Effect of Melatonin on Endometrial Proliferation in Ovariectomized Female Rats

R. Ciortea[1], N. Costin[1], Daria Maria Groza[1], R. Mocan[1], S. Şuşman[2], Cosmina Bondor[3], Carmen Mihaela Mihu[2], D. Mihu[1]

1. Department of Obstetrics and Gynecology II, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca;
2. Department of Histology, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca;
3. Department of Biostatistic&Medical Informatic, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca

Correspondence:
Dr. Răzvan Ciortea
e-mail: r_ciortea@yahoo.com

Presentation:
In ovariectomized female rats the combined treatment of estrogen and melatonin compared to estrogen replacement treatment induces a decrease in endometrial proliferation and prevents the appearance of cellular atypias, in ovariectomized female rats.

Abstract

In the context of endometrial cancer, visceral obesity as a risk factor is associated with a chronic inflammatory process, confirmed by the increase in inflammatory marker levels. At 14 days post-ovariectomy, a time period required for the post-operative validation of ovarian failure, with the experimental induction of artificial menopause, the animals included in the study received estrogen replacement treatment and combined treatment of estrogen and melatonin. The duration of the administered treatment, with products and doses recommended for veterinary use, was 12 consecutive weeks. The combined treatment of estrogen and melatonin compared to estrogen replacement treatment induces a decrease in endometrial proliferation and prevents the appearance of cellular atypias. The presented results suggest that melatonin supplementation can play an important role in the prophylaxis of endometrial cancer in menopause.
Keywords: *melatonin, cancer, endometrial proliferation, rats*

Introduction

Intraabdominal obesity is considered a low level chronic pro-inflammatory state. The adipocyte is the central element that integrates multiple metabolic and endocrine signals. This cell is the source of a large number of bioactive peptides that play an essential role in the modeling of insulin resistance and inflammation: TNFα (tumor necrosis factor), resistin, adipsin, leptin, adiponectin, angiotensin, prostaglandins, IL6 (interleukin).

Inflammatory cells, the production of proinflammatory cytokines (TNFα, IL6, PCR) play an important role in the genesis of endometrial cancer. In the context of endometrial cancer, visceral obesity as a risk factor is associated with a chronic inflammatory process confirmed by the increase in inflammatory marker levels[1].

Melatonin plays a role in neuroendocrine regulation, the increase of immunity, the neutralization of free radicals, the reduction of angiogenesis, the increase of apoptosis, studies on animals and humans demonstrating that melatonin has important oncostatic properties. Blood melatonin levels are reversely correlated with the tumor proliferation index in patients with endometrial cancer[2].

The aim of this study is the experimental exploration of the effects of melatonin and melatonin associated with estrogen on endometrial proliferation in female rats with surgically induced menopause.

Material and method

The researches were performed in white female Wistar rats, with a weight of 160-200 g, from the biobase of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca. The animals were kept under adequate standard zoo-hygienic vivarium conditions: at an environmental temperature of 22±1°C, standardized food and water "ad libitum", with a light-dark cycle of 12 hours.

Motivation of experimental researches in rats:

- Rats are animals that are currently used in the laboratory for experimental models that can be transposed to humans.

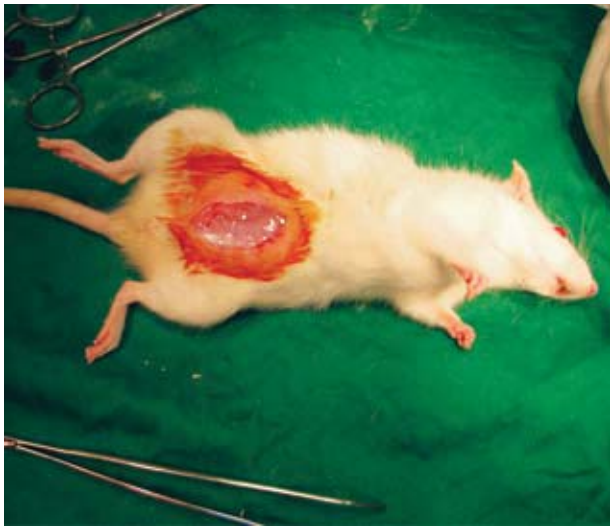


Figure 1. Longitudinal abdominal incision

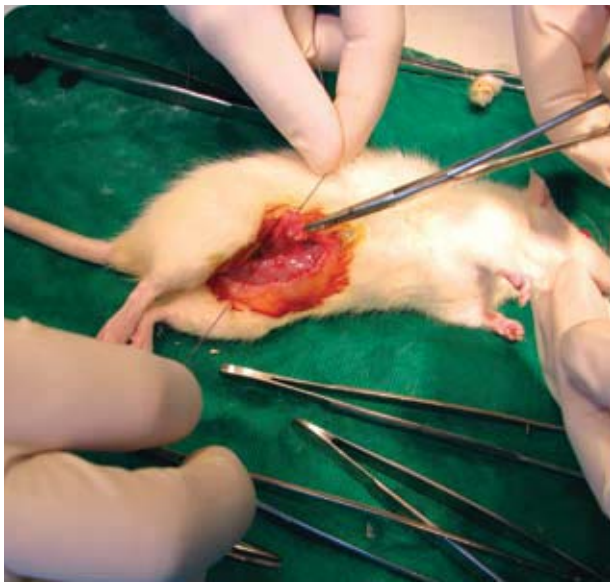


Figure 2. Ligature of the lumbo-ovarian ligament

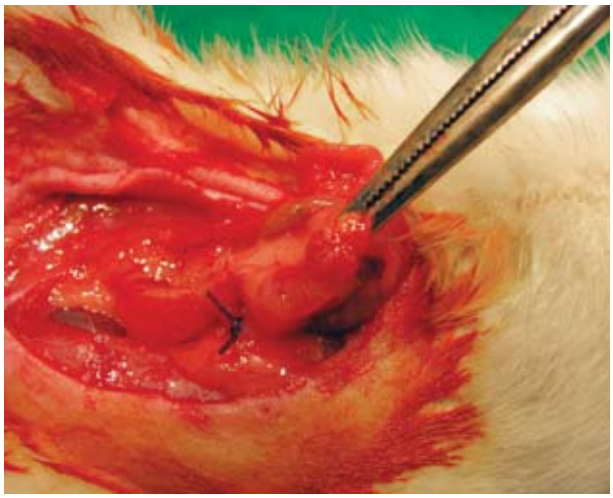


Figure 3. Aspect after unilateral ovariectomy

- Female rats have a short gestation period (approx. 21-25 days) and a life duration of 2-3 years (1 year of life is equivalent to about 30 years of life in humans).
- Female rats reach sexual maturity at 4-5 months (corresponding to the age of 12-14 years in women), when they reach a weight of 160-180 g.

The technique of ovariectomy and anesthesia

The animals were completely anesthetized for 30-60 min with an anesthetic mixture:

- 2/3 ketamine (Ketaminol 10, for veterinary use: 1ml/kg body weight)
- 1/3 xylazine (Xylocontact 50mg/ml), for veterinary use: 0.5 ml/kg body weight)

Bilateral surgical ovariectomy was performed by abdominal approach, applying the technique used in women adapted for female rats.

The operative technique consisted of:

- The narcotized animal was immobilized on the intervention table, after which complete epilation of the abdominal region and partial inguinal epilation was carried out;
- Disinfection with Povidone-iodine (Betadine), 10% solution;
- Longitudinal cutaneous-muscular abdominal incision, at a distance of 2 cm superior to the vulvar area (Figure1);
- Detachment of the parietal peritoneum and its longitudinal section over a 2 cm length corresponding to the white line at the level of the cross section;
- Identification of internal genital organs: in female rats, the uterus has two well developed uterine horns, thin uterine tubes, in the prolongation of uterine horns, and ovaries having the size of a maize grain, situated at the distal end of the uterine tube;
- Successive identification of uterine tubes and ovaries for ovariectomy;
- Surgical subovarian ligature with resorbable 2-0 (Vicryl 2-0) polyglycolic acid suture involving the ligature of the tube and lumbo-ovarian ligament, followed by the excision of the ovary (Figures 2 and 3);
- Introduction of the rest of genital organs in the abdomen;
- Control of hemostasis;
- Washing of the peritoneal cavity, followed by the application of an intraperitoneal antibiotic;
- Suture of anatomical planes in reverse order compared to incision (continuous muscle layer suture, interrupted skin suture);
- Antibiotic administered postoperatively: Ampicillin + Sulbactam daily dose 0.05 g/kg body weight, for 3 days, subcutaneously, in the lateral flank of the animal;
- Application of Azocillin to the operative wound (because the organoleptic properties of this ointment - taste - are not accepted by the rat, and in this way the animals will not tear the sutures) (Figures 4, 5, 6);

- Daily postoperative application of Betadine and Azocillin to the wound;
- Removal of sutures on postoperative day 7.

Groups

At 14 days postovariectomy, a time period required for the postoperative validation of ovarian failure, with the experimental induction of artificial menopause in the studied animals, estrogen replacement treatment (estrogen monotherapy) and combined treatment of estrogen and melatonin were initiated. The duration of the administered treatment, with the products and the doses recommended for veterinary use, was 12 consecutive weeks.

The ovariectomized animals were assigned to 5 groups of 10 animals each:

- **Group I** - control group - healthy ovariectomized animals (surgically induced menopause), without estrogen or melatonin administration;
- **Group II** - healthy ovariectomized animals (surgically induced menopause), receiving estrogen replacement monotherapy (estradiol benzoate - E2b - 10µg/day intramuscular injections for 12 weeks, 5 days/week);
- Group III - healthy ovariectomized animals (surgically induced menopause), receiving estrogen therapy (estradiol benzoate - E2b - 10µg/day intramuscular injections for 12 weeks, 5 days/week) supplemented with melatonin (dissolved in 100% ethanol, added to the drinking water at a concentration of 25 µg/mL - ethanol concentration 0.01%). Melatonin was dissolved in ethanol in order to increase its solubility in water. The bottles with water were covered with aluminium foil in order to prevent photodegradation and were stored at -30°C. The solution was prepared three times a week.
- **Group IV** - healthy ovariectomized animals (surgically induced menopause), receiving estrogen therapy (estradiol benzoate - E2b - 10µg/day intramuscular injections for 12 weeks, 5 days/week) supplemented with melatonin (dissolved in 100% ethanol, added to the drinking water at a concentration of 50 µg/mL - ethanol concentration 0.01%);
- Group V - healthy ovariectomized animals (surgically induced menopause), receiving melatonin (dissolved in 100% ethanol, added to the drinking water at a concentration of 25 µg/mL - ethanol concentration 0.01%).

The commercial products used

- "**Mesalin**" (for veterinary use), vials of 5 ml (0.2 mg/ml estradiol benzoate) with administration in doses of 10µg/day intramuscular injections for 12 weeks, 5 days /week - 0.05 ml in groups II, III and IV.
- **Melatonin** (dissolved in 100% ethanol, added to the drinking water) at a concentration of 25 µg/ml in groups III and V and a concentration of 50 µg/ml in group IV.

The endometrial thickness at the level of the uterine body, as well as of the uterine horns, was ultrasonographically assessed at the initiation of the study and at its end, after 12 weeks of treatment. We mention that rats

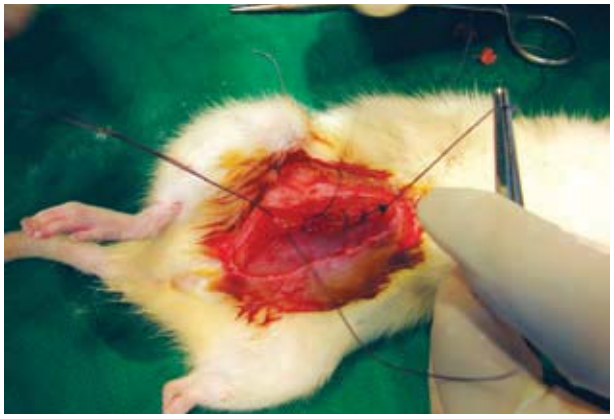


Figure 4. Suture of the muscle plane

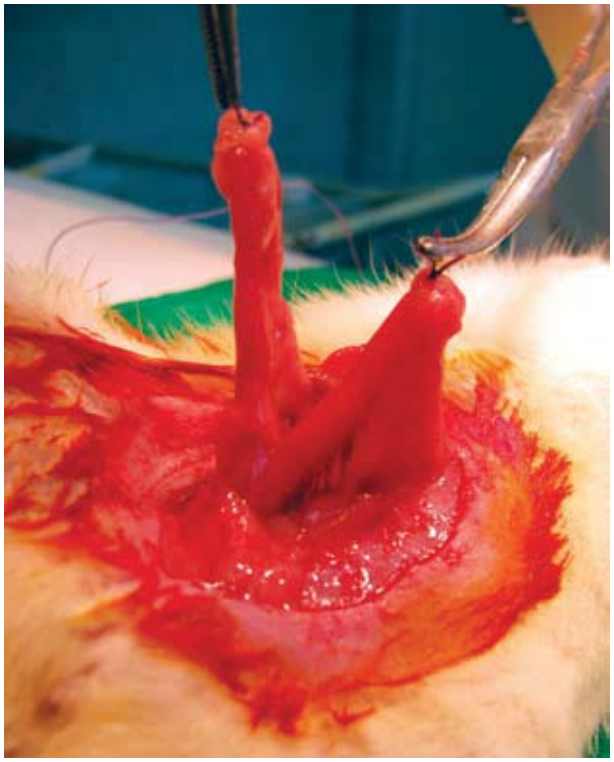


Figure 5. Aspect after bilateral ovariectomy

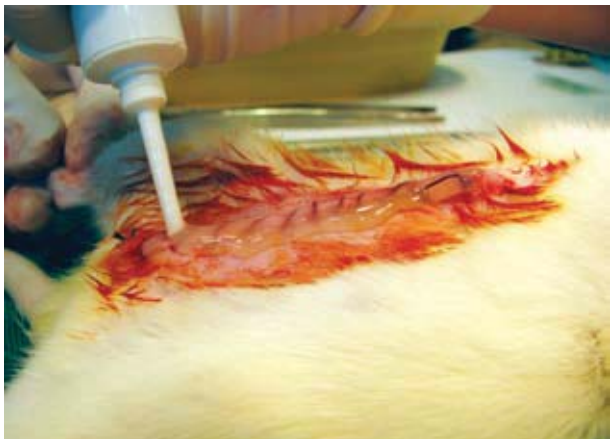


Figure 6. Application of Azocillin

are species with multiple pregnancies that develop at the level of the uterine horns, which is why the endometrium of both the uterine cavity and uterine horns should be considered. Ultrasound examinations were performed at the Department of Reproduction Obstetrics and Gynecology of the Faculty of Veterinary Medicine Cluj-Napoca, using a DC 3 VET ultrasonograph with a 10 MHz transducer.

12 weeks after the initiation of treatment, the female rats were sacrificed by the administration of a lethal dose of Arduan(Pipecuronii Bromidum). Then, the abdomen of the animals was opened along the old scar, the internal genital organs (uterus, uterine tubes) were identified and taken, being subsequently introduced in 7% formol for fixation, with a view to histopathological examination.

Statistical processing in the case of the comparison of two means for independent samples used the Student t test or the Mann-Whitney test for rank comparison. The Anova test or the Kruskal-Wallis test was also used for the comparison of the means for independent samples. For the comparison of two frequencies, the chi-square test was used. For the comparison of the means for paired samples, the Student t test or the Wilcoxon test was used.

Statistical calculations were performed using the applications SPSS 13.0, Statistica 7.0, Microsoft EXCEL. The significance threshold for the used tests was $\alpha = 0.05$.

Results

Of the 50 operated rats, 2 did not survive the anesthetic shock and died during surgery, but these were replaced by others, so that the groups could be maintained equal. Also, one of the rats of group III died, being killed by the other rats after the performance of the second ultrasound examination, because during the shaving of its abdominal hair (for a more conclusive ultrasound examination), a bleeding lesion was induced, which made the other rats in the cage extremely aggressive. The death of this rat did not affect the experiment, as it already was under the effect of hyperestrogenization.

The ultrasound examination performed at the beginning of the study did not detect significant differences in endometrial thickness at the level of the uterine cavity and uterine horns in all the animals included in the study.

The administration of estrogen to female rats with surgically induced menopause replaces the ovarian function, determining endometrial proliferation. Thus, groups I and V, in which bilateral ovariectomy was per-

Table 1 Endometrial thickness at the level of the uterine cavity assessed by ultrasound initially (at the beginning of the study)

		N	Mean	Std. Deviation	Std. Error	Minimum	Maximum	p (Kruskall-Wallis test)
Uterus initially	Group I	10	0,101	0,007	0,002	0,09	0,11	0,96
	Group II	10	0,103	0,008	0,003	0,09	0,11	
	Group III	10	0,102	0,011	0,004	0,09	0,12	
	Group IV	10	0,101	0,010	0,003	0,09	0,12	
	Group V	10	0,103	0,011	0,003	0,09	0,12	
	Total	50	0,102	0,009	0,001	0,09	0,12	

Table 2 Endometrial thickness at the level of the uterine horns assessed by ultrasound initially (at the beginning of the study)

		N	Mean	Std. Deviation	Std. Error	Minimum	Maximum	p (Kruskall-Wallis test)
Uterine horn initially	Group I	10	0,100	0,008	0,003	0,09	0,11	0,64
	Group II	10	0,106	0,010	0,003	0,09	0,12	
	Group III	10	0,102	0,012	0,004	0,08	0,12	
	Group IV	10	0,100	0,008	0,003	0,09	0,11	
	Group V	10	0,101	0,007	0,002	0,09	0,11	
	Total	50	0,102	0,009	0,001	0,08	0,12	

Table 3 Comparisons between endometrial thickness means assessed at the level of the uterine cavity at the end of the study. Group I has significantly smaller endometrial thickness than groups II, III, IV. Group II has significantly greater endometrial thickness than groups III and IV. Group I does not have significantly smaller endometrial thickness compared to group V

Group A	Group B	Group A		Group B		P
		Arithmetic mean	Standard deviation	Arithmetic mean	Standard deviation	
Group I	Group II	0,093	0,017	0,133	0,009	<0,001
Group I	Group III	0,093	0,017	0,122	0,010	<0,001
Group I	Group IV	0,093	0,017	0,119	0,007	<0,001
Group I	Group V	0,093	0,017	0,107	0,012	0,08
Group II	Group III	0,133	0,009	0,122	0,010	0,04
Group II	Group IV	0,133	0,009	0,119	0,007	0,004
Group II	Group V	0,133	0,009	0,107	0,012	<0,001
Group III	Group IV	0,122	0,010	0,119	0,007	0,58
Group III	Group V	0,122	0,010	0,107	0,012	0,01
Group IV	Group V	0,119	0,007	0,107	0,012	0,03

formed, but which did not receive hormone replacement treatment, had the smallest endometrial thickness at the level of the uterine cavity, compared to the rest of the groups that received hormone replacement treatment.

The association of melatonin with hormone replacement treatment induced lower endometrial proliferation at the level of the uterine cavity compared to the group that received hormone replacement treatment without melatonin supplementation, proliferation being reversely correlated with the administered melatonin dose. Thus, endometrial thickness assessed at the level of the uterine cavity was significantly smaller in groups III and IV compared to group II. In group IV, which received a higher melatonin dose compared to group III, endometrial thickness was statistically insignificantly smaller, probably due to the small number of individuals included in the study, which demonstrates the reverse proportionality between the administered melatonin dose and endometrial proliferation in the context of the same hormone replacement treatment.

Endometrial thickness in group V did not significantly differ compared to group I, but was significantly smaller compared to groups II, III and IV, which shows that the administration of melatonin alone (without the association of hormone replacement treatment) does not significantly influence endometrial thickness (Figure 7).

Following the administration of estrogen to female rats with surgically induced menopause, higher endometrial proliferation in the uterine horn was found compared to female rats without estrogen administration. Thus, animals of group I, menopausal but without estrogen administration, had smaller endometrial thickness of the uterine horn compared to animals of groups II, III, IV, which were menopausal and received hormone replacement treatment.

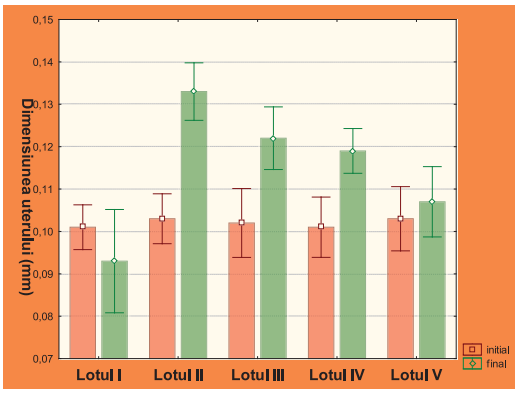


Figure 7. Comparison between initial endometrial thickness and final endometrial thickness at the level of the uterine cavity (mean ± 95% confidence interval)

The association of melatonin with hormone replacement treatment induced lower endometrial proliferation in the uterine horn, which was reversely correlated with the administered melatonin dose. Thus, the endometrial size measured at the level of the uterine horn was significantly smaller in groups III and IV compared to group II.

The administration of melatonin alone (without the association of hormone replacement treatment) significantly influences the endometrial thickness of the uterine horn, which results from the comparison of endometrial

Table 4 Comparisons between endometrial thickness means assessed at the level of uterine horns at the end of the study. Group I has significantly smaller uterine horn thickness than the other groups. Group II has significantly greater uterine horn thickness than the other groups

Group A	Group B	Group A		Group B		p
		Arithmetic mean	Standard deviation	Arithmetic mean	Standard deviation	
Group I	Group II	0,094	0,008	0,146	0,008	<0,001
Group I	Group III	0,094	0,008	0,124	0,008	<0,001
Group I	Group IV	0,094	0,008	0,120	0,007	<0,001
Group I	Group V	0,094	0,008	0,108	0,009	0,004
Group II	Group III	0,146	0,008	0,124	0,008	<0,001
Group II	Group IV	0,146	0,008	0,120	0,007	<0,001
Group II	Group V	0,146	0,008	0,108	0,009	<0,001
Group III	Group IV	0,124	0,008	0,120	0,007	0,35
Group III	Group V	0,124	0,008	0,108	0,009	<0,001
Group IV	Group V	0,120	0,007	0,108	0,009	0,01

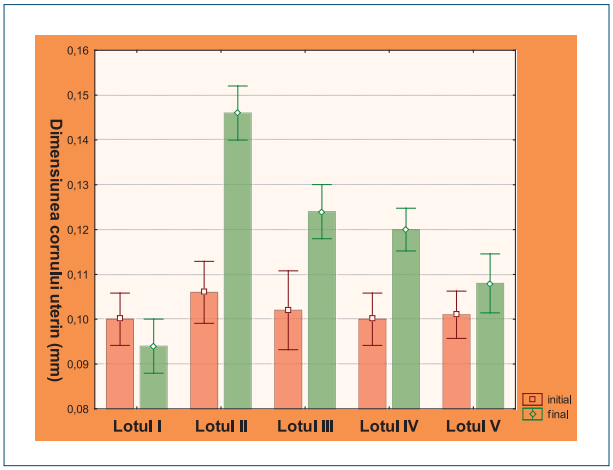


Figure 8. Comparison between initial endometrial thickness and final endometrial thickness of the uterine horn (mean ± 95% confidence interval)

thickness between groups I and V. Unlike the uterine cavity endometrium, which is not influenced by melatonin administered alone, the uterine horn endometrium is significantly influenced by melatonin administration (Figures 8, 9, 10, 11).

The results obtained by ultrasound examination were confirmed by histopathological examination. Thus, animals of groups I and V had endometrial atrophy at the level of both the uterine cavity and uterine horns. In animals of groups II, III and IV, histopathological examination identified endometrial hyperplasia. While in animals of groups III and IV simple or complex endometrial hyperplasia without atypias was detected, in animals of group II complex endometrial hyperplasia with atypias was found. In group II, the presence of a simple serous cyst was found, and in another animal, the presence of an endometrioid adenocarcinoma was detected. An animal of group V developed multiple abscesses located in the uterine tubes (Figures 12, 13, 14, 15, 16).

Discussion

This study was performed in order to extend the previous findings of the literature supporting the oncostatic effect of melatonin and its implication in the pathogenesis of breast cancer, colorectal cancer, prostate cancer, melanoma[3,4,5,6,7]. We also tried to determine whether melatonin administered in association with estrogen as part of hormone replacement treatment reduces the risk of endometrial proliferation.

The endometrium is a tissue submitted to hormonal influences. Under the action of estrogens, a cellular increase and glandular proliferation are identified, which are cyclically counteracted by the effect of progesterone. Although endometrial cancer as well as breast cancer is known to be estrogen dependent, the effect of normal melatonin concentrations on endometrial cancer has not yet been quantified. A deficient pineal gland function is considered a risk factor for the development of endometrial cancer, because melatonin has antiestrogenic properties[8].

These antiestrogenic properties of melatonin are also shown by the present study, in which the association of melatonin with hormone replacement treatment causes lower endometrial proliferation compared to the group with hormone replacement treatment without melatonin, proliferation that is reversely correlated with the administered melatonin dose. This study supports the antiestro-

genic effect of melatonin both by the results obtained on ultrasound examination and the anatomo-pathological aspect of the specimens (uterus, uterine horns), interpreted at 12 weeks from the initiation of treatment.

Cell culture studies have demonstrated that melatonin has different antiproliferative effects on various types of cancer cells, which differ between them by the status of estrogen receptors. Melatonin had no antiproliferative action on the SNG-II cells (without estrogen receptors), but had a significant antiproliferative effect on the Ishikawa cells (with estrogen receptors) at different cell intensities and different incubation times[9].

There are two possibilities by which estrogen inhibits the antiproliferative effect of melatonin: estrogen can compete with melatonin as a ligand to a common receptor or each ligand has its own receptor that interact with each other (by other mechanisms than those related to its own receptor). However, there are also additional, estrogen independent mechanisms that may involve melatonin receptors[10]. Melatonin receptors (MT2) have been identified in the membrane of tumor cell lines responsive to estrogens and it has been demonstrated that the antiproliferative effect of melatonin is mediated by these receptors[9,10].

The reduction of oxidative stress, the increase of apoptosis, the diminution of angiogenesis, the immunomodulating effect are some of the mechanisms that ensure the oncostatic effect of melatonin.

Studies in rats demonstrate that melatonin treatment reduces body weight, intraabdominal adiposity, plasma cholesterol, triglyceride, leptin and insulin levels, all these being considered risk factors for endometrial cancer[11,12,13].

Intraperitoneal adiposity can be considered a low level chronic proinflammatory state, which in its turn is a risk factor for endometrial cancer[14,15]. The reduction of intraperitoneal adiposity in animals treated with melatonin can be explained by a melatonin induced increase in the body temperature. Lipoprotein lipase (an enzyme necessary for the storage of fatty acids in adipocytes) has an increased activity when it is incubated at lower temperatures. This observation has led to the hypothesis that the accumulation of visceral adipose tissue increases with the decrease in the body temperature[16,17].

Conclusions

1. The association of melatonin with hormone replacement treatment administered to female rats with surgically induced menopause determines lower endometrial proliferation and prevents the appearance of cellular atypias.

2. The presented results suggest that melatonin supplementation can play an important role in the prophylaxis of endometrial cancer in menopause.

3. The clinical ramifications of this study require additional investigations in order to determine whether the melatonin level can be a useful biochemical marker in the prevention, diagnosis, treatment and prognosis of endometrial cancer. ■

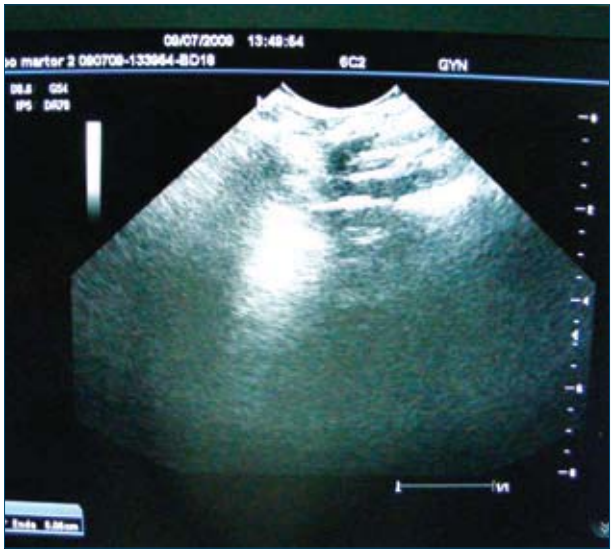


Figure 9. Uterine horn endometrium group I

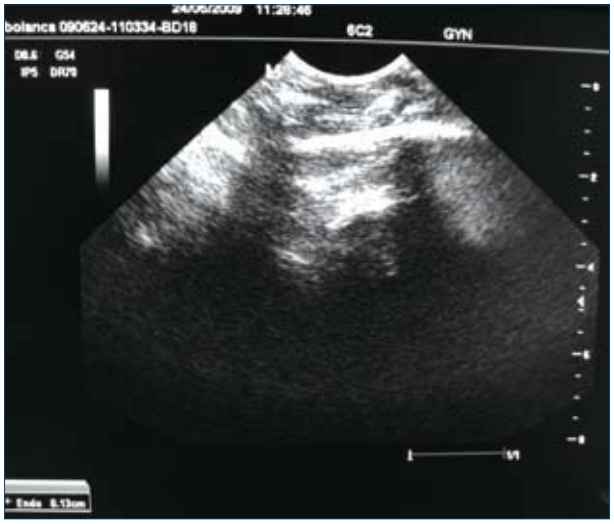


Figure 10. Uterine horn endometrium group IV

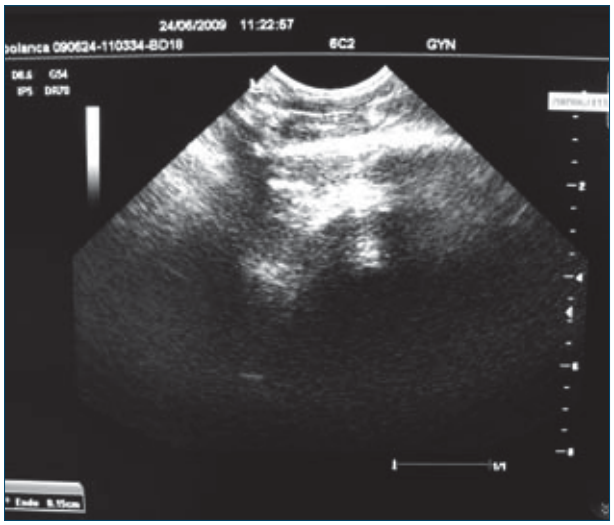


Figure 11. Uterine horn endometrium group II

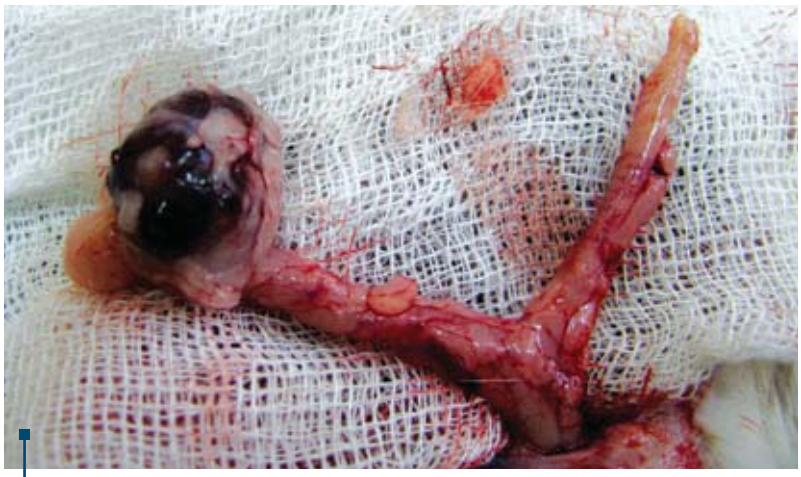


Figure 12. Macroscopic aspect - uterus and uterine horns in group III rat (left) compared to uterus and uterine horns in group II rat (right)

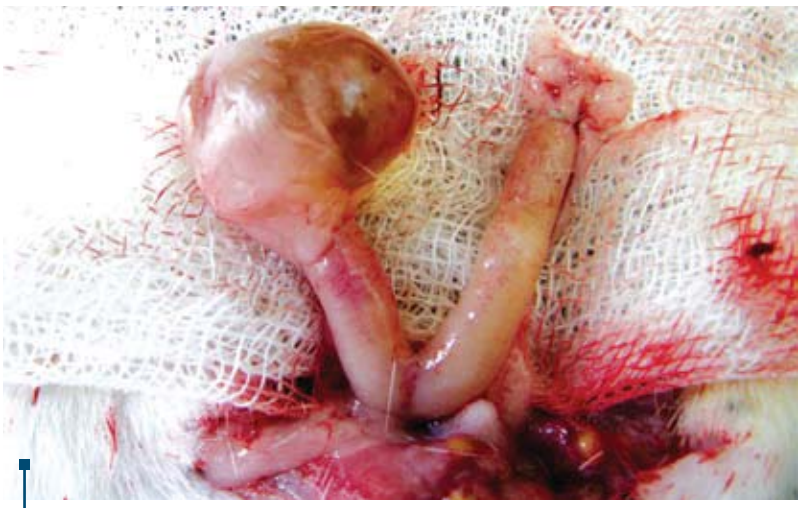


Figure 13. Endometrioid adenocarcinoma (animal of group II)

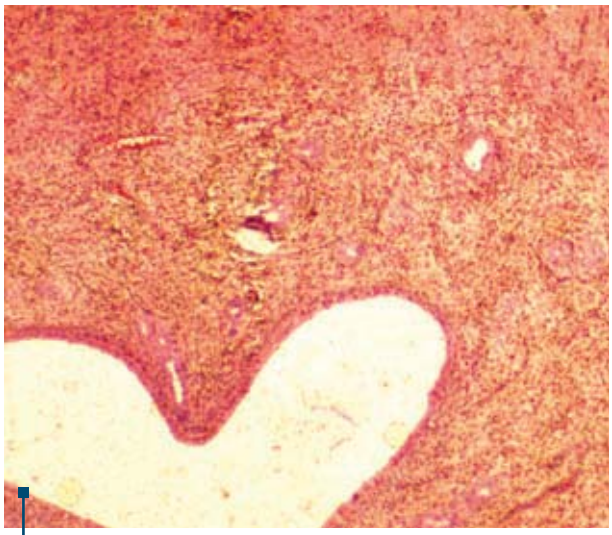


Figure 15. Simple endometrial hyperplasia (animal of group IV)

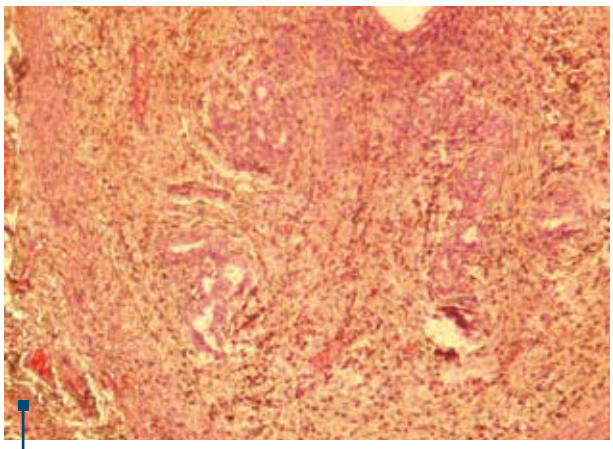
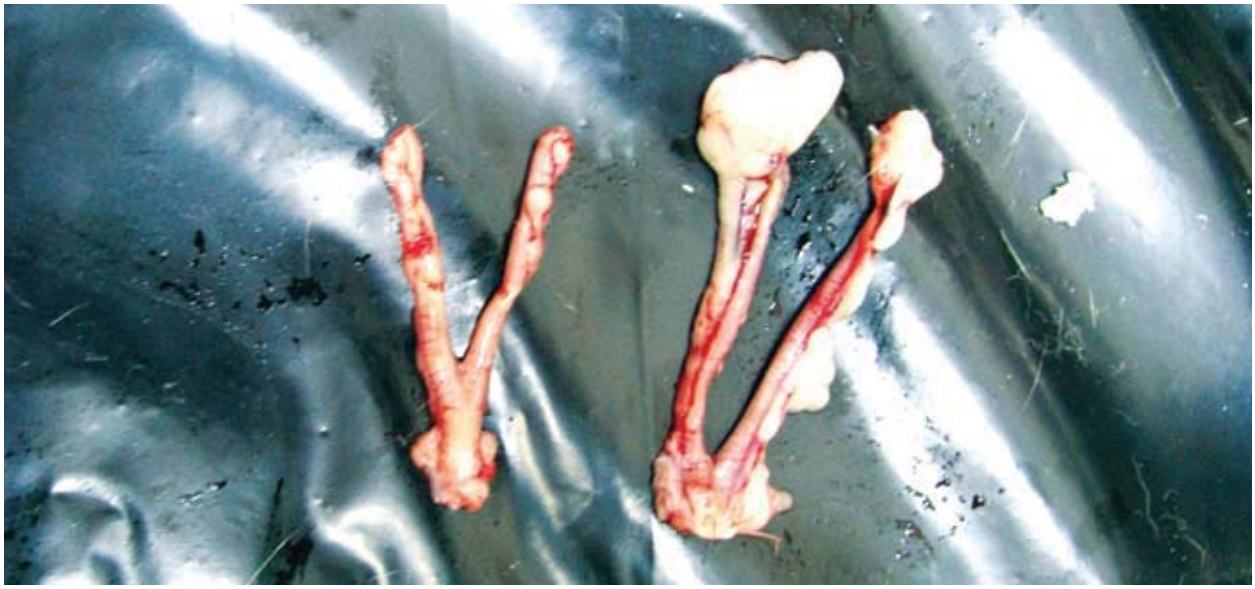


Figure 16. Complex endometrial hyperplasia with atypias (animal of group II)

Figure 14. Simple serous cyst (animal of group II)



References

1. A.S. Greenberg, M.S. Obin. Obesity and the rol of adipose tissue in inflamation and metabolism. The Am. J. of CliClnical Nutrition 2006;2:112-116.
2. Kanishi Y., Kobayashi Y., Noda S., Ishizuka B., Saito K: Differential growth inhibitory effect of melatonin on two endometrial cancer cell lines.J Pineal Res.2000;28(4):227-33.
3. Stevens R.G., Rea M.S.: Light in the built environment: potential role of circadian disruption in endocrine disruption and breast cancer. Cancer Causes Control. 2001;12:279-287.
4. Blask D.E., Dauchy R.T., Sauer L.A., Krause J.A., Brainard G.C.:Growth and fatty acid metabolism of human breast cancer(MCF-7) xenografts in nude rats: impact of constant light induced nocturnal melatonin suppression. Breast Cancer Res Treat. 2003;79:313-320.
5. Farriol M., Venereo Y., Orta X., Castellanos J.M., Segovia-Silvestre T.:In vitro effects of melatonin on cell proliferation in a colon adenocarcinoma line.J Appl Toxicol. 2000; 20(1):21-4.
6. Sainz R.M., Mayo J.C., Tan D.X., León J., Manchester L., Reiter R.J.: Melatonin reduces prostate cancer cell growth leading to neuroendocrine differentiation via a receptor and PKA independent mechanism.Prostate. 2005;63(1):29-43.
7. Walker M.J., Chaudhuri P.K., Beattie C.W., et al: Neuroendocrine and endocrine correlates to hamster melanoma growth in vitro. Surg Forum. 1998. 29:151-152.
8. Karasek M., Kowalski A.J., Zylinska K. Serum melatonin circadian profile in women suffering from the genital tract cancers.Neuro Endocrinol Lett. 2000;21(2):109-113.
9. Kanishi Y., Kobayashi Y., Noda S., Ishizuka B., Saito K: Differential growth inhibitory effect of melatonin on two endometrial cancer cell lines.J Pineal Res.2000;28(4):227-33.
10. Gerdin M.J.,Masana M.I.,Dubocovich M.L.:Melatonin-mediated regulation of human MT(1) melatonin receptors expressed in mammalian cells.Biochem Pharmacol.2004;67(11):2023-30.
11. Wolden-Hanson T, Mitton DR, McCants RL, Yellon SM, Wilkinson CW, Matsumoto AM, Rasmussen DD Daily melatonin administration to middle-aged male rats suppresses body weight, intraabdominal adiposity, and plasma leptin and insulin independent of food intake and total body fat. Endocrinology 2000;141(2):487-97.
12. Puchalski SS, Green JN, Rasmussen DD Melatonin effect on rat body weight regulation in response to high-fat diet at middle age Endocrine 2003;21(2):163-7.
13. Rasmussen DD, Boldt BM, Wilkinson CW, Yellon SM, Matsumoto AM Daily melatonin administration at middle age suppresses male rat visceral fat, plasma leptin, and plasma insulin to youthful levels Endocrinology 2002;143(4):1269.
14. Bray G.A., The Underlying Basis for Obesity: Relationship to Cancer, Journal of American. Society for Nutrition Sciences 2002; 3451S-3455S.
15. Francesmary M, Ness R.B., Chu Chen, Weis N.S., Inflamation and Endometrial Cancer:A Hypotesis, Cancer Epidemiol Biomarkers & Prev 2005;12:2840-2847.
16. Speake BK, Parkin SM, Robinson DS Degradation of lipoprotein lipase in rat adipose tissue.Biochim Biophys Acta. 1984;840(3):419-22.
17. Kahn HS The accumulation of visceral adipose tissue may be influenced by intra-abdominal temperature Obes Res. 1996;4(3):297-9.