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Title: Effect of Melatonin on the Expression of VEGF-A and on the Degeneration of Follicle Reserve in Rat Ovary

Running Head: Melatonin Protects the Ovary

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Abstract

Objective: To analyze the effects of melatonin on vascular endothelial growth factor A (VEGF-A) expression and follicle reserve in rat ovary.

Materials and Methods: A total of 45 female Wistar rats were used in the present study. Rats were divided into three groups: group 1 (control), group 2 (vehicle), and group 3 (melatonin). Rats in the melatonin group were treated with an intraperitoneal injection of melatonin at a dose of 50 mg/kg/day for 56 days. We investigated VEGF-A expression in rat ovary in all the groups using

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Western blot and reverse transcription-polymerase chain reaction. Histopathological parameters were evaluated using light microscopy.

Results: The number of atretic follicles was significantly lower in the melatonin treatment rats than in the control rats ($p<0.05$); however, the number of antral follicles was significantly higher in the former ($p<0.05$). Additionally, we observed a weak immunoblot stain in the melatonin group for VEGF-A protein. Interestingly, melatonin treatment induced a significant decrease in VEGF-A expression in the ovary of group 3 rats ($p<0.05$), whereas no such difference was observed between group 1 and group 2 rats ($p>0.05$).

Conclusion: The present study demonstrates that the protective effect of melatonin on the degeneration of follicles in rat ovary is reduced by decreasing the VEGF-A expression. These results suggest that melatonin is effective against follicular atresia and preserves antral follicles, thus, offering a therapeutic advantage in clinical use.

Keywords: melatonin, antral follicle, atresia, vascular endothelial growth factor A (VEGF-A)

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an important lipophilic amine hormone that is secreted by the pineal gland and considered a powerful free radical scavenger. It plays a critical role in the physiology of the ovary in the reproductive system (1). Some authors have reported that the melatonin receptor is demonstrated in the ovaries, suggesting that melatonin is of great importance to the reproductive system (2, 3). Vascular endothelial growth factor (VEGF) is a chemokine that is the most active endogenous pro-angiogenic factor in humans (4, 5). The VEGF system comprises ligands and receptors that play critical roles in tissue vascularization and endothelial cell growth (6). It is a greater component of the family of angiogenic factors, which contains the placental growth factor, angiopoietin, basic fibroblast growth factor, and the VEGF family (A, B, C, D, and E) (7, 8). Previous studies on cancer have shown that in other tissues, melatonin decreases VEGF secretion (9). Melatonin may affect VEGF, and the relationship between melatonin and VEGF secretion in the ovary is unclear. Thus, we aimed to evaluate the effects of melatonin on the ovarian histology and VEGF expression and on the quantity of follicle selection in adult female rat ovaries.

Materials and Methods

Animals

In total, 45 female Wistar rats, aged between 8 and 10 weeks, with body weights ranging from 150 to 200 g were used. All animals were kept in a controlled environment with artificial light–dark cycle of 12 h lights on and 12 h lights off and received food and tap water ad libitum. The present study was approved by the **** University Institutional Animal Care and Use Ethical Committee. Rats were randomized into three experimental groups as follows: group 1: control, group 2: vehicle, and group 3: melatonin (Table 1).

Melatonin exposure

Melatonin (M5250; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in absolute ethanol, and the final concentration was 50 mg/kg, which is the optimal dose that we had determined in our previous

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study (1). Melatonin was intraperitoneally injected for 56 days in the melatonin group in the same light period (12:00 a.m.), whereas ethanol was administered in the vehicle-treated group (10% ethanol in saline). The melatonin and vehicle group animals continued to receive their usual treatments for 56 days until sacrificed by cervical dislocation.

Histomorphometric analysis of folliculogenesis and follicle count in the ovaries

Histomorphometric analysis was performed by a histologist. The ovaries were preserved in Bouin's fluid for 12 h, followed by immersion in 70% ethanol saturated with lithium carbonate to remove the excess picric acid. After processing, 5- μ m thick serial cryosections were prepared using a cryostat to study the effect of melatonin on the number of follicles. Every fifth section was stained with routine hematoxylin and eosin, and the slides were mounted for further assessment. Approximately 15–20 slides (every fifth section) were evaluated for predicting the follicle count of each ovary. Blind manual counting was exercised by a single observer owing to previous knowledge (10). The follicles with clearly visible nucleus within the oocyte were classified as primordial, antral, and atretic follicles (11). The slides were closely analyzed at 40 \times magnification, and the number of various follicles was individually noted for each animal.

Real-time polymerase chain reaction

Total RNA was harvested from whole ovaries using reverse transcription-polymerase chain reaction (RT-PCR) (n=15). RNA was isolated from whole ovaries using TRIzol reagent (Invitrogen), followed by RNA extraction. Tissue cDNA was assayed in duplicate, and each experiment was repeated three times. RT-PCR for VEGF-A was performed in a 25- μ l final reaction that included 2 μ l of each RT sample within a reaction mix containing 1 \times Buffer D, 10 pmol of each primer, and Taq polymerase. RT-PCR was performed using the following parameters: 1 cycle at 94 $^{\circ}$ C for 5 min (denaturation), followed by 35 cycles (amplification) at 94 $^{\circ}$ C for 30 s, 59 $^{\circ}$ C for 1 min, and 72 $^{\circ}$ C for 45 s for all target genes and control β -actin. Table 2 shows a primer that was used for the amplification of VEGF-A gene.

Western blot

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Briefly, 50 µg of protein lysates were loaded in each lane. Proteins performed electrophoretically by sodium dodecyl sulfate polyacrylamide gel using 10% Tris–HCl gels. After electrophoresis, the proteins were electrotransferred to polyvinylidene fluoride membrane (Bio-Rad Laboratories). Cells were electrotransferred to Immobilon-P transfer membrane (Millipore, Billerica, MA, USA) and Tris-buffered saline that included 5% nonfat milk for 1 h at room temperature. Then, the primary antibody was added overnight at 4°C and washed, and the secondary antibody was used. The same procedure was repeated for β-actin that was used for internal control.

Statistical analysis

Histological data were analyzed using analysis of variance (ANOVA; with post hoc Tukey's test) or Kruskal–Wallis test (post hoc Dunnett's test). RT-PCR results from each group were compared using one-way ANOVA followed by post hoc Holm–Sidak test. Statistical data were examined using SigmaStat for Windows version 3.0 (Jandel Scientific Corp., San Rafael, CA, USA). A p -value<0.05 was considered as significant.

Results

Morphological evaluation

The melatonin, control, and vehicle groups were histologically normal. In the ovaries that appeared to have a normal histology, the follicles were found to have a normal histology at different stages of development, because there was a peripheral cortex, a medullary region displaying normal vessel network regions in the ovarian center (Fig. 1).

Effect of melatonin treatment on antral and atretic follicles

Fig. 2 shows the number of antral, primordial, and atretic follicles in the ovaries of all groups. There was no significant difference between the three groups regarding the primordial follicle ($p>0.05$). The evaluation of atretic and antral follicle counts revealed significant differences between the groups ($p<0.05$). The melatonin group had the highest antral and the lowest atretic follicle count.

Furthermore, the number of atretic follicles in the melatonin group was significantly lower than that in the control group. In addition, the percentage of atretic follicles in the melatonin group was 2.23

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fold less than that in the control group, indicating that melatonin treatment decreases follicular degeneration reserve (atretic, 1.9 ± 0.4 in group 1 vs. 0.85 ± 0.1 in group 3) ($p<0.05$) (Fig. 2). We observed that the percentage of antral follicles was 2.55 fold higher in the melatonin group than in the control group; it was also estimated that signed melatonin treatment supported antral follicle development, preserving the follicular reserve (antral, 1.38 ± 0.2 in group 1 vs. 4.08 ± 0.8 in group 3) ($p<0.05$).

Western blot

No difference in VEGF-A expression was found in the control and vehicle groups. On examining the level of VEGF-A protein expression in the control and melatonin groups, a dramatic decrease in the expression was observed in the melatonin group (Fig. 3).

Real-time polymerase chain reaction

RT-PCR analysis revealed that VEGF-A, the key molecule of angiogenesis, was expressed in all three groups. However, VEGF-A showed a significantly lower protein expression in the melatonin group than in the other groups ($p<0.05$) (figure not shown).

Discussion

The incidence of infertility is gradually increasing worldwide (12). Female infertility can be induced by various processes involving folliculogenesis, ovulation, early embryogenesis, and implantation in most cases. However, a lack of follicle quantity or number with defects in follicular development in the ovary has become the principal cause of female fertility, affecting approximately 1% of women aged under 40 years (13-15). Kandemir et al. showed that melatonin treatment may be effective in treating the female follicular reserve (1). Melatonin has been actively used against oxidative stress and inflammation and to renovate multiple functions of many tissues, such as regulation of fertility (16-18). For example, ovarian activity promotes estrous cyclicity and gonadal atrophy depending on photoperiodic conditions as stimulated by melatonin (19). Previous studies reported that melatonin ensures a protective effect on premature ovarian insufficiency by ovarian and uterus mass and increasing ovarian follicles, decreasing follicular atresia (20-22). Chan et al. established that reactive

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oxygen species and the endoplasmic reticulum are responsible for the increased loss of ovarian reserve (23). Melatonin dose administered in this study was based on our previous study on the effect of melatonin on mammalian target of rapamycin expression in rat ovary (1). In the present study, we also showed that melatonin treatment causes an increase in the number of antral follicles and a decrease in the number of atretic follicles, suggesting that melatonin may inhibit follicular atresia.

Many endogenous activators or inhibitors can affect physiological angiogenesis balance. VEGF-A is the most significant angiogenesis activator that acts through binding of VEGFRs and primary VEGFR-2, which is the most prominent receptor associated with angiogenesis (24). A previous study evaluated the effects of melatonin on angiogenesis and showed that melatonin can significantly decrease VEGFR-2 expression in mice (25). The results of the present study showed a remarkable relationship involving VEGF-A and melatonin in rat ovary. We found that melatonin significantly protected the follicles via the angiogenic factor VEGF-A, which is closely related to the angiogenesis process in the follicle and, thus, to nutrition of stroma and corpus luteum. Additionally, VEGF-A is linked to several cytokines that could be related to the accelerated degeneration of the corpus luteum, leading to attenuated progesterone production (26). Several studies have shown that melatonin decreases VEGF-A and proliferating cell nuclear antigen expression in the epithelial and stromal cells in rat ovary (26, 27). In the present study, melatonin mitigated VEGF-A levels. Our observed loss of VEGF-A may be indicative of an increased protection from atresia in the melatonin group. It can be concluded that a decrease in atresia and an increase in the number of antral follicles can be attributed to diminishing VEGF-A levels.

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TABLES

Table 1. Study groups.

Groups	Ethanol administered	Melatonin treatment	n
Group 1: control	–	–	15
Group 2: vehicle	10 mg/kg/day	–	15
Group 3: melatonin	–	50 mg/kg/day	15

Table 2. Primer list.

Gene name	Sequence
VEGF-A (172 bp)	Sense: 5' GCC CAT GAA GTG GTG AAG TT 3' Antisense: 5' ACT CCA GGG CTT CAT CAT TG 3'

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FIGURE LEGENDS

Fig. 1. Histomorphometric analysis

Histomorphometric assessment of the antral and primordial follicles in rat ovary. Scale bars represent 50 μ m.

Fig. 2. Follicle count

Comparison of the number of primordial, antral, and atretic follicles between the three groups. Data are shown as mean \pm S.E.M.; n=15 rats/group. *, $p<0.05$ indicates significance from respective vehicle values. #, $p<0.05$ indicates significance from respective control values.

Fig. 3. VEGF-A protein expression

VEGF-A protein expression in group 1 (control), group 2 (vehicle), and group 3 (melatonin). Data are shown as mean \pm S.E.M.; n=15 rats/group. *, $p<0.05$ indicates significance from respective control values. #, $p<0.05$ indicates significance from respective vehicle values.

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