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# The Effects of Lapatinib and Trastuzumab in a Rat Model of Endometriosis

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**ABSTRACT**

Trastuzumab and lapatinib are drugs belonging to tyrosine kinase inhibitors family that are used in cancer treatment to prevent cell proliferation. Trastuzumab is an inhibitor of human epidermal growth factor receptor–2 (HER2) tyrosine kinase, and lapatinib is an inhibitor of epidermal growth factor receptor (EGFR). Tyrosine kinase inhibitors have also been investigated for treatment of endometriosis. In the present study, we aimed to investigate the effects of lapatinib and trastuzumab on rat endometriosis model. Endometriosis was surgically induced by the autologous transplantation of endometrial tissue and formation of endometriosis was confirmed via secondary laparotomy in 32 rats. Initially, 4 mg/kg dose of trastuzumab was applied intraperitoneally, and two additional doses of 2 mg/kg were applied 7 days and 14 days after the initial dose. Lapatinib was administered as 100 mg/kg daily doses for 14 days. Rats were randomly divided into four groups and were subjected to lapatinib, trastuzumab, anastrozole (0.004 mg/day, p.o.) and normal saline (0.1 ml, i.p.) treatments for 14 days. Then, endometriosis foci were excised, and endometriosis scores were calculated in a semi-quantitative manner. Immunohistochemical (IHC) examinations were also performed using VEGF, CD117 and Bax antibodies. Both anastrozole and tyrosine kinase inhibitors lowered endometriosis scores. Significant decreases in ovarian follicle numbers were observed in lapatinib and anastrozole groups but not trastuzumab group. Lapatinib and trastuzumab decreased endometriotic foci through suppressing cell proliferation and promoting programmed cell death.

***Keywords:*** Endometriosies, Lapatinib, Trastuzumab, Anastrozole, Tyrosine kinase inhibitors.

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## Introduction

Endometriosis is a progressive, chronic disease characterized by the growth of endometrial tissues out of the endometrial cavity, especially on ovarian visceral and pelvic peritoneal surface [1]. It is a common disease with an estimated prevalence of 6-10% of reproductive-aged women, and patients with endrometriosis may have chronic pelvic pain and impaired fecundity [2]. Endometriosis poses a significant and costly public health problem because of the expense of medical care including the need for surgical procedure and recurrence after surgical treatment [3].

Although multiple theories exist to explain the pathophysiology of endometriosis, Sampson's retrograde menstruation/transplantation theory suggesting the attachment and adhesion of endometrial fragments in peritoneal surfaces is the most widely accepted one [4]. According to Sampson's theory, for endometrial tissues implantation in peritoneal and subperitoneal surfaces, neoangiogenesis, blood supply and endometrial cell proliferation are absolute necessities [5].

Angiogenesis plays a key role in the formation of endometriosis. [6]. Vascular endothelial growth factor (VEGF) and its receptor VEGFR-1, and epidermal growth factor (EGF) and its receptor EGFR are involved in the process of neovascularization in endometrial tissue formation [6]. EGF and EGFR have roles in cell proliferation in ectopic endometriotic foci and it has been reported that gene expressions of EGF and EGFR are different from those in eutopic endometrium tissues and they are associated with the severity of the disease [7].

Although endometriosis is a benign, sex hormone-dependent gynecological disease, its pathogenesis is similar to that of malignant tumoural tissues. Therefore the efficacy of multi-targeted tyrosine kinase inhibitors that may inhibit angiogenesis and cell proliferation and commonly studied in current cancer trials, for the treatment of endometriosis have been investigated [8].

Unlike trastuzumab (Herceptin®), a humanized monoclonal antibody that targets the tyrosine kinase receptor, human EGFR–2 (HER2), lapatinib is an oral dual thyrosine kinase inhibitor of both EGFR and HER2 [9]. Lapatinib reversibly binds to intracellular domains of tyrosine kinase domains EGFR and HER2 causing phosphorylation of tyrosine kinases and drives cells to apoptosis and prevents cell proliferation through inhibition of activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinases (PI3Ks) [10]. The aim of this study was to investigate the role of

anastrozole, a drug used as standard hormonal treatment of endometriosis and tyrosine kinase inhibitors lapatinib and trastuzumab on angiogenesis, apoptosis and endometrial cell proliferation in an experimentally established rat endometriosis model.

## Materials and methods

Wistar-Albino adult female rats weighing 220-240 g were obtained from Laboratory Animal Centre of Cumhuriyet University (Sivas, Turkey). Rats were kept in a light-and temperature-controlled (22°C) room with diurnal lighting without limitation for food and water. There was no difference in the weights of the rats before and after the study. Procedures were performed according to the scientific guidelines for Institutional Care and Use of Laboratory Animals. The study was approved by AnimalResearch Ethics Committee of the Cumhuriyet University (Approval No: 394). Minimal numbers of animals were used in the experiments and every efforts were made to minimize their suffering.

### *Induction of Endometriosis*

To induce endometriosis, uterine tissue was autotransplantated into anterior abdominal wall using the technic suggested by Vernon and Wilson [11]. Ketamine and xylazine were administered intraperitoneally for anesthesia (60 mg/kg and 7 mg/kg, Ketalar 1; Eczacibasi Warner-Lambert, Istanbul, Turkey and Rompun 1, Bayer, Istanbul, Turkey, respectively). The left uterine horns of the rats were ligated and excised via laparotomy, placed in normal saline, then opened longitudinally and dissected into 5 mm squares. The endometrial tissue containing the myometrium was sutured into the peritoneal cavity. The study groups were allowed to recover for 3 weeks, without any medications.

### *Evaluation of Endometriosis*

Twenty-one days after the day endometriosis was induced, a second exploratory laparotomy was performed to observe the growth of endometriosis. Thirty-two rats having confirmed endometriosis were randomly divided into four groups of eight rats each to receive lapatinib (100 mg/kg/day, p.o.), trastuzumab (after the initial 4mg/kg dose of trastuzumab applied intraperitoneally, two additional doses of 2 mg/kg were applied 7 days and 14 days after the initial dose), anastrozole (0.004 mg/d, p.o.) or normal saline (0.1 ml, intraperitoneally). The treatments were applied for fourteen days.

### *Collection of Tissue Samples*

Twenty-one days after the day the development of endometriosis was confirmed, endometriotic and ovarian tissue samples were excised via a third laparotomy. All the rats were euthanized using pentobarbital sodium after completion of the procedures. One of the authors (C.Y) performed all of the surgical procedures.

### *Histopathology*

Formalin 10% was used for fixation the ovaries and the ectopic endometriotic tissues, and then histopathological and immunohistochemical examination (IHC) has done. One of the authors (H.O) who is blinded for the groups made all evaluations.

### *Histopathology of Endometriotic Implants*

Endometriosis scoring was performed according to the description of Keeenan et al. [12] The epithelial lining of the endometrial implants were classified using the following scale: No epithelium, Score 0; Poorly preserved epithelium (occasional epithelial cells only), Score 1; Moderately preserved epithelium with leukocyte infiltrate, Score 2; Well preserved epithelial lining, Score 3 [12].

### *Immunohistochemistry of Endometriotic Foci*

Immunohistochemical staining was performed on the BenchMark XT system (Ventana Medical Systems, Roche) with antibodies against VEGF (Ab-4, Clone BFD31, Lab Vision, USA), CD117 (K69, Lab Vision, USA), and Bax (Ab-2, Clone 5B7, LabVision, USA).

For VEGF immunohistochemical staining, a semi-quantitative method suggested by Donnez et al. [13] was used. VEGF histologic scores (H) were calculated according to the following formula: $H = \Sigma Pi$, i (intensity) varied between 0 (negative cells) and 3 (intensely stained cells), P (percentage of stained cells) for each given i, and P values of staining of <15% of the cells as 1, staining of 15-50% of the cells as 2, staining of 50–85% of the cells as 3, staining of >85% of the cells as 4 and staining of 100% of the cells as 5. For Bax and CD117 immunohistochemical staining, an immunoreactivity score (IRS) obtained by multiplication of the the P (0–4) and i (0–3) was used [14]. The IRSs were as follows: 0, no staining; 1-4, weak staining; 6, 8, 9 or 12, strong staining.

### *Histopathology of Ovaries*

Formalin 10% solution was used for fixation of the rat ovaries. For dehydration and clearing, ethanol and xylene were used. Sections of 6 mm thickness were taken from the tissues for H&E staining. The follicles were classified according to stage into primordial, primary, secondary, and antral and the numbers of each follicles were counted in five sections for each ovary, with a distance of 120 µm between them in order to ensure counting one follicle once.

### *Statistical Analysis*

SPSS version 20.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis. Post Hoc test in ANOVA was used for analyzing the treatment groups. Tukey's Post Hoc test was used to compare the treatment groups for endometriosis scores, ovarian follicle numbers and VEGF, Bax and CD117 immunostaining scores. The scores are

shown as mean ± standard error of the mean and a p-value less than 0.05 (≤ 0.05) was considered as significant.

## Results

The experimental procedures were completed without any apparent side effects. The average weights of the rats before the study entry did not change significantly after the procedures. Evaluation during the second exploratory laparotomy showed that vascularization and cystic appearance of the endometriotic implants were sufficient. Also, the histological examinations confirmed the development of ectopic endometriotic tissue. The histopathological images showing the effect of normal saline, anastrozole, lapatinib and trastuzumab on endometriotic tissues are shown in Figure 1.

The average endometriosis scores, ovarian follicle numbers, and immunostaining scores of VEGF, CD117 and Bax of the normal saline, anastrozole, lapatinib and the trastuzumab groups are shown in Table 1.

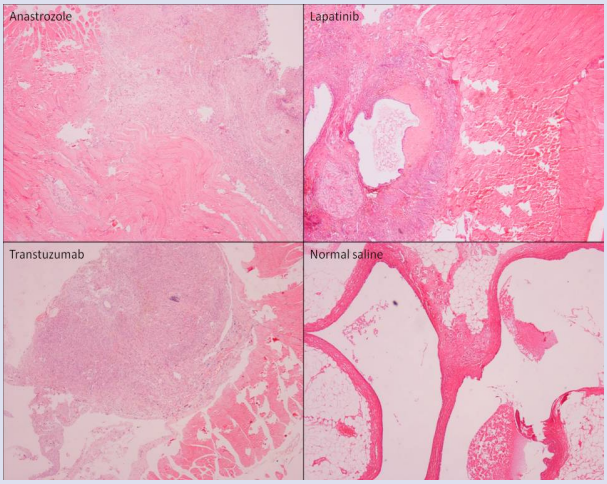


Figure 1. Representative histopathological images (hematoxylin and eosin, _40) of endometriotic tissues obtained from rats administered Anastrozole, Lapatinib, Trastuzumab, and normal saline. Endometriotic tissue shows poorly preserved epithelium in Anastrozole group but moderately preserved epithelium in Lapatinib and Trastuzumab groups, compared to normal saline group.

Table 1. Compresion of histopathologic and immunohistochemical levels of the endometriotic implants

| | SF Mean±SEM | AnastrozoleMean±SEM | Lapatinib Mean±SEM | transtuzumab Mean±SEM | ANOVA | Comparison group | Post hoc p |
|---|---|---|---|---|---|---|---|
| Endometriosis score | 2.750±0.13 | 0.500±0.189 | 1.625±0.33 | 1.250±0.366 | 0.0001 | 1 vs. 2 | 0.0001 |
| | | | | | | 1 vs. 3 | 0.008 |
| | | | | | | 1 vs. 4 | 0.003 |
| | | | | | | 2 vs. 3 | 0.005 |
| | | | | | | 2 vs. 4 | 0.009 |
| | | | | | | 3 vs. 4 | 0.770 |
| Ovarian follicle number | 7.375±0.55 | 4.000±1.210 | 4.000±0.77 | 7.000±0.906 | 0.012 | 1 vs. 2 | 0.005 |
| | | | | | | 1 vs. 3 | 0.005 |
| | | | | | | 1 vs. 4 | 0.993 |
| | | | | | | 2 vs. 3 | 0.991 |
| | | | | | | 2 vs. 4 | 0.099 |
| | | | | | | 3 vs. 4 | 0.009 |
| Apoptosis (Bax) | 3.500±2.00 | 4.000±0.755 | 4.500±1.72 | 4.125±2.295 | 0.000 | 1 vs. 2 | 0.003 |
| | | | | | | 1 vs. 3 | 0.001 |
| | | | | | | 1 vs. 4 | 0.002 |
| | | | | | | 2 vs. 3 | 0.596 |
| | | | | | | 2 vs. 4 | 0.114 |
| | | | | | | 3 vs. 4 | 0.976 |
| Endometriotic proliferation (CD117) | 7.250±1.51 | 6.750±1.752 | 3.925±1.92 | 4.325±2.031 | 0.005 | 1 vs. 2 | 0.119 |
| | | | | | | 1 vs. 3 | 0.0001 |
| | | | | | | 1 vs. 4 | 0.005 |
| | | | | | | 2 vs. 3 | 0.006 |
| | | | | | | 2 vs. 4 | 0.014 |
| | | | | | | 3 vs. 4 | 0.374 |
| VEGF | 6.375±1.86 | 1.750±0.462 | 6.000±1.51 | 5.250±2.439 | 0.0001 | 1 vs. 2 | 0.000 |
| | | | | | | 1 vs. 3 | 0.972 |
| | | | | | | 1 vs. 4 | 0.566 |
| | | | | | | 2 vs. 3 | 0.001 |
| | | | | | | 2 vs. 4 | 0.002 |
| | | | | | | 3 vs. 4 | 0.819 |

Data are expressed as means ± standard error of the mean.
ANOVA: analysis of variance; SEM: standard error of the mean VEGF: vascularendothelialgrowthfactor; NS: normal saline

The average endometriosis scores in the anastrozole, lapatinib and the trastuzumab groups were significantly lower than those in the normal saline group (P=0.000). The endometriosis score of the anastrozole group was significantly lower compared to those of the lapatinib and the trastuzumab groups (p=0.003 and p=0.008, respectively). The endometriosis score did not significantly differ between the the lapatinib and the trastuzumab groups (P=0.770, Figure 2.).

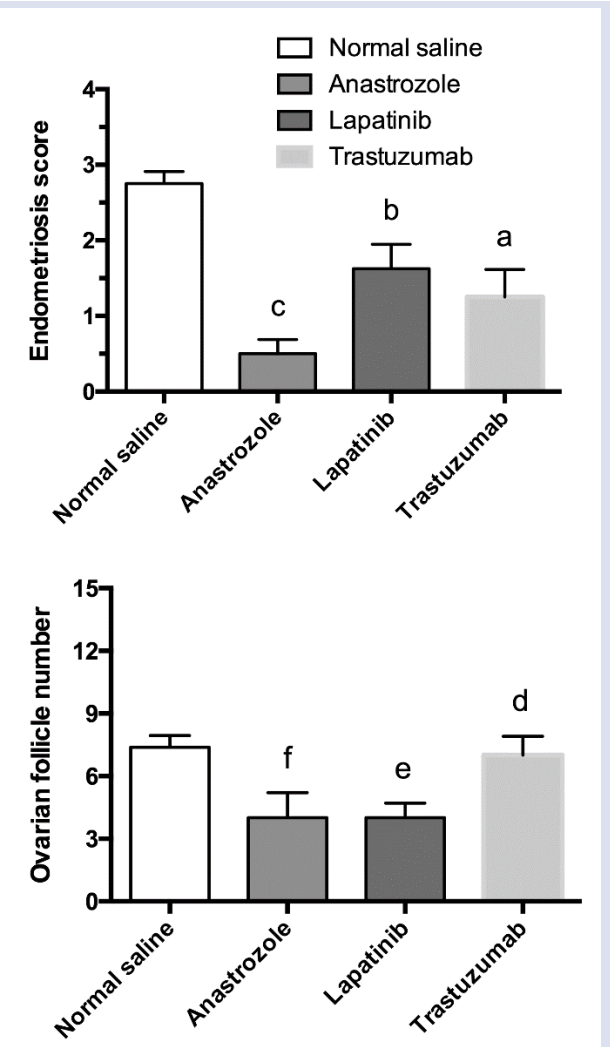


Figure 2. Endometriosis score and ovarian follicle number of normal saline, anastrazole, lapatinib, and trastuzumab groups (n = 8). Data were expressed as mean+ SEM(standard error of the mean).aP< 0.05; trastuzumab vs. anastrozole and normal saline. bP< 0.05; lapatinib vs. anastrozole and normal saline. c,fP< 0.05; anastrozole vs. normal saline.dP< 0.05; trastuzumab vs. lapatinib. eP< 0.05; lapatinib vs. normal saline.

Both the anastrozole and the lapatinib groups had significantly lower ovarian follicle numbers than the normal saline group (P=0.005 for both), the trastuzumab group did not differ from the normal saline group regarding the ovarian follicle number (P=0.991, Figure 2).

The anastrozole group had significantly lower VEGF staging scores than the normal saline group (P=0.000), there were no significant differences for VEGF staging scores between the saline group and the trastuzumab or the lapatinib groups (p=0.972 and p=0.566, respectively, Figure 3.).

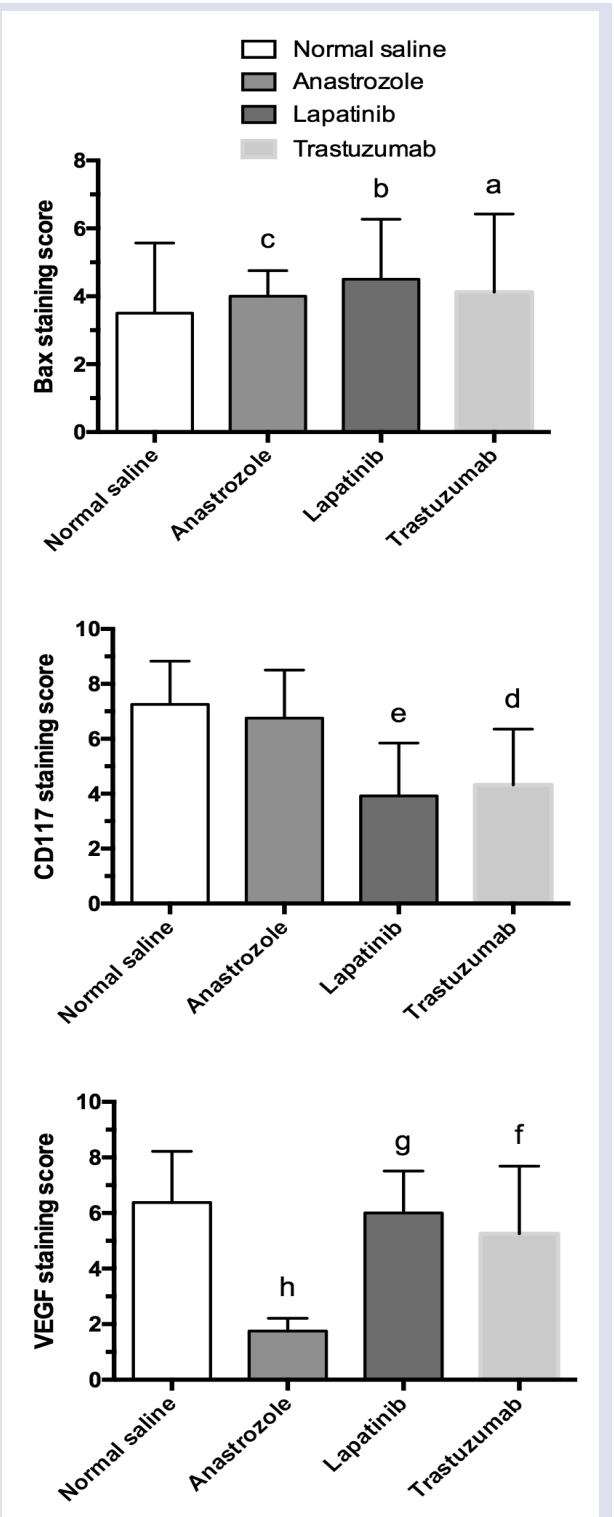


Figure 3. VEGF, CD117, and Bax staining scores of rats administered normal saline, anastrazole, lapatinib, and trastuzumab (n = 8 for each drug). Data were expressed as mean + SEM. a,b,cP< 0.05; trastuzumab, lapatinib, and anastrozole vs. normal saline. dP< 0.05; trastuzumab vs. normal saline. eP< 0.05; lapatinib vs. anastrozole and normal saline. fP< 0.05; trastuzumab vs. anastrozole. gP< 0.05; lapatinib vs. anastrozole.hP< 0.05; anastrozole vs. normal saline.

CD117 staging scores of the anastrozole and the normal saline groups were similar (p=0.119), but the lapatinib and the trastuzumab groups had lower CD117 scores than the normal saline group (p=0.000 and p=0.005, respectively). The difference between the lapatinib and the trastuzumab groups was not significant (P=0.374, Figure 3).

Bax staging scores showed that apoptosis significantly increased in all drug groups compared to the normal saline group (P=0.000), but the differences among the drug groups were not significant (Figure 3.).

## Discussion

Although it may show the characteristics of cancer cells such as spreading to distant organs and surrounding organs, endometriosis is actually considered a benign disease. It can cause chronic pelvic pain, dyspareunia, dysmenorrhea and sometimes infertility. However, it can also cause comorbidities such as adenomyosis, urinary system diseases, gastrointestinal system fasts. For these reasons, it is a disease that both complicates and restricts women's lives and imposes a serious financial burden on the health system. [15]. Despite all these known effects and numerous studies, there are still many question marks about its etiology and treatment options are limited. In the treatment of women with endometriosis, hormonal therapy and surgical treatment or combinations of these are performed. Especially the side effects of long-term hormonal treatment on patients and the inability to always achieve sufficient success in surgical treatment bring along recurrences [16]. Because of these question marks in diagnosis and treatment, endometriosis is still an important disease on which many studies should be done.

With the important developments in molecular biology, targeted therapies have entered clinical use and are increasingly used in treatments. In these new generation therapies, unlike the classical cancer treatment, the targeted therapeutic agents used are directed to the tumor cells, killing the cancer cell while ensuring that the normal cells are not affected [17]. In this process, monoclonal antibodies specific to the epidermal growth factor receptor (EGFR) and tyrosine kinase inhibitors have begun to be used in clinical practice. Blocking the ligand binding to EGFR, blocking the receptor with monoclonal antibodies or inhibiting it with tyrosine kinase activation constitutes an important treatment approach. Trastuzumab is the first targeted drug used in human epidermal growth factor receptor 2 (HER2) positive breast cancer. Lapatinib, on the other hand, is a tyrosine kinase inhibitor that effectively inhibits signaling pathways in cancer cells [18]. Since monoclonal antibodies cannot pass through the cell membrane, they can only act through molecules that are expressed or secreted on the cell surface. Tyrosine kinase inhibitors such as lapatinib interact with the cytoplasmic parts of cell receptors and intracellular signaling molecules. Epidermal growth factor (EGF) initiates a series of intracellular events by binding to specific receptors on cell membranes, and EGFR activation stimulates tumor growth and progression, increases proliferation, and inhibits angiogenesis, invasion, metastasis, and apoptosis [19]. Especially angiogenesis and cell proliferation play an important role in the development of endometriosis. EGFR is found in the corpus luteum as well as granulosa and theca cells in the follicle in the ovaries [20]. It has been reported that EGFR and HER2 are expressed in the endometrial tissue in the menstrual cycle of humans. It has also been suggested that HER2 (c-erbB2 or neu) is expressed in primordial germ cells, granulosa cells, luteal cells, and oocyte in the ovary and plays a role in primordial follicle growth, regulation of granulosa cell function, and oocyte maturation [21].

In our study, histological observations of endometriotic implants in experimentally established rat endometriosis model showed that lapatinib, trastuzumab and anastrozole decreased the development of endometriosis and suppressed the foci. Anastrozole was the drug that most effectively suppressed endometriosis, whereas the efficacy of lapatinib and trastuzumab was similar. Anastrozole and lapatinib administration decreased ovarian follicle number but such an effect was not observed with trastuzumab. Effects of anastrozole, lapatinib and trastuzumab on angiogenesis were evaluated through VEGF immunostaining and it was shown that anastrozole administration significantly lowered VEGF staining. Although lapatinib and trastuzumab administration seemed to lower VEGF scores, the effects were not statistically significant compared to normal saline administration. Cell proliferation, evaluated by CD117 immunostaining, was significantly decreased by lapatinib and trastuzumab administration. On the other hand, anastrozole did not have an effect on cell proliferation. In terms of efficacy on apoptosis evaluated by Bax immunostaining, all drugs were found to increase apoptosis compared to normal saline. In summary, anastrozole lowered endometriosis scores and ovarian follicle number through promoting apoptosis and suppressing angiogenesis, lapatinib and trastuzumab also lowered endometriosis scores, led to a decrease in cell proliferation and an increase in programmed cell death. Lapatinib lowered ovarian follicle number, whereas trastuzumab did not have such an effect.

Anastrozole is widely used in current endometriosis treatment [22]. The drug inhibits the formation of estrogen from androgens in peripheral tissue and decreases cell proliferation [23]. In line with our findings, anastrozole has an apoptosis-enhancing effect on endometrial implants. [24].

Both benign and malignant endometrial diseases are associated with angiogenesis [25]. Angiogenesis process can be defined as formation of new blood veins ensuring blood supply in which VEGF is the key mediator [26]. Patients with endometriosis have higher VEGF levels in both eutopic and the ectopic endometrium tissues than those in healthy women [27].

Anastrozole has been shown to inhibit the development of new vessels in endometriotic tissue via

decreasing VEGF release [28]. Our findings also showed that anastrozole significantly decreased VEGF release in endometriotic foci. On the other hand, trastuzumab, a HER2 tyrosine kinases inhibitor, and lapatinib, an inhibitor of both EGFR and HER2 tyrosine kinases did not have an effect on VEGF staining in our study. These drugs have been shown to suppress angiogenesis in malignant cells and that this effect was principally due to their suppression of HER2 over-expression [29]. The lack of HER2over-expression in endometriotic tissue may be the reason why lapatinib and trastuzumab did not suppress angiogenesis in endometriosis.

The presence of abnormal cell proliferation has been shown in endometriosis [30]. C-kit receptor CD117, a trans-membrane protein, is a tyrosine kinase growth factor receptor, playing role in cell proliferation and growth. C-kit expression has been shown to increase in endometriotic foci compared to eutopic endometrium tissue [31]. Yildiz et al. [32] reported that tyrosine kinase inhibitors pazopanib and sunitinib decreased CD117 level and a restoration of endometriosis score was observed due to the decrease in cell proliferation. In the present study, the effect of tyrosine kinase inhibitors lapatinib and trastuzumab on cell proliferation was evaluated by CD117 immunostaining and it was observed that they decreased cell proliferation in endometriotic foci. Anastrozole, on the other hand, did not have an effect on cell proliferation.

Endometriotic tissue differs from healthy endometrium tissue regarding apoptosis mechanisms, including abnormal expressions of major signal proteins associated with apoptosis such as Fas, Fas ligand, BCL2 and BAX, and decreased programmed cell deaths of endometrial cells [33]. Programmed cell death as a result elevated Bax expression in endometriotic foci is one of the action mechanisms of treatments for endometriosis [34, 35]. Tyrosine kinase inhibitors such as lapatinib and trastuzumab have been shown to increase apoptosis in malignant cells along with the inhibition of HER2 in endometrium, breast and stomach cancers [36, 37]. In our study, lapatinib and trastuzumab promoted apoptosis in endometriotic foci.

The mechanisms of action of current medical treatments with endometriosis include the suppression of ovarian hormones and decreasing the secretion and efficiency of peripheral estrogen [38]. This anti-estrogenic activity causes a decline in ovarian follicle numbers [39]. In accordance with the literature, we observed a significant decrease of follicle number in anastrozole treatment group. We also showed that lapatinib decreased ovarian follicle number in rats, but trastuzumab did not.

As far as we know, the present study is the first study using dual tyrosine kinase inhibitor lapatinib in endometriosis model. There are several limitations of the study that should be noted. We did not evaluate the impact of lapatinib on the eutopic endometrium. In addition, we did not evaluate the effect of lapatinib on ovarian functioning through the regular estrous cycle and/or estrogen levels. Finally, we used only a single standard lapatinib dose and did not evaluate varying doses of the drug on endometriosis regression.

Our study showed that lapatinib and trastuzumab treatments suppress cell proliferation, promote apoptosis process and suppress endometriosis development without a significant effect on angiogenesis. Suppression of endometriotic foci by trastuzumab without any decrease of ovarian follicle number should be further investigated.

## Acknowledgement

A.Z.O., C.Y., and T.K. designed the research study. C.Y. and T.K. performed the research. H.O. performed the histopathological examination. A.Z.O, C.Y. and H.O. analyzed the data. A.Z.O. and C.Y. wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

## Conflict of interest

The authors declare no competing interests.

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