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RESEARCH ARTICLE

Inhibitory Effect of Melatonin on Formation of the Chicken Primordial Follicles

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ABSTRACT

Melatonin plays pivotal roles in controlling photoperiod-related circadian rhythm and regulating the functions of diverse target tissues as a hormone or an antioxidant agent. However, there is little evidence to demonstrate that melatonin is able to regulate the early ovarian development in the chicken. Here we investigated effects of melatonin on the developmental process from ovarian germ cells to primordial follicles in the chicken. Melatonin was administered from the Day 12 of the embryos till Day 6 of the chicks at 1-100 pg/day. Treatment with melatonin (100 pg/day) induced a decrease of germ cell cysts and follicles number, and the ovarian cortex thickness. In addition, melatonin suppressed the ovarian cells proliferation that was demonstrated by decreased proliferation cell nuclear antigen expression and germ cell marker Dazl protein. Furthermore, melatonin increased the expression of hypothalamic gonadotropin-inhibitory hormone (*GnIH*) mRNA, but decreased the expression of all mRNAs of hypothalamic gonadotropin-releasing hormone (*GnRH*) II, pituitary follicle stimulating hormone (*FSH*)-β and ovarian FSH receptor, luteinizing hormone (LH) receptor, estrogen receptors α and β, progesterone receptor, steroidogenesis enzymes *Cyp19a1* and *3βHSDII*. These data indicated that melatonin might inhibit the ovarian germ cells number via suppression of the hypothalamus-pituitary-ovarian axis in the chicken, subsequently resulting in the inhibition of primordial follicles formation.

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INTRODUCTION

Exposing eggs with light during incubation can increase growth, hatchability and decrease incubation time (Shafey, 2004; Archer *et al*., 2009). As a light-sensitive hormone, melatonin plays pivotal roles in controlling photoperiod-related circadian rhythm and regulating the functions of diverse target tissues as a hormone or an antioxidant agent (Liu *et al*., 2011; Singh *et al*., 2012). In addition, melatonin may play an important role in growth and circadian physiological traits of embryos (Özkan *et al*., 2012). However, it is still unclear whether melatonin could regulate ovarian development in the chicken embryos and chicks.

Light illumination melatonin is an important endocrine signal to influence mammalian reproductive system through regulation of a variety of hormones and their receptors (Reppert *et al*., 1994), and melatonin could partially suppress the hypothalamus-pituitary-ovarian axis in rats (Chuffa *et al*., 2011). At embryonic day 12 (ED12) the pinealocytes and supporting cells can be distinguished (Möller and Möller, 1990) and it appeared to act directly on the gonadotropin-inhibitory hormone (*GnIH*) neurons via its receptors to induce hypothalamic *GnIH* expression and release (Chowdhury *et al*., 2010). *GnIH* inhibits the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland (Bentley *et al*., 2009). Meanwhile, a previous study revealed that light-induced low level of melatonin was related with increased plasma gonadotropins (GTH) and decreased plasma PRL level, as well as elevated egg production in 300-day-old hens (Li *et al*., 2015). In addition, monochromatic light up-regulated the expression of estrogen receptor (ER) and progesterone receptor (PR) mRNAs in ovarian follicles of 28-wks-old hens through decreasing melatonin levels (Liu *et al*., 2015). Melatonin prevented the primordial follicles over-activation and had protective effect via antioxidative and antiapoptotic properties on mouse ovary (Cruz *et al*., 2014; Jang *et al*., 2016; Shiroma *et al*., 2016). However, it is unclear whether melatonin plays a similar role during the critical developmental process from embryonic germ cells to primordial follicles.

Chicken embryos provide many advantages in the assessment of the function of hormones on embryonic development as they are independent of maternal influences during incubation. Here, we hypothesized that melatonin could modify the developmental process from the ovarian germ cells to primordial follicles through regulating the hypothalamus-pituitary-ovarian axis, and this critical process is essential for formation of early ovarian follicles. The results would help us to delineate the functions of melatonin to regulate early ovarian development in the poultry.

MATERIALS AND METHODS

Treatments of animals and tissue collection: Fertilized Hyline chicken eggs were incubated at 38.5ºC, 60% humidity. From embryonic day 12 (ED12) to post-hatching day 6 (D6), melatonin (1, 10 and 100 pg/day, Sigma Aldrich, St. Louis, MO, USA) or PBS in 0.1 ml volume was administrated through air sac (embryos) or peritoneum (chicks) at 10:00 am. At Day 6, all chicks were sacrificed after anesthesia, and then the left ovary and blood were collected. All procedures were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of Zhejiang University.

Morphological observation of the ovaries: The ovaries were embedded in paraffin and cut into 5 μm sections. Five serial sections of each ovary were selected for staining with hematoxylin & eosin (H&E) staining and the follicles per cortical area (mm^2) were counted. Follicles were classified as the primordial follicles and the growing follicles. The largest cross-sections were used to count the average thickness of the cortex and the number of germ cell cysts. Meanwhile, the germ cell cysts were graded into three categories, which contained 0-50, 51-100, or over 100 germ cells per cyst, respectively.

Determination of serum melatonin: Serum melatonin levels were assayed using an enzyme-linked immunosorbent assay kit (Cloud-Clone Corp, Houston, USA). Serum was added to the appropriate wells in the antibody pre-coated microplates according to the manufacturer's direction. The optical density at 450 nm was determined using a microplate reader.

Immunocytochemistry of PCNA: The ovarian sections were incubated with mouse antibody against proliferating cell nuclear antigen (PCNA) (Abcam, ab29, Cambridge, UK) overnight at 4ºC. Biotin-goat anti-mouse IgG was used as the secondary antibody and 3, 3'-diaminobenzidine (DAB) as the chromogen. In the negative control the primary antibody was replaced with normal serum.

RNA extraction and real time PCR analysis: Total RNA was extracted using a Trizol reagent (Invitrogen Co., Carlsbad, CA, USA). The cDNA was generated from 2 μg total RNA by the SuperScript First-Strand Synthesis System (Fermantas, Glen Burnie, MD). Then, qPCR was carried out in triplicate to assess the expression of *GnIH,* gonadotropin-releasing hormone *II* (*GnRH-II*), *FSH-β,* FSH receptor (*FSHR*), *Cyp19a1* (Cytochrome P450, family 19, subfamily A, polypeptide 1), *ERα, ERβ,* LH receptor (*LHR*), 3β-hydroxysteroid dehydrogenase type II (*3βHSDII*) and *PR* with a SYBR Premix Ex Taq™ (TaKaRa Bio Inc., Japan). The sequences of primers for qPCR are listed in Table 1. After normalization with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), relative RNA levels were calculated by the delta-delta CT method.

Western blot analysis: The left ovaries were homogenized with 50 mM Tris-HCl buffer containing 150 mM NaCl, 15 mM EDTA, 0.1% TritonX-100, 1 mM PMSF and 2 μg/ml leupeptin. Proteins were separated on a 10% SDS polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membrane was incubated overnight at 4ºC with mouse anti-PCNA primary antibody (Abcam, ab29, Cambridge, UK). Antibody recognition was achieved with the respective secondary antibody conjugated to horseradish peroxidase at room temperature for 2 h. The GAPDH and β-actin bands were adopted as the internal control.

Table 1: Primers for PCR analysis.

Gene	Accession no.	Primer sequences (5'-3')	Product length/bp
ERα	NM.205183.2	TAGTTCCGCTCTACGACCTCTT AGTTGGTTTCGGTTCTCCTCTT	106
ERβ	NM.204794.2	GCGTGTTATGGTCTGCTCCT GGTCCAGTGGTCTCACTTCG	114
Cyp19a1	NM.001001761.2	AACATTGGACGCATGCACAA CCGAGGGCCAAATCCAAATG	117
PR	NM.205262.1	AGGCTTCTGGTTGCCACTAC CACGCTGGACAGTTCTTCCT	148
FSHR	NM.205079.1	ACCTGCCTGGATGAGCTAAA ATCCATGACTTGGCAGGAAG	136
GADPH	M.11213.1	ACTGTCAAGGCTGAGAACGG AGCTGAGGGAGCTGAGATGA	204
GnIH	NM.204363.1	TTGGCTACAGTGGCGTTTCT GACTCCTCTGCTTTTCCTCCA	136
GnRH-II	NM.001039913.1	TTCATTGGTCTGCGACTCCC CAGGAATGGCCTCGGAGAAA	125
FSH-β	NM.204257.1	TGCTTCACAAGGGATCCAGTA GATTCAGGATGGTCACCGCA	122
3βHSDII	NM.205118	ATGCGGTGTTGCGGTGTGGT CACCGTGGGTGCAACGTGGT	179
LHR	NM.204936.1	CGTCCTCATAACCAGCCACT AGCATCCACCGAAGCAAT	115

Statistical analysis: All data were expressed as the mean±SEM and analyzed by t test or One-way analysis of variance (ANOVA) with Duncan's multiple-range tests using the SPSS 21.0 software. $P<0.05$ was considered as a statistically significant difference.

RESULTS

Inhibition of the early ovarian development by melatonin treatment: To confirm if melatonin treatment could affect the chicken ovarian development, we administrated melatonin at 1, 10 and 100 pg/day from ED12 to D6. Melatonin (100 pg/day) treatment decreased the number of ovarian follicles, while the melatonin levels in serum (14.7 pg/ml) increased significantly compared with control group (12.4 pg/ml) (Fig. 1). Statistic result (Fig. 1C) showed that, compared with the control, total follicles, primordial follicles and growing follicles in the cortical area were significantly decreased by 54.79%, 53.72% and 48.12% ($P<0.05$), respectively. However, follicles number in 1 and 10 pg/day melatonin treatment groups showed no significant difference (data not shown).

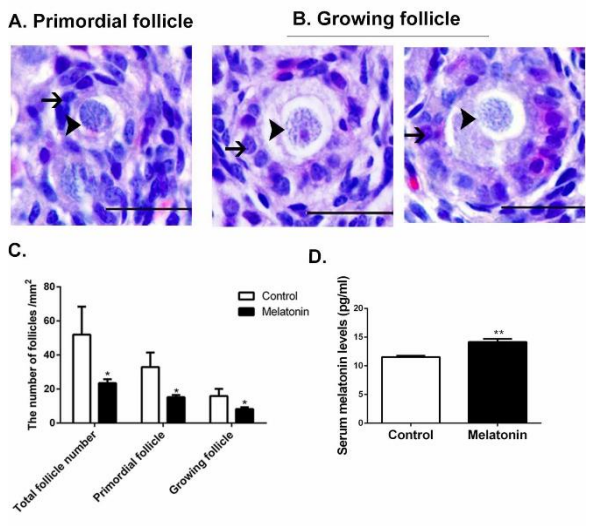

Fig. 1: Effect of melatonin treatment on chicken follicular development. (A) Histology of a primordial follicle with an oocyte surrounded partially or completely by flattened (squamous) pregranulosa cells. (B) Histology of a growing follicle with an oocyte surrounded by one or more cuboidal granulosa cell layers in the chicken. Scale bar: 20 µm. (C) Changes of the number of the primordial and growing follicles after melatonin treatment at 100 pg/day. (D) Serum melatonin concentrations were measured by ELISA using blood samples collected from the chicks after 6 h of melatonin injection at the 6th day. Values are mean±SEM of five experiments. Asterisks indicate significant statistical difference (P<0.05). Arrow: oocyte; Arrowhead: pregranulosa or granulosa cells.

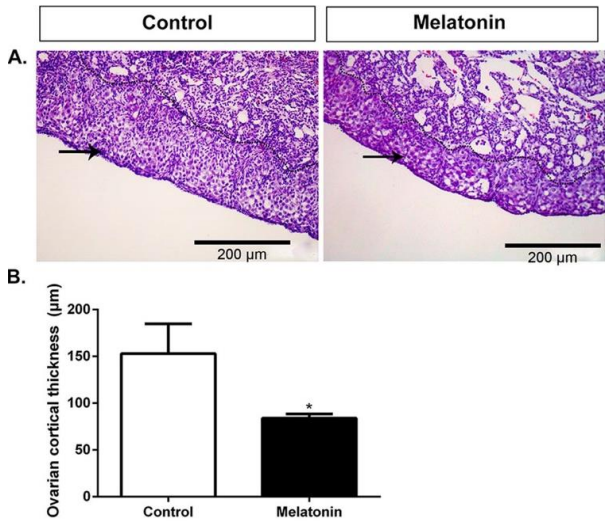

Fig. 2: Effects of melatonin treatment on ovarian cortex thickness in the chicken. (A) Morphological changes of the ovarian cortex from Day 6 chicks after melatonin treatment at 100 pg/day. The black dotted lines represent the boundary between the cortex and medulla, and the arrows represent the ovarian cortex. Scale bar: 200 µm. (B) The average thickness of ovarian cortex in the control and melatonin treatment groups. Values are mean±SEM of five experiments. An asterisk indicates significant statistical difference (P<0.05).

In addition, chick ovarian development was also repressed by melatonin. Compared with the control, melatonin (100 pg/day) treatment could decrease the thickness of ovarian cortex by 45.11% (P<0.05, Fig. 2). The size of germ cell cysts was categorized as small, medium and large class, which contained 0-50, 51-100 and 100- germ cells per cyst, respectively (Fig. 3A). Compared with control, as shown in Fig. 3B, melatonin could decrease the number of total germ cell cyst by 40.74% (P<0.01). Among them, the number of small germ cell cysts was remarkably reducing by 30.58% (P<0.001) after melatonin treatment.

Inhibition of the ovarian cells proliferation by melatonin treatment: Compared with the control, the melatonin group (100 pg/day) manifested less PCNA-positive ovarian cells (Fig. 4). Western blot analysis showed that PCNA expression was significantly lower in the melatonin-treated group than the control (Fig. 4E and F, P<0.001). The ratio between the PCNA-positive and the total germ cells was remarkably lower in the melatonin treatment group than the control (Fig. 5A and B, P<0.01), Meanwhile, the expression of Dazl protein was significantly lower in the melatonin-treated group than the control (Fig. 5C, P<0.05).

Changes in the expression of *GnIH*, *GnRH-II*, *FSH-β* and *GTH* receptors mRNAs after melatonin treatment: Results from the qRT-PCR indicated that melatonin treatment at 100 pg/day could increase the expression of *GnIH* mRNA in the hypothalamus (Fig. 6A, P<0.05). Meanwhile, the mRNA of *GnRH-II* was significantly lower in the hypothalamus in the melatonin-treated group than the control (Fig. 6B, P<0.05). In the pituitary, expression of *FSH-β* mRNA in the melatonin-treated group was significantly lower than the control (Fig. 6C, P<0.05). In the ovary, the mRNAs of both *FSHR* and *LHR* were significantly lower in the melatonin-treated group than the control (Fig. 6D, P<0.05).

Changes of the steroids synthesis and their receptors in the ovaries after melatonin treatment: Expression of *Cyp19a1* and *3βHSDII* mRNAs was significantly lower in the melatonin-treated group (100 pg/day) than the control (Fig. 6E, P<0.05). Furthermore, the expression of all *ERα*, *ERβ*, and *PR* mRNAs was significantly lower in the melatonin-treated group than the control (Fig. 6F, P<0.05).

DISCUSSION

Melatonin could regulate the ovary development via modifying the synthesis and secretion of LH, FSH and E_2 (Dardes *et al*., 2000; Chuffa *et al*., 2011). Meanwhile, our laboratory preliminary study showed that the reproductive axis hormone control the chicken embryo meiosis and mitosis (He *et al*., 2013; Li *et al*., 2016). Furthermore, in birds, melatonin appears to act directly on the *GnIH* neurons or may directly affect ovarian function via the melatonin receptors in the ovary (Tamura *et al*., 2009; Chowdhury *et al*., 2010; Chowdhury *et al*., 2013). Here, we investigated the role of melatonin on the developmental process in primordial follicle assembly using the chicken embryo. Results showed that melatonin treatment at 100 pg/day decreased the number of germ cells and suppressed proliferation of the ovarian cells via endocrine regulation; melatonin enhanced the expression of *GnIH* mRNA in the hypothalamus, or inhibited the synthesis and secretion of steroids and their corresponding receptors. The decreased of ovarian cells might lead to the inhibition of the primordial follicle's formation.

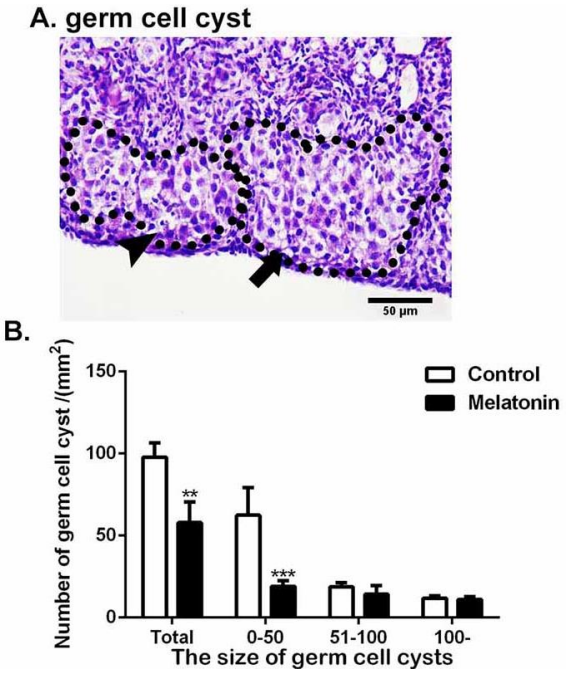

Fig. 3: Effect of melatonin treatment on the number of germ cell cysts. (A) Histology of germ cell cysts with different sizes. Arrow: 50-100 germ cells/cyst. Arrowheads: 0-50 germ cells/cyst. The black dotted lines represent the boundary between the germ cell cysts and the surrounding somatic cells. Scale bar: 50 μm. (B) Changes of the number of germ cell cysts after melatonin treatment at 100 pg/day. Values are means±SEM of five experiments. Asterisks indicate significant differences (** P<0.01, *** P<0.001).

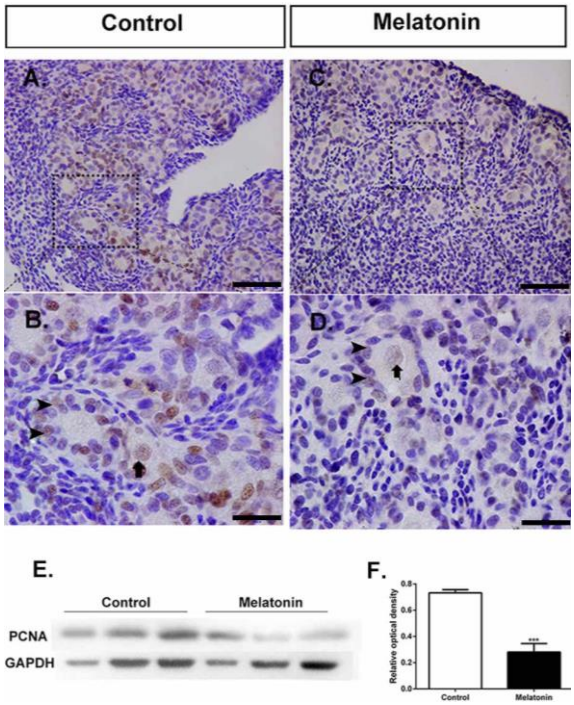

Fig. 4: Effect of melatonin treatment on proliferation of the ovarian cells. The presence of PCNA protein is indicated by a dark brown stain in the ovarian sections. The ovarian section of the control group (A, scale bar: 50 μm) and the enlarged picture (B, scale bar: 20 μm). The ovarian section of melatonin-treated group at 100 pg/day (C, scale bar: 50 μm) and the enlarged picture (D, scale bar: 20 μm). The PCNA staining could be found in the proliferating germ and somatic cells. Arrow: PCNA positive germ cells; Arrowhead: PCNA positive somatic cells. (E) Western blot analysis of PCNA expression in ovaries from Day 6 chicks after melatonin treatment. (F) Grey analysis of the PCNA protein expression after melatonin treatment. Values are mean±SEM of five experiments. Asterisks indicate significant difference (P<0.001).

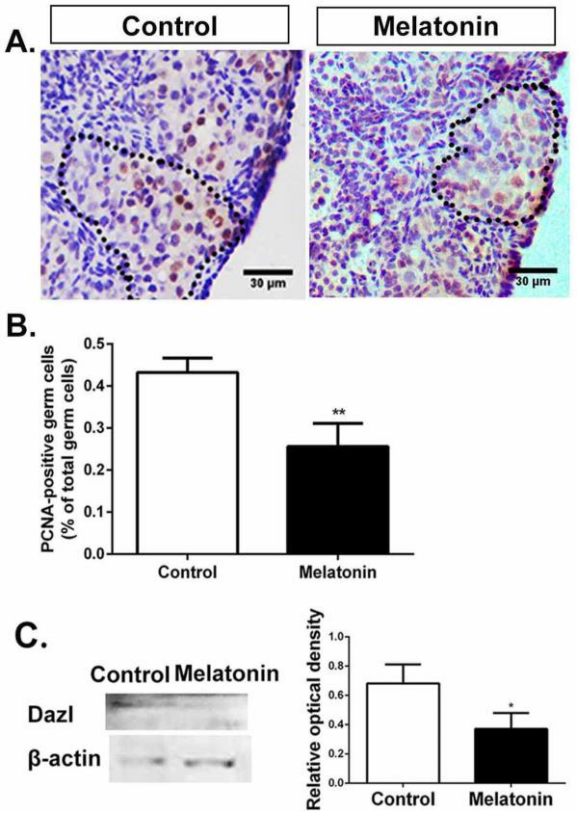

Fig. 5: Effect of melatonin on proliferation of the ovarian germ cells in the cysts. (A) Histology of germ cells with PCNA staining in the control and melatonin-treated groups at 100 pg/day. The black dotted line represents the boundary between germ cells and its surrounding somatic cells. Scale bar: 30 μm. (B) The ratio of PCNA-positive germ cells to total germ cells after melatonin treatment. (C) Western blots analysis of Dazl (germ cell marker) expression in ovaries from Day 6 chicks after melatonin treatment. Values are mean±SEM of five experiments. Asterisks indicate significant difference (P<0.01).

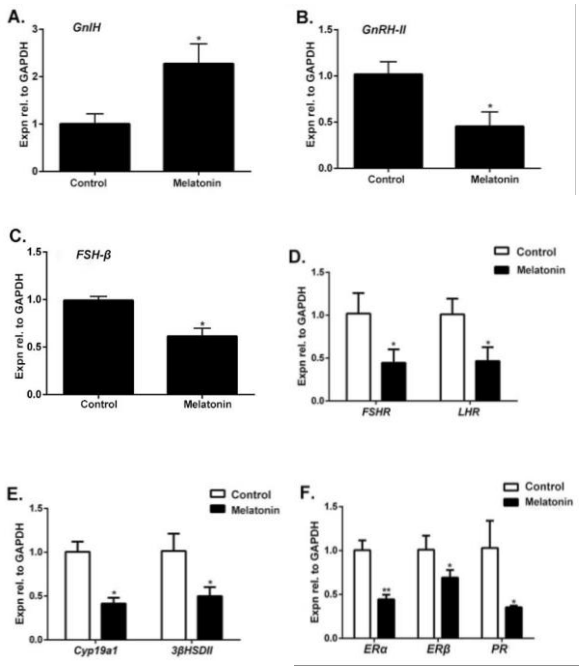

Fig. 6: Effect of melatonin (100 pg/day) treatment on expression of *GnIH* (A), *GnRH-II* (B), *FSH-β* (C) mRNAs, GTH receptors (D), steroid hormone synthetase (E) and steroid hormone receptors (F) mRNAs measured by qRT-PCR in ovaries from Day 6 chick ovaries. Values are the mean±SEM of five experiments. Asterisks indicate significant difference (* P<0.05, ** P<0.01).

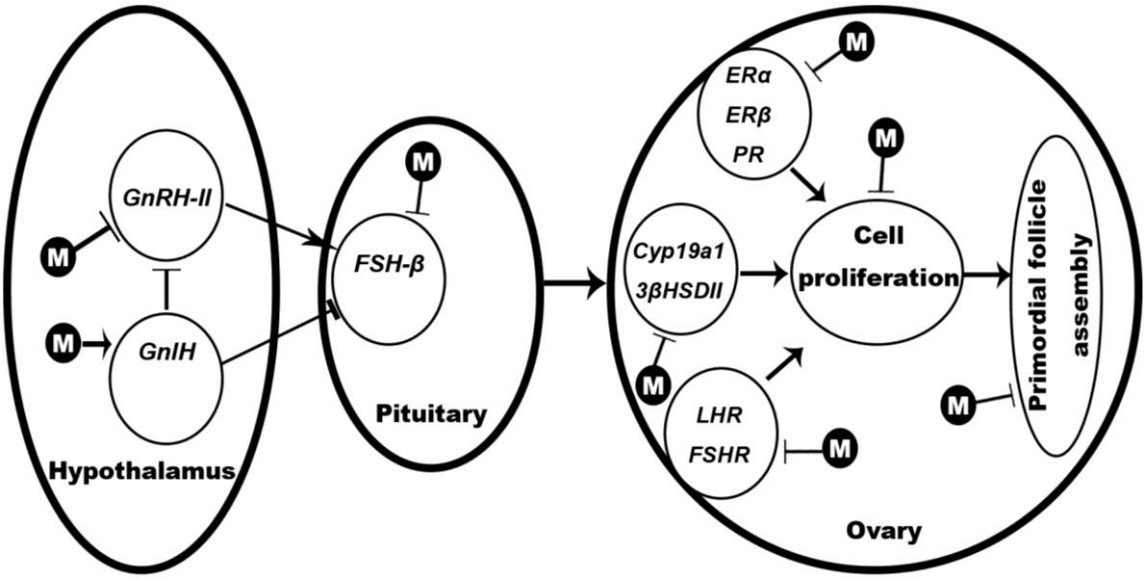

Fig. 7: Melatonin inhibited the primordial follicle formation via inhibiting activity of the hypothalamus-pituitary-ovarian axis and ovarian cell proliferation. During normal folliculogenesis, germ and somatic cells experienced proliferation to form germ cell cysts. After germ cell cysts break-down and assembly the primordial follicles were formed. Melatonin treatment inhibited the germ and somatic cells proliferation, and reduced the germ cell cysts' size and number in the chicken ovaries via enhancing the expression of *GnIH* mRNA, while decreasing the expression of *GnRH-II*, gonadotropins, steroid hormone synthetases and receptors mRNAs, resulting in decreased formation of the primordial follicles. M: melatonin.

Previous studies showed that melatonin or light stimulation could regulate the reproduction in the chicken at post-hatch time (Chowdhury *et al*., 2013; Liu *et al*., 2015). Meanwhile, treatment of the chicken embryos by melatonin may have potentially positive effects on the behavioral and physiological development (Özkan *et al*., 2012). Akasaka *et al.* (1995) reported that melatonin was detected in the chicken embryo as early as ED10, and increasing melatonin content at nighttime was found in the ED13-18 chicken embryos when they were kept under a 12:12 light: dark (LD) cycle. Long-term melatonin treatment showed the decreased of the primordial follicles' number in chicken ovaries, and exogenous melatonin increased the serum melatonin significantly. Furthermore, results from the melatonin treatment indicated that the ovarian cortex thickness decreased significantly, and the number of germ cell cysts showed the inhibition by melatonin treatment. This phenomenon implies that long-term melatonin treatment might inhibit the chicken ovarian development.

FSH promoted cell proliferation via estrogen while LH accelerated the meiotic initiation via elevating P4 production (He *et al*., 2013), which indicated that FSH and LH regulated the number of germ cells. Melatonin could directly or indirectly regulate the hypothalamus-pituitary-ovarian axis. Melatonin stimulated not only *GnIH* expression but also *GnIH* release in the quail (Chowdhury *et al*., 2013). Expression of the *GnIH* precursor mRNA occurred on embryonic day10 (E10). Moreover, *GnIH* could decrease the FSH and LH levels by inhibiting GnRH-II neurons or decreasing *FSH-β* subunit expression in the chicken pituitary (Bentley *et al*., 2009). Melatonin decreased expression of PCNA mRNA and protein, thus to attenuate proliferation of ovarian cells in the chicken and rat (Romeu *et al*., 2011). Here treatment of melatonin decreased the germ cell marker Dazl protein and the ratio of PCNA-positive germ cells to total germ cells, meanwhile, the number of germ cell cysts decreased significantly in melatonin treatment group, which indicated that melatonin might inhibit the germ cells proliferation. In addition, melatonin inhibited the somatic cells proliferation, and decreased the ovarian cells' PCNA protein expression. Meanwhile, the increasing of serum melatonin levels enhanced the mRNA of *GnIH* in the hypothalamus. On the contrary, the mRNA of *GnRH-II* was decreased by melatonin treatment in the hypothalamus. Furthermore, the *FSH-β* subunit mRNA in the pituitary was significantly decreased by melatonin administration. Therefore, melatonin might inhibit germ cells and somatic cells proliferation via enhancing the *GnIH* mRNAs as well as suppressing *GnRH-II* and *FSH-β* mRNAs, leading to inhibition of the germ cell cysts' size and number.

In the present study, melatonin decreased the mRNA abundance of *FSHR* and *LHR* receptors, which indicated that melatonin could inhibit the early ovarian development through inhibiting the expression of the gonadotropin receptors at the ovarian level. It was reported that melatonin might decrease E_2 level during the premenopausal period (Okatani *et al*., 1999) and melatonin can reduce LH and 17 β-estradiol levels, and induce differential regulation of sex steroid receptors in the reproductive tissues during rat ovulation (Chuffa *et al*., 2011). Furthermore, the inhibition of either aromatase or *3βHSDII* resulted in lower oocyte density in the ovary (Grive and Freiman, 2015). In addition, our study also indicated that E_2 promoted the chicken primordial follicle formation (unpublished data). Here, the mRNA of steroid receptors (*ERα*, *ERβ* and *PR*) and the enzymes that convert androgens to estrogens and pregnenolone to progesterone (*Cyp19a1* and *3βHSDII*) were all significantly inhibited by melatonin treatment, which indicated that melatonin inhibit the synthesis of E_2, P_4 and their corresponding receptors, which might inhibit the formation of primordial follicles. Therefore, the study indicated that melatonin suppressed the steroid synthesis

and their corresponding receptors to inhibit the primordial follicle assembly.

Conclusions: This study revealed that melatonin inhibited the germ and somatic cells proliferation, and reduced the germ cell cysts' size and number in the chicken ovaries via enhancing the expression of *GnIH* mRNA, while decreasing the expression of *GnRH-II*, gonadotropins, steroid hormone synthetases and receptors mRNAs, resulting in decreased formation of the primordial follicles (Fig. 7). In addition, melatonin inhibited synthesis enzymes of the steroid hormones and their corresponding receptors to inhibit the primordial follicle assembly.

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Authors contribution: In this paper, G Liu designed the research and wrote the manuscript. G Liu, R Li, C Guo and D Zhao performed experiments, J Li, Y Mi and C Zhang provided the concept and experiment design and revised the manuscript. All authors joined the analysis and interpretation of data and approved the final version of the manuscript.

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