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The effects of melatonin on endometriotic lesions induced by implanting human endometriotic cells in the first SCID-mouse endometriosis-model developed in Turkey

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Summary

Objective: To evaluate the effects of melatonin on endometriotic lesions induced by implanting human endometriotic cells in SCID mice. *Materials and Methods:* Prospective, randomized, controlled, experimental study. Experimental Research Center of Yeditepe University (YUDETAM). Thirty female, non-pregnant, nulligravid severe combined immunodeficient (SCID) mice. Endometriotic cells collected from patients with endometriosis were implanted subcutaneously in 30 SCID mice. These mice were randomized into two study groups: in the first group, mice were administered melatonin (20 mg/kg/day) following induction of endometriosis for four weeks; in the second group, nothing was administered. All the mice were given a high dose of exogenous estradiol (50 µg/kg/d, twice weekly). Four weeks after inoculation, necropsies were performed and endometriotic lesions were collected. All the lesions were evaluated histopathologically and the levels of SOD and MDA were assessed in the lesions. *Results:* Successful implantation was observed in the 28 mice that survived. Mean MDA level was 5.0±1.7 and 8.8±2.6 in the melatonin and control groups, respectively ($p = 0.01$); mean SOD level was 1.1 ± 0.1 and 1.0 ± 0.1 in the melatonin and control groups, respectively ($p = 0.49$). Mean histopathological score was lower in the melatonin group ($p = 0.04$). *Conclusions:* Melatonin was effective in the treatment of experimental endometriosis induced in SCID mice.

Key words: Experimental endometriosis; SCID mice; Melatonin.

Introduction

Endometriosis is an enigmatic disease characterized by the presence and growth of endometrial-like tissue outside the uterine cavity. Endometriosis is a major clinical problem with no cure. Current management of endometriosis is based on pain management, reduction in the volume of endometriotic lesions, and prevention of recurrence. Pain management options include non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics, suppression of ovarian function with hormonal drugs (oral contraceptives, danazol, gestrinone, agonists, dienogest), and surgery (laparoscopic ablation or excision of endometriotic lesions) [1]. If infertility is the problem, then specific treatments like ovulation induction and insemination or in vitro fertilization might be the treatment option. Yet, there is inconclusive evidence to show whether all these modalities cure the disease. These agents also produce varying levels of side effects that in many cases prevent long-term use and can lead to poor compliance [2]. Thus, new pharmaceutical agents are required in the treatment of endometriosis that are non-hormonal and with few and acceptable side effects. In that sense, melatonin is a documented powerful free radical scavenger and a broad-spectrum antioxidant [3]. It has been shown by the present group that melatonin causes regression and atrophy of endometriotic lesions in rats [4].

Severe combined immunodeficient (SCID) mice are perfect experimental animal models for endometriosis research [5-9], since human endometriotic cells collected from endometriotic lesions can be implanted in SCID mice and endometriotic lesions identical to the lesions seen in women can be induced experimentally.

The objective of this project, which was the first research project carried out using SCID-mouse-endometriosis-model in Turkey, was to evaluate the efficacy of melatonin on endometriotic lesions induced by implanting human endometriotic cells subcutaneously in SCID mice.

Materials and Methods

In this project, 30 female, non-pregnant, nulligravid mature SCID mice weighing 21-25 grams were included and the experiments were conducted in the Experimental Research Center of Yeditepe University (YUDETAM). The mice were kept in microisolator cages and housed in a separate barrier facility in a well-controlled pathogen-free environment with monitored ambient temperature and regulated cycles of light and darkness. The mice were fed ad libitum with sterile laboratory chow and water. The

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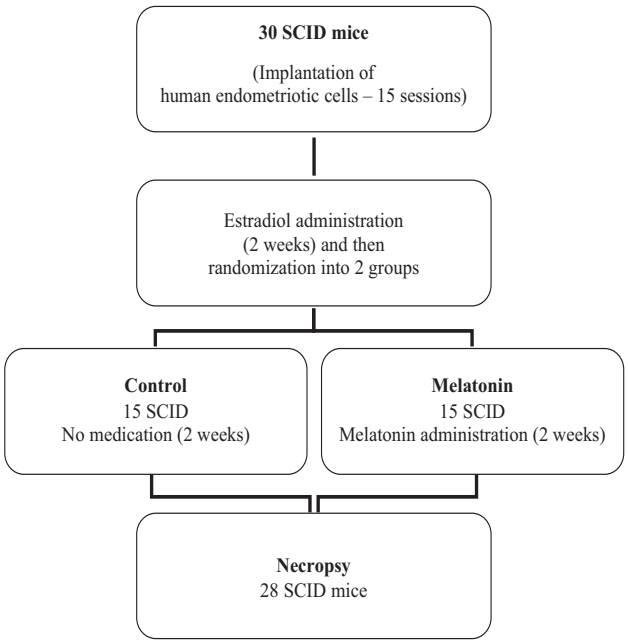

Figure 1. — Study design.

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.

The use of human tissue for this study was approved by the Institutional Review Board of Yeditepe University. Human endometriotic cells were collected from human endometriotic lesions by laparoscopy or laparotomy for endometriosis surgery. The ages of the patients ranged from 21 to 46 years, with a mean of 34 years. None of the patients had received hormonal therapy or used NSAIDs continuously in the three 3 months before they underwent surgery for endometriosis.

Small tissue samples collected from endometriotic lesions were dispersed in one ml of PBS and then they were implanted subcutaneously into the backs of SCID mice using a one-ml insulin syringe. In total 15 implantation sessions were performed and in each session human endometriotic cells were implanted in two SCID mice using an 18-gauge needle. Estrogen therapy was initiated at the time of injection and administered twice a week (50 μg/kg/d, s.c.); two weeks later the mice were randomized into the control group, in which no medication was administered, or in the treatment group, in which all the mice were administered 20 mg/kg/d melatonin s.c., commencing from the end of the second week following the implantation of human endometriotic cells (Figure 1).

Two weeks later all mice were euthanized under anesthesia and the endometriotic lesions were collected. The biopsies were fixed in 10% neutral buffered formaldehyde solution. All pieces were embedded in paraffin after routine dehydration and five-μm-thick sections were made with a microtome. The samples were stained with hematoxylin-eosin (HE). The slides were examined under a light microscope. The pathologist (F.O.) who assessed the samples was blinded to the treatment groups.

Morphological identification of the endometrial glands and stromas was the cornerstone of the microscopic examination. 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].

SOD activity was determined by the NWLSS NWK-SODO$_2$ superoxide dismutase activity assay for mice. Activity was expressed as units per milliliter (U/ml). MDA levels were estimated by the NWLSS NWK-MDA01 assay for mice. Activities were expressed as μM.

The statistical analysis was performed using Student's *t*-test. The data were expressed as mean ± SD and $p < 0.05$ was accepted as significant.

Results

A veterinarian monitored the animals' health on a daily basis. No signs of significant local inflammation at either the operation or injection sites and no systemic reactions or infection were observed in any of the SCID mice included in the study. No significant difference was observed in the weights of the SCID mice. From a total of 30 mice in which human endometriotic tissue cells were implanted, 28 (93.3%) survived the procedure. Two mice died during the study right after estradiol injections.

Successful implantation was observed in 28 mice out of 28 (100%). On gross examination, the implants consisted of well-circumscribed nodules firmly attached to underlying tissue (Figure 2). The mean number of implants was 14 and 14 in the control and melatonin groups, respectively. Mi-

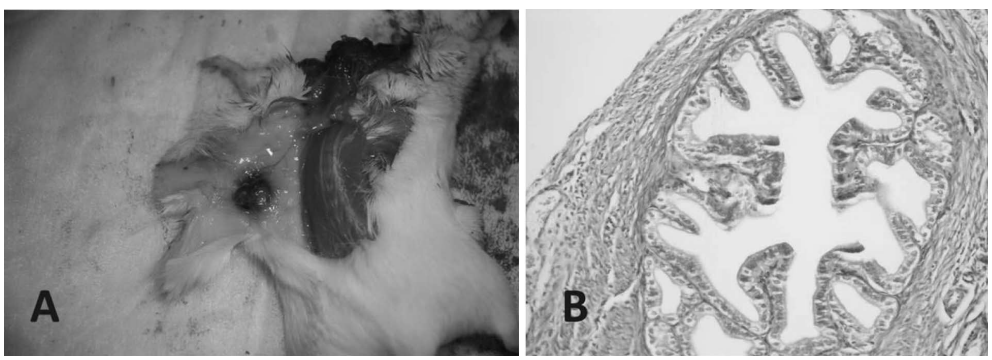

Figure 2. — An endometriotic lesion. A) A gross lesion in the subcutaneous area. B) Histopathological evaluation of the endometriotic lesions in the rats (HE, x40 magnification).

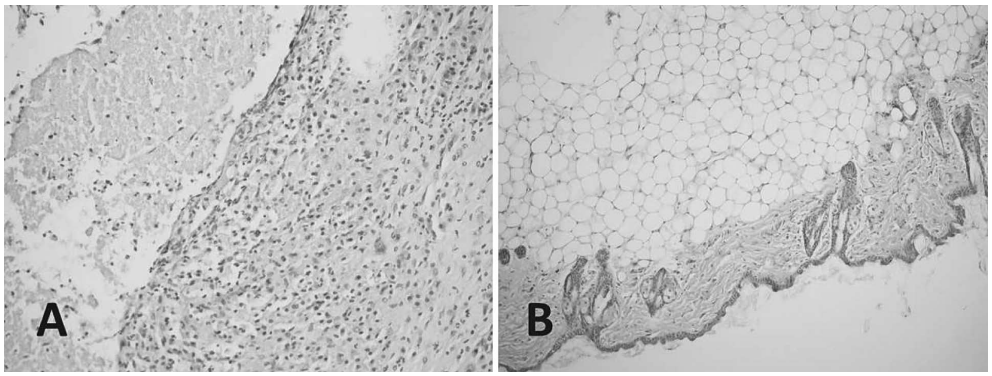

Figure 3. — Regressed endometriotic lesions (HE, x 40 magnification).

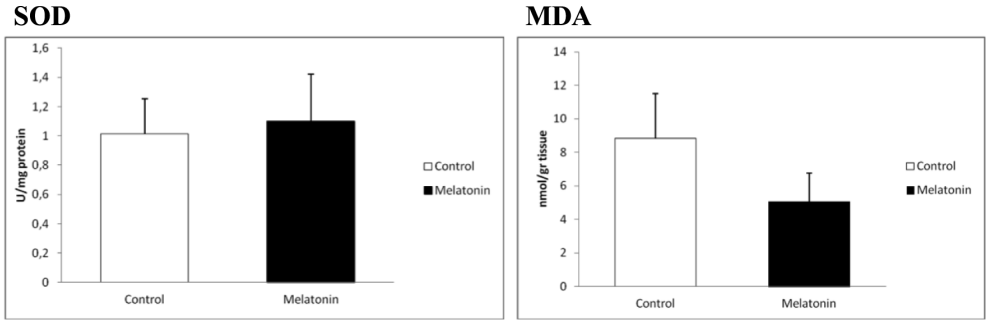

Figure 4. — Comparison of SOD and MDA levels between the control and study groups.

croscopically, HE staining of implant sections demonstrated the presence of endometrial acinar glands in a mixed background of stromal and inflammatory cells. Mean MDA level was significantly lower in the melatonin group (5.0 ± 1.7 in the melatonin group vs. 8.8 ± 2.6 in the control group; $p = 0.01$) (Figure 3). Mean SOD level was higher in the melatonin group (1.1 ± 0.1 in the melatonin group *vs.* 1.0 ± 0.1 in the control group; $p = 0.49$) (Figure 4) but not significantly so, probably due to the number of SCID mice included in the study.

Volume analysis of the lesions was not performed, since the number of endometriotic cells implanted could not be standardized; however, in the examinations during necropsies, the lesions in the control group were found to be larger and more vascularized to some extent. Histopathological examination of the lesions was performed by an experienced pathologist who was blind to the specimens. Mean histopathological score of the lesions in the melatonin-treated group was significantly lower than the mean score in the control group ($p = 0.04$). Mean uterine weights were 0.09 and 0.08 grams in the melatonin and control groups, respectively (p = NS).

Discussion

To the best of the present authors' knowledge, this is the first study carried out to evaluate the effect of melatonin on the endometriotic lesions induced by human endometriotic cells in SCID mice. This is also the first SCID-mouse endometriosis-model developed in Turkey.

As early as the 1940s, Sampson proposed the theory of retrograde menstruation and implantation of endometrial fragments as the origin of endometriosis in women; yet since then only limited progress has been made to define the mechanisms associated with the etiology and pathophysiology of endometriosis. Since retrograde menstruation occurs in nearly all women of reproductive age [11], impairment of some additional factors is needed for the establishment and maintenance of endometriotic lesions. There are many other theories explaining the pathophysiological mechanisms of endometriosis, such as estrogen dependency, the role of a pro-inflammatory environment, and the effects of free radicals. However, at the time of clinical symptoms, most women already have established endometriosis and it is not always possible to carry out studies on the pathogenesis of this disease in humans. In addition, ethical considerations limit the performance of controlled experimental studies in humans, since it is not possible to monitor the progression of endometriosis without performing repeated laparoscopies. Thus, research investigating fundamental mechanisms by which menstrual endometrium adheres, invades, and establishes a functional vasculature to form endometriotic foci can be performed almost only in experimental animal models; moreover, studies on new therapeutic approaches are only possible in these models. Currently available medical therapies are unsatis-

factory, because they focus on relieving the symptoms rather than treating the disease. In addition, they cannot be used for prolonged duration because of severe secondary side effects [12]. This limitation is the main reason for research studies to develop specific and more efficient therapeutic alternatives that eliminate endometriotic lesions, prevent recurrences, and do not interfere with fertility potential.

Humans and non-human primates are the only mammals that spontaneously develop endometriosis; however, the limitations regarding human studies and the expense of primate studies have prompted investigators to pursue the use of small animal models [13]. Non-primate species used commonly for medical research do not menstruate and do not develop endometriosis; nevertheless, a number of investigators have mechanically introduced endometrial tissue into the peritoneal cavity of rabbits, rats, and mice in order to investigate multiple aspects of this disease. This type of study has proven to be valuable for studying some aspects of the disease, but surgical induction of endometriosis does not address the basic cellular mechanisms of endometrial attachment or invasion of ectopic lesions in women. The availability of implanting human endometriotic cells into experimental animals and inducing endometriotic lesions identical to those in women is an attractive approach for researchers who carry out endometriosis studies. Endometriosis-like lesions can be created by the transplantation of human endometrium into chicken chorioallantoic membranes [14], but these lesions can be maintained only short-term in developing embryos. Human endometrium has also been engrafted into the anterior chamber of the rabbit eye but these grafts begin to be rejected seven days after transplantation [15]. 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 in these animal models, which are classified into two types: homologous and heterologous experimental animal models. In homologous models, which are immunocompetent animals, endometriotic lesions are induced utilizing the surgical transplantation of endometrium of the same animal or another syngeneic animal; whereas in heterologous models, which are immunodeficient animals, human endometrial fragments are directly transferred either intraperitoneally or subcutaneously. Although different experimental animal models have been described, the rat endometriosis model, which is a homologous model, has some advantages, such as limited costs; additionally, this model offers the opportunity to perform studies in large groups of genetically similar animals over a long period. It is well-suited for the investigation of mechanisms involved in the peritoneal attachment of endometrial cells as well as the investigation of the effects of therapeutic modalities. Moreover, it allows the evaluation of endometriotic foci at different time intervals and it is readily available in most experimental research centers [16]. In the present authors' previous studies [4, 17], they used a homologous rodent model for the induction of endometriosis; however, since it was a homologous model it was not possible to induce endometriotic lesions using human endometriotic cells, which are identical to the endometriotic lesions in humans. Human endometrium and tissue samples from endometriotic lesions can be transplanted into immunodeficient mice [18, 19]. SCID mice have a combined immunodeficient T- and B-lymphocyte function and highly successful heterotopic transplantation was reported [5]. Immunodeficient nude mice lack a functional thymus and have a greatly reduced number of T lymphocytes but a normal complement of B cells. Greenberg and Slayden [20] subcutaneously engrafted human endometriotic tissue in transgenic recombinase-activating gene-2 knockout [RAG-2/γ(c)KO] mice, which are not only devoid of B and T lymphocytes but also lack NK cells. Phylogenetic differences between species can interfere with our understanding of endometriosis in humans, but the idea of implanting human endometriotic tissue into SCID mice and inducing human endometriosis is attractive and relevant. SCID mice offer the opportunity to create human endometriosis experimentally in a humanized animal model for endometriosis. SCID mice possess a congenital deficiency of B and T lymphocytes and thus cannot efficiently trigger either cellular or humoral immunity [21]. Aoki *et al.* [19] compared the take rate of human endometrium that was transplanted into nude and SCID mice and showed that nude mice rejected 60% xenografts, whereas 100% of the xenografts were accepted by SCID mice. It was shown that endometrial tissue can be implanted successfully on the peritoneum of nude mice [22]; however, Awwad *et al.* reported a higher success rate of implantation of 96.5% in SCID mice peritoneum [5]. In the present study, the authors demonstrated that human endometriotic tissue can be successfully implanted subcutaneously in SCID mice using an 18-G needle with a success rate of 100%. they also showed that the endometriotic lesions were responsive to melatonin treatment.

This is the first research project supported by the Scientific and Technological Council of Turkey (TUBITAK) carried out using SCID mice in which endometriotic lesions were induced by implanting human endometriotic cells. In the present study, the authors implanted endometriotic cells collected from 15 women with endometriosis subcutaneously in SCID mice in 15 sessions. In each session, cells were implanted in two SCID mice and these mice were randomized into treatment and control groups. Using this methodology, a representative sample of cells collected from different clinical endometriotic lesions was used to induce endometriosis in SCID mice. Subcutaneous implantation of human endometriotic cells in SCID mice makes it possible to remove the lesion sur-

gically without sacrificing the animal, so that the effects of hormones or other agents can be assessed over a longer study period. Since the present authors showed that melatonin was effective on recurrence of endometriosis in a homologous rat endometriosis model, they intend to carry out studies in the future using a heterologous animal model (SCID) and the same methodology used in the present study to evaluate the effects of some agents upon recurrence of endometriosis induced by implanting human endometriotic cells in SCID mice.

Because of side effects and high recurrence risk after cessation of treatment, there are ongoing investigations regarding new non-hormonal drugs for the treatment of endometriosis, which is a multifactorial disease. Oxidative stress was proposed as a potential factor in the pathophysiology of the disease [23]. Developing a better understanding of the cellular and molecular mechanisms linking endometrial biology to the pathophysiology of endometriosis remains an important aspect of the present authors' studies; however, another aspect of an experimental disease model is testing the effectiveness of novel therapeutic agents. Therefore, new medical treatments aiming to reduce the oxidative stress with acceptable side-effects and as effective as hormonal treatment are needed. Melatonin seems to be promising in this sense. Melatonin is an endogenous free radical scavenger [24]. Although its mechanism is not clear, some of the steps in this mechanism are known. Melatonin can enter the cells easily because of its high diffusion ability; it can show its effects through its receptors and also without receptors, which makes it one of the most powerful antioxidants. In addition, melatonin may stimulate several anti-oxidative enzymes and inhibit a pro-oxidative enzyme by binding to calmodulin in the intracellular environment [25]. 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. Superoxide anion (O_2^-) seems to be quite important in reactive oxygen species (ROS). Superoxide dismutase (SOD) rapidly decomposes superoxide anion into hydrogen peroxide and oxygen. Superoxide radicals are involved in many physiological and pathophysiological processes [26]. Malondialdehyde (MDA) can be found in most biological samples including foodstuffs, serum, plasma, tissues, and urine, as a result of lipid peroxidation, and has been reported widely for the purpose of estimating oxidative stress effects on lipids [27]. Melatonin is a documented powerful free radical scavenger and a broad-spectrum antioxidant [28]. It has been shown that melatonin causes regression and atrophy of endometriotic lesions in rats [4]. In the present study, the authors found that experimental endometriotic lesions induced in SCID mice, which has several advantages over other homolog experimental models, by using human endometriotic cells, could be used as a tool to test new therapies, such as melatonin, which is a potent antioxidant agent.

In conclusion, the SCID mouse is a promising heterologous animal model for experimental endometriosis studies to evaluate the etiology and pathophysiology of this disease, and to test the efficacy of some pharmaceutical agents on endometriotic lesions induced in this model that are identical to lesions in women. In the present study, melatonin treatment (20 mg/kg/d) was shown to be effective in the treatment of endometriosis induced in SCID mice using human endometriotic cells, in terms of histopathological scores and MDA levels; since possible side effects of this dose of melatonin in humans can be neglected to a large extent, the authors think that it may be high time to start carrying out clinical studies to evaluate the effects of melatonin on endometriosis in women. Due to results of the present study, the authors intend to make an application to the Ethical Committee of the Clinical Trials in order to able to carry out a study to test the efficacy of melatonin in human endometriosis.

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