

Effects of melatonin on histomorphology and on the expression of steroid receptors, VEGF, and PCNA in ovaries of pinealectomized female rats

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Objective: To evaluate the effect of melatonin both on the ovaries of pinealectomized female rats through histomorphometric analysis and on steroid receptors, proliferating cell nuclear antigen (PCNA), and vascular endothelial growth factor (VEGF) expression.

Design: Experimental study.

Setting: Federal University of São Paulo, Brazil.

Animal(s): Forty female rats.

Intervention(s): Forty rats were divided equally into four groups: GI—vehicle without surgery; GII—surgery without removal of the pineal gland (sham); GIII—pinealectomized with vehicle; and GIV—pinealectomized with melatonin treatment. After treatment for 3 consecutive months, the animals were killed and their ovaries removed for analysis.

Main Outcome Measure(s): Estrogen and progesterone receptors, histologic and immunohistochemical analysis.

Result(s): The GIII samples presented signals of proliferation on ovarian surface epithelium and interstitial cells as well as high expressions of PCNA and VEGF in those structures compared with GI, GII, and GIV. Also, the levels of progesterone receptor (fmol/g) in ovaries of GIII (250.6 ± 32.4) were significantly lower than in those of GI (429.0 ± 23.8), GII (442.3 ± 30.2), and GIV (564.1 ± 78.7). The levels of progesterone in GIII were superior to those in GI, GII, and GIV.

Conclusion(s): Our findings suggest that melatonin may attenuate proliferation in ovarian structures and increase the number of luteal bodies as well as the levels of progesterone receptor. (Fertil Steril® 2011;95:1379–84. ©2011 by American Society for Reproductive Medicine.)

Key Words: Melatonin, ovary, VEGF, PCNA, rats

Melatonin may improve sleep in those with delayed sleep phase disorder but has no effect on primary sleep disorder (1). Melatonin may also improve initial sleep quality in older adults with insomnia (2), but its role as a treatment for insomnia in those with Alzheimer disease remains disputed (3, 4). Melatonin may also affect immune function as well as cancer initiation and growth (5). Melatonin is well tolerated with doses of up to 36 mg/d. However, these high doses may produce some adverse reactions: autoimmune hepatitis, confusion, optic neuropathy, fragmented sleep, psychotic episodes, nistagmus, seizures, headache, and skin eruptions (6).

Melatonin acts on the reproductive system by acting on GnRH release (7, 8) and thereby on gonadotropins (9). Some authors (10, 11) have shown evidence of melatonin's direct action on the ovaries. In the reproductive system, melatonin may interact with

sex steroids and interfere with ovarian function. However, the exact mechanism of this process is not well understood. Consequently, research into the mechanisms involved in follicular growth, stromal proliferation and ovarian function is relevant. Pinealectomy resulted in overgrowth of the ovarian stroma (12). However, information on how melatonin may revert this process is scarce.

Studies conducted on cancer showed that, in other tissues, melatonin was able to reduce vascular endothelial growth factor (VEGF) secretion. This fact suggests that melatonin regulates tumor growth (13). Studies also show that melatonin is an antiproliferative agent that decreases proliferating cell nuclear antigen (PCNA) expression in hormone-dependent tumors in mice (14). Therefore, the present study aims to investigate the effects of melatonin on ovarian histology and on VEGF and PCNA expression as well as its action on sex steroids and their receptors in pinealectomized adult female rat ovaries.

MATERIALS AND METHODS

Virgin, adult, female EPM-1 Wistar rats (*Rattus norvegicus albinus*), weighing ~250 g, from the Federal University of São Paulo - Escola Paulista de Medicina CEDEME were used. Rats were handled in conformity with the Ethical Principles for Experimentation. Protocols were approved by the institutional board (#0233/06).

Fifty animals were kept on a diet of Purina ration and water *ad libitum*. Lighting was supplied by a 40-watt daylight fluorescent lamp under control

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TABLE 1**Urinary 6-sulfatoxymelatonin (6-SMT) and serum E₂ and P levels (mean ± SD).**

	GI	GII	GIII	GIV
6-SMT (ng/mL)	93.23 ± 16.62	91.20 ± 19.64	5.78 ± 5.36 ^a	82.99 ± 21.63
E ₂ (pg/mL)	176.41 ± 77.61	175.14 ± 87.33	298.96 ± 99.71	197.21 ± 94.58
P (ng/mL)	14.53 ± 3.57	14.46 ± 8.29	3.92 ± 2.14 ^a	9.98 ± 3.34

^a *P* < .01 compared with other groups.Romeu. *Effect of melatonin on ovary. Fertil Steril* 2011.

of a timer set for an 8:16 photoperiodic cycle with 8-hour light period (lights on 10:00 a.m.). This short photoperiod was used to sensitize the rats to melatonin treatment (15). Temperature was kept at ~22°C, noise was kept to a minimum, and relative humidity of the air was maintained at 40%–70% (11).

The rats with regular estrous cycles (*n* = 40) were randomized into four groups (*n* = 10): GI—10 animals, vehicle without surgery (control); GII—10 animals, vehicle with sham pinealectomy; GIII—10 animals, vehicle with pinealectomy; and GIV—10 animals, melatonin with pinealectomy.

Colpocytologic Examination

After adjustment to the new environment, all of the animals underwent daily collection of vaginal smears for 4 consecutive weeks before the pinealectomy and 4 weeks after the treatment period. The material was processed and stained by the Harris-Shorr method.

Pinealectomy

All animals were anesthetized with 15 mg/kg of xylazine (Rompun) associated with 30 mg/kg of ketamine (Ketalar) via the intraperitoneal route following the method used previously (11, 12, 16). The same procedure was adopted for the sham-pinealectomized animals, except for the removal of the pineal gland. We lost two animals after surgery, one from GIII and one from GIV.

Melatonin Replacement

Daily doses of 200 µg melatonin per 100 g body weight were used. Melatonin (Sigma, St. Louis, MO) was dissolved in a small volume (0.02 mL) of absolute ethanol and properly diluted in 0.9% NaCl to a final concentration of 2 mg/mL. Melatonin injections were performed intramuscularly between 6:00 and 7:00 p.m.

Blood and Urine Collection

In the evening before they were killed, the animals were placed in metabolic cages for night urine collection and subsequent measurement of 6-sulfatoxymelatonin (6-SMT) concentration. On the next day, all of the animals in the proestrus phase were anesthetized with 15 mg/kg of xylazine (Rompun) associated with 30 mg/kg of ketamine (Ketalar) via the intraperitoneal route. Soon afterwards, blood was drawn from the right orbital venous plexus for estrogen (E) and progesterone (P) determinations. The ovaries were removed for histologic and immunohistochemical studies.

Determination of Urinary 6-SMT and Circulating Hormone Levels

The melatonin metabolite in the sample was determined by radioimmunoassay for 6-SMT (WHB, Bromma, Sweden) and expressed in ng/mL, with a detection limit of 0.01 ng/mL (17).

Estrogen (detection limit of 10 pg/mL) and P (detection limit of 0.1 ng/mL) were measured by chemiluminescence with the ACS-180 machine (Bayer, Tarrytown, NY). The maximum percentage of cross-reactivity for other steroids in the E and P determinations was <0.01% (specificity).

Histologic Analysis

Once removed, the ovaries were placed in buffered formaldehyde (10%) for 12 hours and processed for histologic analysis. The sections were stained with hematoxylin-eosin and analyzed under a light microscope.

Morphometric Analysis

The thickness of the surface epithelium as well as numbers of ovarian follicles and interstitial cells were measured using the AxioVision program (Carl Zeiss, Munchen-Hallbergmoos, Germany). Four readings per slide

TABLE 2**Morphometric parameters evaluated in the rat ovaries, estrogen (ER) and progesterone (PR) receptor expression as measured during the proestrus phase, and VEGF expression (mean ± SD).**

	GI	GII	GIII	GIV
Thickness of OSE (×10 µm)	5.7 ± 0.6	6.2 ± 0.9	10.2 ± 0.8 ^c	7.7 ± 2.2
PCNA in OSE ^a	2.45 ± 0.23	2.59 ± 0.21	4.91 ± 0.32 ^c	3.05 ± 0.29
PCNA in interstitial cells ^a	3.18 ± 0.20	3.24 ± 0.21	4.64 ± 0.08 ^c	2.64 ± 0.15
Interstitial cells (n) ^a	152.2 ± 25.2	140.8 ± 18.2	290.1 ± 33.2 ^c	158.4 ± 52.8
ER (fmol/g)	149.0 ± 14.5	142.8 ± 12.5	105.7 ± 10.3	123.2 ± 8.7
PR (fmol/g)	429.0 ± 23.8	442.3 ± 30.2	250.6 ± 32.4 ^c	564.1 ± 78.7
VEGF ^b	1.75 ± 0.46	1.77 ± 0.44	2.88 ± 0.33 ^c	1.75 ± 0.46

Note: OSE = ovarian surface epithelium; PCNA = proliferating cell nuclear antigen; VEGF = vascular endothelial growth factor.

^a PCNA-positive staining among 1,000 cells; *n* = number of interstitial cells in 780 µm².

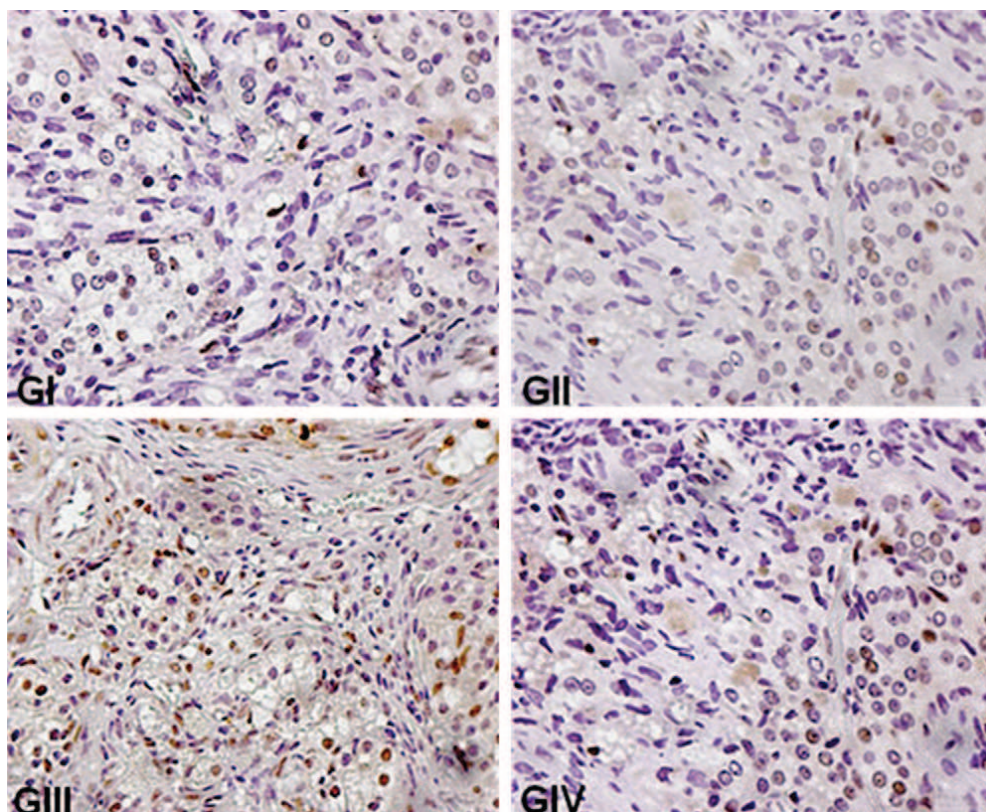
^b VEGF intensity.

^c *P* < .01 compared with other groups. For ER, there were no significant statistical differences among the groups.

Romeu. *Effect of melatonin on ovary. Fertil Steril* 2011.

FIGURE 1

Photomicrographs of histologic sections from adult rat ovaries. Notice the great quantity of interstitial cells in GIII, with higher expression of PCNA than the other groups.



Romeu. Effect of melatonin on ovary. *Fertil Steril* 2011.

where done for each animal. The ovaries were included, and section cuts were made from the central region to the peripheral region. The following two items were analyzed in the ovaries: thickness of surface epithelium and number of interstitial cells in an area of $780 \mu\text{m}^2$.

To determine the thickness of the ovarian surface epithelium in each animal, ten measures were made of the distance between the epithelial limit with the connective tissue (basal membrane) and the outer surface epithelial zone in a straight line perpendicular to the basal membrane.

Immunohistochemical Analysis

To determine the cell proliferation index of the interstitial cells and the surface epithelium, the ovary sections were immunohistochemically stained with anti-PCNA antibody PC-10 (Dako, Glostrup, Denmark). The PCNA-positive nuclei (index) represent values per 1,000 cells.

The ovary sections were immunohistochemically stained with VEGF antibody (Dako). Immunoexpression was assessed using electronic images captured by a light microscope (Carl Zeiss, Munchen-Hallbergmoos, Germany) attached to an AxioCam MRC high-resolution video camera (Carl Zeiss) and a color video monitor (Samsung, Manaus, Brazil). The image analysis software used to produce the images was AxioVision REL 4.6 (Carl Zeiss).

Steroid Receptors

The ovarian tissue fragments were homogenized in a TED buffer (Tris-HCl 10 mmol/L, ethylenediamine tetraacetic acid 1.5 mmol/L, glycerol 10%, and dithiothreitol 2 mmol/L, pH 7.4) three times, for 15 seconds each time, in a Polytron PT 10 (Brinkmann, Duluth, MN).

The homogenate was centrifuged at $105,000g$ for 60 minutes in an A-1256 combo rotor (Sorvall Ultracentrifuge Rotor, St. Herblain, France) to obtain cytosol at -4°C .

Determination of protein concentration followed the conventional method (18) as modified (19) for estimation of receptors. Levels of E receptor (ER) were determined by incubation of $75 \mu\text{L}$ of cytosol with $5 \text{ nmol } ^3\text{H-E}_2$ (79 Ci/nmol; Amersham International, Little Chalfont, Buckinghamshire, England) at 4°C for 18 hours. Levels of a nonspecific ligand were estimated by the addition of 100 times more diethylstilbestrol than is present physiologically. The ligands were separated from the free ligands by the McGuire method (dextran-coated charcoal). Radioactivity was measured by the scintillograph method with Tri-Card 1600 TR (Packard, Downers Grove, IL).

Progesterone receptor (PR) expression was determined as previously described (20) by the replacement of hormone with $12 \text{ nmol } ^3\text{H-ORG 2058}$ (49 Ci/mmol or 79 Ci/mmol; Amersham International) at 4°C for 4 hours. The nonspecific ligand was determined by the 100-fold addition of diethylstilbestrol in combination with nonradioactive ORG 2058.

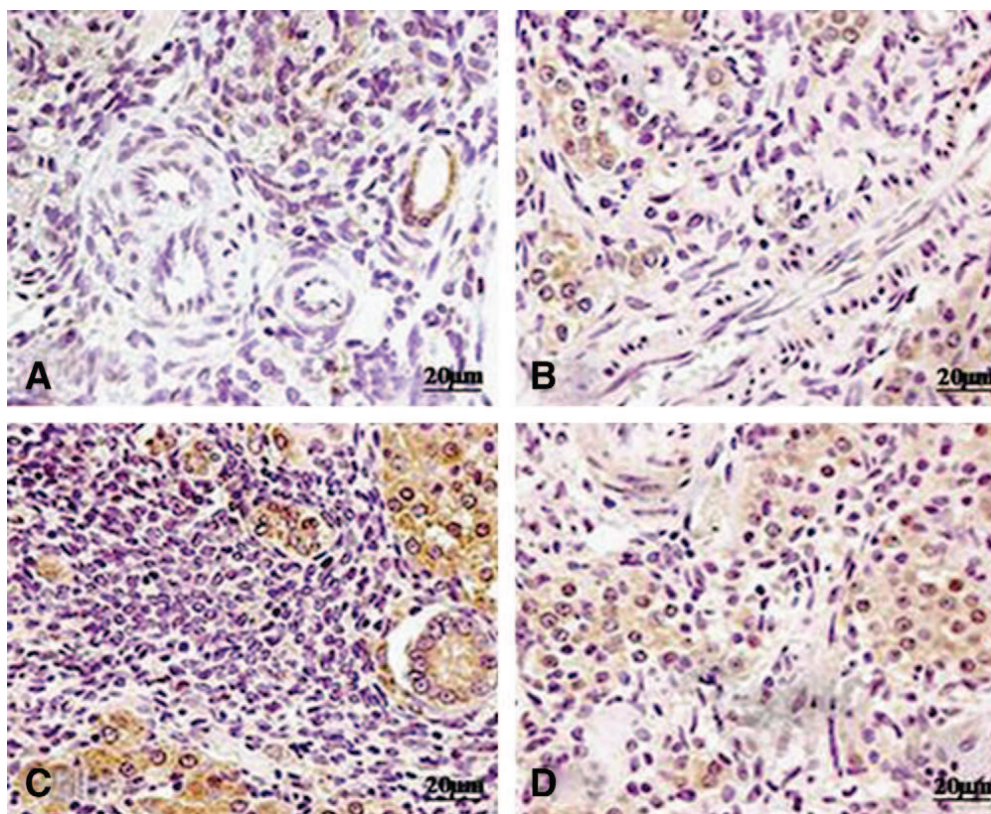
Levels of both ER and PR were expressed as fmol/g protein.

Statistical Methods

The normality tests were applied to all data. After that, the analysis of variance and Tukey multiple comparison tests were used for the identification of significant differences between groups. The level for rejection of the null hypothesis was set at .05. Based on other studies (12), the power calculation of 90% dictated the use of nine rats per group.

FIGURE 2

Histologic sections of representative segments of adult rat ovaries. The purple color indicates positive reaction for VEGF. A large quantity of interstitial cells and remarkably higher VEGF expression are visible in GIII (C) compared with the other images (A = GI; B = GII; and D = GIV).



Romeu. Effect of melatonin on ovary. *Fertil Steril* 2011.

RESULTS

Determination of Serum Sex Steroids (E and P) and of Urinary 6-SMT

The hormone data are summarized in Table 1. GIII animals had the lowest levels of 6-SMT and P ($P < .001$). There were no significant differences in E levels among the groups.

Ovary Morphology

In GI and GII (control and sham), the ovarian surface epithelium was of the simple cuboidal type. The cells had light and eosinophilic cytoplasm with spherical and central nuclei. In GIII the epithelium was of the simple columnar type, consisting of cells with light and eosinophilic cytoplasm, with elongated and heterochromatic nuclei and with a few pseudostratified areas (found in six animals of the group). GIV samples displayed surface epithelium similar to that in GI and GII.

In GIII there was a change in the follicular population, with many developing follicles and, in some cases, follicular cysts. Many of the follicles displayed proliferation of the theca interna cell layer with several mitotic cells and with multiple interstitial cells close to the follicles. There were also many interstitial cell chords in the ovarian stroma. Interstitial cells appeared in large quantities compared with the other groups (GI, GII, and GIV). In GIV, there were mature follicles and corpora lutea, but fewer interstitial cells were observed amid the follicles than in GIII.

Histomorphometry and PCNA

The histomorphometric data on the ovaries from all groups are summarized in Table 2. The thickness of the ovarian surface epithelium as well as the number of interstitial cells in GIII were higher than observed for other groups. Furthermore, PCNA expression in ovarian surface epithelium and interstitial cell density were increased in GIII compared with other groups ($P < .05$; Fig. 1).

Analysis of Steroid Receptors by ^3H Binding and VEGF expression

The results for ER and PR are presented in Table 2. Estrogen receptor levels were similar in all groups, and there was no statistical difference among the groups. The GIII samples had the fewest PRs ($P < .001$). The data on VEGF expression as determined by immunohistochemistry are presented in Table 2 and Figure 2. VEGF expression was highest in GIII ($P < .001$).

DISCUSSION

In a previous study, melatonin was shown to improve ovulation in pinealectomized rats (21). For this reason, we chose to investigate ovarian morphology to elucidate the mechanism of melatonin action. The present data suggest that melatonin affects the ovarian epithelium and stroma of pinealectomized rats and increases peripheral blood levels of P as well as PR density in ovaries.

Soares Jr. et al. (12) showed that a diminution in 6-SMT accompanied the development of cystic ovaries and the increase in interstitial cell density. Other authors (22) produced evidence showing that melatonin rescued the reduction in ovulation that resulted from removal of the pineal gland. However, others (23, 24) suggested that melatonin was also capable of diminishing the number of ovarian cysts observed in pinealectomized animals, owing to the antigonadotropic effects of melatonin (24). There is evidence that melatonin acts through MT1 or MT2 receptors in the ovaries (11). These facts may explain the decrease in the number of interstitial cells.

It should be emphasized that the ovarian stromal-interstitial cells derive from cells of the theca interna or from atretic follicles (25). These structures can produce androstenedione and testosterone (T), giving rise to an androgenic microenvironment that could interfere with follicular growth and the emergence and development of the corpus luteum (25). This effect might be related to decreases in ovulation and fertility (21). One of the endocrine mechanisms underlying these facts may be LH dependent (25), which would also explain the thickening of the ovarian surface epithelium in the pinealectomized animals. Likewise, a disturbance in insulin-like growth factor 1 action may contribute to increased thickness of the epithelium (26). Notably, this growth factor is related to the production of sex steroids (27).

In the present experiment, melatonin diminished VEGF and PCNA expression in the epithelial and stromal cells. Reduced expression of VEGF and PCNA suggests an antiproliferative effect. In fact, some authors suggested that this action may be dependent on the presence of serum and a complex interaction with hormones such as E₂ and/or PRL (28). Regardless of the mechanisms, the effect of melatonin on pinealectomized rats has some benefits: Serum P levels increased, as did the numbers of PRs and corpora lutea. These facts indicate that a greater number of mature follicles could be released. In fact, some authors (21) demonstrated that melatonin replacement increased the number of oocytes in the oviduct as well as the number of embryos in the endometria of pinealectomized adult female rats.

VEGF is closely linked to the angiogenesis process in the follicle, stroma, and corpus luteum. VEGF is also important in the development, maintenance, and degradation of those structures. It is also a major factor in intensifying the vascularization of those same

structures, thus allowing the entrance of nutrients. VEGF is related to the action of LH and angiopoietin produced in the luteinizing cells (29–31). Therefore a disturbance in LH activity may explain our results. Moreover, VEGF is related to several cytokines that could be involved in the accelerated degeneration of the corpus luteum, leading to decreased P production (32). It should be emphasized that accelerated degeneration of the corpus luteum and degenerated follicles in rats may take longer than three consecutive estrus cycles to be detected. This may explain the decrease in the number of luteal bodies in pinealectomized rats. Therefore, the formation of these structures as well as related degeneration may affect the cell population in pinealectomized rats.

PCNA is influenced by the activity of steroids: E increases PCNA production whereas P may decrease it (33). The present data suggest that melatonin diminishes PCNA expression in ovarian cells. One hypothesis is that this effect is due to the rise in P production (33). In our experiment, melatonin is seemingly linked to an increase in P production and PR expression, which may point to a greater effect of melatonin on rat ovaries. One possible explanation is that this hormone acts indirectly to increase levels of P and PR.

Melatonin may also act through ovarian MT1 receptor. In fact, a few authors (14) showed that melatonin was an antiproliferative agent, reducing PCNA expression in hormone-dependent tumors in mouse prostate glands, both in cell culture and *in vivo*. Those authors suggested that the effect of melatonin might be due to the link between melatonin MT1 receptor and neoplastic cells. Blockage of these receptors increases proliferation and PCNA in the cells of the prostate gland. Other authors have suggested that the mechanism of melatonin action was a reduction in the activity of oncogenes such as c-myc and c-jun, which are related to cell mitosis. This process would involve a reduction in cyclic adenosine monophosphate (cAMP), in turn affecting cell signaling (34). In the ovary, T and E increase cAMP, stepping up proliferation (mainly in the theca and stroma cells), whereas melatonin may reduce this second messenger in ovarian tissue (35).

Finally, the present findings suggest that melatonin may attenuate proliferation in ovarian structures, such as epithelial and stromal cells. Melatonin treatment may also increase the number of luteal bodies as well as levels of P and its receptor. Therefore melatonin may play a role in ovarian function.

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