Doxycycline causes regression of endometriotic implants: a rat model

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BACKGROUND: Doxycycline (Dox) has a number of non-antibiotic properties. One of them is the inhibition of matrix metalloproteinase (MMP) activity. The aim of this study was to assess the effects of Dox in a rat endometriosis model.

METHODS: Endometriosis was surgically induced in 40 rats by transplanting of endometrial tissue. After 3 weeks, repeat laparotomies were performed to check the implants and the animals were randomized into four groups: Group I, low-dose Dox (5 mg/kg/day); Group II, high-dose Dox (40 mg/kg/day); Group III, leuprolide acetate 1 mg/kg single dose, s.c.; and Group VI (controls), no medication. The treatment, initiated on the day of surgery and continuing for 3 weeks, was administered to the study groups. Three weeks later, the rats were euthanized and the implants were evaluated morphologically and histologically for immunoreactivity of MMP-2 and -9, and interleukin-6 (IL-6) concentration in the peritoneal fluid was assayed.

RESULTS: Treatment with leuprolide acetate, or high-dose or low-dose Dox caused significant decreases in the implant areas compared with the controls (P = 0.03, P = 0.006, and P = 0.001, respectively). IL-6 levels in peritoneal fluid decreased in Group I (P = 0.02) and Group III (P < 0.05). MMP H scores were significantly lower in the group that received low-dose Dox in both epithelial and stromal MMP-2 and -9 immunostaining when compared with the control group [P = 0.048, P = 0.002, P = 0.007 and P = 0.002, respectively, MMP-2 (epithelia), MMP-2 (stroma), MMP-9 (epithelia) and MMP-9 (stroma)].

CONCLUSIONS: Low-dose Dox caused regression of endometriosis in this experimental rat model.

Key words: endometriosis / rat / doxycycline / MMP / treatment

Introduction

Endometriosis is the adherence and growth of the functionalis layer of the endometrium outside the uterine cavity. This condition is predominantly found in women of reproductive age and affects 7–10% of all women, 71–87% of women with chronic pelvic pain and 38% of all infertile women (Amsterdam et al., 2005). The most widely supported hypothesis for the aetiology of endometriosis is the implantation of endometrial sheddings outside of the uterine cavity via retrograde menstruation (Sampson, 1927). Although the exact mechanism by which refluxed menstrual endometrium leads to ectopic implantation remains unknown, adherence, invasion and disease development all require increased expression of matrix metalloproteinases (MMPs), leading to remodelling of the peritoneal mesothelial layer and angiogenesis (Osteen et al., 1996; Sillem et al., 1998; Osteen et al., 1999; Osteen et al., 2002; Sharpe-Timms and Cox 2002).

The MMPs are a family of zinc- and calcium-dependent endoproteases that are involved in the degradation of the extracellular matrix (ECM) and basement membrane. MMPs influence the outcome of inflammatory reactions, angiogenesis and tissue remodelling by acting as key regulators of ECM turnover, and initiate the release of ECM-bound growth factors and cytokines that regulate many of these processes (Mott and Werb, 2004). These enzymes, therefore, mediate a large variety of normal biological processes including embryonic development, ovulation, menstruation and wound healing. They are also implicated in a number of pathological processes such as cancer, atherosclerosis, inflammatory disorders or endometriosis (Freitas et al., 1999; Nelson et al., 2000). The link between MMP expression and endometriosis pathophysiology has been widely studied (Chung et al., 2001; Cox et al., 2001; Liu et al., 2002; Ria et al., 2002; Wu et al., 2005). Elevated expression of MMPs, leading to increased vascularity, may be a key event in endometriosis progression, facilitating tissue degradation and invasive formation (Yu et al., 2008). Suppression (Bruner-Tran et al., 2002) and down-regulation of MMP expression (Bruner-Tran et al., 2006) inhibits the establishment of ectopic lesions by human endometrium in nude...
mice. Furthermore, MMP inhibitors have been assessed for their therapeutic potential in endometriosis, with encouraging results (Nap et al., 2004).

Doxycycline (Dox), which belongs to the family of chemically modified tetracyclines, is a pluripotent drug that affects many cellular functions including proliferation, migration, apoptosis and matrix remodelling (Bendeck et al., 2002a,b; Lee et al., 2006). Dox and other derivatives of tetracycline are non-specific, potent MMP inhibitors (Golub et al., 2001; Axia et al., 2002; Acharya et al., 2004; Choi et al., 2004; Lee et al., 2004a,b). They act directly on certain MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) (Nip et al., 1993; Utito et al., 1994; Bendeck et al., 2002a,b), independently of antimicrobial activity (Golub et al., 1991; Fife and Sledge, 1995; Ryan et al., 1996; Golub et al., 1998). In addition to inhibitory effects on MMPs, Dox has other, non-antibiotic properties that are anti-angiogenic, anti-inflammatory and anti-apoptotic (Sapadin and Fleischmajer, 2006).

Cytokines are important in mediating acute phase reactions, inflammation and angiogenesis, and their role in the pathogenesis of endometriosis has been investigated (Biffi et al., 1996; Witz et al., 2000). The diagnostic value of the pleiotropic cytokine interleukin-6 (IL-6) has been demonstrated for detecting (Bedaiwy et al., 2002) and identifying (Iwabe et al., 2003) specific forms of endometriosis. Several studies have reported increased concentrations of IL-6 in peripheral blood (Pellicer et al., 1998; Martínez et al., 2007), peritoneal fluid (Keenan et al., 1994; Rier et al., 1995) and ectopic endometrium in women with endometriosis (Salmasi et al., 2008). The concentration of IL-6 has been correlated with the degree of disease severity (Khan et al., 2002). In addition, IL-6 serum level has been proposed as a reliable, non-invasive marker of minimal and mild endometriosis (Martínez et al., 2007).

In the current study, we examine the therapeutic potential of Dox, a non-specific MMP inhibitor, determining the ability of this agent to both down-regulate MMP expression and reduce the ectopic growth of endometrial tissue in an animal model.

Materials and Methods

Animals

Forty mature, female, non-pregnant Wistar Albino rats weighing between 200 and 250 g were used. Animals were provided by Baskent University Animal Reproduction Centre and housed in the 131 Animal Laboratory of Baskent University. They were caged in a controlled environment at 22°C with 12 h light/dark cycles. Standard rat feed and reverse-osmosis-purified water were provided ad libitum. All rats were allowed to acclimatize to this environment for 1 week before the experiment. The Baskent University Committee on the Use and Care of Laboratory Animals approved the experiments, and all investigations complied with the 1996 National Academy of Science’s Guide for Care and Use of Laboratory Animals.

Surgical procedures

Rats were anaesthetized by intraperitoneal administration of 60 mg/kg ketamine hydrochloric acid (Ketalar; Eczacibasi Warner-Lambert Ilaç Sanayi, Levent, Istanbul, Turkey) and 7 mg/kg xylazine hydrochloric acid (Rompun, Bayer Sisli, Istanbul, Turkey). They were immobilized on a standard rat surgery board. Before surgery, the abdominal skin was shaved and antisepsis was obtained with 10% povidone iodine solution. Using sterile technique, a 4–5 cm ventral vertical incision was made to expose the reproductive organs. All rats underwent three consecutive laparotomies.

First laparotomy

Ectopic endometrium was induced surgically in rats by transplanting an autologous fragment of endometrial tissue onto the inner surface of the abdominal wall (Yavuz et al., 2007). Briefly, the left uterine horn was ligated at both the uterotubal junction and the cervical end using 4–0 silk and removed. A 7 mm segment of the excised horn was cut and placed in sterile phosphate-buffered saline (PBS) at 37°C. The endometrium was separated from the myometrium and trimmed to 5 × 5 mm (mean surface area equal to 25 mm²). This piece of uterine tissue was transplanted without removing the myometrium onto the inner surface of the right abdominal wall with the serosal surface apposed, and secured with single non-absorbable 5-0 polypropylene suture in the middle to the abdominal wall. The vertical abdominal incision was closed with the use of two-layer prolene sutures. The skin incision was closed with a horizontal mattress. The duration of surgery was limited to 15–20 min for each rat to minimize tissue drying. After the first operation, all rats were observed for 21 days, during which time they did not receive medication.

Second laparotomy

Animals underwent a second exploratory laparotomy to examine endometrial implants and collect peritoneal fluid for IL-6 concentration analysis. Peritoneal lavage with 3 ml of saline was performed and samples were immediately sent to the laboratory at the beginning of the laparotomy. The length and width of implants were measured macroscopically and the surface areas of the implants were calculated. Then, the laparotomy was closed. Following the second operation, all rats were allowed a resting period of 3 days prior to random allocation to four groups. The rats in Group I (low-dose Dox group, n = 10) were given 5 mg/kg/day oral Dox (Monodoks tablet, Deva, Istanbul, Turkey). The rats in Group II (high-dose Dox group, n = 8) were given 40 mg/kg/day oral Dox. Dosage choices were based on previous studies (Prall et al., 2002; Lee et al., 2006). The rats in Group III (gonadotrophin-releasing hormone (GnRH) agonist group, n = 10) were given a single s.c. injection of leuprolide acetate depot formulation (1 mg/kg body weight; Lucrin; Abbott, Cedex, Istanbul, Turkey). The leuprolide acetate dose was based on a previous study (Dogan et al., 2004). The rats in Group IV (control group, n = 10) were given no medication. The oral medications were given via an orogastric tube by laboratory personnel. All the rats were observed for 21 days.

Third laparotomy

After the end of the medical treatment, rats were euthanized by ketamine anaesthesia and the third laparotomy was performed. During laparotomy, the length and width of the implants were measured macroscopically and the surface areas of the implants were calculated. The implants were then excised and fixed in 10% formalin for histopathological examination. Peritoneal lavage with 3 ml saline was performed again to assess the IL-6 concentration in the peritoneal fluid.

All operations and measurements were performed by physicians blinded to the groups.

IL-6 assessment

The IL-6 concentration in the peritoneal fluid was quantitatively assessed using a commercially available enzyme-linked immunosorbent assay kit (Bender MedSystems®, Vienna, Austria) according to the manufacturer’s instructions. The enzyme immunoassay measures with a sensitivity of
<12 pg/ml; it has an intra-assay variability of <5% and an inter-assay variability of <10%.

Histopathological examination
Formalin-fixed endometriotic foci were embedded in paraffin blocks, sectioned at ~5 mm thickness (four sections per sample), stained with haematoxylin and eosin and examined under a light microscope. The persistence of epithelial cells in endometrial implants was evaluated semiquantitatively as follows: 3, well-preserved epithelial layer; 2, moderately preserved epithelium with leucocytes 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).

Immunostaining was performed on 3 μm thick sections using the avidin–biotin–peroxidase complex (ABC). Following deparaffinization and rehydration, endogenous peroxidase activity was blocked with 0.3% H2O2/methanol followed by non-immune serum for 20 min to block non-specific binding. Sections were incubated with the primary monoclonal antibody against MMP-2 and -9 (MAB3309 and MAB3308, Chemicon) at a dilution of 1/200 for 2.5 h. Sections were washed (three times in PBS) then incubated with biotinylated IgG (1:40 000) for 15 min, washed again (three times in PBS) and incubated with streptavidin–biotin–peroxidase complex (ABC reagent) for 15 min. The reaction was visualized using 3-aminobenzidine carboamide (AEC) chromogen. After counterstaining with haematoxylin, the slides were dehydrated, coverslip-mounted and examined under an Olympus BX51 microscope.

Immunoreactivity was evaluated independently by two observers. Staining reactions were assessed semi-quantitatively using the H-score method. For each section, epithelial and stromal cells of the endometriotic foci were evaluated for labelling intensity in five distinct high-magnification fields (× 400 objective), (0, no labelling; 1+, weak; 2+, moderate; and 3+, strong labelling). H score = [(% at 0) × 0] + [(% at 1+) × 1] + [(% at 2+) × 2] + [(% at 3+) × 3], and the results were set to an H-score range (Keenan et al., 1999).

Statistical analysis
The data were analysed using the Statistical Package for the Social Sciences version 11.0 (SPSS, Chicago, IL, USA). Normally distributed (Shapiro–Wilk test) parametric variables were tested by analysis of variance using the least significant difference test for post hoc analysis. Non-normally distributed metric variables were analysed by the Kruskal–Wallis test and Mann–Whitney U-test with Bonferroni’s correction. The mean surface areas of the endometriotic implants and IL-6 levels in the same group (before and after the medical treatment) were analysed by paired sample t-test since they were normally distributed. P-values <0.05 were considered statistically significant. Values were expressed as mean ± SD.

Results
All rats, with the exception of two in Group II, survived to the end of the study. In Group II, two animals died on the day of randomization due to complications related to surgery. The standardized surgical procedures and drug treatment were well tolerated by the remaining animals. All laparotomy sites were intact. No side effects related to the medication were observed in treatment groups.

At the beginning of the medical treatment, the mean surface areas of the endometriotic implants and IL-6 levels in the peritoneal fluid were comparable in all four groups. Although the baseline mean surface area of implant in GnRHa group was smaller than the others, statistical significance was not observed.

At the end of the treatment period, when compared with the control group, the mean areas of implants were smaller in Group I (P = 0.001), Group II (P = 0.006) and Group III (P = 0.03) (Table I).

The mean areas of implants decreased significantly with treatment in Group I from 31.2 ± 19.4 to 9.5 ± 4.2 mm² (P = 0.006), in Group II from 52.2 ± 46.9 to 13.6 ± 8.7 mm² (P = 0.045) and in Group III from 20.2 ± 12.2 to 10.2 ± 5.9 mm² (P = 0.008). There was no statistically significant change in the control group (P = 0.922) (Table I). Sample views of the endometriotic implants are shown in Figs 1 and 2.

The mean score of the histopathological examination of the implants at the end of the treatment was lower in Group III than in the control group (P = 0.04) (Table I).

IL-6 levels in the peritoneal fluid decreased significantly with treatment from 59.5 ± 24.6 to 41 ± 7.7 pg/ml in Group I (P = 0.02) and from 48.3 ± 19 to 25.2 ± 13 pg/ml in Group III (P = 0.05). However, there was no significant change in Group II or the control group. At the end of the treatment period, peritoneal IL-6 levels were significantly lower in Group III than in the control group (P = 0.008).

Table I Characteristics and results of the groups of rats treated with low (5 mg/kg/day) or high (40 mg/kg/day) dose oral Dox for 21 days, or a single s.c. dose (1 mg/kg) of GnRH agonist

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group I (Dox Group I, low dose)</th>
<th>Group II (Dox Group II, high dose)</th>
<th>Group III (GnRH agonist group)</th>
<th>Group IV (control group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean surface area of the implants at second laparotomy before the medication (mm²)</td>
<td>31.2 ± 19.4</td>
<td>52.2 ± 46.9</td>
<td>20.2 ± 12.1</td>
<td>4 ± 13.8</td>
</tr>
<tr>
<td>Mean surface area of the implants at third laparotomy after the medication (mm²)</td>
<td>9.5 ± 4.2ab</td>
<td>13.6 ± 8.7ab</td>
<td>10.2 ± 5.9ab</td>
<td>33.3 ± 20.4</td>
</tr>
<tr>
<td>IL-6 level in peritoneal fluid before the medication (pg/ml)</td>
<td>59.5 ± 24.6</td>
<td>45.3 ± 7.1</td>
<td>48.3 ± 19</td>
<td>63.3 ± 26.1</td>
</tr>
<tr>
<td>IL-6 level in peritoneal fluid after the medication (pg/ml)</td>
<td>41 ± 7.7ab</td>
<td>65.2 ± 30.9</td>
<td>25.2 ± 13ab</td>
<td>61.1 ± 25.6</td>
</tr>
<tr>
<td>Histopathological score of the implants at the end of the treatment</td>
<td>2.8 ± 0.35</td>
<td>2.6 ± 0.74</td>
<td>0.9 ± 0.6a</td>
<td>2 ± 1.3</td>
</tr>
</tbody>
</table>

GnRH, gonadotrophin-releasing hormone; IL-6, interleukin.
abSignificantly different from control group (P < 0.05).
acSignificantly different from values measured before the medication in the same group (P < 0.05).
concentration was significantly lower in Groups I and III compared with the control group, \((P = 0.0029)\) and \((P = 0.001)\), respectively (Table I).

**Identification and quantification of MMP-2 and -9**

Specific staining of MMP-2 and -9 expression was observed throughout the endometrial tissue, both in the epithelial and in the stromal compartments in the low- and high-dose Dox groups and the control group (Fig. 3). MMP H scores were significantly lower in the Group I for both epithelial and stromal MMP-2 and -9 immunostaining when compared with the control group [MMP-2 (epithelia) \(106.4 \pm 6.9\) versus \(163.1 \pm 96.5\) \((P = 0.048)\), MMP-2 (stroma) \(101.1 \pm 2.2\) versus \(130.5 \pm 47.1\) \((P = 0.002)\), MMP-9 (epithelia) \(124.1 \pm 28\) versus \(220.6 \pm 66\) \((P = 0.007)\) and MMP-9 (stroma) \(102.2 \pm 2.6\) versus \(125.5 \pm 34.8\) \((P = 0.002)\) (Table II).

**Discussion**

We demonstrate here that low-dose Dox significantly reduces the size of experimentally induced endometriotic implants, IL-6 levels in the peritoneal fluid and immunoreactivity of MMP-2 and -9 in a rat model. To the best of our knowledge, this is the first demonstration of the potential therapeutic value of Dox for the treatment of endometriosis.

Treatment with leuprolide acetate, a GnRH agonist, or high-dose or low-dose Dox caused significant decreases in the endometriotic implant size compared with the controls. IL-6 levels of peritoneal fluid and histopathological scoring for MMPs were included to evaluate the effect of Dox treatment, as the only analysis of regression of the mean surface area of the implants may not be objective criteria for evaluation. IL-6 levels in peritoneal fluid decreased in low-dose Dox and GnRH agonist. In addition, MMP H scores were significantly lower in the groups that received low-dose Dox and GnRH agonist in both epithelial and stromal MMP-2 and -9 immunostaining when compared with the control group. This finding suggests to us that low-dose Dox as effective a therapeutic as GnRH agonist.

As in many other pathological conditions, increased or misregulated activity of MMPs in endometriosis has become apparent. Aberrant patterns of MMP protein and mRNA expression in eutopic (Chen et al., 2004; Chung et al., 2001, 2002; Collette et al., 2004; 2006) and ectopic endometriotic tissue have been reported (Chung et al., 2001; Cox et al., 2001; Liu et al., 2002; Gilabert-Estelles et al., 2003; Hudelist et al., 2005; Kyama et al., 2006). The eutopic endometrium from patients with endometriosis may be more invasive and prone to peritoneal implantation because of higher expressions of MMP-2 (Chung et al., 2001) and MMP-9 (Chung et al., 2001; Collette et al., 2006; Pan et al., 2008).

Blockade of MMP activity with MMP inhibitor III, resulting in specific inhibition of ‘classical’ MMPs (MMP-1, -2, -3, -7 and -13), reduces the development of endometriosis-like lesions in an in vitro assay (Nap et al., 2004; Braundmeier et al., 2006). In this regard, systemic delivery of drugs that inhibit pathologically elevated levels of MMPs could be very valuable as an adjunctive treatment/medication in endometriosis.

Dox has various non-antibiotic properties affecting mammalian cell functions in which the signal pathways are still largely unknown, leading to blockage of the inflammation, angiogenesis and/or apoptosis process (Sapadin and Fleischmajer, 2006). Several vascular remodelling and angiogenesis studies in various tissues at various doses demonstrated inhibition of MMP activity by Dox may involve inhibition of Smad, ERK, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (MAPK) signalling pathways, suggesting that the inhibitory effect of Dox could be through mediation of multiple pathways (De Paiva et al., 2004; Lee et al., 2004a, b, 2006; Luo et al., 2004; Kim et al., 2005; Hoyt et al., 2006). A primary function of p38 MAPK is to regulate the mRNA stability of inflammatory mediators, including IL-6. Dox has been shown to influence the inflammatory-related mediators through alteration of p38 MAPK activity (Hoyt et al., 2006) and is proposed as an alternative mechanism by which Dox alters MMP expression in the endometrial cells.
**Figure 3** Immunohistochemical staining

**Table II** Immunohistochemical scoring of MMP-2 and MMP-9 in both epithelial and stromal endometrial tissue among Groups I, II and IV

<table>
<thead>
<tr>
<th></th>
<th>MMP-2 (epithelia)</th>
<th>MMP-2 (stroma)</th>
<th>MMP-9 (epithelia)</th>
<th>MMP-9 (stroma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Dox, 5 mg/kg/day)</td>
<td>106.4 ± 6.9a</td>
<td>101.1 ± 2.2b</td>
<td>124.1 ± 28c</td>
<td>102.2 ± 2.6d</td>
</tr>
<tr>
<td>Group II (Dox, 40 mg/kg/day)</td>
<td>147.5 ± 44.9</td>
<td>105 ± 3.7</td>
<td>182.5 ± 49.2</td>
<td>106.2 ± 3.5</td>
</tr>
<tr>
<td>Group III (agonist group)</td>
<td>102.1 ± 6.1e</td>
<td>104.4 ± 2.2f</td>
<td>101.1 ± 4g</td>
<td>102.4 ± 3.1h</td>
</tr>
<tr>
<td>Group IV (control group)</td>
<td>163.1 ± 96.5</td>
<td>130.5 ± 47</td>
<td>220.6 ± 66</td>
<td>125.5 ± 34.8</td>
</tr>
</tbody>
</table>

*aSignificantly different from the control group (P = 0.048).
*bSignificantly different from the control group (P = 0.002).
*cSignificantly different from the control group (P = 0.007).
*dSignificantly different from the control group (P = 0.002).
*eSignificantly different from the control group (P = 0.037).
*fSignificantly different from the control group (P = 0.012).
*gSignificantly different from the control group (P = 0.002).
*hSignificantly different from the control group (P = 0.02).
(Li et al., 2006), resulting in an enhanced reparative process (Li et al., 2007).

In addition to signalling pathways, Dox is thought to act via both primary direct (Sorsa et al., 1993) and secondary indirect mechanisms on MMP activity and cell proliferation (Golub et al., 2001; Courtman et al., 2004; Burggraf et al., 2007). The potential explanations are that it non-selectively directly inhibits MMPs by binding to the active zinc sites (Sorsa et al., 1994) and also by binding to an inactive calcium site, which causes conformational change (Lovejoy et al., 1994) and loss of enzymatic activity. Secondary mechanisms of inhibition have also been proposed, which include a reduction in activation (Ramamurthy et al., 1993), decreased gene expression (Petricevic et al., 1996) and stabilization of specific and non-specific inhibition (Golub et al., 1994a,b).

By using one or more complex molecular mechanism(s)/pathways, Dox in a dose-, time-, cell- and steroid-dependent manner (Zhang et al., 2000; Curry and Osteen 2003; Acharya et al., 2004; Lee et al., 2004a,b; Bruner-Tran et al., 2006; Li et al., 2006, 2007) may alter or involve different signalling pathways and downstream molecules leading to complete or incomplete inhibition on MMPs that alter their autocrine/paracrine actions possibly leading to endometrial inflammatory, angiogenic and apoptotic responses (Li et al., 2006, 2007). An example of the dose-dependent effect of Dox has been reported in human colorectal cancer cell lines; Dox at 5–10 mg/ml has been reported to reduce MMP-2 and -9 activities, with complete inhibition at 20 mg/ml, without affecting mRNA expression levels (Onoda et al., 2004).

Dox is a potent inhibitor of MMP-2, -9 and -8, is a much weaker inhibitor of MMP-1 and does not inhibit MMP-3 or -7 (Smith et al., 1999; Kivela¨-Rajama¨ki et al., 2003). By comparison, the potent and efficient synthetic MMP inhibitors, such as Batimastat, have a much greater effect, reducing MMP levels to below physiological or protective basal levels, eventually resulting in clinically significant, harmful side effects (Ramamurthy et al., 1993; Utto et al., 2003; Bjornsson et al., 2004). In arthritis and periodontitis, the excessive inhibition of MMPs surprisingly exacerbates rather than alleviates the disease (Coussens et al., 2002; Bjornsson et al., 2004; Folgueras et al., 2004). Therefore, it has been proposed that ‘leaky’, less efficacious MMP inhibitors, such as the tetracycline-based MMP inhibitors, may be safer and more effective at treating certain conditions because they only reduce the pathologically excessive MMPs but not to levels below those required for normal physiological or anti-inflammatory functions (Golub et al., 1994a,b; Preshaw et al., 2004; Sorsa and Golub, 2005). These observations may explain the effect of the low dose in our results.

According to the literature, the daily oral dose of Dox for effective MMP inhibition in rats is 25–100 mg/kg (Ramamurthy et al., 1998; Folwarczna et al., 1999; Lamparter et al., 2002), which is substantially higher than doses given to humans (2–3 mg/kg). Although the high dose used in our study (40 mg/kg) is certainly well within this therapeutic range, it may be associated with major (cytotoxic) side effects (Yao et al., 2007). For calculating equivalent doses between humans and animals, conversion coefficients have been published (Ruiyuan, 1987). When the human/rat conversion coefficient of 56.0 is considered for calculating the equivalent dose of Dox in humans, the dose is 2–3 mg/kg/day (or 200 mg/day, assuming rat and human weights of 200 g and 70 kg, respectively). When converting dose between species, one must also factor in body surface area as well as body weight and rapid metabolism (Prall et al., 2002). In an animal model, 5–10 mg/kg Dox is equal to the 2–3 mg/kg recommended human dose. These doses may have the ideal effect. Accordingly, several animal studies are limited by super-physiological doses of Dox that cannot be extrapolated to humans (Prall et al., 2002). Consequently, further animal studies are needed to optimize the Dox dose for treating endometriosis and to characterize any systemic side effects associated with treatment.

IL-6 is reported to be closely related to the pathogenesis and clinical severity of endometriosis. Elevated IL-6 is demonstrated to be reversed following Dox therapy. This result is consistent with findings that sub-antimicrobicidal doses of Dox reduced the level of IL-6 chronic adult periodontitis (Choi et al., 2004) and in an in vitro model (Brown et al., 2004).

The cell-specific and cytokine-dependent action of Dox in endometrial cells is yet to be elucidated; with differential interaction with ovarian steroids and MMPs, the therapeutic benefit of Dox is likely to involve a complex molecular mechanism(s). Using isolated endometrial stromal cell, glandular epithelial cell and human epithelial cell lines as in vitro models, no significant effect of Dox on the rate of H²-thymidine incorporation or cell proliferation was seen, except with extremely high doses (Li et al., 2006). Hence, this may explain the persisting epithelium score observed in our study.

Progression of lesions in rat endometriosis models peaks between 4 and 7 days and declines at 14 days after uterine autotransplantation. Histopathological features of uterine autotransplantation beyond 14 days are not known (Uchiide et al., 2002). For this reason, the value of semi-quantitative evaluations of persisting epithelium in uterine tissue may be questioned for extended periods such as that in our study.

In addition, the reference study for semi-quantitative evaluation of persisting epithelium in uterine tissue allografts has methodological differences from ours. In the reference study, the tissues used for morphologic and immunohistochemical analysis were collected 2 months after the cessation of treatment, not at the cessation of the study as in ours. Therefore, it is also possible that the uterine explants might be then devoid of specific endometrial structure as in the reference study (Keenan et al., 1999).

A further limitation of our study is that the baseline mean surface area of implants in the GnRH agonist group was smaller than the others; however, this difference was not statistically significant. The small group size in this study is a limiting factor and may mitigate the relevance of this P-value.

Currently, a great deal of effort is being spent on the development of new drugs for the treatment of endometriosis, with the goal of achieving higher efficacy and fewer side effects and, importantly, with the option of long-term treatment. Evidence from preclinical studies has suggested that beneficial effects may be conferred by anti-proliferative, anti-inflammatory or antiangiogenic mechanisms (Cobellis et al., 2004; Lebovic et al., 2004; Chwalisz et al., 2005; Nap et al., 2005). As a pluripotent, non-specific, leaky inhibitor of MMPs, Dox blocks inflammation, apoptosis and/or angiogenesis may therefore be considered a therapeutic option for endometriosis.

Although a number of MMP inhibitors have been synthesized, the major drawbacks of these molecules are the treatment schedule and adverse side effects (Coussens et al., 2002). Dox is frequently used...
in relatively low doses for many months or years and has a good safety and tolerability profile. Furthermore, the cost effectiveness and easy access of Dox justifies additional studies on its efficacy as an adjunct therapy for endometriosis.

Funding

This study was supported by grant no. DA07/19 from Baskent University, Ankara, Turkey.

References


Doxycycline effect on endometriosis in a rat model

Increased peritoneal and endometrial gene expression of biologically relevant cytokines and growth factors during the menstrual phase in women with endometriosis. *Fertil Steril* 2006;85:1667–1675.


