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Effects of repeated propranolol administration in a rat model of surgically induced endometriosis

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ABSTRACT

Objectives: To determine whether propranolol has an inhibitory effect on the angiogenesis of endometriosis in an experimental rat model or not.
Study design: This was an experimental animal model study. Twenty-four female Wistar albino rats (200–250 g) were used to create a model for surgical induction of endometriosis. Two rats died during the surgeries. The rats were randomly divided into treatment (n = 11) and control groups (n = 10), which were treated with daily intraperitoneal propranolol (10 mg/kg) and saline (2 mL), respectively. Study duration was 8 weeks. The volumes and histopathological findings of the implants, and immunochemistry for vascular endothelial growth factor (VEGF), metalloproteinase (MMP)-2, and MMP-9 were evaluated.
Results: Viable endometriotic implants were created in all animals. In the propranolol-treated group, the mean implant volume significantly decreased after treatment (142.5 vs. 32.1 mm^3, respectively; p = 0.008), while the mean implant volume significantly increased in the control group (141.0 vs. 174.2 mm^3, respectively; p = 0.009). There were also significant reductions in VEGF immunoreactivity scores and both stroma and epithelium MMP-2 and MMP-9 immunoreactivity scores in the propranolol-treated group compared with the control group ($p < 0.005$ for all scores).
Conclusions: Propranolol may suppress endometrial tissue by its antiangiogenic activity through inhibitory actions on VEGF, MMP-2, and MMP-9. Therefore, propranolol is a promising candidate drug for effective treatment of patients with endometriosis, which needs to be confirmed with further studies.
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Introduction

Endometriosis is an estrogen-dependent disease affecting 8% to 10% of females of reproductive age. It is diagnosed in 71% to 87% of females with chronic pelvic pain and in 30% of women with infertility [1]. It is defined as adherence and growth of the functional layer of the endometrium outside the uterine cavity.

In endometriotic lesions, the development of new blood vessels from pre-existing ones is necessary to supply oxygen and essential nutrients [2], a process that is coordinated by a sequence of humoral and cellular interactions [3]. Vascular endothelial growth factor (VEGF) is of primary importance as a mediator of angiogenesis in endometriosis in addition to its potent endothelial cell-mitogen-, morphogen-, and vascular permeability-inducing activities [4,5]. Furthermore, metalloproteinases (MMPs) influence the outcome of inflammatory reactions, angiogenesis, and tissue remodeling through regulation of extracellular matrix turnover [6,7].

Hormonal therapies, such as dienogest and danazol, widely used to reduce functional endometrial tissue by causing atrophy, thus inducing regression of the symptoms of endometriosis [8,11]. However, treatment with these hormonal agents is associated with adverse events that limit their use, such as estrogen-withdrawal symptoms (e.g. headache, hot flushes, vaginal dryness, decreased libido, and bone demineralization), androgenic effects, adverse effects on lipids, arterial thrombosis, liver dysfunction, increased glucocorticoid activity, and a high incidence of abnormal menstrual bleeding [10,11]. Therefore, there is a need for alternative treatments that are both effective and safe against endometriosis.

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Propranolol is a non-selective beta-blocker that demonstrates equal affinity for adrenoreceptors and therefore acts on multiple tissues. Propranolol has been found to be potent and safe for treatment of hemangiomas by inhibition of angiogenesis [12]. Beta-blockers also decrease the expression of VEGF, thus preventing angiogenesis [12,13].

Based on these data, we hypothesized that propranolol inhibits angiogenesis, which plays a critical role in the pathogenesis of endometriosis. Thus, the aim of this study was to investigate whether propranolol has an inhibitory effect on the angiogenesis of endometriosis in an experimental rat model. For this aim, we evaluated VEGF, MMP2 and MMP9 immunoreactivity in endometriosis lesions.

Materials and methods

The study was conducted in the Animal Research Center of Ankara Education and Research Hospital with the approval of the Experimental Animals Ethics Committee of the same institution. All procedures were performed in compliance with the international guidelines for the care and use of experimental animals.

Twenty-four female, non-pregnant Wistar albino rats weighing 200 to 250 g were used to create a model for the experimental induction of endometriosis. The rats were caged individually in a controlled environment with a 12-h light/dark cycle and were fed ad libitum. The animals were sexually mature and demonstrated normal estrous cycle changes in uterine histology (data not shown). All rats were observed for several days to ascertain their health before the operation.

Unlike human females, rats do not menstruate, nor do they spontaneously develop endometriosis. Therefore, endometriosis was surgically induced in rats by transplanting an autologous fragment of endometrial tissue onto the inner surface of the abdominal wall as proposed by Vernon and Wilson [14] with modifications by Lebovic et al. [15]. The rats were anesthetized by intramuscular administration of 40 mg/kg of ketamine hydrochloric acid (Ketalar; Eczacıbasi Warner-Lambert, Levent, Istanbul, Turkey) and 10 mg/kg of xylazine hydrochloric acid (Rompun; Bayer, Sisli, Istanbul, Turkey). They were immobilized on a standard rat surgery board [16]. Before surgery, the abdominal skin was shaved, and antisepsis was obtained by 10% povidone iodine solution. A 4- to 5-cm ventral vertical incision 2 cm over the pubis was made to expose the reproductive organs using a sterile technique. The operation was limited to 25 min for each rat to limit the effect of room air on tissue drying. The abdominal incision was closed in a continuous interlocking manner with 2-0 silk sutures. All rats underwent three consecutive laparotomies.

The first operation involved creation of the rat model of endometriosis. Vaginal smears were performed on a daily basis, and only rats exhibiting regular 4- to 5-day estrous cycles were used. Surgery was performed under aseptic conditions as described above. When a rat was in estrous, a distal segment 1 cm in length was resected from the right uterine horn and placed in warm sterile phosphate-buffered saline (PBS). Without removing the myometrium, a 5- × 5-mm piece of this fragment was implanted onto the inner surface of the right side of the abdominal wall close to an artery with the serosal surface apposed and sutured with 4-0 vicryl (polyglactin 910, Medico Huaian Co., Ltd Jiangsu, China) sutures at two edges. Yilmaz et al., secured with non absorbable 4-0 polypropylenesutures at 2 edges and demonstrated successful, viable endometrial implants in their experimental rat model [21]. Attachment of the implant at 2 edges would be less traumatic and it would be less effected by the course of the endometriosis model. Because the endometriosis is also a chronic inflammatory disease.

Before closure of the abdominal wall, 2 mL of saline were administered into the abdominal cavity to prevent drying and to minimize adhesion formation, and the incision was closed in a continuous interlocking manner with 2-0 silk sutures. After this operation, all rats were observed for 4 weeks without medication. Two rats died during this period.

The remaining 22 rats underwent a second laparotomy to examine the endometrial implants for size and viability. One of the investigators measured the endometrial implants in three dimensions ((length × width × height in mm) using a caliper as described by Demirel et al. [31]. The volumes of the implants were calculated using the ellipsoid formula: V (mm^3) = $0.52 \times A \times B \times C$, where A, B, and C indicate width, length, and height, respectively. Tissues were photographed using a digital camera, and all measurements were recorded.

The rats were randomly divided into treatment (propranolol) and control groups. The rats in the treatment group ($n = 11$) were given 10 mg/kg of intraperitoneal propranolol daily (Dideral 40-mg tablet; Sanofi Aventis, France). The drug was dissolved in distilled water and given in a volume of 2 mL. The drug dose was determined based on a previous experimental rat study related to propranolol [17,18]. The rats in the control group ($n = 11$) received daily intraperitoneal injections of 2 mL of saline as a vehicle solution. The injections were applied at approximately the same time each day for 3 weeks.

One rat in the control group died the day after the second laparotomy; thus, the final number of rats in the treatment group was 11 and that in the control group was 10. At the end of the 3 weeks, the rats were euthanized by ketamine anesthesia, and the third laparotomy was performed. The sizes of the endometrial implants were measured again with the same caliper method by the same investigator, who was blinded to the groups. The implants were photographed, then excised and fixed in 10% formaldehyde buffer for histopathologic and immunohistochemical examination.

The formalin-fixed endometriotic foci were embedded in paraffin blocks, sectioned at ~5-μm thickness, and stained with hematoxylin and eosin. All tissues were examined by a blinded observer using a ×40 objective lens on a light microscope (Olympus BX51, Tokyo, Japan). The histologic diagnosis of endometriosis was based on the morphologic diagnosis of endometrial glandular tissue and stroma of the endometrial type, with epithelial lining and luminal formation. Semiquantitative analysis of endometriotic explants was performed according to a scoring system as follows: 3, well-preserved epithelial layer; 2, moderately preserved epithelium with a leukocytic infiltrate; 1, poorly preserved epithelium (occasional epithelial cells only); and 0, no epithelium [19,20].

Immunochemistry

A Leica polymer detection kit was used (DS9800; Leica, Newcastle, United Kingdom) on a Leica BOND-MAX automated IHC ISH system for MMP-2, MMP-9, and VEGF. Kidney, colon, breast cancer, and ovarian cancer tissues were used as positive controls for MMP-2 and MMP-9; corpus luteum, small intestine, and ovary tissues were used as positive controls for VEGF.

Paraffin-embedded tissues were sectioned at 3-μm thickness, deparaffinized in the fully automated systems, boiled in EDTA for 20 min, and incubated with MMP-2 antibody (MMP2-507; Leica Bond, Newcastle, United Kingdom) or MMP-9 antibody (MMP9-439; Leica Bond, Newcastle, United Kingdom) at a 1:40 dilution or in VEGF (C-1) (sc-7269; Santa Cruz Biotechnology, Inc. Dallas, Texas 75220 U.S.A) at a 1:100 dilution for 20 min. For secondary elements, a polymer detection kit was used (DS9800; Leica Bond, Newcastle, United Kingdom). The sections were dehydrated and

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