The combination of letrozole and melatonin causes regression in size not histopathological scores on endometriosis in an experimental rat model

Kombine letrozol ve melatonin tedavisinin deneysel rat endometriosis modelinde lezyon boyutları ve histopatolojik skorlar üzerine etkisi

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Abstract

Objective: To determine the effects of the combination of letrozole and melatonin on surgically induced endometriosis.

Material and Methods: This prospective, randomized, controlled, experimental study was carried out at Yeditepe University Experimental Research Center (YUDETAM). Female non-pregnant, 17 nulligravid Wistar - Hannover albino rats with surgically induced endometriosis were used in this study. Endometriosis was induced by using homologous uterine horn transplantation in the rats. Four operations were performed on each rat. The induction of endometriosis was performed in the first operation. After two weeks of estradiol treatment the second operation was performed and endometriotic lesions were evaluated. Estrogen was then discontinued and in the study groups medications were started. During two weeks the rats were given medications and the third operation was performed for the assessment of the effects of the medications on the endometriotic foci. Then all the medications were stopped and estrogen was started again. Two weeks later all the rats were euthanized and recurrence of endometriosis was evaluated.

Results: The sum of the lesion volumes in the control group was 93.6±31.7 mm^3 at the end of the second week. After the cessation of estradiol it decreased to 85.0±23.8 mm^3 (P=0.31) and increased to 119.7±29.4 mm^3 at the sixth week (P=0.02). A significant reduction in histopathologic scores were seen after cessation of the estradiol (p=0.04). At the end of the sixth week, histopathological scores reached the pretreatment values. In the letrozole and melatonin group the sum of the lesion volumes decreased significantly after the treatment (82.8±21.0 mm^3 and 15.7±8.0 mm^3 respectively). At the end of the sixth week, the mean volume was calculated as 43.9±31.8 mm^3 (p=0.002). Histopathologic scores were 2.3±0.1, 2.0±0.2 and 2.2±0.3 at the end of the second, fourth and sixth weeks, respectively, in the letrozole and melatonin group.

Conclusions: Letrozole and melatonin caused a significant regression in lesion volumes; however, histopathological scores of endometriotic lesions did not change significantly.

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Key words: Endometriosis, melatonin, letrozole, rat endometriosis

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Özet

Amaç: Kombine letrozol ve melatonin tedavisinin cerrahi olarak indüklenmiş endometriosis üzerindeki etkisini belirlemek.

Gereç ve Yöntemler: Bu prospektif randomize kontrollü bir deneysel çalışma olarak dizayn edildi ve Yeditepe Üniversitesi Deneysel Hayvanlar Araştırma Merkezinde (YUDETAM) gerçekleştirildi. Cerrahi olarak endometriosis oluşturulmuş 17 tane nulligravida dişi Wistar – Hannover albino rat kullanıldı. Ratlara homolog uterin boynuz inokülasyonu yapılarak endometriosis oluşturuldu ve iki hafta aralar ile dört operasyon yapıldı. Endometriosis indüksiyonu ilk operasyonda yapıldı. İki haftalık östradiol tedavisini takiben ikinci operasyon yapıldı. Sonra östrojen kesildi ve çalışma grubuna ilaç başlandı. Takip eden iki haftanın sonunda üçüncü operasyon yapılarak endometriyal odaklar üzerinde ilaçların etkinliği araştırıldı. Üçüncü operasyondan sonra bütün ilaçlar kesildi ve östrojen tekrar başlandı. Iki haftalık östrojen tedavisinin ardından tüm ratlara ötanazi yapıldı ve rekürrens oranlarına bakıldı.

Bulgular: Kontrol grubunda tedavinin başında lezyonların volüm ortalaması 93.6±31.7 mm^3 idi. Östradiol tedavisi kesildikten sonra 85.0±23.8 mm^3 (P=0.31) boyutlarına indi ve rekürrens zamanı 119.7±29.4 mm^3'e çıktı (P=0.02). Östradiol kesildikten sonra histopatalojik skorda anlamlı bir düşüş gözlendi (p=0.04) fakat rekürrens zamanı tedavi öncesi boyutlarına ulaştı. L+M grubunda lezyonların volümü tedavi grubunda anlamlı bir şekilde düştü (82.8±21.0 mm^3 ve 15.7±8.0 mm^3). Rekürrens zamanı ortalama volüm 43.9±31.8 mm^3 olarak hesaplandı (p=0.002). Histopatolojik skor ise tedavi öncesi tedavi sonrası ve rekürrens zamanlarında sırası ile 2.3±0.1, 2.0±0.2 ve 2.2±0.3 idi.

Sonuç: Çalışmamızda Letrozol ve Melatonin endometriotik lezyonların boyutlarında gerileme oratya çıkarırken, histopatolojik skorlarda değişikliğe yol açmadı.

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Anahtar kelimeler: Endometriosis, melatonin, letrozole, rat endometriosis

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Introduction

Endometriosis is defined as the presence of a functional endometrial layer, with endometrial glands and stroma, outside the uterine cavity. Although it is common, its ethiopathology is not very clearly known (1).

As early as 1940s, Sampson proposed his theory of retrograde menstruation and implantation of endometrial fragments as the origin of endometriosis, yet since that time limited progress has been made toward defining the mechanisms associated with the etiology and pathophysiology of endometriosis. There are many other theories explaining the pathophysiological mechanisms of endometriosis. Estrogen dependency, role of pro-inflammatory environment, and effects of free radicals are some of these theories. There is much speculation about these topics but the attempts for treatment has mostly been restricted since most treatment are experimental and could not easily be applied to humans As a result, a need to develop alternate models in research animals exists. Many of these experimental animal models were previously described (2, 3). These studies proved that surgically transplanted endometrial tissue in the rat provides an animal model to study the effects of experimental drugs on ectopic endometrial tissue. Recently, we demonstrated a rat experimental model for endometriosis (4, 5). The main advantage of our model is the possibility of assessing the rate of recurrence of endometriotic lesions after cessation of medications.

Experimental and clinical observations suggest that endometriosis is estrogen dependent and that estrogens seem to be important for growth and maintenance of endometriosis (6). The attempt at reducing or antagonizing estrogen was made with GnRh analogs. However, they were not able to block estrogen synthesis appropriately. Recently, aromatase inhibitors have been used for this purpose. Aromatase (estrogen synthetase) is the key enzyme in the synthesis of estrogens and mediates the conversion of androstenedione and testosterone to estrone and estradiol. Letrozole is one of second generation aromatase inhibitors that inhibit 97-99% of estrogen production The importance of estrogen in stimulating endometriotic tissues and the in situ presence of aromatase in these tissues reveal that inhibition of estrogen synthesis is a rational approach to the treatment (7). In our study, letrozol was used for the treatment of endometriotic lesions due to its availability on the market.

It is known that free radicals have a dual role in the reproductive tract and are key signaling molecules for endometriosis. Free radicals mediate their actions through a variety of pro-inflammatory cytokines, with these processes having been proposed as a common underlying factor for endometriosis. Melatonin is a documented powerful free radical scavenger and a broad-spectrum antioxidant (8). It has been shown that Melatonin causes regression and atrophy of endometriotic lesions in rats (3).

Our study evaluated the effects of the combination of letrozole and melatonin on surgically induced endometriotic lesions in a rat model.

Materials and Methods

Seventeen female non-pregnant, nulligravid Wistar-Hannover albino rats weighing 200-250 g were purchased from Yeditepe University Experimental Research Center and used in this study. The rats were caged individually in a controlled environment (the room temperature was 21°C and humidity 60%) with 12 hours light/dark cycles and were fed ad libitum. This study was approved by the Experimental Animals Ethics Committee at Yeditepe University Medical Faculty. All experiments were performed in compliance with international guidelines on the ethical use of animals.

In the first operation; all rats were anesthetized with an intramuscular administration of 60 mg/kg ketamine hydrochloride (Ketalar; Eczacibasi Ilac Sanayi, Levent, Istanbul, Turkey) with 7 mg/kg xylazain hydrochloride (Rompun; Bayer Ilac Sanayi, Sisli, Istanbul, Turkey) as described by us previously (4, 5). Endometriosis was induced surgically under anesthesia as proposed by Vernon and Wilson (2) with modifications by Lebovic (9). Briefly, after adequate anesthesia a vertical incision was made to expose the uterus. Both uterine horns were removed from the cervix up to 1 cm from the ovaries. Electro-coagulation was used for haemostasis. Removed tissue was placed in phosphate- buffered saline at 37°C and split longitudinally. The parametrial tissues were removed, and then both split horns were sectioned into two 6x3 mm pieces. Two of these pieces were transplanted without removing the myometrium onto the inner surface of the right abdominal wall with the epithelial lining opposed to the peritoneal surface. Both ends of the explanted endometrial tissue were secured with non absorbable polypropylene 6-0 suture to the inner abdominal wall. The remaining two pieces were placed in the same manner on the left inner abdominal wall. All tissues were implanted just opposite to both vascular bifurcations on the inner surface of the abdominal wall. The peritoneal cavity was kept moist meticulously with controlled amounts of sterile saline solution throughout the surgery. The midline abdominal incision was closed with 3-0 silk sutures. The skin incision was closed in a continuous interlocking manner with 3-0 silk sutures. All rats were given 50 mg/kg/day cefazolin sodium (IE Ulugay Ilac Sanayi, Istanbul, Turkey) intramuscularly over a 3 day period after the operation. All rats except for Group GC (General Control) were given 50 μg/kg Estradiol -Depot (Jenapharm GmbH&Co, Germany) twice a week intramuscularly until the second operation.

The second operation was performed two weeks after the inoculation. Anesthesia and abdominal entry were carried out as mentioned above. Peritoneal washing was collected for biochemical analysis and stored at -80°C. All the implants were measured by the same author (G.Y.). One of the fourth explants was removed for histopathological analysis. After these operations estrogen was stopped. The rats were randomized to 2 groups. An excel program was used for the randomization. Group C (Control): No medication was given for a two week period after the second operation. Group L + M (Letrozole and Melatonin): These rats were given 0.04 mg/kg/day Letrozole (Femara 2.5 mg tablet, Novartis Pharma AG, Basel, Switzerland) orally with gastric lavage and injected with 10 mg/kg/day melatonin (Melatonin flacon, Acros Organics Co., Geel, Belgium) intraperitoneally and 10 mg/kg/day subcutaneously for two weeks. The melatonin was dissolved in 1:90 absolute ethanol / saline solution.

The third laparotomy was performed two weeks after the second operation. The peritoneal washout was obtained. Measurements of the lesions were taken. One of the remaining three lesions was obtained for histopathological evaluation. The active substances (Melatonin and Letrozole) were stopped. Estradiol was initiated again for the assessment of recurrence.
In the fourth operation; recurrence of the endometriosis was evaluated after two weeks of estradiol treatment. All rats were euthanized under anesthesia and all measurements and tissue collections were performed as described above.
Health conditions of the rats were monitored by a veterinary doctor on a daily basis and by her assistant. Only one animal was lost in the Letrozole and Melatonin group, 2 days after the third operation due to ingesting the gastric lavage tube. The spherical volume of each ectopic uterine tissue was calculated using the prolate ellipsoid formula: V (mm^3) = 0.52 x length x width x height) (all in millimeters). The biopsies were fixed in 10% neutral buffered formaldehyde solution. All pieces were embedded in paraffin after routine dehydration and 5 μm-thick sections were made with a microtome. The samples were stained with hematoxylin and eosin (HE). The slides were examined under a light microscope. The pathologist (F. O.) who assessed the samples was blinded to the treatment groups. At the end of the sixth week, if there were two lesions in a rat, the mean histopathological score of these lesions was recorded. The persistence of epithelial cells in the implants was scored semi-quantitatively as follows: 3 = well preserved epithelial layers; 2 = moderately preserved epithelium with leukocyte infiltration; 1 = poorly preserved epithelium (occasional epithelial cells only); 0 = epithelial line (10).
Statistical analysis was performed using SPSS, version 11.5 (SPSS Inc, Chicago, IL, USA) for Windows. Data were expressed as mean ± standard error of mean, unless stated otherwise. Friedman's Test was used for the evaluation of lesion volumes, histopathological scores and all the biochemical analysis before, after and recurrence time in each groups. When these parameters were evaluated between the groups, Mann-Whitney U test was applied. $P<0.05$ was considered as statistically significant.

Results

Sixty of the 68 implants (88.2%) were formed properly. In the second operation, the pretreatment volumes were compared. The pretreatment volumes were 93.6±31.7 mm^3 and 82.8±21.0 mm^3 in the control and L+M groups, respectively (P=0.64). The sum of the pretreatment histopathological scores were 2.6±0.5 and 2.3±0.1 in the control and L+M groups, respectively (P=0.55). These two results defined the efficient induction and formation of endometriotic lesions (Table 1).
The effects of the medications were assessed in the third operations. The sums of the post-treatment volumes were 85.0±23.8 mm^3 in the control group and 15.7±8.0 mm^3 in the L+M group. The difference was statistically significant (P=0.03). The sum of the post-treatment histopathological scores in the control group was 1.6±1.3; whereas it was 2.0±0.2 in the L+M group (P=0.07).

Table 1. The comparison of endometriotic volumes between the groups

	Control (C) (n=7)	Melatonin + Letrozole (M+L) (n=10)	p
Pretreatment			
Volume (mm^3)	93.6±31.7	82.8±21.0	0.64
Histopathological score	2.6±0.5	2.3±0.1	0.55
Posttreatment			
Volume (mm^3)	85.0±23.8	15.7±8.0	0.03
Histopathological score	1.6±1.3	2.0±0.2	0.07
Recurrence			
Volume (mm^3)	119.7±29.4	43.9±31.8	0.02
Histopathological score	2.5±0.3	2.2±0.3	0.35

The effects of the cessation of the medications were evaluated in the final (fourth) operations. The rat lost after the third operation in the letrozole and melatonin group was removed from statistical analysis at the recurrence time. The sum of the recurrence volumes in the control group was 119.7±29.4 mm^3; whereas it was 43.9±31.8 mm^3 in the L+M group. The difference was statistically significant (P=0.02). The volume gained at the recurrence time were 34.7 mm^3 and 28.2 mm^3 in the control and study groups respectively (P=0.04). The sums of the histopathological scores at that time were 2.5±0.3 and 2.2±0.3 in the control and L+M groups, respectively (P=0.55).
The sum of the lesion volumes in the control group was 93.6±31.7 mm^3 at the beginning. After the cessation of estradiol it decreased to 85.0±23.8 mm^3 (P=0.31) and increased to 119.7±29.4 mm^3 after starting estradiol (P=0.02) (Table 2). A significant reduction in histopathologic score was seen after cessation of the estradiol (p=0.04). At the recurrence time, histopathological scores reached the pretreatment values.
In the L+M group the lesion volumes decreased significantly after the treatment (82.8±21.0 mm^3 and 15.7±8.0 mm^3, respectively). At the time of recurrence the mean volume was 43.9±31.8 (p=0.002). Means of the histopathologic scores were 2.3±0.1, 2.0±0.2 and 2.2±0.3 in the pre- treatment (at the end of the second week), post-treatment (at the end of the fourth week) and recurrence phases (at the end of the sixth week), respectively.

Discussion

The medical treatment of endometriosis has progressed significantly in the last four decades and there are more choices available for clinicians than ever before. However, the ideal medical treatment has not been developed yet. This study was conducted to investigate the effects of the combination of some new drugs such as letrozol (an aromatase inhibitor) and melatonin (an anti-oxidant) on the progression of endometriosis in the rat model. In addition to the evaluation of the efficacy of this combination, our study also focused on the recurrence rate after the cessation of the treatment.

Table 2. The mean volume of endometriotic lesions before and after treatment and at recurrence time

	Pre-treatment (Mean±SEM)	Post-treatment (Mean±SEM)	Recurrence (Mean±SEM)	p
Control with Estrogen (n=7)				
Volume (mm^3)	93.6±31.7	85.0±23.8	119.7±29.4	0.02
Histopathological score	2.6±0.5	1.6±1.3	2.5±0.3	0.04
Melatonin + Letrozole (n=10)				
Volume (mm^3)	82.8±21.0	15.7±8.0	43.9±31.8	0.002
Histopathological score	2.3±0.1	2.0±0.2	2.2±0.3	0.86

We modified and improved the rat endometriosis model developed by Vernon and Wilson (2, 4, 5). Our model is unique, since rats are in hypo-estrogenic status except for estrus state; administration of depot estrogen twice weekly induced endometriosis faster. Additionally, this methodology eliminated the need for a vaginal smear before the second surgery to detect estrus state. The design of the study provided us with the opportunity to detect the recurrence rates after cessation of the treatment. Most of the drugs used for the treatment of endometriosis have to be stopped after 6 months. One of the most important points of all treatment modalities is to prevent recurrence long after they are discontinued. With this model, we were able to detect the recurrence rate of the lesions after termination of treatment. In contrast to humans and non-human primates, estrous animals do not shed their endometrial tissue and therefore do not develop endometriosis spontaneously. However, endometriosis can be induced by transplanting endometrial tissue to ectopic sites. These models are classified into two types, homologous and heterologous models. Homologous models have been employed utilizing surgical transplantation of the endometrium of the same or syngeneic animals in immunocompetent animals, whereas in heterologous models, human endometrial fragments are transferred either intraperitoneally or subcutaneously to immunodeficient mice. In our preliminary study we preferred a homologous rodent model for induction of endometriosis; however we intend to carry out studies in endometriosis research using immunodeficient mice as heterologous models.

In our study, rats were not ovariectomized and were administered exogenous estrogen. Since we wanted the uterine tissues implanted autologously to be endometriotic lesions in all rats; we carried out a pilot study controlling this hypothesis and at the end of the study we concluded that using this methodology resulted in typical endometriotic lesions in all animals.

Endometriosis is an estrogen dependent disease. Many of the medical treatment modalities for endometriosis are targeted at decreasing or antagonizing estrogenic actions. Unfortunately, the ideal medical treatment has still not been developed. Drugs such as GnRH-a (agonists) are widely used for the cure of endometriosis. A wide range of adverse effects and very high recurrence rate after cessation of the therapy limit their long term use. Aromatase inhibitors appear to be the first breakthrough in the medical treatment of endometriosis since the introduction of GnRh-a in the 1980s (7).

Yildirim et al. published the effects of letrozole on endometriosis in a rat model (4). They found that endometriotic lesions regressed in size (p=0.02) and histopathological scores decreased as well (p=0.28) when compared to findings at the beginning of the treatment. They found a significant rise in the lesion volume (p=0.02) and histopathological score (p=0.28) after cessation of letrozole. They hypothesized this to be a rebound phenomenon. The compensatory response to estradiol depletion in the hypothalamus results in higher serum FSH secretion and ovarian stimulation (11). Simultaneously, a strong suppression of estradiol may up-regulate the estrogen receptors in the targeted tissues. The combination of these two possible mechanisms may explain the increase of the endometriotic lesion volumes and scores.

In the current literature there are prospective clinical trials that approach the effectiveness of aromatase inhibitors in the treatment of endometriosis. Aromatase inhibitors with a progestin for the treatment of 10 resistant endometriotic premenapausal women was assessed in one small phase II study and it was found that post-treatment visible endometriotic lesions were smaller when compared to the pretreatment laparoscopic findings. Ninety percent of patients responded to this regimen with decreased pelvic pain (12). It was shown that the combination of an aromatase inhibitor with combined oral contraceptive pills resulted in a effective reduction of pain in the endometriotic women (13).

The main deficiency of such hormonal treatments is the high recurrence rate of the lesions when the treatment is stopped. To solve this problem, the combination of an aromatase inhibitor and a GnRH analogue was used during 24 months for women who have endometriosis and they were followed for two years without giving any medications (14). At the end of the follow-up, the investigators concluded that a novel regimen with an aromatase inhibitor and a GnRH analogue after conservative surgery was effective in controlling recurrence and pain in patients with severe endometriosis. On the other hand, these combinations caused significantly higher bone loss in the spine of the treated patients.

The widespread side effects and high recurrence risk following cessation of the hormonal drugs limit their long term use. There has been some investigation into applying a new non-hormonal management for endometriosis, which is a multifactorial disease, and oxidative stress has been proposed as a potential factor in the pathophysiology of the disease (15). Therefore, new medical treatments are needed which are aimed at reducing the oxidative stress with improved side-effects and being at least as effective as hormonal treatment. Melatonin seems to

be promising in this sense and this is the reason why we used melatonin in our study.

Melatonin (N-acetyl-5-methoxy-triptamine) is an endogenous free radical scavenger (16). Although its mechanism is not clearly explained, some of the steps in this mechanism are known. Melatonin can enter into the cells easily because of its high diffusion ability and can show its effects by entering the cell through its receptors and also without receptors, which makes it one of the most powerful antioxidants. In addition, ME may stimulate several anti-oxidative enzymes and inhibit a pro-oxidative enzyme by intra-cellulary binding to calmodulin (17).

The main reactive oxygen species (ROS) is considered to be superoxide anion (O_2^-). Superoxide dismutase (SOD) rapidly decomposes superoxide anion into hydrogen peroxide and oxygen. Superoxide radicals are involved in many physiological and pathophysiological processes. Activated neutrophiles and macrophages can also produce a large amount during the oxidative burst. Removal of superoxide is a necessary step in cellular defense against these damages (18). Catalase is an ubiquitous antioxidant enzyme present in most aerobic cells. CAT is involved in the detoxification of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS). CAT catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen (O_2) and two molecules of water (H_2O) (19). Polyunsaturated lipids are especially susceptible to this type of damage when in an oxidizing environment and they can react to form lipid peroxides. Lipid peroxides are themselves unstable, and undergo additional decomposition to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides further react to form malonaldehyde (MDA). MDA can be found in most biological samples including foodstuffs, serum, plasma, tissues and urine, as a result of lipid peroxidation, and has become one of the most widely reported analytes for the purpose of estimating oxidative stress effects on lipids (20). The ROS have been shown to be closely related to the inflammatory process and pathophysiology of disease.

There are many suggestions that antioxidant therapy may help the patients suffering from endometriosis. It has been published that if an anti-oxidant agent joined the treatment scheme of the patients, significant clinical improvements may occur (21-23). Yildirim et al. showed that melatonin causes significant regression both in the volume and histopathological scores on endometriosis in a rat model (4). They showed that the recurrence rate in the melatonin group was significantly lower than that observed in the letrozole group. This finding shows that melatonin can prevent recurrence after cessation of the treatment.

Guney et al. published a well designed study and they concluded that melatonin caused regression and atrophy of the endometriotic lesions in rats (3). In their melatonin group, the endometrial explant levels of MDA statistically significantly decreased and activities of SOD and CAT significantly increased when compared with the control group. In our study, SOD, CAT levels were increased and MDA level was decreased in the peritoneal fluids of rats with melatonin. After cessation of melatonin, there was a decrease observed in SOD and CAT levels. No change was observed in the level of MDA after cessation of the melatonin.

We combined letrozole and melatonin in this study. We observed significant volume depletion on the lesions; however, histopathological scores did not decrease. This finding can be extrapolated to humans: the patients who have huge endometriomas can be treated with the combination of letrozole and melatonin before any surgical intervention or prior to an in vitro fertilization program.

In conclusion, the combination of letrozole and melatonin caused regression in the volume of surgically induced endometriotic lesions in a rat model. Unlike letrozol, after cessation of melatonin, the recurrence is very low. Further studies are required to evaluate the effects of this combination.

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