

# Melatonin treatment results in regression of endometriotic lesions in an ooforectomized rat endometriosis model

## *Melatonin tedavisi ooforektomize sıçanlar endometriozis modelinde endometriyotik odaklarda gerilemeye yol açar*

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### Abstract

**Objective:** We aimed to determine the effects of melatonin treatment on endometrial implants in an oophorectomized rat endometriosis model.

**Material and Methods:** This study is a prospective, randomised, controlled experimental study. It was carried out at the Experimental Research Center of Yeditepe University (YUDETAM). Twenty-two, female, non-pregnant, nulligravid Sprague-Dawley albino rats were included in our study. Endometriosis was surgically induced in oophorectomized rats. Rats were randomised into two groups: control group and melatonin group. In the melatonin group, rats were treated with melatonin (20 mg/kg/day) for two weeks. After the operations were performed to assess the regression of the endometriotic lesions, melatonin treatment was stopped. At the end of the sixth week necropsies were performed to assess the rate of recurrence. The volume and histopathological scores of endometriotic foci were examined.

**Results:** Volumes of the endometriotic lesions significantly decreased in the melatonin group. Also, when the melatonin group was analysed within itself, endometriotic lesion volumes decreased and histopathological scores increased significantly.

**Conclusion:** Melatonin causes regression of the endometriotic lesions in rats and improvement in their histopathological scores.

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**Key words:** Endometriosis, oophorectomized rats, volume, histopathological score, melatonin

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### Özet

**Amaç:** Ooforektomize sıçan endometriozis modelinde melatoninin endometriyotik odaklar üzerine etkisini incelemeyi amaçladık

**Gereç ve Yöntemler:** Bu çalışma prospektif, randomize, kontrollü, deneysel bir çalışmadır. Çalışma Yeditepe Üniversitesi Deneysel Araştırma Merkezinde (YUDETAM) yapıldı. Deneysel çalışmamıza 22 adet dişi, gebe olmayan, nulligravid Sprague-Dawley albino sıçan dahil edildi. Ooforektomize edilen sıçanlarda cerrahi olarak endometriozis oluşturuldu. Sıçanlar iki gruba ayrıldı: kontrol grubu ve melatonin grubu. Melatonin grubunda 2 hafta boyunca sıçanlara melatonin (20 mg/kg/gün) tedavisi verildi. Endometriyotik regresyonu değerlendirmek için operasyonlar yapıldıktan sonra melatonin tedavisi sonlandırıldı. Rekürrens oranlarını değerlendirmek için altıncı haftanın sonunda nekropsiler yapıldı. Endometriyotik odakların hacim ve histopatolojik skorları değerlendirildi.

**Bulgular:** Endometriyotik lezyonların hacimleri melatonin grubunda anlamlı derecede azaldı. Ayrıca, melatonin grubu kendi içerisinde incelendiğinde endometriyotik lezyonların hacimlerinin anlamlı derecede azaldı ve histopatolojik skorları anlamlı derecede arttı.

**Sonuç:** Melatonin sıçanlarda endometriyotik odakların gerilemesine ve histopatolojik skorların iyileşmesine neden olur.

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**Anahtar kelimeler:** Endometriozis, ooforektomize sıçan, hacim, histopatolojik skor, melatonin

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### Introduction

Endometriosis is the presence of endometrial tissue with glands and stroma outside the uterus (1). It is a benign disease, but its clinical spectrum varies widely. It is associated

with both pelvic pain and infertility and is prone to progression and recurrence.

Several pathogenic mechanisms including retrograde menstruation and implantation, coelomic metaplasia, direct transplantation, and vascular dissemination have been proposed



in the aetiopathogenesis of endometriosis. However, no one mechanism explains all cases of endometriosis and each is thought to contribute, at least to some extent (2).

Sampson's theory of retrograde menstruation is considered the most commonly accepted theory for the development of the disease, at least in its form of peritoneal implants (3). Although retrograde menstruation occurs in at least 76-90% of women undergoing peritoneal dialysis and laparoscopy, the prevalence of endometriosis is much lower (6.2-8.2%) (4-7). These findings suggest that other factors must determine the susceptibility to developing endometriosis.

It was stated that the ability of endometrial implants to survive in ectopic locations may be due to an aberrant immune response (8). Oxidative stress also has been proposed as a potential factor in the pathophysiology of the disease (9). Inducers of oxidative stress may include erythrocytes, apoptotic endometrial cells, and undigested endometrial cells in the menstrual effluent (10).

Several studies have indicated that antioxidant defences may be altered in endometriosis, as suggested by the aberrant expression of endometrial antioxidant enzymes and lower levels of the antioxidant vitamin E in peritoneal fluid (11-14).

Endometriosis has the unique characteristics of invasion and requires the remodelling of the extracellular matrix (ECM) (15-17). Matrix metalloproteinases (MMPs) are a series of zinc-requiring proteolytic enzymes that are involved in the remodelling and degradation of the ECM. Derangement of MMP regulation is considered to be a critical factor in the development of pathological conditions like endometriosis (18, 19).

Recently, Collette et al. (20) reported that eutopic endometrium of women with endometriosis shows increased activity of MMP-9. Studies also demonstrated that MMP-3 expression is elevated in ectopic endometrial tissues of rats surgically induced to develop endometriosis (21, 22).

Matrix metalloproteinase activity can be modulated by oxidative stress (23, 24). Both MMP-2 and MMP-9 are activated by reactive oxygen species (ROS), and their expression seems to be regulated by oxidant stress (25). The role of ROS in increasing the growth and adhesion of endometrial cells in the peritoneal cavity during endometriosis has been documented (26, 27). Administration of antioxidant enzymes like superoxide dismutase and catalase has been shown to prevent intraperitoneal (i.p.) adhesions of endometriotic tissues in the peritoneal cavity of rabbits (28).

Melatonin (N-acetyl-5-methoxy-tryptamine) is the main pineal hormone synthesised from tryptophan, predominantly during the night (28). Melatonin is critical for the regulation of circadian and seasonal changes in various aspects of physiology and neuroendocrine function (29, 30).

Melatonin is a documented powerful free radical scavenger and a broad-spectrum antioxidant (31). Therefore, it may interfere with the oxidative stress seen in endometriosis.

It may also have an impact on the extracellular matrix remodelling seen in this disease, through the regulation of the zinc-requiring proteolytic enzymes MMPs, as there is indirect evidence that melatonin inhibits the production of adhesion molecules that promote the binding of leukocytes to endo-

thelial cells (32-34). Therefore, melatonin may be an effective treatment modality in endometriosis.

In this study, we investigated the efficacy of melatonin in an oophorectomized rat endometriosis model with continuous high dose exogenous oestrogen administration.

## Material and Methods

Twenty-two reproductive aged female non-pregnant, nulligravid Sprague-Dawley albino rats weighing 200-250 gr, which were bred at Yeditepe University Experimental Research Center (YUDETAM), were used in this study.

The rats were caged individually in a controlled environment. The room temperature was 21°C and humidity was 60%. Rats were fed ad libitum with 12 hour light/dark cycles.

After the induction of endometriosis according to experimental animal model, rats were randomised into two groups (control and 20 mg/kg/day Melatonin group). There were 10 rats in the melatonin group and 12 rats in the control group.

Rats in both groups were administered high dose oestrogen (50 µg/kg; twice weekly) until the end of the study (6 weeks). Melatonin was only given for two weeks to visualise melatonin-related endometriotic implant regression and recurrence.

Oophorectomy was performed to stabilise and standardise the oestrogen levels in rats throughout the study.

This study was approved by the Experimental Animals Ethics Committee of Yeditepe University. All experiments were performed in compliance with international guidelines on the ethical use of animals. Throughout the study period animals were controlled daily by a veterinary doctor and her assistant.

### Surgical techniques

All rats had four operations. In the first operation after oophorectomy, endometriosis was induced by using homologous uterine horn autotransplantation. After two weeks of oestradiol treatment the second operations were performed to visualise endometriotic lesion formation. Then, rats were randomised using a randomisation table into the control and melatonin groups. Melatonin was administered only to the treatment group for two weeks. The third operations were performed for the assessments of the effects of melatonin on the endometriotic foci. After this inspection melatonin was stopped. Two weeks later (at the end of the sixth week) all of the rats were euthanised and the recurrence of endometriosis after melatonin effects was compared to the control group.

### First operation: Endometriosis induction

All rats were anaesthetised with an intramuscular administration of 60 mg/kg ketamine hydrochloride (Ketalar; Eczacıbaşı İlaç Sanayi, İstanbul, Turkey) containing with 7 mg/kg xylazine hydrochloride (Rompun; Bayer İlaç Sanayi, İstanbul, Turkey) as described previously (35-37). Endometriosis was induced surgically under anaesthesia as proposed by Vernon and Wilson, with modifications by Lebovic and Uygur et al. (38-40). After administration of general anaesthesia, the abdominal cavity was opened using a vertical incision. Ovaries and

uterine horns were visualised and all were removed by en block resection. Cauterisation was performed for coagulation, and uterine horns and ovaries were placed in phosphate-buffered saline at 37°C. Ovaries were separated from uterine horns, and parametrial fatty tissue was discarded. Cylindrical-shaped uterine horns were held and cut longitudinally to expose the inner endometrial covered layer. Four pieces of grafts measuring 6x3x1 mm were made by division of the uterine horns. Two of these were implanted with 6/0 non-absorbable polypropylene suture on the peritoneum, where bifurcation of the abdominal wall vasculature was located in the right hypochondrial area. The others were implanted on the left site. During implantation, endometrial surfaces of grafts were attached to the peritoneum. The peritoneal cavity was kept moist with sterile saline solution throughout the surgery. The midline abdominal incision was closed in a continuous interlocking manner with 3-0 vicryl sutures. All rats were given 50 mg/kg/day cefazolin sodium (IE Ulagay İlaç Sanayi, İstanbul, Turkey) intramuscularly for 3 days after the operation. Also, oestrogen (Oestradiol powder, ≥%98, Sigma-Aldrich) treatment was given to all rats (50 µg/kg) two times per week subcutaneously until the end of study.

#### Second operation: Assessment of the endometriotic foci

Two weeks after the first operation (endometriosis induction), the second was performed to assess the endometriotic lesions. Exogenous high dose oestrogen created a hyper-estrogenic state and resulted in well-defined endometriotic lesions. The second operations were performed using the aforementioned methodologies. Before endometriotic lesions were biopsied, all of the implants were measured by the same author (N.C.) in three dimensions (length - width - height in millimetres) using a ruler.

One of the four implants was removed for histopathological analysis using a randomisation table. After this operation, rats were randomised into two groups: control and melatonin treatment groups; 20 mg/kg/day melatonin treatment was administered using intramuscular and intraperitoneal routes in the treatment group. Melatonin was given for 2 weeks (until the third operation).

#### Third operation: The effects of melatonin

In the third operation, which was performed after melatonin treatment (at the end of 4<sup>th</sup> week), volumes of endometriotic foci were measured as previously described. One of the endometriotic lesions was randomly collected for histopathological examination. Melatonin treatment was stopped and oestrogen administration was continued.

#### Fourth operation-necropsy: Evaluation of recurrence

The recurrence of endometriosis was evaluated after cessation of melatonin; during the last two weeks, rats were administered only oestradiol. All rats were euthanised under general anaesthesia and all measurements and endometriotic foci collections (all rest foci) were performed as described before.

#### Volume analysis

The spherical volume of each ectopic uterine tissue was calculated using the prolate ellipsoid formula:  $V \text{ (mm}^3\text{)} = 0.52 \times \text{length} \times \text{width} \times \text{height}$  (all in millimetres) (35, 40).

#### Histopathological analysis

Endometriotic biopsy samples were fixed in 10% neutral buffered formaldehyde solution. After dehydration, all pieces were embedded in paraffin. 3 µm thick sections were made with a microtome. Samples were stained with haematoxylin and eosin (HE), and were examined under a light microscope. The pathologist (FO) who assessed the samples was blinded to the treatment groups. Epithelial cells in the implants were evaluated as described by Keenan et al. (41). Based on this scoring system, lesions were given points as following:

- Point 3: if epithelial surface layers were well preserved,
- Point 2: if moderate leukocytic infiltration was seen,
- Point 1: poor epithelial lining,
- Point 0: if no epithelial lining was seen.

#### Statistical analysis

Statistical analysis was performed by using SPSS, version 11.5 (SPSS Inc., Chicago, IL, USA) for windows. Data were expressed as mean ± standard error of mean. When these parameters were compared between the groups, Kruskal-Wallis Test with Mann-Whitney U test as a post-hoc test was performed. Friedman's Test with Wilcoxon as a post hoc test was used for the evaluation of lesion volumes and histopathological scores throughout the study in each group.  $p < 0.05$  was considered as statistically significant.

#### Results

We operated upon twenty-two rats and implanted 88 uterine graft tissues. Endometriotic foci were seen in each of the implanted grafts at the second operation. No graft failure was seen. Throughout the study there were no complications related to operations or toxic effects of melatonin. Some of the endometriotic foci were cystic in nature and the others were hyper-pigmented thickened lesions.

#### Comparison of the lesions between control and melatonin groups

During the second operation, which was performed 2 weeks after endometriosis induction, mean lesion volumes were (Volume A)  $108.2 \text{ mm} \pm 17.5$  (SEM) and  $124.5 \text{ mm} \pm 14.8$  (SEM), and histopathology scores were (Histology A)  $1.7 \pm 0.1$  (SEM) and  $2.2 \pm 0.2$  (SEM) in the melatonin and control groups, respectively (Table 1). There were no statistically significant differences in the lesion volumes or histopathology scores between the groups.

After this operation, 20 mg/kg/day melatonin treatment was administered using intramuscular and intraperitoneal routes in the treatment group. Melatonin was given for 2 weeks (until the third operation).

Two weeks after melatonin treatment the third operations were performed. Mean lesions volumes were (Volume B)  $25.8 \text{ mm} \pm 3.6$  (SEM) and  $122.4 \text{ mm} \pm 23.1$  (SEM) and histopathology scores were (Histology B)  $2.2 \pm 0.2$  (SEM) and  $2.4 \pm 0.3$  (SEM)

in the melatonin and control groups, respectively (Table 1). The endometriotic lesion volumes were significantly lower ( $p=0.001$ ) in the melatonin group, while there were insignificant differences in histopathology scores between the groups. During the necropsy operations, endometriotic lesion volumes were again significantly lower in the melatonin group ( $p=0.001$ ), while there were insignificant differences in histopathology scores between the groups. Mean lesion volumes were (Volume C)  $32.7 \text{ mm} \pm 6.0$  (SEM) and  $109.2 \text{ mm} \pm 12.8$  (SEM) and histopathology scores were (Histology C)  $2.7 \pm 0.2$  (SEM) and  $2.6 \pm 0.2$  (SEM) in the melatonin and control groups, respectively (Table 1).

### Comparison of the lesions within each group

#### a. 20 mg/kg/day melatonin group

Lesion volumes of this group were (Volume A, B, C);  $108.2 \text{ mm} \pm 17.5$  (SEM),  $25.8 \text{ mm} \pm 3.6$  (SEM) and  $32.7 \text{ mm} \pm 6$  (SEM) at the second, third and fourth operations, respectively. There were statistically significant decreases in lesion volumes of this group ( $p=0.001$ ). "Volume B" values were lower than "Volume A" ( $p=0.03$ ) and "Volume C" values were lower than "Volume A" ( $p=0.03$ ). "Volume C" was insignificantly higher than "Volume B". Histopathology scores were (Histology A, B, C);  $1.7 \pm 0.1$  (SEM),  $2.2 \pm 0.2$  (SEM) and  $2.7 \pm 0.2$  (SEM) at the second, third and fourth operations, respectively. There were statistically significant increases in histopathology scores in this group ( $p=0.009$ ). "Histology C" scores were higher than "Histology A" scores ( $p=0.02$ ). "Histology B" scores were insignificantly higher than

**Table 1. Immunohistochemical staining of Vitronectin and u-PAR of adhesion tissue**

	Melatonin Group		Control Group		P
	Mean	± SEM	Mean	± SEM	
2 <sup>nd</sup> Operation					
Volume A	108.2	17.5	124.5	14.8	0.54
Histopathology A	1.7	0.1	2.2	0.2	0.26
3 <sup>rd</sup> Operation					
Volume B	25.8	3.6	122.4	23.1	0.001
Histopathology B	2.2	0.2	2.4	0.3	0.72
4 <sup>th</sup> Operation					
Volume C	32.7	6.0	109.2	12.8	0.001
Histopathology C	2.7	0.2	2.6	0.2	0.9

"Histology A" scores and "Histology C" scores were insignificantly higher than "Histology B" scores (Table 2).

#### b. Control group

Mean lesion volumes of this group were (Volume A, B, C);  $124.5 \text{ mm} \pm 14.8$  (SEM),  $122.4 \text{ mm} \pm 23.1$  (SEM) and  $109.2 \text{ mm} \pm 12.8$  (SEM); mean histopathology scores (Histology A, B, C) were  $2.2 \pm 0.2$  (SEM),  $2.4 \pm 0.3$  (SEM) and  $2.6 \pm 0.2$  (SEM) at the second, third and fourth operations, respectively (Table 2).

There were no statistically significant differences in lesion volumes (Volume A, B, C) and histopathology (Histology A, B, C) scores in the control group.

### Discussion

Endometriosis is a common disease with a prevalence of 10% in reproductive women; the prevalence of this disease is higher in infertile women (25-40%). Although it is a benign disease, it may cause severe peritoneal adhesions, pelvic distortions, secondary dysmenorrhoea, dyspareunia and infertility (42, 43). Various therapies have been used in an attempt to treat endometriosis, including ovarian suppression therapy, surgical treatment, or a combination of these strategies. The knowledge of the high local oestrogen effects on the severity of disease leads to medical hormonal suppression via progesterone, oestrogen and progesterone combinations, GnRH analogues, danazole and gestrinone (44). Aromatase inhibitors like letrozole and anastrozole are used to inhibit oestrogen formation from androgenic precursors; TNF- $\alpha$  inhibitors are used to inhibit the mechanism of inflammation which leads to propagation of disease. Anti-angiogenic substances and inhibitors of matrix metalloproteinase are used to inhibit further invasion of endometriosis through natural surfaces (44). However, the adverse effects of these treatments limit their long-term use. In addition, recurrence rates after the cessation of therapy are high, and the treatments have had no benefit in endometriosis-associated infertility (45). Therefore new medical treatments, which are as effective as hormone treatments with an improved or no side effect profile, are needed.

Melatonin is a broad-spectrum antioxidant and therefore may interfere with the oxidative stress seen in endometriosis (31). The impact of melatonin on the development of endometriosis through oxidative stress balancing was elegantly demonstrated, by a study in which pinealectomised rats with induced endometriosis developed lesions of a volume that was statistically significantly higher than seen in a control group in which

**Table 2. Summary of results within the melatonin group and control groups**

		2 <sup>nd</sup> Operation		3 <sup>rd</sup> Operation		4 <sup>th</sup> Operation		p
		Mean	± SEM	Mean	± SEM	Mean	± SEM	
20 mg/kg/day Melatonin	Volume	108.2	17.5	25.8	3.6	32.7	6.0	0.001
	Histopathology	1.7	0.1	2.2	0.2	2.7	0.2	0.009
Control Group	Volume	124.5	14.8	122.4	23.1	109.2	12.8	0.20
	Histopathology	2.2	0.2	2.4	0.3	2.6	0.2	0.14



endometriosis was induced with no previous pinealectomy (46). In the pinealectomy group, the level of MDA was statistically significantly higher, and SOD and CAT activity was statistically significantly lower. After the administration of melatonin, lesions in treated animals became similar to those seen in the controls.

Similarly, daily melatonin administration led to a significant reduction in the volume and weight of endometriosis-like lesions in controlled animal studies. Molecular markers of oxidative stress such as malondialdehyde (MDA) were statistically significantly reduced, and antioxidant activity, measured by SOD and catalase (CAT), was statistically significantly increased (35, 47).

Melatonin may also regulate endometriosis by interfering with MMP activity. In the mouse model it was shown that melatonin down-regulated proMMP-9 and MMP-3 expression and activity, and enhanced the expression of a natural inhibitor of MMPs known as TIMP-1 (tissue inhibitors of metalloproteinases) in a dose-dependent manner. The preventive and therapeutic action in endometriosis-like lesions was confirmed, with an increased apoptotic index (34, 48).

Our study showed that treatment with melatonin effectively regressed endometriotic explants in a rat model. A homologous rat endometriosis model was used in our study. Endometriosis was induced surgically under anaesthesia, as proposed by Vernon and Wilson with modifications by Uygur et al. and by us (35, 37, 38, 40, 49).

Oophorectomy was performed to inhibit fluctuation of endogenous oestrogen levels with respect to physiology of rats. Then, high dose of continuous oestrogen (50 µg/kg; twice a week) treatment was started; the 20 mg/kg/day melatonin dose was only given 10 rats which were randomly chosen. After two weeks of melatonin treatment, an additional two weeks of continuous oestrogen was administered to assess the endometriotic recurrence in the melatonin group. During the study, endometrial lesion volumes at the fourth and sixth weeks were decreased in the melatonin group when compared to those in the control group without histopathological differences between groups. The results were consistent with the previous studies in respect to lesion volumes and histology (35, 47).

Furthermore, when the melatonin group was analysed within itself, lesion volumes were insignificantly changed after six weeks, which means that melatonin might lower the recurrence rate. Also, histopathological scores were significantly improved in the melatonin group at the end of the sixth week in comparison with standard endometriotic lesions in the second week with an insignificant increase in the fourth week.

In conclusion, melatonin seems to be a promising non-hormonal treatment agent with obvious effects to minimise endometriosis and with probable effects to reduce the recurrence or to increase the lesion differentiation. In our study, the volumes of surgically induced endometriotic foci were decreased and histopathological scores were significantly improved with melatonin treatment, in spite of high doses of continuous exogenous oestrogen administration throughout the study period.

However further studies using different doses of melatonin or different animal models are required to confirm the safety and efficacy of melatonin.

**Ethics Committee Approval:** This study was approved by the Experimental Animals Ethics Committee of Yeditepe University Medical Faculty. All experiments were performed in compliance with international guidelines on the ethical use of animals.

**Informed Consent:** N/A.

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