Steroidogenesis-related gene expression in the rat ovary exposed to melatonin supplementation

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OBJECTIVE: To analyze steroidogenesis-related gene expression in the rat ovary exposed to melatonin supplementation.

METHODS: Thirty-two virgin adult female rats were randomized to two groups as follows: the control group GI received vehicle and the experimental group GII received melatonin supplementation (10 μg/night per animal) for 60 consecutive days. After the treatment, animals were anesthetized and the collected ovaries were immediately placed in liquid nitrogen for complementary deoxyribonucleic acid microarray analyses. A GeneChip® Kit Rat Genome 230 2.0 Affymetrix Array was used for gene analysis and the experiment was repeated three times for each group. The results were normalized with the GeneChip® Operating Software program and confirmed through analysis with the secondary deoxyribonucleic acid-Chip Analyzer (dChip) software. The data were confirmed by real-time reverse transcription polymerase chain reaction analysis. Genes related to ovarian function were further confirmed by immunohistochemistry.

RESULTS: We found the upregulation of the type 9 adenylate cyclase and inhibin beta B genes and the downregulation of the cyclic adenosine monophosphate response element modulator and cytochrome P450 family 17a1 genes in the ovarian tissue of GII compared to those of the control group.

CONCLUSION: Our data suggest that melatonin supplementation decreases gene expression of cyclic adenosine monophosphate, which changes ovarian steroidogenesis.

KEYWORDS: Melatonin; Steroidogenesis; Gene Expression; Ovary; Rat.

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INTRODUCTION

In rats and in humans, there is evidence of direct melatonin action on ovarian function, steroidogenesis modulation (1,2), primarily during progesterone production (3). In addition, high concentrations of the hormone are found in the preovulatory follicular fluid (4) and associated with the type I (MT1) and type II (MT2) melatonin receptors in rat and human ovarian follicles (5).

Melatonin levels may influence the physiological and neoplastic processes of the reproductive system. Women with neoplastic proliferative activity are known to have much lower than normal melatonin levels (6), suggesting an association between neoplastic growth and melatonin. This is the reason many authors have investigated the molecular mechanism of indoleamine action and tested melatonin in the adjuvant treatment of estrogen-dependent malignant tumors in the female genital system (6). However, high melatonin levels were found in endocrine disorders, such as polycystic ovary syndrome (PCOS) (7). These findings suggest that melatonin exerts an influence on the behavior of the genital system, especially the gonads. Voordouw et al. (7) found that melatonin in association with progestogen acted on the inhibition of ovarian function without affecting the sleep-wake rhythm. The authors suggested employing melatonin for human contraception.

Melatonin has been hypothesized to have the requisites for consideration as an antiestrogenic drug due to its interference with estrogen receptors (8). Additionally, melatonin acts on estrogen synthesis via the inhibition of enzymes (aromatases) that control its interconversion from androgen precursors (8). However, the action of melatonin depends on serum estrogen levels (9).

No potential conflict of interest was reported.

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Experimentally, melatonin acts on the malignant tumors of the reproductive system, especially those that are estrogen dependent. In fact, patients exhibiting tumoral activity have lower than normal melatonin levels. This fact seems to indicate that the production of indoleamine is connected with tumor development. However, the exact mechanisms involved in the action of melatonin on tumor cells are not known (10,11). In addition, women with PCOS have elevated serum melatonin levels (12). Again, the reason for or mechanism involved in this increase is still elusive. In granulosa cell cultures, melatonin receptor expression is dependent on steroid levels, particularly on androgen levels. Thus, there is clear interference of the sex steroids in the function of melatonin in the ovary (13).

Prata Lima et al. (14) showed that a reduction in melatonin levels in rats may trigger the development of micropolycystic ovaries. Soares Jr et al. (15) detected an increase in the number of interstitial cells and proliferation of the theca interna of the ovarian follicle. These changes are similar to those observed in the ovaries of women presenting anovulation with hyperandrogenism. Furthermore, pinealectomized animals exhibited a reduction in fertility with a decrease in oocytes during ovulation (16). Melatonin replacement, however, diminishes the number of cysts in rat ovaries, probably due to melatonin's antigonadotropic effects (17). Moreover, rats that underwent surgery for pineal gland removal developed histological signs suggestive of endometrial hyperplasia, which was also reversed by the use of melatonin (16). In addition, melatonin may decrease estrogen expression in endometrial tissue (18).

Melatonin may have an antiproliferative effect and it acts on the reproductive system (19); therefore, it is essential to search for ovarian proliferation signals and the enzymes that interfere with steroidogenesis. In fact, these goals led us to observe the effect of melatonin supplementation on ovarian gene expression in normal rats, including steroidogenic and ovarian proliferation enzymes.

MATERIAL AND METHODS

Animals

Thirty virgin adult (three-month-old) female EPM-1 Wistar rats (*Rattus norvegicus albinus*), weighing approximately 200 g each, were used in this study. They were housed in plastic cages with metal grids and kept on standard rat chow (Labina Purina, Brazil) and water ad libitum at a room temperature of 22℃. Lighting was artificial and provided by 40-watt daylight fluorescent lamps (Philips, Brazil) placed in a carefully dimensioned and well-ventilated wooden box. The lamps supplied approximately 400 lux in the area occupied by the rats in a light-10:dark-14 photoperiodic cycle (lights on from 8 a.m. to 6 p.m.) throughout the experiment. This study was approved by the local ethics committee (N° 0233/06). The procedures met the criteria for the care and management of experimental animals.

Daily samples of vaginal secretions were taken to assess ovarian function. Only rats with regular estrous cycles were included in the study.

The rats (n = 32) were randomized to two groups: GI, control, received vehicle and GII, experimental, received melatonin supplementation (10 μg/night per animal) for 60 consecutive days.

Vaginal cytological examination

Following a two-week period of adjustment to the new environment, all animals were subjected to daily collection of vaginal secretions for four consecutive weeks. The material was processed and stained by the Harris-Shorr method. Only the rats with regular estrous cycles were included. Samples were also collected throughout the study to identify the estrous cycle.

Melatonin supplementation

Melatonin (Sigma Chemical Co., St. Louis, USA) was dissolved in ethyl alcohol and then in drinking water (final concentration: 0.4 μg/mL); it was then poured into amber bottles and made available to the animals during the dark period (6 p.m. to 8 a.m.). The intake dose of melatonin was calculated to be approximately 10 μg/night per animal. Access to water was restricted during the light period (8 a.m. to 6 p.m.). For GI, the same amount of ethanol was added to the drinking water.

Organ excision

All animals in proestrous were anesthetized intraperitoneally with 15 mg/kg of xylazine (Rompun®) and 30 mg/kg of ketamine (Ketalar®). The ovaries were removed and maintained at -80℃ for subsequent molecular biology analysis followed by immunohistochemical analysis. The animals that were not in proestrous continued to receive the same treatment (vehicle or melatonin according to their groups) and were euthanized upon reaching proestrous.

Isolation, synthesis and labeling of complementary RNA

Total RNA was extracted from ovarian tissue with Trizol® reagent (Invitrogen) according to the manufacturer's instructions. Following total RNA extraction, the sample was purified with the Qiagen RNeasy Mini Kit (USA) according to the manufacturer's instructions and RNA integrity was checked in 1% agarose gels to evaluate the ribosomal band integrity and quantified with a spectrophotometer.

Total RNA (3 micrograms) from the tissue of interest was first converted into complementary DNA (cDNA). The final product of the cDNA synthesis reaction was purified with the GeneChip Sample Cleanup Module System (Affymetrix, Santa Clara, CA); the manufacturer's instructions were strictly followed.

The cDNA was transcribed *in vitro* to biotinylated complementary RNA (cRNA) by incorporating biotin-CTP and biotin-UTP using the Affymetrix IVT labeling kit (One-Cycle Target Labeling Kit; Santa Clara, CA). A sample of 15 μg of biotin-labeled RNA was generated and then fragmented to a 200 bp size by incubation in fragmentation buffer for 35 minutes at 94℃ prior to overnight hybridization. Fragmented RNA was assessed for its relative length in 1% agarose gels. A solution prepared with a hybridization reagent from the GeneChip Hybridization Wash and Stain Kit (Affymetrix) was added to the fragmented cRNA. The resultant solution was placed in the GeneChip Rat 230 2.0 Array chip, which in turn was placed in a hybridization oven at 45℃ and hybridized for 16 hours at 60 rpm. The RNA processing and microarray protocols were carried out in the Molecular Core-Microarray Facility in São Paulo, Brazil.

After hybridization, the chip was placed in the washing and coloring station of the fluidics station (GeneChip Fluidics Station 400; Affymetrix), in which the excess nonhybridized oligonucleotides were retrieved from the chip and cRNA was labeled with biotin. Once effectively connected to the chip probes, biotin was labeled with a solution containing fluorescence-conjugated streptavidin.

Afterward, the chip was analyzed using the GeneChip Scanner 3000 7G connected to the GeneChip Operating Software (Affymetrix). Sign quantification allows the expression of thousands of genes to be compared under various experimental conditions. All samples were analyzed in triplicate. Images were captured, the initial analysis of hybridizations was performed with MicroArray Suite 5.0 (Affymetrix) software and the generated files were saved in the cell format.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Additionally, we conducted qRT-PCR to confirm the data. The resultant cDNAs underwent conventional PCR using a pair of specific primers for the β-actin gene (S: 5′-CGAGGCCCAGAGCAAGAGAG-3′; AS: 5′-AGGAAGAGGATGCGGCAGTGG-3′; GenBank accession number NM_031144.2) to verify the effectiveness of synthesis. Following fragment analysis in agarose gels (Invitrogen), the cDNAs were subjected to qRT-PCR reactions. The oligonucleotides for amplification were the following:

ADCY9 (S: 5′-CAGTGCGGTGGTGGAAAAA-3′; AS: 5′-CAGCGACCTCTGCCAACCT-3′); *INHBB* (S: 5′-TCCTAGTGCCCTGCTGAGAT-3′; AS: 5′-ACCCACAGGGACAACTTCTG-3′); *CREM* (S: 5′-AGTCCCCAGCAACTAGCAGA-3′; AS: 5′-CACAGTCAACCAGGTCCAA-3′); and *CYP17A1* (S: 5′-ACTGAGGGTATCGTGGATGC-3′; AS: 5′-TCGAACTTCTCCCTGCACTT-3′).

All of the primers were designed using the Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) program and synthesized by integrated DNA technology (DNA Technologies, Coralville, IA, USA). Reactions were carried out in duplicate with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a total volume of 25 μl with 450 nM of primers and SYBR Green PCR Master Mix (Applied Biosystems). Fluorescence intensity was measured at the end of the extension phase of each cycle. Relative expression (R) was calculated using the equation $R = 2^{-[\Delta CT\ sample\ -\ \Delta CT\ control]}$. To determine a normalized arbitrary value for each gene, each data point was normalized to the control gene (β-actin) and to its respective controls (20).

Immunohistochemical analysis

All of the ovary samples investigated in the study were tested using the primary goat polyclonal antibodies anti-Per2 against the clock gene Period 2 (1:200; Santa Cruz Biotechnology, USA; H-90; sc-25363); anti-Cyp17a1 (1:200; Santa Cruz Biotechnology, USA; C-17; sc-46081); and anti-Cyp19a1 (1:200; Santa Cruz Biotechnology, USA; H-18; sc-14244) and then incubated with the second antibody and with secondary antibodies from a Dako kit (Dako LSAB + Sys, Peroxidase Universal K0690-1). After 30 minutes of incubation followed by washing, the sections were again incubated with a freshly prepared solution of streptavidin-biotin immunoperoxidase (Dako LSAB kit) according to the manufacturer's protocol. After washing, the bound enzyme was visualized following one additional incubation with the enzyme in the presence of 1% 3, 3′-diaminobenzidine tetrahydrochloride (Dako K3468-1). Bovine serum albumin replaced the primary antibody and was used as a negative control. Other negative controls were applied using non-specific goat antibodies with the same concentration as that of the primary antibody for each immunohistochemical reaction (Per2, Cyp17a1 and Cyp19a1) and for staining the sections with hematoxylin. Afterwards, the image was analyzed using AxioVision (Carl Zeiss) and expression was quantified according to the color intensity in the field. The intensity of the immunoreactions was classified according to proposed criteria that included negative-zero, weak-1, moderate-2, strong/intense-3, or intense-4 (21). Three experienced observers who were blinded to the purpose of the slides carried out all of the assessments. After completion of the study, the same observers reexamined the slides to ensure reproducibility of the semiquantitative assessment.

Statistical methods

To analyze the microarray technique statistically, the data were normalized using the Robust Multi-Array Average (RMA) method. The differentially expressed genes (DEGs) were selected using two distinct methods: Limma and RankProd. Both had p-value<0.05 adjusted for the false discovery rate (FDR) to obtain the significance level. Comparative analyses were performed to identify the differentially expressed genes under distinct experimental conditions; the genes were grouped according to their expression levels and categorized according to their biological functions.

Analysis of variance (ANOVA), along with the post-hoc Tukey test, was used to compare the 6-sulfatoxymelatonin results and those of the immunohistochemical analyses. Differences were considered significant at the $p<0.05$ level. Data are expressed as the mean ± SEM. All statistical tests were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). The power calculation was based on the previous data from Lombardi et al. (22).

RESULTS

Estrous cycles

Vaginal cytology showed estrous cycles lasting from 4 to 5 days in the control group animals (GI). In the experimental group (GII, melatonin supplementation), most animals had 4- to 6-day cycles.

Microarray analysis

Of the 31,100 genes that underwent transcriptional analysis, 835 had a known biological function. A total of 416 genes were transcribed and differentially expressed in the two groups. Of these 416 genes, 279 were expressed less in GII (melatonin supplementation) than in GI (treatment with vehicle). The genes with at least a two-fold increase (upregulation) or a two-fold decrease (downregulation) relative to GII were considered significant in terms of known ovarian function. The genes are listed in Table 1.

Table 1 displays a summary of the genes that were selected. Using the microarray technique and confirming the results using the qRT-PCR technique, differences were found between GI and GII with respect to the following genes: type 9 adenylate cyclase (*ADCY9*), inhibin beta B

Table 1 - List of upregulated and downregulated genes in GI (control, treatment with vehicle) relative to GII (experimental, melatonin supplementation).

Upregulated genes	Symbols	R
Epoxide hydrolase 2, cytosolic	*EPHX2*	↑ 2.950
FC Fragment of IgE, Low Affinity II, Receptor For	*FCER2*	↑ 2.860
Aldehyde Dehydrogenase Family 1, Subfamily A7	*ALDH1A7*	↑ 2.820
Cbp/p300-interacting transactivator 1	*CITED1*	↑ 2.820
Adenylate cyclase type 9	***ADCY9***	↑ **2.560**
Inhibin, beta B	***INHBB***	↑ **2.510**
LIM domain 7	*LMO7*	↑ 2.300
Paraspeckle protein 1	*PSPC1*	↑ 2.280
Plasma membrane calcium-transporting ATPase 1	*ATP2B1*	↑ 2.190
Potassium voltage-gated channel subfamily D member 3	*KCND3*	↑ 2.160
Downregulated genes	**Symbols**	**R**
Epiregulin	*EREG*	↓ 18.918
Nuclear receptor subfamily 4, group A, member 3	*NR4A3*	↓ 10.157
Amphiregulin	*AREG*	↓ 9.181
Sulfiredoxin-1	*SRXN1*	↓ 7.046
Seminal vesicle secretory protein 5	*SVS5*	↓ 6.841
Tumor necrosis factor, alpha-induced protein 6	*TNFAIP6*	↓ 6.472
cAMP responsive element modulator	***CREM***	↓ **6,446**
Cytokine-inducible SH2-containing protein	*CISH-*	↓ 6,249
Cytochrome P450 17A1	***CYP17A1***	↓ **6,135**
Cysteine-serine-rich nuclear protein 3	*CSRNP3*	↓ 6.073
Glutamyl-prolyl-tRNA synthetase	*EPRS*	↓ 5.826

(*INHBB*), cyclic AMP responsive element modulator (*CREM*) and cytochrome P450 family 17a1 *(CYP17A1*). The former two genes were upregulated and the latter two were downregulated relative to control.

Genes expressed by the qRT-PCR analysis

The qRT-PCR results were obtained using the same samples included in the microarray experiment. We selected the *ADCY9, INHBB*, *CREM* and *CYP17A1 g*enes.

There were no significant difference between the two groups with respect to *INHBB* ($p = 0.20$). The *ADCY9* gene was more highly expressed in the group with melatonin supplementation (GII) than in the control group (GI) ($p<0.01$). The opposite was true for the *CREM* and *CYP17A1* genes, which were less expressed in GII than in GI ($p<0.01$). The data are shown in Figures 1A, B, C and D.

Immunohistochemical analysis

Per2 reactivity was more highly expressed not only in the granulosa cells of the ovarian follicle and the blood vessels of the ovarian stroma but also in theca interna cells and interstitial cells in the group with melatonin supplementation (GII) ($p<0.05$). Cyp17a1 also showed greater expression in the theca interna cells and the granulosa cells in the control group (GI) than in the experimental group (GII) ($p<0.05$). There was no difference between the groups with respect to Cyp19a1 (Table 2 and Figure 2).

DISCUSSION

In the United States, melatonin sales are not under government control and the hormone is sold as a dietary supplement to be taken in daily doses of 0.5 to 50 mg. There are no reports in the literature of severe side effects associated with melatonin intake (23). A few authors report cephalgia, nausea and an overall feeling of being unwell following higher doses (24). However, little is known about melatonin's effects on the reproductive system, particularly in women with normal melatonin levels. Consequently, our study aimed to evaluate the potential influence of melatonin supplementation on animals that were not manipulated surgically to assess the interference of the hormone with the female reproductive system, particularly the ovaries.

Our results showed that melatonin may increase cyclin gene expression, especially type 9 and decrease the production of *CREM,* which acts on the cyclic AMP pathway. Concomitantly, we found that melatonin acts on the enzymes involved in steroidogenesis via a decrease in *CREM* (25). It should be noted that this gene represses *CYP17A1* expression (25). Therefore, melatonin may control ovarian function through its influence on the enzymes of steroidogenesis.

The *CYP17A1* family may play a role in cortisol production in humans and in that of corticosterone in rodents, thus establishing a difference between these species, but they are also important in androgen production. In addition, the expression of these genes exhibits circadian variation both in rodents and in humans (26). At this point, melatonin may influence expression via *CREM* (27,28). Our data suggest that melatonin supplementation alters expression of this hormone by inducing changes in ovarian steroidogenesis (27).

Ovarian steroidogenesis generally depends on the interaction between two specialized cells (internal thecae and granulosa cells) that are important for follicular development and ovulation (19,29). In fact, androgen is synthesized from cholesterol in thecal cells in response to luteinizing hormone (LH) and then subsequently converted to estrogen in granulosa cells through the action of follicle-stimulating hormone (FSH). The LH receptor and the Cyp17a1 enzyme, which converts pregnenolone and progesterone into dehydroepiandrosterone (DHEA) and androstenedione, are expressed primarily in theca interna cells and the FSH receptor and aromatase (*CYP19A1*) are expressed in granulosa cells, where androgens are converted into estrogens (27). Curiously, the LH receptor is also expressed in the external portion of the granulosa in response

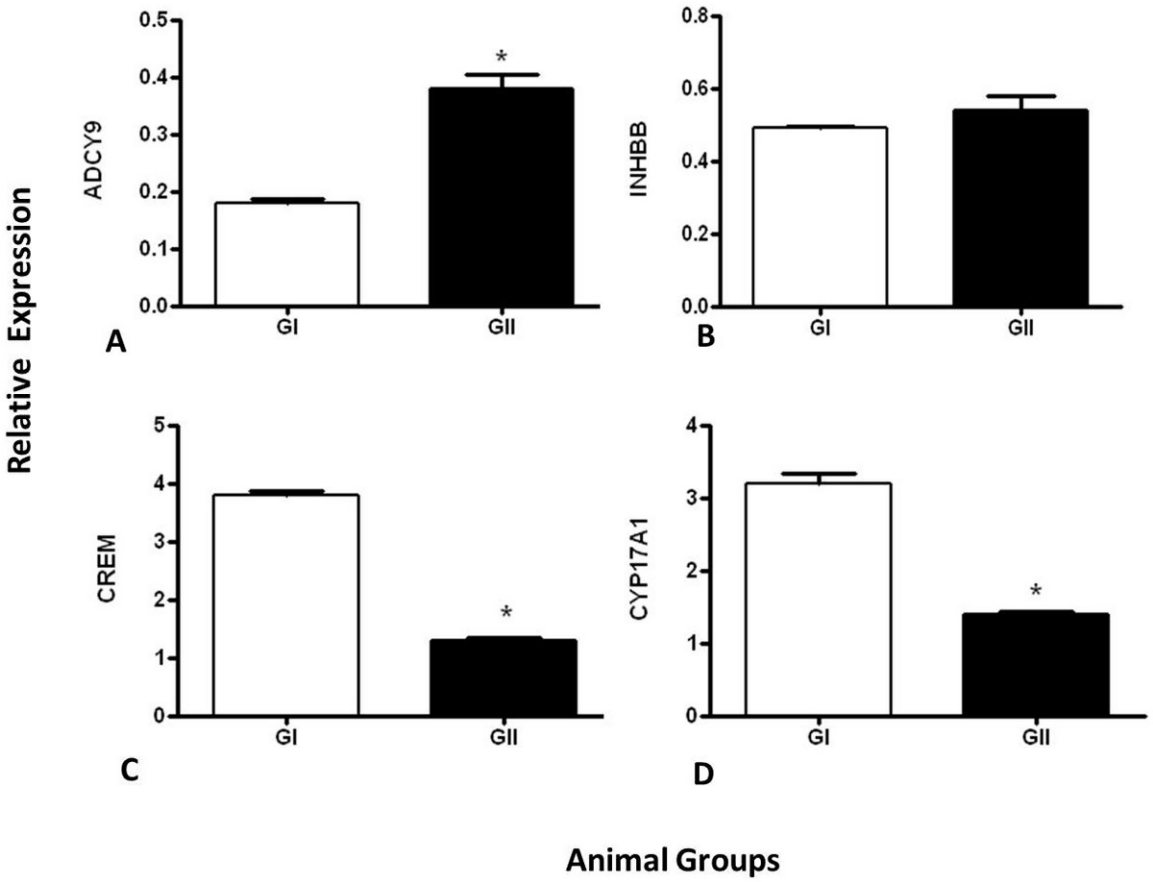

Figure 1 - Values of ADCY9, INHBB, CREM and CYP17A1 gene expression relative to β-actin. A – Mean and standard deviation of 2-ΔΔCt from the qRT-PCR of the ADCY9 gene between GI and GII, using the same samples included in the microarray experiments (ADCY9, *$p<0.01$ compared to GI). B – Mean and standard deviation of 2-ΔΔCt from the qRT-PCR of the INHBB gene between GI and GII using the same samples included in the microarray experiments. No difference was detected (INHBB, $p=0.20$). C – Mean and standard deviation of 2-ΔΔCt from the qRT-PCR of the CREM gene between GI and GII, using the same samples included in the microarray experiments (CREM, *$p<0.01$ compared to GI). D – Mean and standard deviation of 2-ΔΔCt from the qRT-PCR of the CYP17A1 gene between GI and GII, using the same samples included in the microarray experiments (CYP17A1, *$p<0.01$ compared to GI). ADCY9: adenylate cyclase 9; INHBB: inhibin beta B; CYP17A1: 17-alpha-monooxygenase; CREM: cyclic adenosine monophosphate responsive element modulator.

to FSH before LH appears. It should be emphasized that melatonin may both influence LH release through the hypophysis and interfere with the effect of LH on the ovary (19,29).

The FSH and LH receptors are coupled with the stimulatory G protein acting on cyclic AMP and upon activation of its ligand, they stimulate adenyl cyclase, which generates an intracellular increase in cyclic AMP and subsequent activation of protein kinase A (PKA). The increase in cyclic AMP promotes steroidogenesis and not only raises StAR expression but also increases its activity through serine phosphorylation. In addition, cyclic AMP regulates other important proteins in steroidogenesis, such as Cyp19a1 (aromatase) and Cyp17a1 in the granulosa and theca interna cells, respectively (30).

Activation of the subunits coupled with the G protein activate Src, which triggers the Ras/Raf/MEK/MAPK/ERK pathway. LH and FSH stimulation may increase the activity of extranuclear signal-regulated kinase (ERK), which subsequently raises StAR expression in some tissues, such as the adrenal glands. However, Src and MAPK signaling in theca interna cell cultures leads to the inhibition of steroid production and reduces the activity of Cyp17a1 and other enzymes (31). Melatonin supplementation appears to exert an effect that is opposed to that of the gonadotropic receptors, with a decrease in *CREM* and an increase in *CYP17A1*, as our data show.

It should be emphasized that melatonin may become involved in ovarian function indirectly through changes in the gonadotropins (29) or directly in the ovary via activation of its type I or type II receptor (31). The Chinese hamster ovary has been used to study physiological activation of the melatonin receptor (MT1), which potentiates several points of the signal transduction cascade mediated by cyclic AMP (cyclic adenosine monophosphate), by protein G activation, by protein kinase A (PKA) activation and by phosphorylation of the transcription factor of the protein that binds to the element responsive to cyclic AMP (*CREB*). The phosphorylation that binds *CREB* through kinase A is a critical step for promoting DNA transcription and it may reduce *CREM* concomitantly. It is worth noting that this mechanism is the main mechanism conveying information from the melatonin photoperiod to the body and its action is dependent on high levels of nocturnal melatonin (32).

Table 2 - Mean and standard deviation from the mean of Per2 (period circadian clock 2), Cyp17a1 (17-alpha-monooxygenase), and Cyp19a1 (aromatase) expression measurements by immunohistochemistry in GI (vehicle) and GII (melatonin supplementation).

	GI (Vehicle)	GII (Melatonin)
Per2		
Theca interna cells	1.50±0.02	2.30±1.01*
Granulosa cells	1.50±0.03	3.10±0.04*
Interstitial cells	1.50±0.01	2.30±0.45*
Cyp17a1		
Theca interna cells	1.80±0.01	2.90±0.01*
Granulosa cells	0.21±0.11	0.17±0.23
Interstitial cells	0.28±0.82	0.11±0.13
Cyp19a1		
Theca interna cells	0.05±0.01	0.06±0.01
Granulosa cells	1.81±0.19	1.87±0.21
Interstitial cells	0.16±0.42	0.15±0.73

*$p<0.05$ compared to GI.

In ovarian function, the cyclic AMP signaling pathway is involved in many aspects of the differentiation, maturation and function of granulosa cells in ovarian follicles (33). In the ovary, theca interna cells, when completely differentiated, exhibit ultrastructural features of steroid-producing cells. The theca interna cells synthesize androstenedione, which is transported to the granulosa cells and acts under the influence of FSH to synthesize the aromatase enzyme, which transforms androstenedione into estrogen (34). It should be noted that the rise in cyclic AMP is closely related to an increase in steroidogenesis in the granulosa cells due to the increased activity of aromatase cytochrome 450, which converts androgens into estrogens (32). Melatonin may have a modulatory effect on steroidogenesis because when the melatonin receptor is activated, there is a drop in cyclic AMP in the granulosa cells *in vitro* (31). Moreover, melatonin may trigger other cell mechanisms in the ovarian tissue as it interferes in steroidogenesis. Our data based on normal animals show that melatonin may also diminish *CREM* production, which may be a repressor of gene activation when the activator is cyclic AMP and would explain characterization of the Cyp17a1 enzymes (30).

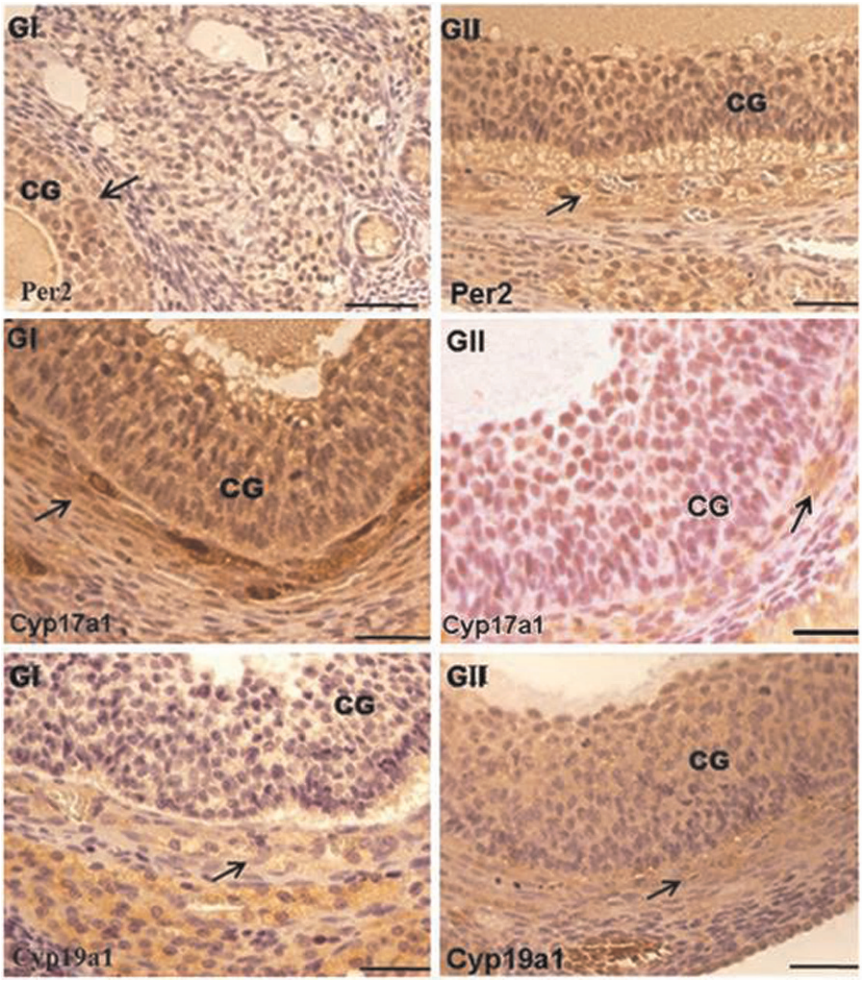

Figure 2 - Photomicrographs of ovarian sections after immunostaining. Expression of Per2 (period circadian clock 2), Cyp17a1 (17-alpha-monooxygenase) and Cyp19a1 (aromatase). GI: control group; GII: experimental group (received melatonin supplementation). Note the greater expression of Per2 in the theca interna cells (arrows) compared to the granulosa cells (CG) and interstitial cells in GII relative to GI. Please also note the intense reactivity for Cyp17a1 in the theca interna (arrows) and granulosa cells (CG) in the GI. Bar = 20 μm, 400 X.

There is some evidence that inhibin expression depends on the pathways of both protein kinase A (PKA) (35) and/or protein kinase C (PKC) (36), as well as on FSH and LH action on the PKA pathway (37); our data also suggest melatonin participation. Physiologically, during follicular growth, high LH concentrations increase the activity of the PKA pathway and activate PKC and they also diminish aromatase activity in rat granulosa cell cultures (38). Melatonin may not exert a strong influence on inhibin B as indicated by real-time PCR and according to our data. However, further studies are necessary to ensure that melatonin does not influence on this pathway.

In short, our study shows that there are molecular alterations in the ovary brought about by changes in steroidogenesis-associated gene expression after melatonin supplementation with an intact pineal. The important point of this study is that melatonin had an impact on ovarian function through Cyp17a1. In clinical practice, melatonin sold as a supplement may influence the reproductive system. Therefore, melatonin should be prescribed with attention to specific melatonin-deficiency symptoms and not to all patients due to its influence on ovarian function. However, our study did not address the repercussions for fertility and pregnancy of these animals, which was a limitation of our study. Consequently, further studies are necessary.

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■ AUTHOR CONTRIBUTIONS

Negro LG designed the study, supervised the experiments, performed the statistical analysis and prepared the manuscript. Maganhin CC and Baracat MC performed the experiments and were involved in the manuscript preparation. Simões RS contributed to the data evaluation and manuscript writing. Sasso GR contributed to the immunohistochemical analysis and manuscript preparation. Fuchs LF performed the experiments and was involved in the manuscript preparation. Simões MJ revised the manuscript Baracat EC provided assistance during the study conception, analyzed the data and critically revised the manuscript for important intellectual content. Soares-Jr JM analyzed the data, revised the manuscript drafting and approved the final version to be published.

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