. Endocrinology, 152: 3952–3962. 2011.

Isobe N and Nakao T. Direct enzyme immunoassay of progesterone in bovine plasma. Animal Science Journal, 74: 369–373. 2003.

Isoe N, Nakao T, Yamashiro H and Shimada M. Enzyme immunoassay of progesterone in the feces from beef cattle to monitor the ovarian cycle. Animal Reproduction Science, 87: 1–10. 2005.

Johnson AL. The avian ovary and follicle development: some comparative and practical insights. Turkish Journal of Veterinary and Animal Sciences, 38: 660–669. 2014.

Johnson PA, Johnson AL and van Tienhoven A. Evidence for a positive feedback interaction between progesterone and luteinizing hormone in the induction of ovulation in the hen, Gallus domesticus. General and Comparative Endocrinology, 58: 478–485. 1985.

Johnson AL, Solovieva EV and Bridgham JT. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. Biology of Reproduction, 67: 1313–1320. 2002.

Mcderment NA, Wilson PW, Waddington D, Dunn IC and Hocking PM. Identification of novel candidate genes for follicle selection in the broiler breeder ovary. BMC Genomics, 13: 494. 2012.

Mehaisen GMK, Saeed AM, Gad A, Abass AO, Arafa M and El-Sayed A. Antioxidant capacity of melatonin on preimplantation development of fresh and vitrified rabbit embryos: morphological and molecular aspects. PLOS ONE, 10: e0139814. 2015.

Moreira AJ, Ordoñez R, Cerski CT, Picada JN, García-Palomo A, Marroni NP, Mauriz JL and González-Gallego J. Melatonin activates endoplasmic reticulum stress and apoptosis in rats with diethylnitrosamine-induced hepatocarcinogenesis. PLOS ONE, 10: e0144517. 2015.

Murayama T, Kawashima M, Takahashi T, Yasuoka T, Kuroyama T and Tanaka K. Direct action of melatonin on hen ovarian
granulosa cells to lower responsiveness to luteinizing hormone. Proceedings of the Society for Experimental Biology and Medicine, 215: 386–392. 1997.

Nakagawa-Mizuyachi K, Takahashi T, Kasai S, Nakayama H and Kawashima M. Calcitonin directly inhibits luteinizing hormone-stimulated progesterone production in granulosa cells of the largest follicle of hen. Journal of Poultry Science, 47: 170–175. 2010.

Onagbesan O, Bruggeman V and Decuyper E. Intra-ovarian growth factors regulating ovarian function in avian species: a review. Animal Reproduction Science, 111: 121–140. 2009.

Pang SF, Li L, Ayre EA, Pang CS, Lee PP, Xu RK, Chow PH, Yu ZH and Shi SY. Neuroendocrinology of melanotin in reproduction: Recent developments. Journal of Chemical Neuroanatomy, 14: 157–166. 1998.

Rangel PL, Rodriguez A, Rojas S, Sharp PJ and Gutierrez CG. Testosterone stimulates progesterone production and STAR, P450 cholesterol side-chain cleavage and LH receptor mRNAs expression in hen (Gallus domesticus) granulosa cells. Reproduction, 138: 961–969. 2009.

Reece WO. Dukes’ Physiology of Domestic Animals. 12th ed. Cornell University Press. Ithaca-London. 2004.

Robinson FE, Etches RJ, Anderson-Langmuir CE, Burke WH, Cunningham FJ, Ishii S, Sharp PJ and Talbot RT. Steroidogenic relationship of gonadotrophin hormones in the ovary of the hen (Gallus domesticus). General and Comparative Endocrinology, 69: 455–466. 1988.

Sechman A. The role of thyroid hormones in regulation of chicken ovarian steroidogenesis. General and Comparative Endocrinology, 190: 68–75. 2013.

Söderquist F, Janson ET, Rasmussen AJ, Ali A, Stridsberg M and Cunningham JL. Melatonin immunoreactivity in malignant small intestinal neuroendocrine tumours. PLOS ONE, 11: e0164354. 2016.

Taketani T, Tamura H, Takasaki A, Lee L, Kizuka F, Tamura I, Taniguchi K, Mackawa R, Asada H, Shimamura K, Reiter RJ and Sugino N. Protective role of melatonin in progesterone production by human luteal cells. Journal of Pineal Research, 51: 207–213. 2011.

Tyagi E, Agrawal R, Nath C and Shukla R. Effect of melatonin on neuroinflammation and acetylcholinesterase activity induced by LPS in rat brain. European Journal of Pharmacology 640: 206–210. 2010.

Vanecek J. Cellular mechanisms of melatonin action. Physiological Reviews, 78: 687–721. 1998.

Wang F, Tian XZ, Zhou YH, Tan DX, Zhu SE, Dai YP and Liu GS. Melatonin improves the quality of in vitro produced (IVP) bovine embryos: implications for blastocyst development, cryotolerance, and modifications of relevant gene expression. PLOS ONE, 9: e93641. 2014.

Wang SJ, Liu WJ, Wu CJ, Ma FH, Ahmad S, Liu BR, Han L, Jiang XP, Zhang SJ and Yang LG. Melatonin suppresses apoptosis and stimulates progesterone production by bovine granulosa cells via its receptors (MT1 and MT2). Theriogenology, 78: 1517–1526. 2012.

Webley GE and Hearn JP. Local production of progesterone by the corpus luteum of the marmoset monkey in response to perfusion with chorionic gonadotrophin and melatonin in vivo. Journal of Endocrinology, 112: 449–457. 1987.

Webley GE and Luck MR. Melatonin directly stimulates the secretion of progesterone by human and bovine granulosa cells in vitro. Journal of Reproduction and Fertility, 78: 711–717. 1986.

Yu GM and Maeda T. Inline progesterone monitoring in the dairy industry. Trends in Biotechnology, 35: 579–582. 2017.

Yu MW, Robinson FE and Etches RJ. Quantification of ovarian steroidogenesis in the domestic-fowl by incubation of intact large follicles. Poultry Science, 71: 346–351. 1992.

Zhang N, Sun Q, Zhang H, Cao Y, Weeda S, Ren S and Ou YD. Roles of melatonin in abiotic stress resistance in plants. Journal of Experimental Botany, 66: 647–656. 2015.

Zhou W, Zhang X, Zhu CL, He ZY, Liang JP and Song ZC. Melatonin receptor agonists as the “perioceutics” agents for periodontal disease through modulation of porphyromonas gingivalis virulence and inflammatory response. PLOS ONE, 11: e0166442. 2016.