Luzindole and 4P-PDOT block the effect of melatonin on bovine granulosa cell apoptosis and cell cycle depending on its concentration

Shujuan Wang¹,²,*, Wenju Liu³,*, Aiyou Wen¹, Bing Yang¹ and Xunsheng Pang¹

¹ College of Animal Science, Anhui Science and Technology University, Fengyang, China
² Anhui Province Key Laboratory of Animal Nutritional Regulation and Health, Fengyang, China
³ College of Life and Health Science, Anhui Science and Technology University, Fengyang, China
* These authors contributed equally to this work.

ABSTRACT

Granulosa cells play an essential physiological role in mediating the follicle development and survival or apoptosis of granulosa cells dictate the follicle development or atresia. The aim of this study was to investigate the role of high dose (10⁻⁵ M) and low dose (10⁻⁹ M) melatonin in bovine granulosa cells, and assess whether MT1 and MT2 inhibitor affect granulosa cells response to melatonin. We found that the high dose (10⁻⁵ M) and low dose (10⁻⁹ M) both could act as an essential role in modulating granulosa cells apoptosis, cell cycle and antioxidant. The beneficil effect could be related to that melatonin promoted the expression of Bcl2, Bcl-xl, SOD1 and GPX4, and inhibited Bax, caspase-3 and p53 expression. Moreover P21 expression was decreased in granulosa cells treated with the high dose (10⁻⁵ M) melatonin and increased in that treated with the low dose (10⁻⁹ M) melatonin. To further reveal the role of MT1 and MT2 in mediating the effect of melatonin on granulosa cells apoptosis, cell cycle and antioxidant, we found that the luzindole and 4P-PDOT did not affect the effect of high dose (10⁻⁵ M) melatonin on regulating Bcl2, Bax, caspase-3, SOD1, GPX4 and p53 expression, while blocked its effect on modulating Bcl-xl and P21 expression. However, luzindole and 4P-PDOT disturbed the effect of low dose (10⁻⁹ M) melatonin on regulating Bcl2, Bax, caspase-3, Bcl-xl, SOD1, GPX4, and p53 expression. In conclusion, these results reveal that the effect of low dose (10⁻⁹ M) melatonin on granulosa cells apoptosis are mediated by MT1 and MT2, and the high dose (10⁻⁵ M) melatonin affect the granulosa cells apoptosis by other pathway, besides MT1 and MT2. Moreover MT1 and MT2 may work in concert to modulate bovine granulosa cells function by regulating cellular progression and apoptosis.

INTRODUCTION

The mammalian ovary is an important organ for females during their whole reproductive life. It can produce oocytes, giving animals the chance to procreate. However, every oocyte
does not have the chance to ovulate, and most of the follicles undergo atresia during their
development (Kaipia & Hsueh, 1997). Many factors affect the ovarian follicle development
process, such as reactive oxygen species (ROS), disease, endocrine and local autocrine/
paracrine systems, as well as the status of granulosa cells (Richards, 2001; Vitt & Hsueh,
2001; Orisaka et al., 2006). Therefore, granulosa cells play an essential physiological role in
mediating the oocytes mature and follicle development. Even the survival or apoptosis of
granulosa cells dictate the follicle development or atresia (Matsuda et al., 2012; Jiang
et al., 2003; Choi et al., 2011).

Granulosa cells act as so important role during folliculogenesis, and many studies
focus on exploring the reason of follicular atresia caused by granulosa cells. Granulosa cells
can secret estradiol and progesterone which are critical for maintaining ovarian function
and inhibiting granulosa cells apoptosis (Arosh et al., 2004; Okamoto et al., 2016).
The luteinization of granulosa cells is essential for pregnancy maintenance, which is a
temporary organ for secretion of progesterone (He et al., 2016a). During follicles development,
granulosa cells completed the preparations for proliferation and differentiation, shifted energy
metabolism to glycolysis, and follicles acquired the capacity for estradiol secretion and
ovulation (Chen et al., 2020). The granulosa cells within the follicle accompanied by the
proliferation, differentiation, and apoptosis during the reproductive period (Chowdhury
et al., 2016; Denkova et al., 2014; Chen et al., 2020). Therefore, the apoptosis cause the change
of granulosa cells function and disturb the state of follicle development. Meanwhile, the
balance between Bcl-2 family and Bax is crucial for granulosa cells survival and apoptosis.
These apoptosis related factors include Bcl2, Bcl-xl, Bax, caspase-3 and p53, which act as
crucial roles in regulating granulosa cells apoptosis (Cruz et al., 2014; Fu et al., 2014; He et al.,
2016b). Endogenous and exogenous factors producing ROS cause oxidative stress, induce cell
membrane and DNA damage, lead to the granulosa cells apoptosis and are involved in
antral follicle atresia (Gupta et al., 2006; He et al., 2016d; Kang et al., 2009).

Granulosa cells undergo many negative factors, and how to overcome these
disadvantages is very important for granulosa cells to avoid apoptosis. Melatonin is present
in follicular fluid, and its concentration is significantly higher compared to that in
peripheral blood serum concentration, and it has also been found that there is positive
connection between melatonin concentration and follicular diameter (Yie et al., 1995;
Tamura et al., 2009, 2012). The main reason is that melatonin can been secreted by
granulosa cells (Tamura et al., 2009; Reiter et al., 2014; Suofu et al., 2017). Recently,
melatonin has exhibited the essential role in directly regulating the ovary function. It is well
known that melatonin has the benefit of promoting the oocyte maturation, embryos
development, as well as hormone secretion of granulosa cells (Dubocovich & Markowska,
2005; Tian et al., 2014; Wang et al., 2012). Moreover, melatonin can mediate the follicle
development and protect the integrity of oocytes and granulosa cells and prevent the
apoptosis by scavenging the ROS (Tamura et al., 2009; Fatehi et al., 2005; He et al., 2016a;
Tanabe et al., 2015). In addition, it is well established that melatonin prevents the apoptosis of
granulosa cells by mediating the apoptosis-related genes, including Bcl2 family, p53 and
caspase-3, which are involved in the granulosa cells apoptosis progress (Cruz et al., 2014;
Fu et al., 2014; He et al., 2016b; Wang et al., 2012). Although numerous studies have
focused the effect of melatonin on granulosa cells, melatonin affecting granulosa cells function through which melatonin receptor is still needed to be investigated.

Melatonin exerts its physiological function primarily through binding to its receptors, MT1 and MT2 (Dubocovich & Markowska, 2005). To confirm the actions of melatonin preventing the apoptosis of granulosa cells and regulating cell cycle are mediated through its receptor, MT1 and MT2. Melatonin receptor antagonist, luzindole and 4P-PDOT were used to further measure the actions of melatonin on granulosa cells, and some crucial genes regulate granulosa cells apoptosis and cell cycle were also determined by qRT-PCR.

MATERIALS AND METHODS

Bovine granulosa cells isolation and culture

Granulosa cells collection was performed as our previously described elsewhere (Wang et al., 2017a, 2017b, 2018; Liu et al., 2018). Bovine ovaries were obtained from Bengbu abattoir (Anhui, China). About 60 bovine ovaries were chose by washing three times using 70% alcohol, and then the ovaries were washed three times by sterile 0.9% NaCl to remove alcohol in this study. The follicular fluid from 3–8 mm antral follicles were chose to isolate the granulosa cells using a syringe and sterile needle puncture method. The follicular fluid also were filtered using a 400 mesh cell strainer. The cell pellets isolated from follicles were digested for 5 min using 0.25% trypsin with 0.025% EDTA (catalog number: 15400-054; Gibco, Grand Island, NY, USA). Then the cell pellets digested were centrifugated at 1,500 rpm for 5 min. The pellets were diluted with Dulbecco’s Modified Eagle Medium (DMEM) (catalog number: 430-2100; Gibco, Grand Island, NY, USA) supplemented with streptomycin (50 µg/ml), penicillin (50 IU/ml) (catalog number: 15070-063; Pen-Strep, Invitrogen, Carlsbad, CA, USA), plasmocin (25 µg/ml, catalog number: ant-pc; Invivogen, San Diego, USA), and 10% fetal bovine serum (FBS, catalog number: SH30070.03; Hyclone, UT, USA). Finally, the separated cells were placed in 60-mm cell culture dishes. The granulosa cells were cultured at 37 °C in an incubator containing 5% CO2. In this study, the protocols for the experiment were reviewed and approved by the Institutional Committee on Animal Care and Use at Anhui Science and Technology University, and the experiments were repeated three times independently.

RNA extraction

To assess the target genes expression level in granulosa cells treated with melatonin within or without 4P-PDOT (catalog number: 134865-74-0; Sigma, Louis, MO, USA) or luzindole (catalog number:117946-91-5; Sigma, Louis, MO, USA), total RNA was extracted from granulosa cells treated with melatonin in the absence/presence of preincubation with luzindole or 4P-PDOT for 48 h using RNAprep pure cell Kit (catalog number: DP430; Tiangen, Beijing, China) according to the manufacturer’s protocols. Moreover, RNase-free DNasel was used to digest the RNA to remove the genomic DNA. The content of RNA was quantified by Nanodrop One (Thermo Scientific, Waltham, MA, USA). The cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (catalog number: K1622; Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s protocols.
Real-time PCR

The target genes expression level were carried out using the quantitative real-time PCR with LightCycler 480 SYBR Green I Master Mix (catalog number: 4887352001; Roche, Penzberg, Germany) according to our previously reported method (Wang et al., 2017b, 2018). The primer pairs designed for measuring the target genes were listed in Table 1. A total of 10 μL reaction volume was prepared as follows: 5 μL SYBR Green I Master Mix, 2 μL cDNA, 0.5 μM forward and reverse primers, and 2 μL RNase and DNase-free water. Real-Time PCR amplification was performed in a LightCycler 480 II System (Roche, Mannheim, Germany), and the procedure was as follows: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, annealing at particular temperatures for 20 s, 72 °C for 20 s. To confirm specific PCR product, the melting curve from 65 °C to 95 °C was performed after real-time PCR reactions. Normalization was performed using β-actin as a control in each sample. The date analysis was using the $2^{-ΔΔCt}$ method (Livak & Schmittgen, 2001).

Western blot Analysis

Western blot analysis was performed as previously described else where (Wang et al., 2017b, 2018). Granulosa cells were collected after treatment for 48 h and lysed in RIPA buffer (catalog number: 89900; ThermoFisher, Rochford, IL, USA), then denatured by boiling for 5 min with bromophenol blue and frozen at –80 °C. The proteins were separated by 12% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Firstly, the membrane were incubated with primary antibody: Bcl2 mouse monoclonal antibody (1:500, sc-7382; Santa Cruz, Dallas, TX, USA), Bax mouse monoclonal antibody (1:500, sc-20067; Santa Cruz, Dallas, TX, USA), caspase-3 rabbit polyclonal antibody (ab13847; Abcam, California, USA) and β-actin mouse monoclonal antibody (1:1000, SC-47778; Santa Cruz, Dallas, TX, USA). Later, the membrane was detected by incubation with HRP labeled goat anti-rabbit secondary antibody (SC-2054; Santa Cruz, Dallas, TX, USA) or goat anti-mouse secondary antibody (1:5000; SC-205; Santa Cruz, Dallas, TX, USA), respectively. Finally, membranes

| Gene | Forward primer sequence (5’→3’) | Reverse primer sequence (5’→3’) | Length |
|------|---------------------------------|---------------------------------|--------|
| Bax  | TGCAGAGGATGATCGCAGCTGTG         | CCAATGTCCAGCCCATCATGGTC         | 198    |
| Bcl2 | CGCATCGTGGCCCTTTTTAGGTT         | GCGGGTTCAAGTACTACGTGAT          | 115    |
| Bcl-xl| ATGGCAGCAGTAGAAGCAAG            | GCCGGTTCAGGATCGAGCAT            | 236    |
| Caspase-3| CAGACAGTGGTGCTGAGAGGA     | GCTACCTTTTCGAGTGAACGGCA         | 211    |
| p53 | CCTCCCCAGAAGACGTCACTCAT       | CTCCGATGCTGTGTCCTCAACT         | 221    |
| GPX4 | TGTGCTCGCTCCATGCGACGA          | CCTGGCTCTGCTGCTCCAA            | 224    |
| SOD1 | GCTGTACCAGTGCCATTCACTCA        | CTTTCCACCTCTGCCCCAAGTC          | 228    |
| CyclinD1 | GCCCTCGGTCTGCTCATCTCA         | ACAGGAAGGCTCAGGCTAGT            | 152    |
| CyclinE1 | CCTCCAAAGTTGACCACTTT         | AGGATACGAGCGCAAGGAGCA           | 195    |
| P21  | CGTCTCAGGAGGACACCCTT          | TCAGTCTGCTGTTGAGTGT            | 159    |
| β-actin | CATCGGCAATGAGCGGTTCC         | CCGTGTTGGCGGTAGGCTCC           | 145    |
was incubated with the Clarity Western ECL kit (catalog number: 170-5060; Bio-Rad Laboratories, Hercules, CA, USA), and scanned in a ChemiDocXRS chemiluminescent imaging system (Bio-Rad, Hercules, CA, USA).

Experimental design
To assess the potential effects of melatonin on granulosa cells, melatonin was added to medium at high dose concentrations (10\(^{-5}\) M) and low dose concentrations (10\(^{-9}\) M). The mRNA level of apoptosis related genes (Bcl2, Bcl-xl, Bax, caspase-3 and p53), cell cycle associated gene (CyclinD1, CyclinE1 and P21) as well as other genes (SOD1 and GPX4) involved in antioxidation were detected by real-time quantitative PCR. To confirm whether melatonin inhibited granulosa cells apoptosis via its receptor, melatonin, melatonin plus 4P-PDOT or luzindole were added to medium in each experimental group, respectively. Therefore, experimental groups were divided as following: DMSO group; melatonin group; melatonin plus 4P-PDOT group; and melatonin plus luzindole group. Furthermore, in each experimental group, we detected the expression level of apoptosis related genes (Bcl2, Bcl-xl, Bax, caspase-3 and p53), cell cycle associated gene (CyclinD1, CyclinE1 and P21) and antioxidation related genes (SOD1 and GPX4). In addition, we also detected protein expression level by western blot. Melatonin, 4P-PDOT, and luzindole were dissolved in DMSO, and then diluted to the final concentration (10\(^{-5}\) M and 10\(^{-9}\) M) with DMEM (catalog number: 430-2100; Gibco, Grand Island, NY, USA) before adding to the cultured granulosa cells. DMSO was added to medium as a control at the same concentration.

Statistical analysis
All data were presented as Mean ± SEM of triplicate experiments (\(n = 3\)). Significant difference was evaluated using Duncan’s multiple comparisons following one-way ANOVA with the General Linear Models Procedure of Statistical Analysis Systems (SAS Inc., Cary, NC, USA). \(P < 0.05\) was considered significant.

RESULTS
Effects of melatonin and melatonin receptor antagonist supplementation on the cell cycle related genes
The effects of melatonin within high dose (10\(^{-5}\) M) and low dose (10\(^{-9}\) M) on regulation of cell cycle processes were investigated by evaluating the expression of cell cycle related genes (CyclinD1, CyclinE1 and P21) after melatonin supplementation in the absence/presence of luzindole or 4P-PDOT (Fig. 1). The expression of CyclinD1, CyclinE1 and P21 were not significantly altered after melatonin supplementation in high dose (10\(^{-5}\) M) with or without luzindole or 4P-PDOT compared with control groups (Figs. 1A–1C, \(P > 0.05\)), except P21 in the melatonin group (Fig. 1C, \(P < 0.05\)). Moreover, CyclinD1 and CyclinE1 expression was not differ and P21 was significantly different among melatonin group, melatonin plus luzindole group and melatonin plus 4P-PDOT group (Figs. 1A–1C, \(P > 0.05\)). The expression of CyclinD1, CyclinE1 and P21 in low dose (10\(^{-9}\) M), however, were different to that of high dose (10\(^{-5}\) M) (Figs. 1A–1C). 

CyclinD1 and CyclinE1...
expression were not significantly different after melatonin supplementation with luzindole or 4P-PDOT compared with control groups (Figs. 1A and 1B, $P > 0.05$). The expression of $P21$ was significantly enhanced after melatonin supplementation with or without luzindole or 4P-PDOT compared with control groups (Fig. 1C, $P < 0.05$), and there was no significant difference among melatonin group, melatonin plus luzindole group and melatonin plus 4P-PDOT group (Fig. 1C, $P > 0.05$). Moreover, the expression of $CyclinE1$ was significantly inhibited after melatonin supplementation (Fig. 1B, $P < 0.05$). These results demonstrated that high dose ($10^{-5}$ M) melatonin inhibited the $P21$ expression, which were affected by melatonin receptor antagonist, luzindole and 4P-PDOT supplementation. However, low dose ($10^{-9}$ M) melatonin increased the expression of $P21$ to regulate the cell cycle, which were not affected by melatonin receptor antagonist, luzindole and 4P-PDOT supplementation.

**Effects of melatonin and melatonin receptor antagonist supplementation on granulosa cells apoptosisrelated genes**

The effects of melatonin in high dose ($10^{-5}$ M) and low dose ($10^{-9}$ M) on regulation of apoptosis related genes, including $Bcl2$, $caspase-3$, $Bcl-xl$, $Bax$ and $p53$, were investigated (Fig. 2). The results showed that melatonin supplementation significantly decreased the expression level of $caspase-3$, $p53$ and $Bax$, while increasing the expression level of $Bcl2$ and $Bcl-xl$ (Figs. 2A–2E, $P < 0.05$). These results revealed that melatonin inhibited granulosa cells apoptosis through regulating apoptosis related genes.

To further confirm whether melatonin inhibits granulosa cells apoptosis via its receptor, the expression level of $Bcl2$, $Bcl-xl$, $caspase-3$, $Bax$ and $p53$ were detected after granulosa cells treated with melatonin within 4P-PDOT or luzindole. The expression of $Bcl-xl$, $caspase-3$, $Bax$ and $p53$ were significantly downregulated and $Bcl2$ expression level was significantly upregulated in the melatonin plus luzindole group and melatonin plus
4P-PDOT group with high dose (10^{-5} M) compared with control group (Figs. 2A–2E, \( P < 0.05 \)). Further analysis found that there were no significant difference among melatonin group, melatonin plus luzindole group and melatonin plus 4P-PDOT group in the expression of \( Bcl2 \), \( caspase-3 \), \( Bcl-xl \) and \( p53 \) (Figs. 2A, 2C–2E, \( P > 0.05 \)) with high dose (10^{-5} M). In contrast, the expression of \( Bcl-xl \) was significantly decreased in the melatonin plus luzindole group and melatonin plus 4P-PDOT group compared with high dose (10^{-5} M) melatonin group (Fig. 2B, \( P < 0.05 \)). These results indicated that the effect of high dose (10^{-5} M) melatonin on regulating the expression of \( Bcl2 \), \( caspase-3 \), \( Bax \) and \( p53 \) was not affected by the melatonin receptor antagonist, luzindole and 4P-PDOT. As for low dose (10^{-9} M), melatonin plus luzindole group and melatonin plus 4P-PDOT group significantly promoted the expression level of \( Bax \) and inhibited the expression level of \( Bcl2 \) compared with control group (Figs. 2A and 2D, \( P < 0.05 \)). The expression level of \( caspase-3 \), \( Bcl-xl \) and \( p53 \) were not significant difference in the melatonin plus luzindole group and melatonin plus 4P-PDOT group compared with control group (Figs. 2B, 2C and 2E, \( P > 0.05 \)). Moreover, the expression level of \( Bcl2 \), \( Bcl-xl \), \( caspase-3 \), \( Bax \) and \( p53 \) were significant difference in melatonin group compared with that of melatonin plus luzindole group and melatonin plus 4P-PDOT group (Figs. 2A–2E, \( P < 0.05 \)). Therefore, melatonin receptor antagonist, luzindole and 4P-PDOT indeed could affect the effect of low dose (10^{-9} M) melatonin on regulating apoptosis related genes.
Effects of melatonin and melatonin receptor antagonist supplementation on granulosa cells antioxidant related genes expression

The $\text{SOD1}$ and $\text{GPX4}$ expression level was assessed after melatonin supplementation with or without luzindole or 4P-PDOT in bovine granulosa cells. The expression level of $\text{GPX4}$ and $\text{SOD1}$ both were significantly upregulated after melatonin supplementation in high dose ($10^{-5} \text{ M}$) with or without luzindole or 4P-PDOT compared with control group (Figs. 3A and 3B, $P < 0.05$). Moreover, no change was observed between the melatonin plus luzindole and melatonin plus 4P-PDOT compared with the control group (Figs. 3A and 3B, $P > 0.05$). Furthermore, the expression of $\text{SOD1}$ and $\text{GPX4}$ were significantly promoted in the low dose ($10^{-9} \text{ M}$) melatonin group compared with control group, melatonin plus luzindole group and melatonin plus 4P-PDOT group, and there was no significantly different among the melatonin plus luzindole, melatonin plus 4P-PDOT groups and control (Figs. 3A and 3B, $P < 0.05$). Therefore, melatonin inhibited oxidative stress in bovine granulosa cells through modulating the expression level of $\text{SOD1}$ and $\text{GPX4}$, and melatonin receptor antagonist, luzindole and 4P-PDOT could affect low dose melatonin mediating the expression of $\text{SOD1}$ and $\text{GPX4}$, however, did not alter the high dose melatonin effect on modulating the expression of $\text{SOD1}$ and $\text{GPX4}$ in bovine granulosa cells.

DISCUSSION

Ovarian follicle continuous development process give animals the chance to procreate, however, many factors affect the ovarian follicle development process, and cause most of the follicle undergo atresia. The high concentration melatonin is present in follicular fluid,
and also it has been found that melatonin plays an important role in follicular
development. In addition to melatonin, granulosa cells also is an important factor, which
synthesize an array of factors, such as steroids, growth factors and cytokines, and affect the
oocytes mature and follicle development. However, how melatonin affects the effects of
bovine granulosa cells through its receptors is still needed to be investigated. Therefore, we
tried to investigate the effect of melatonin with regard to bovine granulosa cells cell cycle,
antioxidant and apoptosis, especially which receptor mediating the effects of melatonin
on bovine granulosa cells. Moreover, we further revealed which receptor affected the
effects of melatonin on granulosa cells by melatonin receptor antagonist supplementation.

CyclinD1 and CyclinE1 are involved in regulating of the cell cycle, and responsible for
mediating the progression from phase G1 to S (M’baye et al., 2015; Zhen et al., 2014;
Liu et al., 2018). On the contrary, P21 is a cyclin-dependent kinase inhibitor and inhibits
the progression from phase G1 to S (Wang et al., 2012; Liu et al., 2018; Han et al., 2013).
In the present study, the expression of CyclinD1 and CyclinE1 were not significantly
altered after melatonin supplementation in high dose (10^{-5} M), and just inhibited the
expression of P21. However, the low dose (10^{-9} M) melatonin decreased the CyclinE1
expression and increased the P21 expression. Melatonin significantly promoted
mesenchymal cells proliferation, and induced the cell arrest at G1 phase (Liu et al., 2011;
Yang et al., 2017; Pan et al., 2018). Melatonin decreased the number of T lymphocytes cells
in the G1/M phase in the patients with premature ovarian failure (Li et al., 2016) and
promoted breast cancer cell apoptosis through blocking the G1/S transition (Cos et al.,
1991). Therefore, melatonin exhibiting different effect may be due to the cell development
state, such as, mouse oocyte vitrification arrested at the G1/S transition in parthenogenetic
zygotes. However, melatonin significantly increased G1/S transitionin parthenogenetic
zygotes for oocyte activation/embryonic development (Pan et al., 2018). During ovulation,
granulosa cells were arrested at G0/G1 (Green et al., 2000), however, melatonin played
a direct role in regulating progesterone production, LH receptor, GnRH and GnRH
receptor gene expression in human granulosa-luteal cells (Woo et al., 2001). Moreover,
the effect of melatonin on cell cycle was related to physiological state, such as health or
pathology (Pan et al., 2018). Furthermore, melatonin receptor antagonist, luzindole and
4P-PDOT altered the effect of melatonin in high dose (10^{-5} M) and low dose (10^{-9} M) on
the expression of P21, and affected the effect of melatonin on the expression of CyclinE1.
It mean that the contradictory effects of melatonin receptor on mediating the melatonin
on modulating the cell cycle may be related to the different melatonin dose and the
mechanism is need to be further elucidated.

Mammalian granulosa cells act as a crucial role during follicle development and oocyte
growth and maturation. Granulosa cells apoptosis or survival is considered as a signal
for follicle development and oocyte growth, thus granulosa cells are essential for cell
proliferation, follicular development, oocyte maturation, follicle ultimately ovulates or
undergoes atresia (Matsuda et al., 2012; Choi et al., 2011; Tanabe et al., 2015; Kadariya
et al., 2015; Chen et al., 2020). Even the granulosa cells state could determine the fate of
follicle, mainly due to its effect on synthesize of steroids, growth factors, and cytokines,
which have been proved to be essential for oocytes mature and follicle development
Therefore, improving the granulosa cells functions become very important for acting its physiological role during oocytes mature and follicle development. Recently, a large number of researches have focused on melatonin, which has exhibited its beneficial effects on anti-apoptosis and antioxidant. It was well known that the Bcl-2 family play important role in mediating the cell apoptosis through modulating the expression of Bcl2 and Bax. Melatonin was well established that its physiological functions in modulating the cell apoptosis was crucial for granulosa cells function, and thus acted as an important role in promoting follicular development and ovulates or inhibiting undergoes atresia (Matsuda et al., 2012; Choi et al., 2011; Kadariya et al., 2015). Moreover, melatonin had exhibited the anti-apoptotic effects in different cells by promoting the expression of Bcl2 or inhibiting the expression of Bax, p53 and caspase3 and protecting the integrity of cells for maintaining optimal mitochondrial function (Fu et al., 2014; He et al., 2016b; Tan et al., 2013; Jou et al., 2010; Radogna et al., 2007, 2008, 2015). Our previous research also found that melatonin suppressed bovine granulosa cells apoptosis through mediating the expression of Bcl2, Bcl-xl, Bax, p53 and caspase3 (Wang et al., 2012, 2017b, 2018; Liu et al., 2018). Consistent with previous reports, in the present study, the high dose (10^{-5} M) and low dose (10^{-9} M) melatonin both could promote the expression of Bcl2 and Bcl-xl and inhibit the expression of Bax, p53 and caspase3. Furthermore, we further investigated the melatonin receptor, MT1 and MT2 mediating the actions of melatonin on preventing the apoptosis of granulosa cells by melatonin receptor antagonist, luzindole and 4P-PDOT. Luzindole and 4P-PDOT could suppress the effect of low dose (10^{-9} M) melatonin on promoting the expression of Bcl2 and Bcl-xl and inhibiting the expression of Bax, p53 and caspase3. However, Luzindole and 4P-PDOT did not affect the effect of high dose (10^{-5} M) melatonin on promoting the expression of Bcl2 and Bcl-xl and inhibiting the expression of Bax, p53 and caspase3. These findings indicated that the effect of low dose (10^{-9} M) melatonin on granulosa cells apoptosis was mediated by MT1 and MT2, and the high dose (10^{-5} M) melatonin affected the granulosa cells apoptosis by other pathway, besides MT1 and MT2. How melatonin receptors modulate the effect of melatonin on granulosa cells apoptosis was not very clear. He et al. (2016b) research showed that MT2 mediate the effect of melatonin on modulating porcine granulosa cells proliferation and apoptosis. However, there was evidence that melatonin modulate the cell apoptosis through MT1 and MT2 acting in a complementary way (Radogna et al., 2007; Espino et al., 2010, 2011). Furthermore, our recent research indicated that MT1 and MT2 might work in concert to modulate bovine granulosa cells function by regulating cellular progression and apoptosis (Wang et al., 2017b, 2018; Liu et al., 2018).

Reactive oxygen species is another factor that can induce cell apoptosis and thus affect the follicular development and oocyte maturation (Matsuda et al., 2012; He et al., 2016a; Chappel, 2013). Melatonin not only directly scavenges the ROS by crossing all cells members, but also can improve anti-oxidant enzymes expression (Tamura et al., 2009; Reiter et al., 2002, 2014), which is consistent with its effects on promoting the survival of granulosa cells, follicular development, and maintaining pregnancy (Matsuda et al., 2012; Choi et al., 2011; Arosh et al., 2004; Cruz et al., 2014; Liu et al., 2018).
study, the high dose ($10^{-5}$ M) and low dose ($10^{-9}$ M) melatonin both could stimulate the expression of SOD1 and GPX4. Moreover, melatonin receptor antagonist, luzindole and 4P-PDOT could affect the effect of low dose melatonin on promoting the expression of SOD1 and GPX4 in bovine granulosa cells. However, did not alter the high dose melatonin effect. Which were consistent with previous reports that melatonin increased the expressions of GPX4 and SOD1 in in bovine embryo (Wang et al., 2014), porcine oocyte (Li et al., 2015), porcine granulosa cells (He et al., 2016a, 2016b) and the mouse granulosa cells (Tanabe et al., 2015). Our recent research revealed that melatonin could stimulate the expression of GPX4 and SOD1 in bovine granulosa cells (Wang et al., 2017a, 2017b; Liu et al., 2018), which was not only depending on MT1 and MT2.

The effect of MT1 and MT2 is well cross talk in vivo and vitro research, and there are not consistent evidence on the role of MT1 and MT2 mediating the response to melatonin. MT1 and MT2 are considered to modulating complex reproductive mechanisms (Dubocovich & Markowska, 2005), however, they exhibit different roles. It has been demonstrated that MT1 plays critical role in regulating mammal gonadal activity (Yasuo et al., 2009). The MT1 receptor is sufficient and necessary to mediate effects of photoperiod-driven changes in melatonin on seasonal changes in behavior and reproductive function in a reproductively photoperiodic mammal (Prendergast, 2010). As well as melatonin and MT1 signaling transfer human chorionic gonadotropin stimulating information to ovary and play important role in regulating female reproduction (He et al., 2016c). On the other hand, melatonin modulates the porcine granulosa cells apoptosis and proliferation through its receptor MT2 (He et al., 2016a). In the knockout mice, MT2 modulates the behavioral effect, immune and inflammatory responses (Drazen et al., 2001; Larson et al., 2006). Moreover, melatonin activates MT2 and then increases serum estradiol level and decreases ovarian GnIHR (Gonadotropin-inhibitory hormone receptor), which promote hens egg-laying rates (Jia et al., 2016). However, recent research indicate that MT1 and MT2 mediate the effect of melatonin through acting in a complementary way. Melatonin modulates the cell life/death balance via interaction with the MT1 and MT2 in human spermatozoa (Espino et al., 2010), human leucocytes (Espino, Rodríguez & Pariente, 2013) and U937 cell (Radogna et al., 2008), bovine granulose cells (Wang et al., 2018, 2017b; Liu et al., 2018). Consistent with presient study, we also found that Luzindole and 4P-PDOT could block the effect of physiological doses ($10^{-9}$ M) melatonin on cell cycle, apoptosis and antioxidant of bovine granulosa cells. Which indicate that MT1 and MT2 could mediate the effect of melatonin through acting in a complementary way in the bovine granulosa cells. What interests us most in this study is that the action mode of high dose melatonin and low dose melatonin were different. The high dose melatonin and low dose melatonin acted a uniform effect on inhibiting the antiapoptosis and enhancing antioxidation of bovine granulosa cells. However, the high dose ($10^{-5}$ M) melatonin still inhibited the apoptosis and enhanced antioxidative relative genes expression within or without Luzindole and 4P-PDOT. Therefore, Luzindole and 4P-PDOT can not block the effect of high dose melatonin on granulosa cells. Luzindole and 4P-PDOT were initially
considered as competitive melatonin receptor antagonists, which could antagonize melatonin potentiation effect. The concentration of 100 nM and above Luzindole or 4P-PDOT, acting as an inverse agonists at MT1 and MT2, exist in a constitutively active form in recombinant MT1 and MT2 systems. The mechanism that melatonin receptors are constitutively active is that Luzindole and 4P-PDOT couple to G protein in the presence or absence of their ligand (Browning et al., 2000; Witt-Enderby & Dubocovich, 1996; Roka et al., 1999; Glaser, Masana & Dubocovich, 1998; Dubocovich et al., 2003). Consistent with these studies, the high dose \(10^{-5} \text{ M}\) melatonin receptors antagonists, Luzindole and 4P-PDOT, indeed exhibit an agonists effect of melatonin on antiapoptosis and antioxidation of bovine granulosa cells. Therefore, the present study indicates how different both Luzindole and 4P-PDOT affect the regulatoin effect of melatonin on cell function. Nonetheless, Luzindole and 4P-PDOT serve as an excellent melatonin receptor antagonists showing pharmacological efficacies at the MT1 and MT2. Therefore the difference must be taken when investigating functional studies mediated by Luzindole and 4P-PDOT that are not fully identical in the target tissue under study.

CONCLUSION

In the present research, we investigated the effects of melatonin supplementation on the cell cycle, apoptosis and oxidative stress in the bovine granulosa cells. Moreover, we further revealed which receptor affected the effects of melatonin on granulosa cells by melatonin receptor antagonist supplementation. The results showed that the high dose \(10^{-5} \text{ M}\) and low dose \(10^{-9} \text{ M}\) melatonin both could act as an essential role in modulating granulosa cells apoptosis, cell cycle and antioxidant. Furthermore, the beneficial effect could be related to mediate the expression of Bcl2, Bcl-xl, Bax, caspase-3, p53, P21, SOD1 and GPX4 in granulosa cells. Our study also emphasized that the effect of low dose \(10^{-9} \text{ M}\) melatonin on granulosa cells apoptosis was mediated by MT1 and MT2, and the high dose \(10^{-5} \text{ M}\) melatonin affected the granulosa cells apoptosis by other pathway, besides MT1 and MT2. Moreover MT1 and MT2 may work in concert to modulate bovine granulosa cells function by regulating cellular progression and apoptosis. The present study benefits to understand that MT1 and MT2 mediate the effect of melatonin on modulating granulosa cells functions.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests
The authors declare that they have no competing interests.

Author Contributions
- Shujuan Wang conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Wenju Liu conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Aiyou Wen analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Bing Yang analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
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REFERENCES
Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J, Fortier MA. 2004. Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function. Endocrinology 145(5):2551–2560 DOI 10.1210/en.2003-1607.

Browning C, Beresford I, Fraser N, Giles H. 2000. Pharmacological characterization of human recombinant melatonin mt1 and MT2 receptors. British Journal of Pharmacology 129(5):877–886 DOI 10.1038/sj.bjp.0703130.

Chappel S. 2013. The role of mitochondria from mature oocyte to viable blastocyst. Obstetrics and Gynecology International 2013(5):183024 DOI 10.1155/2013/183024.

Chen Y, Wang X, Yang C, Liu Q, Ran Z, Li X, He C. 2020. A mouse model reveals the events and underlying regulatory signals during the gonadotrophin-dependent phase of follicle development. Molecular Human Reproduction 26(12):gaa069 DOI 10.1093/molehr/gaa069.

Choi J, Jo M, Lee E, Choi D. 2011. Induction of apoptotic cell death via accumulation of autophagosomes in rat granulose cells. Fertility and Sterility 95(4):1482–1486.
Chowdhury I, Thomas K, Zeleznik A, Thompson WE. 2016. Prohibitin regulates the FSH signaling pathway in rat granulosa cell differentiation. *Journal of Molecular Endocrinology* 56(4):325–336 DOI 10.1530/JME-15-0278.

Cos S, Blask DE, Lemus-Wilson A, Hill AB. 1991. Effects of melatonin on the cell cycle kinetics and "estrogen-rescue" of MCF-7 human breast cancer cells in culture. *Journal of Pineal Research* 10(1):36–42 DOI 10.1111/j.1600-079X.1991.tb00007.x.

Cruz MH, Leal CL, Cruz JF, Tan DX, Reiter RJ. 2014. Essential actions of melatonin in protecting the ovary from oxidative damage. *Theriogenology* 82(7):925–932 DOI 10.1016/j.theriogenology.2014.07.011.

Denkova R, Bourneva V, Staneva-Dobrovski L, Zvetkova E, Baleva K, Yaneva E, Nikolov B, Ivanov I, Simeonov K, Timeva T, Yankov M. 2014. In vitro effects of inhibin on apoptosis and apoptosis related proteins in human ovarian granulose cells. *EndocrRegul* 38:51–55.

Drazen D, Bilu D, Bilbo S, Nelson R. 2001. Melatonin enhancement of splenocyte proliferation is attenuated by luzindole, a melatonin receptor antagonist. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 280(5):R1476–R1482 DOI 10.1152/ajpregu.2001.280.5.R1476.

Dubocovich ML, Markowska M. 2005. Functional MT1 and MT2 melatonin receptors in mammals. *Endocrine* 27(2):101–110 DOI 10.1385/ENDO:27:2:101.

Dubocovich ML, Rivera-Bermudez MA, Gerdin MJ, Masana MI. 2003. Molecular pharmacology regulation and function of mammalian melatonin receptors. *Frontiers in Bioscience* 8(4):d1093–d1108 DOI 10.2741/1089.

Espino J, Bejarano I, Ortiz A, Lozano GM, García JF, Pariente JA, Rodríguez AB. 2010. Melatonin as a potential tool against oxidative damage and apoptosis in ejaculated human spermatozoa. *Fertility and Sterility* 94(5):1915–1917.

Espino J, Ortiz A, Bejarano I, Lozano GM, Monllor F, García JF, Rodríguez AB, Pariente JA. 2011. Melatonin protects human spermatozoa from apoptosis via melatonin receptor and extracellular signal-regulated kinase-mediated pathways. *Fertility and Sterility* 95(2):2290–2296.

Espino J, Rodríguez AB, Pariente JA. 2013. The inhibition of TNF-α-induced leucocyte apoptosis by melatonin involves membrane receptor MT1/MT2 interaction. *Journal of Pineal Research* 54(4):442–452.

Fatehi AN, Roelen BA, Colenbrander B, Schoevers EI, Gadella BM, Beverst MM, Van den Hurk R. 2005. Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. *Zygote* 13(2):177–185 DOI 10.1017/S0967199405003126.

Fu Y, He CJ, Ji PY, Zhuo ZY, Tian XZ, Wang F, Tan DX, Liu GS. 2014. Effects of melatonin on the proliferation and apoptosis of sheep granulosa cells under thermal stress. *International Journal of Molecular Sciences* 15(11):21090–21104 DOI 10.3390/ijms151121090.

Glaser JA, Masana MI, Dubocovich ML. 1998. Selective MT2 melatonin receptor antagonists are inverse agonists on human mt1 melatonin receptor. *FASEB Journal* 12:894.

Green C, Chatterjee R, McGarrigle HH, Ahmed F, Thomas NS. 2000. p107 is active in the nucleolus in non-dividing human granulose lutein cells. *Journal of Molecular Endocrinology* 25(3):275–286 DOI 10.1677/jme.0.0250275.

Gupta RK, Miller KP, Babus JK, Flaws JA. 2006. Methoxychlor inhibits growth and induces atresia of antral follicles through an oxidative stress pathway. *Toxicological Sciences* 93(2):382–389 DOI 10.1093/toxsci/kfl052.
Han L, Wu CJ, Riaz H, Bai LY, Chen JG, Zhen YH, Guo AZ, Yang LG. 2013. Characterization of the mechanism of inhibin α-subunit gene in mouse anterior pituitary cells by RNA interference. *PLOS ONE* 8(10):e74596 DOI 10.1371/journal.pone.0074596.

He Y, Deng H, Jiang Z, Li Q, Shi M, Chen H, Han Z. 2016a. Effects of melatonin on follicular atresia and granulosa cell apoptosis in the porcine. *Molecular Reproduction and Development* 83(8):692–700 DOI 10.1002/mrd.22676.

He YM, Deng HH, Shi MH, Bodinga BM, Chen HL, Han ZS, Jiang ZL, Li QW. 2016b. Melatonin modulates the functions of porcine granulose cells via its membrane receptor MT2 in vitro. *Animal Reproduction Science* 172:164–172 DOI 10.1016/j.anireprosci.2016.07.015.

He CJ, Ma T, Shi JM, Zhang ZZ, Wang J, Zhu KF, Li Y, Yang MH, Song YK, Liu GS. 2016c. Melatonin and its receptor MT1 are involved in the downstream reaction to luteinizing hormone and participate in the regulation of luteinization in different species. *Journal of Pineal Research* 61(3):279–290.

He CJ, Wang J, Zhang ZZ, Yang MH, Li Y, Tian XZ, Ma T, Tao JL, Zhu KF, Song YK, Ji PG, Liu GS. 2016d. Mitochondria synthesize melatonin to ameliorate its function and improve mice oocyte’s quality under in vitro conditions. *International Journal of Molecular Sciences* 17(6):E939.

Jia Y, Yang M, Zhu K, Wang L, Song Y, Wang J, Qin W, Xu Z, Chen Y, Liu G. 2016. Melatonin implantation improved the egg-laying rate and quality in hens past their peak egg-laying age. *Scientific Reports* 6(1):d222–d237 DOI 10.1038/srep39799.

Jiang JY, Cheung CK, Wang Y, Tsang BK. 2003. Regulation of cell death and cell survival gene expression during ovarian follicle development and atresia. *Frontiers in Bioscience* 8(4):d222–d237 DOI 10.2741/949.

Jou MJ, Peng TI, Hsu LF, Jou SB, Reiter RJ, Yang CM, Chiao CC, Lin YF, Chen CC. 2010. Visualization of melatonin’s multiple mitochondrial levels of protection against mitochondrial Ca(2+)-mediated permeability transition and beyond in rat brain astrocytes. *Journal of Pineal Research* 48(1):20–38 DOI 10.1111/j.1600-079X.2009.00721.x.

Kadariya I, Wang J, ur Rehman Z, Ali H, Riaz H, He J, Bhattarai D, Liu JJ, Zhang SJ. 2015. RNAi-mediated knockdown of inhibin α subunit increased apoptosis in granulosa cells and decreased fertility in mice. *Journal of Steroid Biochemistry and Molecular Biology* 152(1):161–170 DOI 10.1016/j.jsbmb.2015.05.006.

Kaipia A, Hsueh AJ. 1997. Regulation of ovarian follicle atresia. *Annual Review of Physiology* 59(1):349–363 DOI 10.1146/annurev.physiol.59.1.349.

Kang JT, Koo OJ, Kwon DK, Park HJ, Jang G, Kang SK, Lee BC. 2009. Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. *Journal of Pineal Research* 46(1):22–28 DOI 10.1111/j.1600-079X.2008.00602.x.

Larson J, Jessen R, Uz T, Arslan A, Kurtuncu M, Imbesi M, Manev H. 2006. Impaired hippocampal long-term potentiation in melatonin MT2 receptor-deficient mice. *Neuroscience Letters* 393(1):23–26 DOI 10.1016/j.neulet.2005.09.040.

Li YM, Liu HL, Sun J, Tian YP, Li CZ. 2016. Effect of melatonin on the peripheral T lymphocyte cell cycle and levels of reactive oxygen species in patients with premature ovarian failure. *Experimental and Therapeutic Medicine* 12(6):3589–3594 DOI 10.3892/etm.2016.3833.

Li Y, Zhang ZZ, He CJ, Zhu KF, Xu ZY, Ma T, Tao JL, Liu GS. 2015. Melatonin protects porcine oocyte in vitro maturation from heat stress. *Journal of Pineal Research* 59(3):365–375 DOI 10.1111/jpi.12268.
Liu WJ, Wang SJ, Zhou JX, Pang XS, Wang LL. 2018. RNAi-mediated knockdown of MTNR1B without disrupting the effects of melatonin on apoptosis and cell cycle in bovine granulose cells. PeerJ 6(5):e4463 DOI 10.7717/peerj.4463.

Liu LF, Zhu Y, Xu Y, Reiter RJ. 2011. Melatonin delays cell proliferation by inducing G1 and G2/M phase arrest in a human osteoblastic cellline hFOB 1.19. Journal of Pineal Research 50(2):222–231.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25(4):402–408 DOI 10.1006/meth.2001.1262.

M’baye M, Hua G, Khan HA, Yang LG. 2015. RNAi-mediated knockdown of INHBB increases apoptosis and inhibits steroidogenesis in mouse granulose cells. Journal of Reproduction and Development 61(5):391–397 DOI 10.1262/jrd.2014-158.

Matsuda F, Inoue N, Manabe N, Ohkura S. 2012. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. Journal of Reproduction and Development 58(1):44–50 DOI 10.1262/jrd.2011-012.

Okamoto A, Ikeda M, Kaneko A, Kishida C, Shimada M, Yamashita Y. 2016. The novel pig in vitro maturation system to improve developmental competence of oocytes derived from atretic non-vascularized follicle. Biology of Reproduction 116:138982.

Orisaka M, Mizutani T, Tajima K, Orisaka S, Shukunami K, Miyamoto K, Kotsuji F. 2006. Effects of ovarian theca cells on granulosa cell differentiation during gonadotropin-independent follicular growth in cattle. Molecular Reproduction and Development 73(6):737–744 DOI 10.1002/mrd.20246.

Pan B, YangHX, Wu ZZ, Qazi IH, Liu GS, Han HB, Meng QY, Zhou GB. 2018. Melatonin improves parthenogenetic development of vitrified–warmed mouse oocytes potentially by promoting G1/S cell cycle progression. International Journal of Molecular Sciences 19(12):4029.

Prendergast BJ. 2010. MT1 melatonin receptors mediate somatic, behavioral, and reproductive neuroendocrine responses to photoperiod and melatonin in Siberian hamsters (Phodopus sungorus). Endocrinology 151(2):714–721 DOI 10.1210/en.2009-0710.

Radogna F, Albertini MC, De Nicola M, Diederich M, Bejarano I, Ghibelli L. 2015. Melatonin promotes Bax sequestration to mitochondria reducing cell susceptibility to apoptosis via the lipooxygenase metabolite 5-hydroxyeicosatetraenoic acid. Mitochondrion 21:113–121 DOI 10.1016/j.mito.2015.02.003.

Radogna F, Cristofanon S, Paternoster L, D’Alessio M, De Nicola M, Cerella C, DiCato M, Diederich M, Ghibelli L. 2008. Melatonin promotes Bax sequestration to mitochondria reducing cell susceptibility to apoptosis via the lipooxygenase metabolite 5-hydroxyeicosatetraenoic acid. Mitochondrion 21:113–121 DOI 10.1016/j.mito.2015.02.003.

Reiter RJ, Tamura H, Tan DX, Xu XY. 2014. Melatonin and the circadian system: contributions to successful female reproduction. Fertility and Sterility 102(2):321–328 DOI 10.1016/j.fertnstert.2014.06.014.

Reiter RJ, Tan DX, Manchester LC, El-Sawi MR. 2002. Melatonin reduces oxidant damage and promotes mitochondrial respiration: implications for aging. Annals of the New York Academy of Sciences 959(1):238–250 DOI 10.1111/j.1749-6632.2002.tb02096.x.
Richards JS. 2001. Perspective: the ovarian follicle—a perspective in 2001. *Endocrinology* 142(6):2184–2193 DOI 10.1210/endo.142.6.8223.

Roka F, Brydon L, Waldhoer M, Strosberg AD, Freissmuth M, Jockers R, Nanoff C. 1999. Tight association of the human Mel1a-melatonin receptor and Gi: precoupling and constitutive activity. *Molecular Pharmacology* 56(5):1014–1024 DOI 10.1124/mol.56.5.1014.

Suofu Y, Li W, Jean-Alphonse FG, Jia J, Khattar NK, Li J, Baranov SV, Leronni D, Mihalik AC, He Y, Cecon E, Webi VL, Kim J, Heath BE, Baranova OV, Wang X, Gable MJ, Kretz ES, Di Benedetto G, Lezon TR, Ferrando LM, Larkin TM, Sullivan Y, Yablonska S, Wang J, Minnigh MB, Guillaume G, Suzenet F, Richardson RM, Poloyac SM, Stolz DB, Jockers R, Witt-Enderby PA, Carlisle DL, Vilardaga JP, Friedlander RM. 2017. Dual role of mitochondria in producing melatonin and driving GPCR signaling to block cytochrome c release. *Proceedings of the National Academy of Sciences of the United States of America* 114(38):E7997–E8006 DOI 10.1073/pnas.1705768114.

Tamura H, Nakamura Y, Korkmaz A, Manchester LC, Tan DX, Sugino N, Reiter RJ. 2009. Melatonin and the ovary: physiological and pathophysiological implications. *Fertility and Sterility* 92(1):328–343 DOI 10.1016/j.fertnstert.2008.05.016.

Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L, Tamura I, Maekawa R, Aasada H, Yamagata Y, Sugino N. 2012. The role of melatonin as an antioxidant in the follicle. *Journal of Ovarian Research* 5(1):DOI 10.1186/1757-2215-5-5.

Tan DX, Manchester LC, Liu X, Rosales-Corral SA, Acuna-Castroviejo D, Reiter RJ. 2013. Mitochondria and chloroplasts as the original sites of melatonin synthesis: a hypothesis related to melatonin’s primary function and evolution in eukaryotes. *Journal of Pineal Research* 54(2):127–138 DOI 10.1111/jpi.12026.

Tanabe M, Tamura H, Taketani T, Okada M, Lee L, Tamura I, Maekawa R, Aasada H, Yamagata Y, Sugino N. 2015. Melatonin protects the integrity of granulose cells by reducing oxidative stress in nuclei, mitochondria, and plasma membranes in mice. *Journal of Reproduction and Development* 61(1):35–41 DOI 10.1262/jrd.2014-105.

Tian X, Wang F, He C, Zhang L, Tan D, Reiter RJ, Xu J, Ji P, Liu G. 2014. Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. *Journal of Pineal Research* 57(3):239–247 DOI 10.1111/jpi.12163.

Vitt UA, Hsueh AJ. 2001. Stage-dependent role of growth differentiation factor-9 in ovarian follicle development. *Molecular and Cellular Endocrinology* 183(1–2):171–177.

Wang SJ, Liu BR, Liu WJ, Xiao Y, Zhang HL, Yang LG. 2017a. The effects of melatonin on bovine uniparental embryos development in vitro and the hormone secretion of COCs. *PeerJ* 5(2):e3485 DOI 10.7717/peerj.3485.

Wang SJ, Liu W, Pang X, Dai S, Liu G. 2018. The mechanism of melatonin and its receptor MT2 involved in the development of bovine granulosa cells. *International Journal of Molecular Sciences* 19(7):E2028.

Wang SJ, Liu WJ, Wang LK, Pang XS, Yang LG. 2017b. The role of melatonin receptor MTNR1A in the action of melatonin on bovine granulosa cells. *Molecular Reproduction and Development* 84(11):1140–1154 DOI 10.1002/mrd.22877.

Wang SJ, Liu WJ, Wu CJ, Ma FH, Ahmad S, Liu BR, Han L, Jiang XP, Zhang SJ, Yang LG. 2012. Melatonin suppresses apoptosis and stimulates progesterone production by bovine granulosa cells via its receptors (MT1 and MT2). *Theriogenology* 78(7):1517–1526 DOI 10.1016/j.theriogenology.2012.06.019.
Wang F, Tian X, Zhang L, Gao C, He C, Fu Y, Ji P, Li Y, Li N, Liu G. 2014. Beneficial effects of melatonin on in vitro bovine embryonic development are mediated by melatonin receptor 1. *Journal of Pineal Research* **56**(3):333–342 DOI 10.1111/jpi.12126.

Witt-Enderby PA, Dubocovich ML. 1996. Characterization and regulation of the human ML1A melatonin receptor stably expressed in Chinese hamster ovary cells. *Molecular Pharmacology* **50:**166–174.

Woo MM, Tai CJ, Kang SK, Nathwani PS, Pang SF, Leung PC. 2001. Direct action of melatonin in human granulosa-luteal cells. *Journal of Clinical Endocrinology & Metabolism* **86**(10):4789–4797 DOI 10.1210/jcem.86.10.7912.

Yang FF, He CJ, Sun XY, Wang J, Luo C, Liu GS, Yang LG, Xiong JJ, Huo LJ. 2017. The regulatory mechanism of MLT/MT1 signaling on the growth of antler mesenchymal cells. *Molecules* **22**(10):1793 DOI 10.3390/molecules22101793.

Yasuo S, Yoshimura T, Ebihara S, Korf HW. 2009. Melatonin transmits photoperiodic signals through the MT1 melatonin receptor. *Journal of Neuroscience* **29**(9):2885–2889 DOI 10.1523/JNEUROSCI.0145-09.2009.

Yie SM, Brown GM, Liu GY, Collins JA, Daya S, Hughes EG, Foster WG, Younglai EV. 1995. Melatonin and steroids in human pre-ovulatory follicular fluid: seasonal variations and granulosa cell steroid production. *Human Reproduction* **10**(1):50–55 DOI 10.1093/humrep/10.1.50.

Zhen YH, Wang L, Riaz H, Wu JB, Yuan YF, Han L, Wang YL, Zhao Y, Dan Y, Huo LJ. 2014. Knockdown of CEBPβ by RNAi in porcine granulosa cells resulted in S phase cell cycle arrest and decreased progesterone and estradiol synthesis. *Journal of Steroid Biochemistry and Molecular Biology* **143:**90–98 DOI 10.1016/j.jsbmb.2014.02.013.