Research Article

Melatonin modulates the *in vitro* angiogenesis of granulosa cells collected from women with marital infertility for IVF

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ABSTRACT

Melatonin concentration is several folds higher in the follicular fluid than that in blood suggesting an important role of this molecule on follicular physiology. However, the actions of melatonin on angiogenesis in granulosa cells are currently unknown. In this study, we have specifically investigated the potential effects of melatonin on the angiogenesis in granulosa cells from female individuals with marital infertility. Sixty patients who were submitted to the *in vitro* fertilization were included. The granulosa-luteal cells of these females were collected for cell culture. The cells were divided into four groups: a) vehicle (control); b) 0.1 µM melatonin; c) 1 µM melatonin; d) 10 µM melatonin treated groups, respectively. After a period of 10 days of culture, expression of genes involved in the angiogenesis signaling pathway were analyzed by Real-Time PCR and Western Blot assays. The results showed that the expressions of *FGF1* (fibroblast growth factor 1), *IL1B* (interleukin 1-beta), *VEGFR-2* (type 2 vascular-endothelial growth factor receptor), and *TGFB1* (tumor growth factor 1- beta) were significantly upregulated in melatonin treated groups compared to the control. In contrast, the expressions of *HIF-1A* (hypoxia-inducing factor 1-alpha), *FGF2* (fibroblastic growth factor 2), *IGF-1* (insulin-like growth factor 1), and *VEGFA* (vascular endothelial growth factor alpha) were significantly downregulated by melatonin compared to the control. The results suggest that melatonin modulates angiogenesis of granulosa cells from women with marital infertility. The underlining mechanism may relate to melatonin maintaining the homeostasis of VEGF, especially at a low dose of melatonin.

Key words: Melatonin, ovary, angiogenesis, infertility, women, granulosa cells

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1. INTRODUCTION

Melatonin is a molecule with multiple functions including actions on the female reproductive system, via influencing ovarian physiology through melatonin receptors 1 (MT1) and/or 2 (MT2). These receptors are distributed in different ovarian cells including follicular cells, mainly granulosa cells (GC) (1-3). Some actions of melatonin on the ovary and uterus do not require receptors, e.g., its free radical scavenging activity (4). Of great interest is that melatonin's concentration in the mature preovulatory follicle is several folds higher than that in the blood (5), suggesting its local synthesis and its importance on ovulation (6, 7). It is well documented that MT1 and MT2 signaling is mainly mediated by cAMP, PKA, and PKC pathways (1-3). Melatonin can affect gene expressions via theMT1 and MT2, both of which are expressed in GCs and oocytes. Therefore, melatonin can potentially alter the cellular functions of GCs by activating the signaling pathway of melatonin receptors (3, 8). Melatonin protects the granulosa cells and avoid the disruption of oocyte maturation induced by Aflatoxin B1 in porcine cells (9). Also, this indolamine ameliorate the damage of oocyte maturation in fish (10) and rescues the development potential of oocytes against ischemic damage during ovary preservation in ovine (11). Furthermore, addition of melatonin into *in-vitro* maturation medium improves cytoplasmic maturation of human immature oocytes and subsequent clinical outcomes in patients under artificial reproductive treatment (12).

It is known that mature follicles are surrounded by a layer of granulosa cells, as well as internal theca cells, which are responsible for ovarian steroidogenesis including estrogen and androgen production (6-8). The matured or newly formed vessels are essential for follicular nutrition and growth, oocyte quality, adequate ovulation, and transportation of hormones produced by the granulosa cells (4-8). Therefore, angiogenesis is critical for granulosa cell physiology (13).

It is reported that melatonin can regulate the angiogenesis of many organs including kidney, liver, and heart (14-15). However, it is not clear whether melatonin also impacts the angiogenesis of ovarian granulosa cells. Granulosa cells play a major role in overall fertility and oocyte maturation. The dysfunction of granulosa cells will be directly or indirectly associated with female infertility. Therefore, it is important to identify the potential actions of melatonin on angiogenesis in the granulosa cells since much higher melatonin level is found in the follicular fluid than that in the serum. For this reason, we have evaluated the effect of melatonin on the expressions of angiogenesis related genes in granulosa-luteal cells of women who undergo IVF due to the marital infertile.

2. MATERIALS AND METHODS

2.1. Experimental design.

This study was carried out from 2012 to 2016. The human granulosa-luteal cells were obtained from follicular fluid aspirates of the collected oocytes and destined for disposal. The granulosa cells were collected from patients submitted to an "*in vitro*" fertilization protocol at the Human Reproduction Sector of the São Paulo Hospital of the Federal University of São Paulo (UNIFESP) and at the Human Reproduction Service of the Hospital das Clínicas of the Faculty of Medicine from the University of São Paulo (HC – FMUSP2.2 Patients). The study included 68 women, aged 20 to 35 years old, they were considered normal with infertile husbands (oligospermia). The

granulosa cells were divided into Control (treated only with vehicle and different melatonin concentration treated groups (0.1m 1 and 10 µM), respectively.

The inclusion criteria of patient selection (donors of granulosa cells) were follows: age between 20 and 35 years; eumenorrheic cycles; without the use of medication or hormone treatment which may interfere with carbohydrate metabolism at last three months or the participant's clinical evaluation including follicle-stimulating hormone (FSH) levels between 3 and 12.5 mIU / ml dosed between the second and fifth days after menstruation; the presence of 5 to 20 follicles on ultrasound on the day of the hCG administration and signing the informed consent form. The exclusion criteria in this study were: Cushing's syndrome; hyperprolactinemia; enzyme deficiency of the adrenal gland (21-hydroxylase); thyroid disorders; anorexia nervosa, bulimia, and depression being treated; adrenal or ovarian tumor; deep-endometriosis; polycystic ovary syndrome; anovulation with a polycystic alteration.

Data such as current age, age at menarche, body mass index, number of pregnancies, race, past use of hormonal medications, breastfeeding period, and endocrine diseases were noted to correlate with the possible findings of this study.

2.2. *In vitro* fertilization program.

Only couples were included where the woman was 35 years old or younger and who had the follicle stimulating hormone (FSH) levels between 3 and 9 µg/ml on day 3 of the menstrual cycle before the treatment cycle. Controlled ovarian stimulation was performed with the use of exogenous recombinant gonadotropins of 225 IU/day (Gonal-F, Merck-Serono, Darmstadt, Germany), from day 2 of the cycle. When the main follicle reached 14 mm in diameter, the endogenous LH release was suppressed with a GnRH analog (Cetrorelix-Cetrotide, Merk-Serono) until the day of hCG administration. When the ovarian follicle reached 17 mm in diameter, a total dose of 250 µg of hCG was administered. The capture of transvaginal oocytes guided by ultrasound was performed at 36 h after the administration of hCG. Shortly after the aspiration of the follicles and the capture of oocytes, aspirates of follicular fluid, such as granulosa cells, without the oocyte, which would normally be discarded, were immediately sent to the preparatory procedures for cell culture.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Escola Paulista de Medicina da Universidade Federal de São Paulo (UNIFESP) (# 2031/09) and Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC – CEP-1.192.888), for studies involving human granulosa cells. The informed consent was obtained from all subjects involved in the study, and written informed consent was obtained from the patients for the publication of this paper.

2.2.1. Retrieval oocyte number and embryo quality.

The number of oocytes retrieved was calculated during the granulosa cell collection. Also, the quality of oocytes and embryos during 1–2 h after warming was examined under the inverted microscope at 400× magnification and the degrees of potential damage were calculated. The levels used to classify embryo damage were set up as 0, 1–25, 26–50, and >50%, respectively. Only the embryos with less than <26% damage were selected. The selected embryos were cultured for an additional 12–18 h. Embryos with no detectable fragmentation on the day of embryo transfer were referred to as good-quality embryos.

2.3 Obtaining granulosa-luteal cells.

After identification and removal of the cumulus-oocyte complexes, follicular aspirates were transferred to 100 mm petri dishes previously warmed to 37ºC and the antral cells were removed under a stereomicroscope. These cells were separated from the oocytes by chemical and mechanical denudation. Chemical denudation was performed by adding hyaluronidase (0.08% in HTF with HEPES [Irvine Scientific® Santa Ana, California, USA]) and mechanical procedure was performed with the aid of two pipettes with 170 µm and 130 µm gauges, respectively (CooK®Urological Inc. Indiana, USA). After separation, the cells were deposited in a transport tube containing PBS, (HBSS (Sigma-Aldrich, São Paulo, SP, Brazil) with a combination of penicillin/streptomycin (Life Technologies, USA).

2.4. Cultivation of granulosa-luteal cells.

The cells were grown in four-well culture plates in M199 (Life Technologies, USA) supplemented with 10% fetal bovine serum (Life Technologies, USA), 100 units/ml penicillin, and 100 µg / ml streptomycin (Life Technologies, USA). The treatments were last for 5 days with four different groups, namely: 1) vehicle; 2) melatonin at 0.1 µM; 3) melatonin at 1 µM; 4) melatonin at 10 µM, respectively and each treatment was in quadruplicate. Cell viability was determined by trypan blue exclusion dye, with a minimum value estimated at 90%. Approximately 200,000 cells were seeded per well, containing 300 µl of previously balanced culture medium. The cells were incubated with 5% CO_2 at 37ºC and maintained for 72 h, with medium replacement every 48 hours.

The cell proliferation assay was carried out by converting the PrestoBlue® Cell Viabillity Reagent for Microplates (Thermo Fisher Scientific). Granulosa cells (2.6 × 10) were cultured in 96-well plates (Corning®) with supplemented medium. After 24 hours of cultivation the cells were treated with different concentrations of melatonin or vehicle. Every 48 hours, melatonin was replaced along with the medium supplemented. The proliferation evaluation was performed during 96 hours of treatment. The 24h and 96h treated cells (avoiding light illumination) were kept in a freezer - 20°C until the time of reading. On the day of the reading the plates were placed at room temperature for 1 hour for the complete thawing. The readings were performed in a GloMax® device -Multi Detection System (Promega). Thereafter, the cells were trypsinized for extraction of total RNA and analysis of the expression profile of the genes involved in the angiogenesis pathway, by quantitative Real-Time PCR (qRT-PCR). The gene products with differential expressions were evaluated by the Western Blot. The processes were illustrated in Figure 1.

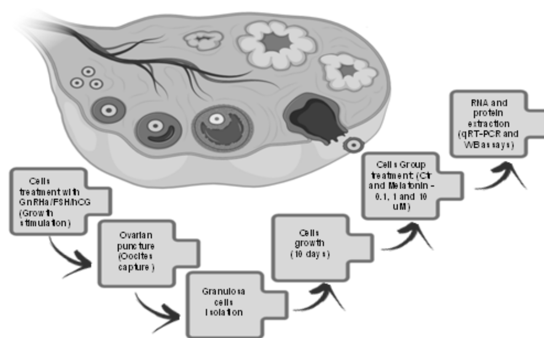

Fig. 1 Schematic representation of the ovulation process, cell collection, and sample processing steps (Designed in the app biorender.com).

2.5. Total RNA extraction and real-time PCR analysis.

The granulosa cells of each individual were collected and the total RNA was extracted by using the Trizol protocol (Life Technologies, USA). Briefly, the cells were lysed in Trizol with the aid of a tissue homogenizer (Polytron, USA) and the lysates were kept at room temperature for 5 minutes to ensure their complete liquefaction, then, 0.2 ml of chloroform (Merck, Rio de Janeiro, Brazil) was added for each 1 ml initial Trizol, for separation of the phases organic and inorganic cell lysate. After centrifugation, the aqueous phase was collected for precipitation of the genetic material, then, the dry RNA precipitates were resuspended in 20µL of RNase-free water. The RNAs were quantified in a spectrophotometer (NanoDrop ND 100, Thermo Fisher Scientific Inc. Co.) and stored at -80ºC until use.

The cDNAs (complementary DNAs) were synthesized from 2µg of total RNA, using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), following the manufacturer's instructions. The efficiency of the reverse transcription reaction and possible contamination with genomic DNA were evaluated by conventional PCR, using specific primers to amplify the β-actin genes (forward - 5´ GGCACCCAGCACAATGAAG 3´ and Reverse - 5´CCGATCCACACGGAGTACTTG 3´). The samples were stored at -20°C and used for qRT-PCR reactions.

The analysis of the expression of genes involved, directly or indirectly, with the angiogenesis pathway was performed using the TaqMan® PCR Master Mix fluorescent probe detection system (Life Technologies), following the manufacturer's instructions, and the reactions were run on the 7500 platforms Real-Time PCR System (Applied Biosystems, CA, USA), containing 96 genes involved. The TaqMan® Array 96 Well-Plates Plate Code 4391524 (Applied Biosystems CA USA) containing pro- and anti-angiogenic genes were selected. The best endogenous control (B2M - beta 2 microglobulin), among those included in the plate, was chosen with the aid of the NormFinder Software program. All reactions were performed in triplicate following the manufacturer's instructions. Quantification of transcripts was performed using the DDCT method, using Expression Suite Software v1.03 (Life Technologies). The results of gene expression are presented as fold change, considering the values obtained for the melatonin treated groups compared to the control group.

2.6. Western blot analysis.

For the analysis of total protein levels related to the angiogenesis pathway, the granulosa cell precipitates were homogenized in microtubes containing a complete cocktail of protease inhibitors, free of EDTA (Roche Applied Science, Mannheim, Germany), and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany) diluted in lysis buffer (50mM Tris-HCl, 150mM NaCl and 1% Triton-x, pH 7.4). The microtubes containing the homogenates were kept on ice for 15 minutes, under constant agitation, centrifuged at 14,000 rpm at 4ºC for 10 minutes and the supernatants were stored at -80ºC for the assay.

The samples (40 mg of protein) and the molecular weight markers (Bio-Rad Life Science, USA) were separated on SDS-PAGE gel (sodium dodecyl sulfate - 15% polyacrylamide gel), transferred to ECL nitrocellulose membrane Hybond for Western blot reaction. For the detection of the different proteins, primary polyclonal antibodies were used as anti-VEGF-A antibodies (dilution, 1: 100; ab51745; Abcam, Cambridge, UK) diluted in 0.1% TBS-Tween, incubated overnight at 4 °C. The biotin-peroxidase detection system was used, revealed by chemiluminescent amplification

(Thermo Fisher Scientific). Protein expression analysis was performed by densitometry (arbitrary units) using the ImageJ 1.440 software (HIH, Bethesda, MD) which determined the relative expression of protein/β-tubulin.

2.7. Statistical analyses.

To analyze the results from microarray assay, the data were normalized by the robust multiarray average (RMA) method. The differentially expressed genes (DEGs) were selected using two distinct methods: Limma and RankProd. Both had P values (< 0.05) adjusted by the false discovery rate (FDR) to obtain the statistical 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. The variance (ANOVA) or Kruskal-wallis analysis followed by the post-hoc Tukey or Dunn test were used for comparing the results of different concentrations of melatonin and vehicle treatments P<0.05 was considered statistically significant.

3. RESULTS

3.1. The quality of the oocyte and embryo.

Table 1 summarized the results of oocyte and embryo quality as well as the granulosa cell proliferation under different treatments. There are not significant differences observed among groups.

Table 1. Summarization of the quality of oocyte and embryo as well as the granulosa cell proliferation under different treatments among groups.

	Control			0.1 µM			1 µM			10 µM		
Variables	Mean ± SD		Median (Q1 - Q3)	Mean ± SD		Median (Q1 - Q3)	Mean ± SD		Median (Q1 - Q3)	Mean ± SD		Median (Q1 - Q3)
RON*	7.49 ± 4.22	**N=81**	6 (5 - 11)	8.34 ± 4.44	**N=79**	7 (5 - 13)	7.89 ± 4.23	**N=76**	7 (5 - 12)	7.45 ± 3.76	**N=80**	6.5 (5 - 9)
EN**	4.11 ± 3.08	**N=81**	3 (2 - 6)	5.11 ± 3.00	**N=79**	4 (3 - 7)	4.89 ± 3.61	**N=76**	4 (2 - 7)	4.13 ± 2.76	**N=80**	4 (2 - 6)
cell/mL* - 24h**	4507.90 ± 734.31	**N=26**	4317.50 (4101 - 5100)	4828.27 ± 1112.45	**N=21**	5065 (4521 - 5462)	4288.54 ± 1315.80	**N=19**	4386 (3541 - 5482)	4611.63 ± 1042.41	**N=21**	4563 (3698 - 5684)
cell/mL* - 96h**	5419.84 ± 778.00	**N=26**	5243 (4988 - 5899)	5020.27 ± 774.83	**N=22**	5214 (4521 - 5421)	5496.86 ± 738.01	**N=22**	5622.5 (4857 - 5874)	5507.0 ± 722.87	**N=22**	5645 (4889 - 5988)

RON= oocyte number per patient; EN**= high quality embryo number per patient; Kruskal-Wallis test was used for comparison among groups; RON, EN: p=0.645 and 0.111, respectively; ***: Anova test was applied on cell proliferation during 24h and 96h: p=0.439 and p=0.069, respectively.*

3.2. Effects of melatonin on the expression of genes related to the angiogenesis pathway.

In this study, 96 genes were evaluated and the expressions of 49 among them had significant differences between the melatonin treated and the control group or among the different melatonin concentration groups. The expressions of 25 genes had no significant differences among groups. Compared to control group, nine genes were significantly upregulated with melatonin treatment and these included *ANPEP* (alanyl-aminopeptidase coder); *FGF1* (fibroblast growth factor 1); *FGFR3* (fibroblast growth factor receptor); *IL1B* and *IL8* (interleukins 1- beta and 8); *VEGFR2-KDR* (Vascular endothelial growth factor - kinase insert domain receptor 2); *PLXDC1* (Plexin domain-containing protein 1) and *TGFB1-2* (transforming growth factor beta 1 and 2) (Table 2). Most of these genes can act in the morphogenesis of endothelial cells and the regulation of folliculogenesis.

Table 2. The summarization of upregulated genes related to the angiogenic pathway in granulosa cells treated with melatonin compared to the control.

Gene	Groups		
	Mel 0.1 µM	Mel 1.0 µM	Mel 10 µM
ANPEP	0.04 ± 0.01	0.03 ± 0.04	0.65 ± 0.01[a]
FGF1	0.53 ± 1.83	-0.09 ± 1.16	2.40 ± 1.81[a]
FGFR3	4.73 ± 0.15[b]	3.12 ± 0.06	4.60 ± 0.08[b]
IL1B	1.89 ± 1.62	0.75 ± 1.06	3.91 ± 0.83[a]
IL8	-0.90 ± 1.56	-0.35 ± 1.25	0.77 ± 1.09[a]
KDR	2.58 ± 0.42[c]	-0.07 ± 1.50	0.60 ± 1.69
PLXDC1	-0.50 ± 0.09[b]	-0.18 ± 0.15	0.23 ± 0.09[a]
TGFB1	-1.41 ± 1.89	0.24 ± 1.20	2.47 ± 1.90[a]
TGFB2	0.20 ± 1.20	-0.03 ±1.17	1.79 ± 2.02

The analysis of variance (ANOVA), along with the post-hoc Tukey test, was used. a- p <0.05 compared to the other melatonin treatment groups (0.1µM and 1.0µM); b- p <0.05 compared to the intermediate melatonin group (1.0 µM); c- p <0.05 compared to the other groups (1.0 µM and 10 µM Melatonin).

The administration of melatonin in high concentration (10µM) induced a significant increase in the expression of the *ANPEP, FGF1, IL1B, TGFB1, TGFB2*, and *IL8* when compared to other melatonin treated groups (0.1 and 1.0µM, p <0.05). The expression of the *KDR* gene in the 0.1µM melatonin group was higher than 1.0 and 10µM groups. The expression of *PLXDC1* at the 1.0 µM melatonin concentration was significantly higher than in the 0.1 µM group. The expression of *FGFR3* gene in 1.0 µM group was higher than those in in 0.1 and 10 µM groups) (p <0.05) (Figure 2).

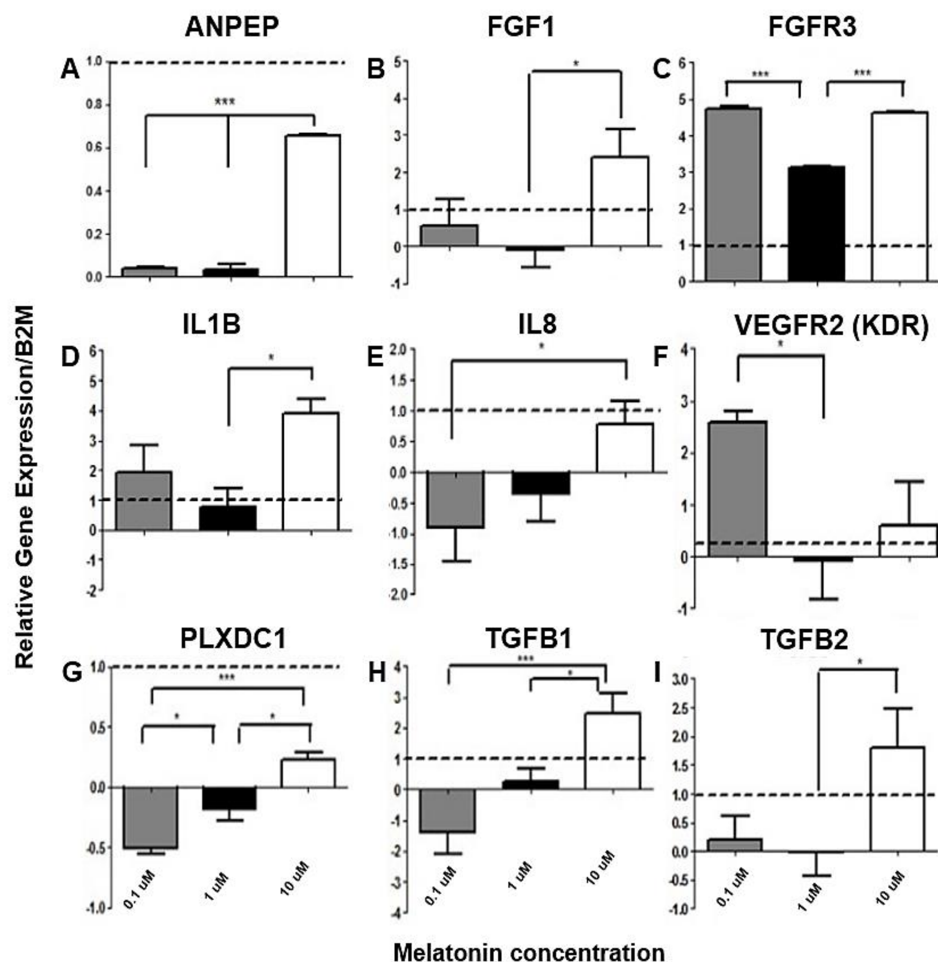

Fig. 2. Effects of different melatonin concentrations on expressions of some genes of granulosa cells.

*Expression values were normalized by the value of β-2-microglobulin gene (B2M). The value of control group was pooled as a reference sample (= 1) indicated as a line in the middle of the graphs. * p <0.05, *** p <0.0001.*

The genes significantly downregulated by melatonin compared to control were *HIF1A, ANGPT2, CXCL5, ENG, FGF2, HANDE2, IGF1, MM2, PDFA, TGFBR1, VEGFA, THBS1*, and *2* (Table 3). In these genes, the *ANGPT2, CXCL5, ENG, MMP2*, and *PDGFA* in the 10μM melatonin treated group had significantly higher expression values than those in the 0.1 and 1.0μM melatonin treated groups. On the other hand, the expression of *IGF1* in 10 μM melatonin treated group was lower than other melatonin concentrations treated groups. The expression values of *HANDE2, FGF2, HIF1A, TGFBR1*, and *VEGFA* in the 0.1μM melatonin treated group was significantly higher than the other melatonin concentrations treated groups. The expression value of *THBS1* in 1.0μM melatonin treated group was higher than the other melatonin concentrations treated groups (p <0.05) (Figure 3).

Table 3 The summarization of downregulated genes related to the angiogenic pathway of granulosa cells treated with melatonin compared to the control.

	Groups		
Gene	Mel 0.1 µM	Mel 1.0 µM	Mel 10 µM
ANGPT2	-2.14 ± 0.12	-4.29 ± 0.21	-1.24 ± 0.04[a]
CXCL5	-2.27 ± 0.12	-1.26 ± 0.13	-0.45 ± 0.13[a]
ENG	0.36 ± 0.01	0.44 ± 0.01	-0.25 ± 0.01[a]
FGF2	1.74 ± 2.12[b]	-0.52 ± 1.78	0.71 ± 1.47
HANDE2	0.34 ± 0.01[b]	-0.36 ± 0.02	-0.51 ± 0.02
HIF1A	0.79 ± 1.08[b]	0.08 ± 0.45	-0.16 ± 0.74
IGF1	-1.82 ± 0,11	-1.52 ± 0,11	-1.10 ± 0,09[a]
MMP2	-1.41 ± 1.89	0.24 ± 1.20	2.47 ± 1.90[a]
PDGFA	-1.31 ± 0.89	-0.17 ± 0.96	-0.07 ± 0.78[a]
TGFBR1	0.98 ± 2.34[b]	-0.95 ± 1.48	-0.32 ± 0.93
VEGFA	1,49 ± 1.82[b]	0.14 ± 0.38	-0.15 ± 0.71
THBS1	-2.14 ± 0.12[b]	0.08 ± 0.66[c]	-0.33 ± 1.04[a]
THBS2	-0.87 ± 0.09	-0.42 ± 0.08	-0.19 ± 0.07

The analysis of variance (ANOVA), along with the post-hoc Tukey test, was used. a- p <0.05 compared to 0.1 and 1.0µM melatonin groups; b- p <0.05 compared to 1.0 µM melatonin group; c- p <0.05 compared to 1.0 µM and 10 µM melatonin groups.

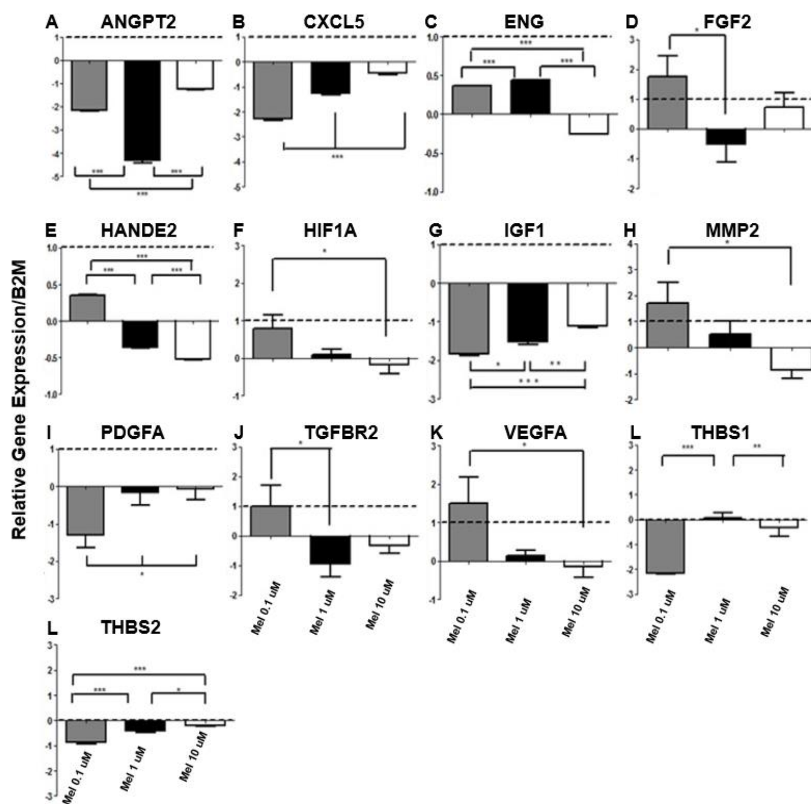

Fig. 3. Effects of different concentrations of melatonin on expression of some genes in the granulosa cells.

*Expression values were normalized by the expression values of β-2-microglobulin gene (B2M). The value of control group was pooled as a reference sample (= 1) indicated as a line in the middle of the graph. * p <0.05, **< 0.01, *** p <0.001.*

3.3. Evaluation of the effects of melatonin on the expression of proteins involved in the angiogenesis pathway.

To validate gene expression data of qRT-PCR), the Western Blot was used to detect the protein expression of these genes. The gene selection was based on their expression levels (p < 0.05 vs control) and their function related to the angiogenesis pathway, specifically, the *VEGF* was selected (Figure 4).

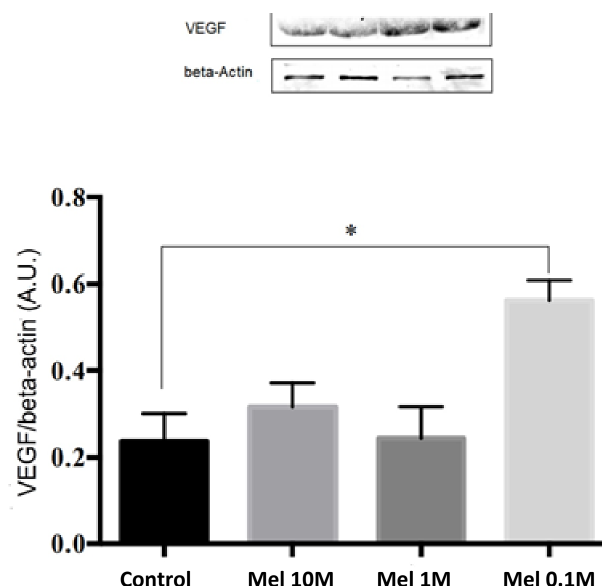

Fig. 4. Effects of different melatonin concentrations on the expression of protein of *VEGF* with Western Blot analysis.

*The values were normalized by the protein of β-actin in granulosa cells Mel: melatonin; * p <0.05 vs control.*

4. DISCUSSION

The research interest on the pleiotropic actions of melatonin in organisms has dramatically increased in recent years. Melatonin researches have been advanced by the emerged new methods which can investigate cell physiology in great detail and characterize additional functions of this molecule. These functions include modulation of humoral immunity, control of glucose metabolism in adipose tissue, modulation of neuronal activity; influence in vascular tone; regulation of reproductive function (15-18). Specific to its role in mammalian reproductive function, its action on the ovaries has not fully understood. The current study will targete this issue, especially on ovarian angiogenesis, which is essential for follicular growth and ovulation (5-8).

In general, angiogenesis associates to the blood flow in tissues. The sufficient blood flow facilitates the supply of oxygen and nutrients, the removal of CO_2 and other toxic metabolic by-products, and transportation of hormones to target cells (8, 18). This process is important for tissue hemostasis. Perturbation of this process compromises organ function and increases cell death (5-8).

The concentration of melatonin in the follicular fluid is more than three times higher than that in the blood (15, 18). The biological significance of this high follicular fluid melatonin is currently unknown but is likely a result of the ovarian synthesis of melatonin (7).
In addition, the ovarian granulosa cells express MT1 and MT2 melatonin receptors in rats (3,19-22). The action of melatonin is estrogen level dependent in cell culture and may have stimulatory or inhibitory effects on cyclic AMP (3, 22). Therefore, melatonin may have the capacity to modulate cell proliferation, mitosis, and angiogenesis (7). The granulosa cells of humans also have melatonin receptors, but their function has not been fully clarified, especially with regard to angiogenesis. The current results showed that melatonin caused alteration of gene expression related to angiogenesis in human granulosa cells.

Ovarian folliculogenesis includes the development of the primordial, primary, secondary, and tertiary or Graaf follicles as well as the corpus luteum. all of these must be accompanied by qualitative and quantitative changes in the microcirculation (15-19). All follicles receive nutrients and oxygen by passive diffusion from the blood vessels of the ovarian stroma. These processes depend on angiogenic growth factors (5-8, 20, 21). Without vascular support, follicular components will stop to develop and finally will result cell death and atresia (5-8). Therefore, it is important to understand the mechanisms of how the endogenous molecule, melatonin, influence angiogenesis in ovary.

During follicular growth, granulosa cells proliferate from a single layer of cells (primary follicle) to several layers (secondary and tertiary follicles). Concomitantly, there is an increase in internal thecal cells, which are an important source of androgens (19-22). This follicular development process is coordinated by the action of estrogen, growth hormones, follicle-stimulating and thyroid hormones and intra-follicular growth factors, such as insulin growth factors (IGFs), members of the transforming growth factor superfamily β and α (TGF-β and TGF-α), fibroblastic factors (FGFs) and vascular-endothelial factor (VEGF), cytokines, prostaglandins, and other components of the immune system (7, 23).

When the secondary follicle has 6 to 12 layers, extensive "gap" communications appear, which are important for the increase of follicular fluid and the enlargement of the antrum (15, 19). This process is highly dependent on nutrients which support the follicle growth since these cells are subjected to the intense metabolic activity and mitosis. At this point, the vascular network is decisive for the survival and maturation of the dominant follicle (24, 25). Therefore, over or under follicular growth will compromise follicular quality and lead to ovulatory disorder. In the current study, we have observed that melatonin has capacity to modulate these actions by modifying the expression of genes related to angiogenesis in granulosa cells. More studies are required to identify the exact role of melatonin played in this process since other hormones including estrogen, progestogen, and androgen, as well as gonadotropic hormones can also influence this process (26, 27).

Capillary endothelium degeneration is a relevant factor in follicular atresia, as it interrupts the supply of nutrients to follicular cells. Although atresia is a normal event in the ovarian cycle, the higher than usual number of atretic follicles cause infertility in some cases, such as in hypothyroidism (29), Cushing's syndrome, hyperprolactinemia, congenital adrenal hyperplasia

and functional pituitary neoplasms (29). In these conditions, the expression and activity of angiogenic related genes are altered, especially the members of the TGF β and α (30, 31). As we reported that melatonin treatment increased the expression of *TGF-beta*. Theoretically, to increase the melatonin level in granulosa cells will retard the atresia.

In the final phase of the development of antral follicle, FSH and estrogens initiate the formation of LH receptors in the granulosa cells, while FSH receptors begin to decrease (15). At this moment, the highest follicular concentrations of melatonin occur (15), which is in the range of the higher concentrations of melatonin used in our study. We also observed a greater number of pro-angiogenic genes were downregulated suggesting the opposite effect of melatonin to the proliferative effect caused by estrogen (7). This relationship between melatonin and estrogen has been previously described in the granulosa cells of rats (3).

In the study, 96 genes related to angiogenesis have been evaluated. Among them, some were up- and some were downregulated with melatonin treatment. The main over-expressed genes were protein-coding genes (ANPEP); fibroblast growth factor 1 (FGF1); fibroblast growth factor receptor (FGFR3); interleukin 1-beta (IL1B); tyrosine kinase receptor (VEGFR-2 (KDR); genes participating in endothelial cell morphogenesis (PLXDC1); folliculogenesis regulating genes (TGFB1 andTGFB2). They act on follicular growth dynamics (32)

The genes of FGF family are pro-angiogenic growth in secondary and tertiary follicles, as well as in the corpus luteum. FGF-1 stimulates the proliferation of endothelial cells, promoting their proliferation and migration (33). This process is important for the growth of the internal thecal and granular layers (33). This growth factor is important for cell survival and secretion of other factors, such as VEGF but it is also regulated by estrogen and FSH (33-36). Melatonin probably can also regulate growth factor since melatonin treatment unexpectedly decreased the expression of VEGF observed in this study.

Interleukin-8 (IL-8) is a cytokine that acts both on the immune system, activating neutrophils, as well as on angiogenesis, negatively modulating the action of steroid hormones and growth hormone (34). Thus, it is important for proper follicular growth. In addition, it participates in the neovascularization process in the formation of the corpus luteum (30). This suggests that melatonin may decrease angiogenesis by increasing the expression of this cytokine.

The members of the β-transforming growth factors (TGF-β) superfamily play important roles in the local regulation of folliculogenesis and oocyte quality or in the development of granulosa and thecal cells (7). It also participates in ovarian steroidogenesis and the follicular atresia process. Our results showed upregulation of this gene with the high concentration of melatonin (10μM) treatment, but with low melatonin concentration treatment this effect disappeared. Therefore, lipidomic and/or metabolomic evaluation (including steroids) in this case is essential to confirm these results and to elucidate the mechanisms involved.

VEGF is a vascular endothelial growth factor. There are seven main types. The first one is identified as VEGF-A. Some types of VEGF exert their functions through the tyrosine kinase system (VEGF-R1 (Flt-1), VEGF-R2 (Flk-1/KDR), and VEGF-R3) (31). VEGF-R2 is mainly expressed in endothelial cells and is important for endothelial proliferation and migration. VEGFR-1 is considered critical for the formation of capillary tubes (31). VEGFR-3 is expressed by lymphatic endothelial cells and regulates lymphangiogenesis (32). All them act on follicular growth and are influenced by ovarian steroids (37). It is unknown whether melatonin acts directly on the tyrosine kinase system in granulosa cells. However, melatonin enhances the action of insulin in other tissues through its receptors (38). We have found that VEGF expression is reduced by melatonin treatment, but the mechanisms remain to be identified.

VEGF synthesis is differentially regulated in each ovarian follicle according to its size. Expression of VEGF mRNA in the primate ovary occurs in cells of antral follicle thecal and granulosa cells closest to the oocyte in the preovulatory follicle (33-39). The permeability of VEGF seems to be essential in the formation of the antrum during follicular development (35). VEGF is also involved in ovulation. The higher expression of VEGF after the administration of an ovulatory dose of gonadotropins correlates with the increase in the levels of prostaglandins, which are known to play a role in ovulation (7). A rapid rise in vascular permeability at the time of ovulation, attributable to the interaction of VEGF with prostaglandins, may favor the leakage of fluid into the follicular antrum and, thus, increase intra-follicular pressure in response to the increase in gonadotropins. In addition, VEGF as a stimulator of some proteolytic enzymes and plasminogen activators in endothelial cells can structurally weaken the follicle wall before its rupture (7).

Excessive action of VEGF impairs ovulation and its balance is important for the normal ovulation. Perhaps, the function of melatonin is to balance this factor. In the study several other genes including *HIF-1A, ANGPT2, CXCL5, ENG, FGF2, HANDE2, IGF-1* were downregulated by high doses of melatonin treatment. Since HIF-A is the main positive regulator of VEGF in primary cultures of primate luteal cells (39) its suppression by melatonin could be a mechanism for reducing VEGF expression. However, our study has a limitation on the mechanism of melatonin on human granuosa cells. In addition, the studies with melatonin receptor antagonists (luzindole, 4P-PDOT) and/or melatonin receptor SiRNA have not been conducted to evaluate whether the effect is mediated by melatonin receptors or by its direct activities.

IGF-1 is a member of the family of IGFs that are related to metabolic activities promoted by insulin (40). IGF-1 in the preantral phase has an endocrine action and the ability to increase androgen production. Excessive androgenic action slows down follicular growth and therefore can lead to anovulation. This fact occurs in polycystic ovary syndrome (40). As observed in the study, melatonin downregulated the IGF-1 expression at its lower concentration in granulosa cell culture.

IGFs act synergistically with FSH to promote follicular growth and estradiol production (40). For ovulation, FSH pulsation increases, followed by the LH peak. This peak is also responsible for the resumption of meiosis to promote oocyte maturation (40-43). Fuhrmeister *et al* (41) demonstrated that IGF-I regulates follicular development in the early stages of folliculogenesis, controlling the oocyte maturation process. Therefore, excessive suppression of IGF-1 can also negatively impact the folliculogenesis (42). Melatonin may have a modulatory role on this factor since the higher concentration of this indolamine increased the expression of this factor. Finally, some of the differentially expressed genes were validated by the Western Blot. The results showed that melatonin at the high concentrations (above 1 μM) exhibited dual functions on some genes of the growth factors and cytokines either by up- or downregulating their expressions. These regulations may be directly or indirectly by physiological compensation mechanism. The overall action of melatonin above 1.0 μM seems negatively modulating angiogenesis of granulosa cells from women subjected to in vitro fertilization.

Recently, Tamura *et al* (44) reported that melatonin treatment was effective for patients with poor fertilization rate in the previous assisted reproductive technology (ART) cycle. In fact, melatonin reduced oxidative stress and protected the granulosa cells in patients with poor ART outcomes. This improvement may relate to VEGF and other oxidative stress genes. The studies from clinical practice suggest that melatonin appears to balance the angiogenesis to improve the quality of ovarian follicles (granulosa cells) and of oocytes (40-44). In conclusion, melatonin modulates angiogenesis associated with granulosa cells from women undergoing *in vitro*

fertilization. Also, the low dose of melatonin may decrease the VEGF expression in the human granulosa cells while the high dose may have the dual activities.

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AUTHORSHIP

CCM participated in the study design, sample collection, molecular studies, data analysis, and writing of the manuscript; MCPB AND CML participated in the study design, sample collection, data analysis, and writing of the manuscript; DB laboratory activities and data analysis, MJBCG design of the protocol, JCN, writing and reviewing the manuscript, RJR - writing and reviewing the manuscript, RSS participated in sample collection, data analysis, and writing of the manuscript; MJS participated in the study design, sample collection, data analysis, and writing of the manuscript; GSC and PM participated in the study design and writing of the manuscript; ECB participated in the study design, data analysis, and writing of the manuscript; and JMSJr participated in the study design and writing of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest with this manuscript.

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