The effects of metformin and letrozole on endometriosis and comparison of the two treatment agents in a rat model

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BACKGROUND: Our aim was to investigate the effects of metformin and letrozole on experimentally induced endometriosis in a rat model.

METHODS: Endometriotic implants were surgically formed, and 38 rats were randomly divided into four groups. Group 1 (control group, 8 rats) was given no medication. Group 2 (metformin group, 10 rats) was given 100 mg/kg/day of oral metformin. Group 3 (metformin group, 10 rats) was given 200 mg/kg/day of oral metformin. Group 4 (letrozole group, 10 rats) was given 0.1 mg/kg/day of oral letrozole. All rats continued to receive the treatment for 4 weeks and then were sacrificed to assess the size of implants and scores of adhesions. The histopathologic scores of implants in excised endometriotic foci were examined by a pathologist.

RESULTS: The mean surface area of endometriotic implants was similar in all groups before the treatment. Although the area was not reduced in controls, it was found to be significantly reduced in all treatment groups (44.50 ± 23.37, 5.90 ± 2.37, 4.30 ± 1.33, 6.90 ± 3.72 mm², respectively; *P < 0.05). The effect was comparable between the treatment groups. The histopathologic assessment revealed that the histopathologic score of implants was lowest after 100 mg/kg/day metformin. Additionally, metformin reduced the severity of adhesions.

CONCLUSIONS: Metformin and letrozole caused a statistically significant regression of endometriotic implants. The effects of metformin on endometriotic tissue were at least comparable to letrozole.

Key words: metformin / aromatase inhibitor / endometriosis / rat model

Introduction

Endometriosis is defined as the presence of a functional endometrial layer with endometrial glands and stroma outside the uterine cavity which induces a chronic, inflammatory reaction and is also linked to pelvic pain and infertility (Kennedy et al., 2005). The morphologic appearance of endometriosis is marked by proliferation, infiltration and severe adhesions around the surrounding tissues. Research into its pathogenesis has focused on anatomic, hormonal, immunologic and genetic factors (Vinatier et al., 2001; Vignali et al., 2002; Nap et al., 2004), although the etiopathology has not been clearly explained yet. However, endometriosis should be accepted as an estrogen-dependent condition because it is seen during the reproductive years and generally disappears after menopause. Aromatase is the key enzyme for conversion of precursor steroids to estrone and estradiol and its activity is detectable in endometriosis (Bulun et al., 2005). In addition, aberrant expression of aromatase and an inflammatory environment stimulates the progression of the disease. Therefore, medical therapy is often aimed at lowering the estrogenic and inflammatory status.

Letrozole is a non-steroidal, highly potent and well-tolerated competitive inhibitor of aromatase enzyme system that is generally used as a second-line agent for the treatment of advanced breast cancer in post-menopausal women (Wasan et al., 2005). Aromatase enzyme has been demonstrated in ovarian granulosa cells, peripheral tissues such as skin, adipose tissue and endometriotic implants (Ailawadi et al., 2004). Local estrogen production by these implants may contribute to the progression of endometriosis even during treatment with GnRH analogs, which only inhibit ovarian production of estrogen (Bulun et al., 1999). Letrozol is a third-generation aromatase inhibitor and it suppresses estrogen production locally and systematically and is effective in the treatment of endometriosis and associated chronic pelvic pain in reproductive age women.
Metformin is a widely used antidiabetic agent that improves insulin sensitivity and is used for the treatment of polycystic ovary syndrome (PCOS) in reproductive medicine (Lord and Wilkin, 2004). Metformin may also reduce inflammation and have an effect on steroidogenesis in ovarian granulosa cells and thecal cells (Mansfield et al., 2003; Isoda et al., 2006). In a recent in vitro study, it was shown that metformin suppresses the inflammatory response, the activation of aromatase and the proliferation in endometriotic stromal cells after culture in a sterile medium (Takemura et al., 2007).

Although, there are only three studies investigating the effect of letrozole on endometriosis in a rat model (Fang et al., 2002; Bilotas et al., 2009; Yildirim et al., 2009), to our knowledge, no studies have been performed to compare the effects of metformin and letrozole on surgically induced endometriosis in an animal model. Additionally, there has been no research to determine the effect of metformin on endometriosis in a rat model. Thus, the aim of the present study was to evaluate the efficacy of metformin and letrozole on endometriotic explants in a rat endometriosis model.

Materials and Methods

Forty female Wistar albino mature rats at 8 weeks, weighing 180–260 g, were used for the study. Animals were fed ad libitum and housed in pairs in steel cages having a temperature-controlled environment (22 ± 2°C) with 12-h light/dark cycles. The experimental procedures were approved by the Committee for Animal Research and the study was conducted in Hakan Cetinsaya Clinical and Experimental Research Center of Erciyes University, Medical Faculty. All animal studies strictly conformed to the animal experiment guidelines of the Committee for Humane Care.

Various methods of induction of endometriosis in rat models have been described. Endometriosis was surgically induced using the method described by Vernon and Wilson (1985) with a minor modification due to our previous experience with this method. All rats were anesthetized, using ketamine hydrochloride at a dose of 50 mg/kg (Ketalar®, flakon, Eczacıbaşı), and 7 mg/kg of xylazine hydrochloric (Rompun®, Bayer) was administered intraperitoneally. The abdomen was opened through a 5 cm midline incision. One uterine horn was ligated at both the uterotubal junction and the cervical end, and removed. The excised horn was immersed into sterile saline solution, the endometrium was exposed by bisecting along its antimesenteric axis, and 5 × 5 mm sections were cut. These explants were then anchored onto the omentum by 7-0 Vicryl® sutures. In all explants, the endometrial surface faced the omentum. Abdominal layers were closed anatomically, using 4-0 Vicryl® and animals were allowed to recover from anesthesia.

At the end of the fourth week following the initial operation, two rats died. The remaining 38 rats, in which endometriosis developed, were surgically examined and the surface area of implants was measured (length × width in millimeters). The tissues were photographed, and the measurements of endometriosis were recorded. After the second operation, all rats were allowed a resting period of 3 days. Then, these rats were randomly divided into four groups. Group 1 (control group, 8 rats) was given no medication, but 4 ml per day of tap water was given by oral gavage. The rats in Group 2 (low-dose metformin group, 10 rats) were given 100 mg/kg/day of oral metformin. The rats in Group 3 (high-dose metformin group, 10 rats) were given 200 mg/kg/day of oral metformin. The rats in Group 4 (letrozole, 10 rats) were given 0.1 mg/kg/day of oral letrozole. Tablets containing 1000 mg metformin (Gilfor, Bilim) and 2.5 mg letrozole (Femara, Novartis) were crushed and suspended in tap water to yield a concentration of 10 mg/ml. According to the weight of each rat, suspended drug solution was completed to 4 ml with tap water. The medications were given via orogastric tubes.

Four weeks after the beginning of the treatments, the third laparotomy was performed, and the rats were sacrificed by ketamine anesthesia. In the third laparotomy, the length and width of implants were measured macroscopically and the surface area of the implants was calculated again. The extent and the severity of adhesions in the operation site of endometriotic implants were evaluated by the same pathologist (F.O.) in a blinded manner, using an established scoring system (Linsky et al., 1987). According to the adhesion system, the extent of adhesions was evaluated as follows: 0, no adhesions; 1, 25% of traumatized area; 2, 50% of traumatized area; 3, total involvement. Fractional scores were given for the extent of adhesions between above grades. The severity of the adhesions was measured as follows: 0, no resistance to separation; 0.5, some resistance (moderate force was required); 1, sharp dissection needed. The total grade was additive, giving a range of adhesion scores from 0 to 4, which represented both extent and severity. These measurements and evaluations were made by one operator blinded to the study.

The implants were then excised and fixed in 10% formalin for histopathologic examination. The formalin-fixed endometriotic foci were embedded in paraffin blocks, sectioned at ~5 mm thickness (four sections per sample), stained with hematoxylin and eosin and examined under a light microscope. The pathologist assessing the samples was blinded to the treatment groups. The persistence of epithelial cells in endometrial explants was semiquantitatively evaluated as follows: 3, well-preserved epithelial layer; 2, moderately preserved epithelium with leukocyte infiltrate; 1, poorly preserved epithelium (occasional epithelial cells only) and 0, no epithelium. This evaluation was based on a previous rat endometriosis study (Keenan et al., 1999).

The statistical analyses were performed using the Statistical Package for the Social Sciences version 11.0 (SPSS Inc., Chicago, IL, USA). Nonnormally distributed metric variables were analyzed by the Kruskal–Wallis test and Mann–Whitney U-test. The mean surface areas of the endometriotic implants between the same group (before and after the medical treatment) were analyzed by Wilcoxon’s signed-rank test. P < 0.05 was considered statistically significant. The statistical comparison of the histopathology of the groups, and adhesion scores were made using one-way ANOVA. Values were expressed as mean ± standard deviation, unless stated otherwise.

Results

In 38 of the 40 rats, endometriosis implants were formed. At the beginning of the medical treatment, the mean surface areas of the endometriotic implants were comparable in all four groups (Table I). However, at the end of the treatment, the mean areas of the implants in all treatment groups were smaller than those of the control group (Table I).

The mean surface areas of implants were similar in all treatment groups. The decrease in the surface area of the endometriotic implants due to the medical treatment was significant in Group 2 (from 45.50 ± 10.68 to 5.90 ± 2.37 mm², P < 0.05), Group 3 (from 46.80 ± 6.81 to 4.30 ± 1.33 mm², P < 0.05) and Group 4 (from 48.80 ± 9.55 to 6.90 ± 3.72 mm², P < 0.05) compared with the control group (from 43.12 ± 17.27 to 44.5 ± 23.37 mm², P > 0.05). Sample views of the endometriotic implants are shown in Fig. 1.

The mean score of the histopathologic examination of the implants at the end of the treatment was lower in Group 2, when compared with the control group and Group 4 (Table I). On the other hand, there was no statistically significant difference between the metformin groups. Although the histopathologic scores were lower in Groups 3
and 4 compared with the control group, there were no statistically significant differences between these groups. Sample views of the histologic scores of endometriotic implants are shown in Fig. 2.

The extent, the severity and the total scores of the adhesions that occurred due to the implants were measured locally after the third laparotomy (Table I). As shown in Table I, the severity scores of adhesions were significantly reduced in the metformin treatment groups compared with those in the letrozole treatment and control groups. Although a statistically significant difference was found between Groups 3 and 4 in the total adhesion score, there were no statistically significant differences between Group 3 compared with the control group or Group 2.

### Table I Treatment results and comparisons of the study groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (control)</th>
<th>Group 2 (100 mg metformin)</th>
<th>Group 3 (200 mg metformin)</th>
<th>Group 4 (letrozole)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats</td>
<td>8</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Mean surface area of implants (mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before medication</td>
<td>43.12 ± 17.27a</td>
<td>45.50 ± 10.68a</td>
<td>46.80 ± 6.81a</td>
<td>48.80 ± 9.55a</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>After medication</td>
<td>44.5 ± 23.37a</td>
<td>5.90 ± 2.37b</td>
<td>4.30 ± 1.33b</td>
<td>6.90 ± 3.72b</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Histopathologic score of implants</td>
<td>2.5 ± 0.53a</td>
<td>1.10 ± 0.73b</td>
<td>2.00 ± 0.67ab</td>
<td>2.20 ± 1.22b</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Extent of adhesion</td>
<td>2.12 ± 0.35ab</td>
<td>2.20 ± 0.63ab</td>
<td>1.90 ± 0.73a</td>
<td>2.70 ± 0.48b</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Severity of adhesion</td>
<td>0.87 ± 0.23a</td>
<td>0.40 ± 0.39b</td>
<td>0.45 ± 0.28b</td>
<td>0.80 ± 0.25a</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total score of adhesion</td>
<td>3 ± 0.46ab</td>
<td>2.6 ± 0.84ab</td>
<td>2.35 ± 0.97a</td>
<td>3.5 ± 0.70b</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Statistically significant difference is not present in groups sharing the same letter.

All data sets of power of performed test with $\alpha = 0.050$: 0.766–1.000.

Figure 1 Macroscopic appearance of endometriotic implants (arrows) in (a) the control group, (b) Group 2, (c) Group 3 and (d) Group 4.
Medical treatments for endometriosis are usually aimed at reducing the endogenous steroid production. Medroxyprogesterone acetate, danazol, oral contraceptives and GnRH-a are all effective in the pain-associated symptoms of endometriosis and are also effective in the regression of the endometriotic lesions. However, their adverse effects limit their long-term use, and recurrence rates after cessation of therapy are high. Additionally, they have no benefit for endometriosis-associated infertility (Hughes et al., 2000). Therefore, new agents, which present synchronous fertility treatment with improved side-effect profiles, are needed. These treatments should also be as effective as hormonal treatments.

GnRH analogs, which suppress ovarian production of estrogen, are the most widely prescribed medical treatment for severe endometriosis. Although treatments have resulted in about a 50% reduction in symptoms in women with moderate and severe endometriosis, the recurrence rate for pelvic pain is as high as 75% at 5-year follow-up (Surrey et al., 2002). Long-term GnRH agonist therapy is not currently practical because of the side effects associated with a hypoestrogenic state, the most serious being bone loss (Pierce et al., 2000). Letrozole might also have similar side effects as a result of hypoestrogenic conditions. Although there has not been any study investigating the effects of metformin on women with endometriosis, metformin therapy may be more beneficial due to the lack of serious side effects.

Aromatase p-450 is the key enzyme for estrogen biosynthesis as it catalyzes the conversion of androstenedione and testosterone to estrone and estradiol. Aromatase expression is consistently found in endometriotic lesions and in the eutopic endometrium from women with endometriosis, whereas it is absent in eutopic endometrium from women without the disease (Meresman et al., 2005). Therefore, aromatase enzyme inhibitors have recently been used for the treatment of endometriosis. Aromatase inhibitors appear to have a promising effect on pain associated with endometriosis, reducing lesion size and probably improving life quality associated with endometriosis (Nawathe et al., 2008). A pilot study using letrozole and norethindrone acetate in premenopausal patients with refractory endometriosis reported marked reduction in pain and laparoscopically visible and histologically confirmed endometriosis (Ailawadi et al., 2004). There are three studies investigating the effect of letrozole on endometriotic lesions in a rat model and all revealed that the treatment with letrozole decreases the size of endometriotic lesions (Fang et al., 2002; Bilotas et al., 2009; Yildirim et al., 2009). In agreement with these investigations, our study also showed that letrozole causes a regression in the size of endometriotic implants in the rat models. On the other hand, the effect of letrozole on adhesion formation was not satisfactory. Although aromatase inhibitors are effective in the treatment of endometriosis and may be a beneficial effect on the treatment of infertility, they have side effects similar to GnRH analogues as a result of hypoestrogenic conditions.
The autotransplantation of uterine pieces into the peritoneal cavity is a well-established method for the induction of endometriosis in rats (Vernon and Wilson, 1985). These autologous endometrial implants are similar to human lesions in vivo and they react in a similar manner to human endometriotic tissues and cells in isolated cell cultures (Sharpe-Timms, 2002; Uchiide et al., 2002). Therefore, this rat endometriosis model permits the study of events involved in the pathophysiology of endometriosis as well as the examination of novel therapeutic approaches for this disorder.

The present study clearly demonstrates that the treatment with a biguanide insulin sensitizer metformin effectively causes regression of endometriotic implants in a rat endometriosis model. The effect is similar to that obtained with letrozole, which is known to be an effective drug used in the medical treatment of endometriosis. To our knowledge, this is the first study investigating and comparing the effects of metformin and letrozole in an experimental endometriosis model.

Metformin has been shown to have an antiproliferative effect on endometrial glands (Session et al., 2003; Takemura et al., 2007; Shen et al., 2008). An antiproliferative effect of metformin has also been demonstrated in leptin-stimulated vascular smooth muscle cells (Li et al., 2005). Abundant aromatase expression and elevated local estrogen levels suggesting local estradiol production by the aromatase enzyme have been demonstrated in endometriotic tissues (Attar and Bulun, 2006). On the other hand, metformin has been shown to inhibit FSH, insulin-stimulated progesterone and estradiol production in granulosa cells (Mansfield et al., 2003). Thus, metformin may inhibit endometriosis through suppression of both ovarian and local production of estrogens.

In a study, endometriotic stromal cells cultured from the human ovarian endometrioma were incubated with metformin for 24 h (Takemura et al., 2007) and the effects of metformin on inflammatory response, estradiol production and proliferation of endometriotic stromal cells were evaluated. Consequently, the favorable effects of metformin on included parameters were demonstrated. Metformin also dose-dependently decreased the cAMP-stimulated aromatase activity in endometriotic lesions. But, in this study, due to the technical limitations, only ovarian endometriotic cells were used. In our study, we demonstrated the regression of non-ovarian endometriosis in the metformin groups similar to that in the group treated with an aromatase inhibitor.

Metformin is an insulin-sensitizing agent used to treat PCOS, which is the most common cause of an ovulation and infertility, affecting 5–10% of women of reproductive age (Franks, 1995). In women with PCOS, metformin treatment restores the cyclic nature of menstruation (Velázquez et al., 1997) and increases ovulation, fertilization and pregnancy rates (Vandermolen et al., 2001). These improvements have been attributed to the decreases in the level of insulin, subsequently attenuating a hyperandrogenic status by increasing SHBG (Moghetti et al., 2000). The putative mechanism of action of metformin in endometriosis is proposed to be a decrease in aromatase enzyme activity (Takemura et al., 2007). In addition to this, it has been shown that SHBG levels are increased after the metformin treatment (Nestler and Jakubowicz, 1996; Moghetti et al., 2000). Therefore, circulating estradiol levels are decreased in response to the increase in SHBG levels. In our opinion, the endometriotic implants in the present study may have been affected by the low levels of estradiol both in endometriotic tissue and in circulation.

One of the goals of our study was to display the comparison of metformin and letrozole effects on endometriosis. The present study demonstrates that endometriotic implants regressed in metformin and aromatase inhibitor groups. Unfortunately, we still do not know the exact mechanism of metformin on endometriosis. Aberrant aromatase enzyme activation may be the most potent known stimulator of endometriotic implants and only one study has shown that metformin may directly decrease the aromatase activity (Takemura et al., 2007). In our study, it has been clearly exhibited that regressions of endometriotic implants in the metformin group were comparable to that in the aromatase inhibitor group.

After inducing endometriosis by a surgical approach, endometriosis had a direct effect on adhesion formation, not on surgery alone (Mohammazadeh et al., 2006). Similarly, in our study, endometrial implants were able to induce adhesions. Metformin caused the regression of endometriotic implants and also reduced the severity of adhesions. This may be due to the anti-inflammatory and antiproliferative effects of metformin which are likely to decrease adhesion formation. However, we did not reveal the same effect on letrozole.

Endometriosis is associated with infertility, as it is a destructive disorder that results in anatomical distortion of pelvic organs due to inflammation and scar tissue (Ozkan et al., 2008). It has been reported that infertile women are 6–8 times more likely than fertile women to have the disease (Mitwally and Casper, 2001). Letrozole is also used for ovulation induction and superior to clomiphene citrate (CC) when comparing side effects, ovulation and pregnancy rates in a general infertile population (Mitwally and Casper, 2001; Fisher et al., 2002; Lord et al., 2003; Atay et al., 2006). Although some authors speculate that metformin has no benefit for ovulation induction, a meta-analysis recommended that metformin added to CC might improve the ovulation rates (Lord et al., 2003). Therefore, when the strong relation between endometriosis and infertility is discussed, anti-endometriotic drugs usage may be seen as beneficial for the ovulation induction in patients with endometriosis, but no studies have yet examined the effects of letrozole or metformin for ovulation induction in infertile patients with endometriosis.

The present study confirms that both metformin and letrozole are effective in the treatment of endometriosis as measured by a decrease in the size of endometriotic implants. However, there was not any statistical difference between the drugs. Additionally, metformin decreased the severity of adhesion formation that is associated with endometriotic foci. Metformin may be a helpful anti-inflammatory and antiproliferative agent in the treatment of the disease. More experiments on animal models and clinical trials would be helpful in determining the utility of compounds for women with endometriosis.

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