Research Article

Melatonin regulates the expression of Bone Morphogenetic Protein 15 (*Bmp-15*), Growth Differentiation Factor 9 (*Gdf-9*) and LH receptor (*Lhr*) genes in developing follicles of rats

Lia Alencar Coelho[1, 2] *, Daniella do Carmo Buonfiglio[2,3], Wilson Mitsuo T Kuwabara[2], Jéssica Andrade-Silva[2], Patrícia Rodrigues Lourenço Gomes[2], Julieta Helena Scialfa[2], Rafael Peres[4], Fernanda Gaspar Amaral[5], José Cipolla-Neto[2]

[1] Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering (FZEA), University of São Paulo (USP), Pirassununga-SP, Brazil
[2] Department of Physiology and Biophysics, Institute of Biomedical Sciences (ICB), University of São Paulo (USP), São Paulo-SP, Brazil
[3] Department of Pharmacology and Toxicology and Neuroscience, Institute, Morehouse School of Medicine, Atlanta, Georgia, USA
[4]Department of Anatomy, Biochemistry and Physiology, John A. Burns School of Medicine, University of Hawaii at Mãnoa, Honolulu, Hawaii, USA
[5] Department of Physiology, Federal University of São Paulo (UNIFESP), São Paulo-SP, Brazil
*Correspondence: liac@usp.br, Tel: + 55 11 30917466.

Running title: Melatonin during the follicle development

Received: July 9, 2020; Accepted: September 16, 2020

ABSTRACT

This study investigated the effects of melatonin supplementation on the daily mRNA expression of *Bmp-15*, *Gdf-9*, FSH (*Fshr*) and LH (*Lhr*) receptor genes and the effect of gonadotropin supplementation on protein expression of melatonin-synthetizing enzymes (ASMT and AANAT) during *in vitro* maturation (IMV) of rat follicle-enclosed oocytes. We also studied the effects of pinealectomy on the mRNA expression of *Bmp-15*, *Gdf-9*, *Fshr* and *Lhr* genes in immature oocytes and cumulus cells and on the protein expression and immunolocalization of *AANAT* and *ASMT* in immature ovaries. Ovaries were collected from sham-pinealectomized (INTACT) and pinealectomized (PINX) females during the light and dark phases of the 12h:12h light-dark cycle. Melatonin increased the *Bmp-15* and *Gdf-9* mRNA expression in follicle-enclosed oocytes during dark phase and *Lhr* mRNA expression during the light phase. The daily mRNA expression of *Lhr* was negatively correlated with the daily mRNA expression of *Bmp-15* and *Gdf-9* in follicle-enclosed oocytes supplemented with melatonin. There were no phase differences in oocytes mRNA expression of *Bmp-15* and *Gdf-9* from PINX females. Pinealectomy did not altered the mRNA expression of *Lhr* gene in cumulus cells. There were no phase differences in *ASMT* and *AANAT* expression in ovaries from INTACT and PINX females and their immunolocalization was seen in most of ovarian cells. *AANAT* expression in follicle-enclosed oocytes supplemented with LH from INTACT and PINX females was greater during the dark phase. The results suggest that melatonin regulates the daily *Bmp-15*, *Gdf-9* and *Lhr* mRNA expression during the rat follicle development. There is also an indicative that melatonin plays an important role in the relationship

between the daily expression of *Lhr* and the daily expression of *Bmp-15* and *Gdf*-9 in follicle-enclosed oocytes during oocyte maturation process.

Key Words: gonadotropin receptors, melatonin AANAT, ASMT, oocyte maturation, transforming growth factor-β.

---

1. INTRODUCTION

Oocyte maturation involves nuclear and cytoplasmic modifications that make the oocyte capable of undergoing fertilization and embryo development (1). The *in vivo* maturation process requires the pre-ovulatory peak of luteinizing hormone (LH) (LH surge), which activates cumulus cells expansion of the ovulated cumulus-oocyte complexes - COCs (2, 3). Mural (distant from the oocyte) and cumulus (near the oocyte) granulosa cells-oocyte interactions support nuclear and cytoplasmic maturation of oocytes (1, 4, 5) as well as the presence of LH receptors in mural and cumulus granulosa cells (6). In all stages of folliculogenesis, the development and differentiation of granulosa cells and their communication with the oocyte depend on several oocyte-derived growth factors including Bone Morphogenetic Protein 15 (BMP-15) and Growth Differentiation Factor 9 (GDF-9) belonging to Transforming Growth Factor-β (TGF-β) superfamily (7-9). These growth factors are responsible for the oocyte-cumulus cell regulatory interaction after LH surge (7), induction of cumulus cell expansion (10) and they also stimulate the *in vitro* maturation of COCs by increasing the nuclear maturation rates (11) or modulating the mRNA expression of embryo development related-genes in cumulus cells (12). Additionally, these growth factors are responsible for regulation of mRNA expression of follicle-stimulating hormone (FSH) (13) and LH (14) receptors in growing follicles.

Melatonin, a secretory product of the pineal gland (15), plays an important role in ovarian function, particularly during final step of the follicle development (16-18). During the maturation process, pineal melatonin and melatonin from the follicular fluid (19, 20), probably secreted by mammalian oocytes (21, 22) and/or mural and cumulus granulosa cells (23), protect the pre-ovulatory follicles and the COCs from oxidative stress (17, 19, 22, 24), control the granulosa cell apoptosis (20, 24) and the transcription of steroid synthesis-related genes including LH receptors (25). It was also reported that melatonin controls BMP-6 regulation of steroidogenesis in rat immature growing follicles by altering the expression of FSH receptors (26).

Considering the relationship between BMP system and melatonin in the ovary (27) and the synergistic effect of BMP-15 and GDF-9 (13) in improving *in vitro* maturation of COCs (7, 11), the effects of melatonin on expression of *Bmp-15*, *Gdf-9*, FSH (*fshr*) and LH (*Lhr*) receptor genes in rat follicle-enclosed oocytes during *in vitro* maturation should be investigated. Before the *in vitro* experiments, we investigated the effect of pinealectomy on the expression of *Bmp-15* and *Gdf-9* in immature oocytes and FSH (*Fshr*) and LH (*Lhr*) receptor genes in immature cumulus cells. Moreover, we tested whether the protein expression of the two melatonin synthetic enzymes (AANAT and ASMT) (28) in the ovary was affected by pinealectomy and then identify their potential localizations in the ovary from intact and pinealectomized animals with immunohistochemistry. We also studied the effects of melatonin supplementation on mRNA expression of *Bmp-15*, *Gdf-9*, *Fshr* and *Lhr* genes in isolated rat follicle-enclosed oocytes and also the effect of melatonin supplementation in maturation medium on the possible correlations between mRNA expression of gonadotropin receptors and the mRNA expression of *Bmp-15* and

Gdf-9. Subsequently, we investigated whether the ASMT and AANAT expression in rat follicle-enclosed oocytes are affected by gonadotropins (FSH and/or LH) supplementation since these hormones promote the *in vitro* maturation of the oocyte in the intact follicle (29, 30). In all experiments, the influence of the phases of light-dark cycle was analyzed.

2. MATERIALS AND METHODS

2.1. Animals.

Female Wistar rats (3-month-old) were housed on a 12L:12D light-dark cycle, in a temperature-controlled room (23°C ± 2°C), with food and water available *ad libitum*. All the procedures involving animals were authorized by the Animal Care Committee of Ethics in Animal Experimentation of the Institute of Biomedical Sciences, University of São Paulo. The approved protocol number was CEUA 199/116f.

2.2. Hormones and Antibodies.

The equine chorionic gonadotropin (eCG - Folligon® 5000 UI) and LH (Chorulon 5000 UI) were obtained from MSD Saúde Animal Inc., São Paulo, SP, Brazil. FSH (Folltropin) and melatonin (M5250) from Vetoquinol Inc., Mairiporã, SP, Brazil and Sigma-Aldrich, (St. Louis, MO, USA), respectively. The GnRH analogue (Buserelin Acetate) was obtained from Ourofino Saúde Animal Inc., (Cravinhos, SP, Brazil). Polyclonal rabbit anti-AANAT and anti-ASMT antibodies were a donation from Dr. D.C. Klein, (NIH, Bethesda, USA). The monoclonal mouse anti-γTUBULIN antibody (T5326) was obtained from Sigma-Aldrich, and the secondary antibodies, Alexa Fluor 790 (A11369) and Alexa Fluor 680 (A10038), were obtained from Invitrogen, (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Hormonal stimulation of the females and collection of ovaries, FOs, oocytes and cumulus cells.

Forty-eight hours before euthanasia, the females were stimulated with 20 U eCG for induction of development of a cohort of immature follicles. Ovaries were removed and placed in the manipulation medium (H-199), composed of Tissue Culture Medium (TCM) 199 with HEPES (Gibco®/12350-039, Invitrogen), supplemented with 1µL/mL gentamicin (G1272, Sigma-Aldrich), and 0.1% polyvinyl alcohol (P8136, Sigma-Aldrich). COCs were isolated from the ovarian follicles by dissection and the oocytes were denuded from cumulus cells by vortexing for 5-8 min. The denuded oocytes were incubated for 5 min in H-199 medium with 0.1% pronase (P8811, Sigma-Aldrich) for removal of the zona pellucida. After the oocytes (80/sample) and cumulus cells were collected, the separated samples were put into 800 µL of lysis solution for RNA isolation and stored at -80 °C and the ovaries were immediately stored at – 80 °C. The FOs were cultured in appropriate maturation media and then stored at – 80 °C.

2.4. Culture of ovarian follicle-enclosed oocytes.

For *in vitro* maturation studies, after three washes in manipulation medium, groups of isolated ovarian FOs were transferred to 6-well plates (3 mL/well) containing maturation medium (B-119

medium) composed of TCM 199 medium without HEPES and with sodium bicarbonate (Gibco®/11150-067, Invitrogen), supplemented with 1 µL/mL gentamicin and 0.1% polyvinyl alcohol. The cultures were incubated at 37°C under 5% CO_2 in humidified air for 16 hours. Pools of 25 to 30 follicles per sample were either put into 800 µL lysis solution for RNA isolation and stored at - 80°C or subjected to protein extraction procedures and then stored at - 80°C.

2.5. RNA extraction and qRT-PCR.

Total RNA was extracted from the samples using guanidine isothiocyanate based reagent (TRIzol®, 15596-018, Invitrogen) as instructed by manufacturer. First-strand cDNA was synthetized from 1µg of total RNA pellet diluted in RNase-free water using reverse transcriptase (Superscript III, 18080-044, Invitrogen) and random primers (48190-011, Invitrogen). The Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) was performed on QuantStudio 6 Flex Real-Time PCR equipment (Applied Biosystems, Inc, Foster City, CA, USA) using the relative analyses ($2^{-\Delta\Delta CT}$ method) (31) and reported as arbitrary units. All measures were performed in duplicate and *Rpl37a* was used as reference gene (32). The primers used in this study are listed in Table 1.

Table 1. List of qRT-PCR primers used in the present research.

Genes	Accession Number*	Primer sequence 5´- 3´	Base Pairs (bp)
Gdf-9	NM_021672.1	F: (5'- 3') – CAATACCGTCCGGCTCTTCAG R: (5'-3') – AGGGGTCCTGTCATCTGGTTG	72
Bmp-15	NM_021670.1	F: (5'-3') – TTCTCAGAGGTCCTTGGCAT R: (5'-3') – TGATGGCGGTAGACCACAGT	100
Fshr	NM_199237.1	F: (5'-3') – GGAACGCCATTGAACTGAGG R: (5'-3') – CCAGGTCTCCAAATCCAGCA	81
Lhr	NM_012978.1	F: (5'-3') – TTCCCAGGAGCAAGTAAGCC R: (5'-3') – TAACGCTCTCGGTGGTATGG	84
Rpl37a	NM_001108801	F: 5'- 3') – TTGAAATCAGCCAGCACGC R: 5'- 3') – TGCCAACGGCTCGTCTCT	74

*F: Forward, R: Reverse, * Accession Number is provided by the National Center for Biotechnology.*

2.6. Protein extraction and western blotting.

For ASMT and AANAT protein expressions, the ovaries, follicle-enclosed oocytes and pineal glands (used as a positive control) were lysed and homogenized in 0.1 M sodium phosphate buffer pH 6.8 containing 1% Triton X-100, protease (P8340) and phosphatase (P 5726 and P0044 inhibitors (1:100) were from were from Sigma-Aldrich. The total protein concentration was measured by using Pierce™ BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, USA) and 50 µg (ovaries) or 20 µg (FOs) of total protein samples were diluted in 4X Laemmli (161-0747) with 10% 2-mercaptoethanol (161-0710) (Bio-Rad Laboratories Inc., Hercules, CA, USA). After boiling (90ºC) for 5 min, the samples were loaded in a 12% TGX™ FastCast™ Acrylamide Kit (#1610175, Bio-Rad Laboratories Inc.) and transferred to nitrocellulose membrane

using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories Inc.). The membranes were blocked with Tris-buffered saline (TBS + 5% BSA) for 1 hour and incubated overnight at 4ºC with primary ASMT (1:4000), AANAT (1:1000) and γTUBULIN (1:1000) antibodies and then washed in Tris-Buffered Saline (TBS) solution; the membranes were then incubated with appropriated secondary antibodies for 1 hour at room temperature. After successive washing, the membranes were scanned in Li-COR Odissey system.

2.7. Immunohistochemistry.

The ovary samples were fixed in 4% buffered paraformaldehyde, submitted to in graded series of ethanol and xylol solutions and embedded in paraffin. Sections (5 μm) mounted onto silanized slides were diaphanized, rehydrated and blocked with 3% BSA in phosphate-buffered saline (PBS). Then, the slides were washed and incubated overnight with rabbit primary ASMT (1:200) or AANAT (1:200) antibodies. The immunoreaction points were detected using the chromogen 3,3'-diaminobenzidine (DAB), using the EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436, Abcam, Cambridge, UK), following the manufacturer's recommendations. Sections without the primary antibody were used as a negative control of the immunoreaction protocol. Finally, sections were counterstained with hematoxylin, dehydrated with an increasing series of ethanol, diaphanized in xylene and coverslipped with DPX as the mounting medium (Millipore Corporate Headquarters, Billerica, MA, USA). The sections were examined with EVOS microscope (Thermo Fisher Scientific).

2.8. Experiment 1.

This experiment investigated the effects of pinealectomy on the daily distribution of mRNA expression of *Bmp-15*, *Gdf-9* genes in oocytes and *Fshr* and *Lhr* genes in cumulus cells and *ASMT* and *AANAT* protein expressions in ovaries obtained from intact or pinealectomized females either during the light (6 h after lights on - ZT6) or the dark (6 h after lights off - ZT18) phase of 12h:12h light/dark cycle. The option of collecting all the samples only at two zeitgeber times (ZTs) was based on daily profile of *Aanat* activity and *Asmt* and *Aanat* mRNA and protein expressions in rat pineal gland (33) which reflects melatonin synthesis by pineal gland (28).

The animals were intraperitoneally anesthetized with ketamine and xylazine (0.15 mL/100 g body weight) and submitted to the surgery according to Hoffman and Reiter (34) procedures with some adaptations (35). Animals were divided in two groups: sham-pinealetomized (INTACT), and pinealectomized (PINX).

2.9. Experiment 2.

This experiment investigated the effect of addition of melatonin during *in vitro* oocyte maturation process on the mRNA expression of *Bmp-15*, *Gdf-9*, *Fshr* and *Lhr* genes. In this study, follicle-enclosed oocytes (immature follicles) were submitted to different conditions for 16 h culture: a) Immature follicles cultured only in B-199 medium without melatonin (Control - C); b) Immature follicles cultured in B-199 medium with 10^{-3} M melatonin (M-3); c) Immature follicles cultured in B-199 medium with 10^{-6} M melatonin (M-6); d) Immature follicles cultured in B-199 medium with 10^{-9} M melatonin (M-9). Immature follicles were obtained from ovaries of intact females euthanized either during the light phase (ZT6) or during the dark phase (ZT18).

2.10. Experiment 3.

This experiment investigated the effect of gonadotrophins during the *in vitro* oocyte maturation process on the *ASMT* and *AANAT* protein expressions in follicle-enclosed oocytes from ovaries obtained from euthanized intact or pinealectomized females either during the light (ZT6) or the dark phase (ZT18) of the 12h:12h light/dark cycle. The immature follicle-enclosed oocytes were subjected to different culture conditions for 16 hours: a) Immature follicles cultured only in B-199 medium without gonadotrophins (Control - C); b) Immature follicles cultured in B-199 medium with 10 µg/mL FSH (F); c) Immature follicles cultured in B-199 medium with 100 ng/mL LH (L); d) Immature follicles cultured in B-199 medium with 10 µg/mL FSH and 100 ng/mL LH (FL); e) Immature follicles cultured in B-199 medium with 1 µg/mL GnRH analogous (G). The option of using the GnRH analogous was due to its beneficial effect on the *in vitro* maturation process of rat follicle-enclosed oocytes (29).

2.11. Statistical analysis.

Data results were plotted as mean ± SEM, calculated from at least five to six replications and analyzed using two-way ANOVA followed by Bonferroni´s post-test. $P < 0.05$ was considered as statistical significance. These analyses were carried out using GraphPad Prism (GraphPad Software version 8.30; San Diego, CA, USA).

3. RESULTS

3.1. Effects of pinealectomy on daily mRNA expression of *Bmp-15, Gdf-9, Fshr* and *Lhr* genes in immature oocytes and cumulus cells and *ASMT* and *AANAT* protein expression and their immunolocalization in ovaries.

Considering the *Bmp-15* and *Gdf-9*, *Fshr* and *Lhr* mRNA expressions in immature oocytes and cumulus cells from INTACT and PINX rats (Figure 1), the light-dark distribution of *Bmp-15* (Figure 1A) and *Gdf-9* (Figure 1B) mRNA expressions in immature oocytes was abolished by pinealectomy. There were no light-dark phase differences in oocytes of PINX animals whereas in INTACT animals the *Bmp-15* and *Gdf-9* mRNA expressions were greater during the dark phase. On the other hand, absence of pineal melatonin did not alter the daily distribution of *Fshr* (Figure 1C) and *Lhr* (Figure 1D) mRNA expressions in immature cumulus cells. In both INTACT and PINX females the mRNA expression of *Lhr* in cumulus cells was greater during the dark phase and no phase differences were observed on *Fshr* mRNA expression.

The melatonin synthetizing enzyme (ASMT and AANAT) proteins were expressed in ovaries of both INTACT and PINX rats and no light-dark phase differences were detected either in INTACT or PINX rats (Figure 2). The immunohistochemistry analyses of ASMT (Figure 3) and AANAT (Figure 4) in immature ovaries from INTACT (Figures 3A and 4A) and PINX animals (Figures 3B and 4B) revealed an immunoreaction for ASMT and AANAT in primary (preantral), secondary and tertiary (antral) follicles and corpus lutea. The immunostaining of ASMT was weakly observed in most follicular cells and in the medullar region (Figure 3: A2, A4, A5, B2, B4, B4 and B5). The immunoreaction of AANAT was detected in follicular cells (mainly in theca cells) and corpus luteum but, a strong AANAT immunostaining was observed in oocytes (Figure 4: A2, A4, B2 and B4).

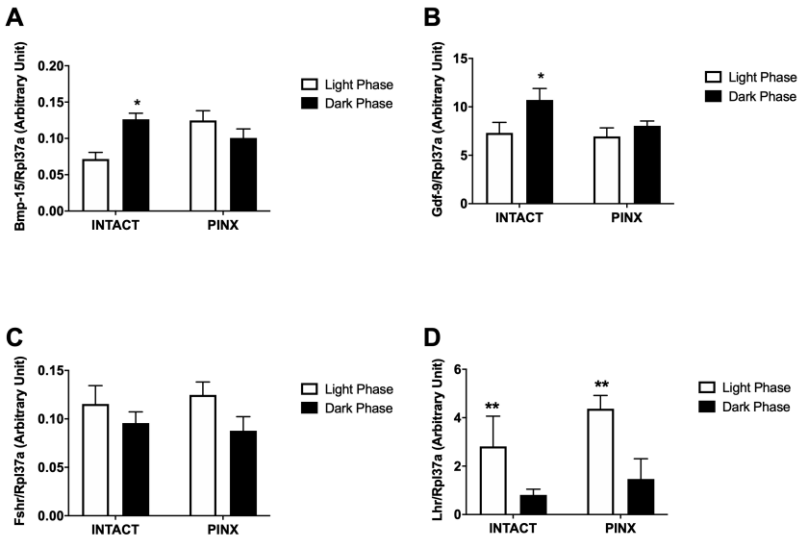

Fig.1. Effects of pinealectomy on daily mRNA expression of *Bmp-15* (A), *Gdf-9*, *Fshr* and *Lhr* genes in immature oocytes (A and B) and cumulus cells (C and D).

*INTACT: sham-pinealectomized rats (50 females:25/phase); PINX: pinealectomized rats (50 females:25/phase). Each sample (5 replications/group) represents a pool of 80 COCs from five females. Data are expressed as mean ± SEM. Light phase (ZT6: 6 h.after lights on) and Dark phase (ZT18: 6. h after lights off). *P<0.05; **P<0.01 vs each other. Reference gene: Rpl37a.*

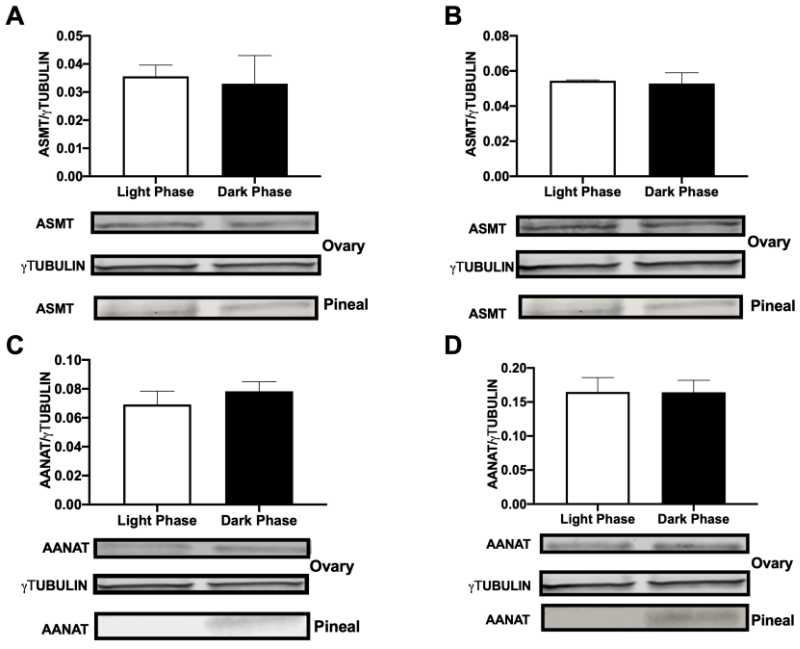

Fig. 2. Effects of pinealectomy on daily *ASMT* (A and B) and *AANAT* (C and D) protein expression in ovaries from INTACT (A and C) – PINX (B and D) rats.

INTACT: sham-pinealectomized rats (6 females/phase); PINX: pinealectomized rats (6 females/phase). Data are expressed as mean ± SEM. Light phase (ZT6 6 h. after six hours after lights on) or dark phase (ZT18: 6 h. after lights off). Positive control: pineal gland.

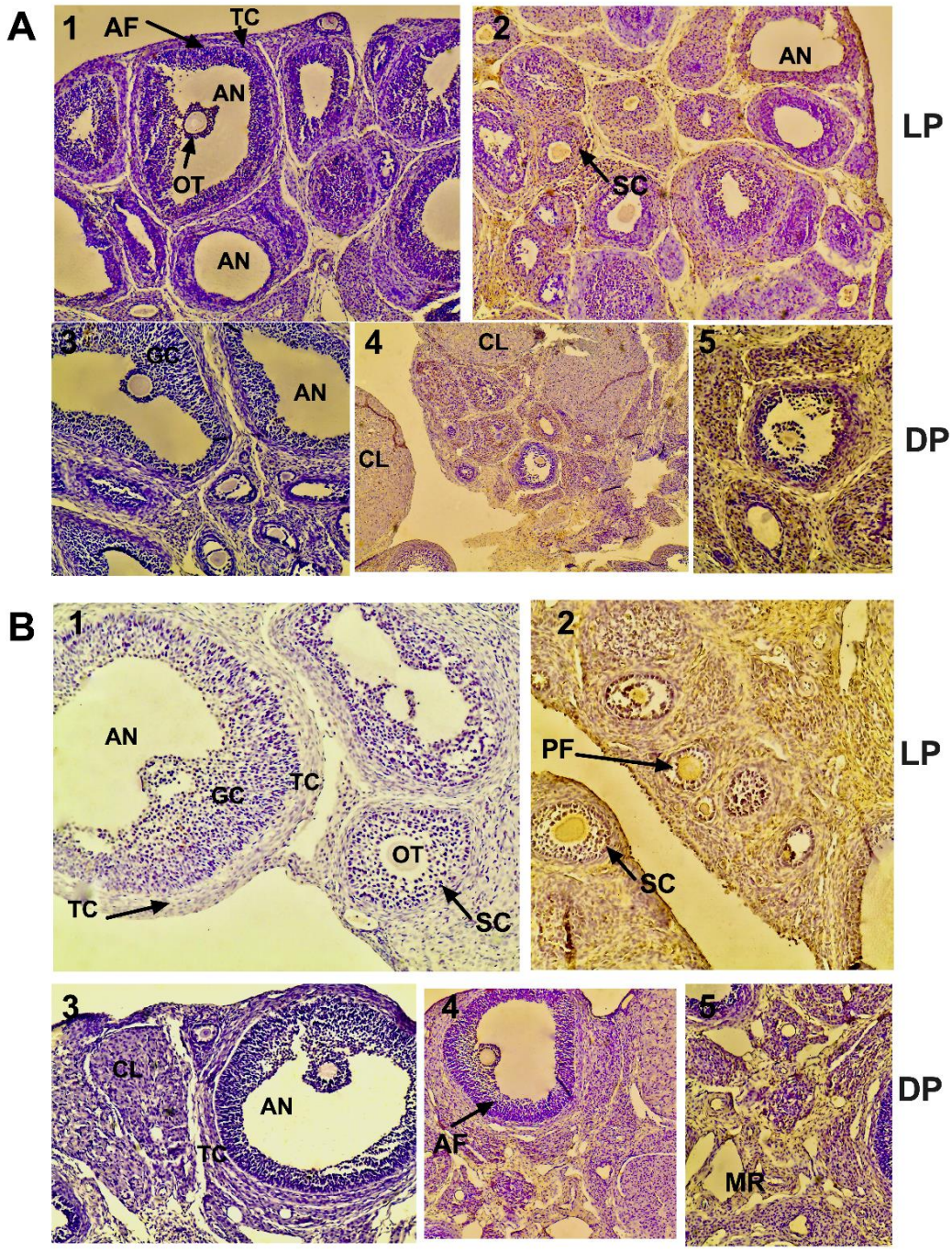

Fig. 3. Immunohistochemical staining of ASMT in ovaries from – INTACT (A) and PINX (B) rats during Light Phase (LP) and Dark Phase (DP) of the light-dark cycle.

Negative controls: A1, A3, B1 and B3. The yellow-brown color indicates the immunoreactivity of ASMT: A2, A4, A5, B2, B4 and B5 (Medulla Region - MR). The sections were visualized in EVOS microscope at X 10 (A1, A4, B4, B5 and B6) and X 20 (A2, A3, A5, A6, B1, B2 and B3). PF = Primary Follicle; SF = Secondary Follicle; AF =Antral Follicle or Tertiary Follicle; IAF = Initial Antral Follicle; CL = Corpus Luteum AN = Antrum. Inside the follicles: TC = Theca Cells; GC = Granulosa Cells.

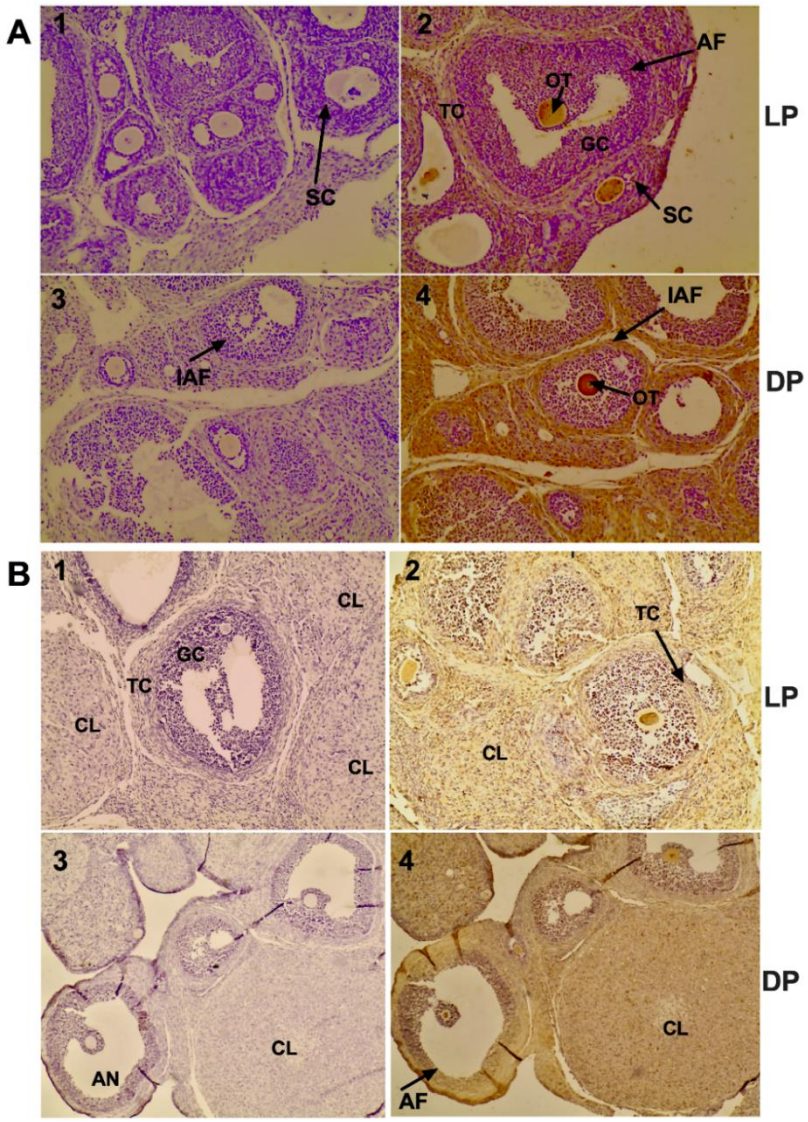

Fig. 4. Immunohistochemical staining of AANAT in immature ovaries from INTACT (A) and PINX (B) rats during Light (LP) and Dark Phase (DP).

A1, A3, B1, B3: negative controls. A2, A4, B2, B4: AANAT positive staining (yellow-brown color) visualized under EVOS microscope at X 10 (A1, A3, A4, B3, B4) and X 20 (A2, B1, B2). SF = Secondary Follicle; AF =Antral Follicle or Tertiary Follicle; IAF = Initial Antral Follicle; CL = Corpus Luteum; AN = Antrum, OT = Oocyte; TC = Theca Cells; GC = Granulosa Cells.

3.2. Effects of melatonin supplementation in maturation medium on mRNA expression of *Bmp-15, Gdf-9, Fshr* and *Lhr* genes in FOs.

The results showed that the addition of melatonin to the maturation culture medium altered the *Bmp-15*, *Gdf-9* and *Lhr* mRNA expressions in FOs according to the phase of the light-dark cycle (Figure 5). Melatonin increased the *Bmp-15* (Figure 5A) and *Gdf-9* (Figure 5B) gene expressions during the dark phase and the *Lhr* gene expression during the light phase (Figure 5D). The *Fshr* expression was not affected by melatonin to the cultures (Figure 5C).

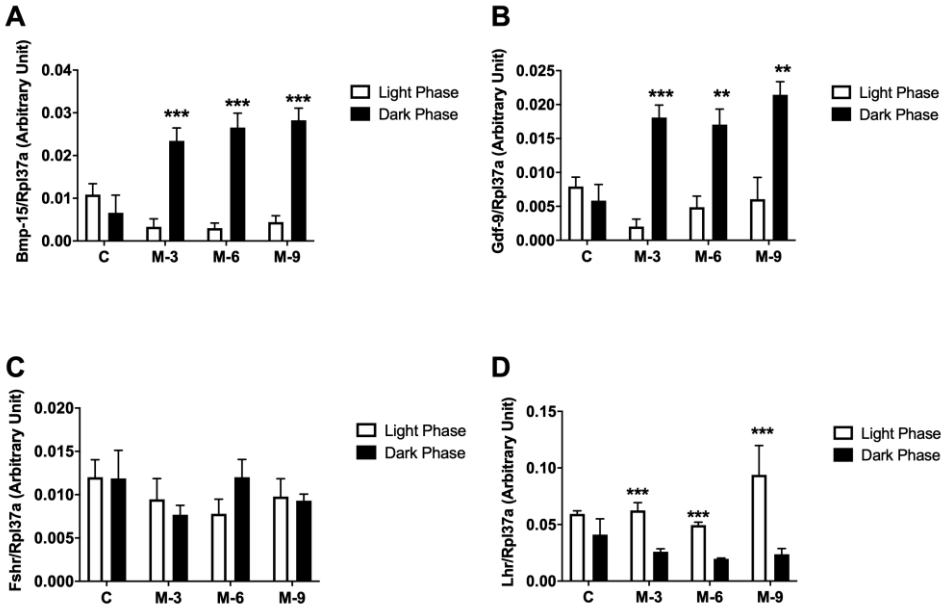

Fig. 5. Effects of melatonin supplementation in maturation medium (B-199) on mRNA expression of *Bmp-15* (A), *Gdf-9* (B), *Fshr* (C) and *Lhr* (D) genes in rat FOs.

*FOs were collected in different phases: Light (ZT6: 6 h after lights on) and Dark Phase (ZT18: 6 h after lights off) and cultured in B-199 medium without (C) or with 10^{-3} M (M-3), 10^{-6} M (M-6) and 10^{-9} M (M-9) melatonin for 16 h. Data are expressed as mean ± SEM. Each sample (5 replications) represents a pool of 30 FOs from 5 females. Reference gene: Rpl37a. **P<0.01; ***P<0.001 vs each other.*

Correlation analysis (Table 2) showed a negative correlation between the daily mRNA expression of *Lhr* and the daily mRNA expression of *Bmp-15* or *Gdf-9* in FOs when melatonin was added to maturation medium. The daily *Fshr* mRNA expression in FOs supplemented with melatonin was not correlated with daily mRNA expression of *Bmp-15* or *Gdf-9*.

Table 2. Correlation of mRNA expression both *Bmp-15* and *Gdf-9* between *Fshr* or *Lhr* in FO cultured without (C) or with 10^{-3} M (M-3), 10^{-6} M (M-6) and 10^{-9} M (M-9) melatonin for 16 hours during the light and dark phases (* P<0.05; ** P<0.01 vs control)**.**

Correlations	*Bmp-15* (Y)		*Gdf-9* (Y)	
	Pearson r	P values	Pearson r	P values
Fshr (X)				
C	0.188	0.628	-0.215	0.5785
M3	0.088	0.821	-0.046	0.906
M6	0.323	0.395	0.377	0.317
M9	-0.042	0.912	-0.389	0.300
Lhr (X)				
C	0.042	0.907	-0.237	0.509
M3	-0.745	0.013*	-0.796	0.005**
M6	-0.867	0.001**	-0.775	0.008**
M9	-0.697	0.036*	-0.719	0.044*

3.3. Effects of gonadotropin supplementation in maturation medium on protein expression of *ASMT* and *AANAT* in FOs from immature ovaries of INTACT and PINX animals.

Gonadotropin supplementation had no significant effect on *ASMT* expression in FOs from INTACT (Figure 6A) or PINX (Figure 6B) animals according to the light/dark cycle. Although there are no differences in most of gonadotropin treatments in the expression of *AANAT* (Figure 7), the addition of LH to the *in vitro* maturation medium significantly altered *AANAT* expression in FOs according the phase of the light-dark cycle. *AANAT* expression in FOs from intact (Figure 7A) and pinealectomized (Figure 7B) females was greater during the dark phase.

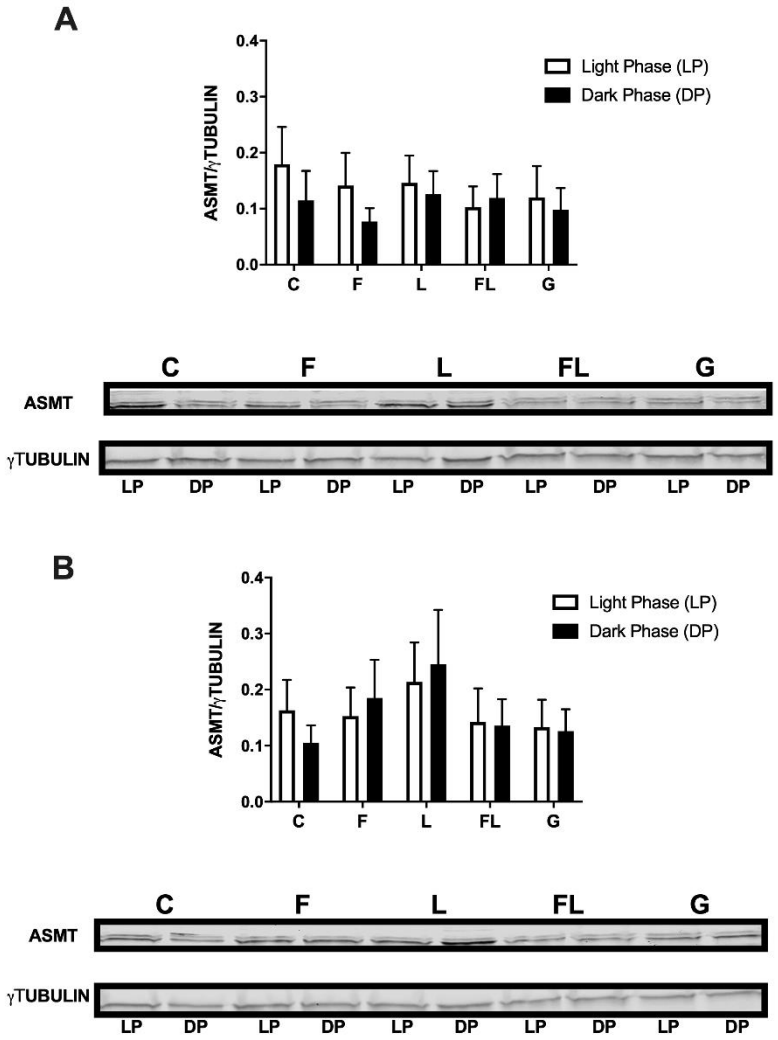

Fig. 6. Effects of gonadotropin supplementation in maturation medium (B-199) on protein expression of *ASMT* in FOs from– INTACT (A) and PINX (B) rats.

Follicles were collected in different phases of light-dark cycle: Light Phase (LP) and Dark Phase (DP) and cultured in B-199 medium only (C) or B-199 medium with 10 µg/mL FSH (F), or with 100 ng/mL LH (L), or with 10 µg/mL FSH and 100 ng/mL LH (FL) or with 1 µg/mL GnRH (G) for 16 hours. Each sample (5 replications) represents a pool of 30 FOs from 5 females (50 INTACT females and 50 PINX females). Data are expressed as mean ± SEM).

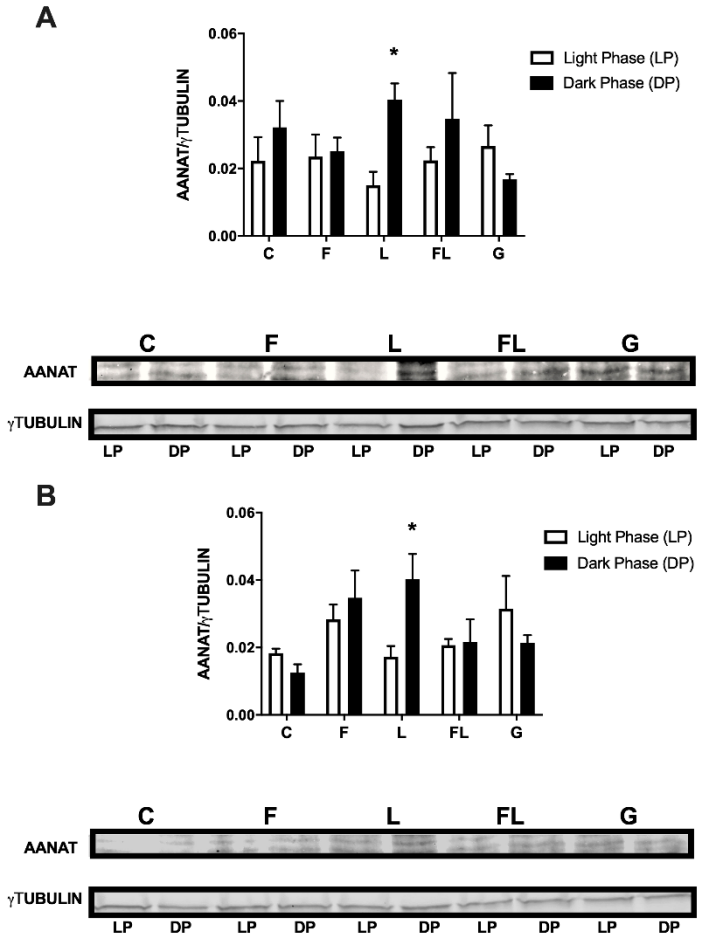

Fig. 7. Effects of gonadotropin supplementation in maturation medium (B-199) on protein expression of *AANAT* in FOs from – INTACT (A) and PINX (B) rats.

*Follicles were collected in different phases of light-dark cycle: Light Phase (LP) and Dark Phase (DP) and cultured in B-199 medium only (C) or with 10 µg/mL FSH (F), or with 100 ng/mL LH (L), or with 10 µg/mL FSH and 100 ng/mL LH (FL) or with 1 µg/mL GnRH (G) for 16 hours. Each sample (5 replications) represents a pool of 30 FOs from 5 females (50 INTACT females and 50 PINX females). Data are expressed as mean ± SEM. *P<0.05.*

4. DISCUSSION

The present study investigated the effects of melatonin supplementation during *in vitro* maturation of rat FOs on the mRNA expression of *Bmp-15*, *Gdf-9*, *Fshr* and *Lhr* genes and tested whether these effects would be altered by the photoperiodic changes. Since pineal gland is considered as a master-regulator of light/dark cycle, the effects of pineal gland on the mRNA expressions of *Bmp-15*, *Gdf-9* in immature oocytes, *Fshr* and *Lhr* in immature cumulus cells and the protein expressions of *AANAT* and *ASMT* in ovary were also investigated, respectively. Moreover, the effects of gonadotropin supplementation during *in vitro* maturation of FOs on protein expression of *AANAT* and *ASMT* in intact and pinealectomized females were also studied. The addition of melatonin to the FOs maturation medium increased the mRNA expression of *Bmp-*

15 and *Gdf-9* genes only during the dark phase of the light-dark cycle. These results indicate a daily mRNA expression of *Bmp-15* and *Gdf-9* genes are under the control of circulating pineal-derived melatonin. This was confirmed using immature COCs from pinealectomized animals. Pinealectomy abolished this phase different expression of *Bmp-15* and *Gdf-9* and it suggested their daily expression rhythm throughout follicular development. The immunostaining of BMP-15 and GDF9 in human oocyte and granulosa cells increase with follicular growth (36) and their presence is important for the heathy mature oocytes (36-39). In fact, studies focusing on mRNA (37-38) and protein (36, 39) expressions of BMP-15 and GDF9 in growing follicles of women with polycystic ovary syndrome (PCOS) have identified an abnormal pattern of their expression which probably influences the nuclear (37) and cytoplasmic (40) maturation of cumulus- oocyte complexes. The reduced expression of these genes in oocytes from PCOS patients could be also associated to impaired developmental competence of the oocyte (41) subjected to *in vitro* fertilization in assisted reproductive technologies (ART) (42). Melatonin improves the nuclear and cytoplasmic maturation in COCs from PCOS mouse model (43) and in oocytes from PCOS patients (44).

Herein, the mRNA expression of *Fshr* in FOs was not altered by melatonin supplementation during either light or dark phase, but melatonin supplementation increased the *Lhr* mRNA expression during the light phase**.** The results suggest that melatonin participates in the regulation of LH by modulating the *Lhr* mRNA expression in granulosa cells during the maturation process. Studies with large mammalian ovaries have demonstrated that melatonin added to the culture medium increases the *Lhr* mRNA expression in buffalo granulosa cells (45) and the upregulation of *Lhr* mRNA expression in bovine granulosa cells is correlated with final stage of the follicle development (6), when the oocyte maturation is stimulated by the LH surge (46, 47). In rat, the timing of the proestrus LH surge, which coincides with the LH receptor mRNA levels (48), is controlled by pineal melatonin (49, 50). We also observed that the daily mRNA expression of *Lhr* was negatively correlated with the daily mRNA expression of *Bmp-15* and *Gdf-9* in FOs supplemented with melatonin, suggesting interactions among melatonin, genes mentioned above and LH receptor during maturation process. These interactions have also occurred in rat preovulatory follicles (8, 14) with an unknown physiological mechanism.

Our results also suggest that the *Lhr* mRNA expression in immature cumulus cells is also regulated by melatonin, showing a similar expression phase pattern as observed in mature FOs. However, the daily *Lhr* mRNA expression pattern in immature cumulus cells was not altered by pinealectomy, suggesting a putative participation of ovarian melatonin synthesis in growing follicles. In fact, extrapineal melatonin synthesis has been described in literature (51, 52) and the mitochondria seem to be the potential source of melatonin synthesis in peripheral tissues (53). It has been also shown that melatonin is synthetized from serotonin in rat oocytes where AANAT protein is predominantly expressed (21). When we analyzed the immunolocalization of melatonin-synthetizing enzymes, both ASMT and AANAT proteins were localized in oocytes and granulosa cells of the antral follicles and also corpus luteum in ovaries from intact and pinealectomized females confirming the potential of the ovary to synthesize melatonin, but more research is necessary to confirm this hypothesis. The significant phase differences of *Lhr* mRNA expression in immature cumulus cells from pinealectomized females observed in this study could be consequence of the pinealectomy or melatonin treatment on the modulation of LH blood levels (54, 55), which is a reflection of *Lhr* mRNA expression in rat granulosa cells during the follicle development (48). Additionally, pinealectomy changes the serum estradiol and progesterone levels and ovarian progesterone receptors during estrus stage (56) that could alter the temporal pattern of regulation of estrous cycle (54, 56) by suprachiasmatic nuclei (57).

To detect potential phase differences regarding to the mRNA expression of *Bmp-15*, *Gdf-9* and *Lhr* in COCs and FOs the samples were collected both light and dark phases which likely reflects a daily profile of their expression associated with pineal melatonin. The time of sample selection was based on the results of daily mRNA and protein expressions of *AANAT* (analyzing eight time points) in rat pineal gland with the nocturnal peak expression at ZT18 and diminished expression during the light phase (33). In the same study, it was also observed that the nocturnal increase of ASMT expression is not obvious and no significant difference with its expression during the light phase (33). The results are compatible with those reported by Simonneaux and Ribelayga (28) in pineal gland and by Coelho *et al*. (35) in testes, respectively. Our findings show no phase differences in protein expression of two melatonin synthetic enzymes in ovaries of both intact and pinealectomized females suggesting that, in this case, the analysis of only two time points was not enough to show the effect of pinealectomy on the daily protein expression of these enzymes. On the other hand, our previous work analyzing four time points documented that the daily mRNA expression of *Aanat* and *Asmt* genes in immature COCs (58) is driven by pineal melatonin.

Considering the effects of gonadotropin supplementation during *in vitro* maturation on the protein expression of *ASMT* and *AANAT* in FOs, the results showed that LH significantly increased the *AANAT* expression during the dark phase in FOs from both intact and pinealectomized females suggesting the role of LH in the modulation of *AANAT* expression during maturation process. It has been demonstrated that, under *in vivo* conditions, the mRNA expression of this melatonin-synthetic enzyme in mice cumulus cells increases after hCG (equivalent LH) injection (59).

We hypothesize that melatonin modulates the mRNA expression of *Lhr* gene in granulosa cells of preovulatory follicle during the light-dark transition (timing of the proestrus LH surge and maturation process) (57) and then, during the dark phase, the LH surge stimulates the *AANAT* expression to promote melatonin synthesis in the preovulatory follicle. This hypothesis requires more data to support. Nevertheless, it was reported that melatonin from follicular fluid was synthesized by the ovary under the influence of LH since melatonin levels in the mouse ovaries significantly increased after hCG injection but not altered simultaneously the serum melatonin levels (59). It seems that ovarian melatonin synthesis would explain the reduced fertility observed in pinealectomized female rats (60, 61) which show some physiological alterations during the follicular development such as an increase of the number of atretic follicles (18), abnormal steroidogenic gene expression (62), and alteration of the LH surge timing (50).

The current findings demonstrate that melatonin act in the regulation of mRNA expression of *Bmp-15*, *Gdf-9* and *Lhr* genes during maturation process. Finally, the present research also shows that melatonin has the potential to enhance the competence of the oocyte to develop into embryo after fertilization by modulating the mRNA expression of these genes, under *in vitro* maturation conditions. Moreover, these results emphasize the importance of the clinical application of melatonin in ART programs which should be given to infertile women to improve oocyte maturation and fertilization rates (63) or should be used as supplementation to *in vitro* maturation medium (44) to improve the implantation rates in PCOS patients.

ACKNOWLEDGMENTS

This work was financially supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (2014/17830-9), FAPESP (2014/50457-0), São Paulo, Brazil. We would like to thank Dr. D.C. Klein, NIH, Bethesda, USA for the AANAT and ASMT antibodies.

AUTHORSHIP

LC conceived and design the research and, wrote the paper. DB, WK, JA-S, PG, JS and FA performed the experiments. RP analyzed data and RR wrote and revised the paper. JC-N conceived the research and wrote the paper.

CONFLICT OF INTEREST

We declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

REFERENCES

1. Tripathi A, Prem Kumar KV, Chaube SK (2010) Meiotic cell cycle arrest in mammalian oocytes. *J. Cell. Physiol.* **223**: 592-600.
2. Nagyova E, Kalous J, Nemcova L (2016) Increased expression of pentraxin 3 after in vivo and in vitro stimulation with gonadotropins in porcine oocyte-cumulus complexes and granulosa cells. *Dom. Anim. Endocrinol*. **56:** 29-35.
3. Epigg JJ. FSH (1979) stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature* **281**: 483-484.
4. Ge L, Sui H-S, Lan G-C, Liu N, Wang J-Z, Tan J-H (2008) Coculture with cumulus cells improves maturation of mouse oocytes denuded of the cumulus oophorus: observations of nuclear and cytoplasmic events. *Fertil. Steril.* **90:** 2376-2388.
5. Ouandaogo ZG, Haouzi D, Assou S, Dechaud H, Kadoch IJ, Vos JD, Hamamah S (2011) Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage. *Plos One* **6:** e27179.
6. Robert C, Gagné D, Lussier JG, Bousquet D, Barnes FL, Sirard M-A (2003) Presence of LH receptor mRNA in granulosa cells as a potential marker of oocyte developmental competence and characterization of the bovine splicing isoforms. *Reproduction* **125:** 437-446.
7. Su Y-Q, Wu X, O'Brien MJ, Pendola FL, Denegre JN, Matzuk MM, Eppig JJ (2004) Synergistic roles of BMP15 and GDF9 in the development and function of the oocyte-cumulus cell complex in mice: genetic evidence for an oocyte-granulosa cell regulatory loop. *Dev. Biol.* **276:** 64-73.
8. Otsuka F, McTavish K, Shimasaki S (2011) Integral role of GDF-9 and BMP-15 in ovarian function. *Mol. Reprod. Dev.* **78:** 9-21.
9. Castro FC, Cruz MHL, Leal CLV (2016) Role of growth differentiation factor 9 and Bone morphogenetic protein 15 in ovarian function and their omportance in mammalian female fertility – A review. *Asian Australas. J. Anim. Sci.* **29:** 1065-1074.
10. Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM (1999) Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Mol. Endocrinol.* **13:** 1033-1048.
11. Garcia P, Aspee K, Ramirez G, Dettleff P, Palomino J, Peralta OA, Parraguez VH, De los Reyes M (2019) Influence of growth differentiation factor-9 and bone morphogenetic protein 15 on in vitro maturation of canine oocytes. *Reprod. Dom. Anim.* **54:** 373-380.
12. Velásquez A, Mellisho E, Castro FO, Rodríguez-Álvarez L (2019) Effect of BMP15 and or AMH during in vitro maturation of oocytes from involuntarily culled dairy cows. *Reprod. Dom. Anim.* **84:** 209-223.

13. Moore RK, Shimazaki S (2005) Molecular biology and physiological role of the oocyte factor, BMP-15. *Mol. Cell. Endocrinol.* **234:** 67-73.
14. Vitt UA, Hayashi M, Klein C, Hsueh AJW (2000) Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biol. Reprod.* **62:** 370-377.
15. Cipolla-Neto J, Amaral FG (2018) Melatonin as a hormone: new physiological and clinical insights. *Endocr. Rev.* **39:** 990-1028.
16. Tamura H, Nakamura Y, Korkmaz A, Manchester LC, Tan D-X, Sugino N, Reiter RJ (2009) Melatonin and the ovary: physiological and pathophysiological implications. *Fertil. Steril.* **92:** 328-343.
17. Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L, Tamura I, Maekawa R, Asada H, Yamagata Y, Sugino N (2012) The role of melatonin as an antioxidant in the follicle. *J. Ovarian Res*. **5:** 5. doi:10.1186/1757-2215-5-5.
18. Maganhin CC, Fuchs LFP, Simões RS, Oliveira-Filho RM, Simões MJ, Baracat EC, Soares JM Jr (2013) Effects of melatonin on ovarian follicles. *Eur. J. Obstet. Gynecol. Reprod.* Biol. **166:** 178-184.
19. Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Reiter RJ, Sugino N (2008) Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J. Pineal Res.* **44:** 280-287.
20. Wang Y, Zeng S (2018) Melatonin promotes ubiquitination of phosphorylated pro-apoptotic protein Bcl-2-interacting mediator of cell death-extra long (Bim_{EL}) in porcine granulosa cells. *Int. J. Mol. Sci*. **19:** 3431. doi:10.3390/ijms19113431.
21. Sakaguchi K, Itoh MT, Takahashi N, Tamuri W, Ishizuka B (2013) The rat oocyte synthesizes melatonin. *Reprod. Fertil. Dev.* **25:** 674-682.
22. He C, Wang J, Zhang Z, Yang M, Li Y, Tian X, Ma T, Tao J, Zhu K, Song Y, Ji P, Liu G (2016) Mitochondria synthesize melatonin to ameliorate its function and improve mice oocyte's quality under in vitro conditions. *Int. J. Mol. Sci.* **17:** 939. doi:10.3390/ijms17060939.
23. Itoh MT, Ishizuka B, Kuribayashi Y, Amemiya A, Sumi Y (1999) Melatonin, its precursors, and synthesizing enzyme activities in the human ovary. *Mol. Hum. Reprod.* **5:** 402-408.
24. An Q, Peng W, Cheng Y, Lu Z, Zhou C, Zhang C, Su J (2019) Melatonin supplementation during maturation of oocyte enhances subsequent development of bovine cloned embryos. *J. Cell Physiol.* **234:** 17370-17381.
25. Woo MMM, Tai C-J, Kang SK, Nathwani PS, Pang SF, Leung PCK (2011) Direct action of melatonin in human granulosa-luteal cells. *J. Clin. Endocrinol. Metab.* **2086:** 4789-4797.
26. Nakamura E, Otsuka F, Terasaka T, Inagaki K, Hosoya T, Tsukamoto-Yamauchi N, Toma K, Makino H (2014) Melatonin counteracts BMP-6 regulation of steroidogenesis by rat granulosa cells. *J. Steroid Biochem. Mol. Biol.* **143:** 233-239.
27. Otsuka F (2018) Interaction of melatonin and BMP-6 in ovarian steroidogenesis. *Vitam. Horm.* **107:** 137-153.
28. Simonneaux V, Ribelayga C (2003) Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacol. Rev.* **55:** 325-395.
29. Hillensjö T, LeMaire WJ (1980) Gonadotropin releasing hormone agonists stimulate meiotic maturation follicle-enclosed rat oocytes *in vitro*. *Nature* **287:** 145-146.

30. Sela-Abramovich S, Chorev E, Galiani D, Dekel N (2005) Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology* **146:** 1236-1244.
31. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25:** 402-408.
32. Vandesompele J, De Preter K, Pattyn F, Poppe B, Roy NV, Paepe AD, Speleman F (2002) Accurate normatization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3:** RESEARCH0034.
33. Amaral FG, Turati AO, Barone M, Scialfa JH, Buonfiglio DC, Peres R, Peliciari-Garcia RA, Afeche SC, Lima L, Scavone C, Bordin S, Reiter RJ, Cipolla-Neto J. (2014) Melatonin synthesis impairment as new deleterious outcome of diabetes-derived hyperglycemia. *J. Pineal Res.* **57:** 67-79.
34. Hoffman RA, Reiter RJ (1965) Rapid pinealectomy in hamsters and other small rodents. *Anat. Rec.* **153:** 19-21.
35. Coelho LA, Andrade-Silva J, Motta-Teixeira LC, Amaral FG, Reiter RJ, Cipolla-Neto J (2019) The absence of pineal melatonin abolishes the daily rhythm of *Tph1* (Tryptophan Hydroxylase 1), *Asmt* (Acetylserotonin O-Methyltransferase) and *Aanat* (Aralkylamine N-Acetyltransferase) mRNA expressions in rat testes. *Mol. Neurobiol.* **56:** 7800-7809.
36. Wei L-N, Huang R, Li L-L, Fang C, Li Y, Liang X-Y (2014) Reduced and delayed expression of GDF9 and BMP15 in ovarian tissues from women with polycystic ovary syndrome. *J. Assit. Reprod. Genet.* **31:** 1483-1490.
37. Wei L-N, Fang C, Huang R, Li L, Zhang M, Liang X (2012) Change and significance of growth differentiation factor 9 and bone morphogenetic protein15 expression during oocyte maturation in polycystic ovary syndrome with ovarian stimulation. *Zhonghua Fu Chan Ke Za Zhi.* **47** (11): 818-22.https://pubmed.ncbi.nlm.nih.gov/23302121.
38. Teixeira Filho FL, Baracat EC, Lee TH, Suh CS, Matsui M, Chang RJ, Shimasaki S, Erickson GF (2002) Aberrant expression of growth differentiation factor-9 in oocytes of women with polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* **87:** 1337-1344.
39. Zhao S-Y, Qiao J, Chen Y-J, Liu P, Li J, Yan J (2010) Expression of growth differentiation factor-9 and bone morphogenetic protein-15 in oocytes and cumulus granulosa cells of patients with polycystic ovary syndrome. *Fertil. Steril.* **94:** 261-267.
40. Beli M, Shimasaki S (2018) Molecular Aspects and clinical relevance of GDF9 and BMP15 in ovarian function. *Vitam. Horm.* **107:** 317-348.
41. Qiao J, Feng HL (2011) Extra- and intra-ovarian factors in polycystic ovary syndrome: impact on oocyte maturation and embryo developmental competence. *Hum. Reprod. Update* **17:** 17-33.
42. Kollmann M, Martins WP, Lima MLS, Craciunas L, Nastri CO, Richardson A, Raine-Fenning N (2016) Strategies for improving outcome of assisted reproduction in women with polycystic ovary syndrome: Systematic review and meta-analysis. *Ultrasound Obstet. Gynecol.* **48:** 709-718.
43. Nikmard F, Hosseini E, Bakhtiyari M, Ashrafi M, Amidi F, Aflatoonian R (2017) Effects of melatonin oocyte maturation in PCOS mouse model. *Anim. Sci. J.* **88:** 586-592.
44. Kim MK, Park EA, Kim HJ, Choi WY, Cho JH, Lee WS, Cha KY, Kim YS, Lee DR, Yoon TK (2013) Does supplementation of in-vitro culture medium with melatonin improve IVF outcome in PCOS? *Reprod. Biomed. Online* **26:** 22-29.

45. Riaz H, Yousuf MR, Liang A, Hua GH, Yang L (2019) Effect of melatonin on regulation of apoptosis and steroidogenesis in culture buffalo granulosa cells. *Anim. Sci. J.* **90:** 473-480.
46. Nagyova E, Kalous J, Nemcova L (2016) Increased expression of pentraxin 3 after in vivo and in vitro stimulation with gonadotropins in porcine oocyte-cumulus complexes and granulosa cells. *Dom. Anim. Endocrinol.* **56:** 29-35.
47. Epigg JJ (1979) FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature* **281:** 483-484.
48. Nakamura K, Minegishi T, Takakura Y, Miyamoto K, Hasegawa Y, Ibuki Y, Igarashi M (1991) Hormonal regulation of gonadotropin receptor mRNA in rat ovary during follicular growth and luteinization. *Mol. Cell. Endocrinol.* **82:** 259-263.
49. Walker RF, McCamant S, Timiras PS (1982) Melatonin and the influence of the pineal gland on timing of the LH surge in rats. *Neuroendocrinology* **35:** 37-42.
50. Chiba A, Akema T, Toyoda J (1994) Effects of pinealectomy and melatonin on the timing of proestrous luteinizing hormone surge in the rat. *Neuroendocrinology* **59:** 163-168.
51. Acuña-Castroviejo D, Escames G, Venegas C, Díaz-Casado ME, Lima-Cabello E, López LC, Rosales-Corral S, Tan D-X, Reiter RJ (2014) Extrapineal melatonin: sources, regulation, and potential functions. *Cell. Mol. Life Sci.* **71:** 2997-3025.
52. Stefulj J, Hörtner M, Ghosh M, Schauenstein K, Rinner I, Wölfler A, Semmier J, Liebmann M (2001) Gene expression of the key enzymes of melatonin synthesis in extrapineal tissues of the rat. *J. Pineal Res.* **30:** 243–247.
53. Reiter RJ, Rosales-Corral S, Tan DX, Jou MJ, Galano A, Xu B (2017) Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. *Cell. Mol. Life Sci.* **74:** 3863-3881.
54. Dardes RC, Baracat EC, Simões MJ (2000) Modulation of estrous cycle and LH, FSH and melatonin levels by pinealectomy and sham-pinealectomy female rats. *Prog. Neuro-Psychopharmacol. Biol. Psychiat.* **24:** 441-453.
55. Chuffa LGA, Seiva FRF, Fávaro WJ, Teixeira GR, Amorim JPA, Mendes LO, Fioruci BA, Pinheiro PFF, Fernandes AAH, Franci JAA, Dellela FK, Martinez M, Martinez FE (2011) Melatonin reduces LH, 17 beta-estradiol and induces differential regulation of sex steroid receptors in reproductive tissues during rat ovulation. *Reprod. Biol. Endocrinol.* **9:** 108.
56. Soares JM, Simões MJ, Oshima CTF, Mora OA, Lima GR, Baracat EC (2003) Pinealectomy changes rat ovarian interstitial cell morphology and decreases progesterone receptor expression. *Gynecol. Endocrinol.* **17:** 115-123.
57. Barbacka-Surowiak G, Surowiak J, Stoklosowa S (2003) The involvement of the suprachiasmatic nuclei in the regulation of estrous cycles in rodents. *Reprod. Biol.* **3:** 99-129.
58. Coelho LA, Peres R, Amaral FG, Reiter RJ, Cipolla-Neto J (2015) Daily differential expression of melatonin-related genes and clock genes in rat cumulus-oocyte complex: Changes after pinealectomy. *J. Pineal Res.* **58:** 490-499.
59. He C, Ma T, Shi J, Zhang Z, Wang J, Zhu K, Li Y, Yang M, Song Y, Liu G (2016) Melatonin and its receptor MT1 are involved in the downstream reaction to luteinizing hormone and participate in the regulation of luteinization in the different species. *J. Pineal Res.* **61:** 279-290.
60. Teixeira AAC, Simões MJ, Teixeira VW, Soares JM Jr (2004) Evaluation of the implantation in pinealectomized and/or submitted to the constant illumination rats. *Int. J. Morphol.* **22:** 189-194.

61. Dair EL, Simões RS, Simões MJ, Romeu LRG, Oliveira-Filho RM, Haidar MA, Baracat EC, Soares JM Jr (2008) Effects of melatonin on the endometrial morphology and embryo implantation in rats. *Fertil. Steril.* **89**: 1299-1305.
62. Manganhin CC, Simões RS, Fuchs LFP, Sasso GRS, Simões MJ, Baracat EC, Soares JM Jr (2014) Melatonin influences on steroidogenic gene expression in the ovary of pinealectomized rats. *Fertil. Steril.* **102:** 291-297.
63. Tamura H, Jozaki M, Tanabe M, Shirafuta Y, Mihara Y, Shinagawa M, Tamura I, Maekawa R, sato S, Taketani T, Takasaki A, Reiter RJ, Sugino N (2020) Importance of melatonin in assisted reproductive technology and ovarian age. *Int. J. Mol. Sci.* **21**: 1135. doi:10.3390/ijms21031135.

Please cite this paper as:
Coelho, L., Buonfiglio, D., Kuwabara, W., Andrade-Silva, J., Gomes, P., Scialfa, J., Peres, R., Amaral, F. and Cipolla-Neto, J. 2020. Melatonin regulates the expression of Bone Morphogenetic Protein 15 (Bmp-15), Growth Differentiation Factor 9 (Gdf-9) and LH receptor (Lhr) genes in developing follicles of rats. Melatonin Research. 3, 4 (Oct. 2020), 515-533. DOI:https://doi.org/https://doi.org/10.32794/mr11250076.